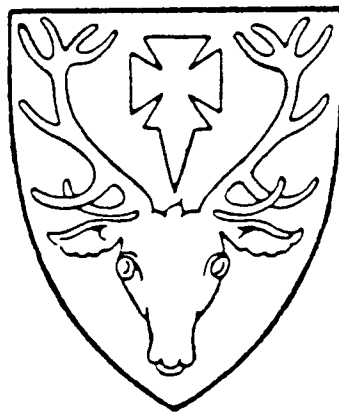


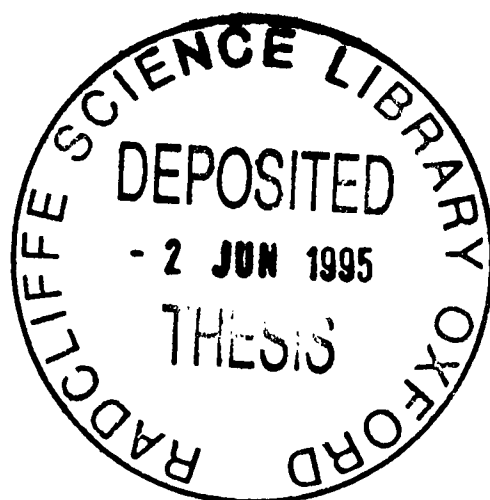
Multiple Mechanisms in the Establishment of Thalamocortical Innervation

Thesis submitted for the degree of Doctor of Philosophy
Oxford University

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Szüleimnek

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Statement

The vast majority of the experiments and analysis contained in this thesis are my own work, though they have benefited enormously from practical instruction and advice that I have received from many colleagues.

For the confocal microscopy reported in Chapter 3 and 4, I received help and instruction from Richard Adams and William Hinkes.

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John Mitrofanis of the Department of Human Anatomy at Oxford very kindly allowed me to examine his collection of material stained for NADPH-diaphorase and parvalbumin. My analysis of these specimens is described in Chapters 5 and 6, together with that of some additional material that I prepared, with technical advice from Dr Mitrofanis.

No part of this thesis is being submitted, or has already been accepted, for any other degree at this, or any other, university.



Oxford, 01 July 1994

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Abstract

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Multiple Mechanisms in the Establishment of Thalamocortical Innervation

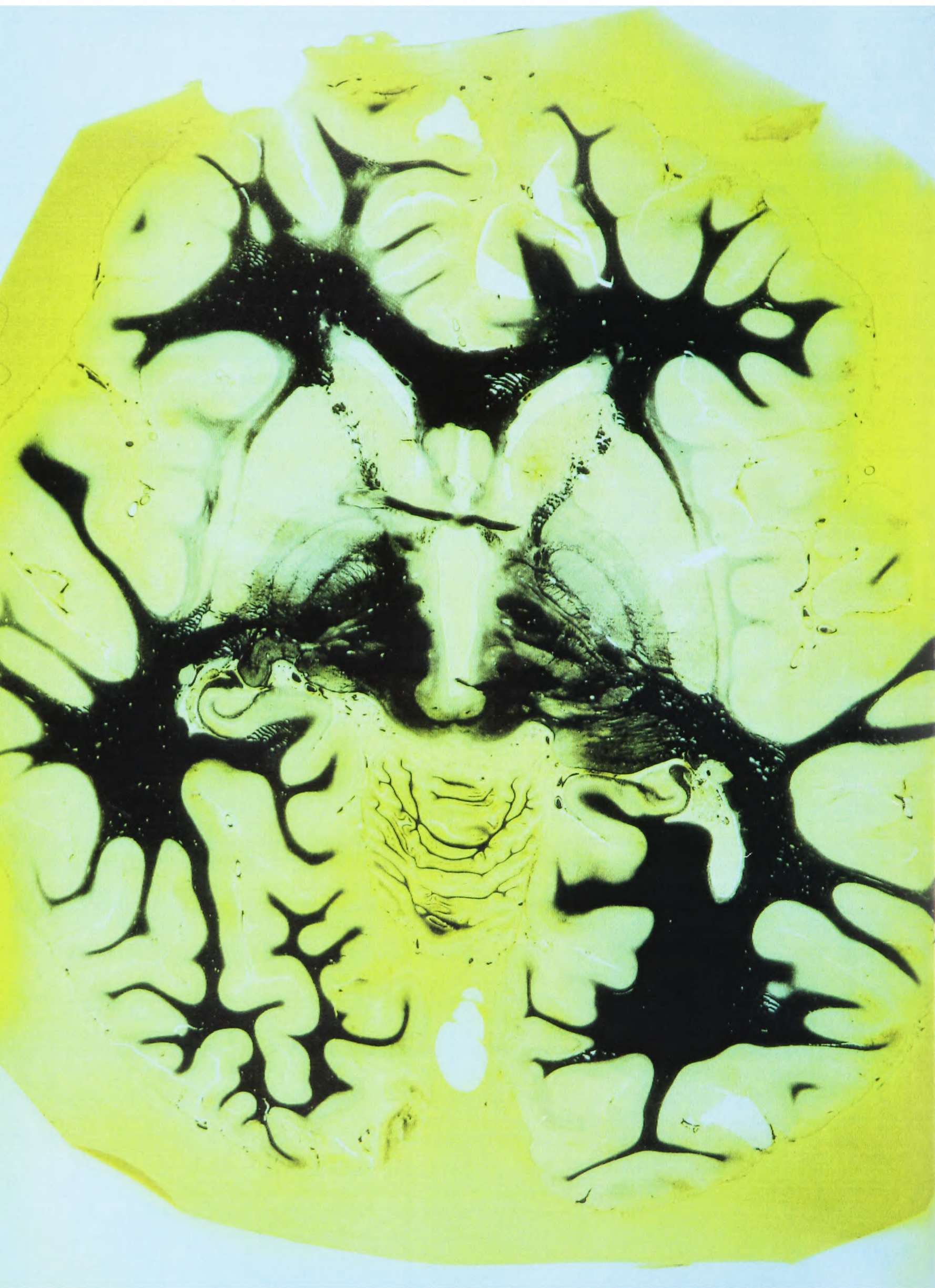
This thesis describes studies of the mechanisms by which regional specification, selective thalamic innervation and topographic organization of the rodent neocortex are achieved during development, and proposes that a sequence of simple steps contributes to the construction of the complexity of the adult mammalian neocortex.

Experiments involving organotypic co-culture of explants of E16-17 rat thalamus with slices of neocortex from animals of various ages revealed that: (1) embryonic cortical slices younger than E19 promote the outgrowth of axons from nearby thalamic explants (presumably through the influence of a diffusible, growth promoting agent) but permit little ingrowth of those axons; (2) for cortex between E19 and P3, thalamic fibres invade the slice (implying that the cortex now expresses a growth-permissive factor) and after a few days *in vitro* they extend radially through the entire thickness of the cortex without obvious termination; (3) in cortical slices taken from pups older than P3, thalamic axons invade (regardless whether they entered the slice from the ventricular or from the pial surface) and mostly arborize and terminate about 300µm below the pial surface, implying that a 'stop signal' is expressed in layer 4 from about P3. Presumably the various age-dependent factors revealed by these experiments influence thalamic fibre outgrowth, accumulation below the cortical plate, invasion and then termination *in vivo*. *In vitro*, any region of the rat thalamus (at E15-17) innervates any region of the cortex (E16-P11): no distinct positional preference is apparent even when the thalamic explant is given a choice of various cortical targets.

Axon-tracing with carbocyanine dyes in fixed brains showed that there is roughly synchronized, topographically ordered outgrowth of pioneering axons from the first-generated cortical preplate cells and from the corresponding region of the thalamus (at E14 for occipital cortex and LGN). The two wave-fronts meet and intermingle in the primitive internal capsule. By E16, fibres from the LGN have reached the appropriate subplate region by taking routes through the intermediate zone closely related to their corticofugal counterparts. Morphogenetic gradients, simple mechanical factors and *chronotopic* fibre ordering, in particular, may play an important role in imposing topography in the initial stages of growth of both sets of axons, when the structure of the forebrain is very simple. The scaffold of subplate axons may then serve to guide thalamic axons through the telencephalon, as cerebral growth distorts the earlier simple pathway, and to ensure that they accumulate in the subplate layer, from about E16-19. At E19 they begin to invade the cortical plate and they terminate, mainly in layer 4, each arborizing laterally over about 300µm.

Similar studies of the early projections in Reeler mutant mouse, in which the layers of the cortex are basically inverted and the early-generated cells all remain superficial, revealed the same basic algorithm of development. The mutation does not affect the formation of the preplate, outgrowth of a topographically ordered array of axons from the preplate, synchronized outgrowth of thalamic axons through the primitive internal capsule, their tangential distribution over the scaffold of 'superplate' corticofugal fibres or their period of 'waiting' in the 'superplate' layer, over the cells of origin of that scaffold. Thus, thalamic axons establish and maintain their relationship with the axons and cells of the displaced 'superplate' layer, leading to their innervation of the appropriate cortical areas.

Histochemical methods were used to examine the distribution of cells in rat cortex containing the enzyme NADPH-diaphorase, which is a synthase of nitric oxide (NO), an intercellular messenger that may regulate cell growth, death and synapse formation and plasticity. NADPH-diaphorase appears around the time of birth in polymorphous cells of the subplate and it is subsequently expressed in progressively more superficial layers of the cortex, in what appear to be aspiny interneurons. Although these cells are very sparsely scattered, there is an elaborate and ubiquitous meshwork of NADPH-diaphorase-positive processes. Therefore NO-producing cells are in a strategic position to influence the entire cortex during the major phase of dendritic maturation, synaptogenesis, cell death and synaptic plasticity. Immunohistochemistry revealed that the calcium binding protein, parvalbumin, is expressed in an unusual pattern - first in the subplate, around the time of birth, then in aspiny interneurons, starting in layer 4 and moving outwards through all layers. Its appearance may be associated with cell death and with the regulation of synaptic plasticity in the developing cortex.



"There is but one safe way to avoid mistakes: to do nothing, or at least, to avoid doing something new. This, however, in itself may be the greatest mistake of all. The unknown lends an insecure foothold and venturing out into it, one can hope for no more than that the possible failure will be a honorable one."

"See what every one sees, but think what no one else thinks"

"Research is rarely guided by logic. It is guided mostly by hunches, guesses and intuition. All the same, once we get somewhere and present our results, we like to present them as a logical sequence."

Albert Szent-Györgyi
(1893 - 1986)

MULTIPLE MECHANISMS IN THE ESTABLISHMENT OF THALAMOCORTICAL INNERVATION

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CHAPTER 1

GENERAL INTRODUCTION

TO THE DEVELOPMENT OF THE CEREBRAL CORTEX

The emergence of the mammalian cerebral cortex is arguably one of the most significant events in phylogeny, and the development of this complicated structure during individual ontogeny is no less spectacular. A neural sheet, initially just a monolayer of cells, becomes a three-dimensional, laminated, extremely complex structure on which the body musculature, sensory organs and much of the rest of the brain are mapped in intricate ways by the afferent and efferent projections. These connections start to become established while neurons destined to contribute to the cortex are still being generated and subsequently there is extensive cell migration and cell death. The orchestration of the events is fascinating - everything is in the right place at the right time.

To understand the mechanisms of each step, though itself a considerable challenge, is far simpler than to explain why Nature uses each particular solution, each particular logic, at each particular stage, in each particular species. Comparative developmental studies can bring us closer to understanding the various strategies discovered by different species to solve the same problems. Each solution to a new problem and each new solution to an existing problem are likely to have exploited and built on already existing mechanisms. Obviously, different processes could have evolved to solve the same kind of problem in different species, and some solutions may even employ redundant parallel mechanisms to give the system more stability and resistance to disturbance. This makes it difficult to envisage being able to compose universal

hypotheses of cortical development. We have to be careful even with minor generalizations between different systems and between different species. The only generalization that is almost certain to be valid is that the ontogenesis and phylogenesis of the neocortex use very diverse mechanisms.

Even the examination of the development of one particular structure in one chosen species can create difficult problems. Often, different temporally coincident but possibly independent mechanisms are responsible for the establishment of a certain structure. Then is it reasonable to ask which is the most important? Even if we could perturb the system in a clear-cut way (and often this is not the case) it might not be easy to choose from the many interpretations of the results. This makes studying the development of the nervous system an exciting but frustrating challenge.

I am interested in the development of cortical connectivity. The cerebral cortex might not be the simplest or most easily accessible model system in which to ask developmental questions, but it is certainly a very important one. The differentiation of the cerebral cortex presumably underlies the highest human functions and therefore the solution to the puzzle of how each region of the cortex becomes specialized may eventually even provide insight into the mechanisms of perception, thought and language and the way in which they have evolved. Specification of the structure of a functional brain, able to interact meaningfully with the outside world, involves a great deal of information. Millions of neurons have to interconnect in highly specific patterns. The genome surely cannot hold all the information required as a blueprint for the precise wiring of every single cell in the brain. Rather than determining every connection and every cellular commitment, broad principles seem to characterize the development of the nervous system. In the brain, perhaps especially the cerebral cortex,

remarkable amplification and supplementation of the information in the genome are brought about by self-organizational phenomena. They result mainly from the regulation, in space and time, of the expression of genes, influenced by other events and signals unfolding in the immediate environment of the structure in question. To reduce the genetic load, Nature does not construct invariant patterns of organization; to some extent it creates redundancy and later eliminates the unwanted. The precise nature and timing of developmental events are not always rigidly genetically determined, they often depend on an interaction of genetic predispositions and epigenetic influences, such as external chemical signals and mechanical forces (many of those dependent on other events in and influences on the growing embryo). Only a part of the sequence of these signals is specifically coded genetically, giving a certain stability and flexibility to the system at the same time. To get a better understanding of the enduring question of "Nature or Nurture" - to understand the contribution of precise genetic commands and those that are modulated by environmental effects (in the broadest sense) - we have to challenge the system.

My goal is to understand the mechanisms that underlie cell-cell interactions during the development of neural connections, with special attention to the steps that might be influenced by the extracellular environment of those cells, including diffusible substances, neighbouring surface properties and including electrical and/or chemical signals from neighbouring neurons, which in some cases are related to or initiated by the outside world. This thesis considers the mechanisms involved in early cortical development, focusing on the significance of thalamocortical connectivity in the development and differentiation of the cortex.

1.1 Specification of cortical areas

The adult mammalian neocortex consists of numerous areas distinguished from one another largely on the basis of differences in cytoarchitecture, connections and neurophysiological properties. The different areas are specialized to perform different functions, and damage to a particular area usually results in a specific functional loss. The most important problem in the field is to understand the mechanisms by which different cortical areas achieve their identities. Other related questions follow. Are those identities predetermined and immutable? Do the different regions of the neuroepithelium give rise to regionally specified, area-unique lineages of neurons or do they generate a uniform, uncommitted cortex?

The importance of thalamo-cortical connections in cortical development

The question of how different areas of the cerebral cortex develop their unique characteristics has led to the emergence of two opposing theories - basically genetic and epigenetic in emphasis, respectively.

According to Reznikov et al.(1984) and Rakic (1988) the proliferative cells within the ventricular zone constitute a mosaic or fate-map, which generates a **proto-map** of prospective cytoarchitectonic areas (in radial units): the different areas would be predetermined from very early stages, according to the fate of the daughter neurons.

Others (e.g. Driesch 1914, O'Leary & Stanfield 1989, O'Leary 1989) suggest that the cortical plate is initially a homogeneous '**protocortex**', a more or less equipotential structure, committed only to a basic pattern of laminar organization but without any inherent regional differentiation. Area specificity would depend on some **extrinsic signal**, which would trigger regional differentiation and determine the characteristic local discrimination and target-specific outgrowth from the cortex.

The second hypothesis seems more consistent with recent progress in the field. Although the cortical lamina for which each immature neuron is destined is apparently determined very early, during mitosis (McConnell 1988, 1989), no molecular or genetic manifestation of regional parcellation has been described prior to the arrival of the first afferent input that reaches the cortex during and after neural migration. By marking clonally related neocortical cells with retrovirus-mediated transfer of a histochemical marker gene, Walsh and Cepko (1992) demonstrated widespread dispersion of neuronal clones across different functional regions of the cerebral cortex. This finding, together with the more recent developmental studies on the dispersion of proliferative layers of the cerebral cortex (Fishell et al., 1993; Walsh and Cepko, 1993; also argue against the 'proto-map hypothesis' of cortical generation and against the idea that the cortex is generated as strict radial units. Very recently Tan and Breen (1993) investigated cortical cell dispersion using transgenic mice in which half of the brain cells contain the transgene after random inactivation of the X chromosome. The inactivation is completed by day 12.5 of gestation (E12.5). The initial strict radial cell dispersion (where all cells in a radial column were still either labelled or unlabelled) was later degraded by an even distribution of tangentially spreading cells, but the radial bands were still apparent. This experiment suggests that both radial mosaicism and tangential cell migration are involved in cerebral cortical development.

The importance of the complement of afferents in generating certain cortical properties is increasingly apparent, at least for the sensory areas of the posterior half of the cerebral hemisphere. I shall mention here just a few examples to demonstrate the importance of the input in promoting cortical differentiation.

In rodents the whiskers provide a very important sensory input. Each whisker

has its own well defined representation (a neural "barrel") in the somatosensory cortex and these barrels do not develop fully until several days after birth (after the arrival of thalamic afferents) (Erzurumlu and Jhaveri, 1990; Jhaveri et al., 1991). Changes in the sensory input during the first postnatal week (produced by cauterizing some of the whisker follicles or blocking the inflow of sensory afferentation by cutting the trigeminal nerve of the mouse) prevents formation of those barrels corresponding to the missing sensory input (Van der Loos and Woolsey, 1973; Welker and Van der Loos, 1986). Schlaggar and O'Leary (1991) recently transplanted late embryonic visual cortex into the position of somatosensory cortex and demonstrated barrel formation within the transplanted cortex.

Rakic (1988) produced another striking example of the organizing influence of the sensory input. Removal of the eyes very early in embryonic life in primates results in the degeneration of a large proportion of the cells of the lateral geniculate nucleus (LGN). The shrunken LGN then innervates the occipital cortex, but the size of the primary visual cortex (where LGN fibres terminate), recognized by its distinctive cytoarchitecture, is substantially reduced, and the entire sulcal pattern of the occipital lobe is changed. We should note that in all these experiments the effect of interference with afferent input (through whisker removal or enucleation) has a dramatic effect on the developing brain only during a certain period of vulnerability.

The patterns of output characteristic of different cortical regions in adults is also not an inherent property of the cortical tissue. In the rat, layer 5 cells from all cortical regions initially project to the spinal cord, but with subsequent development, the occipital cortex loses this projection through axon withdrawal (O'Leary and Stanfield, 1986), and only the collateral innervation of the pontine nuclei and the superior

colliculus remains. But when a piece of cortex from the occipital region of a late embryonic rat is transplanted to another region of the hemisphere of a neonatal host, it adopts the output characteristics of the host site, retaining its projection to the spinal cord (O'Leary and Stanfield, 1989).

These results emphasize the influence of the afferentation on the formation of local cytoarchitecture and the determination of output projections. The afferent input to each cortical area from the thalamus might provide the extrinsic signal that determines regional differentiation of sensory areas, though it must be emphasized that these experiments do not allow us to say whether it is merely the **presence** of afferent axons in the cortex that triggers local differentiation, or whether the pattern of **activity** in those axons plays a crucial role.

The thalamus is a major relay structure in the diencephalon. It transmits nearly all sensory input destined for the cortex. Each sense organ (except the olfactory) and major subcortical motor centres provide input to one or more **specific** nuclei of the thalamus, and these nuclei have well defined reciprocal interconnections with particular cortical areas. There is a strict relationship between the various specific thalamic nuclei and the cortical fields with which they are interconnected. The connections in both directions between the major thalamic nuclei and the cerebral cortex are specific not only for the area of cortex that receives or gives them, but also for the laminae in which they terminate or originate. Layer specificity is remarkably similar for all cortical territories and is conserved between species. In all areas in which the early stages of thalamic innervation have been studied, the specific thalamo-cortical input terminates mainly in layer 4 (with some termination in layers 1 and 6). A high proportion of neurons of layer 6 of each area send corticofugal projections back to the corresponding

thalamic nucleus.

The most obvious way in which events in the outside world could influence the development of the cortex is through the pattern of neural activity arriving in each cortical region through the thalamic afferents. Over the past 20 years or so, there has been great interest in the extent to which such influences actually occur and the ways in which they might be mediated (e.g. Blakemore 1978; Movshon and van Sluyters, 1981; Rakic and Singer, 1988; Rauschecker and Maler, 1987; Singer, 1987; Merzenich et al., 1990; Shatz, 1990). There is now a consensus that activity-dependent plasticity is a fundamental property of sensory cortical areas, that it takes place during quite well defined 'sensitive periods' and some plastic phenomena are due to modulation of the strength of individual synapses through forms of long-term potentiation and/or depression (see Toyama et al., 1991). However, before such activity-dependent plasticity can occur, thalamic axons must obviously be in place and have formed synaptic connections in an appropriately organized area of cortex.

There is also the possibility that thalamic afferents exert influences on cortical regional differentiation even at very early stages. Recent work (including that reported in Chapter 3) has established that thalamic axons are already distributed in remarkable topographic order, directly below the cortical plate, from shortly after the arrival of the first cells that contribute to the cortex proper. Moreover, thalamic axons invade the putative sensory regions of cortex before the regional differentiation of cytoarchitectonic and output characteristics. In the broad sense, the thalamic input is part of the environment of the developing cortex and indeed the arrival of thalamic axons is the first such environmental event that is regionally specific. Thus, the thalamic afferentation could constitute the extrinsic signal required, in the uncommitted

'protocortex' hypothesis, to trigger many of the early morphogenetic processes in the cortex. This gives special importance to the study of the early development of the thalamocortical system and especially the mechanisms by which these axons are guided to the appropriate cortical area. The entire process of regional differentiation of the cortex could depend on the way in which thalamic axons become distributed beneath the developing cortical plate and the way in which they invade it and terminate in it.

1.2 Neurogenesis and the formation of the cortical plate

The basic principles of the cortical neurogenesis are very similar in all mammals. The cells are born in the ventricular and subventricular zone. From this 'mitotic factory' the first-generated postmitotic neurons migrate to the cortical surface and form the primordial plexiform zone (the preplate). The subsequently formed neurons migrate and take up their position in the middle of the preplate, splitting it into the marginal zone at the top and the subplate at the bottom (Nomenclature: Boulder Committee 1970; Marin-Padilla, 1971; Luskin and Shatz, 1985a,b) (Fig.1.1.B). The cortical plate proper, sandwiched between the two components of the preplate (Luskin and Shatz, 1985a,b), steadily thickens as subsequent waves of arriving neurons migrate through the existing cells of the plate and take up their position under the marginal zone in an inside-first outside-last fashion (Berry and Eayrs, 1963; Berry and Rogers, 1965; Rakic, 1974; Lund and Mustari, 1977)(Fig.1.1.A.).

In the embryonic cortex of mammals, the subplate forms a substantial layer. In the adult, however, layer 1 and the white matter under the cortex contain few neurons (Cajal, 1909). The relative paucity of neurons in the adult in regions corresponding to the original preplate is surely one of the reasons why the subplate, and subplate cells, were largely ignored until relatively recently (see chapter 7). Luskin and Shatz

Figure 1.1 The pattern and timing of cortical neurogenesis

A.: Early thymidine autoradiographic birthdating studies revealed the inside-out pattern of cortical assembly (Sidman et al., 1958; Uzman, 1960; Angevine and Sidman, 1961). The earlier generated neurons migrate to the most superficial position, but they are subsequently displaced to deeper levels by those that arrive later. Berry and Rogers (1965) summarized the inside-out sequence of the development of cortical layers in this diagram but, at that time the existence and special nature of the preplate had not been recognized. Each column represent a narrow sector of the rat cortex of a given age (the ages are marked under the bottom of each column). Cell proliferation, which extends from E12 to E21 in the rat, (Berry and Rogers, 1965; Berry et al, 1964, Hicks and D'Amato, 1968), is over at birth, but cell migration continues until the end of the first postnatal week. (The gestation period is 21/22 days in the rat.)

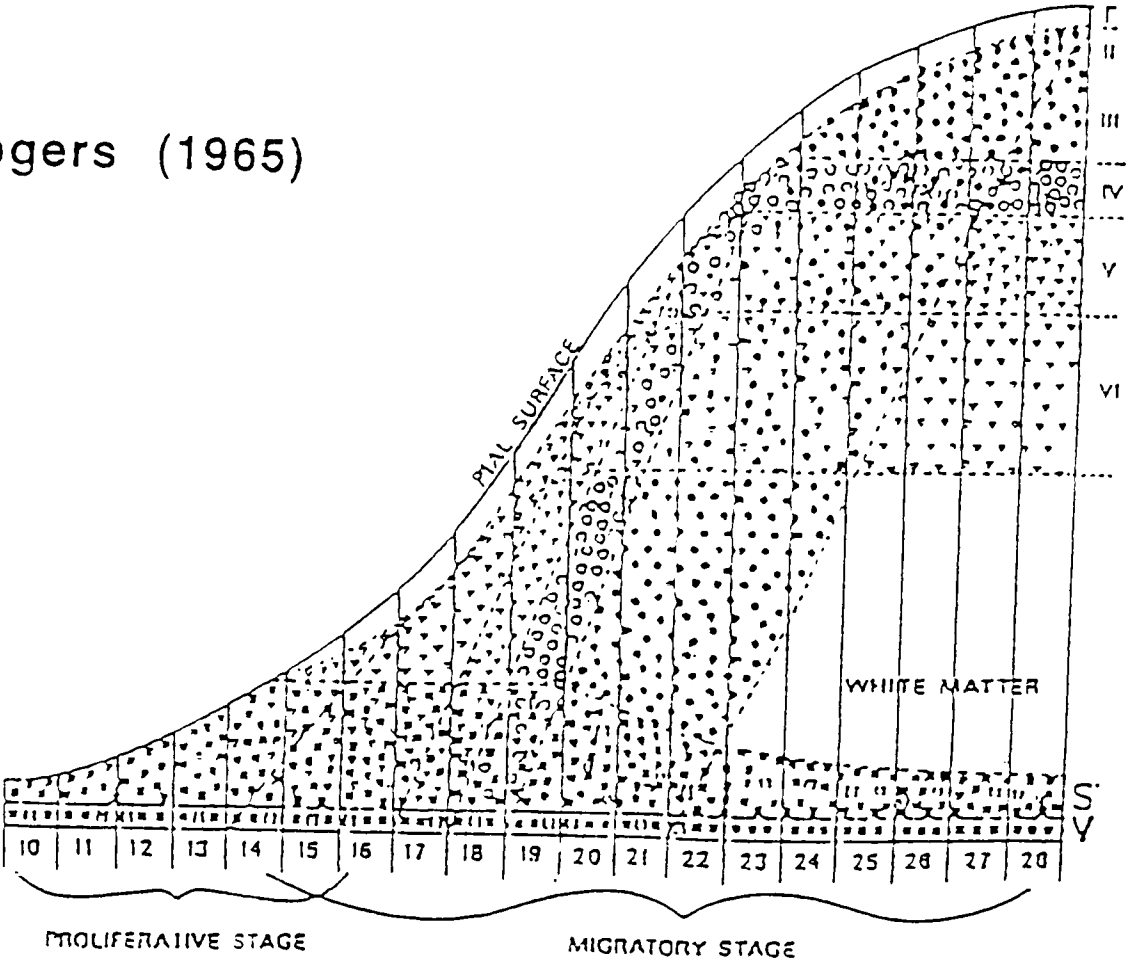
B.: Later the simple inside-out concept of cortical development had to be modified. Marin-Padilla, based on his Golgi studies (Marin-Padilla, 1971, 1972, 1988), first suggested that the cortical plate develops within the preplate. Luskin and Shatz's (1985) autoradiographic studies confirmed that the cortical plate does indeed split the first-generated cell layer of the primordial plexiform zone (or preplate) into two, as Marin-Padilla originally suggested. This camera lucida drawing of a Golgi preparation (25-day-old cat embryo cortex, taken from Marin-Padilla, 1988) demonstrates the splitting of the preplate into marginal zone (I) and subplate (VII) by the developing cortical plate (CP).

C.: The early autoradiographic studies on cortical cell generation revealed the relative delay in development of the medial-occipital cortex compared with the lateral-frontal (Berry and Rogers, 1965; Lund and Mustari, 1977). In the rat there is a maturational difference of 1.5-2 days, but in at least some marsupials it is more than 30 days. This relatively slow development of the marsupial cortex was exploited by Sandersen and Weller (1990) when they described the areal pattern of the histogenesis of the marsupial brain. The diagram (taken from Sanderson and Weller, 1990) demonstrates the regional pattern of neurogenesis in the opossum cortex (which is probably more or less similar in sequence to that of the rodent cortex). The strips represent the ages (numbers in postnatal days) at which neurogenesis was complete for all neurons in the given segment (called isogenetic stripes). The strips of synchronously generated cells run roughly parallel to the rhinal fissure.

Fig. 1.1

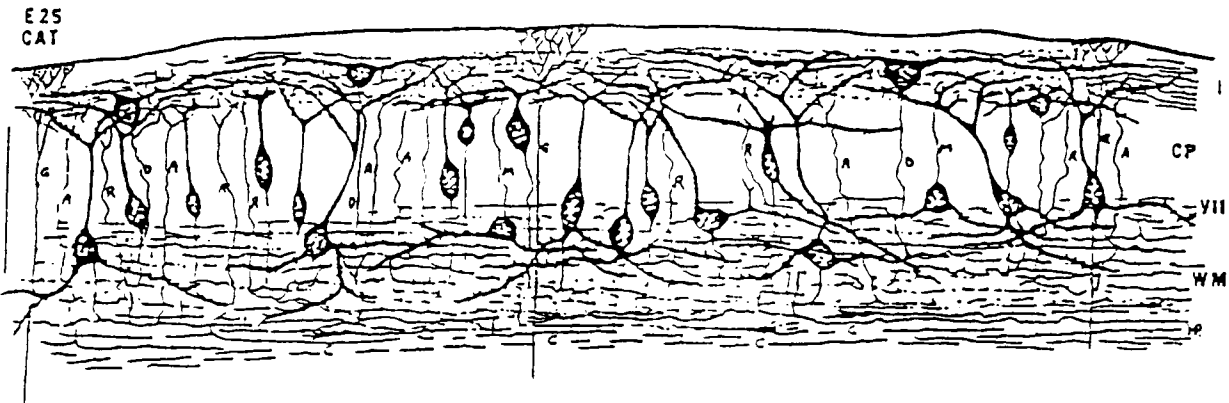
A

Berry and Rogers (1965)



B

Marin-Padilla (1971)



C

Sanderson and Weller (1990)

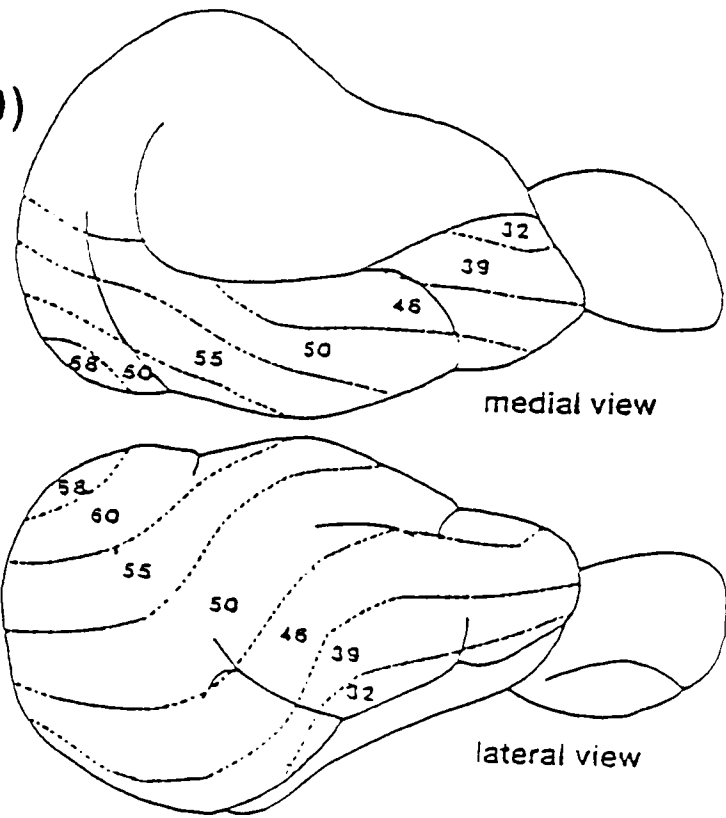


Fig. 1.2

Figures adapted from **Luskin and Shatz (1985b)**

A and B:

Camera lucida drawings of representative autoradiographs to show the distribution of heavily labelled cells (black dots) in the visual cortex of the cat at birth resulting from [³H]thymidine injection on E27 (A) or E33 (B), as seen in coronal sections coinciding with the midpoint of the lateral geniculate nucleus. Roughly comparable numbers of labelled cells are found after an injection on E27 (332) and E33 (335), although they are situated within the cortical plate (CP)/(layer 6) after the E33 injection (B), and predominantly below it (subplate), with a few in the marginal zone, after the E27 injection. Labeled cells are also present in layer 1 after the E27 injection, but not after the injection on E33. Dorsal (D) is up and medial (M) is to the left. WM, white matter. Calibration bar at A refers to both drawings.

C and D:

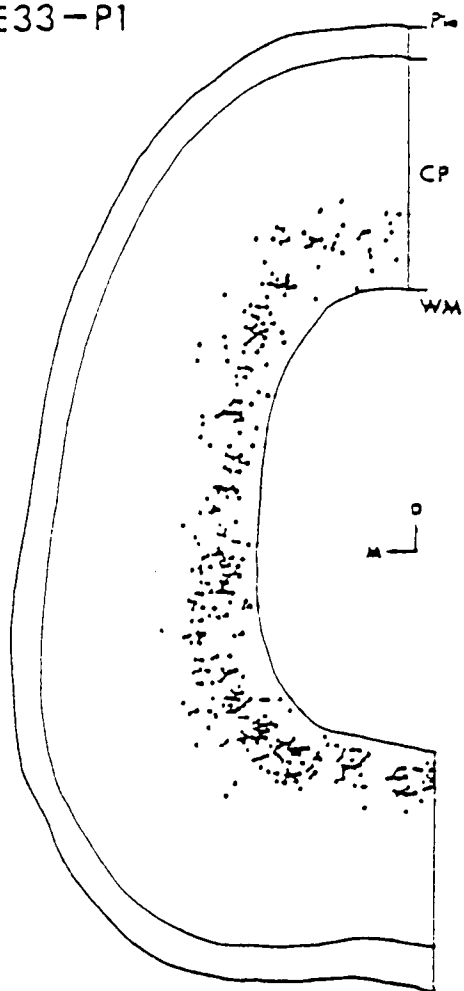
Camera lucida drawings of comparable representative autoradiographs of adult cat primary visual cortex (delimited by radial double lines) to compare the distribution and density of heavily labelled cells (black dots) resulting from [³H]thymidine injection on E28 (C) or E33 (right). Note the much greater density of heavily labelled cells remaining in layer 6 after the E28 injection that remain below the cortex after the E28 injections, despite the fact that E28 section was purposely chosen because an unusually large number of labelled cells compared with other sections. Both sections were selected from around the coronal plane passing through the midportion of the lateral geniculate nucleus. Numbers indicate layers 1 to 6. (Boxes indicate the locations of photomicrographs in Figure 2 of the Luskin and Shatz (1985b) paper.)

Fig. 1.2

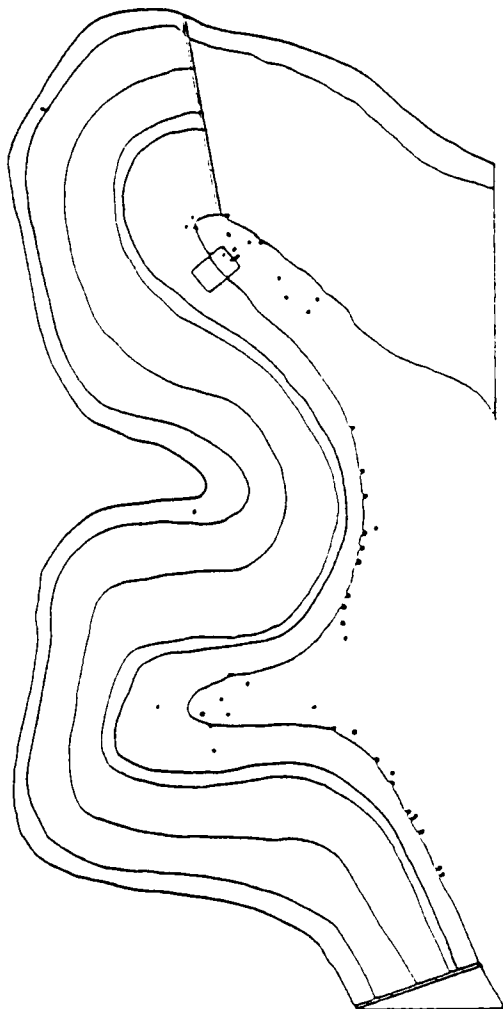
A E27-P0



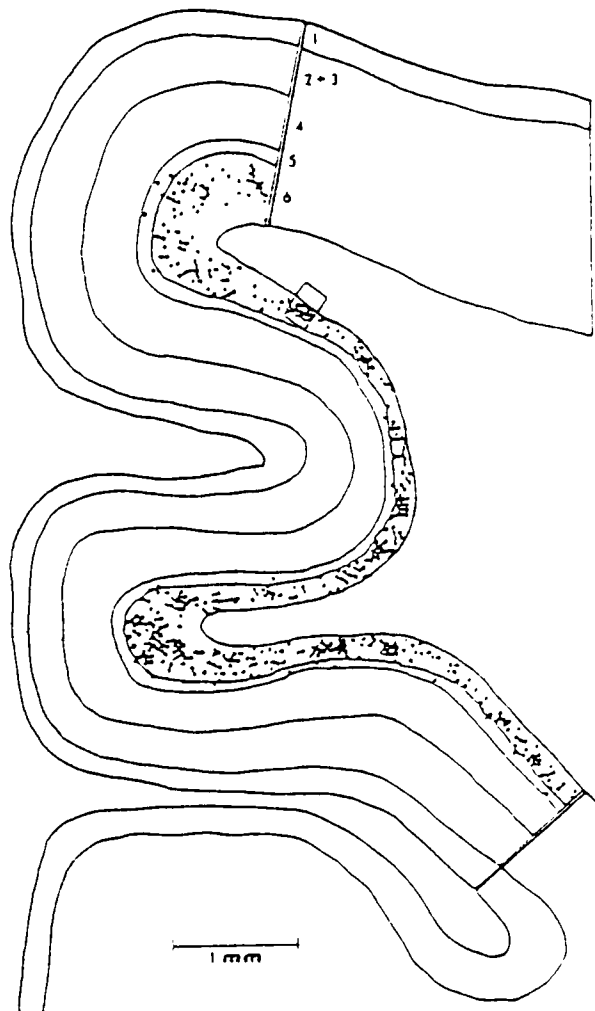
B E33-P1



C E28-P78



D E33-P62



(1985a,b) in the cat confirmed Marin-Padilla's original suggestion (Marin-Padilla, 1971) that cells of the subplate and the marginal zone are co-generated and form the primordial plexiform zone. This was the first study to demonstrate that the subplate and marginal zone cells have a common origin and that they both decline in number after birth compared with cortical plate cells (Fig. 1.2).

In the rat, thymidine birthdating at E12 labels only cells of the subplate and layer 1 (Lund and Mustari, 1977; Valverde et al., 1989; Bayer and Altman, 1990). Recent work shows that these earliest born cells in rodents (originally residing in the premordial plexiform zone) also die to a greater extent than cells of the cortical plate (Wood et al., 1992; Derer and Derer, 1992), although relatively more of them seem to survive than in cats and primates (Kostovic and Rakic, 1990). Unfortunately, for primates and rodents there are no comprehensive birthdating studies similar to that performed by Luskin and Shatz, 1985 (a,b) for the cat (Fig. 1.2), and this makes it impossible to compare quantitatively the amount of cell death in different species.

In the rat, the first cells of the cortical plate itself are born on fetal day 14, the last at E21. Thus cortical cell generation finishes before birth and the subventricular zone disappears by postnatal day 3 (P3), but cell migration into the upper layers continues until the end of the first postnatal week (Berry and Rogers, 1965; Lund and Mustari, 1977; Miller, 1988).

In the tangential direction across the developing cerebral cortex, there is another distinct neurogenetic gradient. The anteroventral areas antecede the caudodorsal in maturity (Berry and Rogers, 1965; Lund and Mustari, 1977). The most detailed studies of these gradients were performed in marsupials because of the prolonged neurogenesis of the cortex (Sanderson and Weller, 1990; Mark and Marotte, 1992; Fig.1.1.C.).

Autoradiographic birthdating studies in these species with protracted cortical neurogenesis suggested that the cortex can be divided, according to the time of its generation, into strip-shaped loci that develop synchronously (Sanderson and Weller, 1990). Corticogenesis begins first in the ventromedial region adjacent to the paleocortex and archicortex, and there is a simple orderly wave of maturation, from rostral and ventral to caudal and medial. Lines defining the locus of points at an identical stage of development lie roughly parallel to the rhinal fissure and are termed isogenetic strips (Sanderson and Weller, 1990)(Fig.1.1.C.).

1.3 Introduction to the development of thalamic nuclei

It was Edinger (1885) and His (1893, 1904) who established the first terminology for the gross differentiation of the diencephalon, based on their work on human embryos (Jones, 1985). They described the way in which the hypothalamic sulcus (an early sulcus on the medial wall of the diencephalon) divides the diencephalon into dorsal and ventral parts. The ventral part later develops into hypothalamus, the dorsal forms the epithalamus, thalamus and metathalamus (metathalamus = geniculate bodies; Edinger, 1885).

Based on comparative studies in amphibian embryos, Herrick (1918) made similar, but slightly different descriptions. To emphasize the different developmental history of the thalamus and metathalamus from the epithalamus and hypothalamus he divided the thalamus into dorsal and ventral thalamus. Herrick's (1918) fundamental subdivision of the developing diencephalon was confirmed for mammals (in the rabbit) by Droogleever-Fortuyn (1912) and it eventually became widely accepted and is still used (Jones, 1985).

Even before the application of autoradiographic birthdating techniques two

phases of thalamic growth were distinguished: (i) an early phase of cellular differentiation during which the epithalamus, dorsal thalamus and ventral thalamus differentiate from one another, followed by (ii) a later phase during which the individual nuclei differentiate within the three larger subdivisions.

(i) In the rat, the thalamus and the hypothalamus can be distinguished after the appearance of a ventricular groove (anterior diencephalic wall) at the 12th embryonic day of gestation (Cogeshall, 1964). On E13, a wedge-shaped enlargement appears in the diencephalic wall with further furrows defining the developing dorsal and ventral thalamus. These furrows become less apparent as the thalamus further increases in size.

(ii) At embryonic day 16/17 nuclear differentiation is commencing in the epithalamus and ventral thalamus but not in the dorsal thalamus (Cogeshall, 1964; Jones, 1985). Within the thalamus nuclear differentiation starts posteriorly and laterally and proceeds anteriorly and medially (Rose, 1942, Cogeshall, 1964; Jones, 1985). In view the growing evidence that there are transient cell populations in and around the developing thalamus itself (Mitrofanis and Guillery, 1991; Mitrofanis, 1992) it is important to re-examine the development of the thalamus in relation to the birthdates of its cells in embryonic and early postnatal material rather than drawing conclusions in the mature brain. These studies should be re-addressed in detailed autoradiographic developmental series to define the nature, position and possible function of transient cells in and near the developing thalamus.

Autoradiographic studies show that cellular proliferation precedes nuclear differentiation by several days. Virtually all thalamic neurons are born in the rat between E13 and E19 (McAllister and Das, 1977; Altman and Bayer, 1979) coinciding with the period of generation of cells of the preplate of the occipital cortex. The

postmitotic neurons migrating outwards from the ventricular and subventricular zones come to rest in regions that are determined by their time of birth, and not by nuclear boundaries. The lateral geniculate nucleus (LGN) is generated over 2 days, between E12 and E14, coinciding with the period of generation of cells that form the preplate of the occipital cortex (Bruckner et al., 1976; Lund and Mustari, 1977). Jones (1985) describes three **neurogenetic gradients** in the developing thalamus: (1) posterior to anterior in that the cells of the posterior nuclei are born before those of anterior nuclei (referring to the relative position of nuclei in the adult thalamus). (2) A second operates from lateral to medial so that lateral nuclei are born before the medial ones. (e.g. neurons in the reticular nucleus are born before those of the mediodorsal nucleus). (3) The third thalamic neurogenetic gradient operates from ventral to dorsal (Jones, 1985). It is not yet established whether these gradients exist within individual nuclei as well as across the thalamus as a whole (Hickey and Cox, 1979; Hickey, 1980; Shatz, 1981, 1983; Hickey and Hitchcock, 1984; Altman and Bayer, 1979). Also it is important to know how these gradients are distributed across the embryonic thalamus, since there are considerable changes in the orientation of the thalamus as a whole during development in some species (Rakic, 1977; Jones, 1985).

1.4 Axonal pathfinding and cell-cell recognition during development

The ontogenesis of the nervous system relies on numerous cell-cell interactions. The appropriate cells have to recognize each other during their development. The morphogenetic role of the preference shown by any type of cell for associating with other cell types is an old concept (Wilson 1907, Humphreys 1967, Burger 1974).

There have been many direct experimental approaches, in vivo and in vitro, to the study of axonal path finding, target recognition and synapse formation in the

nervous system (see Jacobson, 1991; Lumsden and Cohen, 1991). As early as the forties, Weiss (1941, 1947) proposed that the intercellular recognition of nerve cells during the assembly of nerve circuits is the consequence of their cytochemical properties. Steinberg (1963) observed that chicken embryonic cells from various tissues, reaggregating in vitro, tend to regain the intercellular relations typical of the original tissue, through a hierarchical order of affinity. This concept formed the basis for Sperry's (1950, 1963) influential chemoaffinity theory of neuronal specificity, which suggested that selective innervation in the developing nervous system is due to selective chemical signals, attracting growing axons towards their appropriate targets. Because of the improbability of every target cell carrying its own unique chemical tag, it was proposed that **gradients** of such **affinity substances** across target tissues interact with whole populations of incoming fibres, imposing topographic order on them, rather than defining precisely where each should terminate. These questions have been most intensively studied in the retinotectal system. The dominant general view is that growing retinal axons are indeed sensitive to a gradient distribution of guidance cues across the tectum. No candidate molecule has been identified yet (for a critical review see Sterling, 1991).

Distant cells can influence each other's growth by releasing diffusible factors. The idea of **chemotaxis** was originally proposed by Cajal (1909). Target cells are thought to produce some chemical substance that diffuses to the growing cells and stimulates their outgrowth. Trophic and tropic effects are distinguished. **Trophic** factors enhance the growth and the survival of cells and axons (NGF, BDNF, CNTF, NT3 and FGF are trophic factors). Unfortunately the nomenclature does not include separate expressions for survival-promoting and growth-promoting effects and the usage of the

Fig.1.3

According to the pattern of the fibre outgrowth co-cultures in 3-dimensional collagen gels, Lumsden (1992) proposed that trophic, tropic and combined (trophic and tropic) effects can be distinguished.

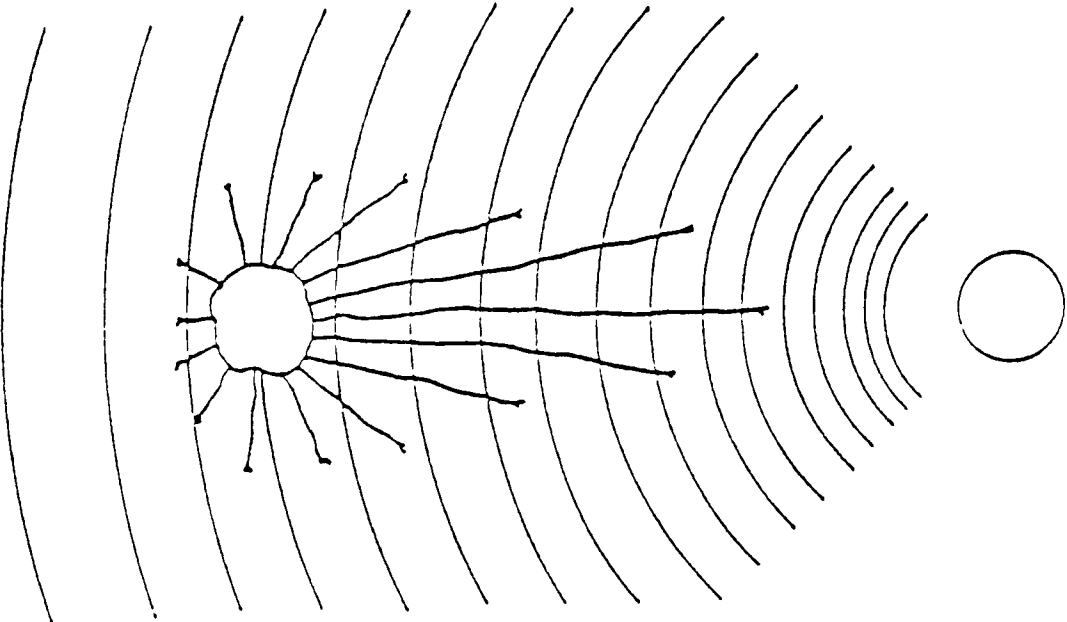
A.: A **trophic** agent emanates from one of the co-cultured explants, diffuses within the collagen matrix and establishes a concentration gradient (represented by the concentric lines). Because of the concentration drop across the tested explant, the axons of cells closer to the target explant (receiving more concentrated trophic influence) extend longer fibres than those on the opposite side. The fibre outgrowth is simply radially directed.

B.: In the presence of a hypothetical purely **tropic** agent, there is no distinction in the length and intensity of fibre outgrowth throughout the explant and the tactic factor has no effect on cell survival. Only the direction of fibre outgrowth is affected.

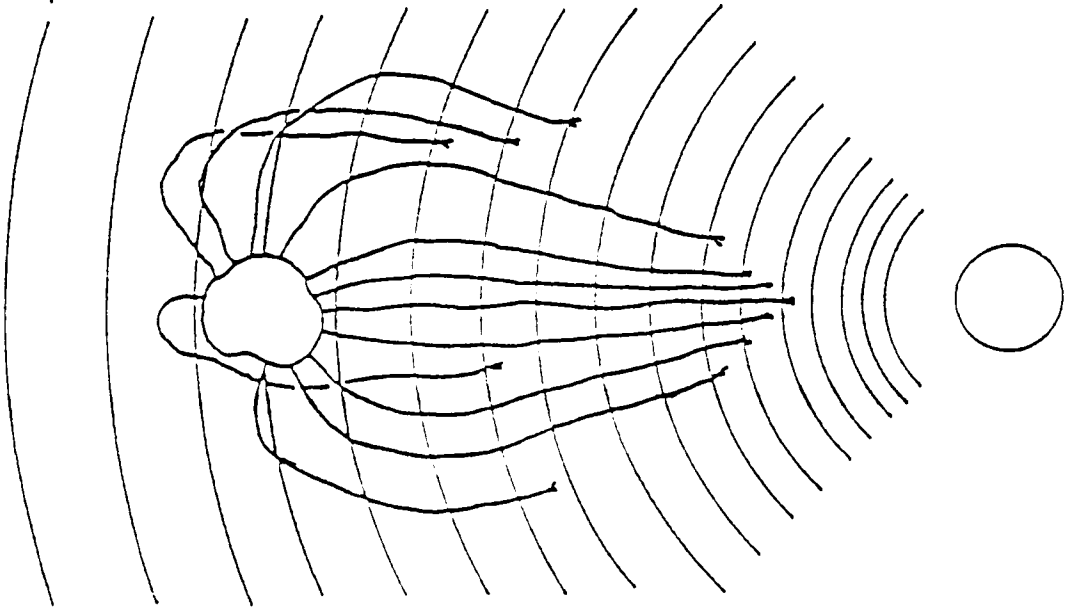
C.: Lumsden (1991) distinguishes a third **combined** (trophic and tropic) effect, but in my opinion a trophic (growth-promoting) factor alone (depending on the distance, steepness of the concentration gradient, etc) could produce such an outgrowth pattern, without a specific tropic (purely directional) influence.

Fig. 1.3

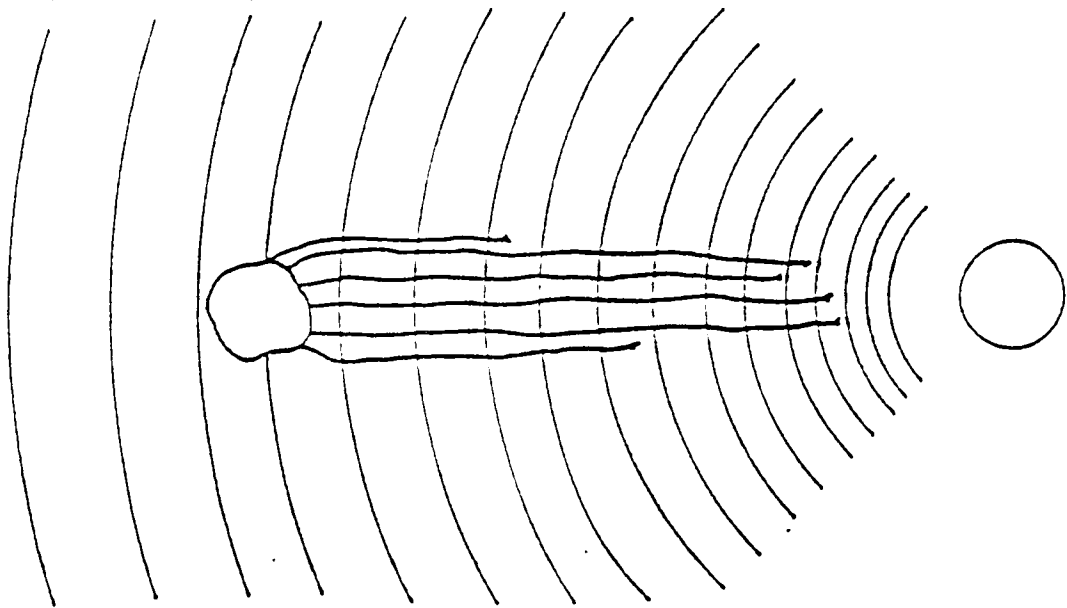
A: TROPHIC



B: TROPIC



C: COMBINED



term trophic (literally = ‘nourishing’) is therefore ambiguous and can be misleading. In order to distinguish the different meanings of the term ‘trophic’, two separate expressions might be used: ‘survival-promoting’ or ‘**soteric**’ and ‘growth-promoting’ or ‘**auxetic**’. Both short forms have Greek origin¹: soteric derives from σωζω ‘to be saved or survive’ and auxetic comes from αυξω ‘to cause, to increase or grow’. Prefixes (e.g. somo-, dendro-, or axo-) could even be employed to describe the specific sites of the soteric or auxetic effects, depending whether they are exerted on the cell body or different cell processes.

According to the classical view, **tropic** factors cause directed axonal outgrowth specifically towards the source by producing a directional response of growth cones towards the vector of the concentration gradient. No specific tropic factor has yet been identified.

In the literature the difference between these two classical concepts (trophism and tropism) is usually sharp, and it is widely accepted that the two can be distinguished experimentally by the observation of neurite growth unconstrained by surface properties in three-dimensional collagen gels, which are believed to capture diffusible factor gradients (Lumsden, 1992). In such cultures, tropic and trophic interactions are assumed to produce different outgrowth patterns (Fig.1.3). However a very steep concentration gradient of a purely trophic factor might produce asymmetric, directed growth. In the three dimensional collagen matrix, A. Pini (personal communication) recently found that effects on outgrowth can appear either tropic or trophic depending on the size of the explant, outgrowth from large explants appearing

¹ I am very grateful to Eleanor Dickey and Thomas Braun for their suggestions for these terms.

very asymmetric presumably because of the difference in concentration of the growth-promoting substance on the two sides of the explant. By culturing dorsal root ganglion with a gel containing different concentrations of conditioned media or NGF, Pini demonstrated that the phenomenon is indeed concentration-dependent. NGF is probably the best studied growth factor (Levi-Montalcini, 1952; 1982).

One aspect of NGF behaviour that is still difficult to clarify, however, is the mode by which NGF directs axonal outgrowth. Various studies demonstrated that NGF acts as both a tropic and trophic factor (discussed in Hankin and Silver, 1986; Hankin and Lund, 1991). The sharp distinction between tropic and trophic factors has to be reconsidered, at least temporarily, until a genuine tropic factor (different from any of the known trophic molecules) is definitively identified.

Even the loudest advocates of chemotropism recognize the influence of local cues. "The distant chemotaxic attractions act in the mechanical guides and constraints imposed by tissue spaces and planes" (Lumsden, 1992). The earliest axons in a pathway may be directed towards their target by chemoattractants, but the later-growing projections could then simply follow the surfaces of pre-existing ones, using the guidance of the immediate neighbourhood. This form of guidance has been termed **contact guidance**. Cajal himself was very much aware of the potential guiding role of the physical environment for growing axons. He made fundamental observations about the surrounding milieu in regenerating axonal pathways (Cajal, 1928). Indeed some of the pre-existing fibres can be essential for the further development. If (i) a fibre tract extends the first axons along a particular trajectory through the extracellular space; (ii) later-growing axons follow the same pathway, and (iii) the pre-existing pathway is essential for the later-growing axons to find their targets, then the first axons are called

pioneers (for more details see Chapter 3). Strictly, then, the term pioneer should be used only for initial fibres that have a subsequent **guiding** role. Although the term pioneer is quite widely employed, the guidance function of pre-existing fibres has only been sufficiently demonstrated in insects where the study of mutants is allowing the underlying genetic mechanisms to be worked out (Bate, 1976; Bentley and Keshishian, 1982; Goodman et al., 1984).

Occasionally fibres make characteristic sharp changes of directions as they reach specific groups of target cells or 'stepping stones' (Bate, 1976). The literature uses the expression **guide post cells** for particular cell-types exerting a guiding role at a particular circumscribed point, during the course of the establishment of a pathway. These 'stepping stones' or 'guidepost' neurons (Bentley and Keshishian, 1982) are in strategic position to orchestrate the pathfinding of developing projections through sequential target recognition.

The commonly used term '**scaffold**' has a less well defined meaning (see McConnell et al., 1989). The term implies a three-dimensional structure of axons that could mediate a variety of different types of interaction with later axon projections (e.g. growing over the scaffold in the same direction as the pioneer fibres or in the opposite direction). The term scaffold is used in various way by different authors.

Morphogenetic gradients

The generation of cells in a given structure is usually orderly and follows a certain spatio-temporal gradient (Bayer and Altman, 1991). Fibre outgrowth in many neuronal structures follows the neurogenetic and morphogenetic gradient (e.g. spinal cord and retina) and fibres can occupy positions in a projecting bundle according to their time of generation within the whole population (His, 1904; Herrick, 1941, 1942;

Gaze and Grant, 1978; Easter et al., 1981; Reh et al., 1983; Walsh and Guillery, 1985). This type of fibre ordering pattern in which position is a reflection of time of birth has been termed chronotopic. Bayer (1980; 1985; 1986) and Bayer and Altman (1987; 1991) pointed out that many of the major anatomical interconnections of the telencephalon can be related to the order of neurogenesis and to neurogenetic gradients of either the neurons of origin (entorhinal cortex, olfactory bulb mitral cells), the receiving neurons (lateral septal nucleus, cortical nuclei of amygdala, anterior olfactory nucleus, primary olfactory cortex) or both (magnocellular basal nuclei, striatum). The extent to which chronotopic fibre ordering plays a part in the initial sorting of the connectivity and in establishing topographic order in different target structures is still a controversial issue, but it probably contributes, to various extents in different systems, to the multiple mechanisms by which the final mapping patterns are established (Horder and Martin, 1987; Hankin and Silver, 1986; Blakemore and Molnár, 1990; Shatz, 1992a; Chapter 3).

The obvious conclusion we can draw by examining the development of various systems is that **none** of the above mentioned mechanisms (affinity gradients, chemotaxis, contact guidance, spatio-temporal waves in neurogenesis or ordered fibre outgrowth) can alone adequately explain **all** aspects of the formation of orderly interconnections and map formation. This is not to say that one of the distinct factors can not be dominant at a particular stage (the initial establishment of a fibre path may be governed by different mechanisms from those determining the final synaptic targeting, and different again from mechanisms controlling the **regeneration** of a pathway). But in general it is likely that **multiple mechanisms** act simultaneously or in an overlapping cascade of mechanisms. To understand the final result we have to

examine the development of a pathway step by step.

1.5 Experimental strategy: the aim of the present studies

In my thesis I shall describe and discuss several factors involved in the establishment of specific interconnections between the thalamus and cerebral cortex. In chapters two to four of this thesis I shall attempt to formulate soluble questions about a particular aspect of thalamocortical development.

In Chapter 2 I shall examine the possibility that, during development, different cortical areas exert a specific attraction on thalamic axons from the distinct thalamic nuclei with which they will establish connections. I shall also consider the mechanisms that might be responsible for the development of layer-specific innervation patterns. I address these questions using an in vitro organotypic culture system, culturing together different parts of the thalamus with various cortical areas. This technique allows one to create many paradigms under controlled conditions. I shall focus especially on the regional selectivity and laminar specificity of thalamic innervation.

In Chapter 3 I shall describe the establishment of reciprocal connections between different thalamic nuclei and cortical areas in vivo in the rat. The development of lipid-soluble fluorescent dyes for axonal tracing (Honig and Hume, 1986; 1989; Godement et al., 1987) now makes it possible to study the ordering of fibres in the earliest projections without performing intrauterine surgery or using immunohistochemical markers. In this chapter I shall focus on the basic question of orderliness of the earliest projections. Are they organized topographically from the very beginning or are they initially exuberant and lacking precise organization? At what time is the adult-like areal distribution of thalamic fibres achieved?

In numerous systems the temporal sequence of neuron production and

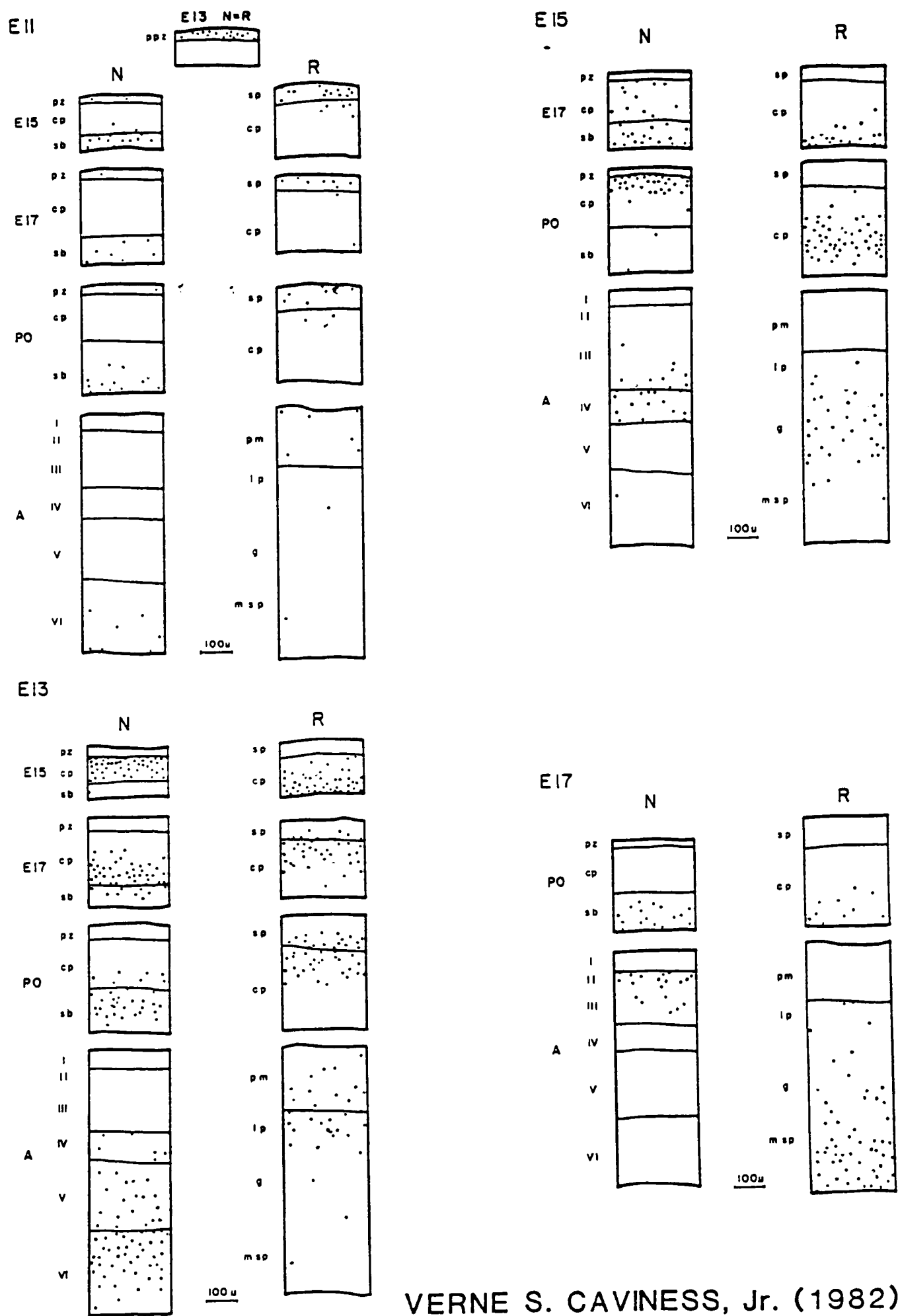
differentiation in the interconnecting structures is correlated with the temporal sequence of formation of connections between them. The translation of a temporal gradient into topography of connectivity has been proposed for a number of structures (Bayer and Altman, 1987). Bayer and Altman (1987) pointed out that many of the major anatomical interconnections of the telencephalon can be related to the order of neurogenesis and to neurogenetic gradients (see above). What is the case for thalamocortical connections? Is there a spatial gradient in the establishment of thalamic connections with different cortical areas? The timing of establishment of initial thalamocortical interconnections as well as other cortical connections (callosal, corticospinal and mature cortico-thalamic projections) will be discussed in Chapter 3 and their ordering and relationship to each other will also be considered. The plausibility of the proposal that these temporal gradients are indeed important in the formation of orderly interconnections rests on showing that the maturational gradients are matched within the various interconnecting structures, that fibre outgrowth follows a spatio-temporal gradient and that fibres are ordered during their outgrowth and arrival. I consider the possibility that, at the site of innervation, the arriving thalamic fibres occupy their place on a 'first-come-first-served' basis, establishing a map based on chronotopy.

Chapter 4 will examine the hypothesis that subplate cells play a central role in the deployment of thalamocortical connections (Shatz et al 1990; Blakemore and Molnár, 1990) by observations on an 'experiment of Nature'. The autosomal recessive mutation in the 'Reeler' mouse has its primary effect on the migration of immature neurons (Caviness and Sidman, 1973), disrupting the process that normally leads to the layers of the cortex being formed in an 'inside-out' sequence (Berry and Eayrs 1963; Rakic 1974; see above). The first-generated postmitotic neurons migrate to the cortical surface,

Fig. 1.4

The figure (adapted from Caviness, 1982) demonstrates the distribution of sequentially formed neurons as they achieve their final positions in the developing neocortex of normal (N) and reeler (R) animals. Neurons formed at 48-hourly intervals at gestational ages E11, E13, E15 and E17 were autoradiographically labelled by a single injection of [³H]thymidine. Plots of the distribution of heavily labelled cortical cells are demonstrated at successive developmental stages E11-17, P0 and adult in normal and reeler animals. ppz primordial plexiform zone, vz ventricular zone, cp cortical plate, sb subplate, sp superplate. In the adult reeler cortex pm, lp, g and msp indicate the zones of polymorphic, large pyramidal, granule and small and medium pyramidal cells, respectively.

Fig. 1.4



VERNE S. CAVINESS, Jr. (1982)

as in normal mammals, and form the primordial plexiform zone (the preplate). But in the Reeler mutant, the cells of the cortical plate itself fail to invade and split the population of first-generated polymorph cells, which stay together at the top of the cortex as a single layer (termed the 'superplate'). The cortical plate develops below the superplate, with its layering inverted (Fig. 1.4). In spite of this gross disturbance of cortical genesis, Reeler develops relatively normal thalamocortical connectivity. Previously this was taken as an example of axons finding malpositioned targets on the basis of remote chemoaffinity, despite their having to traverse abnormal pathways (Caviness et al., 1988). I shall examine the development of the reciprocal thalamocortical connections in Reeler step-by-step from early embryonic ages and shall show that precisely the same developmental program occurs as in normal animals, and the same pathways are followed, the only difference being the relative positions of cortical plate cells and the cells that should have formed the subplate.

Chapters 5 and 6 are descriptive, providing an account of the development of two molecular species that might play a part in cortical development.

Chapter 5: Nitric oxide (NO) is a newly described inter- and intracellular-messenger. In view of the potential power of NO to regulate the growth of cellular processes, the conductivity of gap junctions and cell death, and both to control synaptogenesis and to modulate existing synapses, I was interested in the possibility that NO-producing neurons might play an important role in regulating the formation of cortical circuitry, neuronal excitability and local differentiation of cortical fields. In chapter 5, I document the distribution of NADPH-d-positive neurons (i.e. capable of synthesizing NO) in the developing rat cortex.

In the early postnatal period there is a considerable rearrangement in the cortical

connectivity and activation pattern as thalamic fibres find their ultimate target cells in layer 4 (Friauf and Shatz, 1991; Callaway and Katz, 1992). In Chapter 6 I shall discuss the age-specific patterns of the expression of the calcium-binding protein, parvalbumin. In view of the fact that calcium is known to be an extremely important intracellular signal involved in a number of second-messenger cascades that could lead to changes in growth and synaptic modification, parvalbumin could play an important role in the formation and transformation of neuronal circuitry during development. Parvalbumin is a calcium-binding protein and its appearance might correlate with the changing activity patterns during the transformation of transient neuronal circuitry during cortical development.

In Chapter 7 I shall compare ontogeny and phylogeny to try to get an insight into the evolutionary origin of the subplate system and the way in which it has come to play its different roles in cortical development. Early cell death is now known to be a very widespread phenomenon in the developing brain and it is clearly more exaggerated in some areas and cell types than for others. I shall argue that specific populations of transient cells (including the first-generated border cells in the spinal cord (Knyihár et al., 1978) and those in the thalamoreticular and perireticular nuclei (Mitrofanis, 1992a) and the optic chiasm (Sretavan, 1993)) might have an evolutionary origin similar that of the subplate system. Their examination is important in the understanding of the developing brain.

I shall also discuss whether my previous observations in the rodent can be generalized to species with gyrencephalic brains. In primates, the morphology and the arrangement of the various thalamic nuclei is rather different from that in rodents. I shall compare the anatomy and relationship of the different thalamic nuclei at the same

developmental stage between rodents and primates during the establishment of their specific interconnections.

By examining these questions I hope to give an overview of the **multiple mechanisms** involved in the (areal and laminar) specification of thalamocortical connections, pointing out the periods during which extrinsic mechanisms can build on and interact with intrinsic ones.

CHAPTER 2

DEVELOPMENT OF THALAMOCORTICAL CONNECTIONS IN VITRO

INTRODUCTION

2.1 Aim of the study

My overall goal is to gain insight into the nature of the mechanisms responsible for the development of specific interconnections between thalamus and cerebral cortex. Obviously this is a complex process and many different mechanisms are likely to be involved. The various forms of cell-cell interaction during the formation of connections can be studied in several different ways. Description of the anatomy of normal development gives much useful background information but often not sufficient to reveal mechanisms. Transplantation and lesion studies in vivo can provide substantial information, but their interpretation can be complicated by variability in the survival of grafts and the extent of and reaction to lesions. The technique of organotypic co-culture make it possible to create conditions under which neurons can develop, differentiate and interconnect, but at the same time to allow very specific manipulations.

I was interested to see what features of the timetable of normal development could be replicated in vitro, in the hope of gaining insights into the mechanisms of the development of area- and lamina- specific connections in the living animal. I was particularly concerned to see whether the 'waiting period' and the layer-specific termination pattern within the cortex might be duplicated in culture and whether different thalamic nuclei and cortical areas when co-cultured demonstrate regional selectivity in vitro.

The outgrowth of axons from the developing thalamus of the rat was first studied by Lund and Mustari (1977), using degeneration techniques. They suggested that

fibres start to grow out of the putative LGN at about E16, just as the nucleus becomes clearly recognisable in Nissl-stained sections. Lund and Mustari (1977) suggested that these axons reach the occipital cortex some 2 days later but do not substantially penetrate the cortical plate until around the time of birth, some 3-4 days after they begin to accumulate under the cortical plate. This observation led to the concept of a 'waiting period', which has been reinforced by the observation of much longer periods of accumulation of thalamic axons in Carnivores (Shatz and Luskin, 1986) and Primates (Rakic, 1976; 1977).

The introduction of the sensitive tracing of axons with carbocyanine dyes has led to revision of Lund and Mustari's (1977) timetable, but has confirmed the basic pattern of growth, waiting and invasion. For a detailed description of the in vivo developmental timetable see Chapter 3. In summary, the first fibres start to grow out of the posterior, dorsal thalamus (putative LGN) of the rat around E14, before the various thalamic nuclei can be clearly differentiated, and they reach the occipital pole of the cortex around E16 (Chapter 3; Molnár and Blakemore 1990a, Blakemore and Molnár 1990, DeCarlos and O'Leary, 1990, 1992). They initially accumulate under the cortical plate for a brief period (E16-19; Chapter 3), a somewhat shorter, as well as earlier, period than that originally described by Lund and Mustari (1977). Catalano et al. (1991) have also reported that thalamic fibres arrive under the somatosensory cortex earlier and enter the cortical plate before birth. Indeed they even questioned the existence of a waiting period during which the thalamic fibres do not invade the cortical plate. My own results (Chapter 3) clearly indicate that there is a period of axon accumulation, at least for the occipital cortex. The final pattern of thalamic innervation, with the typical dense termination in layer 4, sparser termination in layer 6 and a little

in 1, is achieved only at the end of the first postnatal week (Lund and Mustari 1977; Wise and Jones, 1978; Chapter 3). The peak of synaptogenesis, some of which may well be due to continued formation of thalamocortical contacts, is around the time of eye opening in the second postnatal week (Cragg, 1975; Kristt, 1978; Wise et al., 1979).

The intention of these in vitro experiments was to identify some of the various forms of cell-cell interactions that might play a part in the establishment of layer-specific and area-specific interconnections between thalamus and cortex. I hoped that the use of an in vitro model system would simplify the task of studying axonal guidance, neuron-neuron recognition and the possible role of chemoattractants and trophic interactions during the formation of the thalamocortical connections.

My plan was to use numerous paradigms (see Table 2.1A-G below) of co-culture, combining different thalamic regions with different cortical (and other) areas, taking the explants at different ages, arranging the cultured structures in many different orientations with respect to each other or giving choice to the thalamic or cortical piece by culturing three structures at once.

2.2 Historical remarks:

development of organotypic tissue culturing

Wilhelm Roux succeeded in maintaining the medullary plate of a chick embryo in vitro as early as 1885. However, it later became possible not only to keep the tissue alive, but also to promote cell growth and differentiation. Harrison reported the first successful, reproducible technique for growing nerve cells under in vitro conditions in 1907. The techniques for the maintenance of organotypic cultures and co-cultures of explants of immature nervous tissue are now well developed (see Bornstein, 1958, 1964, 1973; Crain, 1972, 1974, 1976; Gahwiler, 1988; Romijn et al., 1988).

Organotypic cultures (the term was originally introduced by Maximov in 1925) preserve the basic structural organization of their tissue of origin. In such cultures of nervous tissue, cells not only differentiate but also form neural circuits typical of the structure in question (for review see Gahwiler, 1988). Numerous techniques have been developed to maintain the necessary nutrition and gas exchange within the tissue piece and to allow development of the whole organotypic tissue block at the same time: Maximov depression slide chambers (for so-called 'lying-drop' preparations); roller-tube techniques; polyamide gauze carriers in plastic culture dishes (for a practical manual see Freshney, 1986). Each technique has its own advantages and shortcomings. My choice settled on the **interphase-type, stationary culture system**, for the following reasons:

- 1) The tissue does not flatten to monolayer thickness even after several weeks in vitro. I feel that this is important because neural growth and the formation of local circuitry are likely to be more natural in three-dimensional substrates than in flattened monolayers.
- 2) The cultures are stationary, so, as in vivo, the fluid environment of the tissue is not mixing continuously. This is especially important when considering whether diffusible trophic or tropic factors are present and whether they produce directed axonal outgrowth (Lumsden, 1992), though it must be said that diffusional characteristics are likely to be rather different within a homogeneous fluid culture medium compared with the extracellular space of the developing brain.
- 3) The conditioned medium can be collected, and the medium can easily be changed.
- 4) One has direct access to observe the specimen during culturing, allowing the possibility of time-lapse photographic techniques, electrophysiological recording, optical recording, etc. (However, the cultures have to be resectioned for conventional light

microscopy and can be transferred into an electrophysiological recording set-up only after cutting the membrane around them.)

2.3 Development of serum-free, chemically defined nutrient media for culturing of nerve tissue

Up to the 1950s there was no alternative to the use of media containing blood serum, amniotic fluid, or embryo extracts to provide the rich nutritional environment needed for neural culture. In vivo, the central nervous system (with the exception of few areas) is normally protected from direct contact with serum by the blood-brain barrier and therefore serum probably almost never comes into close contact with nerve cells. Nevertheless, serum has been the most widely used culture medium supplement, although there are considerable disadvantages in its use. There is frequently variation in growth-promoting properties between different serum batches. Serum contains a large number of active factor, some inhibitory or toxic to neurons. It contains neurotoxic and neurite outgrowth inhibiting factors whose effects becomes more apparent when serum is applied in high concentration. Paradoxically in lower concentration serum stimulates excessive neurite outgrowth. Cytostatic agents must be applied to long-term cultures in serum-enriched medium to prevent uncontrolled proliferation of glial cells (for review see Rominj, 1988). Culturing with serum always involves striking a balance between its toxic and its growth-promoting properties. When culturing with medium containing serum enriched with growth factors one has to be especially cautious about the interpretation of 'spontaneous' axonal outgrowth (Hankin and Lund 1991).

To avoid these shortcomings in the use of serum-enriched media, chemically defined, serum-free growth media have been developed. After the pioneering work of Evans (1956), Eagle (1959) and Ham (1965), Luduena (1973) performed the first

successful culturing of neural tissue under serum-free conditions. Many different chemically defined media were subsequently developed to provide optimal conditions for growth of particular tissue or cell types. The most widely used media for culturing neural tissue were developed by Bottenstein and Sato (1979), Snyder and Kim (1979), Honegger et al. (1979) and Rominj et al. (1988). For rat neocortical cultures the N-2 and the R-16 media proved to be the best (Bottenstein and Sato, 1979; Rominj et al., 1988; Yamamoto et al., 1989; Brunner et al., 1982). For all of the co-culture experiments described here, I used N-2 medium.

2.4 Application of the technique (advantages and limitations)

Only recently has it become possible to maintain embryonic and neonatal cerebral cortex in culture (Rominj et al., 1988 and Caesar et al., 1989). In successful cultures, neurons mature remarkably normally, developing characteristic morphological, electrophysiological and pharmacological properties (e.g. Caesar et al., 1989). The electrophysiological properties of cultured cortical slices do, however, show interesting differences when compared to acute preparations. For example, after several days in vitro, cortical cultures develop rhythmic, multi-unit discharges with high levels of activity not seen in the initial period of culturing (Wolfson et al., 1989; Gutnick et al., 1989 and Baker et al., 1989). The time-course over which this activity appears might reflect the process of synaptogenesis in vitro but the selective loss of GABA-ergic neurons (because of their particular sensitivity to hypoxic damage) has also been suggested as the cause of the high spontaneous activity (Caeser et al., 1989).

In some co-culture systems, selective interconnection, following general rules of specificity similar to those operating in the living animal, has been demonstrated (e.g. Frotcher and Gahwiler, 1988; Gahwiler, 1988). The first successful co-cultures of

thalamus and neocortex were reported by Yamamoto et al. (1989).

There are several reasons for the increasing popularity of organotypic co-culture techniques. Certain questions that cannot be addressed in acute slice preparations (because of the inevitable de-afferentation during the cutting of the slices or the need for a long period of study) became accessible in co-cultures. Long-term, controlled pharmacological manipulations during synapse formation are feasible only in co-cultures. It is relatively easy to examine interactions between remote areas of the brain or interactions between areas at different developmental stages. In acute slice preparations recordings are made from neurons that have just undergone mechanical damage and extreme alterations in their immediate environment. Cultures provide mechanical stability, which facilitates intracellular recording as well as direct microscopic observation, and the application of activity-imaging techniques.

Organotypic co-culture techniques now provide a potentially powerful method to address basic questions about the nature of neuronal specificity and the cellular and molecular mechanisms underlying the formation of afferent and efferent cortical connections.

METHODS

2.5 Localization of embryonic sub-cortical structures

Many of my co-culture experiments involved the use of explants from known regions of the thalamus, preferably from individual nuclei, particularly the LGN. It was essential then to learn to recognize the relative positions of thalamic nuclei, and especially the LGN, from surface features of the fetal diencephalon. To learn how to do this, I started by exposing several E16 fetal rats by hysterotomy and, using a glass pipette I injected one eye with horseradish peroxidase conjugated with wheatgerm agglutinin (WGA-

HRP) of every individual of 4 litters (of which I processed 12 to reveal the retinofugal projections). For the intraocular injection I used 1µl of a freshly made 4% WGA-HRP solution. After 8-10 hours of survival these fetuses were delivered by Caesarian section and perfused transcardially with 1% paraformaldehyde, 1.25% glutaraldehyde solution. Sections of the brains (50µm, cut into PBS) were processed with TMB (Mesulam, 1978) to reveal the terminal fields of optic nerve axons in the LGN. Once the LGN had been clearly identified, it was fairly simple to locate the other major divisions of the thalamus and to relate them to surface landmarks visible during dissection. The same type of experiments were later repeated with carbocyanine dyes (DiI, DiA and DiAsp) applied as small crystals directly on the retina or optic nerve in paraformaldehyde-fixed fetal brains, eliminating the need for difficult intrauterine surgery. (For a detailed description of the developing retinofugal projections in the rodent see Lund, 1965; Lund and Bunt, 1976; Horsburgh and Sefton, 1986; and Colello and Guillery, 1990).

During the early co-culture experiments, I proved the reliability of my identification of the LGN from surface landmarks by cutting, staining and examining sections of the **remaining** part of the diencephalon, after removal of the explant assumed to contain the LGN, to be sure that I had removed the desired region and little more for culture (using Coggeshall's atlas (1964) of the developing diencephalon to identify the remaining structures).

2.6 Dissection

Fetal rats were obtained from time-mated females by Caesarian section under pentobarbital anaesthesia (100 mg/kg, intraperitoneal). In these experiments Hooded Lister rats were used, with the exception of a few initial cases when only albino Sprague-Dawley rats were available. (Where exactly comparable experiments were

performed on tissue from the two breeds, the results did not differ in any obvious way). All the experimental animals were bred in the Animal House of the University Laboratory of Physiology, Oxford. The 'plug date' was taken as embryonic day zero (E0) for the dating of fetuses.

Under sterile conditions the anaesthetized fetuses were exposed, removed, chilled then decapitated. The whole brain was dissected and immersed in chilled medium. At E16, the commonest date at which thalamic explants were taken, the entire thalamus is approximately 1.3 mm antero-posterior, 1.25 mm medio-lateral, 0.8 mm dorso-ventral. Explants of the thalamus, as small as 0.5 x 0.5 x 0.3 mm, were dissected from the dorsolateral aspect of the posterior diencephalon (LGN), from an equivalent position more anterior in the thalamus (corresponding to nuclei that become connected to frontal cortical fields), or from other parts of the developing thalamus. In experiments in which axon growth into or out of different thalamic nuclei was to be compared, each pair of thalamic explants was dissected from the same side of an individual brain, to ensure that they consisted of different thalamic nuclei (Fig. 2.1.).

The dissection was performed with the aid of an operating microscope under a sterile hood. The blocks were gently transferred (with a modified Pasteur-pipette) into a Petri dish containing a few drops of Hank's balanced salt solution supplemented with extra glucose to a final concentration of 6.5mg/ml, before transfer to the culture chambers. Preincubation at room temperature (for 60 mins) reduced necrosis in the centre of explants (Rominj, 1988).

To obtain cortical explants from embryonic 15-16 day old brains a 'tissue-ribbon' (3-4 mm x 1-2 mm x entire depth of the embryonic cortex) was cut in the coronal plane. Then smaller (1-2 mm x 0.5mm x entire depth of the embryonic cortex)

slices were cut from the end of the ribbon, ie in a parasagittal plane (Fig. 2.22).

In older animals, after about E19, slices approximately 350 μm thick were cut directly from the whole brain with a hand-held tissue chopper. Surgery on postnatal animals was performed under hypothermia or (after P5) under I.P. pentobarbital anaesthesia. After decapitation, the head was immersed into 2 baths of chilled 70% ethanol in quick succession, for 1-2 seconds each (to reduce the danger of possible contamination during further dissection) and dried with a sterile gauze. Under a sterile hood the whole brain was quickly removed and placed into a drop of chilled glucose-supplemented (final concentration of 6.5mg/ml) Hank's balanced salt solution in a Petri dish. After the careful removal of the pia, the brain was held stationary in the dish with a pair of forceps in one hand, while the other hand was employed to push a tissue chopper down quickly, perpendicular to the convexity of the required area of the hemisphere, roughly in the coronal plane. The tissue chopper was constructed (in the laboratory workshop) from five razor blades separated by 350 μm -thick stainless steel metal spacers, mounted in a metal holder. The 4 resulting coronal brain slices (containing both hemispheres, striatum or hippocampus together with mesencephalon and diencephalon) were transferred into another Petri dish containing a few drops of chilled Hank's balanced salt solution. The neocortical cortical explants were then dissected from the dorsolateral aspect of these whole brain slices with a microsurgical knife (Weck Ophthalmics, Bristol-Myers Squibb, Jacksonville, USA). The explants were 2-5 mm long, 300-350 μm thick and spanned the entire cortical depth from the pial surface to the ventricular side (Fig. 2.1.).

For some experiments, explants of hippocampus were also dissected from the coronal whole-brain slices; and in some cases roughly parasagittal slices were cut

directly from the cerebellum with the tissue chopper (Fig. 2.1). After preincubation (Rominj et al, 1988) for 1 h in glucose-supplemented Hank's balanced salt solution (at room temperature) the slices were transferred to the tissue culture chamber.

2.7 Culturing conditions

Tissue culture chamber

The cultures were placed on the surface of a microporous (pore size 0.4 μm), collagen-treated membrane in commercially available Transwell-COL culture chambers (Costar: Cambridge, MA). I used the 24.5 mm diameter inserts on one 6-well cluster plate. The arrangement of the individual chambers (Fig. 2.1) allowed the cultured slices to be maintained in a manner similar to that employed in the type of interphase recording chamber frequently used for in vitro electrophysiological recording. The level of the medium was adjusted so that only a very thin film covered the surface of the explants: this arrangement seemed to optimize the efficiency of oxygenation and nutrition (Fig. 2.1.). The cultures were kept stationary and were not rotated or rocked (tilted).

For most experiments, tissue was taken from fetal animals or animals younger than 1 week postnatal. To maintain tissues older than about P10, higher partial pressure of O_2 is needed. Since there was no incubator with high O_2 concentration, (and it would have been extremely costly to provide high pp O_2 within the present incubators) the culture chambers were placed in smaller incubator chambers (Modular Incubator Chamber, Flow Laboratories) filled with humidified carbogen (5% CO_2 and 95% O_2 , at 100% humidity). The gases were changed daily in the chamber.

Coating the membrane

In most cases I used Transwell-COL tissue culture chamber inserts, whose membranes are already treated with an equimolar mixture of Types I and III **collagen** (derived from

bovine placenta). No precoating or other treatment was required (only a brief preincubation with the medium for 1-2 h, to moisturize the collagen on the surface). In a few cases another culturing surface, **laminin**, was also tested. Then the membranes of the Transwell dishes were treated overnight with poly-Lysine solution (1 mg/ml in Hank's balanced salt solution) in order to facilitate the binding of the laminin (1-2 h, 20 µg/ml in Hank's balanced salt solution).

The Transwell membranes have microscopic grooves on their surface, which can influence the direction of axonal outgrowth, especially when the co-cultured explants are relatively far from each other. For the 'choice' experiments (see below), in which comparisons were made of the pattern of ingrowth from one explant into two different neighbouring explants, the three explants were lined up parallel to the grooves, so that any influence of the orientation of the grooves on axon growth would apply equally for the two targets. To control for the possible effects of the grooves on the Transwell membranes, I repeated all the basic experimental paradigms with other culture dishes without grooved membranes (Petriperm, GmbH, purchased from Bachofer). In these dishes the cultures were placed on a 25µm thick, gas permeable foil-membrane, which formed the bottom of the dish, and the medium was placed above the cultures. All the general findings described here were confirmed with this alternative form of culturing.

N-2 medium

The N-2 chemically defined, serum-free medium (Bottenstein and Sato, 1979) is made up as follows: 1:1 mixture of Dulbecco modified Eagle's medium and Ham's F 12,

supplemented with:

- Insulin - 5 µg/ml
- Transferrin - 100 µg/ml
- Progesterone - 20 nM
- Putrescine - 100 µM
- Selenium (as Na₂SeO₄) - 30 nM

These components were usually made up into "stock-solutions", filter-sterilized (pore size 0.2 μm) and then added to the medium individually. In 1990 GIBCO marketed a 1:100 concentrated N-2 stock solution (GIBCO BRL, Grand Island, N.Y.) and this has proved to be much more convenient to use. The N-2 medium was stored at 4°C under sterile conditions. If the prepared medium was not used up within a month, it was supplemented with additional L-Glutamine (12.5 ml commercially available stock solution/l/month) every 4 weeks. All the chemicals whose source is not specified were obtained from Sigma (U.K.).

During culturing, the medium was changed every 4-6 days. It proved to be crucial to keep the level of the nutrient medium so that only a very thin film covered the membrane. Therefore the cultures were checked (once or twice each day) and any necessary adjustments made.

2.8 Anatomical processing

Labelling connections in the cultures

Depending on the duration of culturing, I used two techniques to reveal axonal connections.

1) For less than 6 days of culturing:

One of the explants in each co-culture group was prelabelled by brief immersion in a solution of DiI (Molecular Probes, Eugene, OR) in alcohol and dimethyl sulphoxide (DMSO) (25 mg DiI was dissolved in 10 ml of 5% DMSO and 95% alcohol mixture) before it was rinsed quickly in Hank's balanced-salt solution. This caused minute crystals of DiI to form on the surface of the explant. This procedure presumably labelled most of the cell bodies within the whole explant, and any axons growing out were intensely labelled with the

fluorescent dye for a period of about 5 days (after which the label became concentrated back in the cell body membranes, presumably because of membrane recycling; Honig and Hume, 1986; Fig. 2.2A-B). Although this labelling technique should make it possible to follow the fibres in living cultures (which Yamamoto et al, (1992) and I have recently exploit for confocal microscopic time-lapse imaging of fibre ingrowth), for all the experiments reported here, I examined the labelled axons with fluorescence microscopy after fixing the co-cultures in 4% paraformaldehyde (30 min in 0.1 M phosphate buffer, pH 7.4). Interestingly, after several days, cells sending projections from the unlabelled explant into the labelled co-explant start to accumulate fluorescent dyes in their cell body by retrograde diffusion. (For instance, cortical layer 6 cells accumulate DiI after being cultured for 10-20 days with a prelabelled LGN block). This phenomena can be used to examine the distribution of cells sending projections out in culture (Yamamoto et al., 1991; 1992). Counterstaining with bisbenzamide (10 min in 2.5 µg/ml solution in 0.1 M phosphate buffer) allowed me to visualize the outline of the explants and some of the laminar boundaries under ultraviolet illumination (see Fig. 2.8).

2) For more than 6 days of culturing:

I exploited the fact that these fluorescent dyes will diffuse along axons in fixed tissue (Godement et al., 1987). The tracing was performed after the termination of the culturing by fixation with buffered 4% paraformaldehyde (pH 7.4, 1-2 h at 4°C). Small crystals of DiI were placed on one of the co-cultured structures, then, after storage in fixative at room temperature or at 37°C for 2 to 4 weeks, the culture was counterstained (as in 1 above) and mounted in Hydromount

(National Diagnostics). Tracing with DiI in fixed tissue labels equally well the anterograde and the retrograde projections (Fig. 2.2C and D).

I also tried other labelling and tracing techniques (which I had previously used successfully in vivo) to try to reveal connections in co-cultures (*Phaseolus Vulgaris* Leucoagglutinin, Biocytin, Tetramethylrhodamine, Rhodamine B isothiocyanate, HRP, Rager Silver Staining). Unfortunately, in my hands at least, these methods were not consistently successful and I abandoned them for routine experiments, where reliability and consistency of labelling were important.

Nissl staining

For Nissl staining, the slices were fixed with 4% formalin in phosphate buffer (0.1 mM, pH 7.4) for 1-2 h at 4°C. Cultures were rinsed in phosphate-buffered saline and embedded in paraffin (ascending alcohol line, 30 min each, then methyl salicylate overnight, then paraffin 2x2 h). 10-µm-thick slices were cut, mounted on subbed slides, stained with an aqueous solution of cresyl violet (1%) for 5 min, differentiated in acetic-acid alcohol, dehydrated in alcohol, cleared in xylene and embedded in DPX.

Semithin preparations

In order to prepare semithin sections, some cultured explants were fixed in 5% glutaraldehyde in sodium cacodylate buffer (300 mOsm; pH 7.4) for 1.5 h, and postfixed in 1% OsO₄ in the same buffer (1.5 h). The explants were dehydrated in a graded ethanol series and embedded in Epon. 1 µm-thick slices were counterstained with toluidine blue.

RESULTS

2.9 General characterization of the cultures

Macroscopic appearance

Over the culturing period, the cortical slices became fan-shaped with a prominent convex pial surface and a more irregular, narrower ventricular aspect (Figs 2.6 and 2.7). The tendency for the pial surface to become convex and the overall change in the shape of the cultured slice may partly be due to the changes occurring during preincubation (slices tend to curl) or/and the continued migration of immature neurons from the intermediate zone and the lower part of the cortical plate, up to the top of the plate causing the pial surface to expand. My own results provide indirect evidence for some continuation of migration in culture, since a cortical slice taken around P1 (when only layers 6 and 5 are fully formed) and placed in culture continues to thicken, and the upper layers are fairly similar in appearance to those in normal animals of the same chronological age as the cultured slice (Fig. 2.6B and 2.7B). The radially cut edges of the cortical slices taken from younger animals (E20-P3) tended to curl over onto the cut top surface of the slice. This was not observed in slices older than P4-5.

The gross structure of the explant remained compact and tightly organized, with sharp boundaries, throughout the culturing period. Moreover, the cortical slice remained organotypic, even to casual observation: distinct cell and fibre layering could be seen from pial to ventricular surface, even in unstained cultured slices.

The slices tended to become somewhat thinner in culture, especially over the first day in vitro, but not to the same extent as with the roller tube technique (Gahwiler, 1988), where cortical cultures are "always less than 4 cell layers thick and often monolayers are obtained" (Caeser et al., 1989). Cortical slices taken at younger ages

flattened relatively more than older ones. As with the roller tube technique, the flattening was unequal for different cortical layers: the pial side of the cortical slices tending to flatten less than the ventricular. However, even the thinnest regions of the explant remained over 150-200 μm thick after several weeks in culture. Typically, 15-20 cell layers or more could be distinguished through the whole thickness of a cultured cortical slice, from top to bottom surface, when sectioned perpendicular to the microporous membrane (see Fig.2.9A). The thalamic blocks, which were mainly taken from E16 embryos, flattened somewhat more than the older cortical slices.

While the maintenance of three-dimensional structure provided a more natural environment for axon growth within the tissue and for the formation of neural circuits, it made processing of the tissue for conventional light microscopy more tedious: the explants had to be resectioned and stained before examination (Figs. 2.6 and 2.7)).

Phase-contrast microscopy: cell migration out of the explants

During culturing the slices were occasionally viewed by phase-contrast microscopy. This did not require any staining or removal of the culture from the culture chamber. Around the edges of each explant, some cells were seen to have migrated out of the block, across the surface of the culture chamber membrane. The majority of such migrating cells were seen at the edges of the thalamic blocks and especially at the ventricular edge of the cortical explants. The degree of cell migration was strongly dependent on the nature of the surface of the microporous membrane (confirming the results of Nagata and Nakatsuji, 1990), laminin being much more permissive (Fig. 2.2 and 2.3) than collagen (Fig. 2.5). On laminin-coated membrane, after 7 days in vitro, the maximum distance of cell migration from a P0 cortical slice was as far as 2 mm on collagen and 5 mm on laminin. Most of the migrating cells appeared to be neurons but

definitive identification with neuron- or glia-specific immunohistochemical markers was not performed.

Nissl-stained and semithin sections: maturation of cell types and lamination

The cultured cortical slices (with or without accompanying explants) were observed in Nissl-stained 10-30 μ m sections cut horizontally (tangential to the microporous membrane) (Fig.2.6 and 2.7) and in toluidine-stained semithin (1 μ m thick) preparations (Fig. 2.9B). The semithin sections demonstrate cell morphology in more detail, including subcellular organelles and compartments (nuclei, nucleoli and cytoplasm)(Fig.2.9B). To study the histological appearance through the thickness of the cultures, I also prepared sections perpendicular to the membrane (Fig.2.9A). These showed that, even after long periods in culture, cortical slices remained 15-20 or more cell layers thick. The upper face of the slice (which had occupied the medium-gas interface) was covered with a thin (10-15 μ m thick) layer of tangled cell processes and other debris. Some pyknotic profiles were seen, characteristic of dying cells, almost exclusively at the lower surface of the slice, in contact with the microporous membrane. This was most evident in the sections cut perpendicular to the plane of the microporous membrane (Fig.2.9A). This result suggests that some cellular degeneration and cell death was still taking place at the lower surface of the slice even after several weeks in culture, in spite of the considerable preservation of overall thickness and cell number.

More recently a confocal microscopic viability assay has been developed, using combined staining with acridine orange and propidium iodide to allow the identification of depolarized, presumed dying cells in cultured and fresh slices (Adams, R. et al; 1993). This method confirmed the observations described above, showing that cells that are probably dying are seen at the lower margin of cultured cortical slices. However,

cells appeared to be healthy and similar in their appearance throughout the whole of the rest of the depth of the slice, between these thin boundary regions.

In Nissl-stained transverse sections (i.e. parallel to the slice) the distribution of cell types within the cortical grey matter varied with the age of the animal at the time the slice was taken and with the time in culture. Fig 2.7 compares the appearance of coronal sections through occipital cortex taken acutely from perfused animals and sections from slices maintained in vitro for various periods of time.

In rodents, the cells that are destined to make up the superficial layers (layer 4, 3 and 2) have not yet migrated into position at the time of birth (Fig. 2.7A). Slices taken at this age and cultured for 7 days are certainly not suspended in their original form: maturation continues to some extent. The cortex taken at P0 and cultured for a week has thickened, the dense cortical plate has thinned and the cells of the upper layers are larger and more sparsely distributed than at birth (Fig. 2.7B). After culturing for an additional week the lamination of the cultured cortical slice became even more apparent (Fig. 2.7B).

These observations on the thickening of the cortex and the evolution of the upper layers in culture (similar but not identical to maturation in the living animal) imply that cell migration continues in vitro, but probably not to the same extent as in vivo (see discussion for birthdating experiments from other studies).

Table 2.1

The Table gives a summary of the co-culturing experiments. The columns represent the arrangement and identity¹ of the co-cultured structures, the number of days in vitro and the number of cultures examined. A star (*) in the first column indicates those cultures in which connections were revealed with DiI application after fixation (see Methods section). All co-cultured explants were separated by <2mm, except for those indicated by >2mm in the first column. ‘inv’ indicates inverted cortical slices, ‘side’ indicates that the thalamic explant was placed on the cut side of the cortical slice. ‘stripe’ indicates long cortical ribbons. ‘MAM’ labels the cortical slice cultures taken from pups treated with methylazoxymethanol (MAM) at embryonic day 16-17. All the Petriperm dishes were coated with laminin. The Transwell dishes were coated with collagen, with the exception of a few that were laminin-treated: (‘lam’ in the table.)

¹

Abbreviations:

LGN	-	dorsolateral thalamus containing the lateral geniculate nucleus
CTX	-	cerebral cortex
Occ	-	occipital
Fr	-	frontal
AntThal	-	anterior thalamus
Hipp	-	hippocampus
Cereb	-	cerebellum
CP	-	cortical plate
SP	-	subplate

Arrangement	Co-cultured structures	Days <u>in vitro</u>	Number
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A Remote influence of cortex on thalamic outgrowth in vitro Petriperm dishes

>2mm	E16 LGN	/	P0 Occ CTX	6	12
	E16 LGN	/	P0 Occ CTX	6	6
	E16 LGN	/	E16 Occ CTX	6	6
	E16 LGN		-	6	6
	E16 LGN	/	P3 Occ CTX	3.5	6
	E16 LGN	/	P3 Occ CTX	3.5	6
	E16 LGN	/	P3 Occ CTX	4	4
	E16/17 LGN		-	4	4
	E16/17LGN		-	4	10

B	Conditioned medium (cond. med.)
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9
10	10
11	11
12	12
13	13
14	14
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90	90
91	91
92	92
93	93
94	94
95	95
96	96
97	97
98	98
99	99
100	100

E16 LGN (in P0 CTX cond. med.)	4	12
E16 LGN (in P0 CTX + E16 LGN cond. med)	4	12

C **P21-P3**

Growth of thalamic axons into cortical slices

Emergence of laminar selectivity for thalamic termination

	g	y			
	E16 LGN	/	P0 Occ CTX	1	4
	E16 LGN	/	P3 Occ CTX	1	4
	E16 LGN	/	P3 Occ CTX	1.5	10
	E16 LGN	/	P0 Occ CTX	2	4
	E16 LGN	/	P2 Occ CTX	4	8
	E16 LGN	/	P3 Occ CTX	4	20
	E16 LGN	/	P1 Occ CTX	5	4
*	E16 LGN	/	P3 Occ CTX	10	8
	E16 LGN	/	P3 Occ CTX	4	6
>2mm	E16 LGN	/	P3 Occ CTX	4	6
	E16 LGN	/	P0 Occ CTX	4	14
	E16 LGN	-		4	6
	E16 LGN	/	P0 Occ CTX	7	8
	-		P0 Occ CTX	14	6
	E16 LGN	/	-	20	6
	E16 LGN	/	P3 Occ CTX	10	6
	P2 Occ CTX	/	P2 Occ CTX	4	4
side	E16 LGN	/	P3 Occ CTX	4	6
inv.	E16 LGN	/	P0 Occ CTX	5	8
inv.	E16/17 LGN	/	P3 Occ CTX	4	6
inv.	E16 LGN	/	P1 Occ CTX	22	8
	P0 LGN	/	P0 Occ CTX	21	6
	E21 LGN	/	E21 Occ CTX	4	4
	E16 LGN	/	E21 Occ CTX	4	8
	E16 LGN	/	P11 Occ CTX	4	6

Arrangement	Co-cultured structures		Days <u>in vitro</u>	Number	
D	P3-P11				
	Experiments on the cause of termination in layer 4				
	P6 Occ CTX	-	4	8	
	E16 LGN	/ P5 Occ CTX	4	5	
	E16 LGN	/ P6 Occ CTX	4	10	
	E16 LGN	/ P11 Occ CTX	4	5	
	E16 LGN	/ P3/P6 Occ CTX	4	5	
side	E16 LGN	/ P6 Occ CTX	4	8	
inv.	E16 LGN	/ P8 Occ CTX	4	6	
*	inv.	E16 LGN	/ P6 Occ CTX	10	4
	inv.	E16/17 LGN	/ P6 Occ CTX	4	6
	MAM	E16 LGN	/ P6 Occ CTX MAM	4	10
E	E16-E19				
	Experiments on the absence of a waiting period in co-culture				
	E16 LGN	/ E16 Occ CTX	4	6	
	E16 LGN	/ E19 Occ CTX	4	8	
	E18 LGN	/ E18 Occ CTX	7	6	
inv.	E18 LGN	/ E18 Occ CTX	7	6	
inv.	E16 LGN	/ E19 Occ CTX	4	6	
F	Do subplate and cortical plate stimulate outgrowth and invasion				
	E16 LGN	/ ‘SP’ CTX P0	4	4	
	E16 LGN	/ ‘CP’ CTX P0	4	4	
	E16 LGN	/ ‘CP’ & ‘SP’ P0	4	4	
	E16 LGN	/ ‘SP’ Stripe P0	4	4	
	E16 LGN	/ ‘CP’ Stripe P0	4	4	
G	Target selectivity of thalamic innervation in culture				
	E16 LGN	/ P3 Hipp	4	8	
	E16 LGN	/ P6 Hipp	4	8	
	E16 LGN	/ P3 Hipp & Occ CTX	4	4	
	E16 LGN	/ P6 Hipp & Occ CTX	4	4	
	E16 LGN	/ P5 Hipp & Occ CTX	5	4	
	-	P0 Cerebellum	4	6	
	E16 LGN	/ P3 Cerebellum	4	15	
	E16 LGN	/ P8 Cerebellum	4	4	
	E16 LGN	/ P1 Cereb & Occ CTX	5	4	
	E16 LGN	/ P3 Cereb & Occ CTX	4	6	
	E16 LGN	/ P6 Cereb & Occ CTX	4	8	
	E16 LGN	/ P8 Cereb & Occ CTX	5	4	
	E16 LGN	/ P5 Hipp & Occ CTX	22	4	
	E21 LGN	/ E21 Cereb & Occ CTX	4	4	
	E18 LGN	/ E18 Cereb & Occ CTX	4	4	

H

Lack of regional selectivity for thalamocortical innervation in culture

E16 LGN	/	P3 Fr. CTX	4	10
E16 LGN	/	P3 Fr. & Occ. CTX	4	5
E16 LGN	/	P1 Fr. & Occ. CTX	2.5	6
E16 Ant. Thal./		P1 Fr. & Occ. CTX	2.5	6
E16 Ant. Thal./		P3 CTX	4	4
E16 LGN	/	P5 Fr. & Occ. CTX	5	4
E16 LGN	/	P6 Fr. & Occ. CTX	5	10
E16 LGN	/	P8 Occ CTX.&FrCTX	4	5
E16 Ant. Thal./		P8 Occ. & Fr. CTX	4	5
E15 LGN	/	E19 Fr. & Occ. CTX	4	4
E15 Ant	/	E19 Fr. & Occ CTX	4	4
E21 LGN	/	E21 Fr. & Occ CTX	4	4
E16 LGN	/	E20 Fr. & Occ. CTX	4	4
E16 Ant	/	E20 Fr. & Occ CTX	4	4

I

Early corticothalamic projections in culture

E18 CTX	/	E18 LGN	4	4
E16 Occ CTX	/	E16 LGN	4	6
E16.5 Occ CTX	/	E16 Ant. Thal.	4	4
E16.5 Occ CTX	/	E16 Ant. Thal.	4	4
E16.5 Fr CTX	/	E16 LGN	4	4
E15 Fr CTX	/	E15 Ant.Thal&LGN	2.5	6
E15 Occ CTx	/	E15 Ant.Thal&LGN	2.5	6
E14.5 Occ CTX/		E14.5 Ant.Thal&LGN	4	6
E14.5 Occ CTX/		E14.5 Ant. Thal&LGN	4	6
E16 Fr CTX	/	E16 Ant. Thal. & LGN	4	6
E16 Occ CTX	/	E16 Ant. Thal. & LGN	4	6
E16 Occ CTX	/	E16 Ant. Thal. & LGN	4	6

2.10 Remote influence of cortex on thalamic outgrowth in vitro

In most co-culture experiments (see Table 2.1) I took thalamic explants (most commonly the region of the LGN) from E16 embryos - old enough for me to be able to differentiate between the different sectors of the diencephalon in stained sections and to detect the position of the LGN from surface landmarks; but young enough for the thalamus to be in a state of active axonal growth. In all but 12 co-cultures, where wider separation was used (see Table 2.1), these thalamic explants were placed close (< 2 mm) to the ventricular aspect of slices of cortex (most commonly occipital cortex, assumed to be the precursor of area 17) taken from other animals, aged between E16 and P11, spanning the entire natural period of thalamic fibre outgrowth, arrival, accumulation and ingrowth. I shall concentrate on the patterns of ingrowth of axons from LGN to occipital cortex, but all other combinations of thalamic and cortical explants produced indistinguishable behaviour (see 2.14 below).

To examine the possibility of a remote growth-promoting effect of the cortex I examined thalamic fibre outgrowth on collagen-coated and laminin-coated dishes (see Table 2.1A,B and C for details). There was little axon outgrowth from thalamic explants of any age when they were cultured alone, in serum-free medium, on a collagen coated-membrane (Fig. 2.5). Even when co-cultured with a slice of the appropriate region of cortex (occipital, putative visual cortex for LGN), there was little axon extension if the gap between the two exceeded about 2 mm on collagen and about 4 mm on laminin (Figs. 2.2 - 2.5 demonstrate examples which are representative for all cultures examined). However, if an LGN explant (E16) was placed closer than these distances to the ventricular surface of an occipital cortical slice (E16-P11), within a day axons were seen streaming out of the thalamic explant, especially on the side closest to the

cortex. If the two explants were very close (0-0.5 mm), the gap between them became filled with a mass of fibres. But with larger gaps (up to 2 mm on collagen, 4 mm on laminin), after 2-3 days in vitro, the co-cultured structures developed one or more distinct tissue bridges between them (Fig.2.2.A). These tissue bridges consisted of large bundles of axons but also contained migrating cells.

Experiments with conditioned medium

I attempted to demonstrate directly the presence of a diffusible cortex-derived growth-promoting factor, by testing the effect of medium taken from culture wells containing cortex (P0) or cortex-LGN co-cultures (P0 CTX, E16 LGN). Medium was harvested from cortical (n=12) or cortex/LGN co-cultures (n=12) after 4 days in vitro and used immediately as the culturing medium for freshly prepared LGN explants taken at E16, cultured on collagen. (Each co-culture provided 1-1.5 ml of conditioned medium, which was enough to test one thalamic culture.) In no case (out of 12 cultures for both paradigms) was there obvious excessive sprouting of axons from the thalamic block, similar to that seen in the presence of the cortex, even after 4 days in vitro with conditioned medium.

2.11 Growth of thalamic axons into cortical slices

When fetal thalamic explants are cultured with cortical slices older than E19 (see Table 2.1) axons grow out of the thalamic block on the side of the cortical slice, initially at a speed of about 1 mm per day, and invade the slice. The axons grow through the cortical slice (not just over its surface). In the photomicrographs (e.g. Fig. 2.11) the plane of focus was adjusted to be near the middle of the thickness of the section to show the axons within the tissue. The culture demonstrated in Fig. 2.11 was also examined in the confocal microscope; projection of the 3-D dataset showed that

individual fibres were growing within the depth of the culture (Fig. 2.12B). These results imply that the cortex not only produces a diffusible growth-promoting factor, but also acts as an attractive, permissive medium for thalamic axon growth after the age of about E19 (for occipital cortex).

2.12 Emergence of laminar selectivity for thalamic termination

By combining E16 LGN blocks with cortical slices taken at different ages (E16-P11) I observed three distinctly different ingrowth patterns according to the age at which the cortical block was taken. I shall present the results in this three groups (i; E16-19; ii; E21-P3; iii; P3-P11); see Table 2.1C,D and E for details.

E16 to E19: The E16 thalamic explant combined with E16-19 cortical explants (see Table 2.1C for details) sent out axons, but more than three quarters of them did not enter the cortical explant. In all (n=8) of the E19 cultures, the fibres mainly grew extensively on the collagen membrane, in a circular pattern around the border of the cortical slice (Fig. 2.16). The cut edges and the pial and ventricular surfaces were outlined with the numerous labelled fibres running along them Fig. 2.16A. At least some of the fibres still had clear growth cones at their tips (recognized as enlargements of the tip with irregular outlines) after 4-5 days in vitro (Fig. 2.16B). Some of the thalamic fibres (fewer than one quarter of them) did penetrate the cortex, especially the older slices in this group, but grew right through it, emerged from the opposite border of the cortical slice, and joined the fibres coursing around the cortical slice (Fig. 2.16B). This suggests that most thalamic fibres do not find the embryonic cortex an attractive environment to penetrate and that they prefer the edge of the slice to the collagen coated membrane as a surface for growth.

In two of the eight E19 cortical explants, there was noticeably more axonal

ingrowth than in the other explants in this group. In these cases roughly half of the thalamic axons grew in through the ventricular surface of the cortical slice and radially into the cortex itself, as for the E21-P3 group described below.

E21 to P3: Fibres grew profusely out of the E16 thalamic block when combined with cortex taken at E21-P3 (see Table 2.1C for details). Some, especially on the side opposite the cortical slice, merely extended short distances across the culture chamber membrane, usually parallel to the grooves in its surface, but the majority of them entered the ventricular surface of the cortical slice, running radially through the intermediate zone and on through the subplate layer (the lowest cell zone directly below the cortical plate), without any obvious termination or hesitation.

The thalamic axons continued radially into the cortical plate, as a fairly uniform array across the whole width of the slice. Growing at approximately 1 mm per day, they passed through the layer of immature neurons that forms the dense cortical plate and reached the marginal zone after about 3-4 days in culture (Fig. 2.13A and 2.14A). Some axons (less than one tenth) turned and ran, taking independent paths, through the marginal zone. But the vast majority of the fibres penetrated the surface, turned 90°, and ran over it, directly below the pia itself, forming a fasciculated band of fibres similarly to the circling axons seen at younger stages (Figs. 2.14A; 2.15A are representative of all of the cultures at similar stages). Even after 5 days in vitro most fibres still had obvious growth cones at their tips (Fig. 2.13B). There was no sign of any preference for a particular sector of the cortical plate and no evidence of active branching and termination in any layer. In about half of the P3 (total n=36) cortical explants, the thalamic axons behaved differently and tended to branch and terminate below the dense cortical plate in what appeared to be layer 4, as for older explants (see below).

I cultured eight P1 occipital cortical explants with E16 LGN for 22 days (see Table 2.1C). Then, after fixing them, a small crystal of DiI was placed on the LGN block. Interestingly, even after this long period of culturing the majority of thalamic fibres still appeared to reach to the pial surface. The fibres labelled from the thalamus had characteristic varicosities which might represent synapses (Fig. 2.10C).

P5 to P11: With half of the P3 explants and with all older slices of cortex (see Table 2.1D), corresponding to the time over which axons are reaching and terminating in layer 4 in vivo, the initial invasion by thalamic axons in co-culture was somewhat slower, but after only 4 days in culture, many axons appeared to have branched locally, lost their growth cones and terminated around 300 μm below the pial surface, at a depth corresponding to layer 4. Layer 4 is indicated on Figures 2.6; 2.7B, 2.8A for various different cultures, which are representative of all cultures of the same age. Just as in vivo, occasional axons (fewer than 10%) still extended to the marginal zone and at least some seemed to terminate there.

This general pattern of innervation occurred for **all** later postnatal slices of occipital cortex, between P5 and P11, the oldest age at which explants were taken. To be sure that these results with older slices (even though extensively replicated: P3 n=36; P5 n=5; P6 n=10; P8 n=10; P8 n=6; P11 n=5; see table 2.1) were not accounted for by chance differences in the culturing conditions, I employed the **choice paradigm**, culturing a single E16 LGN explant flanked by two cortical slices, one P3, the other P6 (n=5). Both were innervated by the LGN but with the different characteristic patterns, as found with single co-cultures. Thus, it appears that sometime around P3-4 the cortex starts to express a specific signal (which I shall call a 'stop signal') that makes thalamic fibres branch and terminate in layer 4 and prevents the majority from continuing to

advance to the pial surface.

Experiment on the absence of a ‘waiting period’ in co-culture

I considered why it should be that, for all ages of cortical explant, thalamic axons fail to demonstrate a high affinity for the subplate zone (as they do in vivo between about E16 and E20 for occipital cortex; see Chapter 3). At E15-19 the fibres mainly run around the cut edges of the cortical slice and along the pial and ventricular surfaces, and do not reach or terminate in the subplate. After E19, thalamic fibres pass through the ventricular zone and reach the subplate layer but pass on without hesitation, into the cortical plate.

I wondered whether the unusual direction of entry of the axons in culture, from the ventricular surface, might interfere with normal mechanisms by which thalamic axons wait in the subplate and form temporary terminals on subplate cells (Shatz et al., 1988). I therefore cultured occipital cortex (P3, n=6; P6, n=8) with an explant of 16 LGN placed at the **side** of the cortical slice, next to the radially cut white and grey matter, in the hope that axons would grow in the normal direction along the intermediate zone, and would be encouraged to stop in the subplate. Axons did indeed enter, through both white and grey matter, but they did not accumulate and wait below the plate. They simply ramified tangentially and then the majority turned up, running radially through the plate and terminating around layer 4.

Do both subplate and cortical plate stimulate outgrowth and invasion?

In vivo, axons start to grow out from the thalamus at E14 (Chapter 3), when the only cells that have migrated into position in the cortex are the first-born preplate neurons destined to become marginal zone and subplate cells. Even when the thalamic axons arrive under the cortex at E15/16, only the cells of the lowest layers of the cortex

proper have been born (Miller, 1988) and have moved into position (see Introduction). Given the current interest in the possible role of subplate cells in guiding and acting as temporary targets for thalamic axons (see 2.15 and Chapter 3), I considered whether the subplate might be a more potent source of growth-promoting influence and might be more permissive for thalamic fibre ingrowth than the cortical plate itself. I wanted to test whether the subplate is better in attracting outgrowth of thalamic axons and encouraging their invasion.

In 20 occipital cortical slices (taken at P0; 4 cultures for 5 different paradigms, see table 2.1F) I separated the subplate from the cortical plate itself by making a pia-parallel cut through the cortical plate along a line definitely above the top of the subplate (Fig. 2.17). This produced two tissue ribbons: one (the **plate ribbon**) consisting of upper cortical plate with no subplate; the other (the **subplate ribbon**) contained subplate, intermediate and ventricular zones and a little of the lowest part of the cortical plate (perhaps most of layer 6). I then co-cultured these ribbons with E15/16 LGN explants in a variety of configurations.

In four co-cultures, the LGN block was placed against the lower surface of one or other ribbon; in others (n=4) it was placed against the radially cut end of the ribbon. The choice paradigm was also employed (n=4), with the LGN explant flanked by a subplate ribbon on one side and a plate ribbon on the other. In no case did I see any obvious difference in the outgrowth of thalamic axons or their tendency to invade the cortical fragment. The extent of axonal growth at the end of the 4 day in vitro period was also very similar in the two tissue pieces (Fig. 2.17 demonstrates examples characteristic of all these cultures).

Experiments on the cause of termination in layer 4

There are two obvious possible classes of mechanism that could explain the cessation of thalamic axon growth in layer 4 in cortex older than about P3-4:

- 1) Some cell type in layer 4 could express a substance (or substances) that causes axons to branch, growth cones to collapse and terminals to form (a stop signal); or
- 2) The supragranular layers could produce a factor that discourages the entry of thalamic axons, which react by branching and terminating in layer 4 (a growth inhibiting signal).

I tested these two (not mutually exclusive) hypotheses. In the most direct approach, I cultured explants of E16 LGN next to the cleaned **pial surface** (axons were unable to penetrate the intact pia) of occipital cortical slices (P0, n=8; P1, n=8; P3, n=6; P6, n=10; P8, n=6). The behaviour of thalamic axons was essentially the same as for the more normal entry from the intermediate zone, except for the direction of growth. If the cortex was younger than P3, axons entered the supragranular layers and ran, mainly radially, but **downwards**, through all the layers of the cortical plate and subplate, and on into the intermediate and ventricular zones, where most of them were found ramifying, with growth cones at their tips, after 4-5 days in culture (Fig. 2.18 is representative of all cultures at P0; n=8). Even with cortex older than P4, thalamic axons still streamed into the cortex and grew profusely through the superficial layers before branching and terminating in the middle of the cortical plate, at just the same depth as if they had entered from below (Fig. 2.18 is representative of all the cortical cultures, P8, n=6; P6 n=8).

In a further experiment on the nature of the stop signal, I attempted to prevent

the normal generation of the neurons of layer 4 by application of the cytotoxic agent methylazoxymethanol (MAM; Chen and Hillman, 1986; Yurkewicz et al., 1984) at the fetal age at which the majority of those cells are born (around E16-17; Lund and Mustari, 1977; Miller, 1988). I gave 2 doses of MAM (15 mg/kg I.P. to the mother), 8 hours apart, on embryonic day 16-17, to cover the whole mitotic cycle when most of the daughter neurons destined for layer 4 are being generated. Surprisingly the cortical layering in the four MAM-treated litters appeared almost normal; only the overall cortical thickness was slightly reduced (Fig. 2.19). Culturing with 10 of these cortical slices (taken at P6) I saw in **all** of these cultures the same ingrowth pattern (with branching and termination some distance below the pial surface) as in normal cortices.

2.13 Target selectivity of thalamic innervation in culture

The most parsimonious interpretation of the basic result of my co-culture experiments is that a piece of occipital cortex, older than E19 (Table 2.1), is a more attractive medium for LGN axon invasion than the collagen-coated membrane that makes up the rest of the thalamic explant's environment in the culture chamber. However, the unwillingness of axons to enter younger cortical explants implies that the occipital cortex develops some particularly attractive property between E19-21. To test whether this influence is specific to occipital cortex, I cultured E16 LGN with slices of neural tissue from other sites (hippocampus, n=32; cerebellum, n=53; age ranging from E18 to P8 see Table 2.1G for details). In fact, I used the choice paradigm to compare the innervation of different structures, in the hope of revealing even subtle preferences.

Figs 2.20 and 21 demonstrate examples characteristic of **all** the results of culturing E16 LGN for 5 days with various choices. The most dramatic result is the contrast between the florid invasion of P6 occipital cortex and the virtual avoidance of

P6 cerebellum (Fig. 2.21B-D). In more than 53 cultures involving LGN and cerebellum (from P1-8, E18, n=4; E21, n=4; P1, n=4; P3, n=21; P6, n=8; P8, n=4), I have seen only a tiny number of axons actually penetrating the cerebellum. Axon growth was mainly restricted to a few fasciculated bundles running around the edge of the cerebellar slice or across its surface.

Given the choice between occipital cortex and hippocampus (P3, n=4; P5, n=4; P6, n=4), LGN axons showed no preference in terms of the density of axons invading the tissue (Fig. 2.20D; typical of all cultures examined). Hippocampal slices were innervated almost as densely as neocortical slices, but, interestingly, the axons mostly continued up to the surface of the hippocampus and did not tend to terminate specifically in the middle layers, as in occipital cortex of this age. This implies that the hippocampus is permissive to axon invasion, as is the neocortex, but has no precise equivalent to the stop signal expressed by neocortical layer 4.

2.14 Lack of regional selectivity for thalamocortical innervation in culture

My starting question concerned the mechanisms by which thalamic axons from different nuclei find their way to the appropriate cortical fields. I have demonstrated a axon-growth-promoting influence of occipital cortex on LGN (indeed of neocortex on thalamus, in general) at E16 (which might appear even earlier), and a growth-permissive property that appears around E19 for the occipital cortex. If these signals produced by the cortex were specific for each particular field and acted preferentially on the equivalent thalamic nucleus, they might account for the selective guidance of thalamic axons to and into the correct cortical area. Following Sperry's (1963) notion of chemoaffinity, a mere gradient of one or more molecular species from one part of the hemisphere to the opposite pole might be sufficient to establish topographic order in the

input.

I tested these ideas by using the choice protocol, culturing explants of either LGN or some other region (more anterior or medial) of the developing thalamus, each flanked by slices of two different regions (frontal and occipital) of cortex (Fig. 2.20A-D demonstrate examples characteristic for all of the 42 cultures with LGN: E20, n=4; E21, n=4; P1, n=6; P3, n=5; P5, n=4; P6, n=10; P8, n=5 and 23 cultures with anterior thalamus (Ant Thal): E19, n=4; E20, n=4; P1, n=6; P3, n=4; P8, n=5). The result was unequivocal: the pattern of innervation was indistinguishable whatever regions of thalamus and cortex were combined. Fig. 2.20C is an example representative of all of these cultures (P6, n=10), demonstrating the very similar axonal outgrowth from an E16 LGN explant into P6 occipital cortex and P6 frontal cortex (which the LGN never innervates in vivo). Equally, explants of E16 anterior thalamus and more ventral thalamus sent axons in a similar pattern into occipital, frontal, or any other region of cortex (Fig. 2.20B demonstrates an example characteristic of all of the P1 cultures; n=6).

One might imagine that regional specificity of the diffusible factor alone, evident only at an earlier age when thalamic axons are leaving the thalamus and finding their way through the intermediate zone to the correct cortical region, might be sufficient to explain their selective guidance. However, I saw no hint of any difference in the degree of axonal outgrowth from thalamic explants, even when cultured with slices of E19 cortex, whatever the site of origin of thalamic and cortical explants.

These results, if they apply in vivo, make it unlikely that a remote regionally specific influence of cortical areas on thalamic nuclei is responsible for the topography of thalamocortical projection (see General Discussion).

2.15 Early Corticothalamic Projections in Culture

Recently, McConnell et al. (1989) have shown that cells of the cortical subplate, the first postmitotic neurons of the developing cerebrum, send a pioneering projection down toward the thalamus in the cat and the ferret before the cells of the lower layers of the cortical plate proper have even been born. They suggested that this pioneering pathway might play a part in guiding subsequent thalamocortical innervation (and possibly other later corticofugal projections too). This is an attractive notion, which would remove the need for any positionally specific cortical trophic influence on thalamic axons. However, it remained to be established that this subplate projection is positionally ordered, with each cortical zone sending its subplate axons only towards to the appropriate region of the thalamus and interacting only with axons from that nucleus. If this is the case (and results in Chapter 3 show that it is partly true), it displaces the need for a mechanism of positional guidance from thalamocortical to corticothalamic projections. Perhaps instead of searching for a selective remote effect of cortex on thalamus, one should expect to find a regionally specific influence of developing thalamus on the outgrowth of subplate axons.

I tested this possibility by culturing rat thalamic explants with slices of embryonic cortex, taken as early as E14.5 (E14.5, n=12; E15, n=12; E16, n=24; E16.5, n=12; E18, n=4), when the first neurons of the cortex proper are just being born (Miller, 1988) and only the preplate cells have completed their migration. In these experiments, I preincubated the cortical slice with DiI to label corticofugal axons. Fig. 2.22 summarizes the results by presenting representative examples to all of the cultures (Table 2.1I). In the absence of a neighbouring thalamic explant few if any axons grew out of these embryonic cortical fragments. But in co-culture, axons, presumably from

subplate cells, did grow out of the cortex, especially on the side of the thalamic block. The fibres from E14.5 cortical slices did not enter the thalamic blocks in large numbers, they grew around the edges of the thalamic explant rather than penetrating it (Fig. 2.22A-D). At E16 there was more abundant outgrowth and the fibres also penetrated the thalamus, although the innervation was still less profuse and much more variable in its pattern than the thalamocortical innervation described above (Fig. 2.22 E and F). However, again there was no obvious positional preference. Any region of embryonic cortex innervated equally well any region of developing thalamus, even when confronted by both correct and inappropriate thalamus in the choice paradigm.

It could be argued that, although only preplate cells were in position and mature at the earliest ages (E14.5-16) at which cortex was taken for these experiments, the connections to the thalamus seen here might have originated from neurons that migrated into position in other layers during the 4-5 days of culturing. In particular, they might have come from conventional corticothalamic cells of layer 6, which are in position and send out axons by about E17 in vivo (Chapter 3). Such an outgrowth from cells of the cortical plate proper might have obscured a regionally specific outgrowth of true subplate cells. Although hard to dismiss completely, I think this possibility unlikely. Even from E14.5 cortices axons left as early as the first day in culture and they had grown as far as 1.5-2 mm after 4 days in culture (Fig 2.22). To resolve this issue back-labelling studies, combined with birthdating, would be required.

If these results apply in vivo, and if fibres from subplate to thalamus nevertheless establish ordered guidance paths along which thalamic axons grow to the correct areas of cortex (Chapter 3), they must do so by means of some mechanism other than a positionally specific gradient of attraction from the thalamus.

Fig. 2.1

Schematic diagram of the procedure for the collection of tissue for co-culture. At the top are drawings of the neonatal rat brain (*left*) and the fetal brain (*right*). Small thalamic blocks were taken from E14.5-17 rat fetuses, usually from the dorsolateral aspect of the diencephalon, as shown at the top right. The posterior blocks (shown very schematically in light stippling) contained almost exclusively LGN, whereas the more anterior blocks (coarse stippling) definitely excluded LGN and consisted of fragments of thalamic nuclei that would normally have connected to more frontal areas of cortex. In some cases, more ventral and lateral thalamus was explanted. The thalamic explant was placed on the collagen-coated microporous membrane of a Costar Transwell-COL culture chamber (*lower diagram*) close to one or more slices of tissue from fetal (E14.5-21) or postnatal (P0-11) rats. The upper left illustration of the neonatal rat brain shows the positions from which slices were cut. Slices of, occipital (OCC) cortex (putative area 17) frontal (FRO) cortex (putative sensorimotor cortex) or hippocampus (HIP) were excised, as shown by the stippled regions, from coronal sections cut at the anteroposterior levels indicated. In some experiments sagittally cut slices of cerebellum (CER) were used.

The lower photograph shows the 6-well tissue culture dish. The diameter of each well is 3.5 mms.

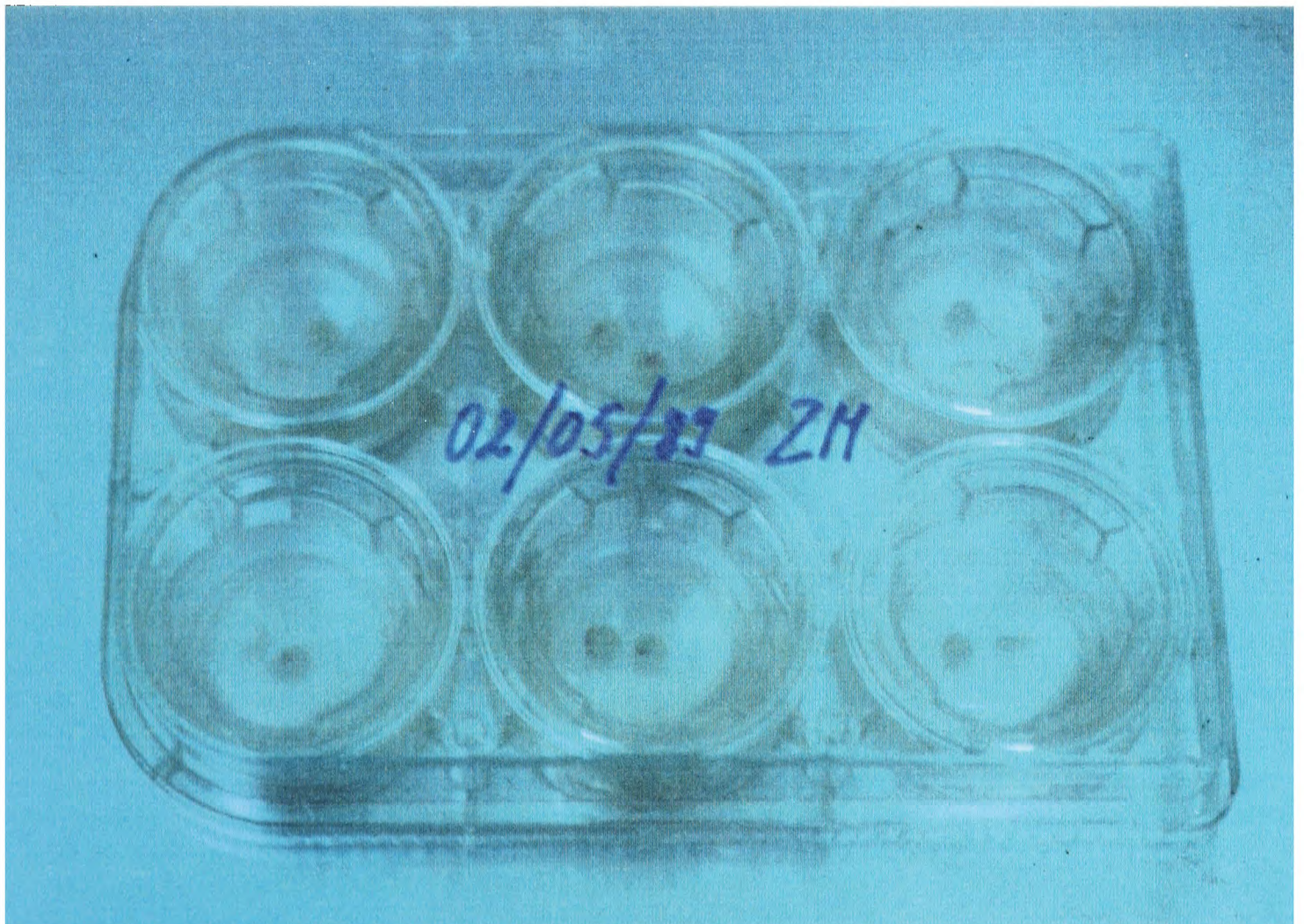
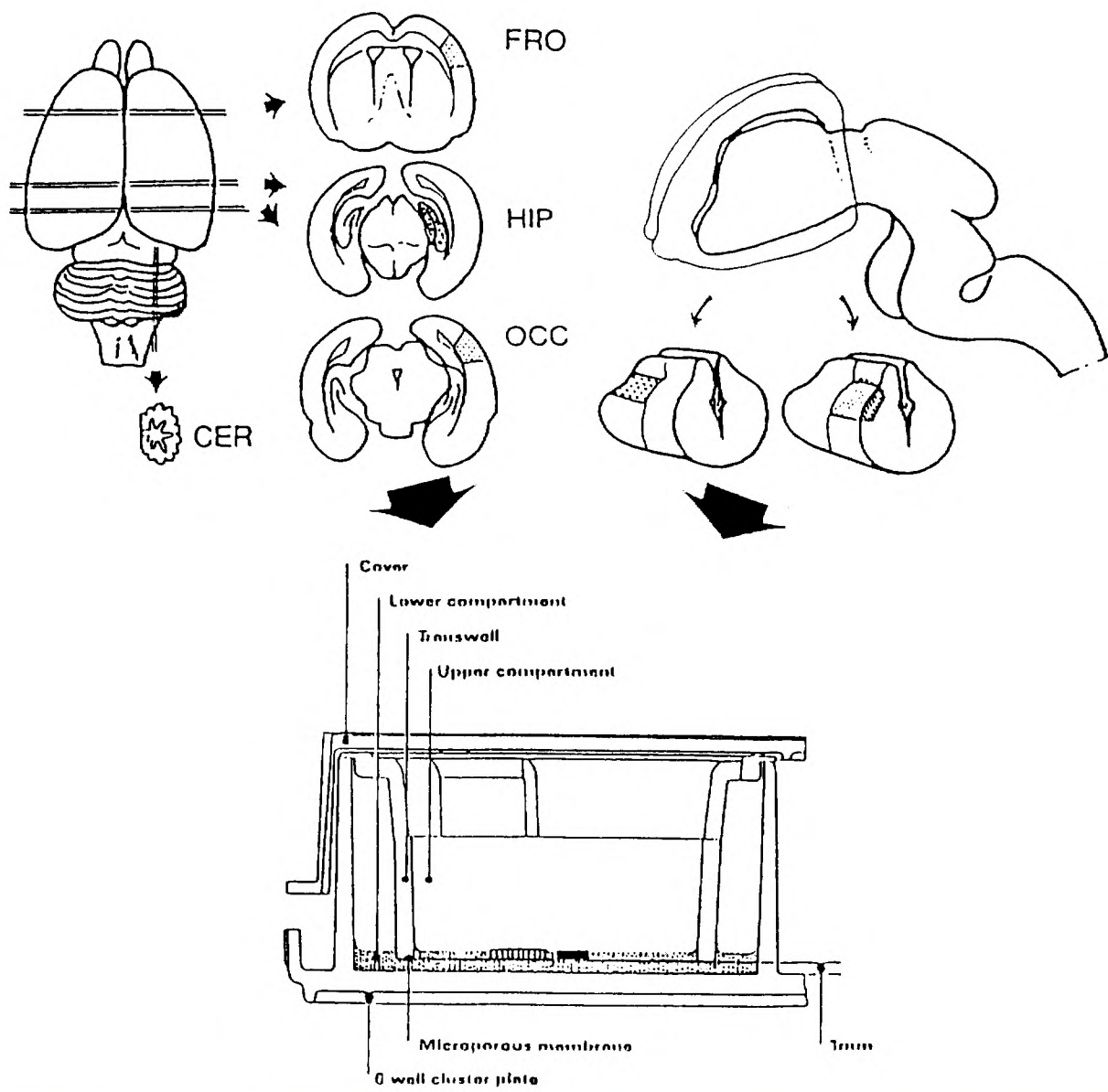


Fig. 2.2

Under phase-contrast microscopy the fibre outgrowth from outside tissue blocks could be examined and followed in living slices.

A.: A 'tissue bridge' (arrow), containing cells and fibres, formed between the co-cultured explants after 6 days in vitro on a laminin-coated Petriperm tissue culture dish. The E16 LGN block is to the right (LGN), the ventricular surface of a slice of P0 occipital cortex (CTX) is to the left. There is a considerable spread of the cells on the membrane below the plane of the tissue bridge (not in focus).

B.: Cells migrated into the space between the explant of a P0 cortex (CTX) and a E16 LGN (LGN; 6 days in vitro) on a laminin-coated Petriperm membrane. There is more cell spread from the LGN in the direction of the cortical slice, whereas fibres leave the LGN block in all directions, though more densely from the side facing the cortical slice (see Fig. 2.3 for higher magnification). Scale bar in A: 100µm; in B: 500µm.

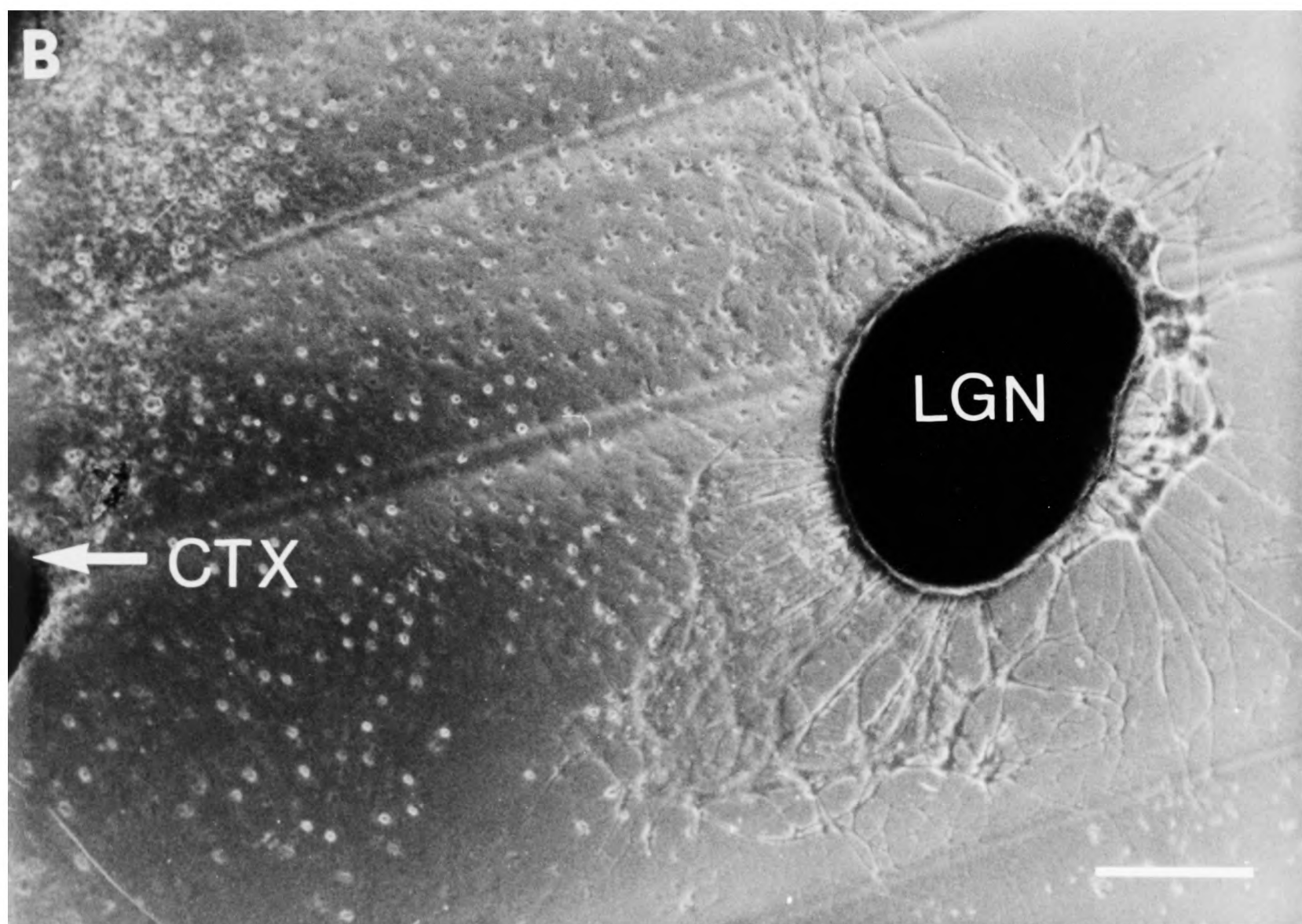
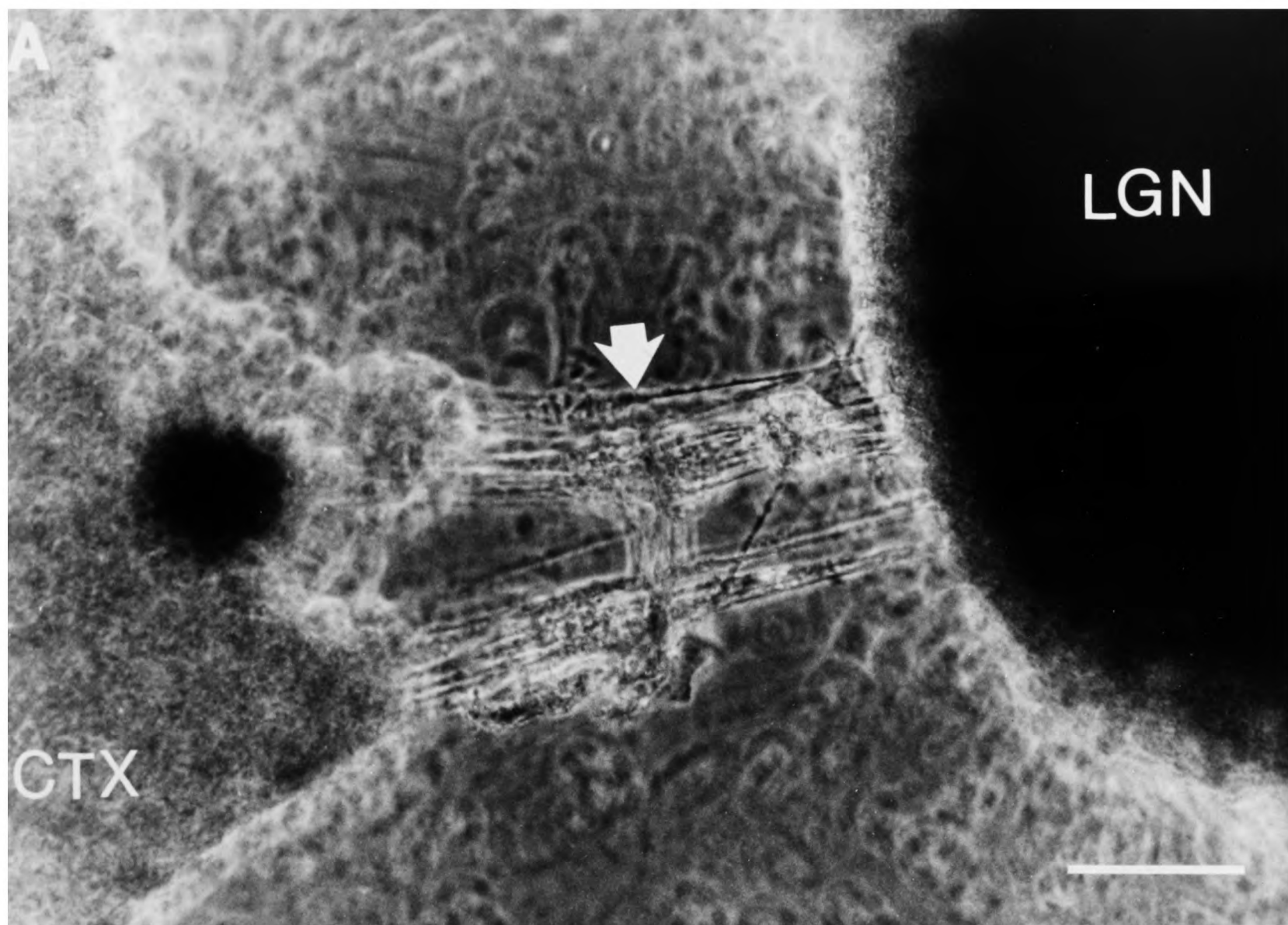


Fig. 2.3

Higher-power phase-contrast montage from Fig. 2.2B showing cell migration into the space between the explants for a co-culture of P0 cortex (CTX) and E16 LGN (LGN) on laminin-covered Petriperm membrane (6 days in vitro). There is a considerable amount of cell migration, as well as fibre outgrowth, in the direction of the cortex (arrow, CTX). There is much less cell migration on the other side of the LGN explant and close examination shows that the density and mean length axon outgrowth from that side are also lower than from the side facing the cortex. Individual fibres extend as far as 2mms from the LGN block in the direction of the cortical slice.

Scale bar: 500µm

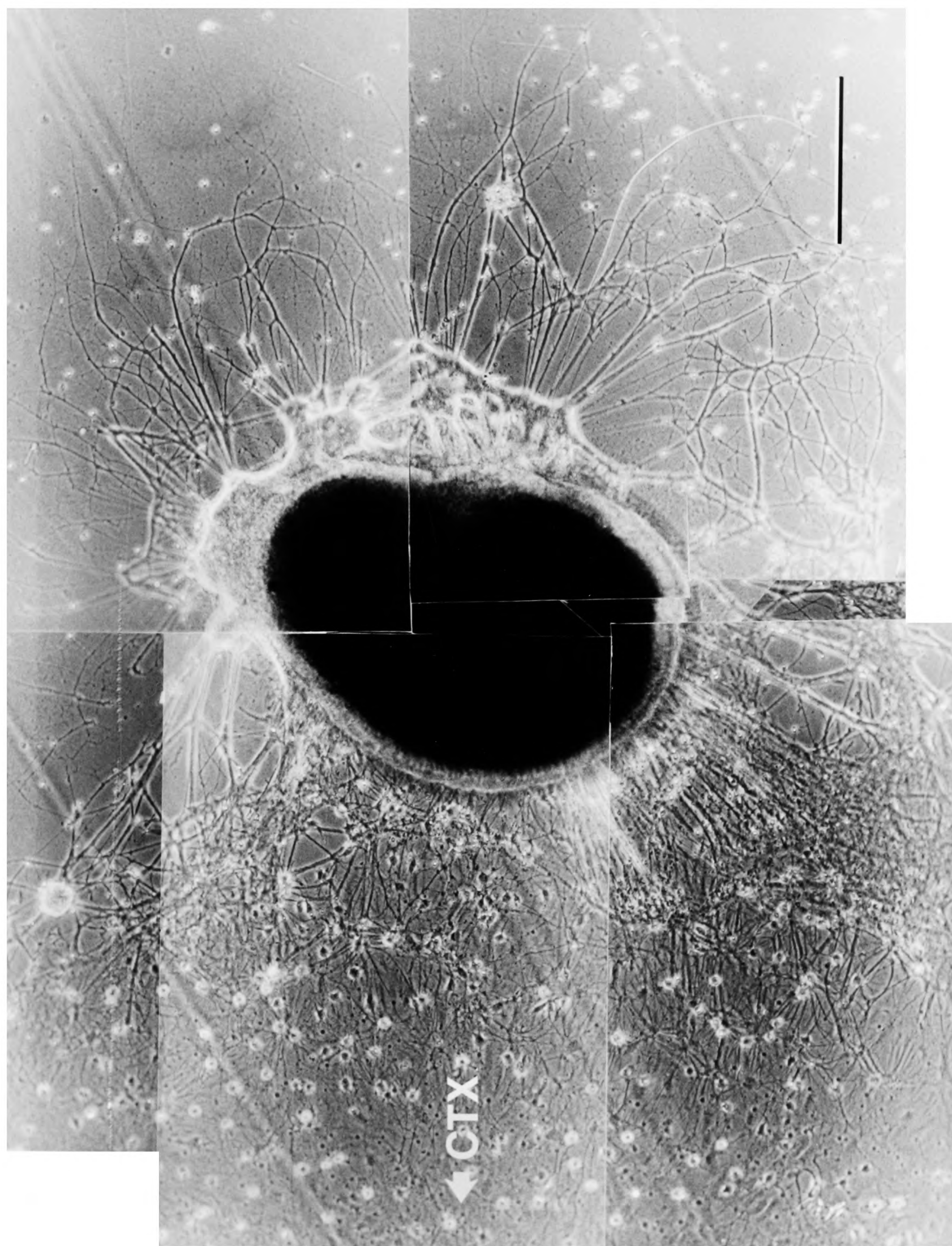


Fig. 2.4

An E16 LGN block was cultured on a laminin coated Petriperm dish for 6 days with (A,B) and without (C,D) cortical slices (taken at E16) in the dish. The cultures were fixed with 4% paraformaldehyde and examined in a phase-contrast microscope.

A: At the end of 6 days in vitro cells and fibres are seen to have left the E16 LGN block, which was surrounded with 6 pieces of occipital cortical slices, arranged around the circumference of a 5mm diameter circle, with the thalamic explant in its centre.

B: Higher-power view of fibres taken from the region indicated by the square in A.

C: At the end of 6 days in vitro thalamic fibre outgrowth and cell migration was much less abundant from the thalamic explant when the Petriperm dish did not contain any cortical slices.

D: Higher-power picture, taken from the region indicated with the square in C.

Scale bar in A and C: 300µm. In B and D:50µm

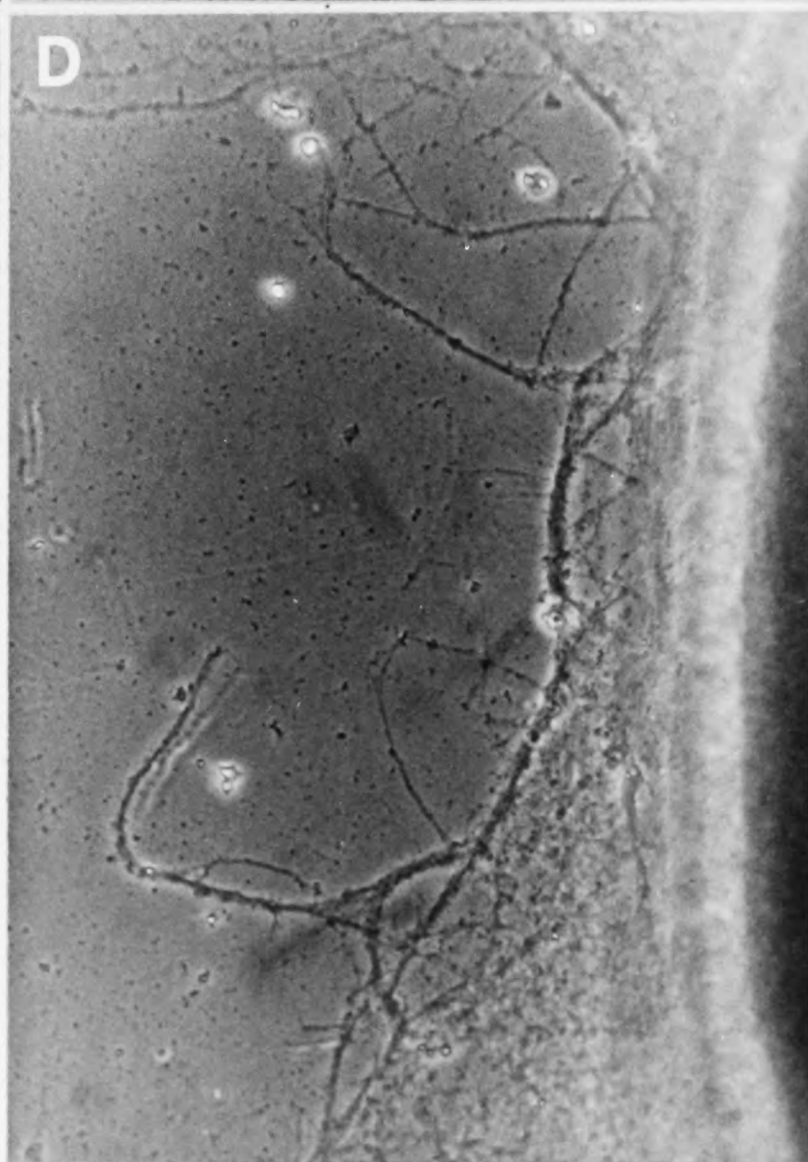
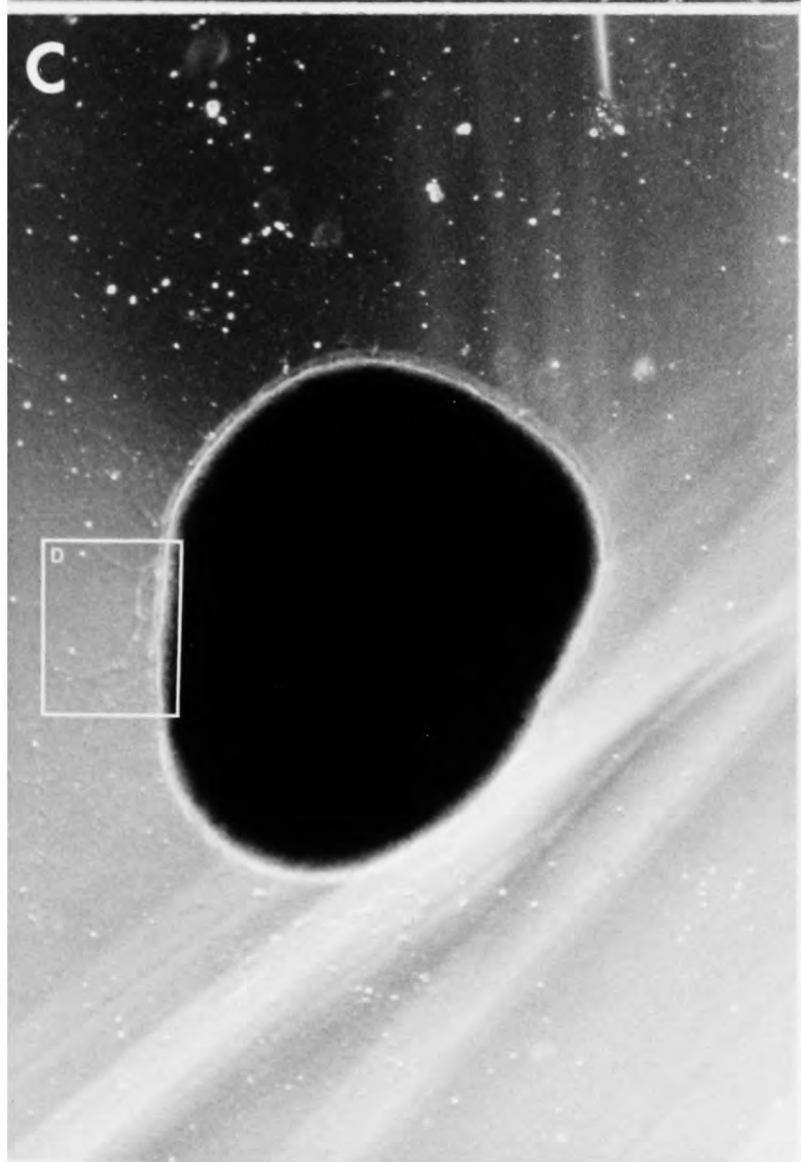
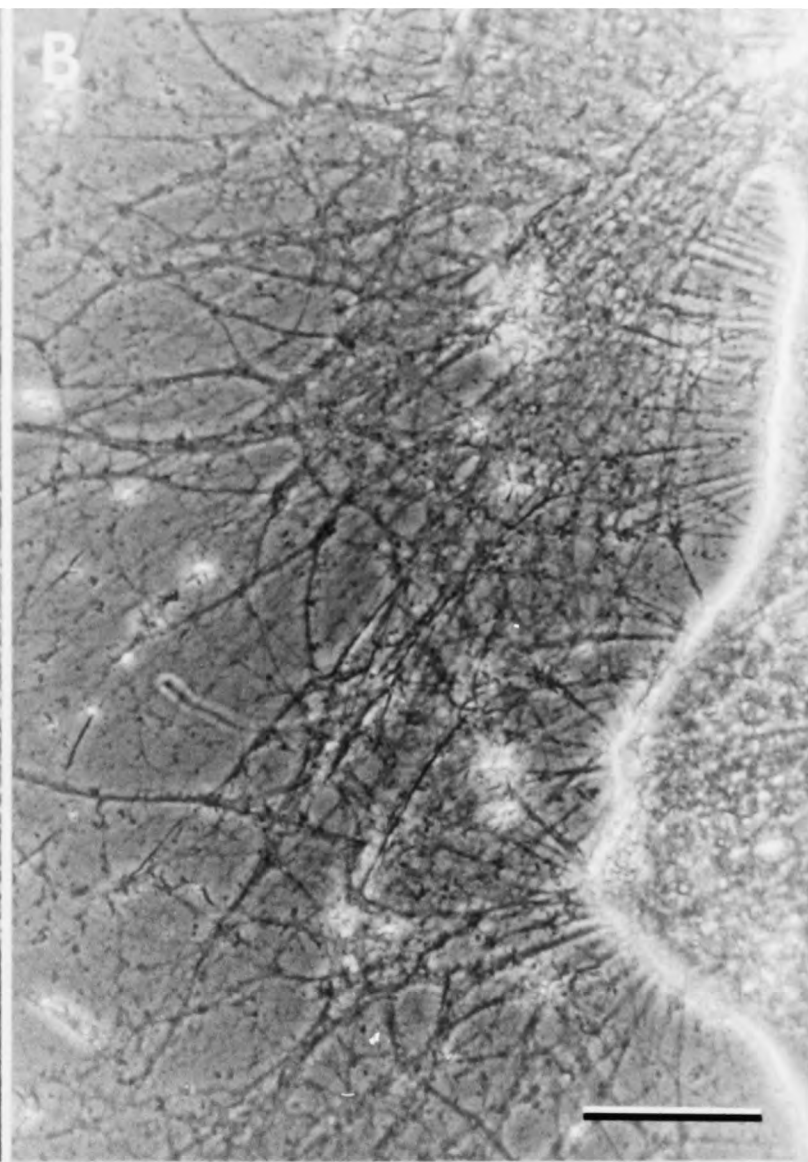
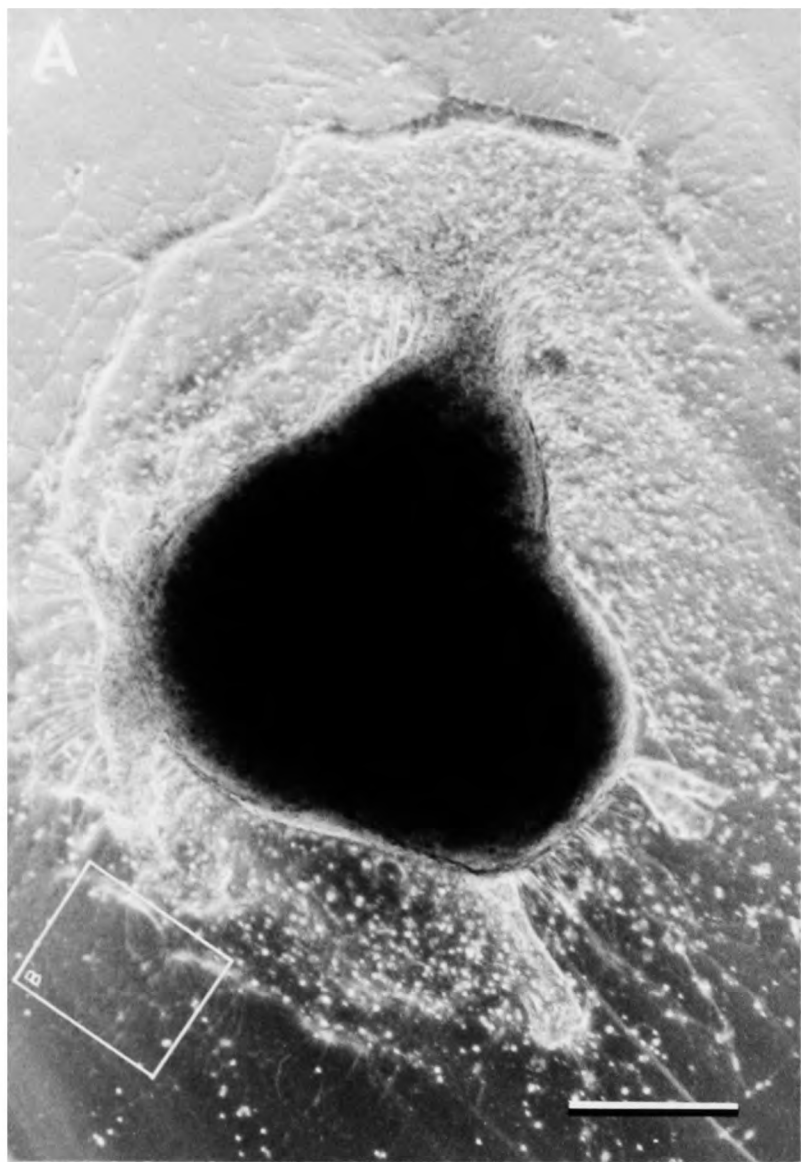


Fig. 2.5

Fibre outgrowth appears rather different on collagen-coated (Transwell Col) membranes to that observed on Petriperm dishes (see Figs. 2.2-2.4).

A and C: An E16 thalamic block was prelabelled with DiI, flanked with two occipital cortical slices (taken at P0) and cultured for four days. The borders of the cortical slices (CTX) are marked with dotted lines. Many fibres grew out onto the membrane and extended over a distance more than 500 μ m. C: High-power fluorescent micrograph from the region indicated with a square in A, centered some 300 μ m from the edge of the thalamic explant.

B and D: A DiI prelabelled E16 thalamic block was cultured on its own for 4 days under the same conditions as A but without cortex in the dish. D: High-power picture from the region indicated with a square in B at the very edge of the thalamic block. The fibre outgrowth that does occur is much less shorter than with cortex present (compare C). Note that the high-power pictures were taken at different distance from the edge of the thalamic explant.

Both in laminin-coated (Petriperm) and collagen-coated (Transwell Col) dishes thalamic outgrowth was stimulated by the presence of cortical explants in the same dish.

Scale bar at A and B: 300 μ m; C and D: 50 μ m.

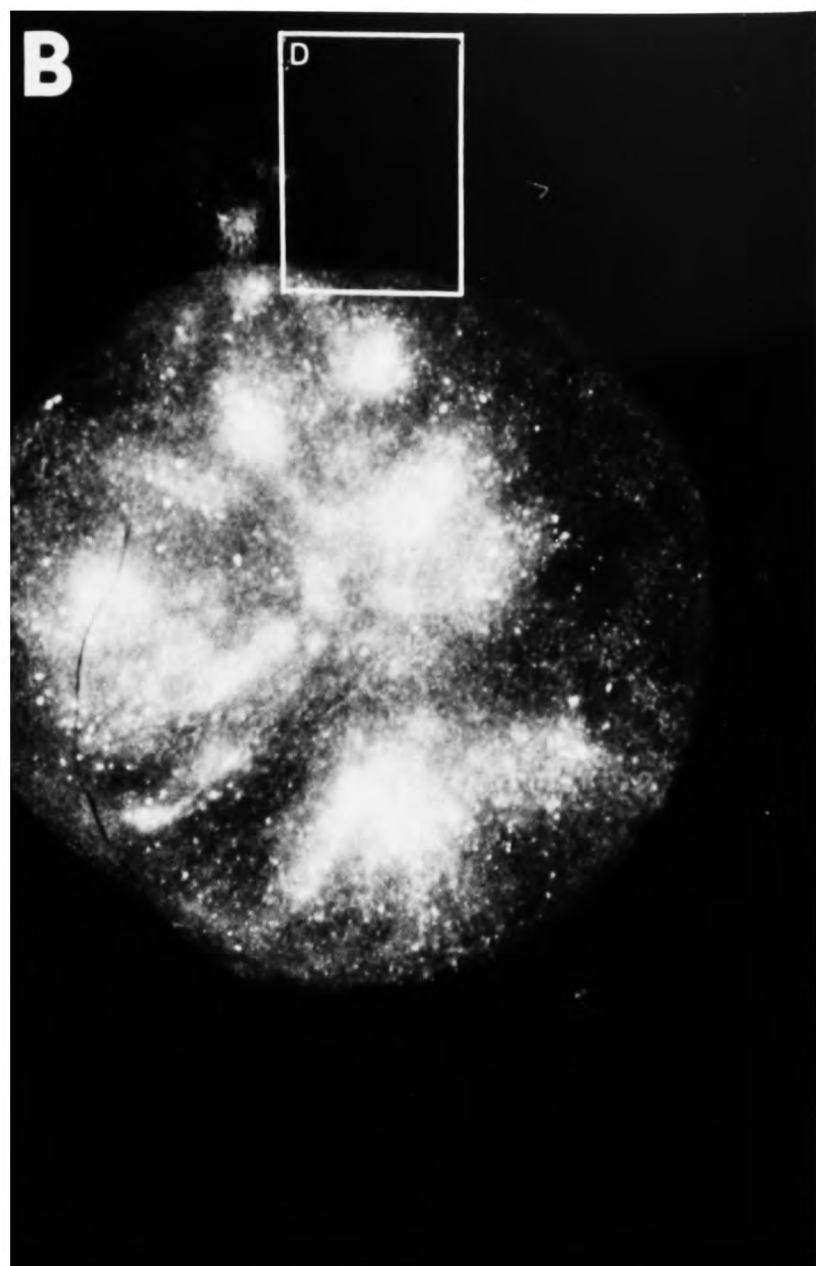
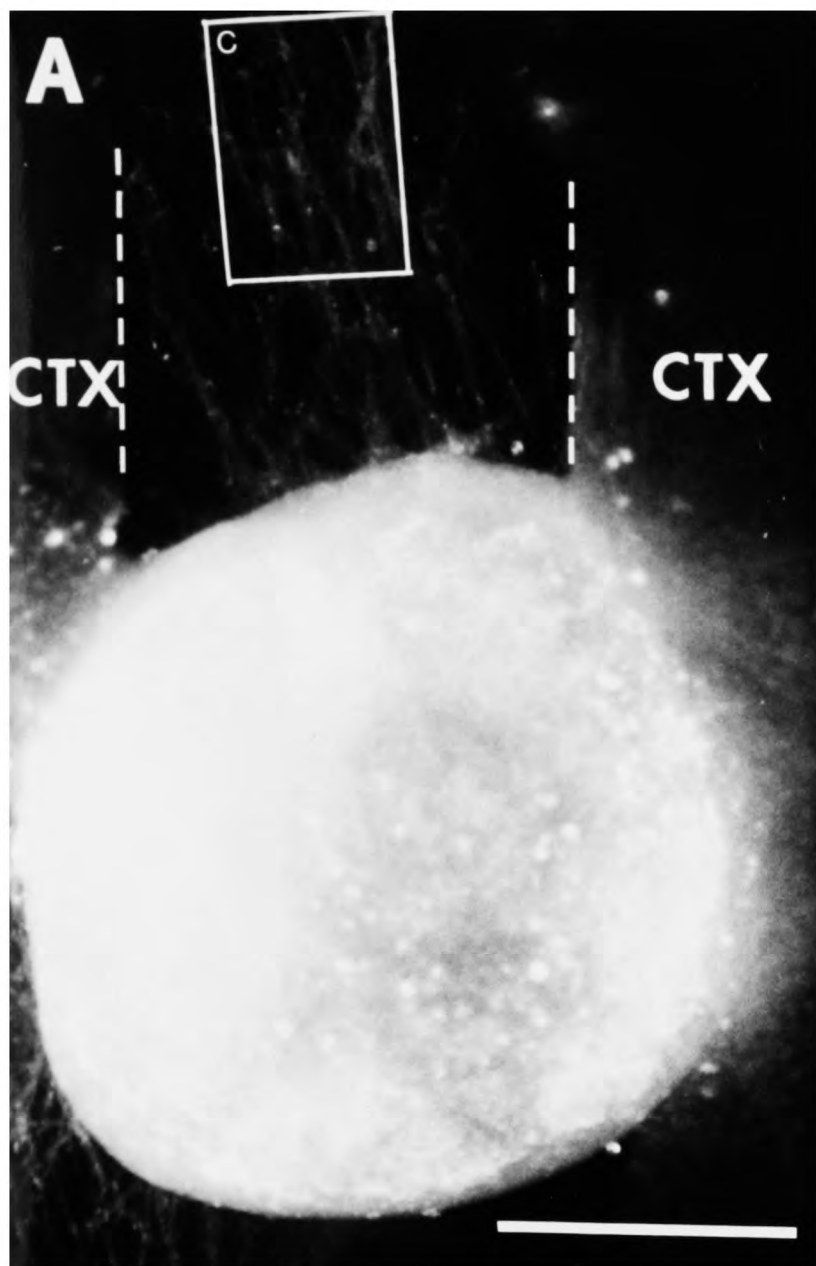


Fig. 2.6

These photomicrographs were taken from a Nissl-stained section of a co-culture (P0 cortex; E16 LGN) after 7 days in vitro.

A: The culture was fixed, cryoprotected and 30µm thick sections were cut parallel to the culture chamber membrane to reveal the histological appearance. The LGN (left) is closely fused to the cortical slice (right). The white matter (wm) and the different layers (1-6) are labelled on the cortical culture.

B: High-power view of the cortex. The layering is marked.

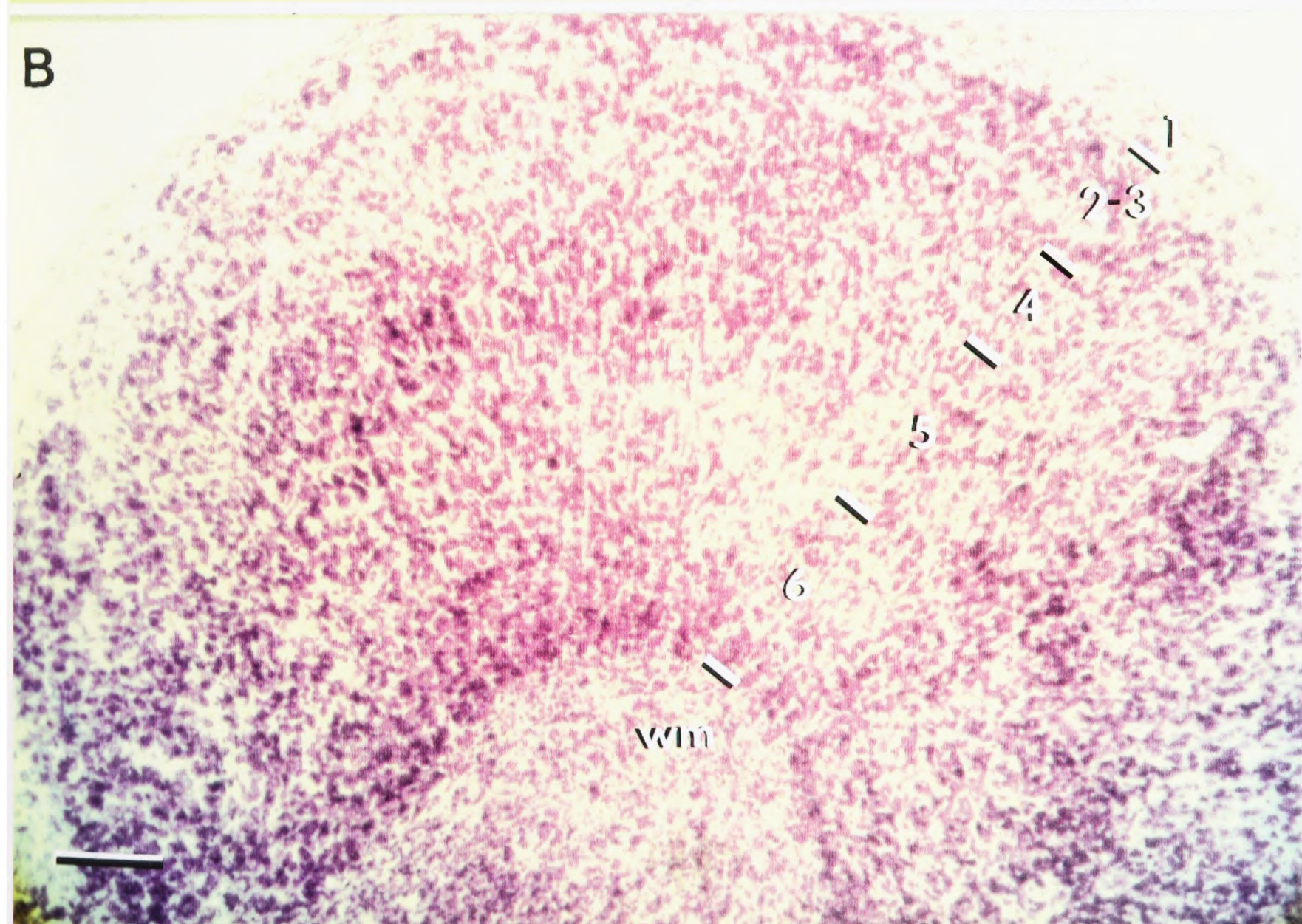
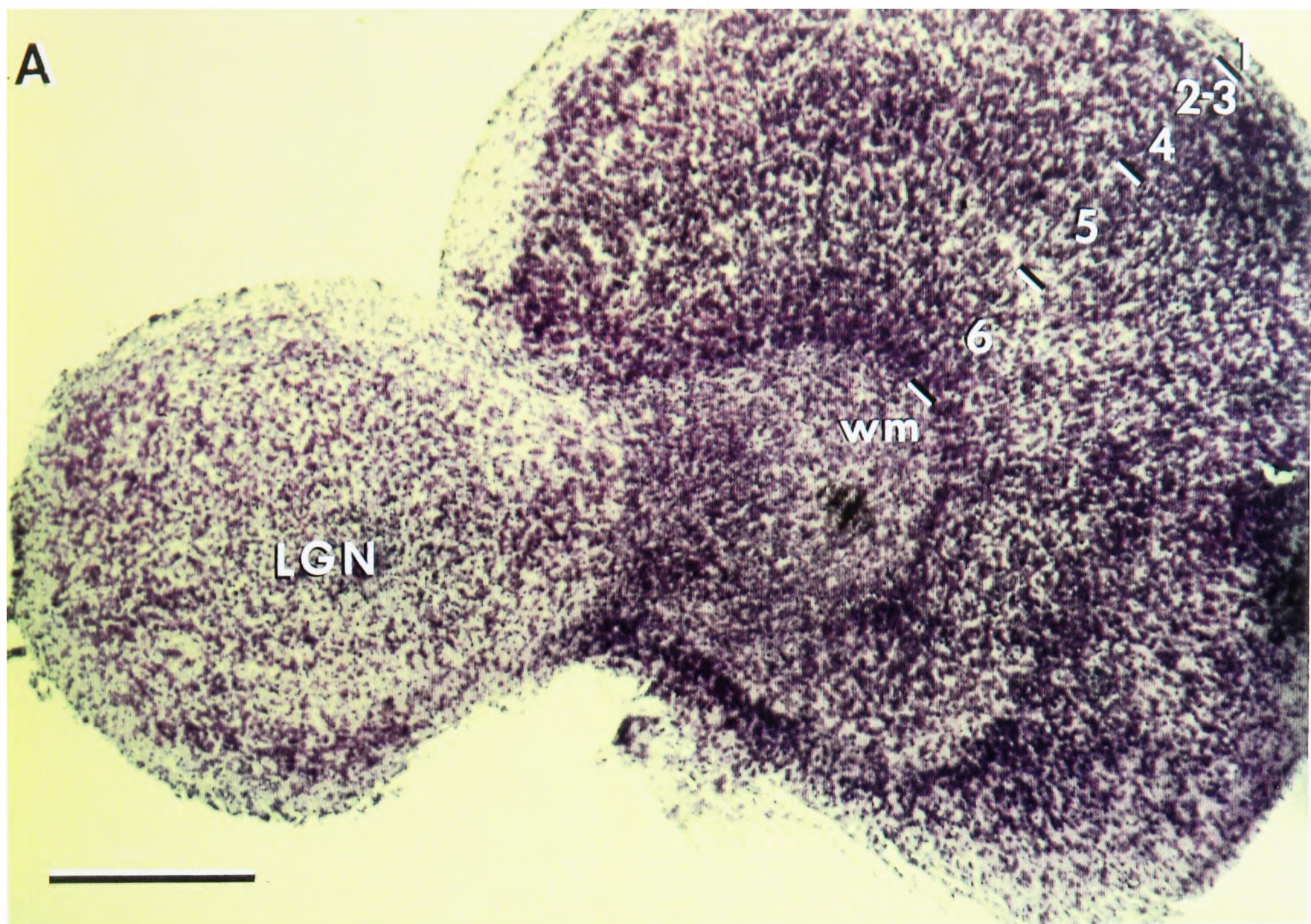


Fig. 2.7

Comparison between the formation of layering in vitro and in vivo. To match the cortices developed in vivo and in vitro, the cultured slices, 150-200µm thick, were frozen and then resectioned (20µm) with a cryostat in the original coronal plane (tangential to the microporous membrane) and compared with normal coronal sections from animals of various ages. The sections are oriented with their pial surface up and ventricular surface down.

A: 20µm-thick, Nissl-stained coronal section taken from the occipital pole (putative visual cortex) of a newborn rat. Layer 6 and 5 are in position, whereas the upper layers have yet to be formed. (WM, white matter; dcp, dense cortical plate; MZ, marginal zone).

B: A 20µm-thick section of an occipital cortical culture taken at birth and cultured alone for 2 weeks. By comparing the normal and cultured slices (A and B) it is evident that changes have occurred in vitro, with the upper layers becoming thicker and differentiated into layer 4 and layers 2/3. Some of the thickening of the upper layers is simply due to enlargement of the neuropil and consequent separation of individual cells. However it seems likely (in view of other evidence; see text) that some migration has continued in culture.

Scale bars.: 100µm

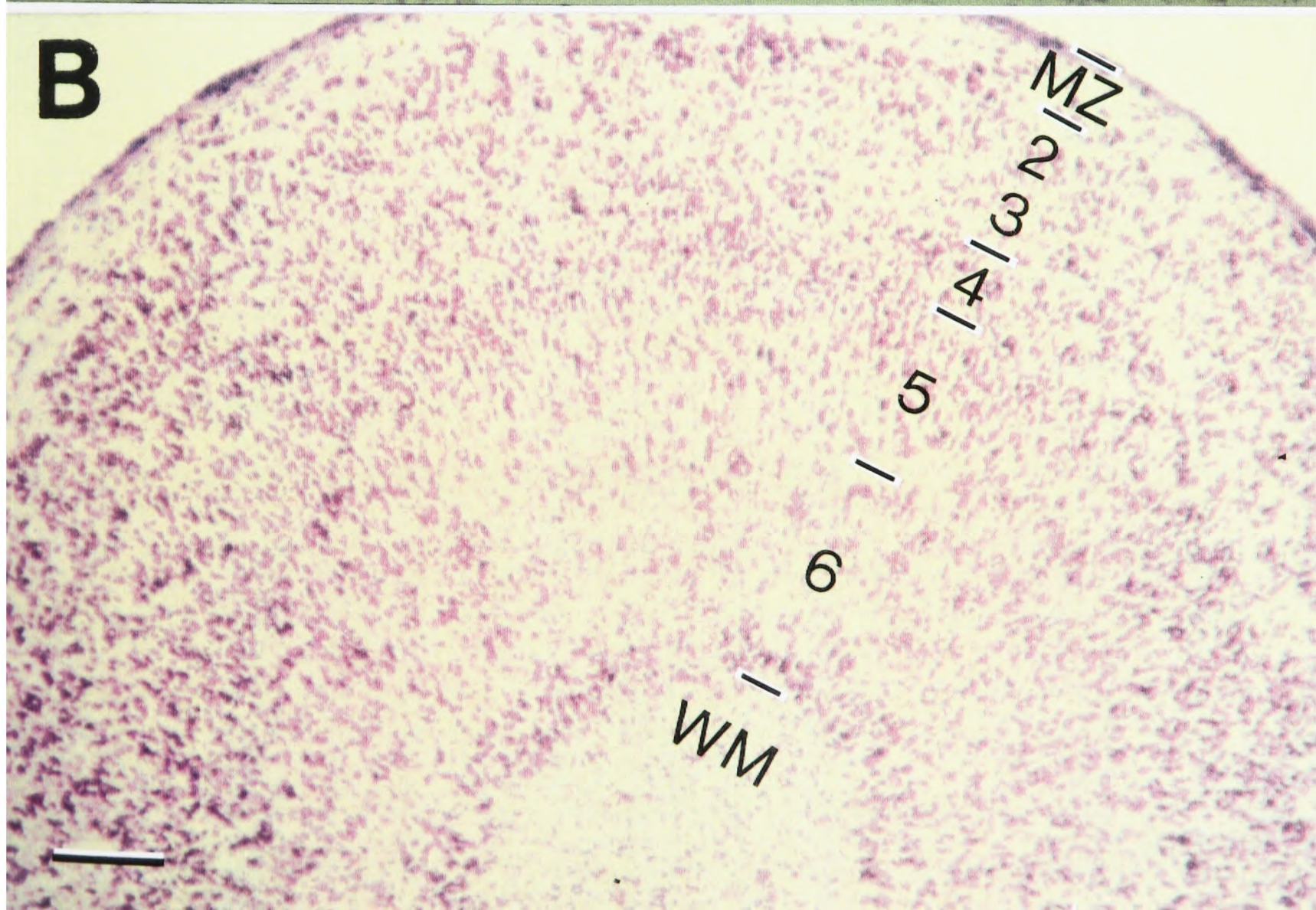
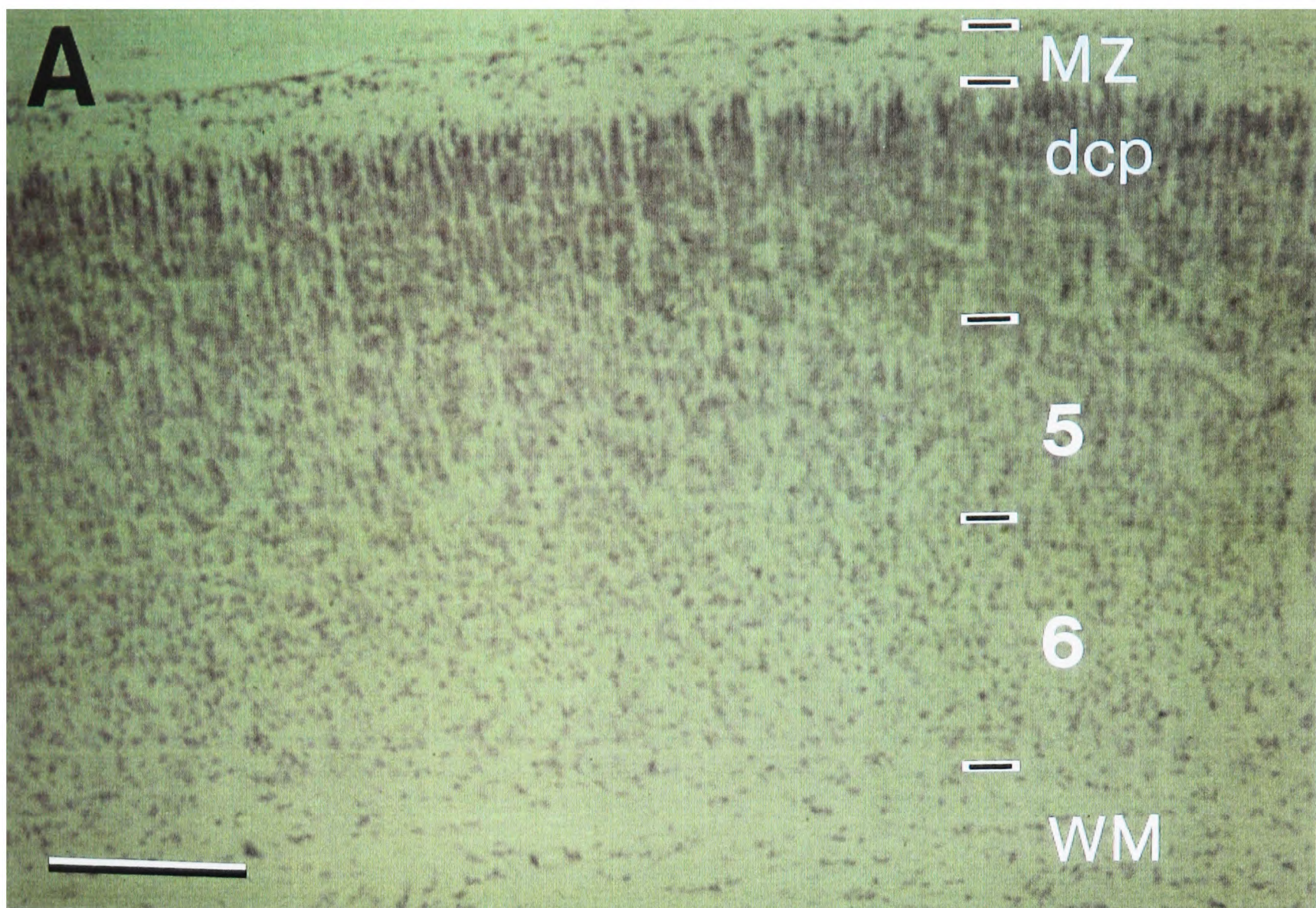


Fig. 2.8

The fluorescent chromatin stain, bizbenzimidazole, was used to reveal the layering of cultures under uv illumination.

A: Dark-field fluorescent uv photomicrograph of a cultured occipital cortical slice, taken at P6, after 4 days in vitro. The layering is more advanced than in a cortical slice, taken at birth, after 7 days in culture (see Fig. 2.6B). Scale bar: 100µm.

B: Parasagittal sections of newborn cerebellum were cultured for 4 days. The bizbenzimidazole counterstaining revealed the white matter, the so-called laminae albae (LA), within the cerebellar folia. Scale same as A.

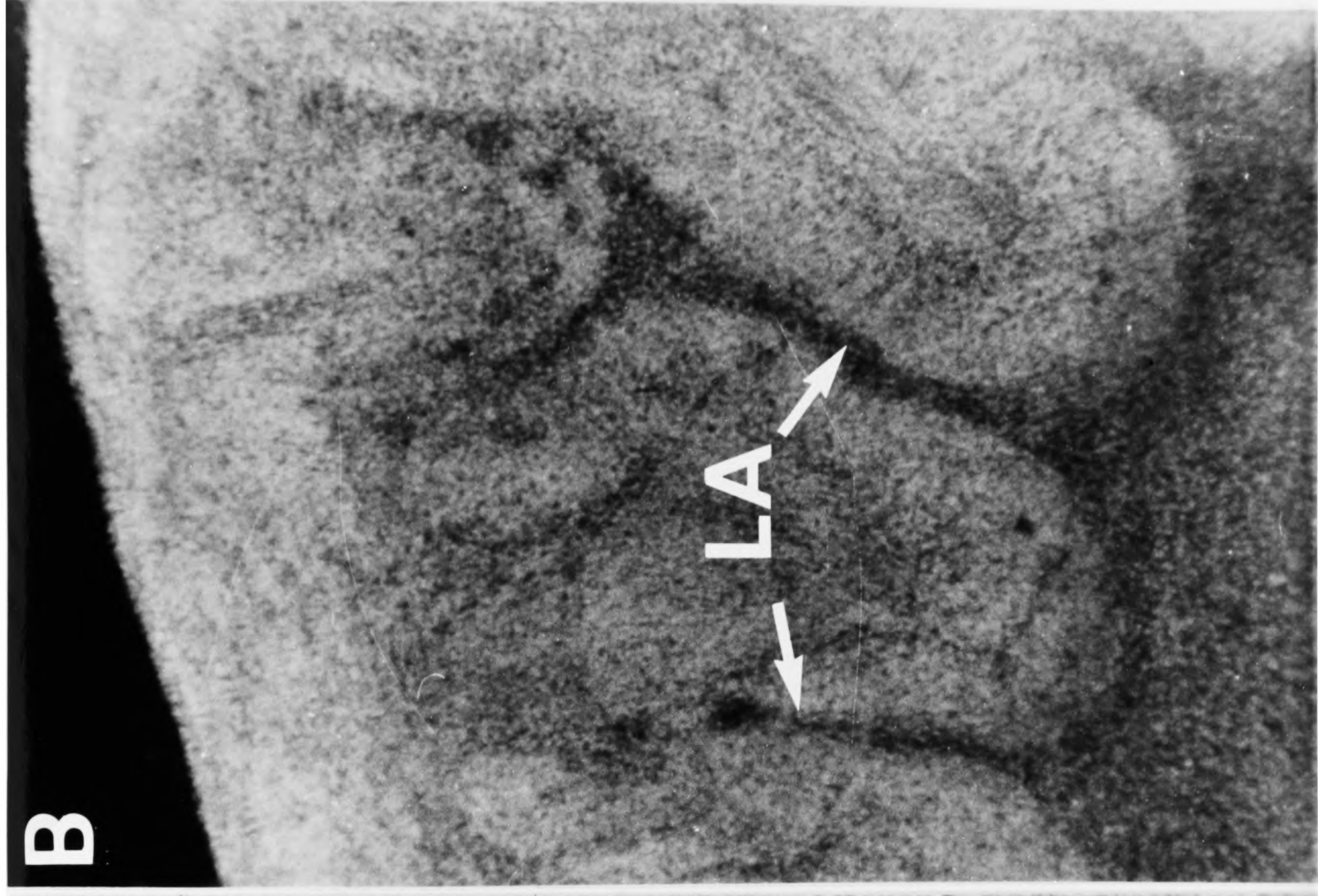
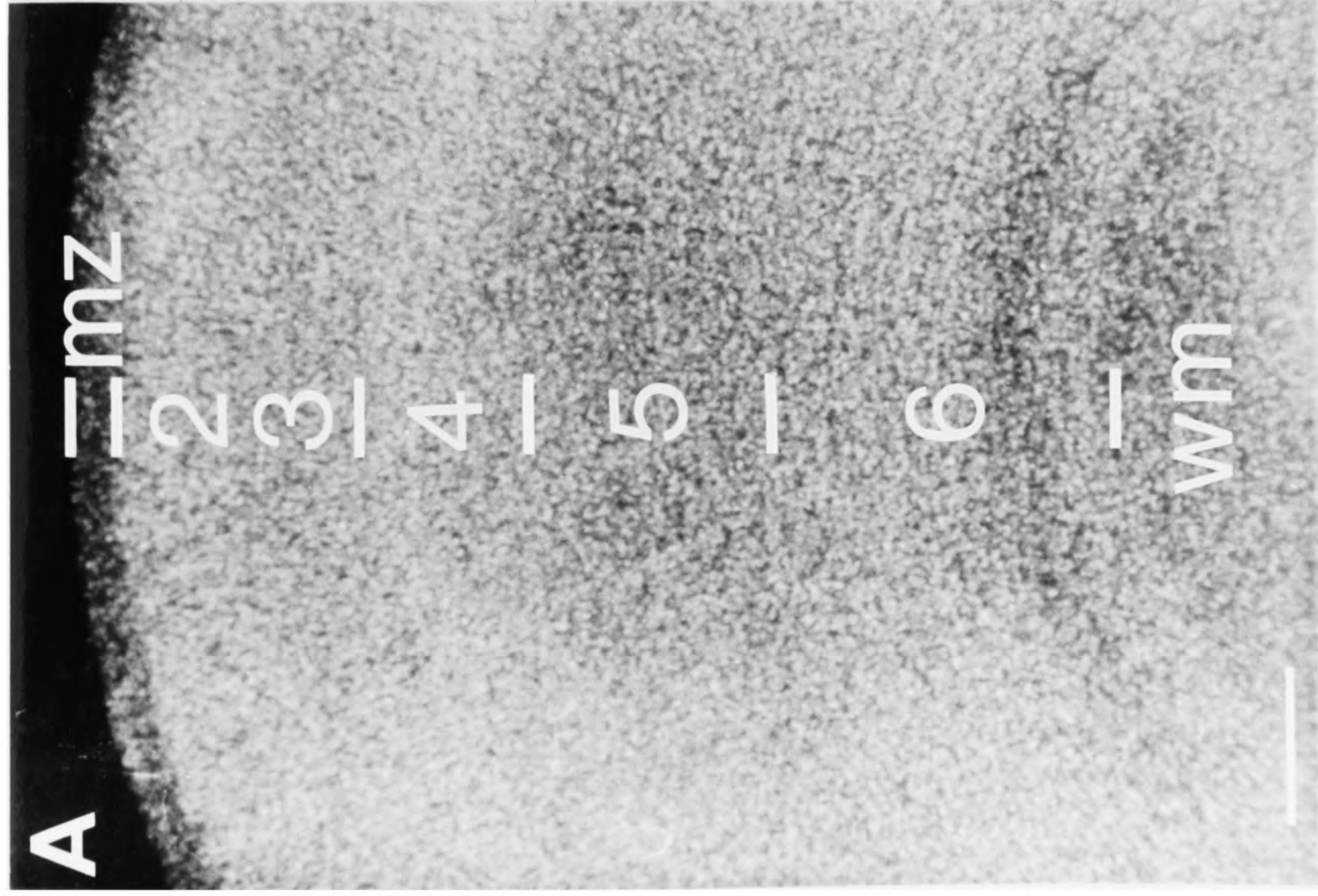


Fig. 2.9A

This photomicrograph was taken from a 10 μ m-thick section of P0 cortex which was cultured for a week, then, after paraffin embedding, sectioned perpendicular to the membrane to reveal the histological appearance through the whole thickness of the culture. The surface that had occupied the medium-gas interphase is up, the surface contacting the membrane is down. The preparation shows that there is no obvious gliosis in the culture, and that the culture is more than 20 cell layers thick. In the lowest 20 μ m of the slice, which was in contact with the microporous membrane, we see smaller dense nuclei (which might indicate degenerative changes), but cells above, throughout the interior of the slice, appear healthy.

Fig. 2.9B

The cell organelles (nuclei, nucleoli, cytoplasm) and cell morphology are even more apparent in this semithin preparation of P0 occipital cortex, after 7 days in vitro. 1 μ m-thick slices were cut parallel to the culture surface and then counterstained with toluidine blue.

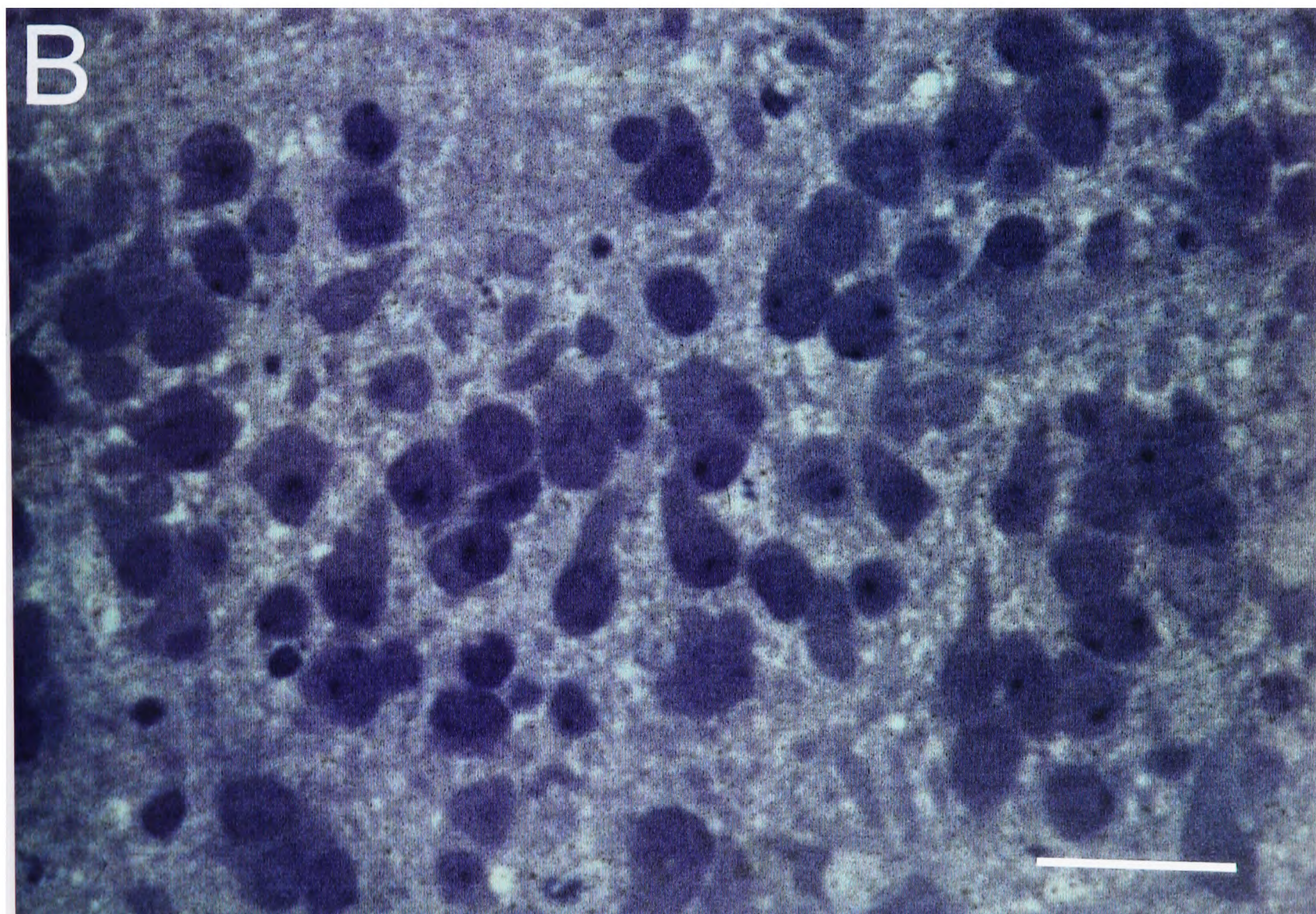
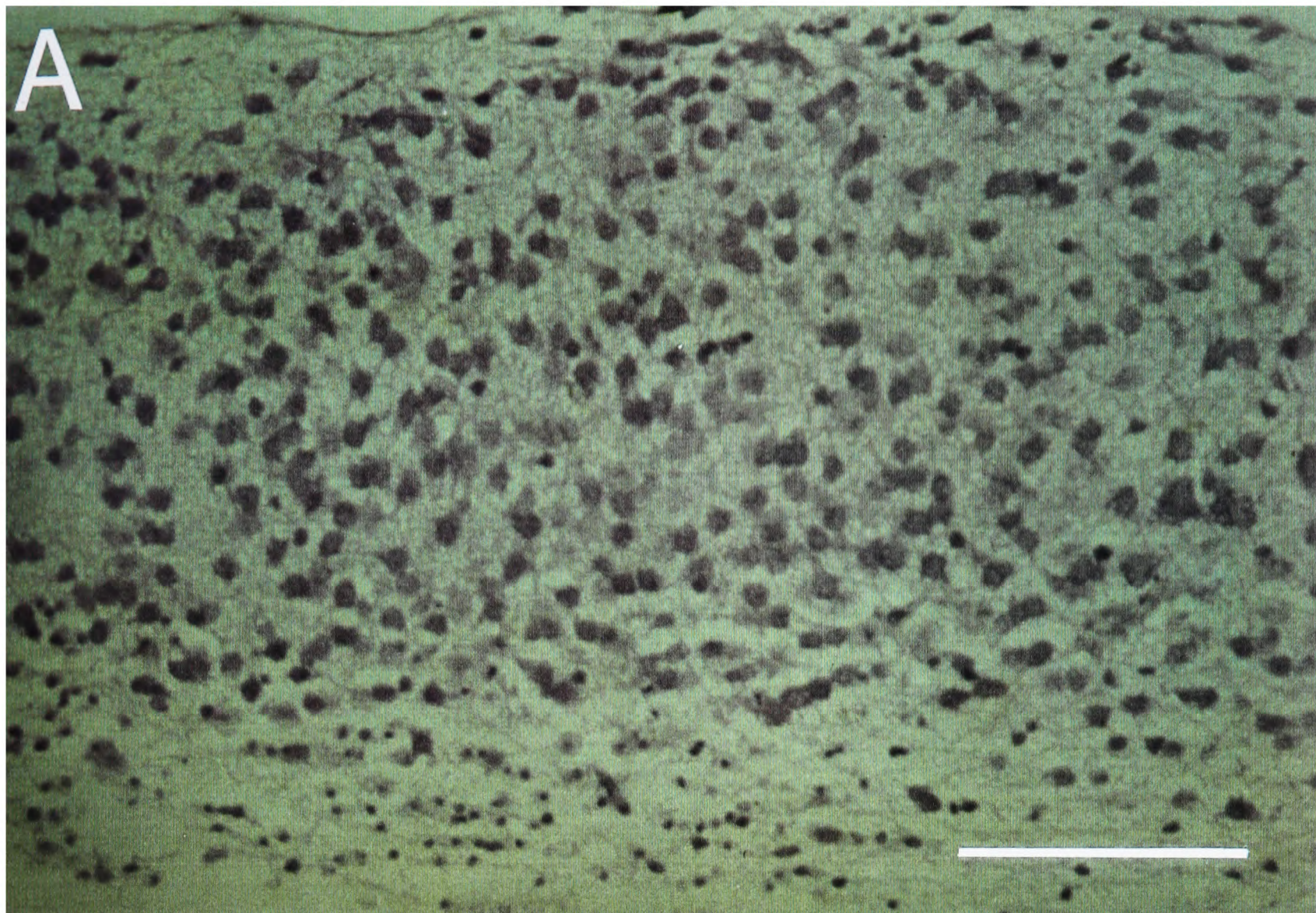


Fig. 2.10

The carbocyanine lipid-soluble dye, DiI, was used to reveal connections in living (A and B) and fixed (C and D) cultures.

A.: Fibres leaving a DiI-prelabelled LGN block (intense homogenous labelling at right upper corner) towards the cortical explant to the left (not seen). The fibres run in fascicles on the collagen coated microporous membrane (E16 LGN and P3 occipital cortex, co-cultured for 4 days).

B.: This photomicrograph was taken from the edge of a pre-labelled LGN explant after 20 days of culturing. The cell processes have lost their labelling (probably through membrane recycling): only the cell bodies contain the fluoroprobe.

The connections can be revealed in fixed tissue by applying a small crystal of DiI on to the cultures, which after incubation of 2 weeks at room temperature, labels the anterograde and the retrograde projections equally well.

C.: Anterogradely labelled thalamic fibres (from E16 LGN) within a cortical explant (P3) after 10 days in vitro. The crystal was placed on the LGN explant. The fibres show varicosities which might represent en passant synapses.

D.: Retrogradely labelled cell body in layer 6 (arrow) of a P2 cortical explant cultured with a P2 LGN block for 4 days. The culture was fixed in 4% paraformaldehyde, then a crystal of DiI was placed on the LGN. After 2 weeks incubation at room temperature the DiI revealed the cell bodies from which the corticofugal fibres originated.

Scale bar: 25µm for all

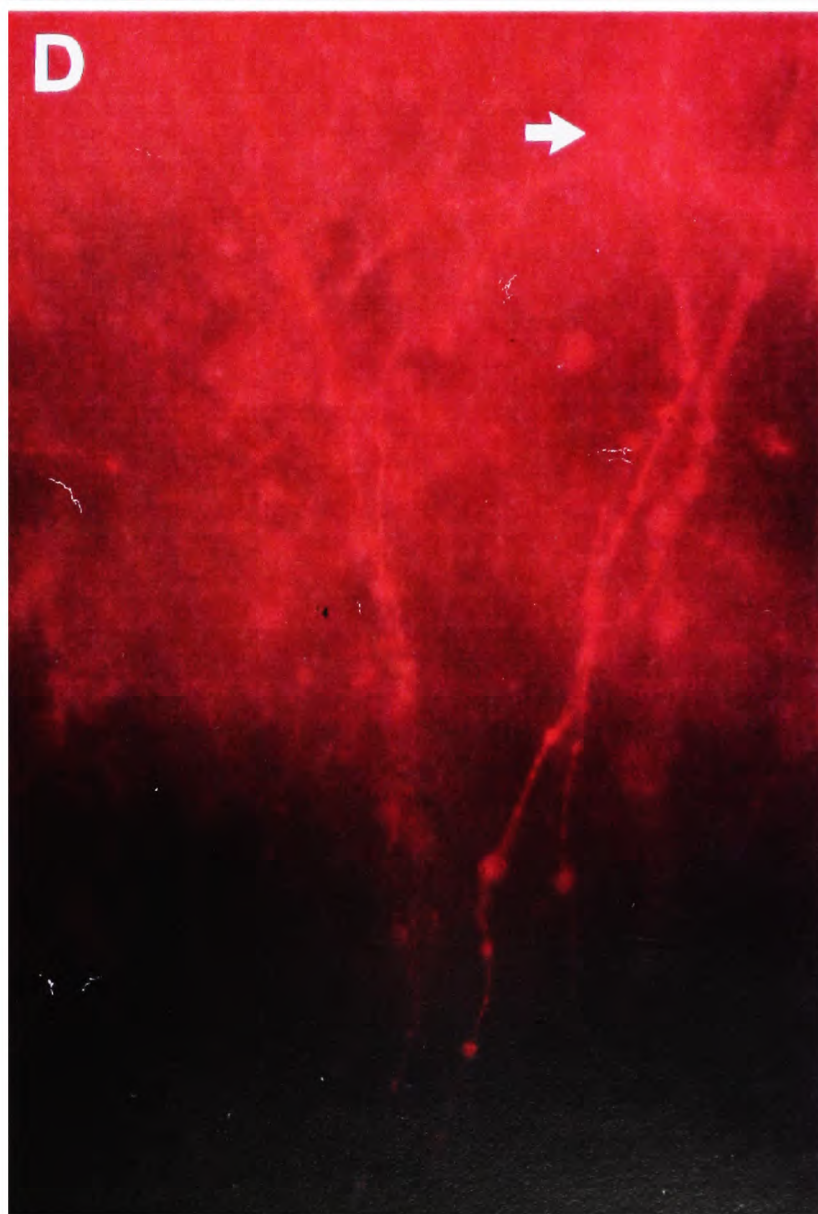
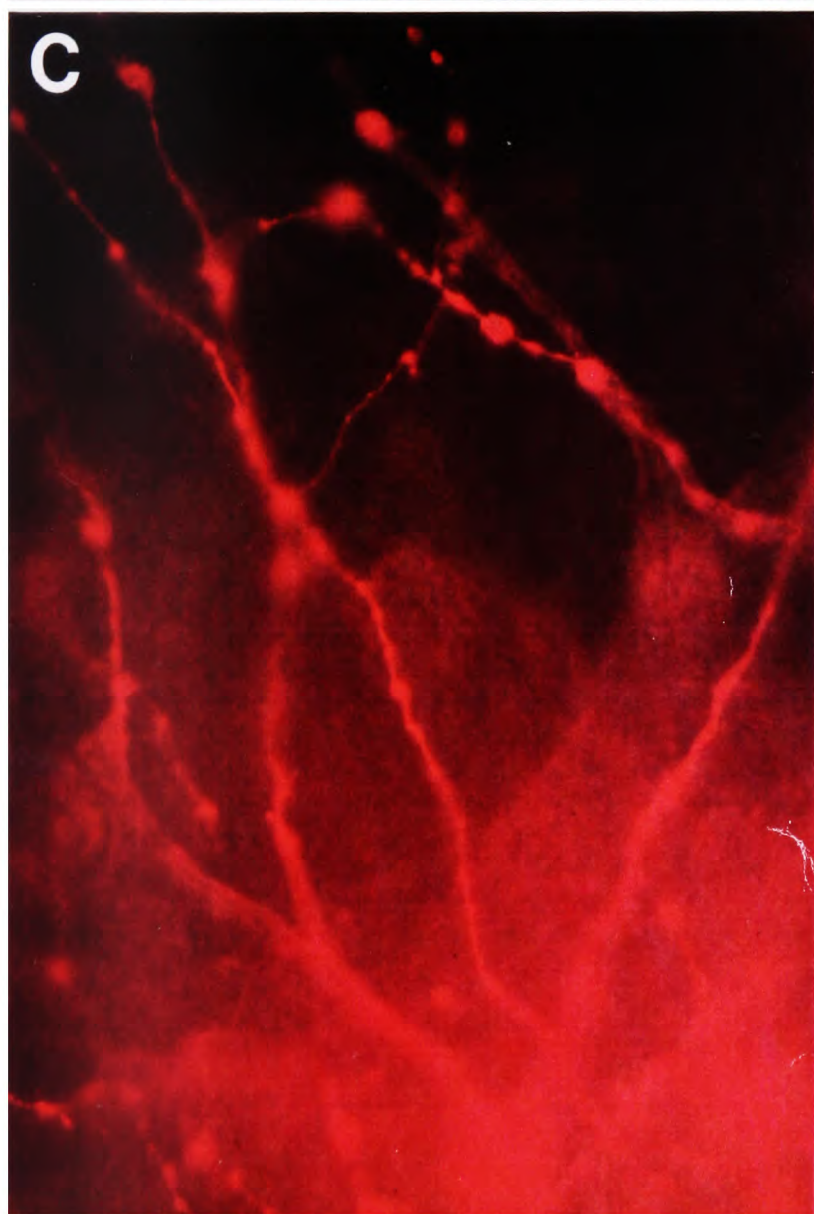
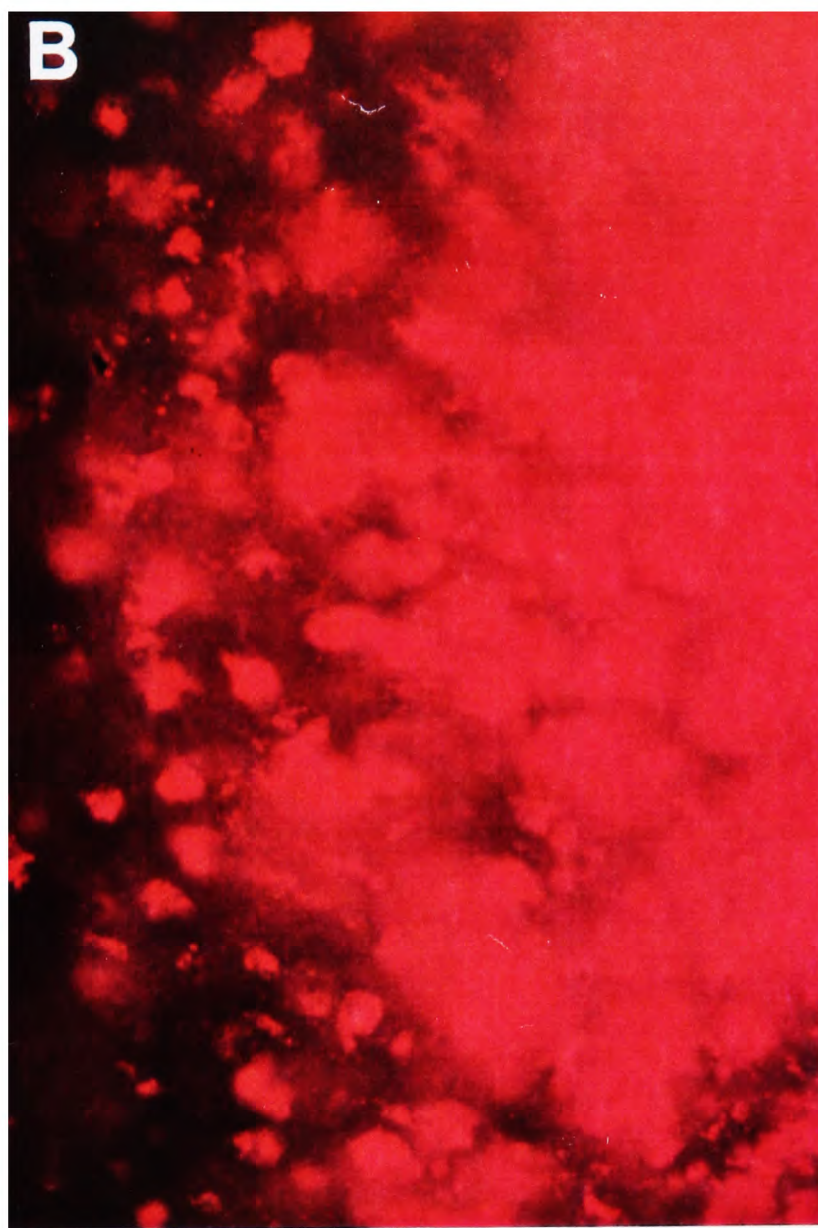
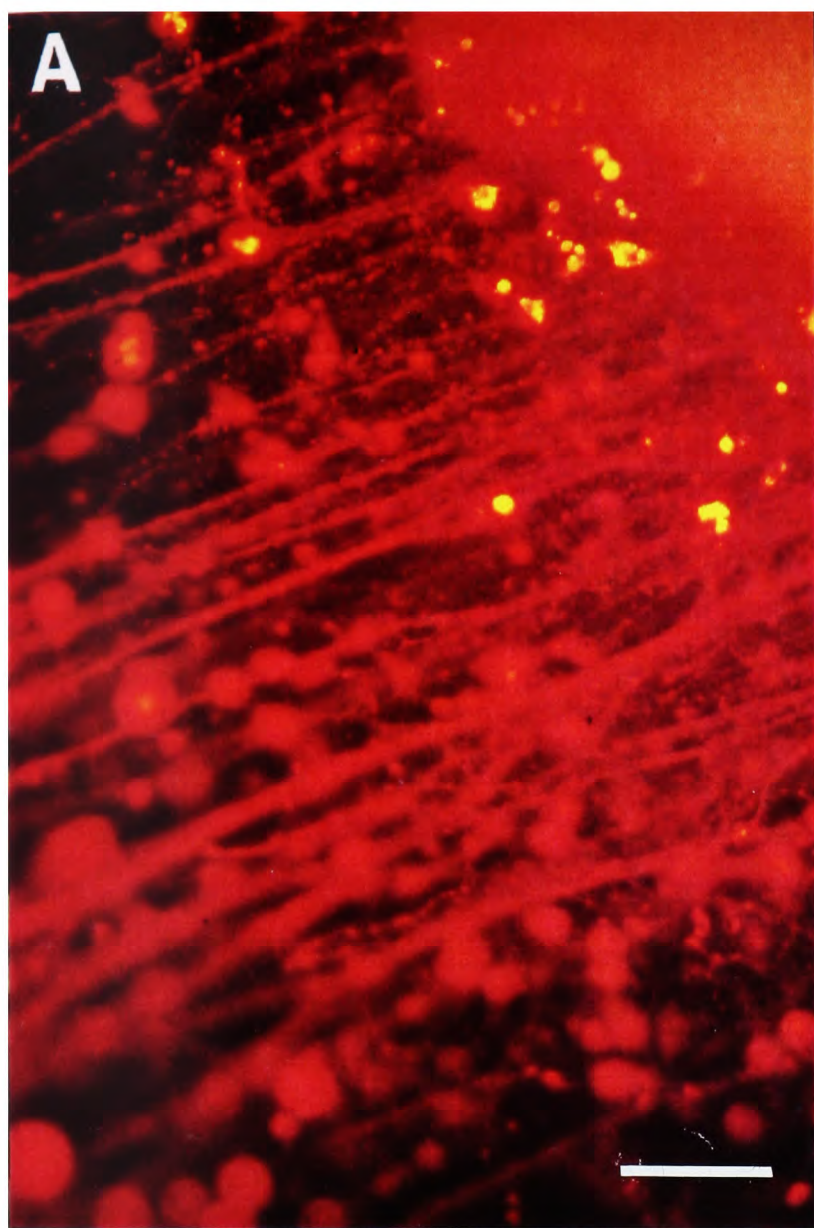


Fig. 2.11

Darkfield micrographs under epifluorescent illumination, showing DiI-labelled axons from an E16 LGN explant invading a cortical slice (taken at P3) after 1.5 days in culture.

A.: The thalamic fibres have invaded the P3 occipital cortex densely. Axons are running radially upwards through the cortical plate with growth cones at the tip of many of them (see C and D). The majority of the fibres have reached layer 4 within 1.5 days in vitro.

B.: The same as A but viewed under ultraviolet illumination to reveal the cortical layering and the pial surface, after staining with bisbenzimidazole (mz: marginal zone, dcp: dense cortical plate, wm: white matter).

C, D.: High-power view from the regions indicated with small squares at A showing growth cones at the tips of many axons.

Scale bars: A and B: 250µm; B and C: 50µm.

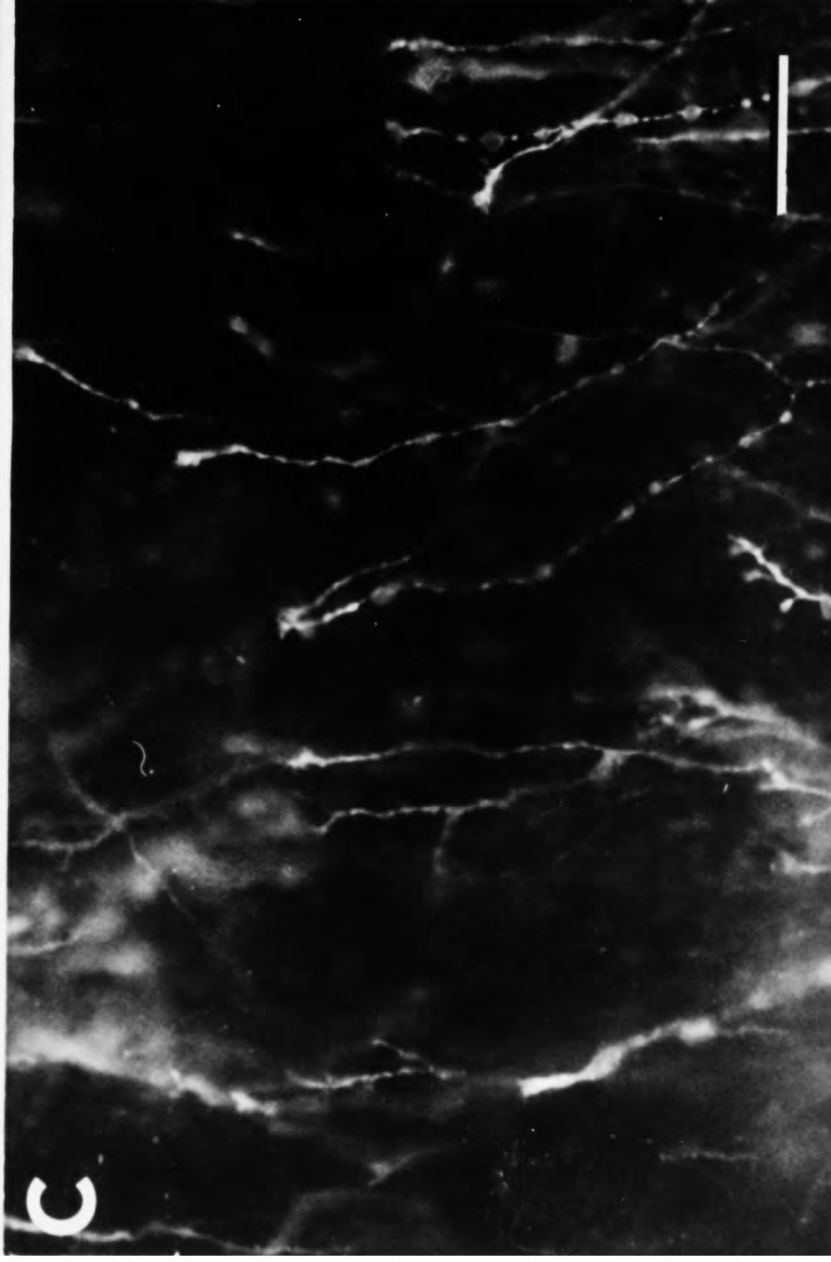
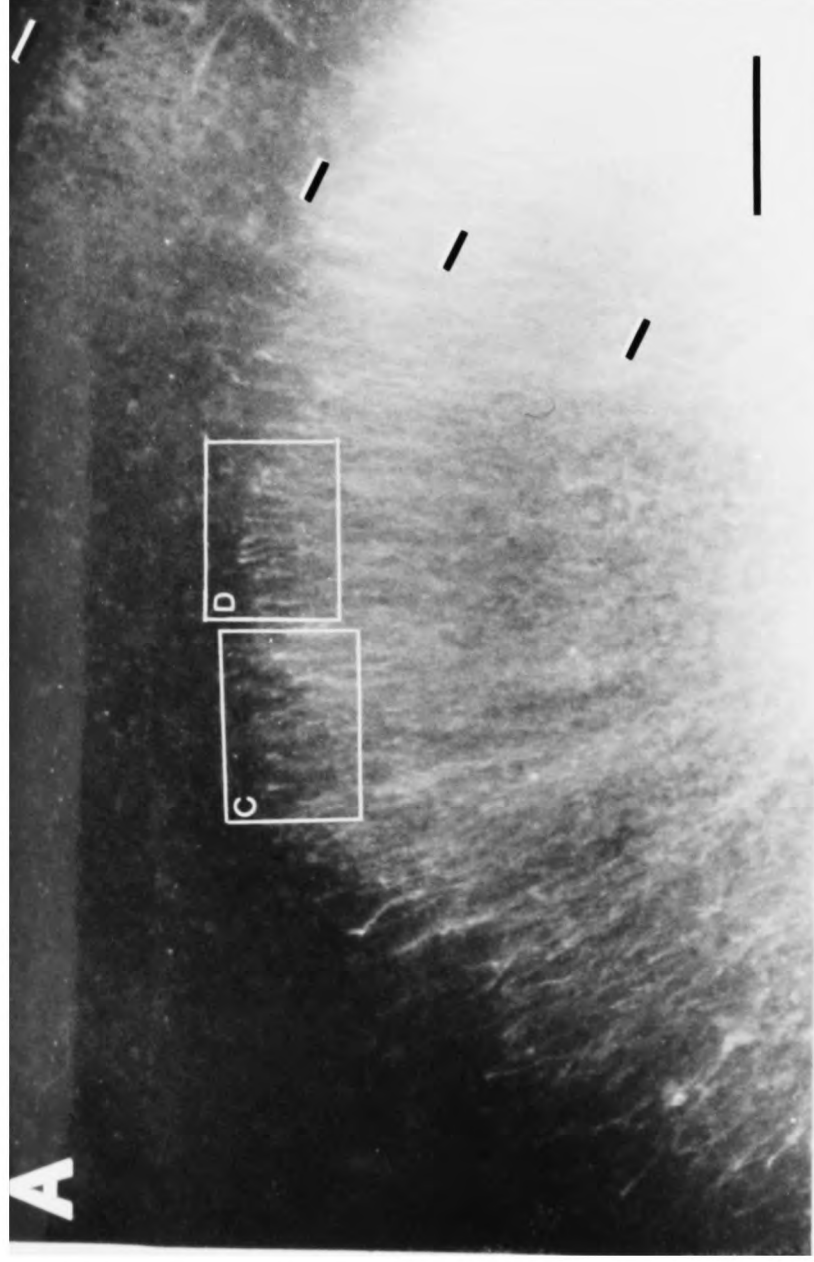


Fig. 2.12

High-power confocal microscopic reconstruction of the fibre ingrowth from the culture demonstrated in Fig. 2.11. 31, 1µm-thick, serial optical sections were collected of thalamic axons within the cortical slice and a 3-D dataset was created.

A: To demonstrate the fibre ingrowth in 3-D, a two-colour stereo anaglyph was created from the dataset. (Please view with the red/green glasses provided.) The image demonstrates the detailed morphology of the growth-cones at the tips of the thalamic fibres.

B: From the same 3-D dataset a *lateral* view was created to illustrate that the fibres run within the depth of the cortical slice culture and not just on the surface.

C: Acridine orange counterstain demonstrates the distribution of the cell nuclei within the same segment as B.

Scale bar: 25µm

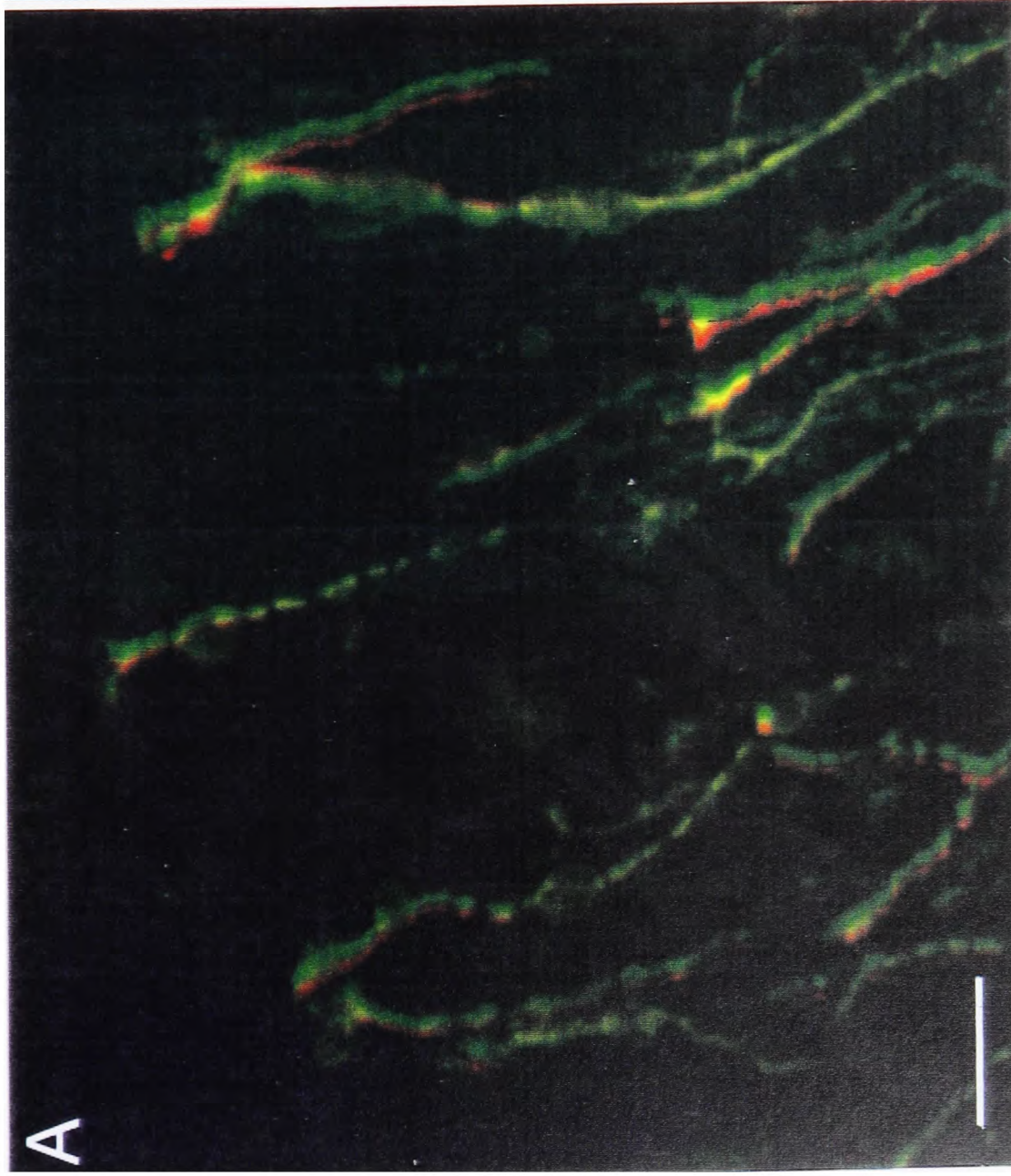


Fig. 2.13

Darkfield micrographs under epifluorescent illumination, showing DiI-labelled axons from an E16 LGN explant invading cortical slices after 4 days in culture.

A.: In P0 occipital cortex, axons are seen running radially or obliquely up through the cortical plate to the marginal zone, where some have turned laterally to run over or beneath the pial surface. One axon (far right) has turned 90° and coursed horizontally more than 1.5 mm: at its tip is a growth cone. Scale bar: 0.25 mm.

B.: High-power view of growth cones at the tips of axons below the cortical surface in the same (A) co-culture. Scale bar 50 µm.

C.: With P6 occipital cortex, axons from an E16 LGN explant have invaded densely and most are branching and losing their growth cones about 300 µm below the pial surface. Scale bar: 0.25 mm.

D.: The same as C but viewed under ultraviolet illumination after staining with bisbenzimidazole, to reveal the pial surface and cells within the slice. Scale bar 0.25 mm.

Comparison between Nissl-stained sections of such cortical cultures and of fixed occipital cortex from P6 animals indicated that the depth of termination of thalamic axons corresponded to layer 4.

E.: High-power view from C, showing individual axons branching and apparently terminating. Scale bar 50 µm.

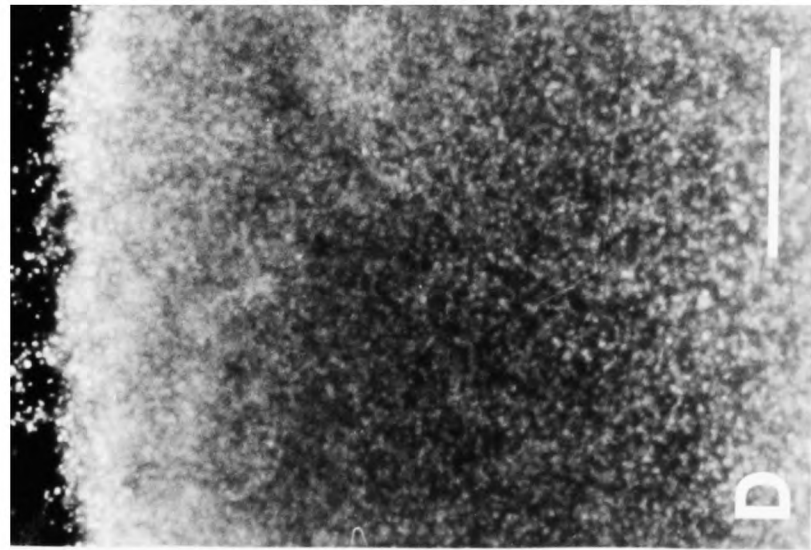
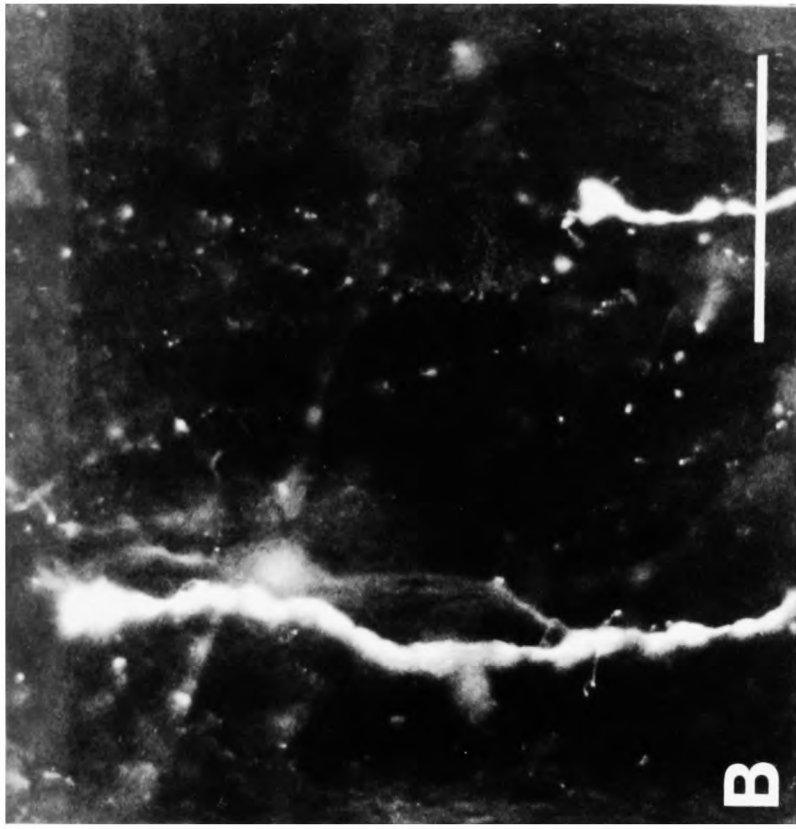
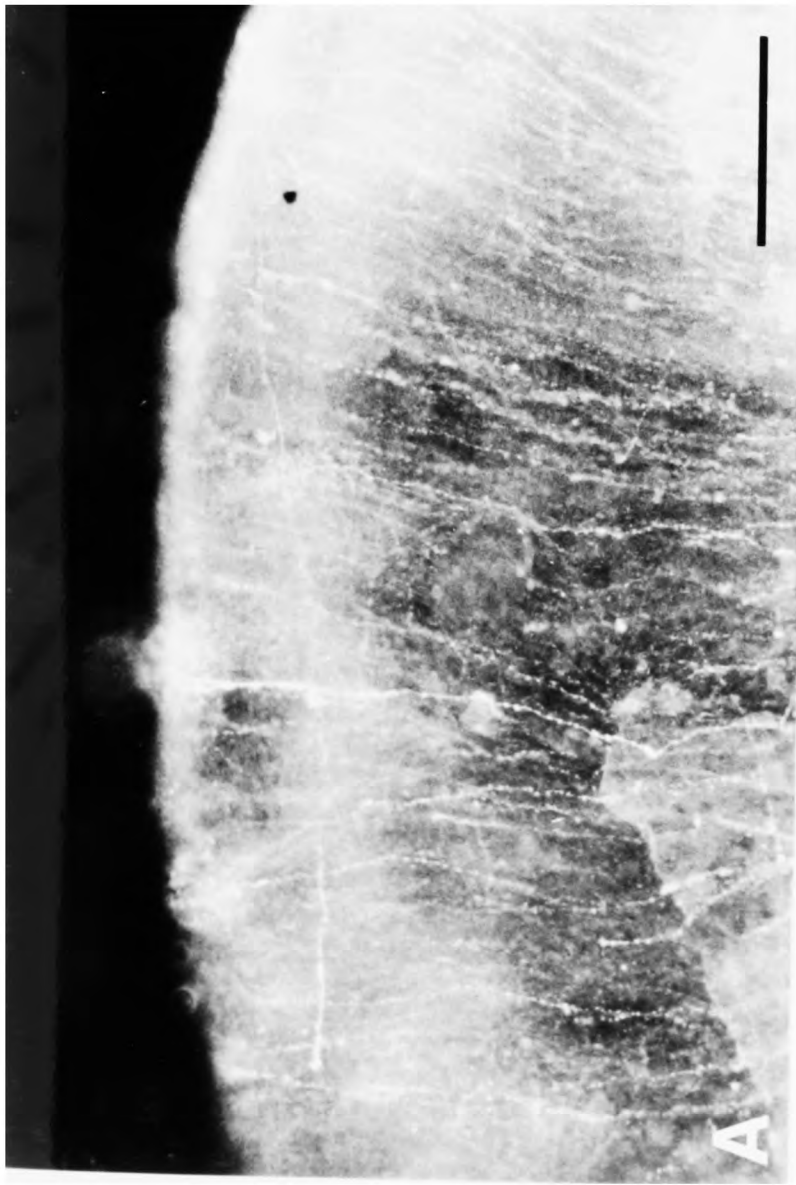


Fig. 2.14

Confocal micrographs from P0 and P2 cultures after 4 days in culture (same magnification).

A: In P0 occipital cortex, axons are seen running radially or obliquely up through the cortical plate to the marginal zone and the pial surface, where many have turned laterally to run over or beneath the pial surface forming a thick band of parallel fibres.

B: Under the same conditions, few thalamic fibre reach the pial surface in a P2 occipital cortical slice but the majority of axons terminate about 300µm below the pial surface.

Scale bar 100µm

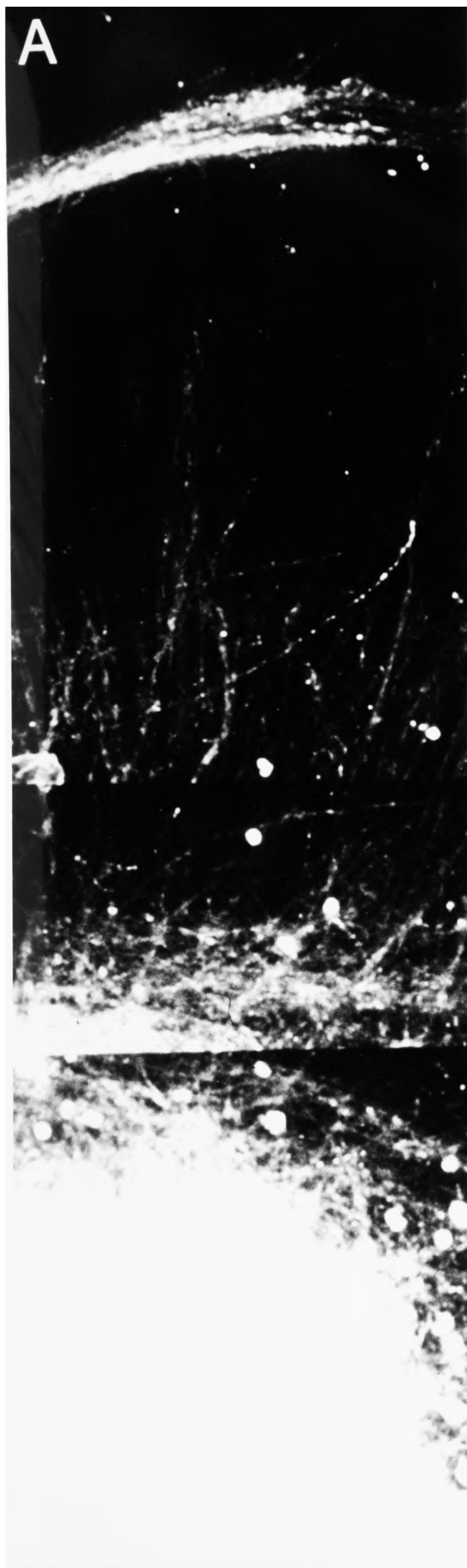


Fig. 2.15

High-power 3-D, two-colour stereo anaglyph of the ends of the fibres in the cultures of Fig. 2.14. (Please view with the red/green glasses provided.)

A: In P0 occipital cortex, fibres reach to the marginal zone and the pial surface, where most have turned laterally to run over or beneath the pial surface, forming a band of parallel fibres and not extending further over the collagen coated membrane.

B: Under the same conditions only a few thalamic fibre reach the pial surface in a P2 occipital cortical slice. Most axons terminate about 300µm below the pial surface.

Scale bar 100µm for both.

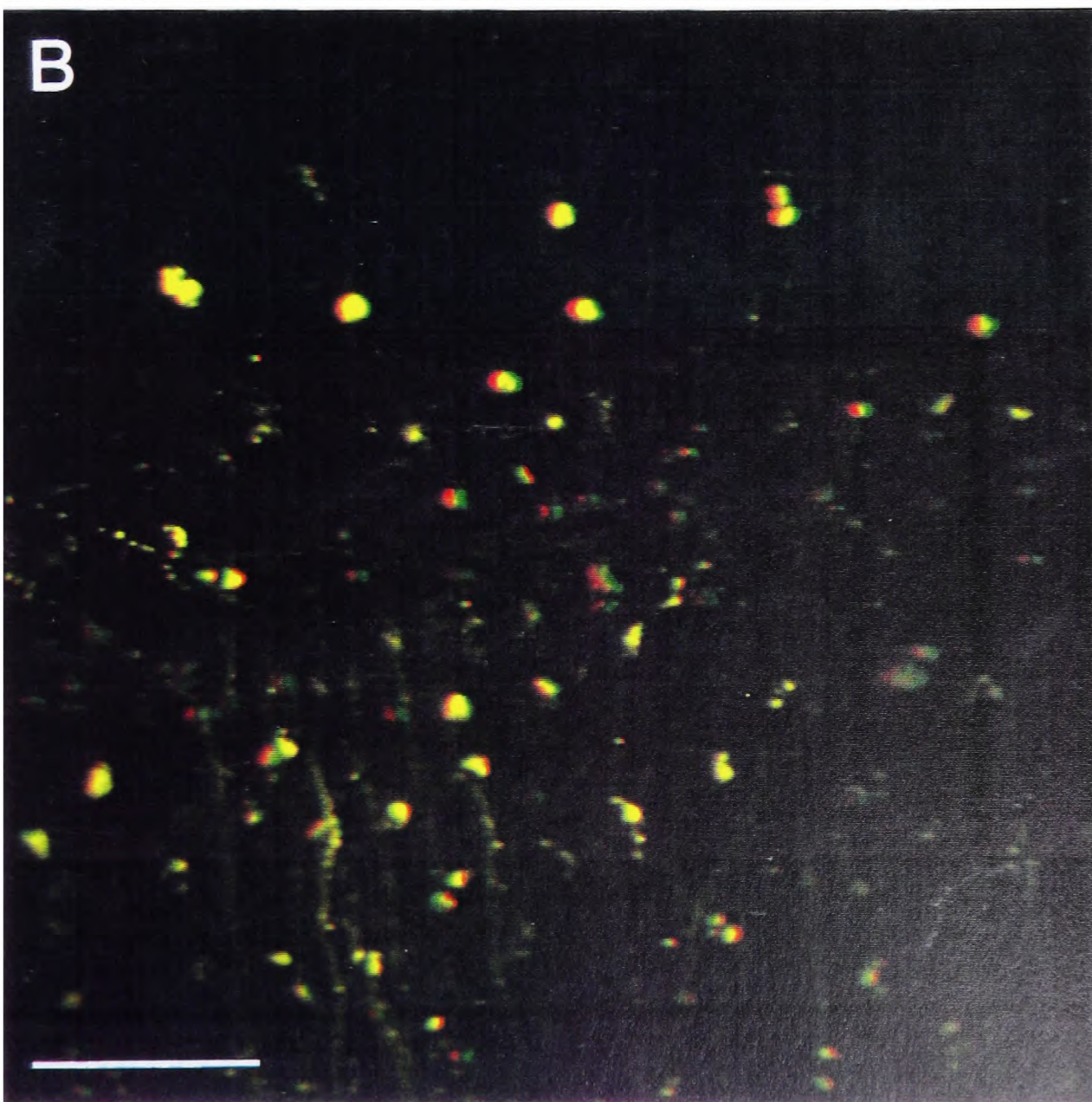
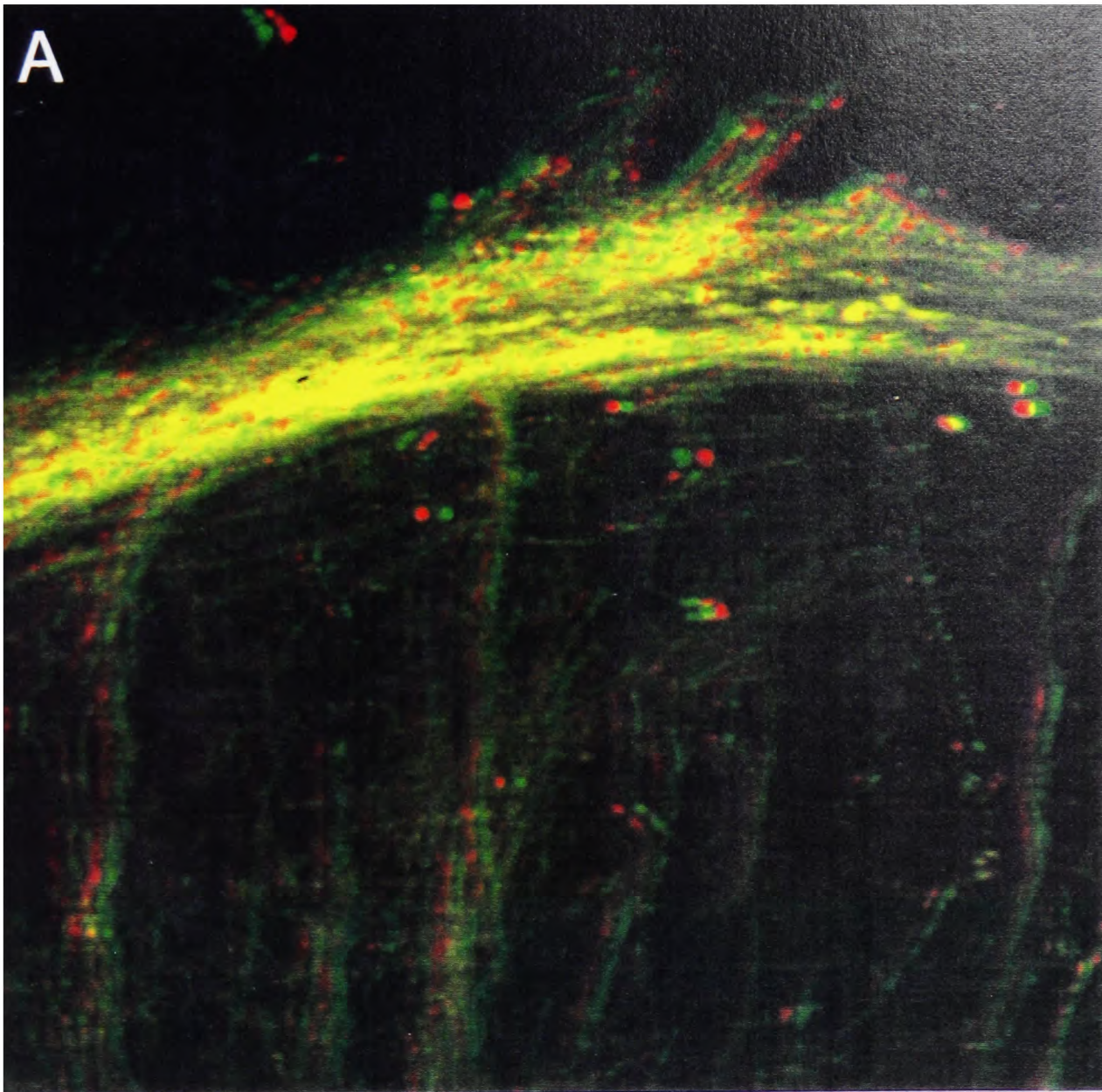


Fig. 2.16

A.: Partial fibre ingrowth from an E16 thalamic block into an E19 occipital cortex explant after 4 days in vitro. A few fibres course through the cortical slice then emerge to join the thick intensely stained layer of axons (upper right corner) that are coursing around the edges of the cortical slice. Typically, in combination with E19 explants, thalamic explants send out axons but the majority of them do not enter the cortical explant. Instead the fibres grow around the border of the cortical slice in a circular fashion. The white arrowheads mark one of the few axons which has entered the cortical plate and the white arrow indicates the growthcone.

B.: High-power view of the edge of the same culture, demonstrating the fibres growing around and not within the cortical slice. The bright enlargement of the fibre tip represent a growth cone (white arrow).

Bar at A: 200µm; B: 100µm.

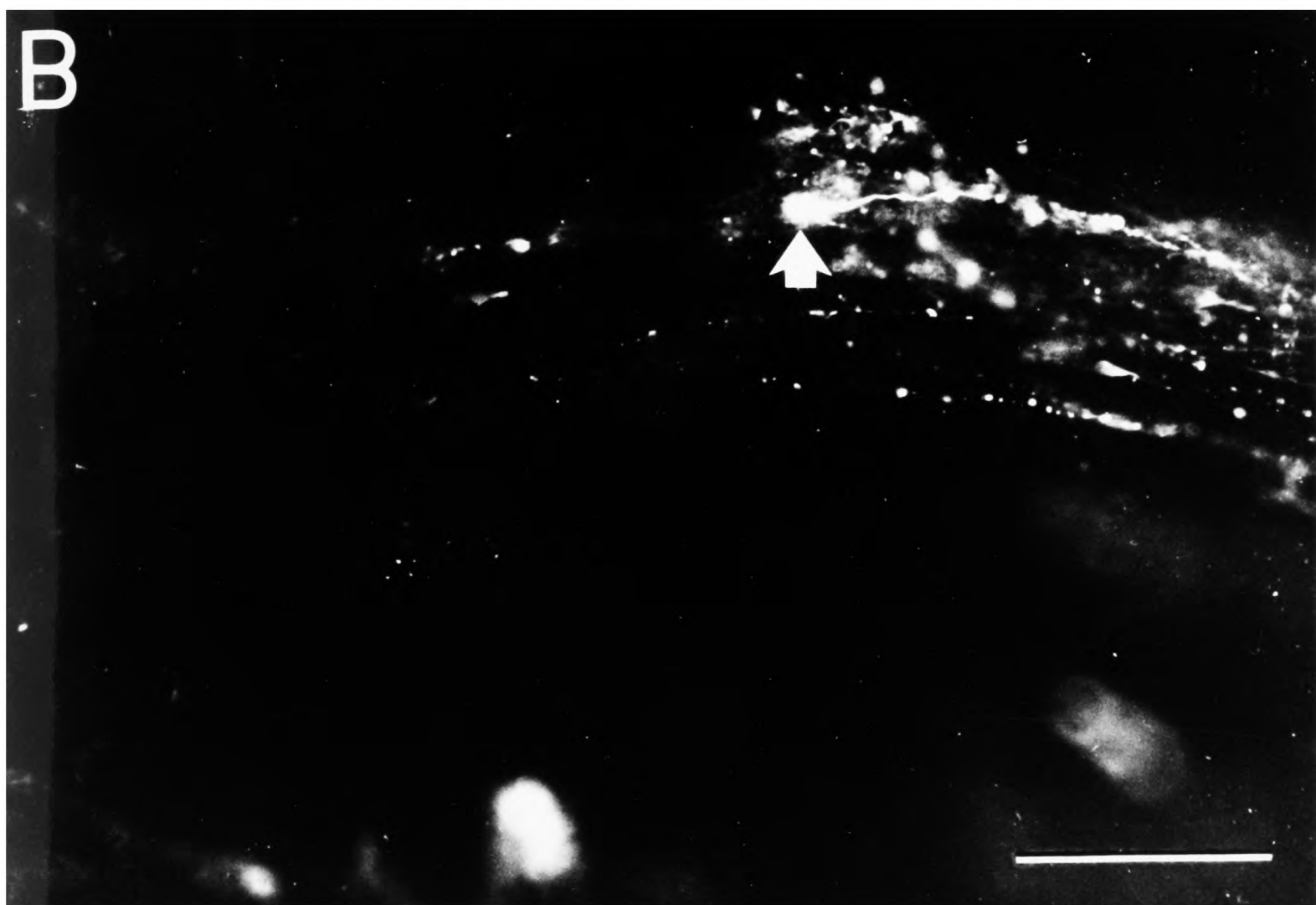
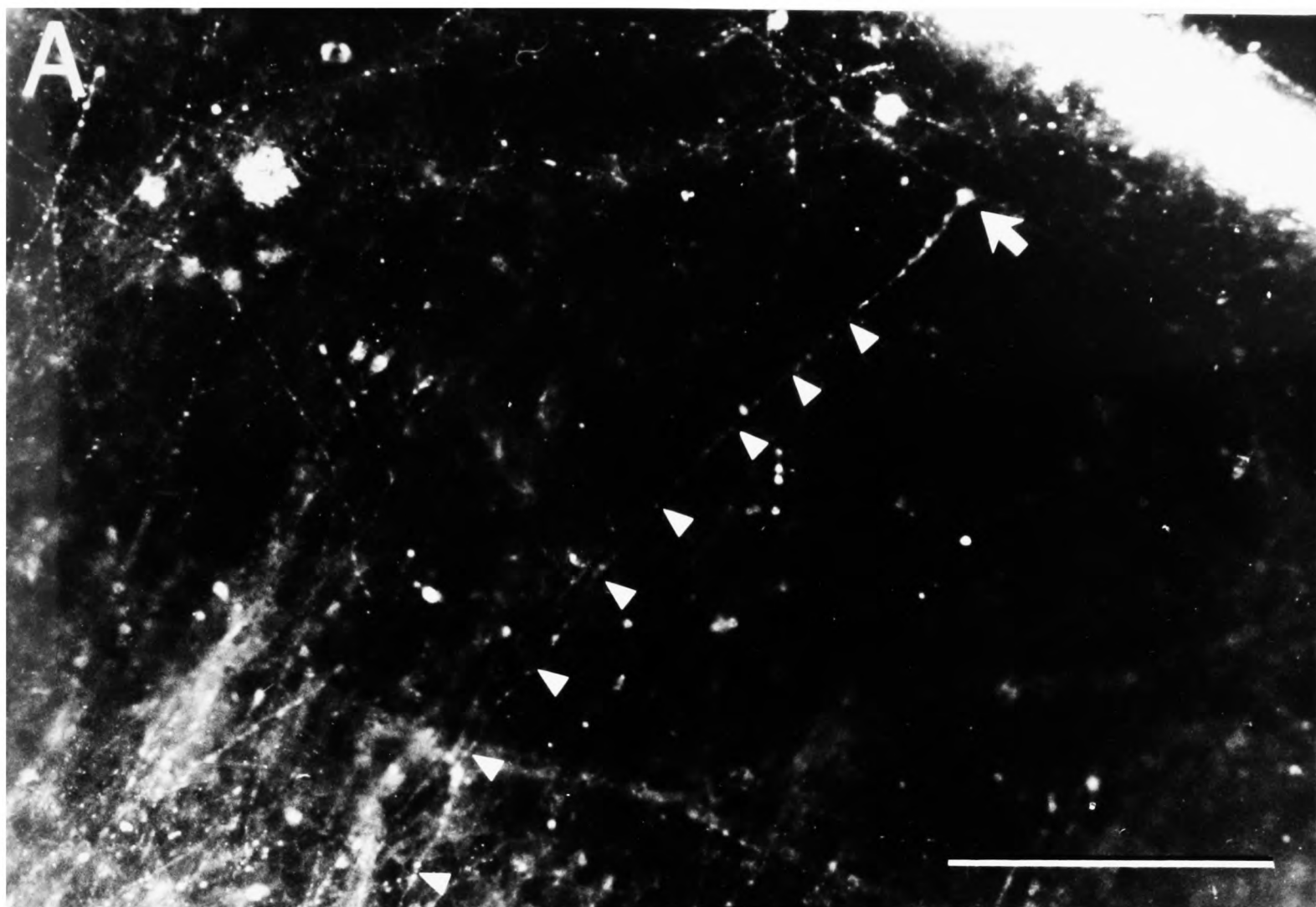


Fig. 2.17

The upper schematic diagram demonstrate the procedure for making a pia-parallel cut through the cortical plate (CP) along a line definitely above the top of the subplate (SP). This cut produced a '**cortical plate ribbon**'(consisting of upper cortical plate with no subplate; only the marginal zone contaminates the purity of the cortical plate) and a '**subplate ribbon**'(also containing a thin segment of the lowest part of cortical plate, plus intermediate and ventricular zones).

These ribbons then were co-cultured with E16 LGN explants in a variety of configurations. The LGN block was placed against the lower surface of the 'cortical plate ribbon' (upper-left) or the 'subplate ribbon'(upper-right). In others it was placed against the radially cut end of the ribbon (lower diagrams). Then after 4 days in culture the thalamic fibre outgrowth was examined and camera lucida drawings were made.

I saw no obvious difference in the amount or velocity of outgrowth of the thalamic axons or the pattern of their invasion into the two different P0 cortical fragments.

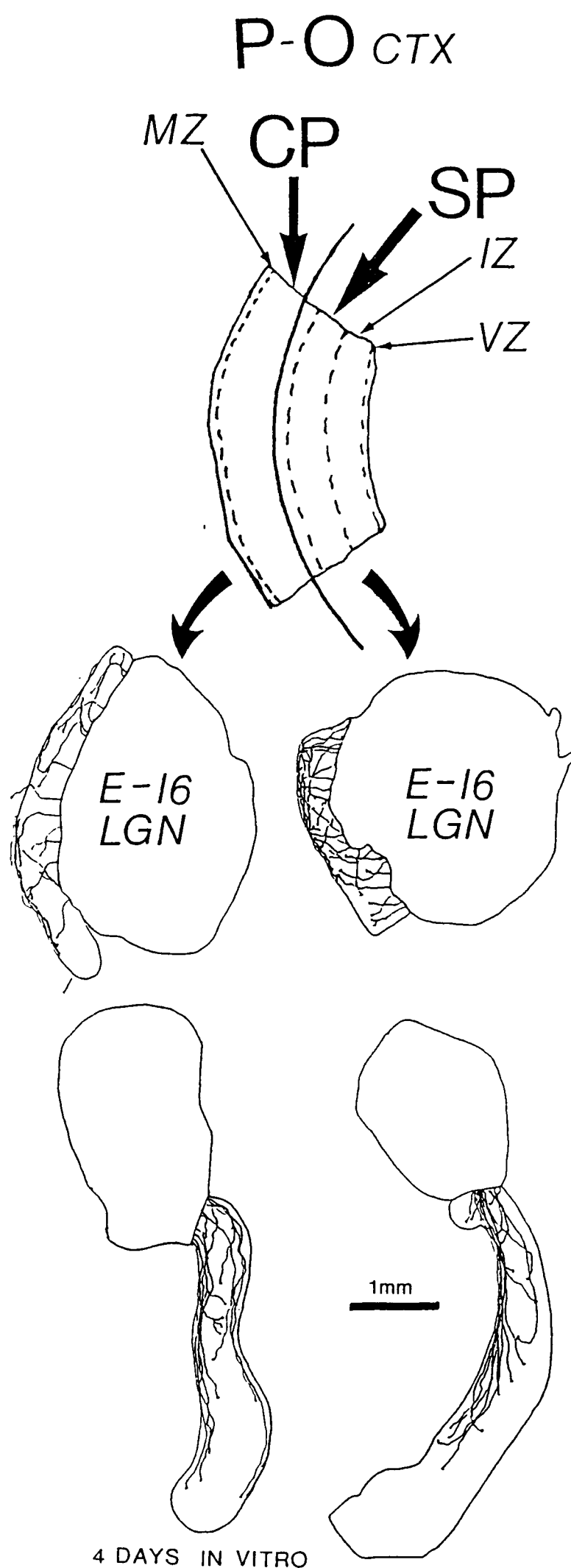


Fig. 2.18

An E16 LGN explant was placed next to the cleaned pial surface of a P0 (A and B) and a P6 (C and D) occipital cortical slice and cultured for 5 days.

A & B were taken from the same field, viewed under rhodamine (A) and uv (B) illumination to reveal the DiI-labelled fibres and the bizbenzimidazole counterstaining. In the P0 cortex, the thalamic fibres pass through the entire depth of the cortex from the pial surface (PS) and reach the ventricular surface (VS). An interrupted line indicates the cortical plate/white matter (WM) border. Scale bar; 250µm.

C: Dark-field micrograph under epifluorescent illumination, showing the front of the DiI-labelled axons from the LGN explant invading a P6 cortical slice from the pial surface after 5 days in vitro. The fibres do not reach the ventricular surface (VS), but stop about 300µm below the pial surface (PS). The zone of fibre termination is indicated by the horizontal white arrow. Bar; 500µm.

D: High-power view from the region marked with a square in C showing the fibres at their termination zone. Only a few of the DiI labelled fibres are in focus since the culture is more than 150µm thick.

mz: marginal zone, dcp: dense cortical plate, WM: white matter

Bar: 100µm.

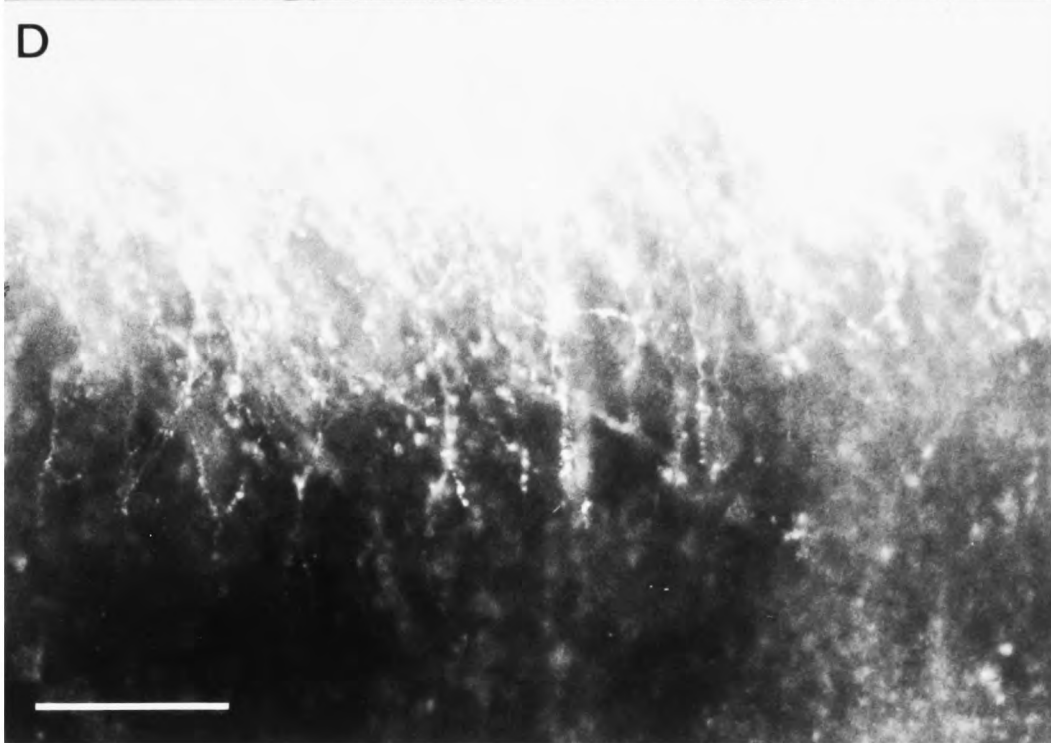
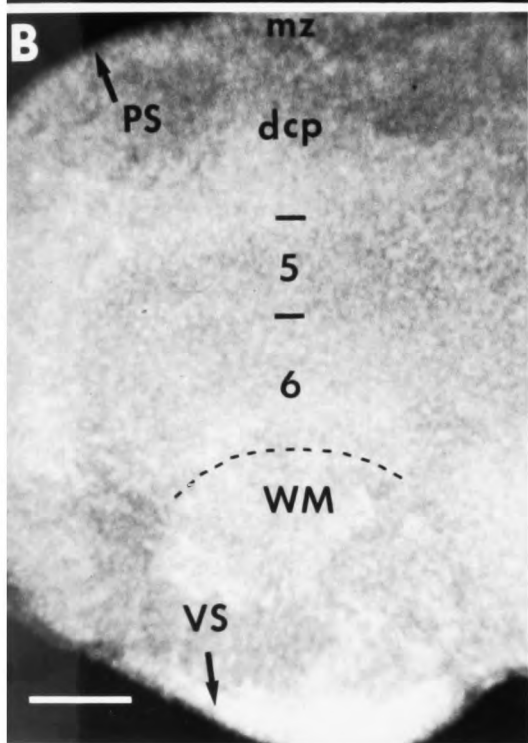
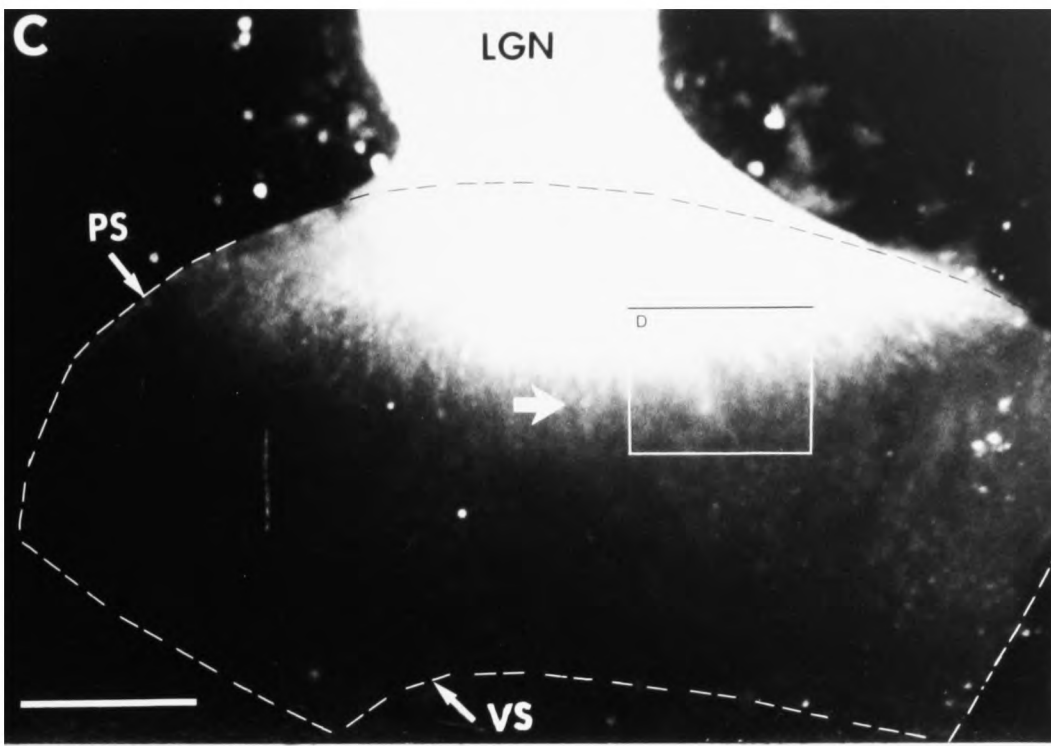
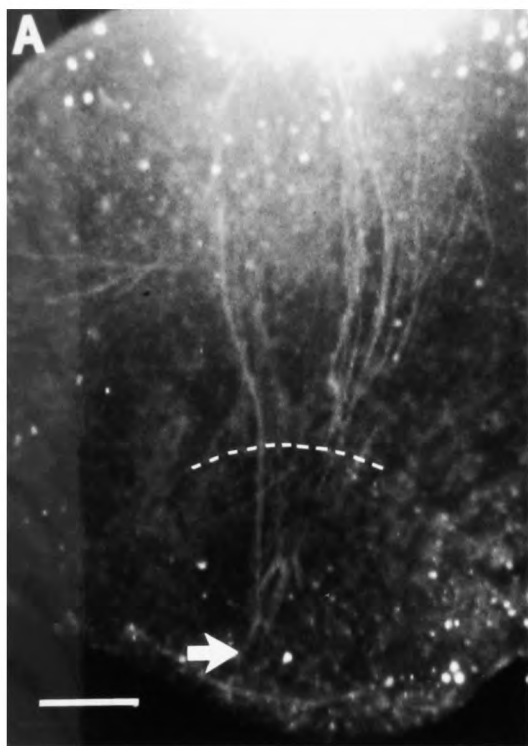


Fig. 2.19

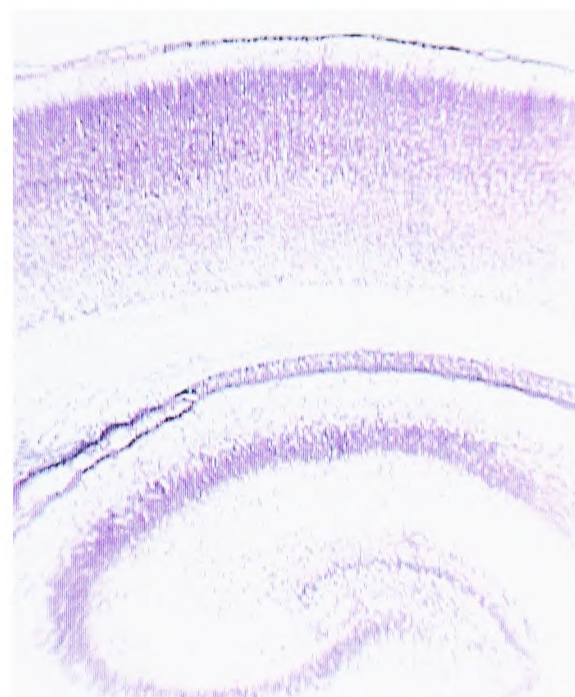
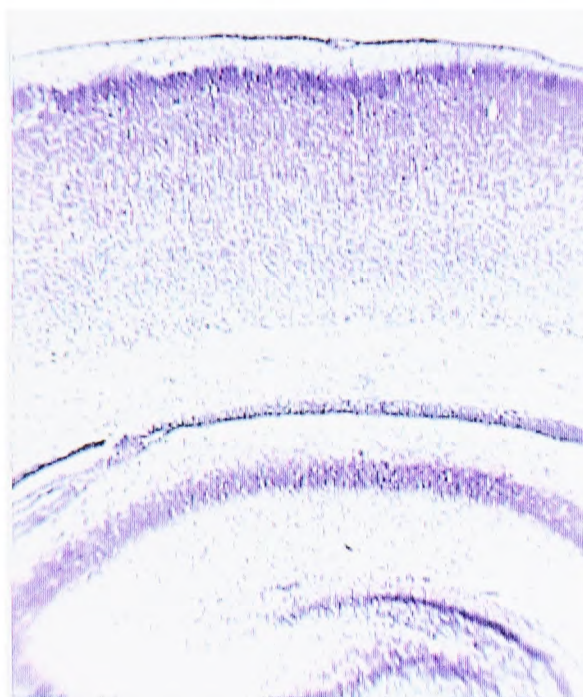
To investigate the relationship between the generation of layer 4 and subsequent synaptogenesis and formation of layer-specific thalamic innervation I attempted to eliminate (or at least to reduce the number of) layer 4 cells by the application during neurogenesis of methylazoxymethanol acetate (MAM), a cytotoxin that destroys dividing cells. On the 16th day of pregnancy, time-mated pregnant rats were given two i.p. injections of 15mg/kg MAM with the intention of disrupting the generation of layer 4. To be sure of covering the whole of one mitotic cell cycle I waited 8 hours between the two injections. The litters were born at the normal age and the lamination of the cortex was examined in the offspring at various postnatal ages.

The photomicrographs compare the cortical layering of the somatosensory cortex at two postnatal ages; P3 (upper half) and P8 (lower half). The normal controls (N) are on the left, the MAM-treated animals (MAM) on the right. There was only a slight reduction in the overall thickness of the cortex in the MAM-treated animals. Otherwise, the lamination appeared remarkably normal.

N

MAM

P3



P8

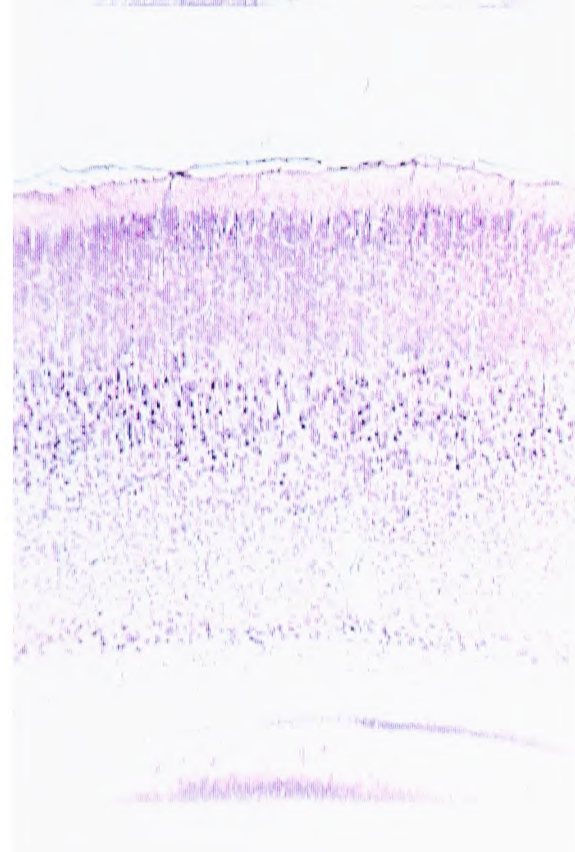
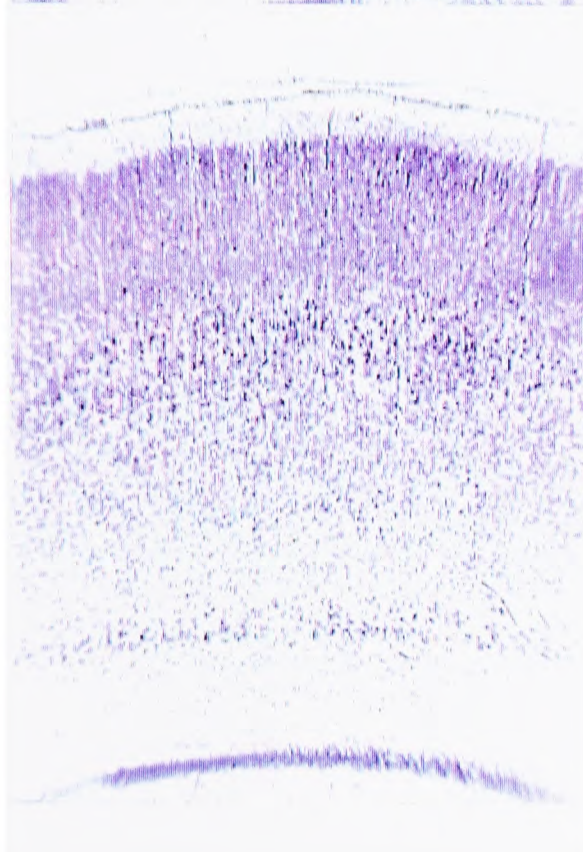


Fig. 2.20

Results of the 'choice' paradigm in which each explant of E16 LGN was cultured next to two different slices. Camera lucida drawings show DiI-labelled thalamic axons after 2.5 (A-B) or 5 (C-D) days in vitro.

A-B.: Confronted by P1 occipital cortex (OCC) and frontal cortex (FRO), geniculate (LGN) (A) or anterior thalamus (ANT) (B) innervation was indistinguishable. Most axons ended in growth cones, and a few already had reached the pial surface after only 2.5 days in vitro. There was a slight preference for the fibres to enter the cortical slice from the ventricular surface rather than growing around the edge and into the other layers from the cut margin.

C.: 'Choice' paradigm similar to A but with P6 occipital (OCC) and frontal (FRO) cortex (after 5 days in vitro). Again there is no hint of preference for occipital cortex. Only a few axons have reached the marginal zone, and most have terminated in what appears to be layer 4, about 3-400 μm below the surface.

D.: Given the choice between P6 occipital cortex (OCC) and P6 hippocampus (HIP), LGN axons showed little preference in terms of density of invasion; after 4 days in vitro, hippocampal slices were innervated almost as densely as neocortical slices. However, interestingly, many axons continued up to the surface of the hippocampus and they did not tend to terminate specifically in a particular layer.

Bar: 1mm.

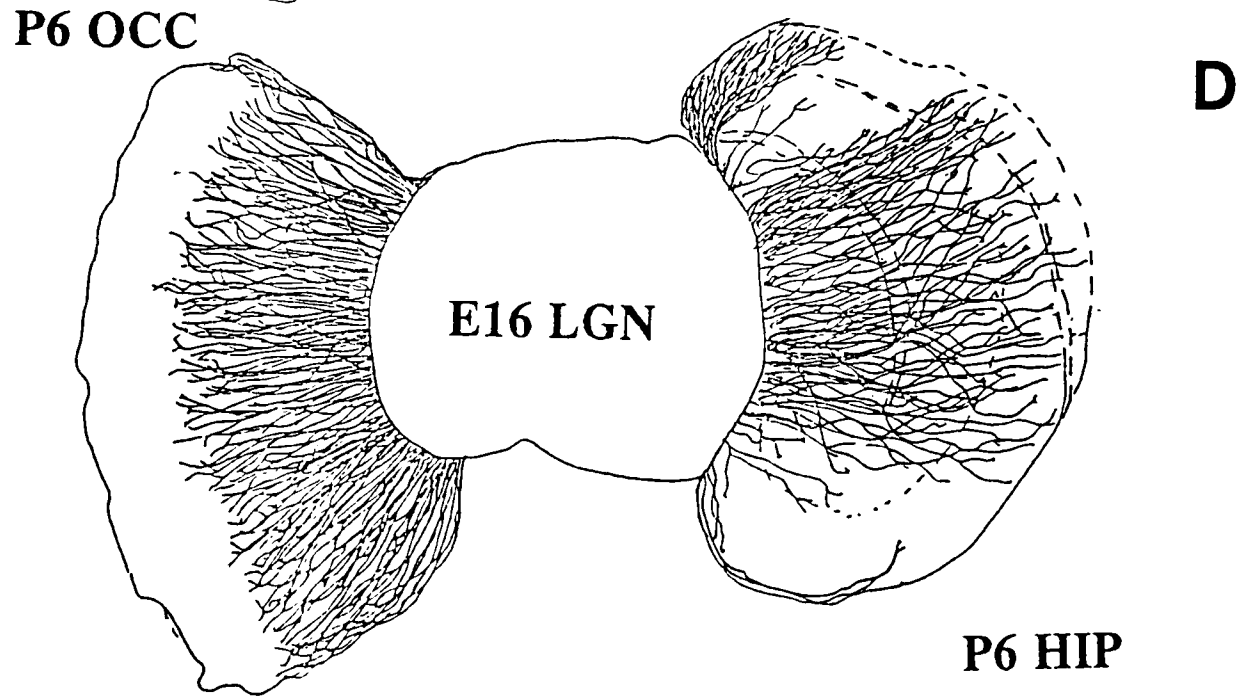
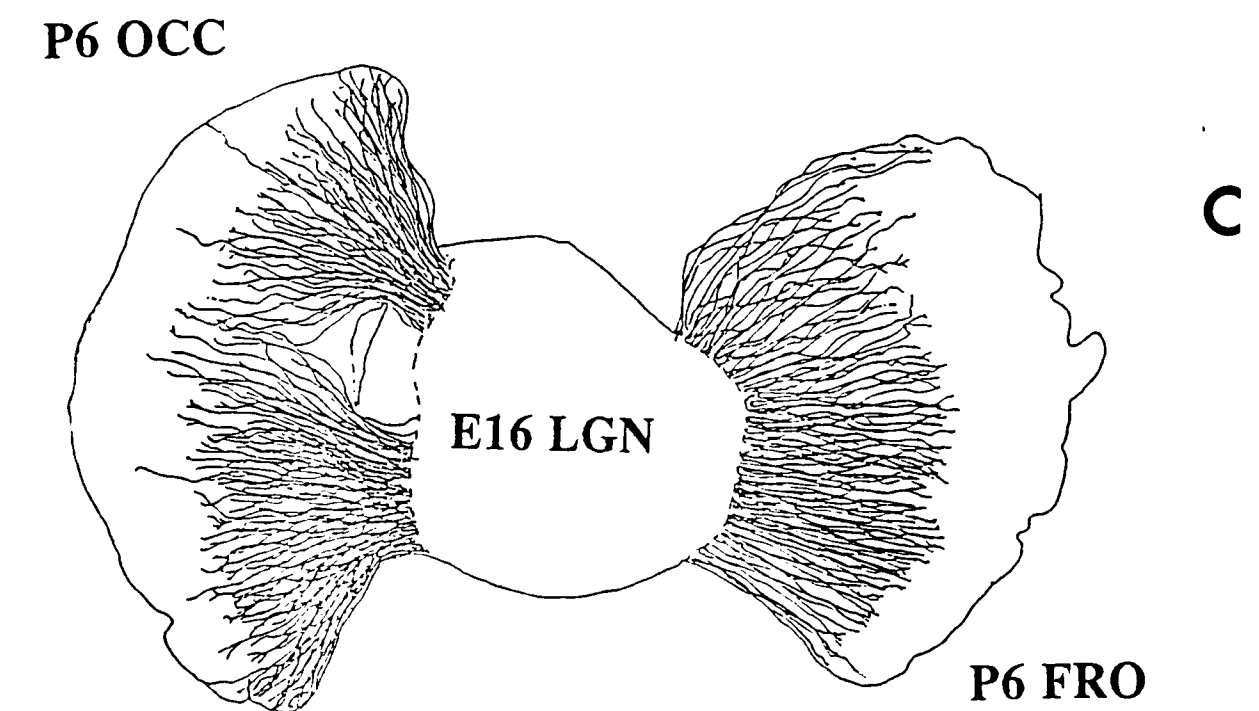
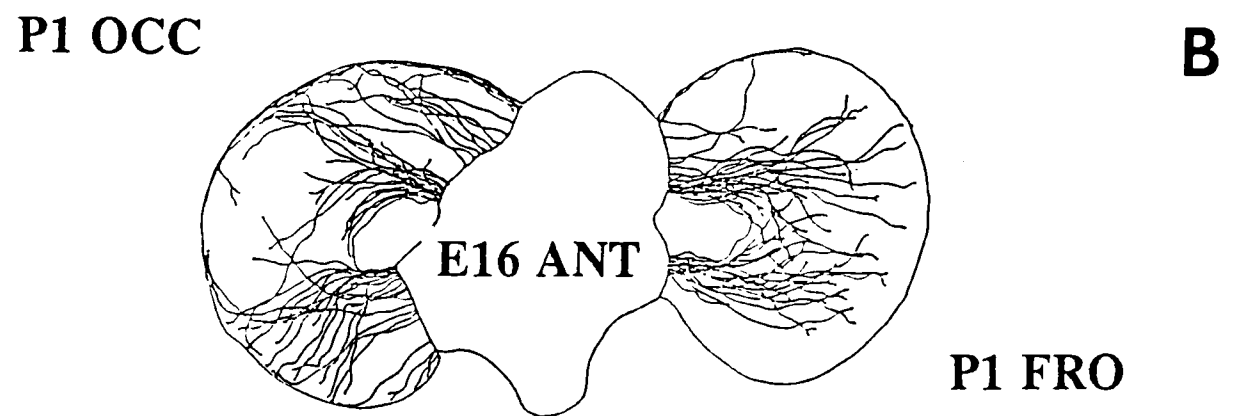
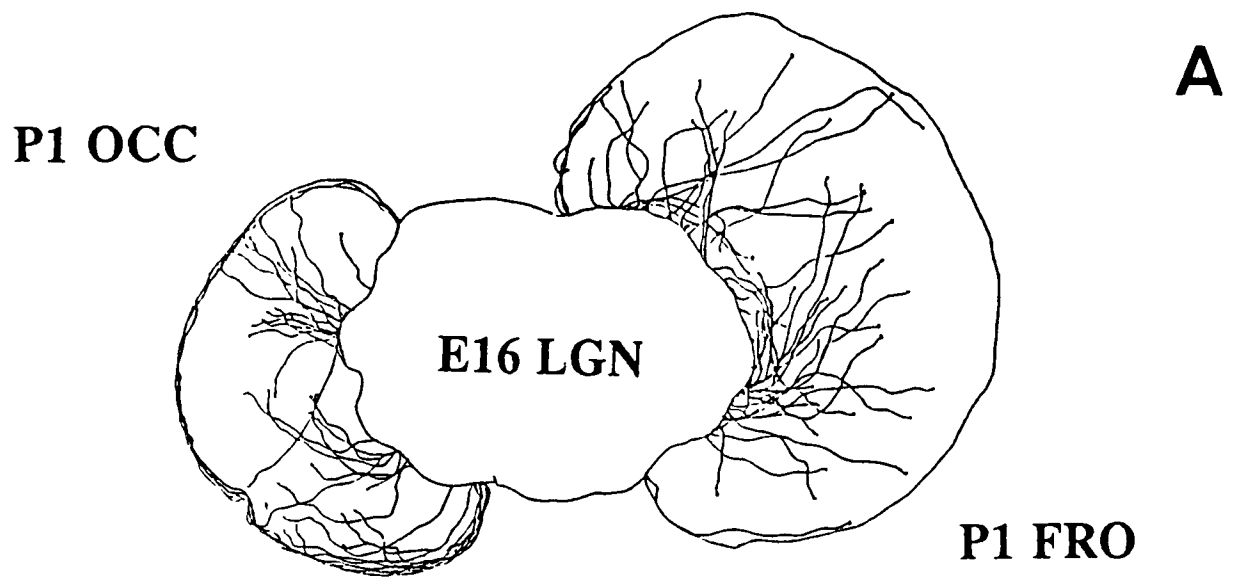


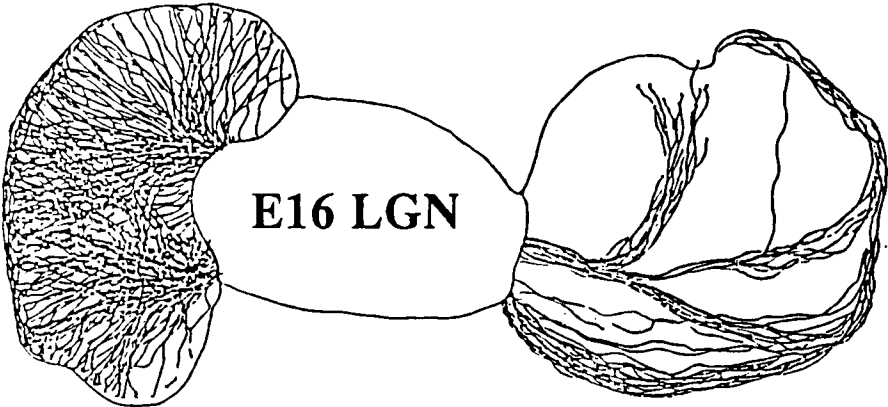
Fig. 2.21

A-D.: In a 'choice' paradigm in which each explant of E16 LGN was cultured next to a cerebellar and an occipital cortical slice the geniculate innervation was largely restricted to the slice of neocortex. In the cortex, according to its age, the fibres either run up to the pial surface (A, P1) or terminate approximately 300µm below the surface (B-D, P6). Of the few axons that sprouted toward the cerebellar slice, most ran around one border of the slice or in fascicles across the surface of the slice to reach and course around the opposite edge.

Bar: 1mm.

P1 OCC

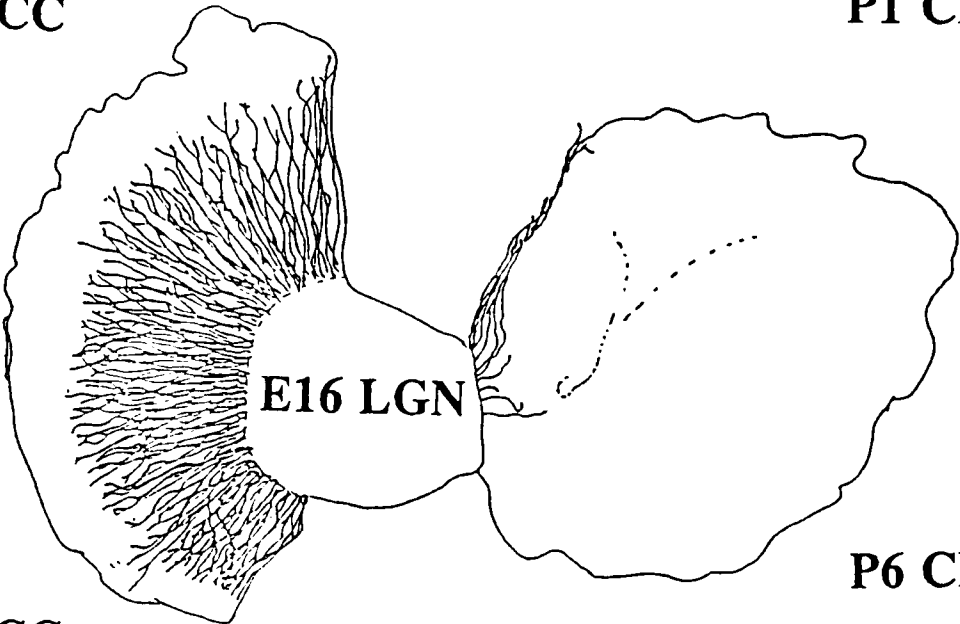
A



P6 OCC

P1 CER

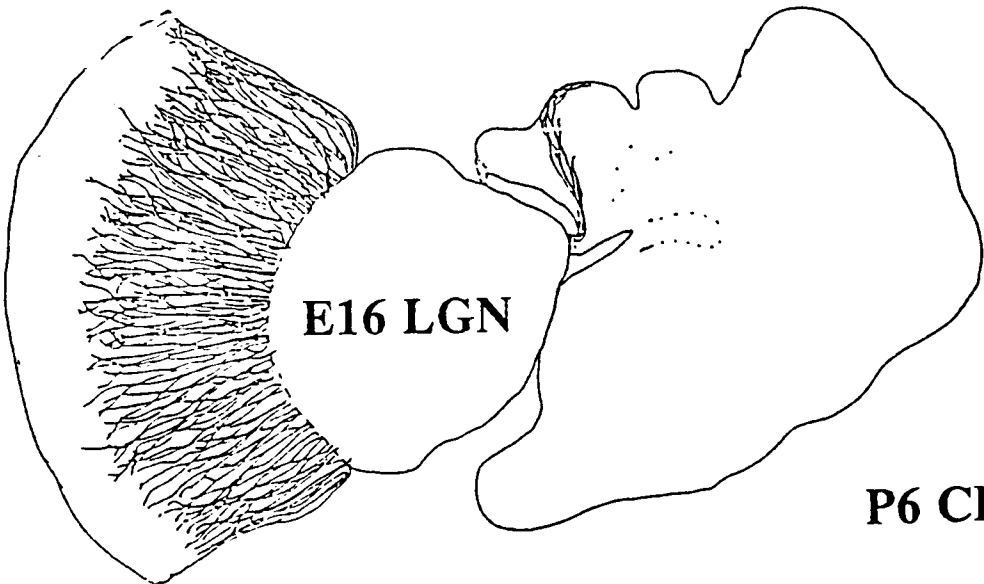
B



P6 OCC

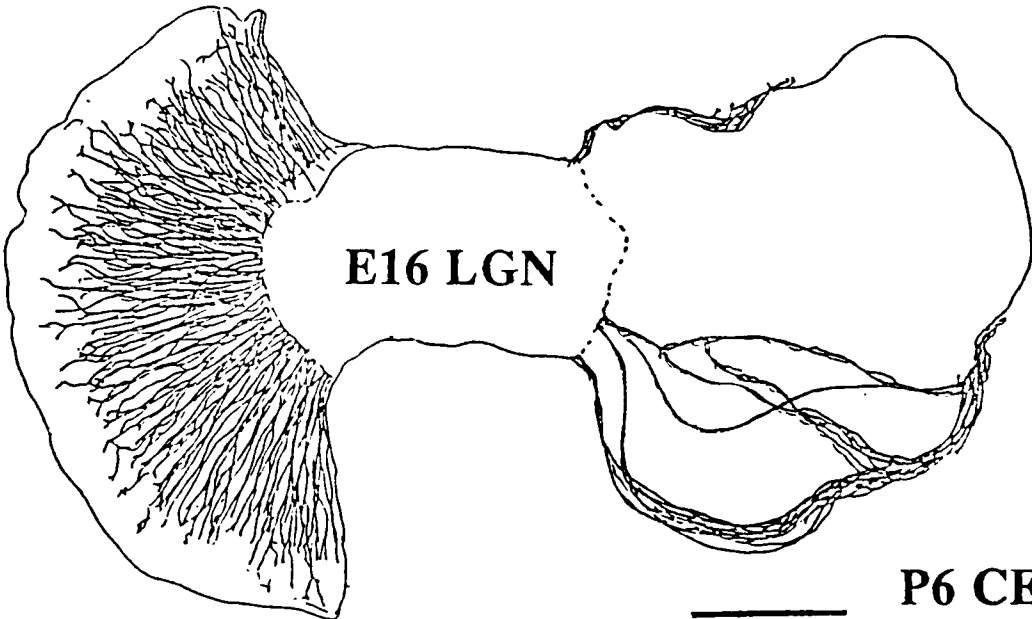
P6 CER

C



P6 OCC

D



P6 CER



Fig. 2.22

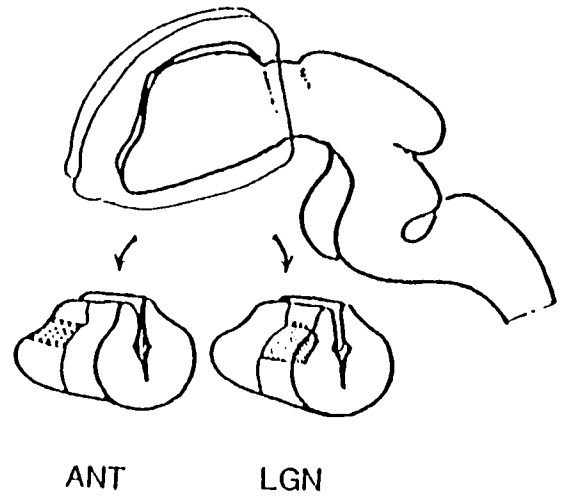
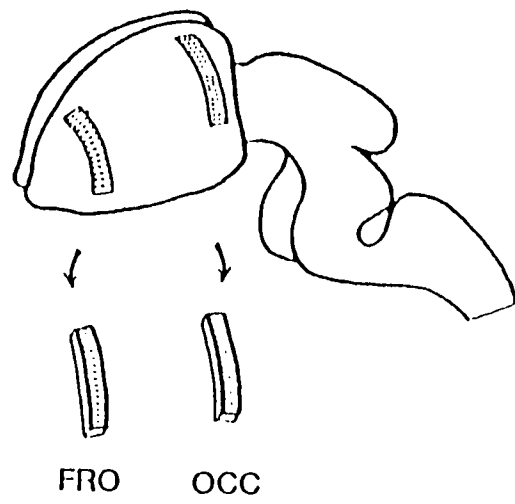
Upper schematic diagram summarizes the dissection procedure to provide explants of embryonic cortex as well as thalamus. In these experiments, each pair of thalamic explants was dissected from the same side of an individual brain, to ensure that they consisted of different thalamic nuclei (*right*). To obtain cortical explants from different regions of the embryonic 14.5-16 day old cortex narrow 'tissue ribbons' were cut from the occipital or the frontal part of the cortex in the coronal plane (*left*). (For a more detailed description of the dissection of LGN and anterior thalamus also see Fig. 2.1) Camera lucida drawings of axon outgrowth from E16 cortical explants, pre-labelled with DiI, cultured for 4 days with a choice of thalamic explants of the same age.

A & C: Occipital cortex (OCC) sends small numbers of axons (presumably mainly if not exclusively from subplate cells) into both lateral geniculate nucleus (LGN) and anterior thalamus (ANT), some cortical axon do not enter the thalamic explants but run on the edge. There is no obvious difference in the pattern of axon growth on the two sides.

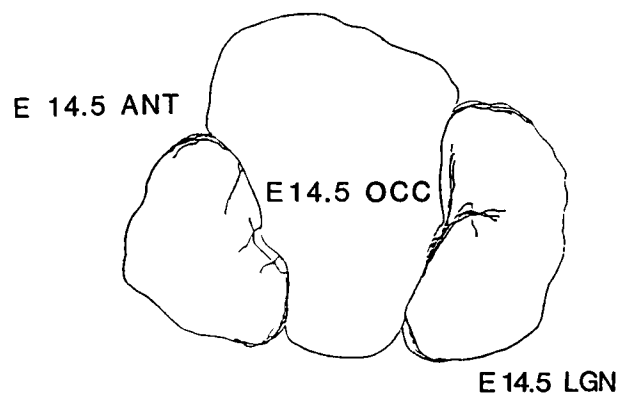
B & D: Frontal cortex (FRO) also innervates LGN and anterior thalamus very similarly.

E & F: From E16 frontal and E16 occipital cortex there was more fibre outgrowth (into E16 thalamic blocks) but again there was no difference in the innervation of 'appropriate' and 'inappropriate' target structures.

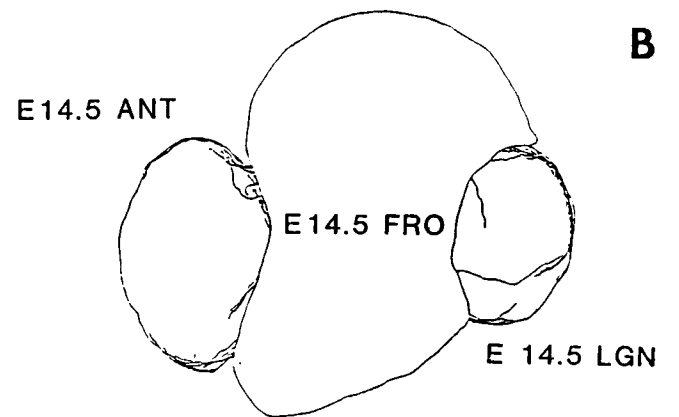
The pattern of cortical innervation of thalamic explants was more variable than that of thalamic cortical ingrowth in vitro. In some cases, cortical axons grew in fairly uniformly, but in others they tended to form fascicles or to favour certain segments of the thalamic explant. These examples, from 122 co-cultures involving E-14.5-16 cortex and thalamus, illustrate the range of variation, which did not correlate with the sites of origin of the explants. Scale bar 1mm.



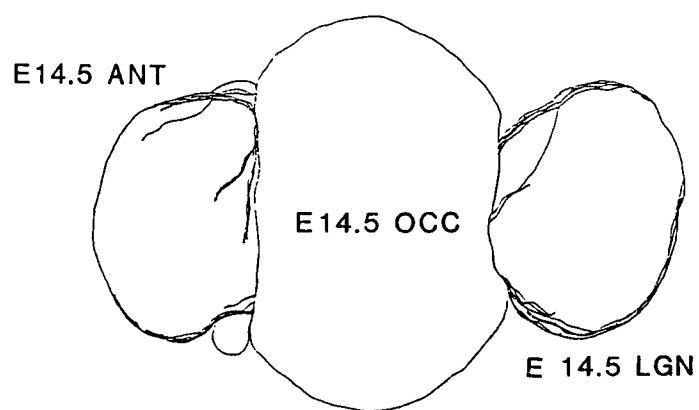
A



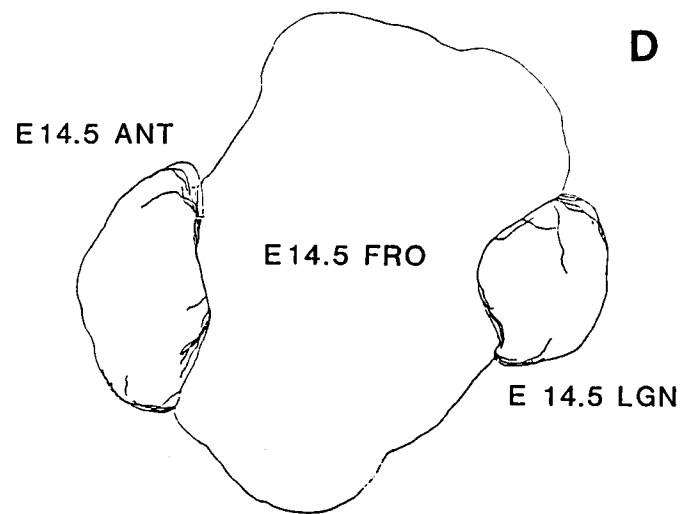
B



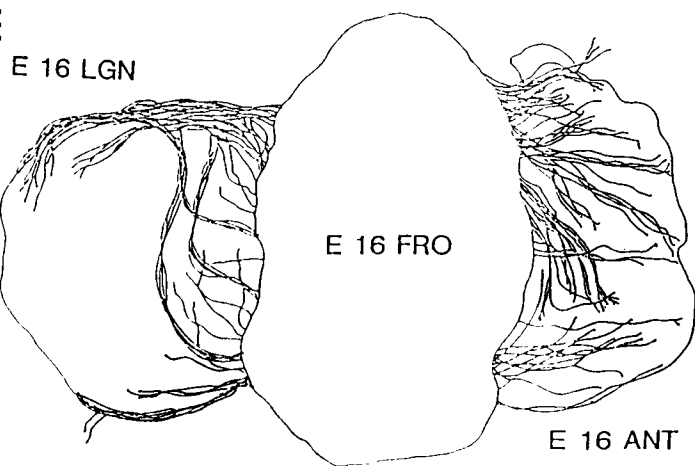
C



D



E



F

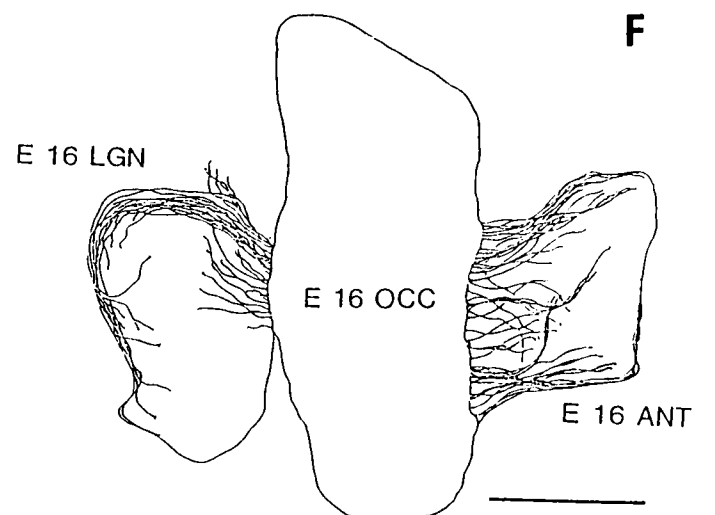


Fig. 2.23

Summary diagram of the thalamic fibre ingrowth patterns in thalamocortical co-cultures. Occipital cortical slices were taken at E19, P0 and P6 and cultured for 5 days with E16 LGN blocks. In the left column the schematic drawings demonstrate the fibre ingrowth from the ventricular surface, and in the right column from the pial surface. Depending on the age of the cortical slice different fibre ingrowth patterns were observed.

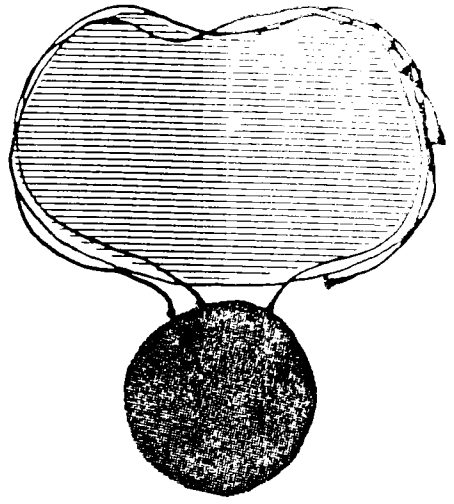
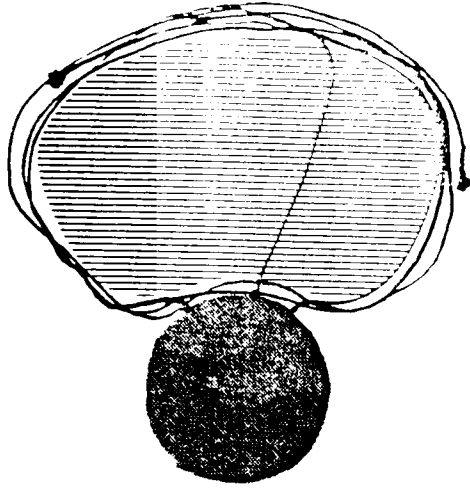
E19: Cortical slices at E19 (or younger) do not accept thalamic fibres. Most grow around and not within the cortical slice. The distribution is similar in the slices in the thalamic block was placed on the ventricular side and on the pial surface.

P0: In newborn cortical slices the thalamic fibres run radially or obliquely through the cortical plate to the marginal zone, where some turn laterally to run over or beneath the pial surface. The ingrowth pattern of the thalamic fibres entering from the pial surface was similar to that of axons entering from the ventricular surface: the fibres grew through, all the way to the opposite side.

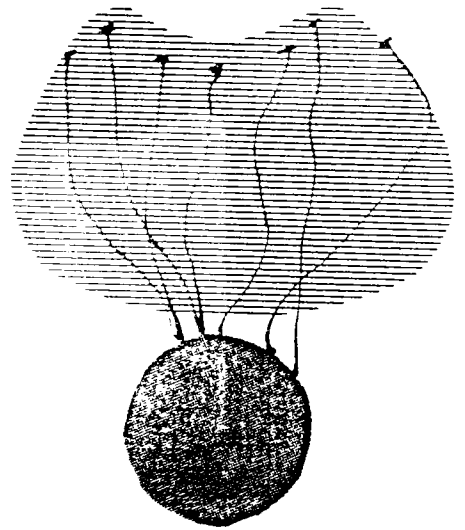
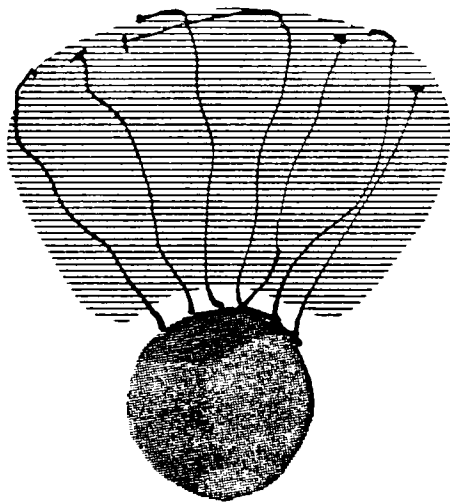
P6: In cortical slices taken from a six day old pup, most thalamic fibres branched and terminated some 250-300µm below the pial surface and very few invaded the upper layers. This sub-pial region does not seem to be actively inhibitory for axon ingrowth because it was invaded by the thalamic fibres when the thalamic block was placed against the pial surface. The fibres grew in and branched and arborized within the same termination region, 250-300µm below the pial surface.

These experiments imply that the cortex starts to become ingrowth-permissive at about E19 and that a layer-specific stop signal is expressed before the end of the first postnatal week.

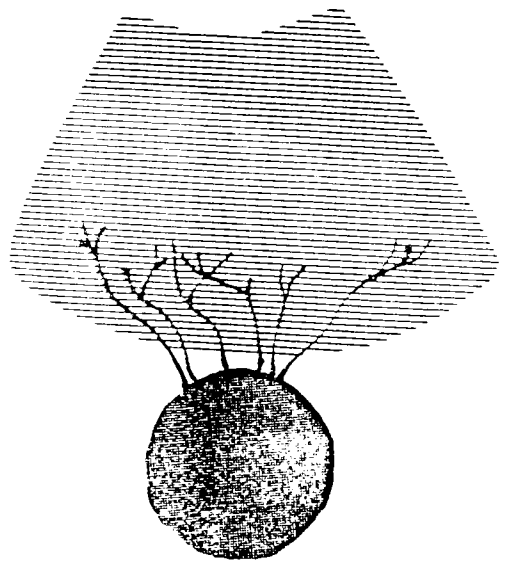
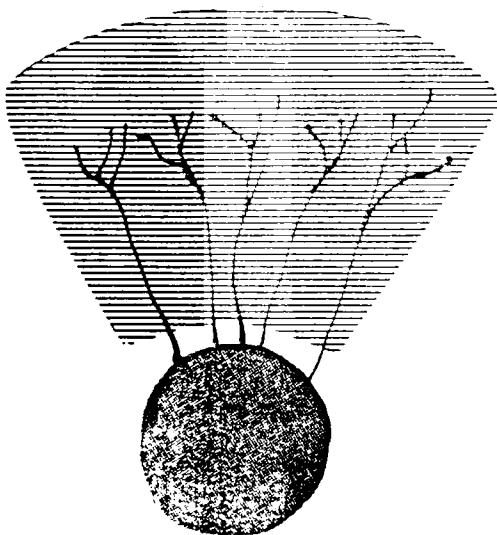
E19



P0



P6



GENERAL DISCUSSION

Long range effects between thalamus and cortex

Chemotropism as a mechanism of axon guidance was originally suggested by Cajal (1893, 1909). The theory was reformulated as the specific chemoaffinity hypothesis by Sperry (1963). Recently, interest in chemotropism has re-emerged with quite convincing examples of this mechanism operating in various parts of the peripheral and central nervous system (see Lumsden, 1992). In the light of recent work with co-culture we are in a position to ask questions about the nature of the cell-cell interactions underlying the formation of area- and layer-specific thalamocortical connections.

After I had begun the experiments described here, Yamamoto et al. (1989) reported that LGN and occipital cortex form layer-specific inter-connections after 2 weeks in vitro, geniculate axons terminating in layer 4 and cells of layer 6 sending axons into the LGN. In their study, P1 to P3 cortical slices were combined with small E16 thalamic blocks. In my study I examined thalamic ingrowth mostly after 2 to 5 days in vitro and the age at which the cortical slice was taken covered a much larger range (E16-P11, see Table 2.1).

In cortex of different ages, thalamic fibres exhibited different behaviour that could be related quite convincingly to the situation in vivo. First, with the culturing methods that I used, few axons grew out of thalamic or cortical explants, on either collagen or laminin, in the absence of an appropriate neighbour. This allowed me to assess the presence of remote influences of one explant on another and I found that cortex does exert a clear growth-stimulating influence on thalamus at appropriate ages. The interaction seems best described as trophic rather than tropic (see Lumsden, 1992), though classically, trophism implies an interaction that is essential not only for the

growth but for the survival of the cells in question (see Chapter 1.). A survival-promoting influence has not been shown by any of the studies in co-culture (though it would be an interesting topic for future investigation with more sensitive viability assays; Adams R. et al; 1993). Cunningham et al (1987), have provided evidence for a classical (death-preventing) trophic influence of cortex on thalamus in the rat in vivo. They showed that the degeneration of the LGN that normally follows removal of the occipital lobe in newborn rats could be partially prevented by replacing the ablated cortical tissue with a gel impregnated with medium conditioned by cultured cortex. The soluble fraction responsible was heat-labile and they believed it to be proteinaceous.

The results that I obtained with conditioned media were disappointing but are entirely compatible with the fact that outgrowth is stimulated in co-culture only when the gap between the two explants is small compared with the diameter of the culture well; presumably the concentration of the factor in the whole volume of medium was insufficient to promote growth (although there are other possible additional explanations, including rapid deterioration of the factor). This subject clearly deserves further study.

Nevertheless the results suggest that a cortical slice in culture produces some substance that diffuses through the medium and, if the thalamic explant is close enough for the concentration of the substance to be sufficiently high, stimulates axons to leave the thalamic explant. Growth of thalamic axons within the thalamic block can be quite different (see Placzek et al., 1990; Tessier-Lavigne and Placzek, 1991). Whether the effect is *trophic* (simply enhancing the growth and/or survival of those axons closest to the source of the factor) or *tropic* (causing growth specifically towards the source by producing a directional response of growth cones up the concentration gradient) is

problematical to determine in my culture system, but obviously of considerable importance (Lumsden, 1992). In principle, the difference between the two classical concepts is quite sharp, and they can be distinguished experimentally, for instance by the observation of neurite growth in three-dimensional collagen gels, unconstrained by surface properties (Lumsden, 1992). Such experiments will be needed on thalamocortical co-cultures to be sure how the growth-promoting factor actually works (see Fig. 1.2. of chapter 1). However, in any situation (in vitro or in vivo) where very steep concentration gradients of a purely trophic factor are established, it can produce directed growth, and therefore mediate a form of chemotaxis.

The functional significance of soluble growth-promoting substances is especially difficult to interpret for explants in culture, because the tendency to establish gradients will obviously depend on the extent to which the medium is mixing or being changed. With the roller-tube technique, the medium is constantly in motion. Indeed, Bolz et al. (1992) have reported that they see no directed outgrowth from thalamus to cortex in co-culture, using the roller-tube method. He even suggests that there is no diffusing influence at all, because he sees no difference in axon outgrowth for thalamic explants with and without a neighbouring piece of cortex. However, serum-enriched medium, of the type used by Bolz et al. (1992) stimulates considerable (and variable) axon outgrowth even from an isolated thalamic explant, which makes it difficult to interpret the effect of any added cortical slice. Since, in the conditions that I have used, little or no outgrowth occurs from a thalamic explant without a nearby cortical explant, my experiments seem to provide firm evidence for a remote influence.

D. Caric, D. Price and S. Rennie, using culturing methods identical to mine, confirmed my observations on remote growth-stimulating influences in co-cultures of

mouse thalamus and cortex (Caric et al., 1991; Rennie et al., 1994). They observed and quantified axon outgrowth under phase-contrast microscopy in living cultures and concluded that it is dependent on the distance of the cortical explant, up to a distance of 2 mm on collagen. Therefore, with a sufficient gap between the two explants, outgrowth from the thalamic explant is denser and axon elongation faster on the side facing the cortical slice.

Age-dependent, ingrowth-permissive property of cortical slices

Bolz et al (1992), who has confirmed the failure of thalamic axons to invade very young cortex, have reported evidence that older cortex has a membrane-bound factor that encourages axon ingrowth (Bolz et al, 1992; Götz et al, 1992; Bolz and Götz, 1992). They deposited a membrane suspension extracted from cortical explants onto the surface of a culture chamber on which they grew thalamic explants. If the cortex came from E16 embryos, there was little or no axon outgrowth, but if it came from P7 cortex there was florid outgrowth on the membrane-treated surface.

The nature of this membrane-bound factor deserves investigation. Godfraind et al., (1988) and Chung et al. (1991) demonstrated rapidly changing immunoreactive patterns in the developing rodent brain. Interestingly the growth-supporting factor L1 was found to be (almost exclusively) associated with the subplate and subplate fibres. Chun and Shatz (1988) also described transient fibronectin expression in the subplate in the developing cat cortex.

Bolz and Götz (1992) have recently reported that this membrane-bound influence can vary slightly from region to region: outgrowth from an LGN explant is somewhat more vigorous on a surface treated with the membrane fraction from P7 occipital cortex than from P7 frontal cortex. At first sight, this result seems to contradict to my general

conclusion that there is no obvious regional specificity in any of the interactions that I and others have seen in co-culture (Molnár and Blakemore, 1991; Yamamoto et al 1991; 1992), which Bolz also confirms in similar paradigms. However, it must be pointed out that Bolz agrees that the growth-permissive nature of cortex is not yet present at E16 by which time thalamic fibres in vivo have already made their way in topographic order to their cortical target areas (Chapter 3; Molnár and Blakemore, 1990a; Blakemore and Molnár, 1990; Molnár et al, 1993), or even at early postnatal ages. Therefore even if there is slight regional variation in the nature of the permissiveness of the cortex at much later stages, it cannot play a part in the basic process of ordered guidance of thalamic axons to their cortical target areas.

The slight difference in the influence of membrane fractions from P7 occipital and frontal cortex on outgrowth from LGN might conceivably be due to the presence of thalamic axons in the explants of cortex at that late age. There might indeed be a tendency for axons from any one thalamic nucleus to prefer to grow on axons from the same nucleus rather than others. Indeed such selective adhesion and fasciculation might contribute to the maintenance of fibre order in the advancing front of thalamocortical afferents in vivo (see Chapter 3). Certainly, the tendency of axons to fasciculate preferentially on existing axons is a well-described phenomenon (Cajal, 1928) and they can be selective for particular, similar axons. Description of the location of membrane bound factors during thalamocortical pathfinding is obviously important, but P7, the age used by Bolz and Götz (1992) is too late to define factors important in initial axon guidance.

There is evidence that the molecule or molecules responsible for the growth-permissive influence are not the same as the diffusible factor. When E16 LGN was

cultured with explants of occipital cortex taken from embryos younger than about E19, axons grew out of the LGN block but few invaded the cortical fragment (Fig. 2.16). Instead they mostly ramified around the edges and to some extent over the surface of the cortical explant without penetrating it (see below). Clearly this implies that a diffusible factor is produced by the cortex at least as early as E16, but that some additional property is required of the cortex to encourage axon invasion, and this is not expressed until about E19.

In co-culture with young embryonic cortex, thalamic fibres grow around the edges of the cortical slice but rarely enter it. In late fetal and early postnatal cortex, thalamic fibres grow through the whole thickness of the cortical slice but still fail to terminate within the cortex and tend to grow up to the pial surface and fasciculate over it. But in slices taken from animals older than P3 they stop and arborize near the middle of the cortical plate after 4 days in vitro, and they do so even when the fibres enter through the supragranular layers (for a schematic summary diagram see Fig. 2.23).

This sequence correlates convincingly with that which occurs in vivo. Axons do indeed start to grow out of the thalamus at around E14 (Chapter 3) and it is conceivable that this is at least partly due to a remote (but not regionally specific) influence from the subplate. At E14, subplate cells are in place and they (or some other element in the cerebral wall) might generate a diffusible growth-promoting factor capable of producing some degree of stimulus for growth in culture. Equally, the synchronized outgrowth of subplate axons towards the thalamus in vivo might be influenced by a (non-regionally-specific) growth-promoting factor from the thalamus, revealed by the outgrowth from embryonic cortex that occurs in culture in the presence of thalamus of similar age (Fig 2.22).

There is, of course, the possibility that positionally specific signals normally expressed by cortex in vivo are lost or inadequately expressed in vitro. But if these in vitro results do apply in vivo, some additional mechanism must account for the topographic nature of the early thalamocortical projection. Thalamic axons arrive under the correct region of cortex at about E16 - a stage at which the cortex itself is not yet permissive for ingrowth of axons in culture. Indeed, in vivo, the thalamic axons accumulate below the cortical plate and may form temporary synapses on cells of the subplate (Shatz et al., 1988; Herrmann et al., 1991). At about E19, as judged by its properties in culture, the cortex itself starts to become permissive to thalamic axon invasion and, in vivo, small numbers of axons do grow into the cortical plate proper at this stage, but the majority remain in the subplate for 2-3 days after the cortical plate becomes permissive to invasion (E19-birth). This waiting period is one characteristic of the natural pattern of innervation that I could not duplicate in culture. Perhaps thalamic axons have to arrive at the subplate cells by fasciculated growth along their particular axons in order to be able to form synapses on them and hence to be temporarily captured in that layer (see Chapter 3 and 4 for further discussion).

It would be worth repeating with less mature cortical slices the co-culture experiments in which LGN explants are placed at the side of the cortical slice, in close proximity to the intermediate zone, to encourage thalamic axons to associate closely with subplate axons as they grow towards the cortex. In the preliminary experiments that I did with this procedure, thalamic axons did grow into cortical slices from the side, via the intermediate zone, but they did not wait in the subplate layer. However the slices of cortex came from relatively old animals (P3, P6), taken after the normal termination of the waiting period. It would be very interesting to continue these

experiments with cortical slices taken at E15 or E16, after subplate axon outgrowth but before the end of the waiting period.

The experiments in which thalamic explants were co-cultured with ribbons of subplate or cortical plate (see Fig. 2.17) suggest that in postnatal cortex both subplate and upper cortical layers are capable of remotely stimulating thalamic outgrowth and permitting ingrowth and that prior exposure to the environment of the subplate is not necessary in order for axons to enter the plate proper.

It would obviously be interesting to repeat these experiments with E16-19 cortical slices, but unfortunately younger cortical slices are extremely difficult to handle. Alternatively ‘tissue printing’ (Derer and Derer, 1992) from the surface of the cortex of the fetal Reeler mouse (containing only preplate neurons; see Chapter 4) might overcome these problems. However, the fact that thalamic axons do grow out when combined with cortical slices as young as E16 (see Fig. 2.4), when mostly subplate cells, ventricular and subventricular cells are present, strongly suggests that the subplate alone can generate a remote growth-stimulating factor (even though thalamic axons are reluctant actually to **enter** and grow through cortical explants at such young ages).

Layer-specific innervation; the stop signal

The parallel between the natural pattern and events in culture becomes convincing again after birth. In vivo, the majority of thalamic axons start to detach from the subplate, to turn sharply and to grow into the cortical plate shortly before birth (at least for the occipital cortex: see Chapter 3) - a stage at which the cortex is indeed highly attractive to thalamic axons in vitro. In vivo, they grow up through the deep layers and reach layer 4 by about P2-3 when that layer is starting to express its stop signal in vitro. Thalamic axons that enter somewhat precociously in vivo tend to grow up to the

cortical surface just as they do with cortex younger than P3 in vitro; odd axons are seen in vivo extending into the marginal zone, as if they have managed to grow through the cortex before the stop signal appears. This tendency for early thalamic axons to avoid being 'captured' by layer 4 may be the origin of the disproportionate early innervation of layer 1 of the kitten cortex (Kato et al., 1984; Henderson and Blakemore, 1986). Possible mechanisms will be discussed further in Chapters 3 and 4.

When thalamic explants are cultured close to the pial surface (rather than the ventricular surface) of a cortical slice they grow down through the superficial layers and either terminate in layer 4 (after about P3: see Fig. 2.18) or continue throughout into the intermediate zone (before P3). Therefore the upper cortical layers are not inhibitory to axon growth and the signal expressed around P3-4 is not one that simply discourages axons from entering the upper layers. It seems likely that neurons of layer 4, which have reached their correct position and matured considerably by that age, express a specific **stop signal**, which encourages thalamic axons to arborize and terminate. The molecular nature and localization of this signal deserve further investigation.

Even though this experiment strongly suggests that the superficial layers do not entirely resist growth into them, it is still conceivable that growth inhibition (perhaps within layer 4 itself) plays a part in encouraging termination in layer 4. There might be some kind of interaction between local growth-terminating and growth-inhibiting signals.

Appearance of layer 4 coincides with the stop signal

One requirement for this kind of tight comparison between culture and real life is that the upper laminae of the cortex should continue to develop, at least to some extent, in culture. There are few systematic studies on this matter. Bolz et al. (1990) have shown,

by means of birthdate labelling with bromodeoxyuridine (BrDU), that cells born on E16 and E18 have distributions through the cortex that look fairly similar in normal animals more than 12 days old and in slices taken shortly after birth and cultured (by the roller tube technique) for 2-3 weeks. I was especially interested in the position of layer 4 cells but, unfortunately, there is a huge overlap in the generation of the cells contributing to different layers in rodents (Miller 1988). Although the peak production of cells destined for layer 4 occurs on E16, cells born between E14.5 and E18 contribute to this layer (Lund and Mustari, 1977; Miller, 1988). Therefore it cannot be completely and exclusively labelled by thymidine or BrDU birthdating.

The extended period of generation of cells destined for a particular layer of the rodent cerebral cortex makes the selective and total elimination of one layer by means of cytotoxic agents impossible. Injection of methylazoxymethanol (MAM; a cytotoxin that destroys dividing cells, Chen and Hillman, 1986; Yurkewicz et al., 1984) only twice on E16 cannot possibly interfere selectively, exclusively and completely with the generation of layer 4 (Fig. 2.19). Therefore it is not surprising that in co-culture with late postnatal cortical slices from such MAM-injected rats, thalamic axons still stop in the middle of the cortical plate, just as normal tissue. Perhaps more complete elimination of layer 4 could be achieved in species with more protracted period of cortical neurogenesis (see Fig. 1.1C in Chapter 1).

At E20, only the cells of the uppermost layers are still being born in the rat. By injecting BrDU on this day and culturing the cortical slices from P3 for several weeks Bolz et al. (1990) showed that there is indeed migration in culture, and these cells take up their expected position below the marginal zone. However, slices taken at or before birth and cultured for several weeks show a retarded distribution in similar experiments,

indicating that migration and the maturation of laminae is disturbed in such young tissue in vitro (Bolz et al, 1990). My own results confirm this picture. Slices taken at birth certainly continued to thicken and showed obvious differentiation of cells in the upper layers, but the laminar pattern above layer 5 was not exactly the same as that in a normal animal of the same chronological age. The later the slice is taken, the more normally it appears to complete its development.

De Jong et al. (1988) examined the cytoarchitecture of cultured rat neocortex explants taken at P6 and cultured (by the interphase-type tissue culture technique) up to 13 days. They reported major changes in the layering of the cortical slices during the first 2 days in culture. The thickness of the pial-ventricular axis increased and this was due to an increase in the proportion of layers 2, 3 and 4. In spite of this evidence of continued migration and/or enlargement of cells and neuropil in the upper layers, a distinct layer 4 could not be consistently identified in that study, perhaps because of the degeneration of its thalamic afferents in the cortex-alone culture. In addition, neuronal migration has been recently been observed directly in similar cultures (O'Rourke et al, 1991), but its extent is difficult to judge.

All these experiments were performed with cortical slices which were removed from normally developing brains at various ages. Some of these cortices therefore had already been exposed to thalamic fibres and their development could therefore have been affected by those thalamic fibres. It would be interesting to repeat some of these experiments with 'virgin' cortical slices of various ages, which have never been exposed to thalamic fibres.

During the preparation of cultures, explants suffer extreme alterations in their environment. It is therefore of interest to consider to what extent intrinsic connectivity

remains intact and continues to develop normally in the slice cultures. In cultures of hippocampus, Zimmer and Gähwiler (1984) have described reorganization of nerve connections similar to that observed after lesions in vivo. De Jong et al. (1988) did not see obvious widespread cell death in any layers of their cultured slices, when examining them several times up to 13 days in culture; only a few individual degenerating cells were seen, especially in layer 5. On the other hand, in hamsters with the posterior thalamus lesioned at birth, a decrease in the number of cells in layer 4 of the occipital cortex has been reported (Miller et al., 1991; Windrem and Finlay, 1991). In my own collaborative work (Molnár et al., 1991) on the in vivo development of the cortex deprived of thalamic input by the embryonic disruption of the thalamocortical pathways, the isolated cortex develops its basic laminar organization. However, Nissl staining alone is obviously a very crude technique to examine these questions in detail. Combined birthdating and cell filling studies would be needed to be confident about cell fate and morphology (see Katz and Callaway, 1992). Nevertheless, both in vivo and in vitro the results suggest that the thalamic input might contribute to and play an important role in the formation of circuitry, at least in layer 4, and might help to sustain cells in that layer.

Experiments with thalamic and cortical organotypic co-cultures led to the conclusion that three molecular signals are expressed by the cortex at different ages: (1) a trophic effect on the thalamus, (2) an axon-growth-permissive property from about E19; and (3) a 'stop signal', specific to layer 4, from about P3. These factors presumably influence thalamic fibre outgrowth, accumulation below the cortical plate, invasion and the termination. In vitro any region of the rat thalamus (E16) innervates any region of the cortex (E16-P11): no positional preference is apparent even when the

thalamic explant is given a choice of various cortical targets.

The most important question that remains, with no hint of a mechanism from the co-culture experiments, is: How are thalamic axons guided in an orderly fashion to their own cortical areas? That is the issue tackled in the next chapter.

CHAPTER 3

MECHANISMS UNDERLYING THE ESTABLISHMENT OF TOPOGRAPHICALLY ORDERED EARLY THALAMO-CORTICAL CONNECTIONS IN THE RAT

INTRODUCTION

3.1 Importance of thalamocortical innervation in the development of the cerebral cortex

The adult mammalian neocortex consists of numerous areas that can be distinguished from one another on the basis of differences in the cytoarchitecture, input and output connectivity as well as their functional properties (revealed by recording neuronal activity and by observing the behavioural effects of lesions). The embryonic cortex looks strikingly homogeneous in contrast with the heterogeneity in the adult. A central issue in understanding neocortical area differentiation is determining the relative contributions of genetic and epigenetic factors in the emergence of area-specific features during neocortical development. When and how do the areas of the cerebral cortex become specified?

The orderly topographic maps formed by many projections constitute one of the most remarkable features of the adult brain. Axons begin to reach the cortex even at a very early stage, before the majority of cortical neurons have been born. Thus, the arrival of these early axonal projections, if they are topographically distributed and distinctive for each particular area, could be the first obvious regionally differentiated event. Indeed, the earliest axonal inputs to the cortex could be capable of constituting an *extrinsic signal* that plays a part in triggering other specific regional differentiation,

perhaps even leading to most of the repertoire of areal specializations. In the extreme version of such a proposal, the cortical mantle might be generated as an entirely uncommitted 'protocortex' (O'Leary, 1989) from which regional specialization is carved out by the influence of axons arriving at early stages. This hypothesis has aroused considerable interest in the relationship between the development of the first extrinsic connectivity of the cortex and its cytoarchitectonic differentiation.

In this context, the rodent somatosensory cortex has been extensively studied. The distinctive feature of this cortical area is the representation of individual whiskers as individual patternings of layer 4 cells and incoming fibres to form the whisker barrels, provided an excellent model system for numerous studies. These demonstrated that the postnatal maturation of the barrel field (Van der Loos and Woolsey, 1973) requires an intact sensory periphery and intact afferentation during a critical period (Woolsey and Wann, 1976; Belford and Killackey, 1980; Jeanmonod et al., 1981). Application of more sensitive tracing techniques, including lipophilic carbocyanine dyes (Erzurumlu and Jhaveri, 1990) and acetylcholinesterase histochemistry (which provides an early transient marker of thalamocortical axons; Robertson, 1987) has revealed the periphery-related patterning of thalamocortical afferent termination at least 1 day earlier than previous markers for barrels (reviewed by Schlaggar and O'Leary, 1993). Another group of experimental results in support of the external influence on cortical differentiation comes from transplantation studies. If an explant of E17 rat visual cortex is transplanted into the position of the putative barrel field in a newborn rat, then it is capable of developing at least some of the cytoarchitectonic features of barrels (patterned distribution of thalamocortical afferents, layer 4 cells and matrix molecules) ordinarily unique to the primary somatosensory cortex (Schlaggar and O'Leary, 1991).

A number of other cortical transplantation studies in rodents have demonstrated that heterotopic cortical afferent (Chang et al., 1986; O'Leary et al., 1992) and efferent (Stanfield and O'Leary, 1985; Barbe and Levitt, 1991) connections develop appropriate for the new location, rather than the original site of the cortical explant.

Increasing evidence suggests that although a certain basic intrinsic cortical organization can develop without any afferent connectivity, some cytoarchitectural attributes of at least the major sensory cortical regions are somehow imposed by the influence of specific thalamocortical fibres (Woolsey et al., 1979; Killackey and Shinder, 1981; Welker and van der Loos, 1986; O'Leary, 1991). This generalization appears to be valid even for the primate. Dramatic changes in the macroscopic appearance of the occipital pole and the dimensions of the striate cortex have been demonstrated in monkeys as a result of early prenatal enucleation, which leads to a decrease in the number and distribution of LGN axons (Rakic, 1988; Dehay et al., 1989).

If one accepts the considerable evidence that thalamic input plays a part in triggering regional cortical differentiation and in defining the boundaries of major cortical fields, unravelling the mechanism by which thalamic axons are directed towards their target regions is important in understanding how cortical organization is determined. There are many questions that need to be answered. For instance, are the earliest thalamocortical projections distributed in topographic order and without gross exuberance? If so, how is that order established and maintained? And how do the earliest input fibres interact with the developing cortex? Understanding the guidance of thalamocortical projections may be the key to understanding cortical organization.

3.2 Adult topography of the thalamocortical projections

Thalamocortical projections have distinct and characteristic areal and laminar specificity (see Chapters 1 and 2). The representation of the three-dimensional thalamic volume on the continuous planar sheet of the cortex has been studied extensively in the rodent (Caviness and Frost, 1980; Crandall and Caviness, 1984). These studies demonstrated that all areas of the rodent neocortex receive a thalamic projection (Caviness and Frost, 1980). In the **adult**, neighbourhood relationships within the thalamus do not always correspond precisely to the distribution in the cortex. In most cases adjacent neocortical fields receive their projections from adjacent thalamic nuclei, and proximity relationships correspond in the cortex and the thalamus; but occasionally, non-adjacent thalamic nuclei project to neighbouring cortical regions, with no overlap, separated sharply at the borders of their cortical regions (Caviness, 1988). The relative positions and neighbourhood relationships of the fibres originating from different thalamic regions are, to a considerable extent preserved on their course to the cortex within the characteristic fan-shaped radiation of thalamic fibres (Caviness and Frost, 1980; Caviness, 1988; Agmon and Connors, 1991), but this does not appear so convincing at the level of individual fibres, which exhibit mixing and twisting along their path (Bernardo and Woolsey, 1987). However, these studies do not necessarily provide information about the mechanism of establishment of connections. For one thing, all of these studies used only a single tracer, but the synchronous application of two or more distinguishable tracers into neighbouring thalamic or cortical regions is needed to reveal topographic relations on a more global scale. More important, all these observations were made in the adult (Caviness and Frost, 1980; Bernardo and Woolsey, 1987) or in the postnatal animal (Crandall and Caviness, 1984). They, therefore did not address the

crucial question of topographic relationships within the embryonic thalamus during the differentiation of the individual nuclei (see Chapter 1) nor the mapping of the ascending embryonic thalamic fibres at the earliest stages of outgrowth.

3.3 Chronotopy

In many systems the temporal sequence of neuron production and differentiation in interconnecting structures is correlated with the order of establishment of connections between the structures (Bayer and Altman, 1987). The translation of a temporal gradient into topographically ordered connectivity was first suggested for the retina after its neurogenetic and morphogenetic gradients were revealed (Horder and Martin, 1978; Martin and Perry, 1983). Bayer and Altman (1987) pointed out that many of the major anatomical interconnections of the telencephalon can be related to the order of neurogenesis and to neurogenetic gradients of either the source neurons (entorhinal cortex, olfactory bulb mitral cells), receiving neurons (lateral septal nucleus, cortical nuclei of amygdala, anterior olfactory nucleus, primary olfactory cortex) or both (magnocellular basal nuclei, striatum) (Bayer 1985; 1986; 1980; Bayer and Altman 1987; Bayer and Altman 1991). The attractive possibility, that fibre ordering is based on the relative time of their establishment and that temporal events are translated into spatial distributions, is termed *chronotopic* fibre ordering. It could provide (or at least contribute to) a mechanism for the establishment of mapped projections without the need for any specific chemical matching between the interconnecting structures.

The possibility that chronotopy plays a part in the establishment of ordered thalamocortical projections assumes greater significance, in view of the co-culture experiments reported in Chapter 2, which suggest that in vitro there are no powerful, regionally specific attractive signals between cortex and thalamus. However, the

plausibility of the chronotopic hypothesis rests on showing that:

- 1) fibre outgrowth follows a spatiotemporal gradient; and
- 2) fibres remain ordered, according to their times of generation, during their outgrowth and arrival.

Only if these conditions exist would it be conceivable that at the site of innervation the arriving fibres could simply occupy their places on a 'first-come-first-served' basis, establishing a map based on chronotopy.

I became interested in the possibility that such a mechanism plays a role in establishing thalamocortical connections and examined the timing and organization of reciprocal fibre outgrowth from thalamus and cortex. In this chapter the pattern, relationship and precision of topography of different fibres with known origin and termination were examined in Hooded Lister rats. For the tracing of the early reciprocal connections different fluorescent dyes from the carbocyanine family were used in aldehyde-fixed whole brains.

3.4 Carbocyanine dyes in embryonic tracing

Until recently, the only information about the development of thalamocortical connectivity came from studies using degeneration techniques (e.g. Lund and Mustari, 1977) or transneuronal transport of label from the eye (Rakic, 1976). In the past couple of years, however, the fluorescent carbocyanine dyes (Honig and Hume, 1986, 1989) have revolutionized the tracing of embryonic pathways, because they can be employed in fixed tissue (Godement et al, 1987). Although some aspects of the issue of the development of thalamocortical projections were addressed more than a decade ago (e.g. Lund and Mustari, 1977; Wise and Jones, 1978) the greater versatility and sensitivity of the new techniques promised a greater depth of understanding.

The ingrowth of thalamic afferents has now been studied with carbocyanine dyes in fixed brains by several groups (Catalano et al, 1991; Reinoso and O'Leary, 1990; Erzurumlu and Jhaveri, 1990, 1992; Blakemore and Molnár, 1990; Molnár and Blakemore 1990a,b, 1992; Kageyama and Robertson, 1993; Miller et al. 1993). In most respects this recent work has confirmed the essential findings of the earlier studies, but has added further precise information.

3.5 The role of the subplate

Another reason for re-addressing these issues is provided by the new knowledge of the earliest corticofugal projections. The first postmitotic neurons of the mammalian cerebral cortex are largely a transient population, which come to reside above and below the developing cortical plate (Marin Padilla, 1971; Luskin and Shatz, 1985a,b). Some speculate that during their brief presence, these neurons, especially the so called 'subplate' population, participate in numerous cell-cell interactions that are important in cortical development (Shatz et al., 1990; Blakemore and Molnár, 1990). McConnell et al. (1989) drew our attention to the fact that subplate axons (as originally described by Marin-Padilla, 1971,1972) pioneer the first axon pathways to subcortical targets, before neurons of layers 5 and 6 (which form the adult subcortical projections) extend their axons. Thus, thalamocortical axons (and conceivably later-growing corticothalamic fibres) might navigate their way to distant targets along pathways laid down by pioneering axons of subplate neurons.

To support the hypothesis that the pioneering subplate axons provide topographic guidance for thalamocortical projections, two further pieces of evidence are essential. First, the subplate projection must itself be topographically ordered, providing guidance pathways between each thalamic nucleus and the appropriate cortical area. Second, it

must be shown that thalamic axons grow in intimate association with their corresponding subplate fibres. Neither of these requirements for the theory was established by McConnell et al. (1989). I became interested in the role of the subplate during the development of this pathway in the rat and began to study the development of the early reciprocal connections.

Not long ago it was not universally accepted that rodents have a true subplate, comparable to the distinct, precociously maturing, transient population in cats and primates (Shatz et al. 1990; Kostovic and Rakic, 1990). However recent evidence suggests that there are numerous similarities between the rodent subplate and that of other mammals. Using tritiated thymidine birthdating in the rat it was demonstrated that the first cortical neurons are born on E12, the 12th postconceptual day (Valverde et al., 1989; Bayer and Altman, 1990). Valverde et al (1989) showed that cells generated on E12 (before the start of proliferation of the true cortical plate) come to lie almost exclusively in layer 1 and sublayer 6b and that they decline in number postnatally. Ferrer and collaborators (1992) derived very similar conclusions from the quantitative study of pycnotic profiles in the developing rat cerebral cortex. Woo et al. (1990) and Annis et al. (1990) reported that cell loss is more pronounced in layer 6b of rodents (compared with layer 4 and the LGN). More recently Wood and colleagues (1992) provided rigorous quantitative evidence that the first generated cells constitute a partly transient cell population in the mouse. This all suggests that layer 6b of the rodent may be considered the equivalent to the subplate of the cat (Luskin and Shatz, 1985a,b; Chun et al., 1987; Shatz et al., 1988) and monkey (Kostovic and Rakic, 1980). The major difference is that although there is disproportionate cell death in the rodent subplate (Ferrer et al., 1992; Wood et al, 1992), a substantial population of subplate

cells persists, forming an intact and distinct layer 6b in adult rodents. In many respects, however, it is now widely accepted that the rodent subplate is similar to that of carnivores and primates.

The aim of the study described in this chapter was to explore the timing of establishment and the orderliness of the first corticofugal and thalamocortical fibres, by examining the relationships among and between the two axon arrays on the way to their targets. I was also interested in the possibility that the earliest-generated cells of the subplate and their axonal projections play a role in guiding and hence determining the topography of the thalamic connections, which in turn might influence the differentiation of a given cortical area.

METHODS

Hooded Lister rats, whose gestation period is 21-22 days, were time-mated at the University Laboratory of Physiology, Oxford. Assessment of the embryonic age (E) of the fetuses was based on the plug date, defined as E0. Fetuses were removed under pentobarbital anaesthesia (100 mg/kg I.P. to the pregnant female) at various stages, from the 13th to the 21st or 22nd post-conceptual day. They were immediately chilled on ice and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) delivered through a glass micropipette under a dissecting microscope. Postnatal animals of various ages were anaesthetized by chilling (up to P4), or by overdose of sodium pentobarbital I.P. (if older than P4), prior to perfusion.

The brains were removed and after overnight post-fixation (in 4% buffered paraformaldehyde) single crystals of fluorescent carbocyanine dyes ("DiI" C18 [D-307]; "DiA" 4-di-16ASP [D-3883] and 4Di10ASP [D-291], Molecular Probes, Inc. Eugene, OR, U.S.A.) were inserted into different parts of the cortex or diencephalon under an

operating microscope. (Other similar carbocyanine dyes, DiO, DiS, etc., were also tested, but the transport of these dyes was not so impressive; see Fig. 3.18D below). Table 1 lists the number of brains (both hemispheres) used for the different studies at each developmental stage (E13 to P8).

For each cortical placement, a single small, sharp carbocyanine crystal was inserted into a tiny slit made in the cortex with the tip of a pair of fine forceps, or, in embryonic brains, with a thin stainless steel wire. The depth to which the crystal was inserted into the cortex varied with age, from as little as 0.1 mm at E14 to 0.5mm in the oldest animals. In half of the brains, I made deposits of a number of different dyes, at different points across the cortex, usually spaced 1-2 mm apart in either a parasagittal or a coronal row in one hemisphere. For most of these experiments, I used alternating placements of DiI and DiA (the two dyes that are best transported), which are clearly distinguishable under different wavelengths of illumination.

Even fetuses that are members of the same litter, and therefore of the same age, may show differences in size according to their position in the uterine horn (caudal fetuses are usually larger). To control for such variation between individuals, in at least 3 brains from each age group, crystal placements were made in the cortex of one hemisphere and into the thalamus of the other, in the same brain, for direct comparison of thalamic and cortical projections.

For crystal placements in the diencephalon (usually into the LGN), I first transected the brainstem coronally, rostral to the superior colliculus, to expose the posterior thalamus. For insertions into deeper and more rostral thalamic nuclei (e.g. the ventrobasal complex, VB), I bisected the brain sagittally and cut away the medial part of the diencephalon to expose the relevant part of the thalamus. Then the crystal was

Table 3.1 Numbers of animals studied

Age	Carbocyanine dye implantation sites ¹					
	Thal	CTX	C+T	IC	TRN	CP
E13	-	6	-	-	-	-
E13.5	-	6	-	-	-	-
E14	14	6	-	6	-	-
E14.5	5	4	8	6	-	-
E15	7	5	10	6	-	-
E15.5	8	4	8	-	-	-
E16	8	12	8	6	6	-
E18	6	-	-	6	-	-
E19	6	7	-	6	-	-
E20	6	12	-	6	-	-
P0	8	-	-	-	-	-
P2	8	4	-	6	-	8
P4	6	-	-	-	-	3
P8	6	-	-	6	-	4

1 Thal - Thalamus
 CTX - Cerebral Cortex
 C+T - Cortex and Thalamus within the same hemisphere
 IC - Internal Capsule
 TRN - Thalamic Reticular Nucleus
 CP - Cerebral Peduncle

inserted into a small slit made in the tissue with the tip of a fine pair of forceps under an operating microscope.

After crystal insertion, the brains were stored in fixative or in phosphate-buffered saline (PBS) containing 0.1% Na azide, to prevent contamination, at room temperature (22°C) or at 37°C. The incubation period to allow diffusion of the dye (ranging from 1 to 10 weeks, see Table 3.2) was dependent on the age and on the temperature. I had a slight preference to use room temperature because of the slightly lower background labelling. At the end of the incubation period the brains were embedded in 5% agar and 75-250 µm coronal or horizontal sections were cut on a vibratome (Oxford Instruments). All sections were counterstained with bisbenzimidazole (10 minutes in 2.5 µg/ml solution in PBS) or in another chromatin stain, acridine orange (10 µg/ml in PBS). The sections were coverslipped under Hydromount (National Diagnostics) or glycerol-phenylenediamine solution (0.1% p-phenylenediamine, 10% PBS and 90% glycerol, made up and stored according to the method of Johnson et al., 1982). For the 3-D serial reconstruction experiments, I coverslipped the sections under PBS and sealed the coverslip with Paraseal (Raymond A Lamb, London) and imaged them within 4 hours to prevent the differential shrinking and distortions of the individual members of the serial sections.

From the serial sections, double- or triple-exposure colour micrographs were taken (Kodak Ektachrome 400 ASA colour slide film) and camera lucida drawings made under epifluorescent illumination using different filters to reveal the various dyes. Initially, in each brain (and in other control brains not used for dye tracing), some sections were subsequently stained with cresyl violet, to help with identification of the major divisions of the thalamus and to reveal the full thickness and lamination pattern

of the cortex. Counterstaining with bisbenzimidazole and acridine orange helped me to orient the slides by revealing the contours and the outlines of obvious cell patterning (e.g. cortical layer I, layers in the hippocampus, etc.) or confirmed whether a labelled structure contained nuclear chromatin or not. Some sections, selected in conventional fluorescence microscopy, were subsequently examined and photographed with a laser scanning confocal microscope (Biorad MRC-600 or Leica). High resolution reconstructions were made from fine optical sections (1-5µm) to create 3-D datasets. The datasets were projected with 14 degree disparity (using different colours, red and green, for the two eyes' images) to create left-right anaglyphs. For the 3-D effect these pictures should be viewed with the glasses enclosed at the back of the thesis.

Table 3.2 Incubation periods for carbocyanine dye transport
 Incubation periods in weeks (at different temperatures)

Age	22°C	37°C
E13-14.5	2	1
E14.5-16	3-6	2-4
E18-P0	6	4
P0-P8	6-10	4-8

RESULTS

For the sake of clarity I shall present the results in four parts according to the tracer placement site. (i) First, I shall describe thalamic tracing experiments to demonstrate the timing of the thalamocortical fibre outgrowth, arrival at the cortex and cortical plate invasion. (ii) The origin of the corticofugal projections reaching the internal capsule at different developmental stages will be then examined by the presentation of results obtained from internal capsule tracing experiments. (iii) Then I shall describe the data obtained from cortical dye placements, demonstrating the timing and topography of the first corticofugal projections (anterograde transport) and then the topography of the early thalamo-cortical connectivity (retrograde tracing from cortex). (iv) Early corticofugal and corticopetal fibres both pass through the internal capsule (at E14.5 - 16). In the final part I shall describe their relationship by presenting the results of the combined cortical and thalamic tracing experiments.

3.6 Development of thalamocortical projections

Thalamocortical fibre outgrowth

Small crystal implantation into the dorso-lateral part of the thalamus reveals thalamic fibres reaching the internal capsule at E14 (Fig. 3.1 demonstrates a representative example for all experiments performed at this age, n=14). The thalamic fibres run through the primitive internal capsule and under the developing corpus striatum but do not reach the intermediate zone of the ventralmost cortical areas until E15.5 days of gestation (Fig. 3.2). In addition to staining thalamocortical fibres, DiI implantation in the thalamus labelled numerous cells within the ventral thalamus (within the thalamic reticular nucleus) and a few within the internal capsule (Fig. 3.1), in the so-called perireticular nucleus (Mitrofanis, 1992).

Thalamic fibre accumulation below the cortex

Fibres from the dorso-lateral thalamus reach the intermediate zone beneath the ventral cortex around E15.5, and the occipital cortex itself at E16 (Figs. 3.3 and 3.4 are representative examples of all experiments E15.5, n=8; E16, n=8). As they leave the diencephalon, the thalamocortical fibres form distinct fascicles, which open up in a fan shaped fashion. Viewed in coronal sections the ordering of the fibres is such that the inferior lateral fascicles are destined for the ventral part of the cortex, while the superior medial fascicles turn upwards and head towards more dorsal cortical areas. The trajectories of the fascicles remain parallel as they turn up into the intermediate zone: they do not cross each other extensively, although individual axons and small axon bundles do appear to switch from one fascicle to another along their course (Figs. 3.4 and 3.7). Examination of cross sections of these fascicles (labelled from the dorsal thalamus at E15.5) with a confocal microscope shows that they are not entirely compact: their optical cross sections reveal holes and the bundles have rather irregular edges (Fig. 3.8).

At the border of the corpus striatum and the intermediate zone the thalamic fibres defasciculate and break up into a fairly uniform array of individual fibres. Within the intermediate zone the fibres run parallel to each other in an organized fashion. Similarly to the fascicles, the ordering of the individual fibres is such that the inferior lateral fibres are destined for the more ventral cortical segment, while the more medial fibres, situated deeper in the white matter, head towards more dorsal cortical segments (Figs. 3.3, 3.4 and 3.7).

Invasion of the cortex

Although thalamic fibres from the region of the LGN reach the occipital cortical regions

around E16, they do not substantially invade the cortical plate for some time. Most of the labelled fibres remain restricted to the intermediate and subplate zones until E19. During this period, thalamic fibres accumulate and develop local branches, but these are also mainly restricted to the subplate region (Fig. 3.5). Using extremely long incubation periods (10 weeks at 22°C) the processes of radial glia become stained, probably transcellularly from the mass of thalamocortical endings (Fig. 3.6).

The migration of cortical plate neurons is at its peak at the beginning of this 'waiting period' for thalamic axons. The migrating cells therefore have to pass through the mass of accumulating fibres waiting in the subplate layer, as well as between the subplate cells themselves. Before about E19-20 very few thalamic axons appear to have grown into the occipital cortical plate itself. Invasion suddenly begins, on a massive scale, at about late E20, just a day or so before birth. The course taken by these invading thalamic axons is distinctive and highly ordered: they make virtually a 90° turn from their trajectory in the white matter and the vast majority of the fibres initially grow radially, straight up into the cortex directly above the region of the subplate in which they have been waiting. Only a small portion take more erratic routes, running somewhat obliquely upwards.

Establishment of laminar termination patterns

By birth a substantial number of thalamic fibres has invaded the cortical plate. Within the cortical plate the fibres follow a much less regular pattern of ascent than it was apparent in the white matter. A few fibres reach the marginal zone, but the vast majority respect the uppermost sector of the cortex, the dense cortical plate (Fig. 3.9). Individual axons were followed in 200µm-thick sections and drawn via a *camera lucida*: this revealed that at P2 their arborizations typically extended over an area with a

diameter of about 300 μ m (Figs. 3.10; 3.17B).

This extensive lateral spread of the terminal arbors of thalamic axons contrasts with the remarkable order with which they are arranged at the junction between white and grey matter. In favourable preparations whole arrays of individual axons can be seen entering the cortex and their strict parallel arrangement is most impressive (Fig. 3.17A). The fibres heading for ventral areas run superficial to fibres destined for more dorsal cortical segments. Since the earlier fibres enter the more ventral cortical areas first, and since these areas develop relatively earlier, this type of fibre ordering reflects the timing of its establishment. It is as if the topographic array of arriving fibres, established in the intermediate zone and held during the waiting period in the subplate layer, is 'read out' as the fibres invade the cortex, to transform the array into a precise two-dimensional topographic pattern of axons entering the cortical sheet (Fig. 3.13).

Some individual thalamic axon can be seen to give branches in layer 6a and within the lower third of the dense cortical plate, but most of the terminal arbors are in the putative layer 4. By about P2, most axons have bifurcated at least once at around the top of layer 5, have arborized quite widely directly below the dense cortical plate, in what is presumably destined to become layer 4, and appear to have lost their growth cones. A few fibres grow all the way up through the dense cortical plate to the marginal zone, where they branch and appear to terminate within a fairly narrow laminar range.

Between P2 and about P8 (when the cortex has achieved its mature lamination) the arborizations of thalamic afferents in putative layer 4 become progressively displaced downwards as migrating neurons continue to arrive and the supragranular layers form (Fig. 3.12). By implanting different carbocyanine crystals into different

regions of the thalamus in the same brain, I observed labelled fibres from different thalamic nuclei invading distinct sectors of the cortex (Fig. 3.11).

During the periods of thalamocortical fibre outgrowth, accumulation below the cortex and invasion of the cortical plate very few if any cortical cells were observed to be backlabelled by dye placement within the body of the thalamus (Figs. 3.3; 3.4; 3.7 and 3.9). I was interested in determining when the corticofugal projections reach the internal capsule and which cortical neurons project to the thalamus itself.

Tracing from the internal capsule

At E14 a small crystal placement within the internal capsule back-labels numerous cells together with their processes, within a segment of the presumptive entorhinal and lateral cerebral cortex. It also labels a segment of the striatal radial glia above and below the implantation site, and neurons within the internal capsule itself (Figs. 3.12 and 3.13 demonstrate representative results for all experiments, n=6). The majority of the back-labelled cortical cells are in the preplate, but even by E14-14.5 this population of first-generated cells has been split into marginal zone and subplate by the emerging cortical plate (Fig. 3.13C). Interestingly both marginal zone and subplate cells are back-labelled from the internal capsule at E14.5. Occasionally I observed a few back-labelled cells within the cortical plate itself but the vast majority were in the marginal zone and especially the subplate (Fig. 3.14). At these early stages, only the most mature ventralmost cortical segments contained back-labelled cells after crystal placement in the internal capsule (Figs. 3.13 and 3.14) but with maturity the segment of back-labelled cortical cells expanded dorsally, indicating that the axons from more dorsal areas reach the crystal placement site progressively later than those from more ventral regions.

Since crystal implantation into the dorsal thalamus before E16 labelled

very few cells in the cortex, but internal capsule crystal placement did, I suspected that many subplate axons stop growing somewhere in the lateral part of the diencephalon, medial to the primitive internal capsule, and do not extend into thalamic nuclei. I was interested to compare the pattern of retrograde labelling from the dorsal thalamus and internal capsule with that from crystal placement in the thalamic reticular nucleus at the same age (E16). To permit critical comparison I applied crystals at different points on the two sides in individual brains and applied the same incubation conditions for the two hemispheres. Interestingly, crystal implantations in the thalamic reticular nucleus back-labelled numerous cortical cells, some of which were exclusively located in the subplate, but some in the lowermost part of the cortical plate (Fig. 3.15). No marginal zone cells were labelled at this stage.

Carbocyanine crystals implanted into the internal capsule at later stages, after E16, when cortical lamination was more advanced, back-labelled cells in both layer 5 and layer 6, indicating that axons of corticofugal projection neurons in these layers had reached the implantation site in the internal capsule (Fig. 3.16). Cells of layer 6 project to thalamus, while those of layer 5 cells project to the tectum, pontine nuclei and spinal cord through the cerebral peduncle. By applying DiI to the cerebral peduncle it was indeed possible selectively to back-label layer 5 cells (3.17 C and D). It is interesting to note that the axons of layer 5 are very highly ordered and parallel in the white matter (Fig. 3.17C and D), in the same manner as the thalamic fibres described earlier. The corticofugal fibres from ventral areas run superficial to fibres originating from more dorsal areas.

3.7 E14 to E15: Anterograde labelling of the earliest corticofugal projections. Establishment of the subplate scaffold

The exact appearance of the cerebral wall and the state of axon outgrowth, as revealed by crystal placements in the cortical surface, varied with position across the hemisphere, at any particular age, because of the rostrolateral-to-caudomedial wave of cortical maturation. The rostrolateral cortex is 1.5-2 days ahead of the caudomedial cortex throughout the period of study (Fig. 3.19C and D). Unless otherwise stated, the following descriptions apply to crystal placements in the occipital cortex (putative area 17).

Crystal placement into the surface of the occipital cortex early on E14 results in the staining (to varying extents) of the pial surface of the cortex, radial glia and a population of bipolar cells with radial processes extending from the ventricular surface to the pial surface (Fig. 3.16A and 3.17B). These cells are presumably early postmitotic, immature neurons, since nucleated cell bodies are seen at various positions along these extended processes. No outgrowing axons are seen at this earliest stage in the formation of the cortex.

During the 14th embryonic day the early-born cells accumulate at the cortical surface to form the preplate. Crystal placement in the cortex at successively later times during a period of a few hours early on E14 produces progressively more intense superficial staining, presumably concentrated in the membranes of preplate cells. The first axons are seen growing out from these preplate cells at mid-E14 (Fig. 3.18B and 3.19E and F) and they appear, virtually without exception, to be directed ventrally into the intermediate zone, heading towards the ventral telencephalon and the primitive internal capsule (Fig. 3.18B). A significant population of axons is seen growing from

each labelled region, and they form a fairly tight bundle of fibres running within or directly under the subplate zone.

All the axons at the advancing front of the stained bundle are seen to end in expanded growth cones, which exhibit complex filopodial morphology and are exceptionally large (roughly 10µm across) as they descend in the nascent intermediate zone (Fig. 3.19F). Crystal placements on the occipital cortex on E14 (n=6) never resulted in the back-labelling of thalamic cells, indicating that no thalamic fibres have yet reached the site of crystal placement. The bundle of axons originating at any particular point on the cerebral wall maintains its coherence fairly tightly and follows a direct path towards the PIC. At the advancing front however, the growing tips of the axons are more dispersed than the diameter of the bundle behind them (Fig. 3.18B), suggesting that some of the foremost pioneer axon explore outside their territory, before pulling back to assume their final trajectory.

Topography of the scaffold

Examination of brains with multiple cortical dye placements, arranged in a row, parasagittally or coronally, clearly reveals that the preplate of the anterior, lateral part of the hemisphere sends out projections first, and the occipital, dorsal part last. These multiple injections also demonstrate that the early pathways are already topographically organized as soon as they appear. Along the rostrocaudal axis, topography is maintained simply by virtue of the fact that the axons tend to follow direct courses towards the primitive internal capsule, and hence form a fan-shaped array, converging on that region. In the orthogonal axis, topography is also preserved, despite the fact that all fibres from any mediolateral strip of cortex share the same narrow portion of the intermediate zone, because labelled axon bundles appear not to cross their neighbours.

Thus, at any part of the intermediate zone, the axons derived from more medial (dorsal, later-maturing) parts of the hemisphere lie deep to those from more lateral (ventral, earlier-maturing) regions. Thus the whole array of axons approaching the ventral telencephalon forms a two-dimensional representation of the entire surface of the hemisphere.

3.8 Retrograde labelling of thalamic neurons

At E16, fibres from the dorsolateral thalamus have reached the occipital pole of the cortex, as revealed by dye implantation in the LGN (Fig. 3.4 is representative for all experiments, n=8). In agreement with this, a single carbocyanine crystal placement on the occipital cortex leads to the labelling of cell bodies in the LGN at this age, as well as to the staining of a fibre bundle extending through the telencephalon and diencephalon, linking the two structures (Figs. 3.18.C; 3.19G and 3.20). Thus, both anterograde and retrograde labelling of thalamocortical projections show that their axons have reached the occipital cortex (indeed all regions of cortex) by E16. Therefore any single, small dye crystal placed into the cortex from E16 onwards will inevitably label a *mixed population* of fibres, including both anterogradely labelled (corticofugal) subplate axons and retrogradely labelled thalamocortical axons. If these two axon arrays take different routes through the telencephalon, a single crystal should give rise to two distinct, separate stained bundles. I therefore examined the form of the axon array stained by single small dye placements at various points on the cortex, at ages from E16 onwards, following the axons in serial coronal or horizontal sections with conventional fluorescence and confocal microscope. 3-D reconstructions from fine optical sections as well as serial sections were performed on these specimens to reveal more of the fine and gross topography.

The results of these experiments are very clear. Even after E16 (until E18/19), the bundle (including subplate and thalamic fibres) labelled by a small dye placement in the cortex remains discrete and single throughout its trajectory through the intermediate zone and primitive internal capsule, with no tendency for the fibres to separate into two distinct fascicles at any point in the telencephalic portion of the pathway (Fig. 3.20). From serial optical sections, high-resolution confocal microscopic reconstructions were made to reveal the fine topography of individual fibre arrangements within the bundle, though even this technique did not quite allow me to follow **individual** fibres all the way from the implantation site to the thalamus. These experiments suggest (compare Figs. 3.7 and Fig. 3.21) that the topography of the scaffold of early corticofugal axons is extremely similar to that of the corresponding thalamocortical fibres, and that corresponding ascending and descending axons run together through the telencephalon, in very close association. Axons (including corticofugal fibres) within the white matter display an organized, parallel distribution with apparently little rearrangement. Within the corpus striatum the axons reorganize into fascicles. There is some exchange of individual axons between adjacent fascicles but no gross intermingling or crossing. Entering the internal capsule the fascicles labelled by a single crystal in the cortex coalesce into a tight bundle of closely associated, strictly parallel axons. The gross topography is maintained along the entire fibre pathway for both corticofugal and corticopetal axons (see Fig. 3.23 below).

The most compelling evidence for the topographic arrangement come from the experiments involving multiple cortical dye placements (Fig. 3.22-26), in which the arrays of fibres labelled by each crystal can be followed towards the primitive internal capsule and back to the thalamus. Each distinct, single axon bundle is clearly separate

from the others, even as they converge on and pass through the primitive internal capsule (Fig. 3.23). The ascending thalamic axons appear, within the accuracy of this method, to follow exactly the same topographically ordered paths through the telencephalon as their counterparts descending from the subplate.

It is obviously not possible to identify the individual components of the mixed fibre population labelled from a cortical implantation site. However, important inferences can be drawn from the patterns of retrograde labelling of thalamic cell bodies (Fig. 3.22 and 23). In horizontal sections the thalamic cells labelled by a single crystal in the cortex line up along an antero-posterior longitudinal slab. With different crystals placed along a parasagittal line as early as E16, tracing revealed that more anterior cortical regions are connected with more medial thalamic slabs, whereas more posterior cortical regions were connected to more lateral slabs. The two back-labelled cell groups revealed by any two implantation sites along a coronal line seem to form a continuous slab within the thalamus. These experiments reveal that adjacent cortical fields receive their projections from adjacent groups of thalamic cells, although the arrangement of corresponding regions on the surface of the cortex and within the volume of the thalamus is rather different. The interlinking fibres (within the accuracy of the technique) preserve their neighborhood relationships along the whole length of the pathway. As soon as thalamic fibres arrive below the cortical plate the general ordering of thalamocortical connections is established.

The capacity of the single crystal technique to reveal **separate** fibre paths is established by the fact, that after the age of about E18/19, such single dye placements do indeed reveal two distinct fibre bundles in the ventral telencephalon and brainstem: one (presumably a mixture of thalamocortical, subplate and at this stage, true

corticothalamic axons) still extends through the primitive internal capsule and is directed towards the thalamus; the other appears to diverge into the posterior extension of the primitive internal capsule, the putative cerebral peduncle (Fig. 3.26). At this stage, dye placed in the posterior primitive internal capsule exclusively labels cell bodies in layer 5 of the cortical plate itself, presumably the cells of origin of corticotectal, corticopontine and corticospinal projections (3.16). Thus, it seems that these non-pioneering, later-descending projections from layer 5 follow trajectories in the ventral telencephalon different from those taken by the earlier subplate axons but that the latter are closely followed by the ascending thalamic fibres, and probably, by the layer 6 corticothalamic axons too.

Relationship between thalamocortical and interhemispheric projections

By E20-21, dye placement in anterior cortical regions (depending on the site of the placement) labels fibres in the corpus callosum. It is interesting to examine the relationship between thalamocortical and callosal projections (Fig. 3.27). The thalamic fibres apparently lie superficial to the callosal fibres belonging to the same cortical segments. It is relevant to note that the thalamic fibres arise before the callosal axons to a particular cortical region. Thus the pattern of axons within the white matter again suggests chronotopic fibre ordering, the first-established fibres taking a more superficial position in the white matter.

3.9 Combined cortical and thalamic tracing.

Evidence that thalamic fibres mingle with the subplate scaffold

within the internal capsule and intermediate zone

Crystal placement into the posterodorsal thalamus at mid-E14 (the same age at which the first axons are seen leaving the occipital preplate) reveals fibres just leaving the

thalamus through the primitive internal capsule (Figs 3.18B and 3.1). Late on E14, they are seen to have passed through the primitive internal capsule and to be growing under and partly through the anlage of the corpus striatum. At the same time cortical crystal placements revealed that the early corticofugal fibres are also advancing towards the primitive internal capsule. By early on E15, the two synchronized, advancing fronts of pioneering axons, from occipital subplate (and marginal zone) and LGN invade a region in the ventral telencephalon, just outside the primitive internal capsule (as revealed by experiments previously described). Thus, the outgrowth of the two corresponding sets of axons, corticopetal and corticofugal, are remarkably synchronized and co-ordinated in their timing and spatial patterns.

The single cortical dye implantation experiments were very suggestive that thalamo-cortical and early corticofugal projections take very similar routes. Therefore I was especially interested in examining the possibility whether the two fibre systems actually grow over each other. In particular, I wanted to try to label corresponding regions of thalamus and cortex, with different dyes, to see whether the two fibre systems literally contact each other at early stages, before thalamic fibres have reached their target region. True correspondance cannot be established until E16 when the thalamic fibres have reached their target area and thalamic cells can be backlabelled from the cortex; nevertheless I shall use the term 'corresponding' or 'matching' regions before this period to refer to small areas within cortex and thalamus that will eventually become linked. Small dye crystal placements label only small bundles of fibres and therefore it is impossible to be **certain** prior the experiment that two such dye placements in thalamus and cortex are indeed in precisely corresponding structures: negative results in such experiments, as reported by Miller et al. (1993), are not

Fig. 3.1

A small crystal of DiI was implanted into the putative primordial dorsal lateral geniculate nucleus (LGN) in the dorsolateral part of the left thalamus of a fixed E14 brain. After 4 weeks incubation 100µm-thick coronal sections were cut, counterstained with bisbenzimidazole and examined in a fluorescent microscope under uv and rhodamine optics.

A: Unfilled arrow marks the crystal implantation site in the dorsolateral part of the thalamus (DT), putative lateral geniculate nucleus, under uv illumination to show bisbenzimidazole labelling (VT - Ventral Thalamus).

B: The same section as A under rhodamine illumination to reveal DiI. Labelled fibre bundles extend from the dorsal thalamus to the ventral thalamus. There are numerous backlabelled cells below the implantation site within the thalamic reticular nucleus of the ventral thalamus (indicated with a filled arrow) and few above, in the epithalamus (filled arrow above the implantation site). The mass of axons within the dorsal thalamus is presumably a mixture of the ascending axons of these thalamic reticular cells and the orthogradely labelled thalamofugal fibres from the presumed LGN.

C: High-power view of the backlabelled thalamic reticular cells from the region indicated with the lower filled arrow at B.

D: Two sections anterior to A the geniculofugal labelling reaches its full length more lateral, just lateral to the primitive internal capsule (IC). The photomicrograph at D was taken with double exposure (uv and rhodamine filters).

E,F: The high-power view of the fibre endings revealed a small number of backlabelled cells within the internal capsule, in the so called perireticular nucleus (filled white arrows).

Scale bars: 500µm at A,B and D; 100µm at C and E; 50µm at F.

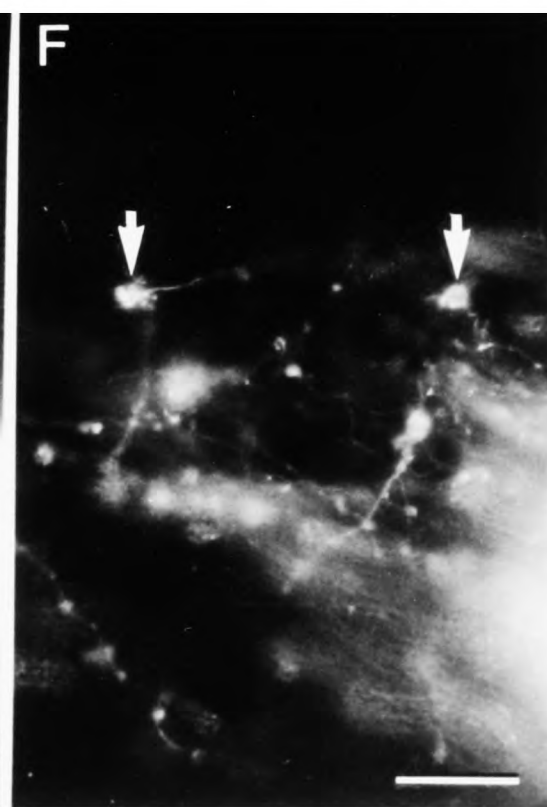
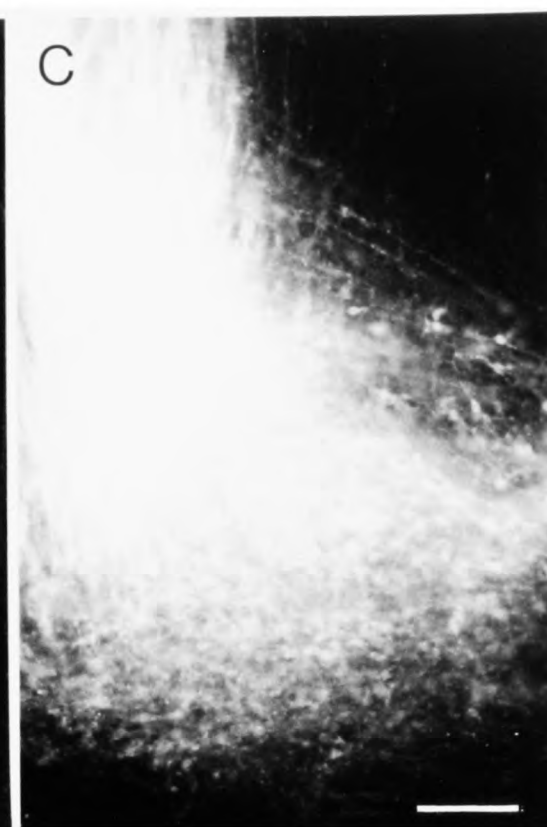
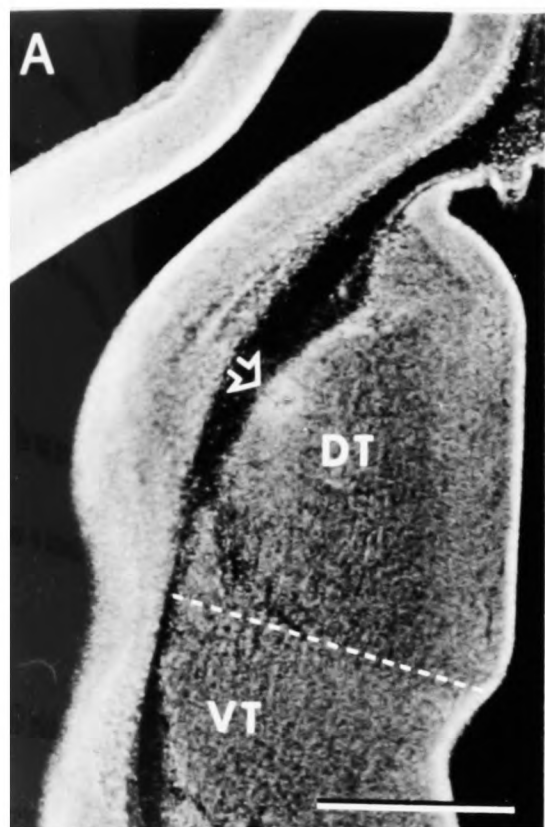


Fig. 3.2

A DiI and a DiA crystal were inserted 200µm apart (DiI lateral to the DiA) into the dorsal thalamus of an E14.5 brain. After 3 weeks incubation at room temperature, 100µm-thick coronal sections were cut and counterstained with bisbenzimidazole. Fluorescent micrographs were taken by exposing the same film three times using rhodamine, fluorescein and ultraviolet filters.

The labelled thalamic fibre bundles remain separated as they descend in the ventral thalamus (B) and as they pass through the internal capsule. Fibres originating from more lateral thalamic regions (filled arrow) lie below the fibres originating from more medial thalamic region (empty arrow) as they pass under the striatum (D), and they head more caudally within the hemisphere.

Bar: A: 100µm; B-D: 500µm

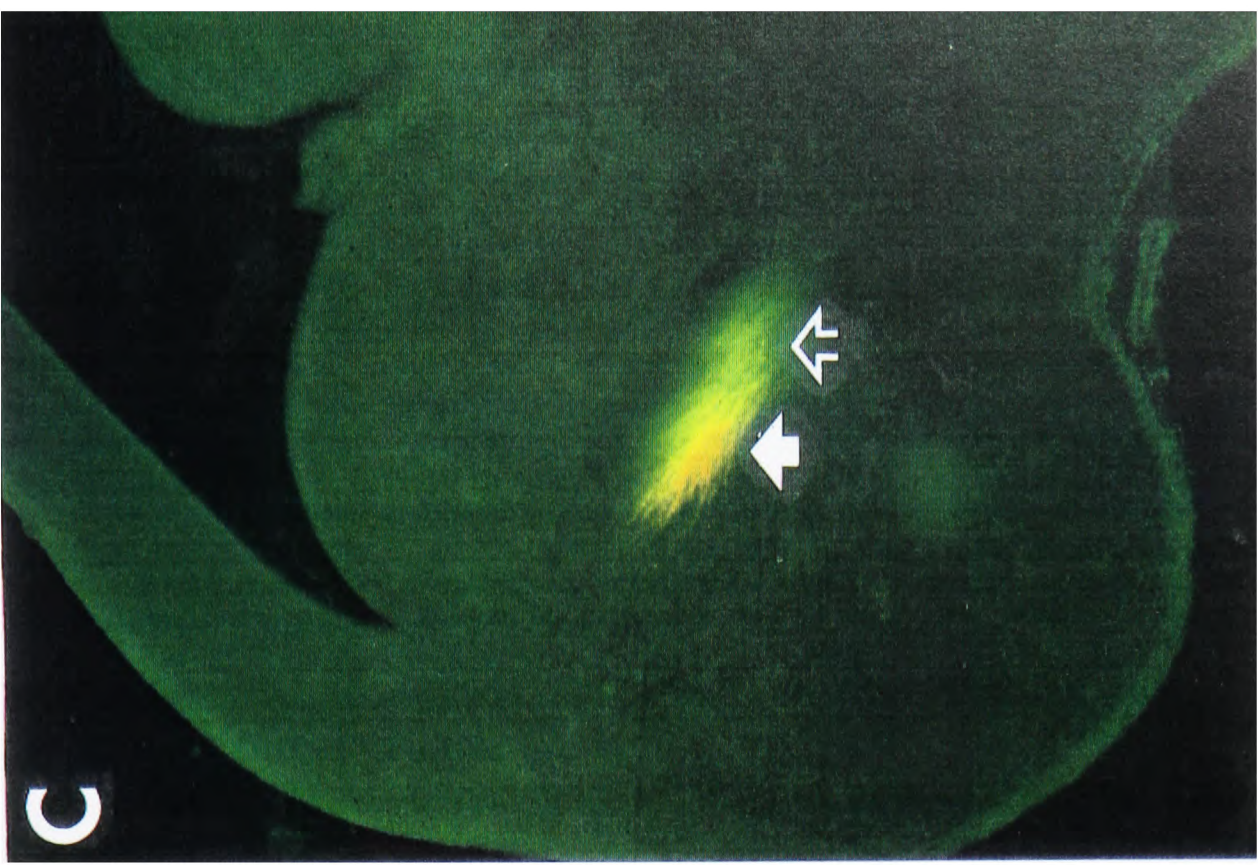
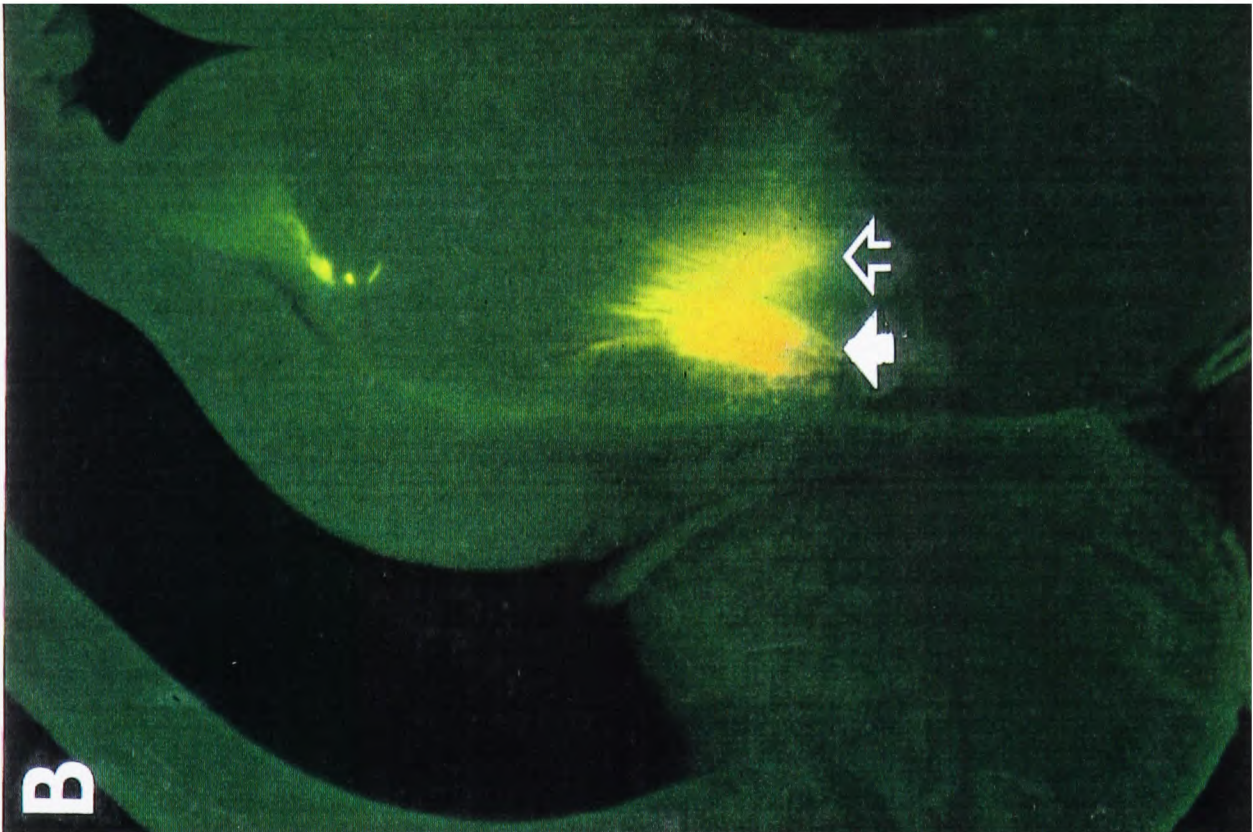
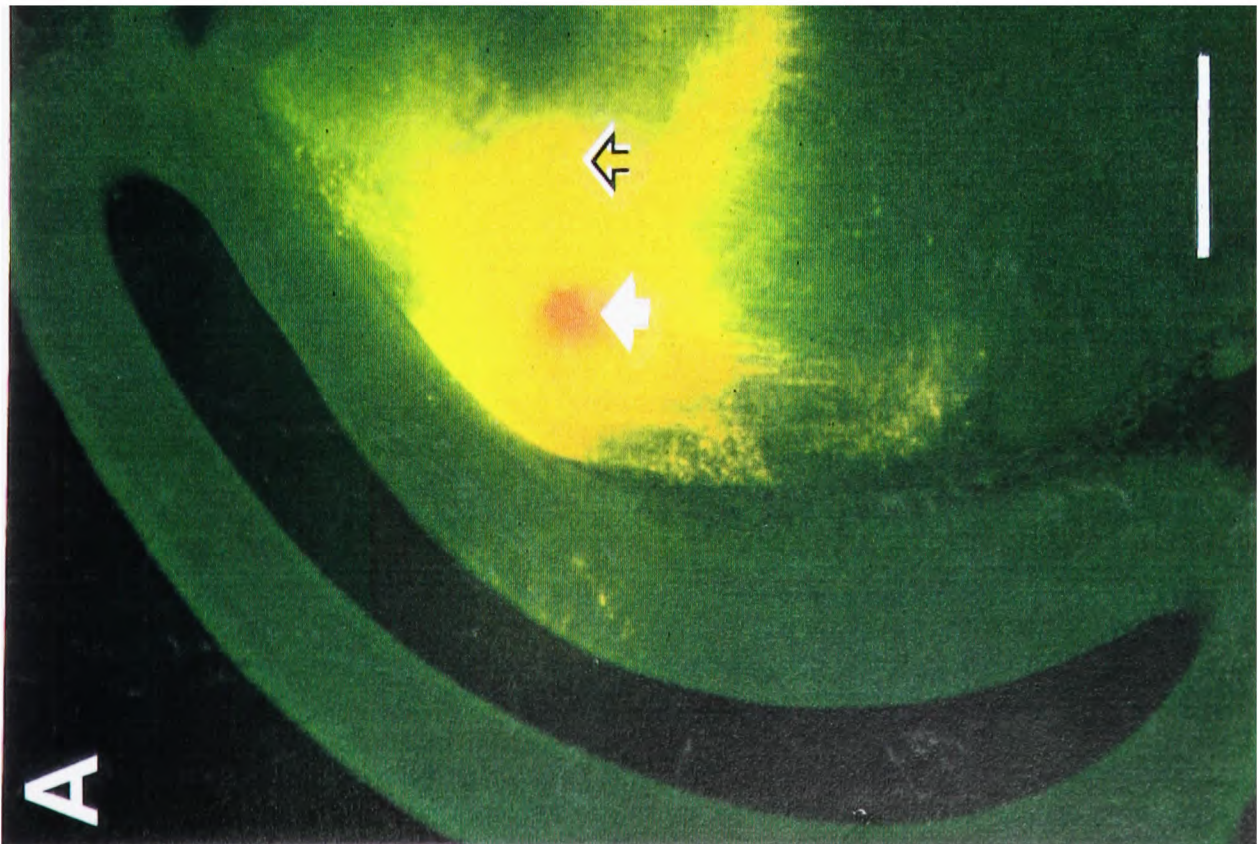


Fig. 3.3

The advance of the thalamocortical fibres towards the cortex is revealed with a crystal placement into the putative LGN in the left dorsal thalamus (DT) of fixed brains at E15 (top row, A-D) and E15.5 (bottom row, E-H). After 2-6 weeks incubation at 37°C, 100µm coronal sections were cut and counterstained with bisbenzimidazole. For each row, the first two panels demonstrate the thalamic crystal implantation site (A,E under the rhodamine filter and B,F the same sections under uv illumination). The crystal placement sites (unfilled arrows) are located in the dorso-lateral aspect of the thalamus. Numerous cells are labelled in the reticular nucleus of the ventral thalamus (VT) (white filled arrows). The right-hand pairs of panels show the tips of the thalamic fibres at a more rostral level (C,G and D,H under rhodamine and uv illumination respectively). The first thalamic fibres reach to the cortex around E15.5, and extend to the convexity at E16. (LV - lateral ventricle; ST - striatum; IC - internal capsule, demarcated by unfilled arrows in D and H)

Scale bar: 500µm for all panels.

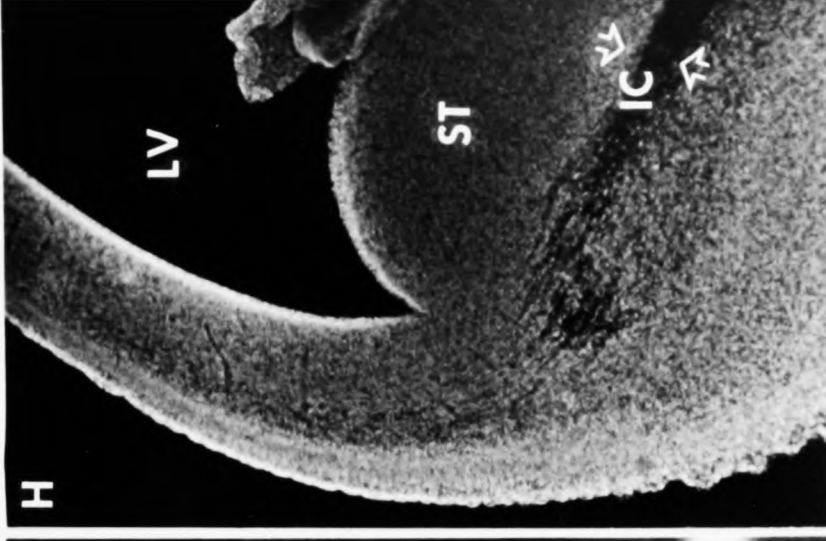
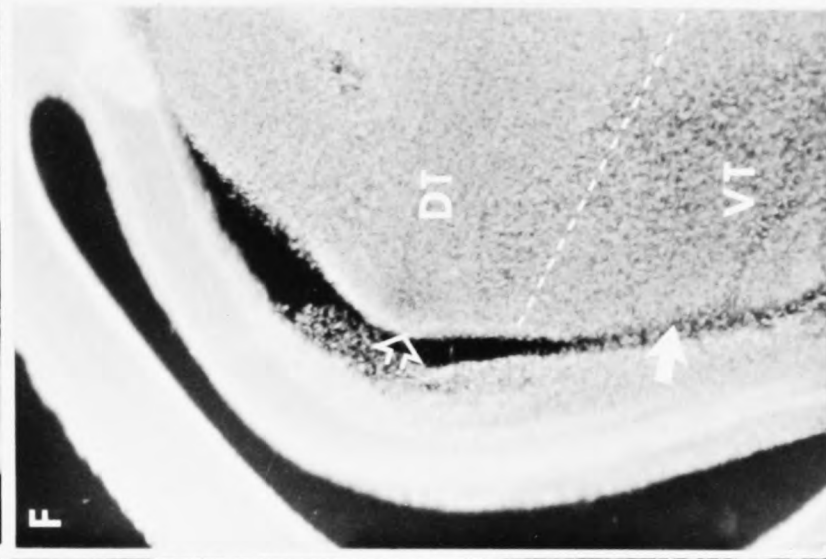
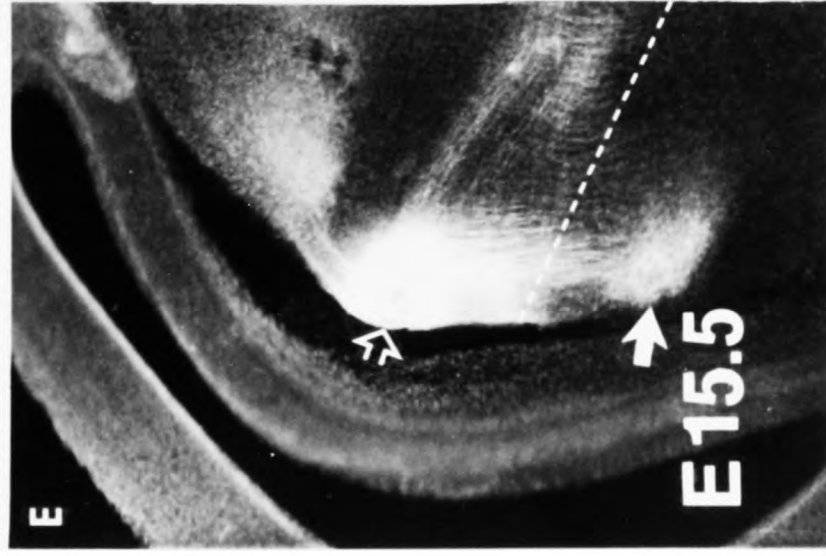
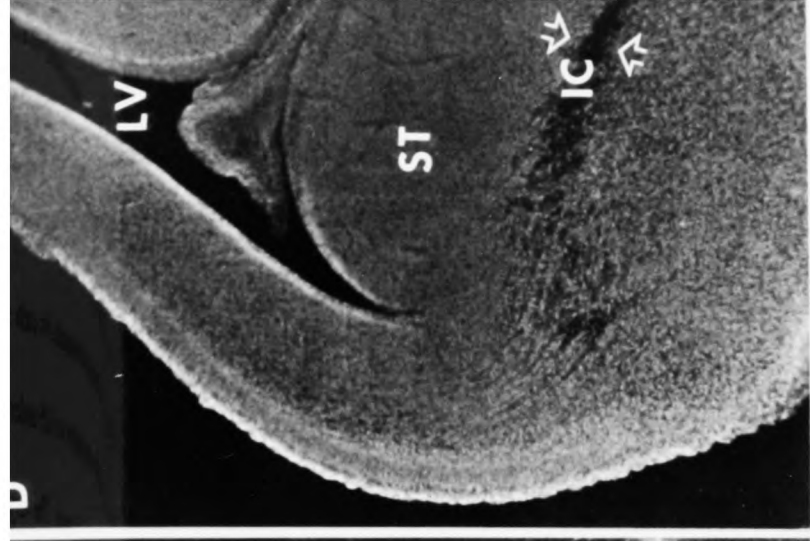
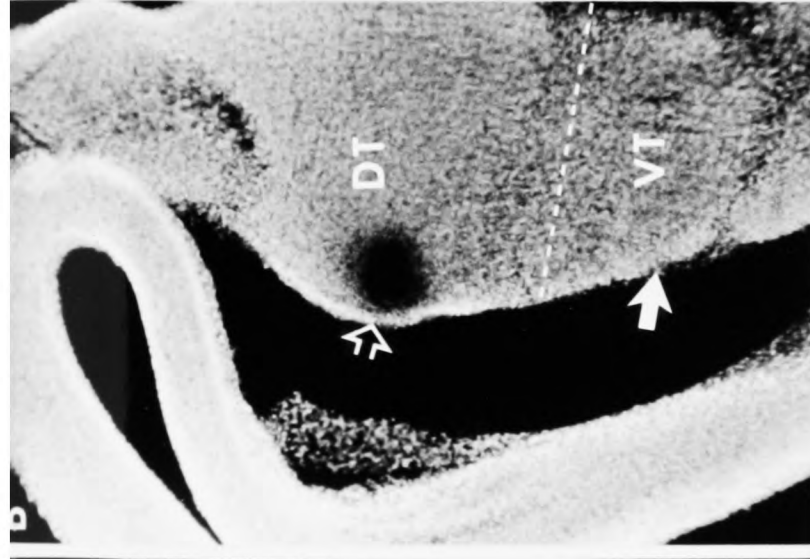


Fig. 3.4

The further progress of thalamocortical axons at E16 (top row) and E19 (bottom row) is illustrated exactly as in Fig. 3.2. Fibres from the dorsolateral thalamus have reached the subplate layer of the occipital cortex by E16 but there is very little advance into the cortical plate itself even at E19. Scale bar: 500µm for all panels.

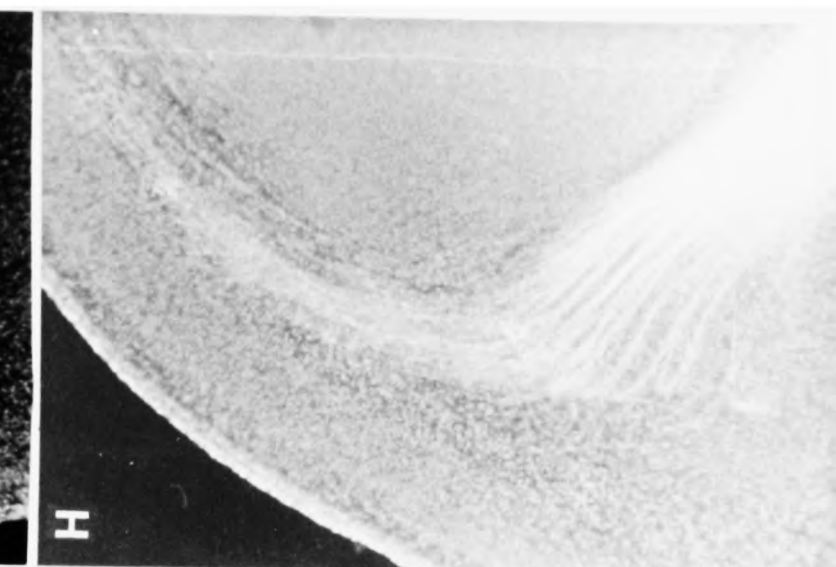
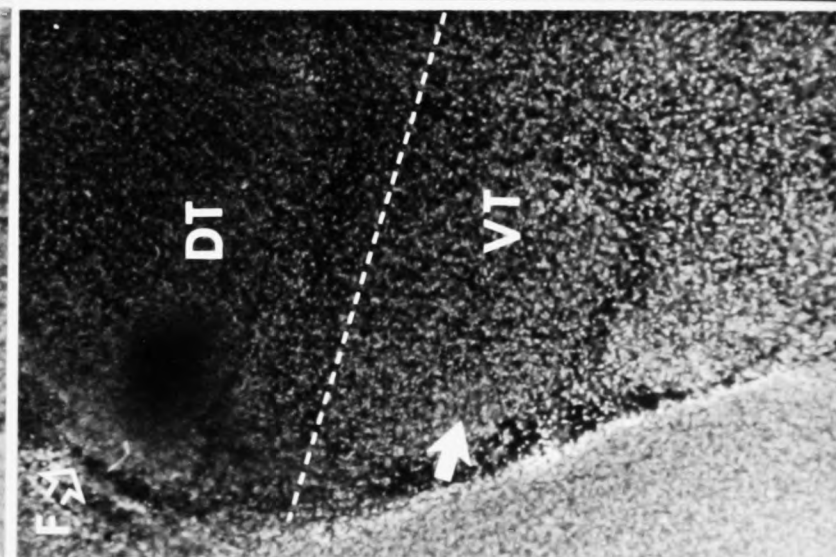
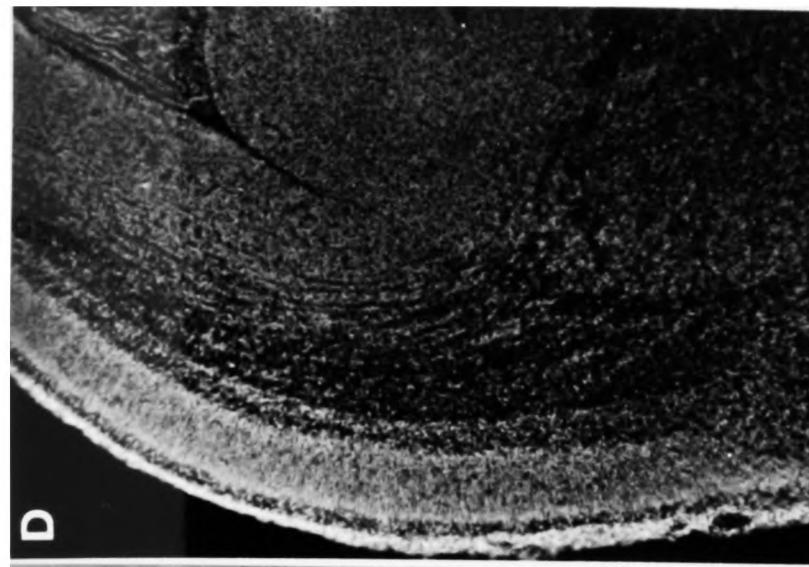
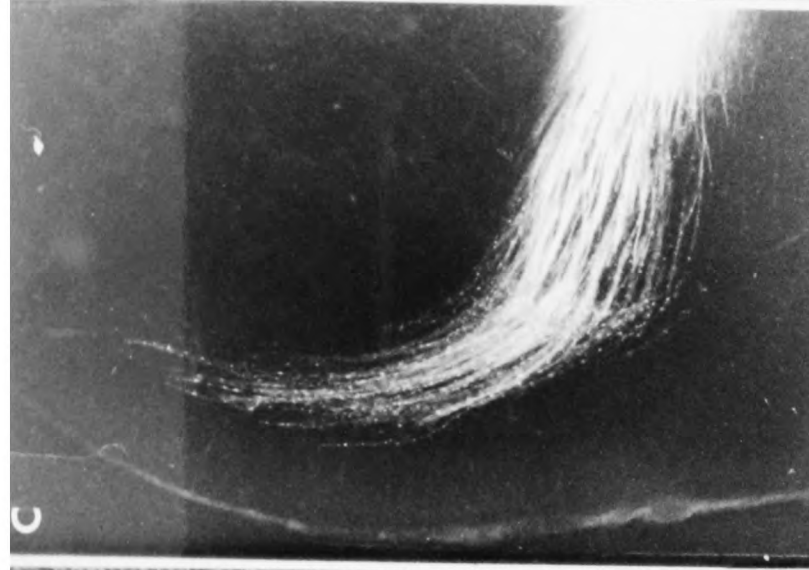
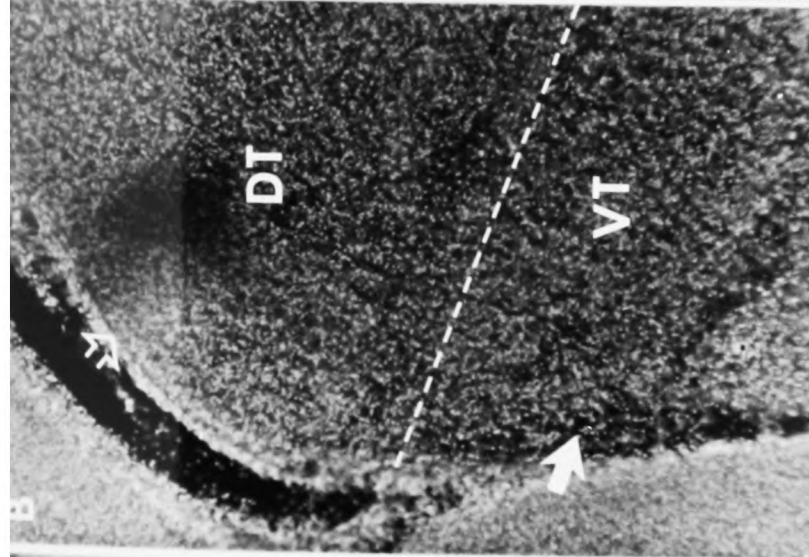
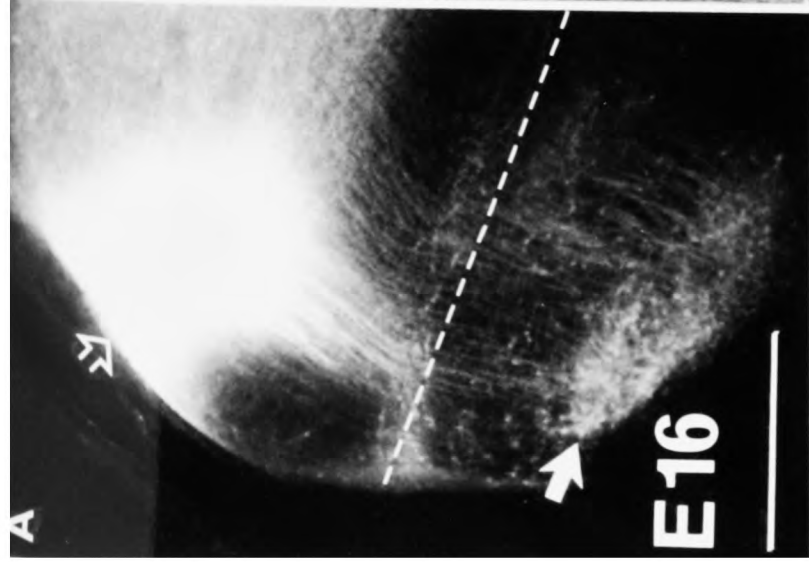


Fig. 3.5

Higher-power fluorescent photomicrographs (from 3.4 C and G) of the thalamic fibres below the cortical plate at stages E16 (top row) and E19 (bottom row). A,C bisbenzimidazole labelling under uv illumination; B,D DiI labelling in the same sections.

The thalamic fibres reach the occipital cortex at E16, but they remain restricted to the intermediate (IZ) and subplate zones (SP). At E19 some fibres have developed side branches (D) but the vast majority still lie within the same regions (in the SP and IZ), and have not entered the cortical plate (CP). MZ = marginal zone.

Note that, with discrete crystal placements restricted to the dorsolateral thalamus, no cell bodies are back-labelled in the cortical plate or the subplate, even at E19 and after 6 weeks of incubation period at room temperature.

Bar: 100µm for all figures

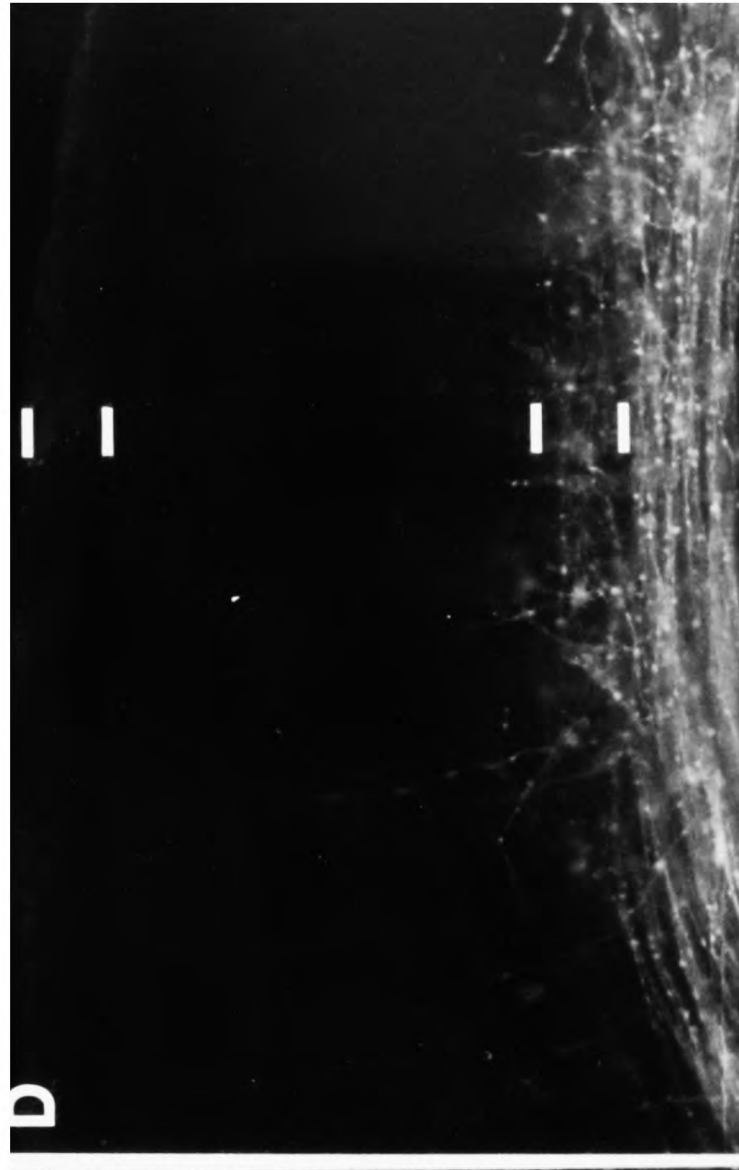
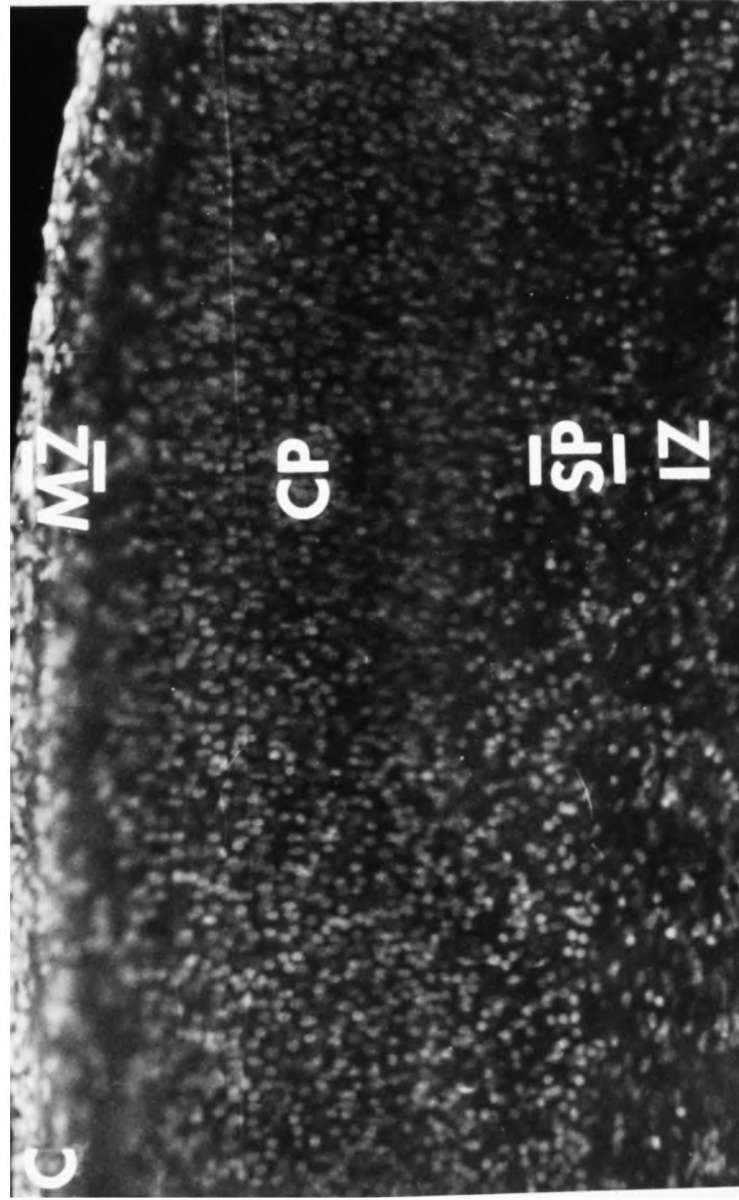
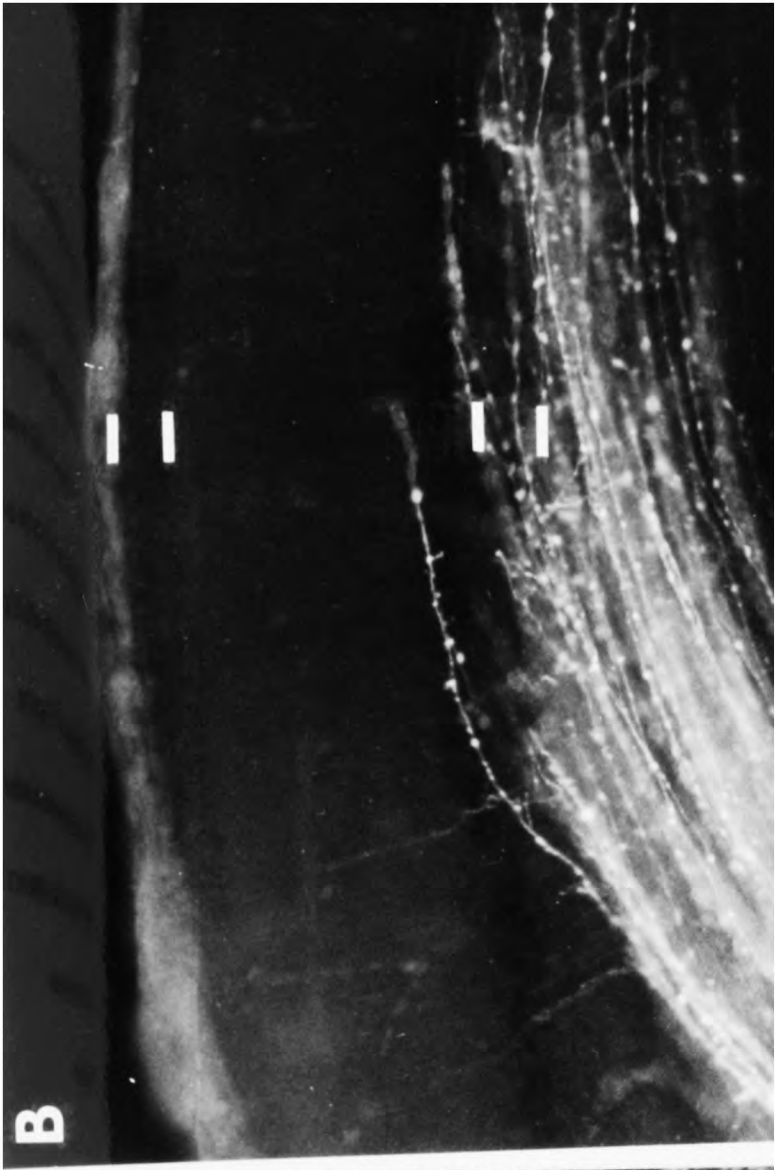
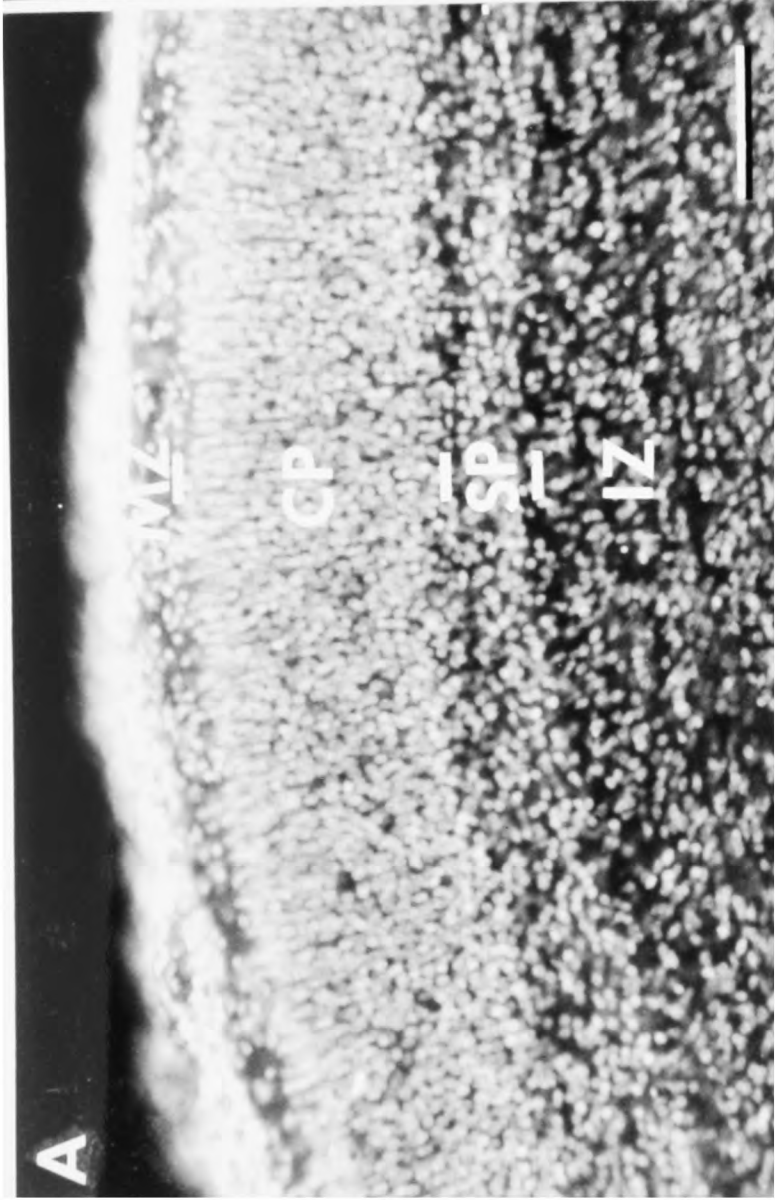


Fig. 3.6

Three stages of thalamic fibre ingrowth, at E16, E19 and P2. The thalamic fibres were stained with a crystal placement into the dorsal thalamus of fixed brains. After 3-6 weeks incubation at 37°C, 100µm coronal sections were cut and counterstained with bisbenzimide. They were viewed under rhodamine illumination (left panels) to reveal DiI labelling and under uv illumination (*right* panels) to reveal the bisbenzimide staining, showing the layering of the cortex.

The first thalamic fibres arrive in the intermediate zone of the occipital pole at E16 (top row) and accumulate within the subplate (SP) (from E16-19) but do not enter the cortical plate (CP) before E19 (B). (In more anterior and ventrolateral areas this happens a day earlier.) The thin radial processes seen extending up to the marginal zone (MZ) at E19 do not have the appearance of axons and are presumably the processes of radial glia since they are identical in appearance to the processes of such cells labelled by DiI staining of the cortex itself (see below). After E19 (occipital cortex) or E18 (ventrolateral cortex) the lower layers of the cortical plate are suddenly invaded by the thalamic fibres, although axons still stop short of the dense cortical plate (DCP). Very few fibres reach the marginal zone (MZ), but most of them terminate and arborize about 100-200 µm below. The bottom row demonstrates the distribution of thalamic fibres at P2. Note the very regular, parallel axon arrays in the white matter, visible in the examples at E16 and P2, compared with the somewhat disorganized growth of the axons into the cortical plate at P2.

Bars are all 100µm.

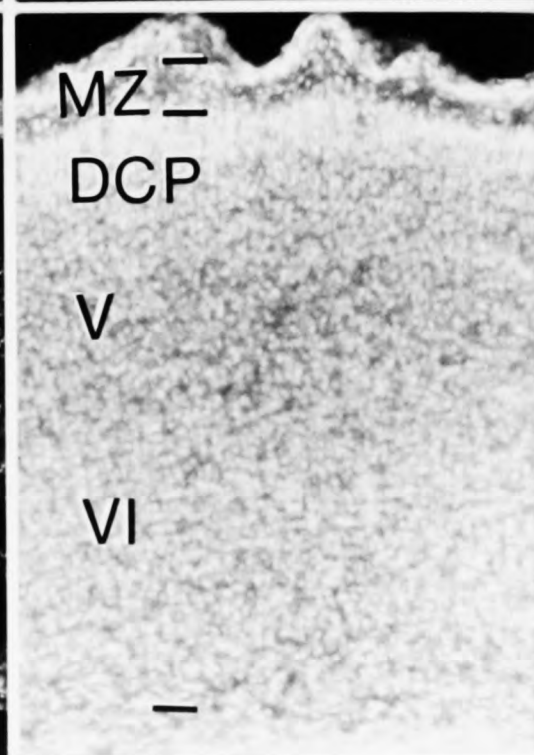
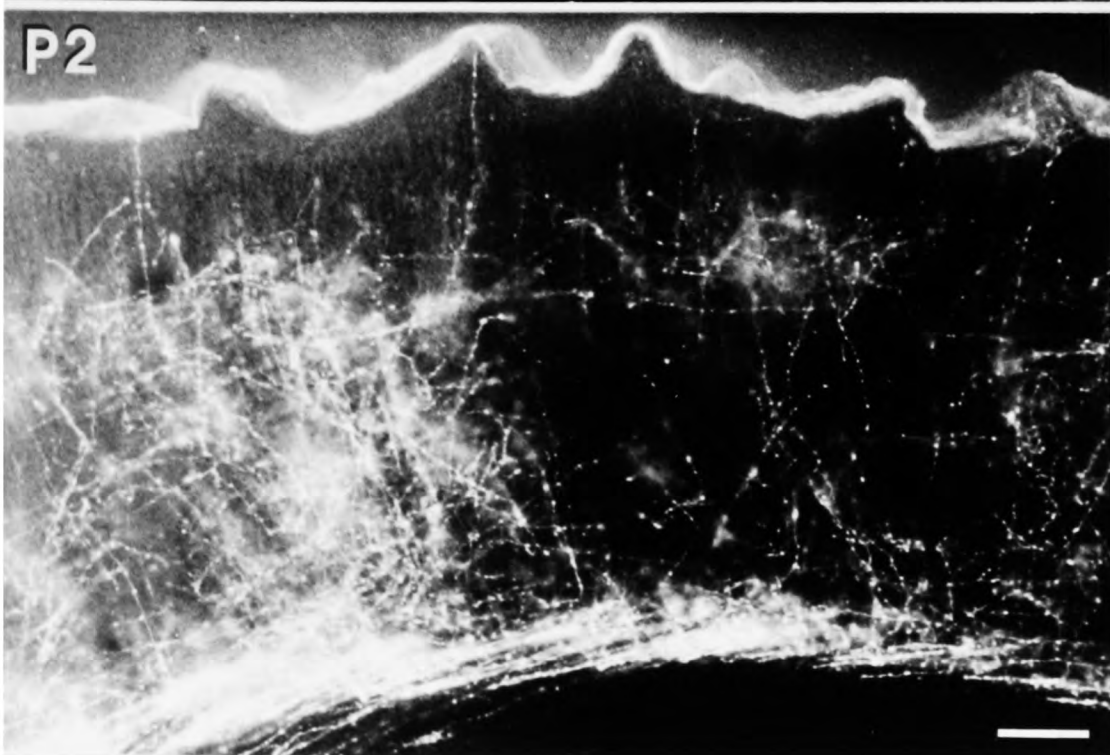
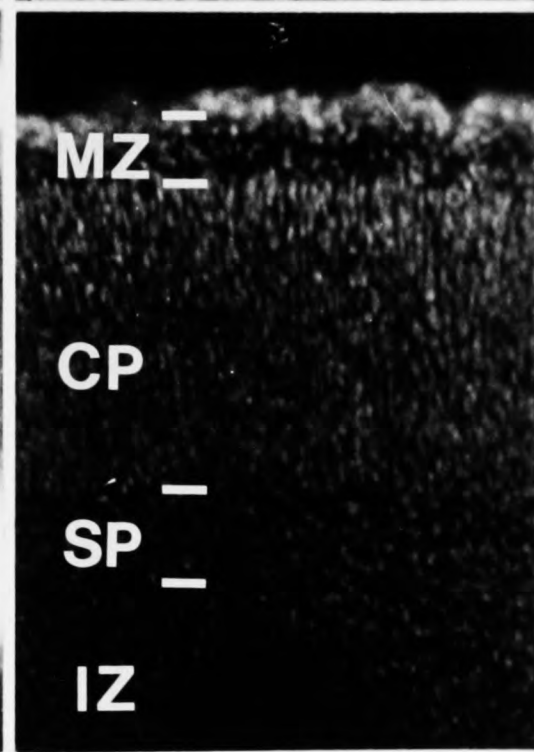
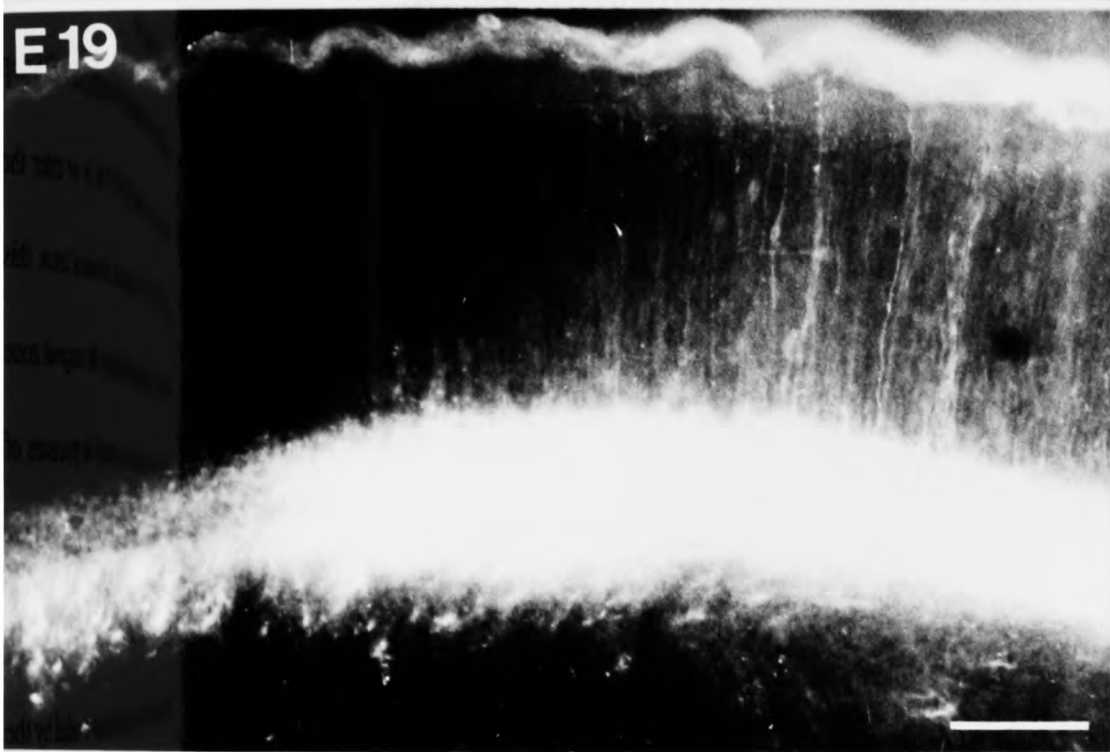
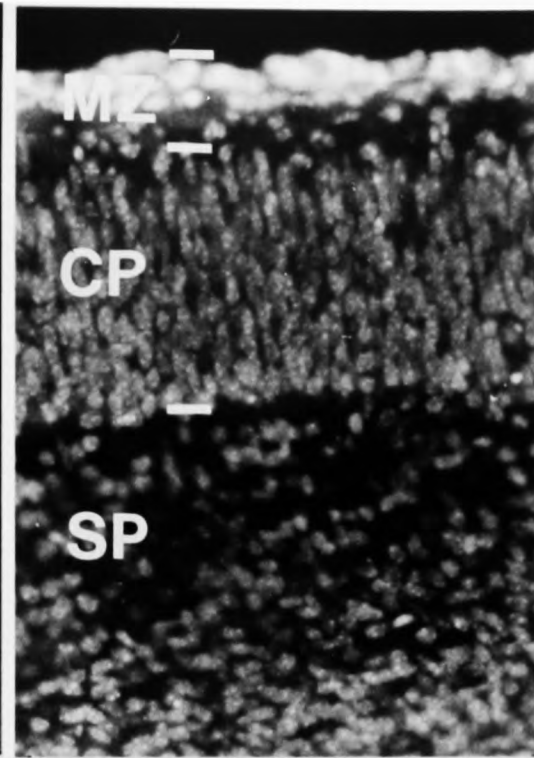
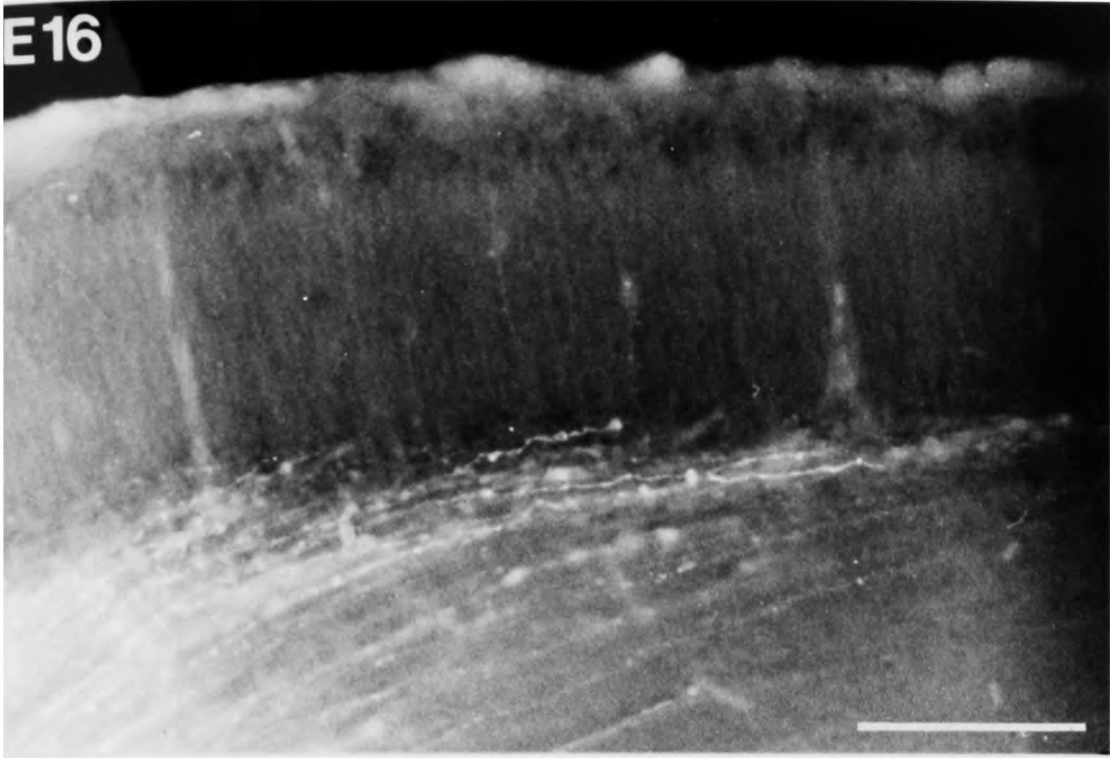


Fig. 3.7

A: Crystal placement into the dorsal thalamus reveals the first fibres reaching the intermediate zone beneath the ventralmost part of the intermediate zone by E15.5 (half a day before they reach the occipital pole itself). Leaving the diencephalon, the fibres form fascicles which open up in a fan shaped fashion. Viewed here in a coronal section, the ordering of the fibres is such that the inferior-lateral fascicles are destined for the more ventral part of the cortex, while the superior medial fascicles turn upwards and head towards more dorsal cortical areas. The trajectories of the fascicles remain parallel as they turn up into the intermediate zone: they do not cross each other extensively.

B: Same as A, but taken with higher power from the region where the fascicles break up into individual fibres as they reach the intermediate zone.

To examine the fibre ordering further the contralateral hemisphere (with identical crystal placement to that in A) was sectioned perpendicular to the fibre path. C,D and E are sections at levels corresponding to the labelled arrows in A.

E: The fibres leave the diencephalon through the primitive internal capsule. The bundle is at its narrowest at this point. No fasciculation is apparent in this region.

D: As the fibres run under the corpus striatum, they form 30-50µm-thick fascicles, slightly separated from each other. The labelled fibre array loosens up, and expands compared to E.

C: The fibre bundles defasciculate as they reach the intermediate zone and turn under the cortical plate. The right side of the section still contains some fascicles, while the left demonstrates the defasciculated fibres running parallel to each other in the intermediate zone.

Scale bars: A: 300µm; B: 50µm; C-E: 100µm

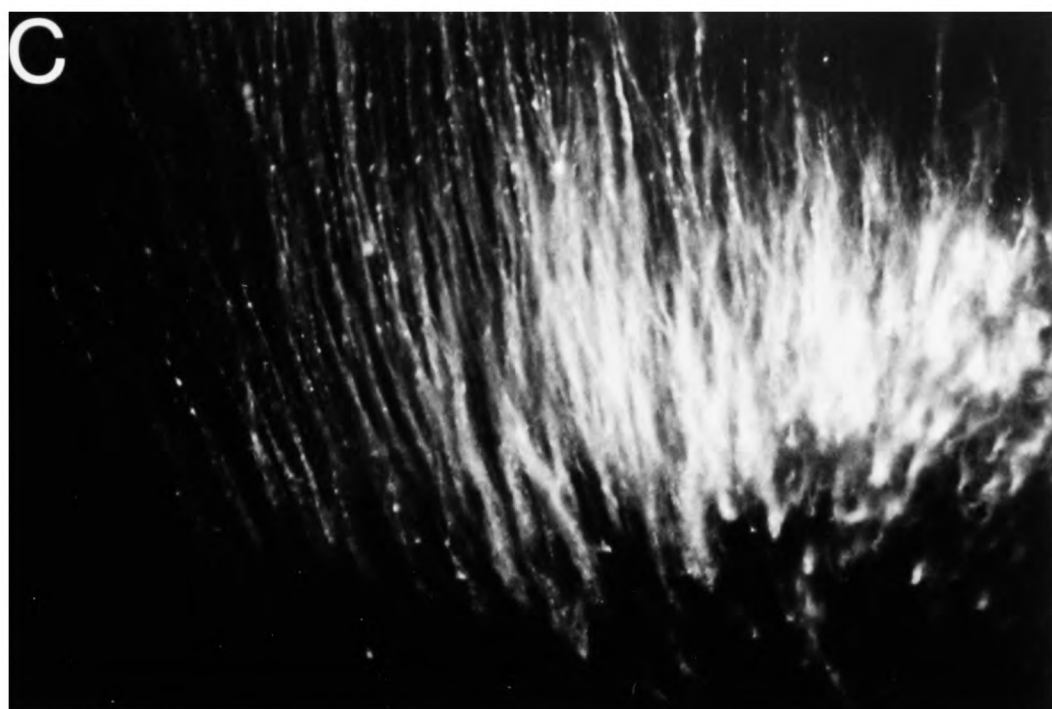
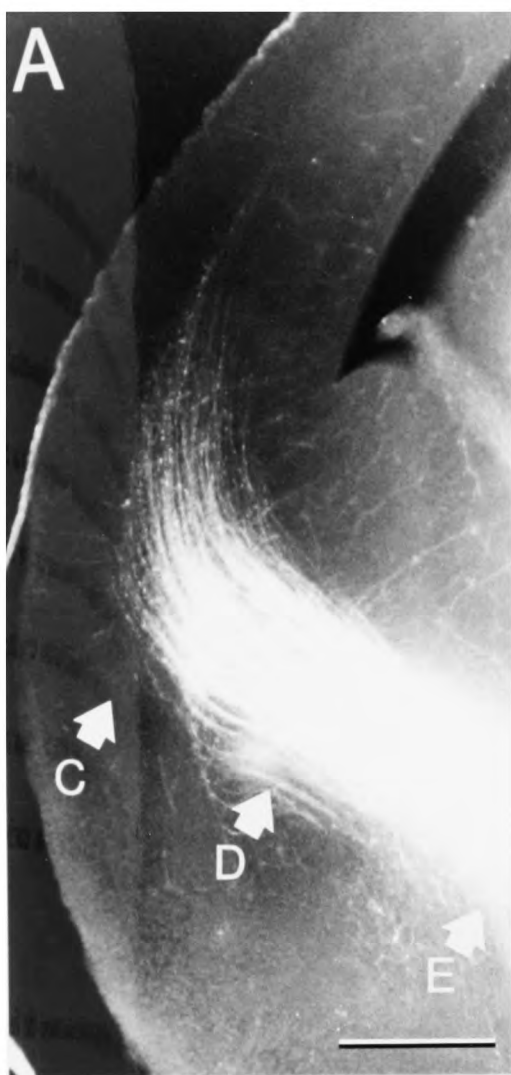


Fig. 3.8

The individual fascicles of Fig. 3.6D are seen here in cross-section with confocal microscopy.

A: Optical cross-section through the fascicles labelled from the dorsal thalamus at E15.5. White arrow indicates a single fascicle.

B, C and D: Optical sections along (B) and perpendicular (C,D) to the trajectories of labelled thalamic fascicles. It is apparent that within each fascicle the axons are not homogeneously distributed and are not compact. The profiles contained unlabelled 'holes'.

Scale bar: A: 100µm; B-D: 50µm.

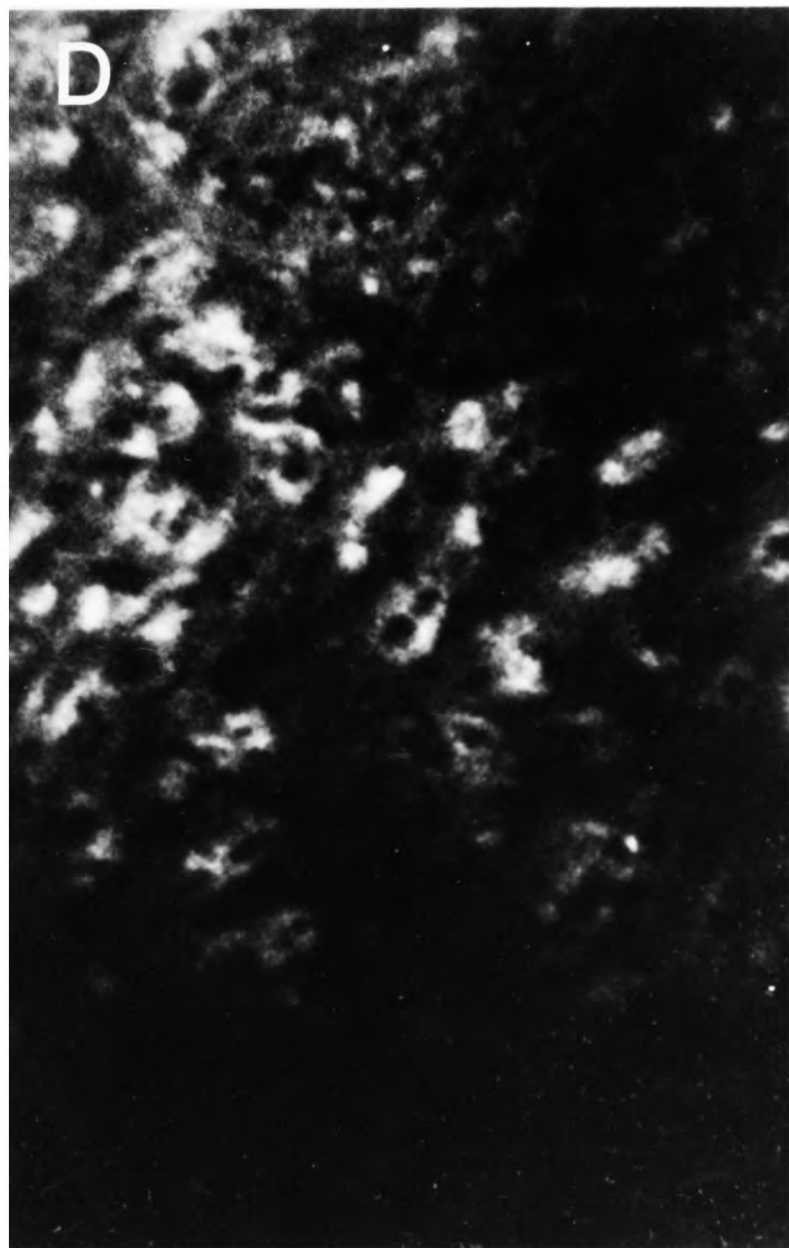
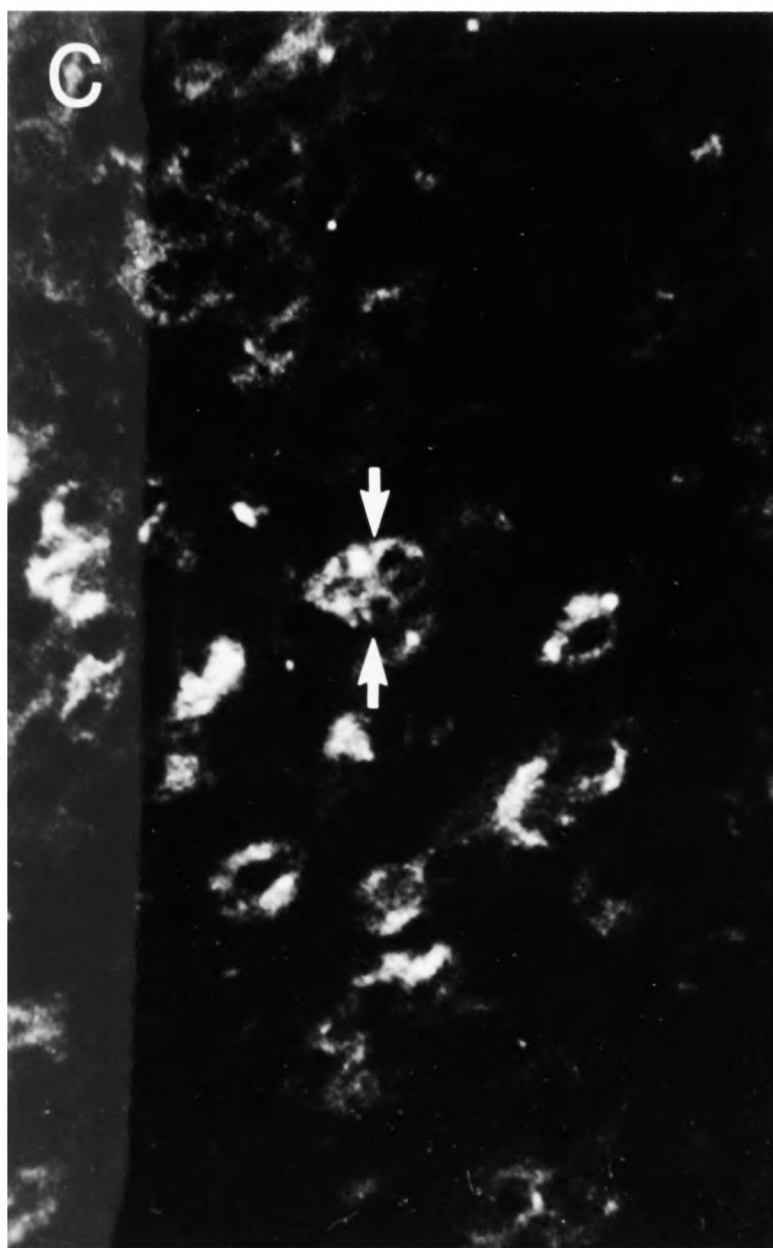
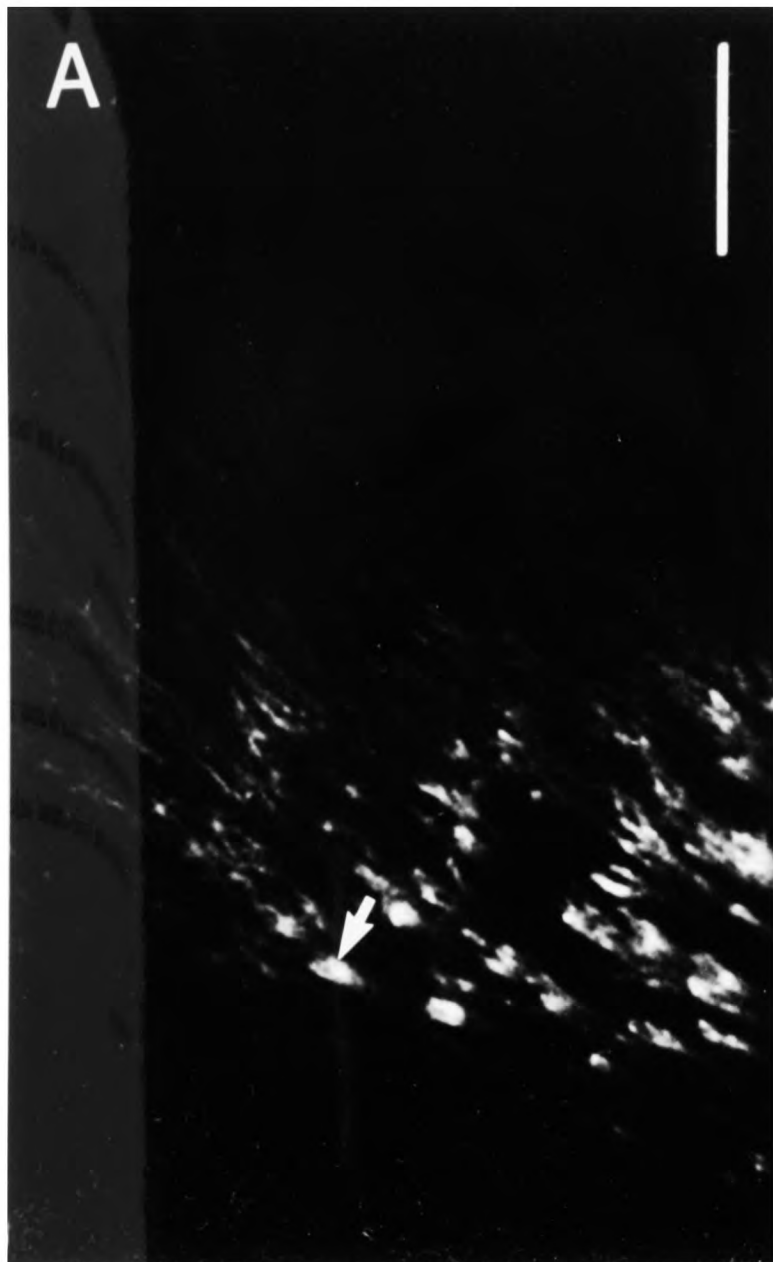


Fig.3.9

Montage of photomicrographs taken (under epifluorescent illumination) from a coronal section of a P2 hemisphere containing the caudalmost part of the somatosensory cortex. A DiI crystal was placed in the thalamus, then after 6 weeks of incubation at 37°C, 100 µm coronal sections were cut. The insert (*lower right corner*) represents the entire coronal section of the hemisphere, and was photographed at low power, under uv illumination. The bisbenzimidazole counterstain reveals the outline of the section and the contours of various distinctive structures (layer 1, hippocampus, etc). The limits of the cortical territory shown at high power under a rhodamine filter (*upper left montage*) are indicated on the insert by white arrows at the pial surface. The black star indicates the site of the crystal placement.

The labelled fibres form a narrow band in the white matter immediately below the cortex. At the white matter/cortical plate border the fibres turn up into the cortex and enter the cortical plate at approximately 90° to their trajectory in the white matter. They then follow less regular patterns of ascent. A few fibres reach the marginal zone, but the vast majority avoid the uppermost 200-250 µm of the cortex. At the relatively more mature ventral part, the superficial layers, above the level of branching axons, are relatively thicker than in the more dorsal sectors, indicating the ventrodorsal temporal gradient of maturation of the cortex. The fibre-dense areas in the dorsolateral aspect of the section may represent developing whisker 'barrels'. Only two back-labelled cells (*lower left corner*) were observed in the entire section, which suggests that the crystal placement site was not penetrated by corticofugal projections.

(Bar in the insert is 1mm, in the montage is 0.5mm.)

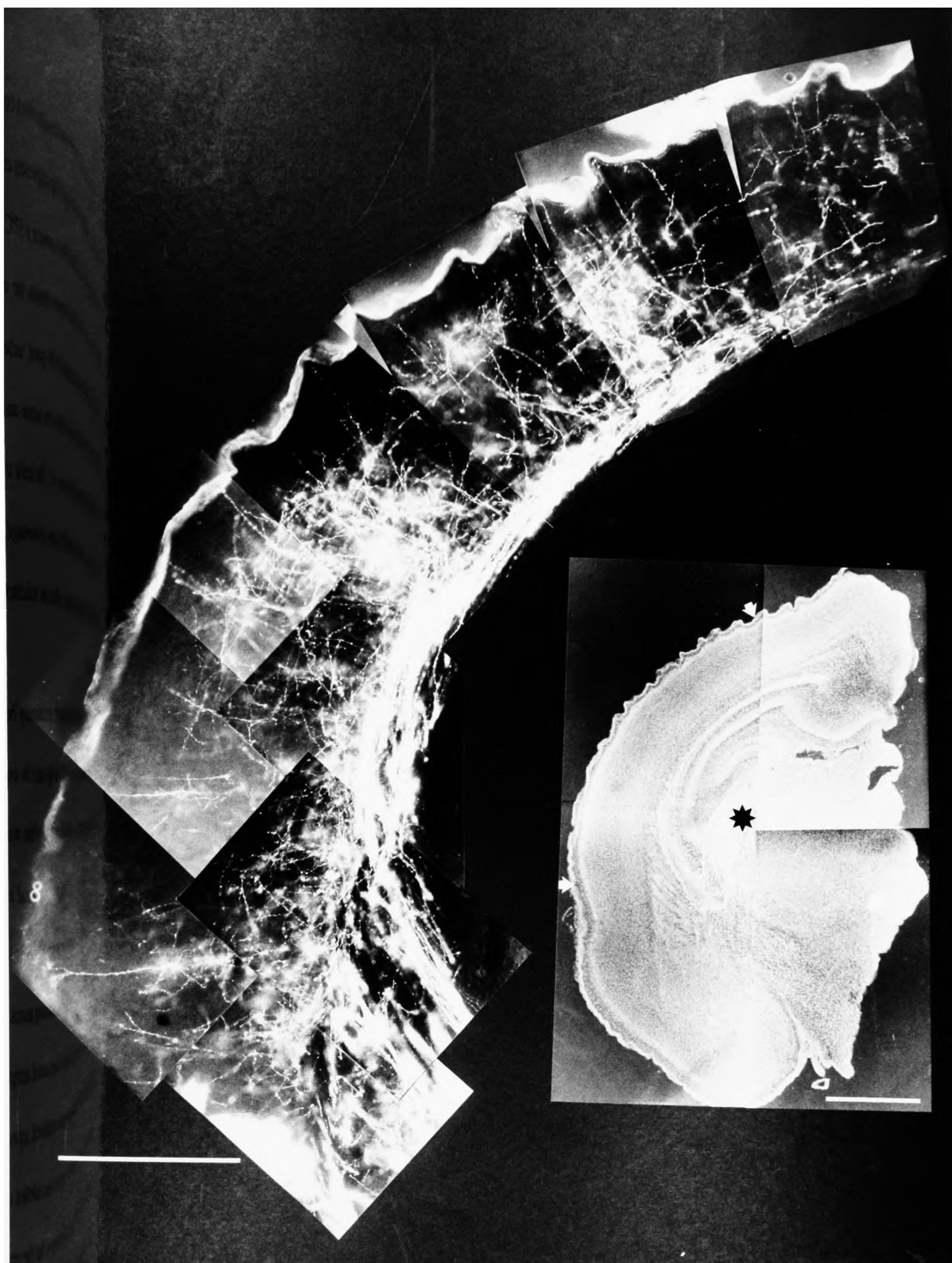


Fig. 3.10

To gain a general impression of the size of individual thalamic fibre arbors just after they have entered the cortex, individual arbors seen in a 200µm-thick coronal section of a P2 cortex, under epifluorescent illumination, were drawn with the help of a *camera lucida*. In each drawing the upper continuous line marks the pial surface while the interrupted line shows the upper boundary of the white matter. It is quite likely that many individual arborizations extended outside the thickness of the 200µm section since, within the plane of the section, some arbors cover areas up to about 500µm in diameter (though some branch more locally, over about 250µm). Unfortunately the fibres could not be followed far within the white matter because they run out of the plane of the section.

Bar 500µm.

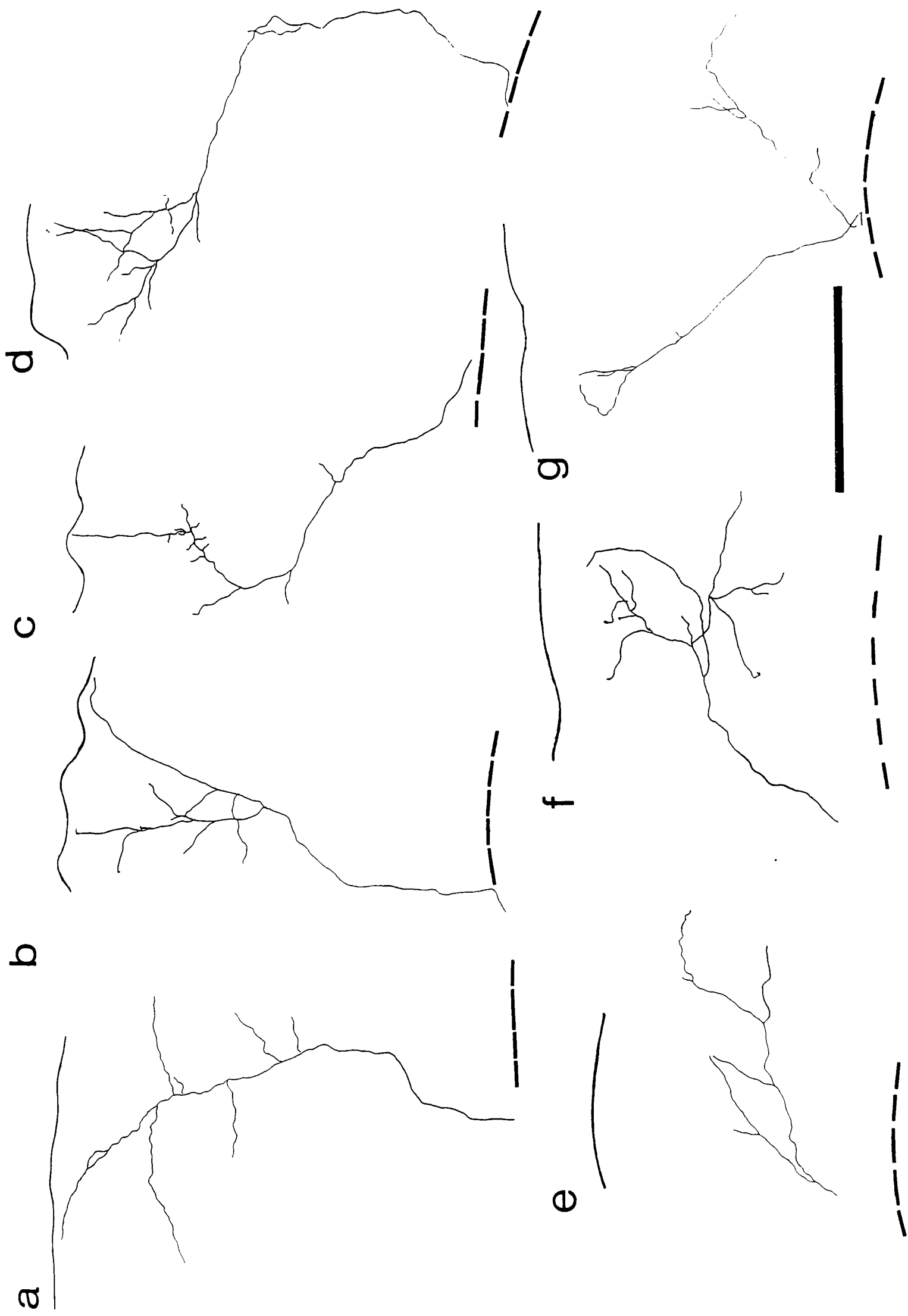


Fig. 3.11

To estimate the precision of the topographic relationships between fibres derived from different thalamic nuclei and their innervation patterns, crystals of different dyes were implanted at two different locations in the thalamus of a P2 brain. A DiI crystal was placed into the dorsolateral part of the thalamus (corresponding to the dLGN, black star) and a DiASP crystal into a ventromedial area (green star). The stars indicate the centres of the labelling. Every third section (100µm, coronal) was drawn via a *camera lucida* using epifluorescent illumination and they are shown here in caudo-rostral sequence.

The two different crystals revealed two different fibre bundles terminating in different sectors of the cortex. The DiI crystal (black) labelled mainly fibres projecting to the putative visual area in the occipital cortex but in addition part of the projection to the future somatosensory and auditory cortex was also labelled. The DiAsp crystal (green transparent page) labelled fibres terminating in more anterior sections. Scale bar 1 mm.

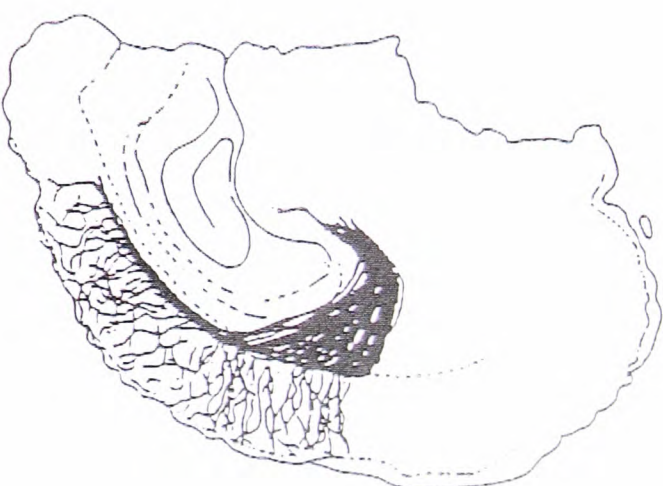
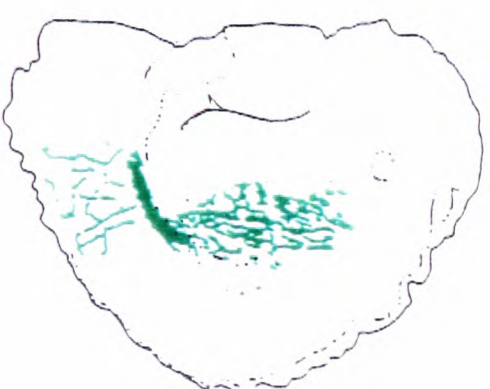
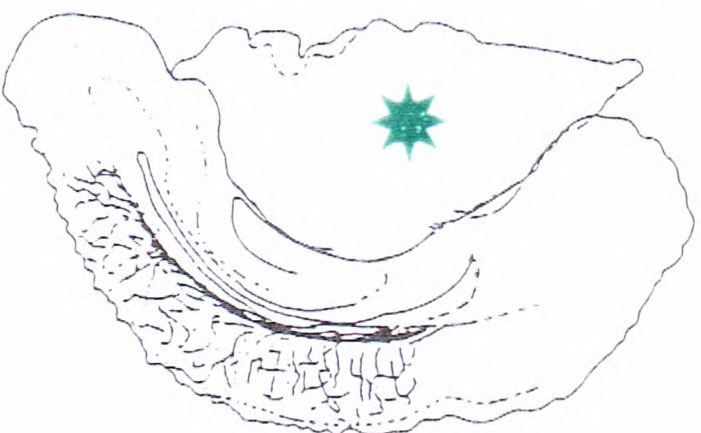
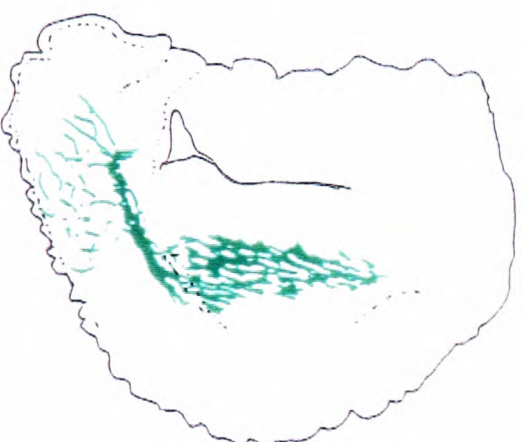
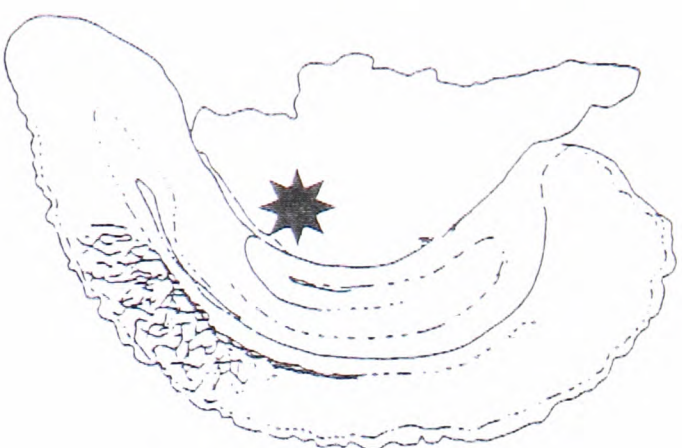
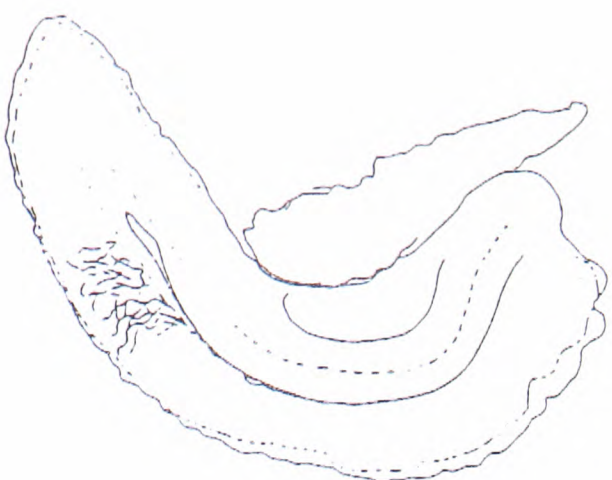


Fig. 3.12

A DiI crystal was implanted into the internal capsule of an E14 fixed embryonic brain, rostral to the thalamus. After 4 weeks incubation at room temperature 100µm-thick coronal sections were cut and counterstained with bisbenzimide. Two sections are shown here, each photographed using uv (A,C) and rhodamine (B,D) filters.

A,B: This section shows the crystal placement (white arrow in A, black in B), numerous labelled radial glia in the striatum (ST), and back-labelled cells in entorhinal and lateral cortex (CTX).

C, D are high-power views from the lateral part of A and B to demonstrate back-labelled neurons, lying mainly at the base of the cortical plate in presumptive entorhinal and lateral cortex. Note that some of these cells have processes extending up into the cortical plate.

Scale bar: 500µm for A and B; 100µm for C and D.

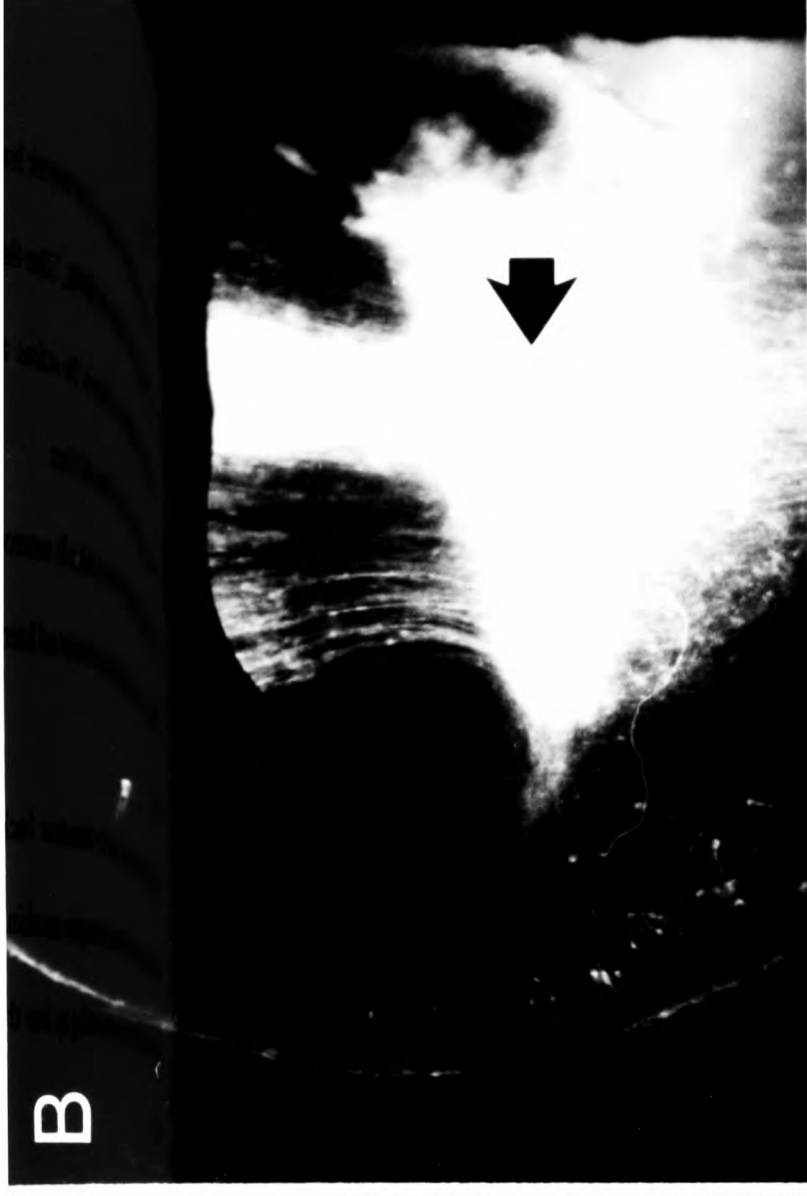
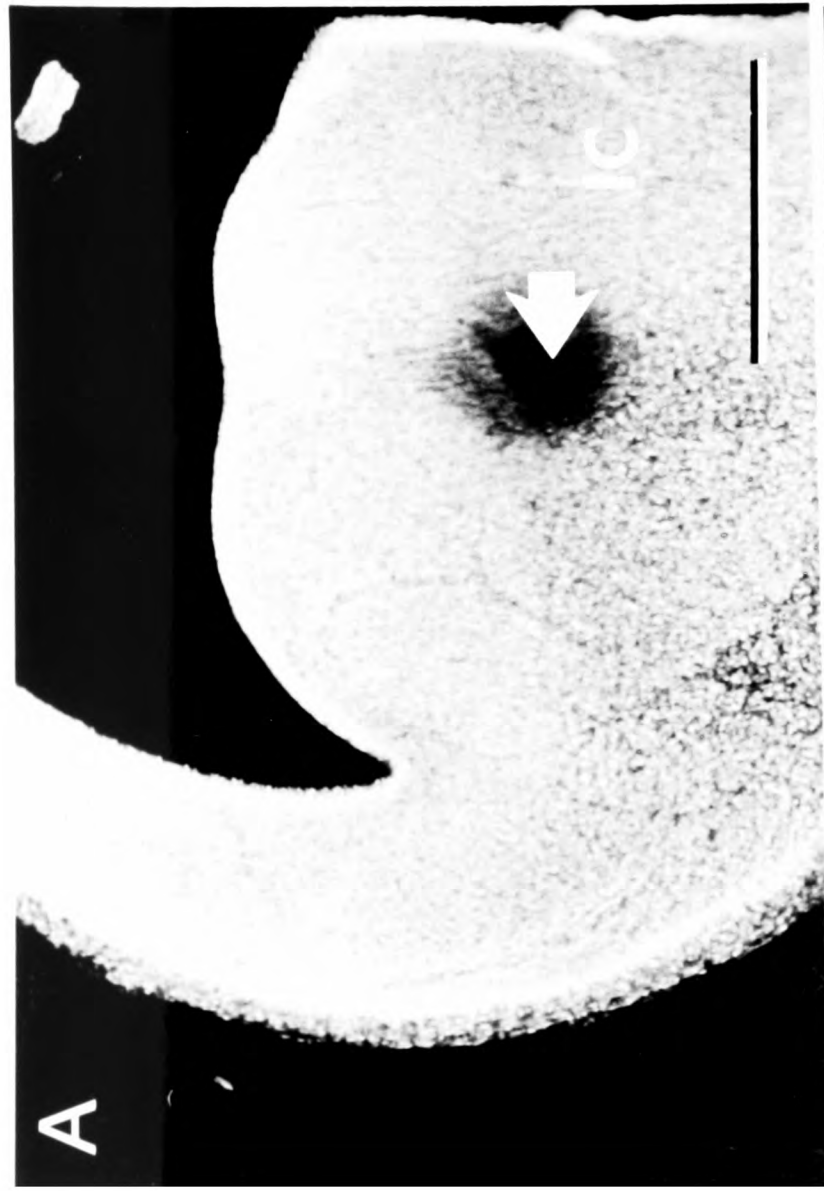


Fig. 3.13

This section was adjacent and rostral to the one shown in Fig. 3.12. As in Fig. 3.12 back-labelled cells appear in entorhinal and lateral cortex, and in this section the crystal placement site does not mask the large group of labelled cells within the internal capsule (IC). The radial glia of the striatum (ST), extending through these labelled cells, are also more noticeable. A and B are uv- and rhodamine-illuminated views of the same section in low power. C and D were taken with high power from the same section, showing the region above and below, respectively, the cloud of labelled cells within the developing internal capsule (the so-called perireticular nucleus). White arrows of different shape indicates identical points in B, C and D.

Scale bar: 500µm at A and B; 100µm at C and D.

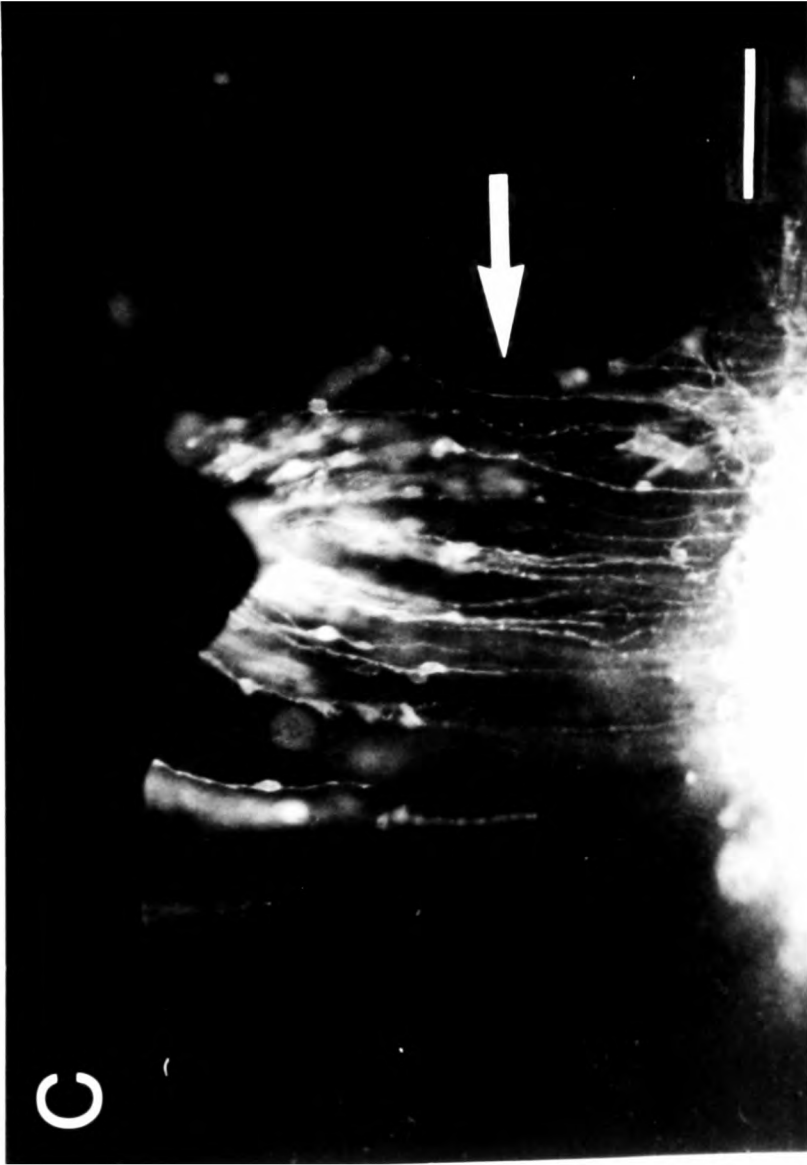
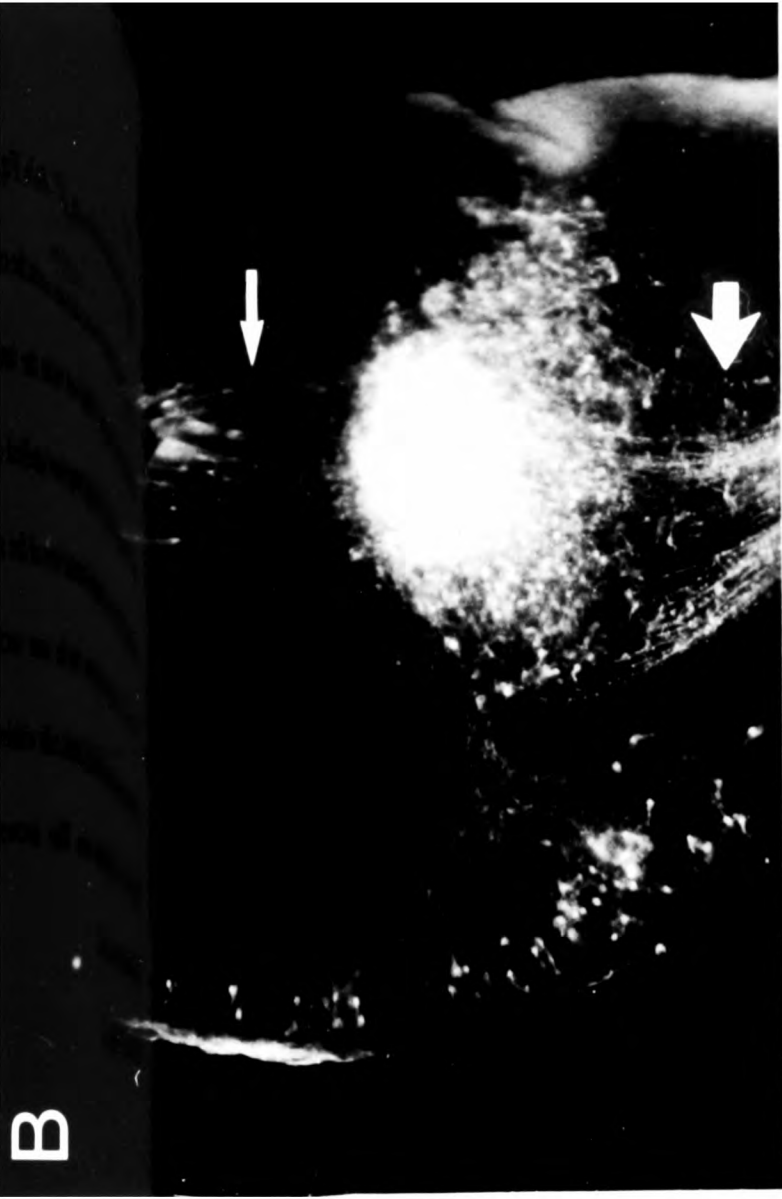


Fig. 3.14

A: 100µm-thick coronal section from an E14.5 rat brain in which a DiI crystal had been implantated into the internal capsule of the right hemisphere (black arrow). White arrows indicate the segment of cortex containing back-labelled cells.

B: The same section at higher magnification to demonstrate that the majority of the back-labelled cells are situated in subplate and in marginal zones, but there are also a few backlabelled cells within the emerging cortical plate.

C and D were taken from the same field with different filters. The true cortical plate (CP), only a few cells thick, is sandwiched between the marginal and subplate zones (MZ and SP, respectively).

Scale bar: 500µm in A, 100µm in B and 50µm in C and D.

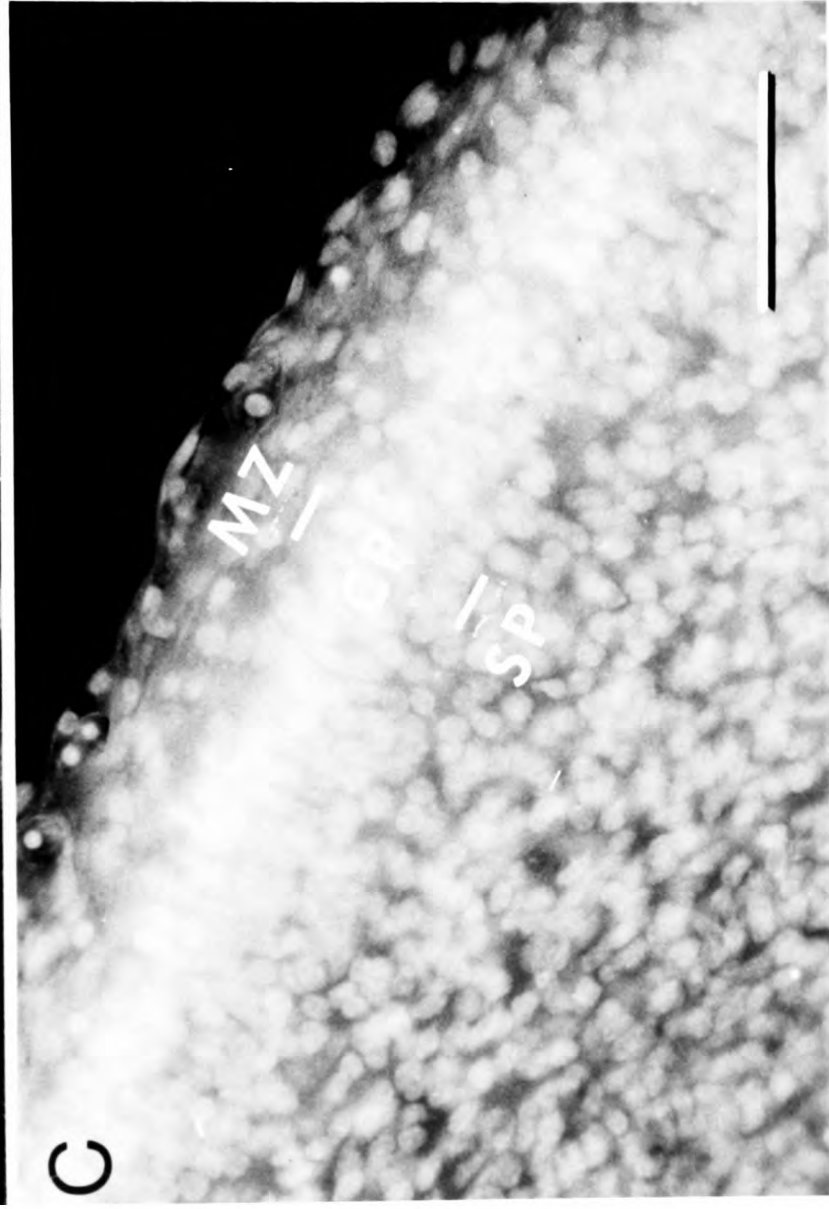
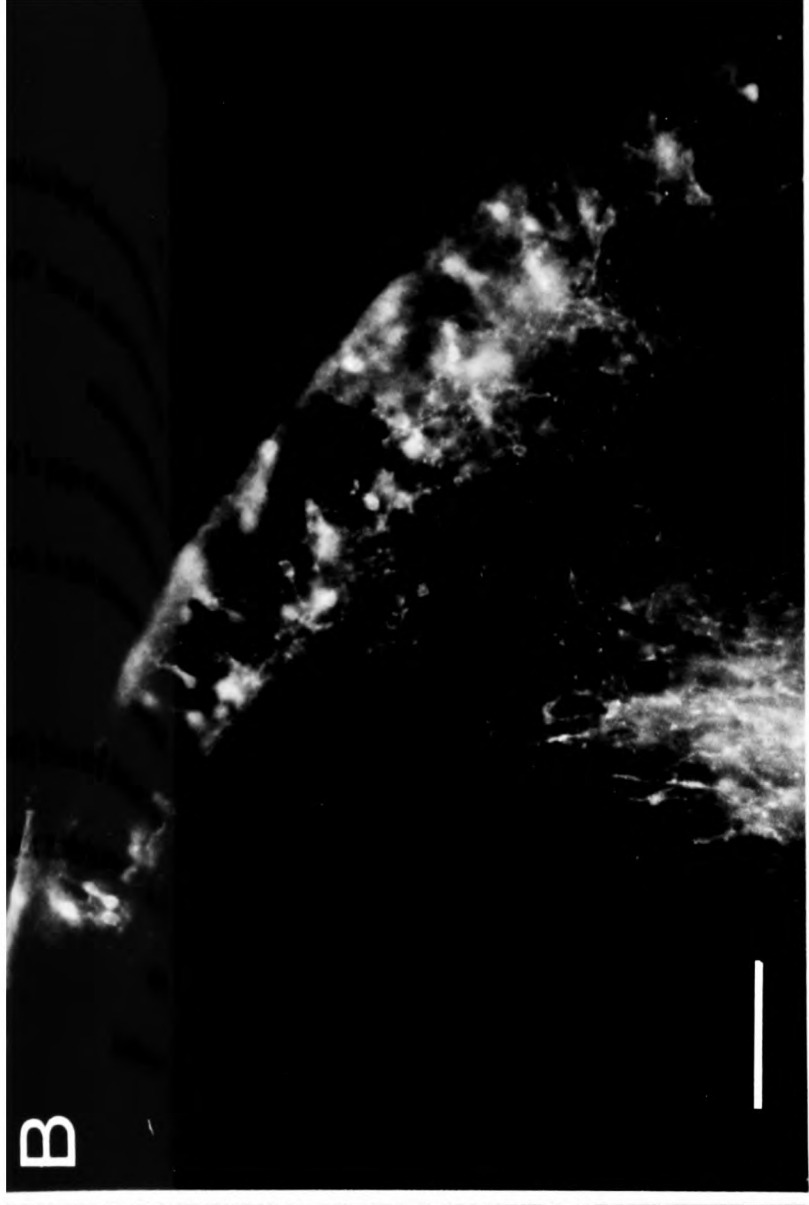
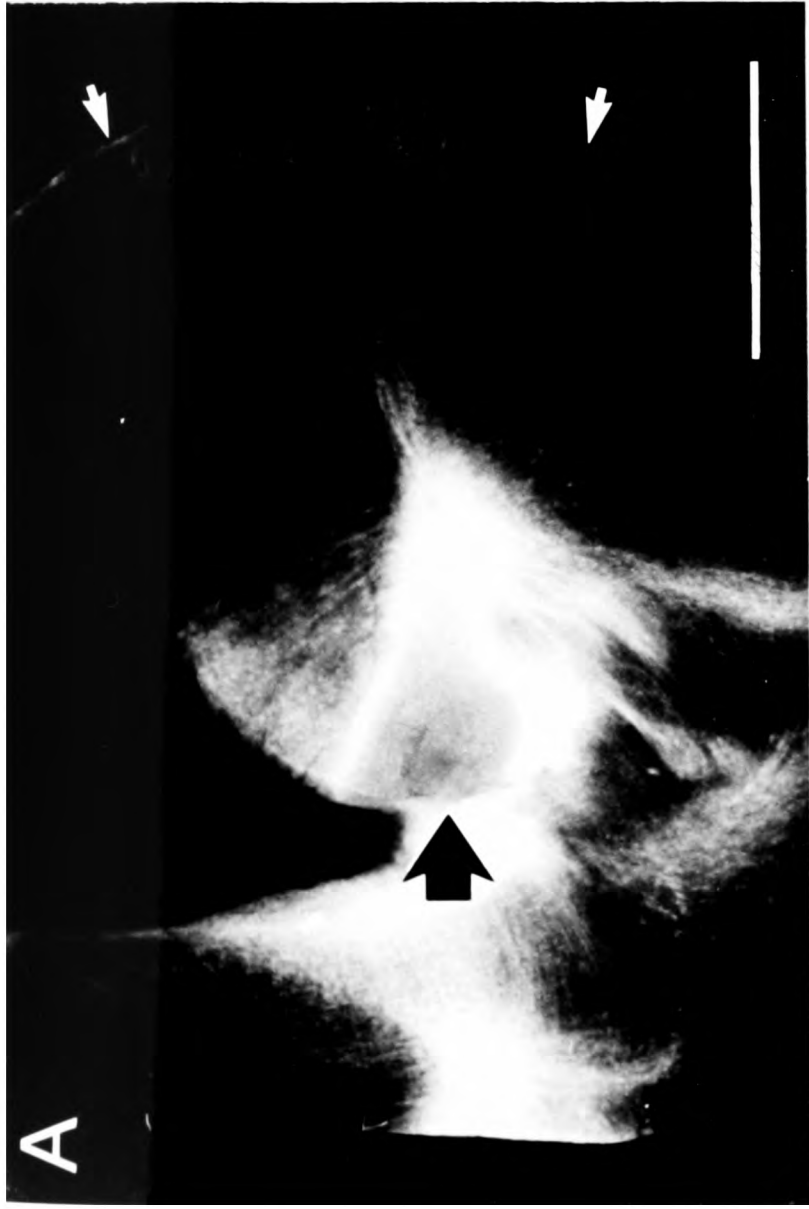


Fig. 3.15

Crystal implantation low in the ventral thalamus at E16 produces labelling very similar to that seen after dorsal thalamic placements, except that more backlabelled cells are seen in the cortex. The same incubation conditions (4 weeks at 37°C) were used for both sets of experiments.

B: DiI placement site (unfilled arrow) into the reticular nucleus in ventral thalamus (VT) viewed under rhodamine optics.

A: Same field as in B but viewed under ultraviolet illumination.

C: The bisbenzimidazole counterstain of the lateral cortex revealed the marginal zone (MZ), cortical plate (CP), subplate (SP) and intermediate zone (IZ).

D: Same view as in C but under rhodamine illumination. Back-labelled cells are seen in the lower part of the cortical plate and in the subplate. Small crystal placements within the ventral thalamus, in contrast with those in the dorsal thalamus, revealed thalamic fibres and back-labelled cells. Some of the cells were exclusively located in the subplate but some lay in the lowermost part of the cortical plate. If the crystal placement was strictly restricted to the body of the thalamus, then very few if any subplate cells were labelled (see Figs 3.4 and 3.5). This suggests that the subplate projection (and even that from layer 6 of the true cortical plate at this early age) does not extend far into the thalamus, but ends just outside it.

Scale bar 500µm at A and B; 100µm at C and D.

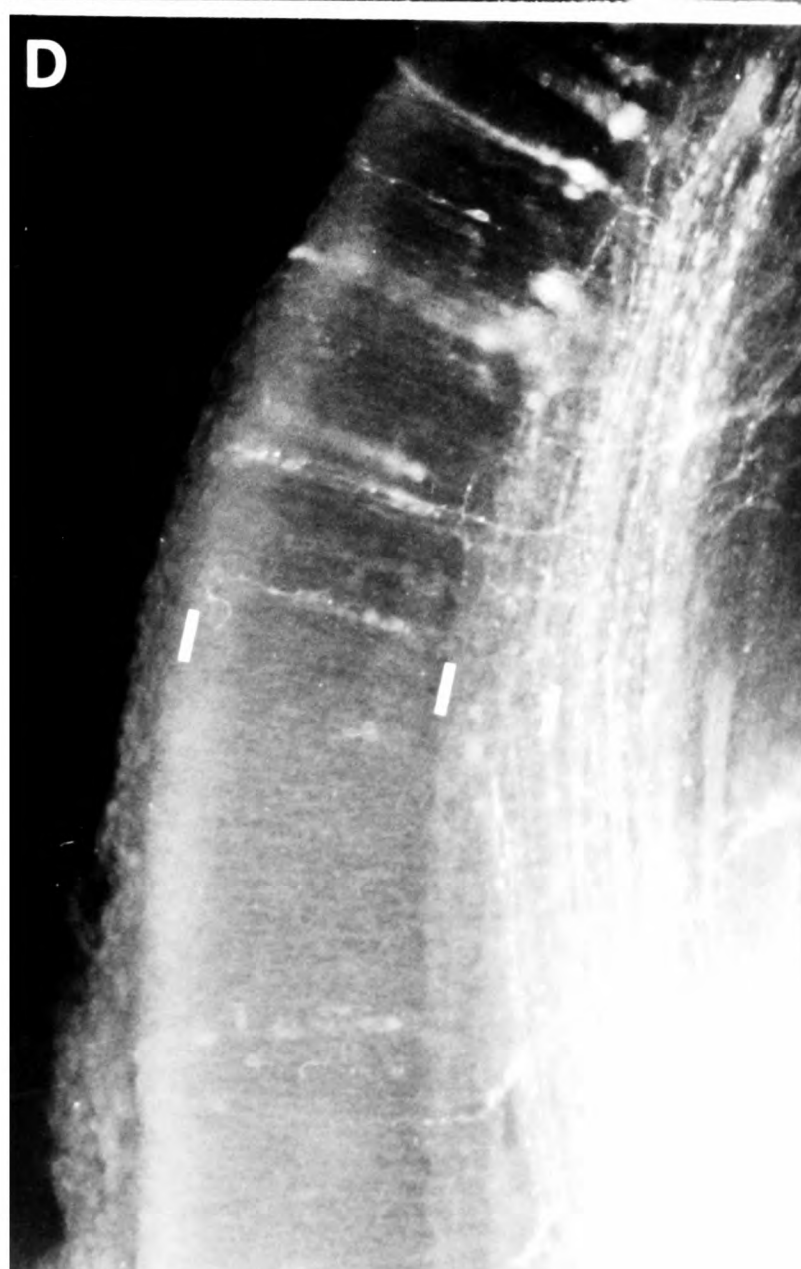
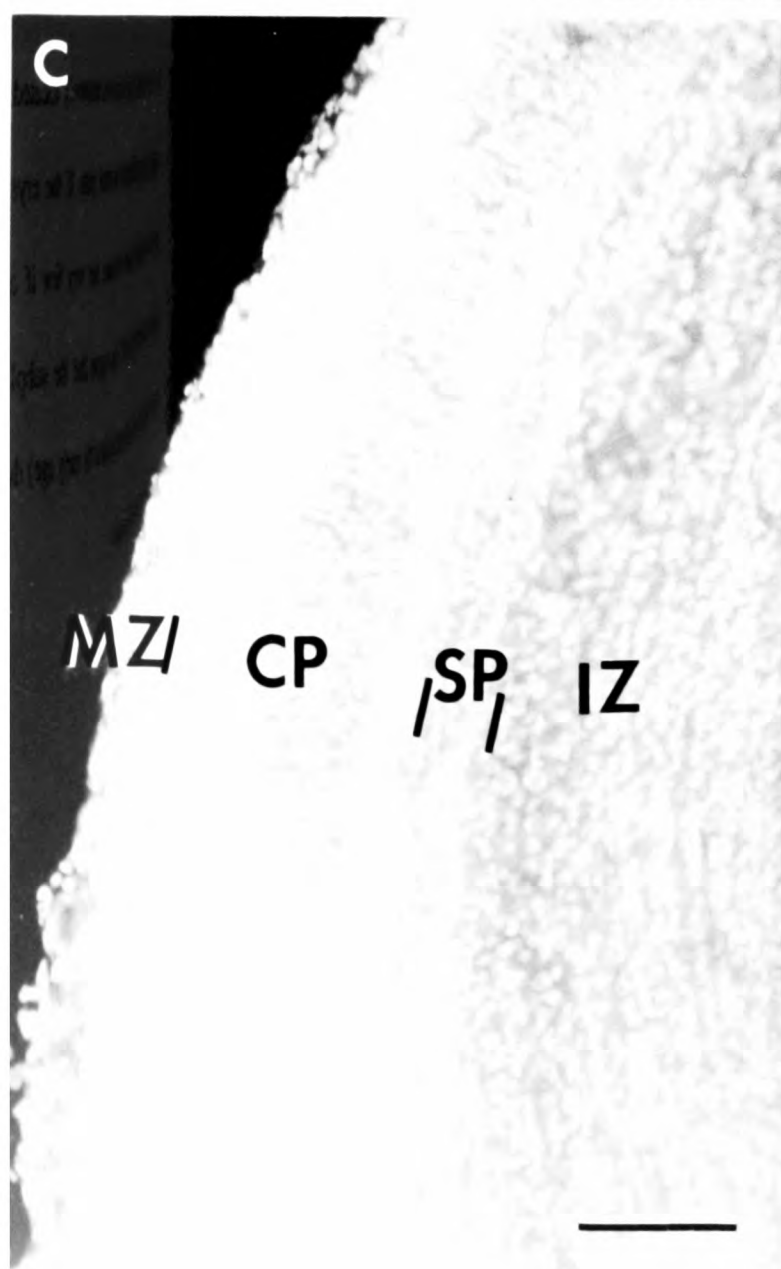
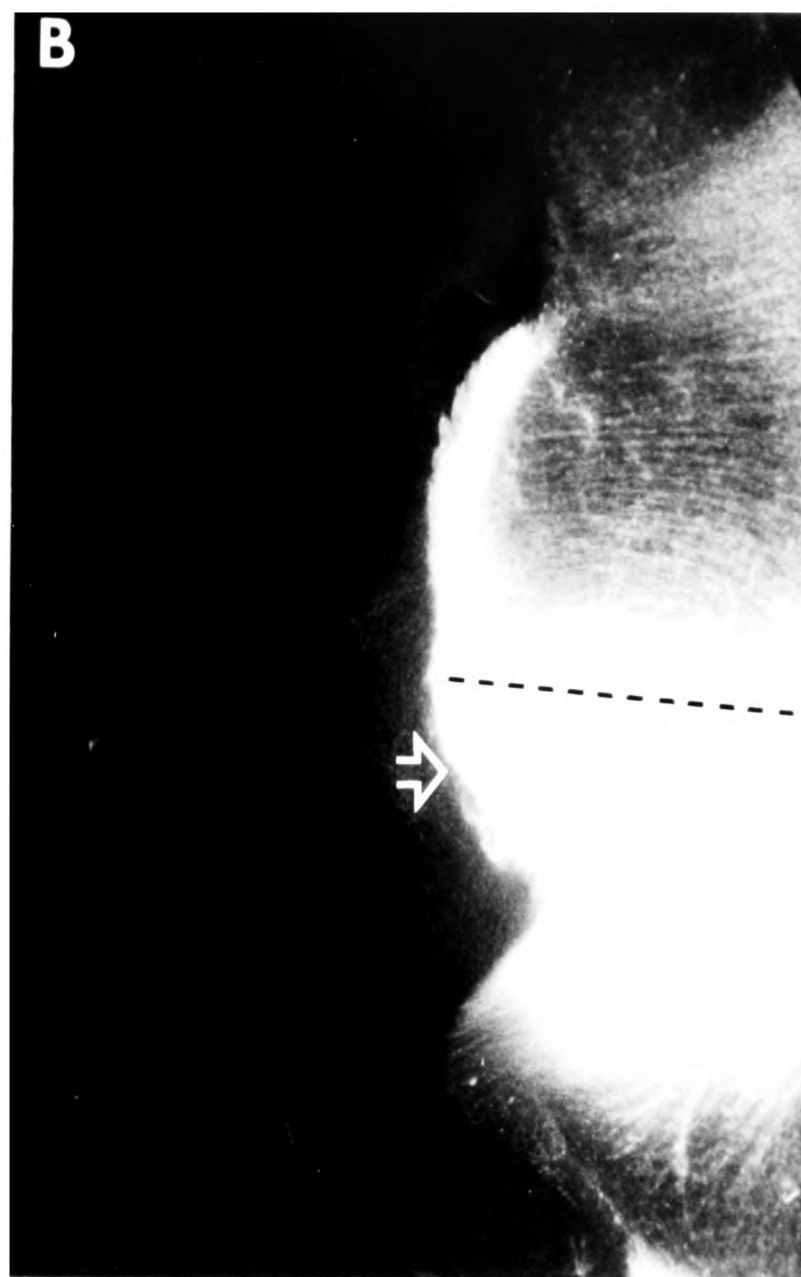
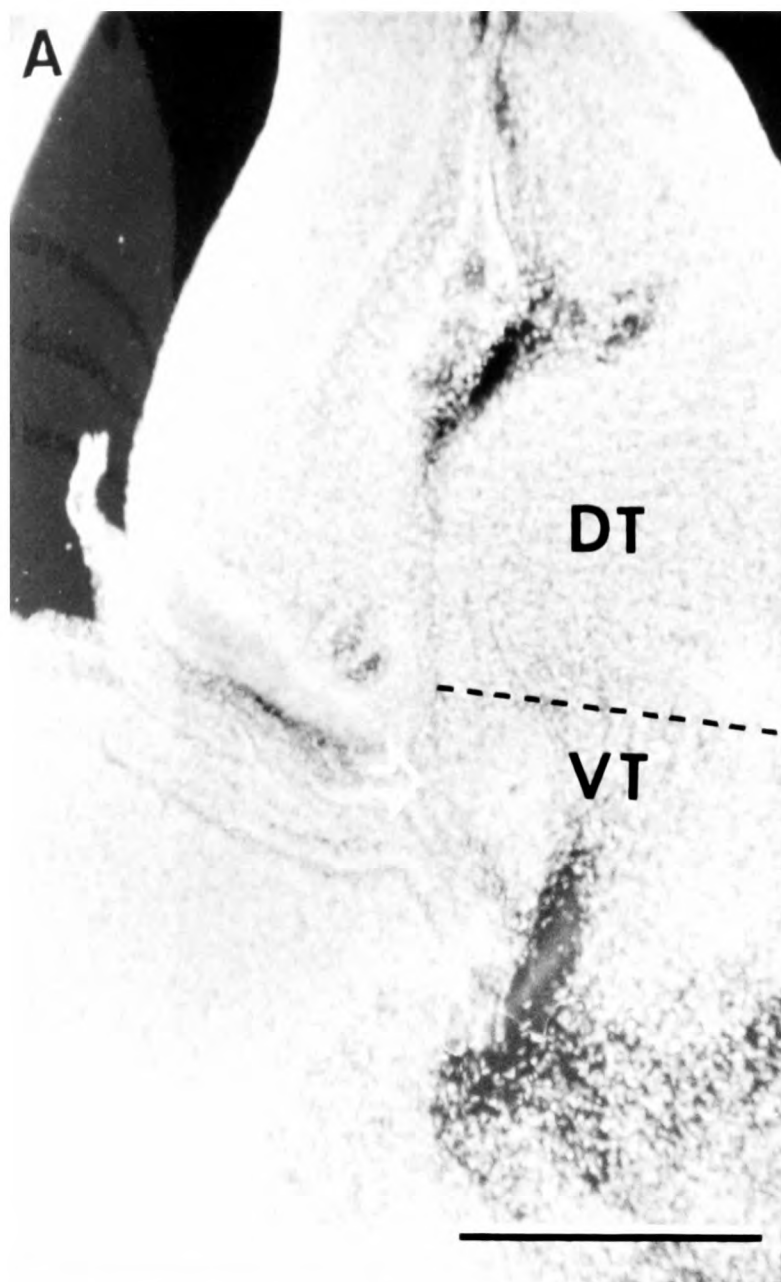


Fig. 3.16

Placement of a crystal of DiI into the lateral part of the diencephalon of a P8 brain (just lateral to the site of separation of the thalamic and peduncular fibres; see Fig. 3.25) back-labels cells of both layers 5 and 6. The thalamic fibres are almost completely masked by the back-labelled cell processes. The layer 6 cells project to the thalamus, while layer 5 cells project to the colliculus, pontine nuclei and spinal cord through the cerebral peduncle. The layer 5 cells possess thick apical dendrites, branching in the marginal zone. Right photomicrograph is of the same section as the left but viewed under ultraviolet illumination. The layering of the cortex (boundaries marked with white arrows) is now close to adult maturity.

Scale bar: 250µm.

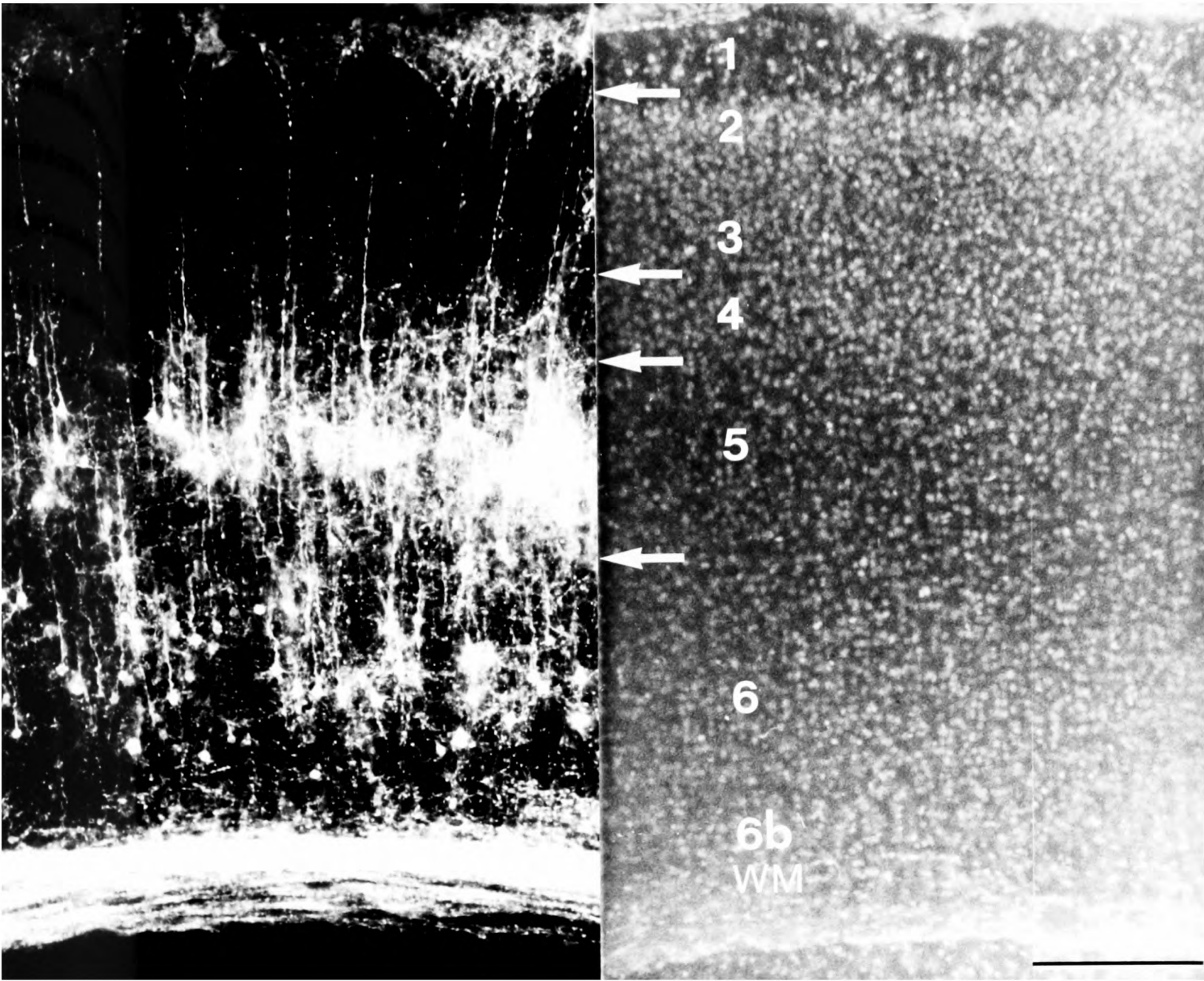


Fig. 3.17

Photomicrographs of the occipital cortex of a P2 rat in which a DiI crystal had been placed in the dorsal thalamus (A, B) or the cerebral peduncle (C,D).

A: The thalamic fibres are parallel to each other until they enter the cortex. There they follow more irregular patterns as they ascend to the middle layers, where they arborize.

B: The photomicrograph was taken from the same section as A, but focusing on a single arbor extending over a large area, some 200µm below the pial surface.

C and D: Inserting a single crystal of DiI into the cerebral peduncle leads to the labelling of layer 5 pyramidal cells (D). Their axons are highly ordered in the white matter (C).

In both the thalamocortical and the corticospinal projections, the fibres are so arranged, that the fibres belonging to ventral areas run superficial to fibres destined for, or originating from, more dorsal areas. Since the earlier fibres enter the more ventral cortical areas first, and since these areas develop relatively earlier, this type of fibre ordering reflects the timing of its establishment. There is *chronotopic* fibre ordering within the white matter.

By following individual axons of the labelled layer 5 cells within the white matter and in layer 6, varicosities of the axons could be observed, which might correspond to *en passant* synapses on subplate and to layer 6 cells. By electron microscopy Shering and Lowenstein (1992) recently demonstrated in the cat that axons of layer 5 and 6 neurons form asymmetrical synapses on subplate cells. The varicosities seen in the photomicrograph in C (arrows) suggests a similar arrangement in the rat. Scale bars: A,B: 200µm; C: 100µm; D: 500µm.

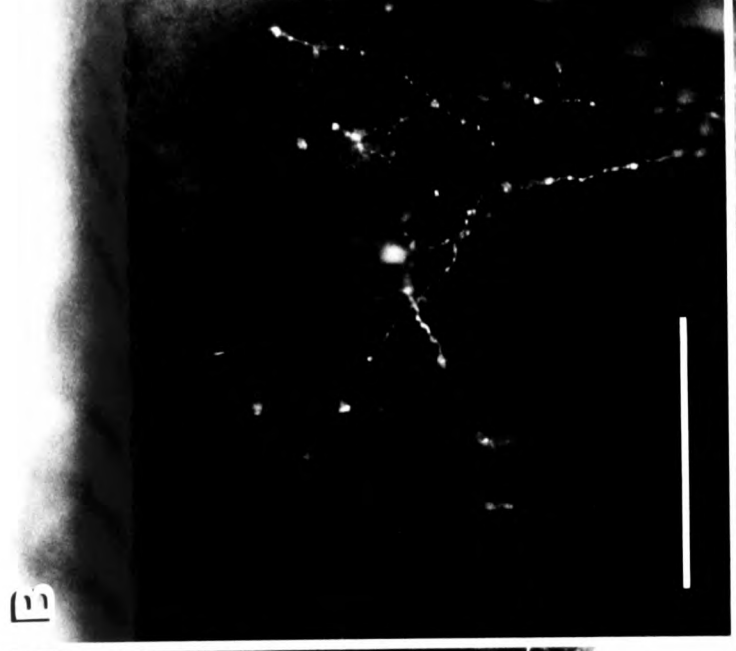


Fig. 3.18

A: A DiI crystal was placed in the pial surface of the occipital cortex a E13.5 embryo (* indicates the DiI placement site). After 4 weeks of incubation the brain was sectioned horizontally and examined in the fluorescent microscope with rhodamine optics. The dye labelled many spindle shaped immature neurons with their processes extending from the pial surface to the ventricular zone (VZ). The narrow lateral ventricle (LV) separates the medial and lateral cortical sheets. No outgrowing axons are evident.

B: On this coronal section of a mid-E14 brain the cortical DiI crystal placement (*) revealed the first corticofugal projections descending towards the primitive internal capsule (PIC).

C: At E16, DiI crystal placement in the occipital cortex revealed backlabelled cells in the putative lateral geniculate nucleus (LGN). In this coronal section the labelled fibre bundle is not continuous: a large segment of its most lateral portion lies in the plane of more posterior sections. The fibre bundle has several curves as it runs through the internal capsule (IC).

D: Although only small crystals were used for tracing there was a halo of diffuse labelling surrounding the placement site with a radius extending for several hundreds of microns. This can be best demonstrated on horizontal sections in the plane of the crystal placement sites. Triple exposing the uv, rhodamine and fluorescein image, this micrograph demonstrates the extent of the background labelling around four different carbocyanine dyes (a:DiAsp, b:DiI, c:DiS, d:DiO) on this horizontal section of an E20 embryo after 6 weeks incubation at room temperature. Rostral, top; Lateral, left.

Scale bar: A: 100µm; B and D: 350µm; C: 500µm;

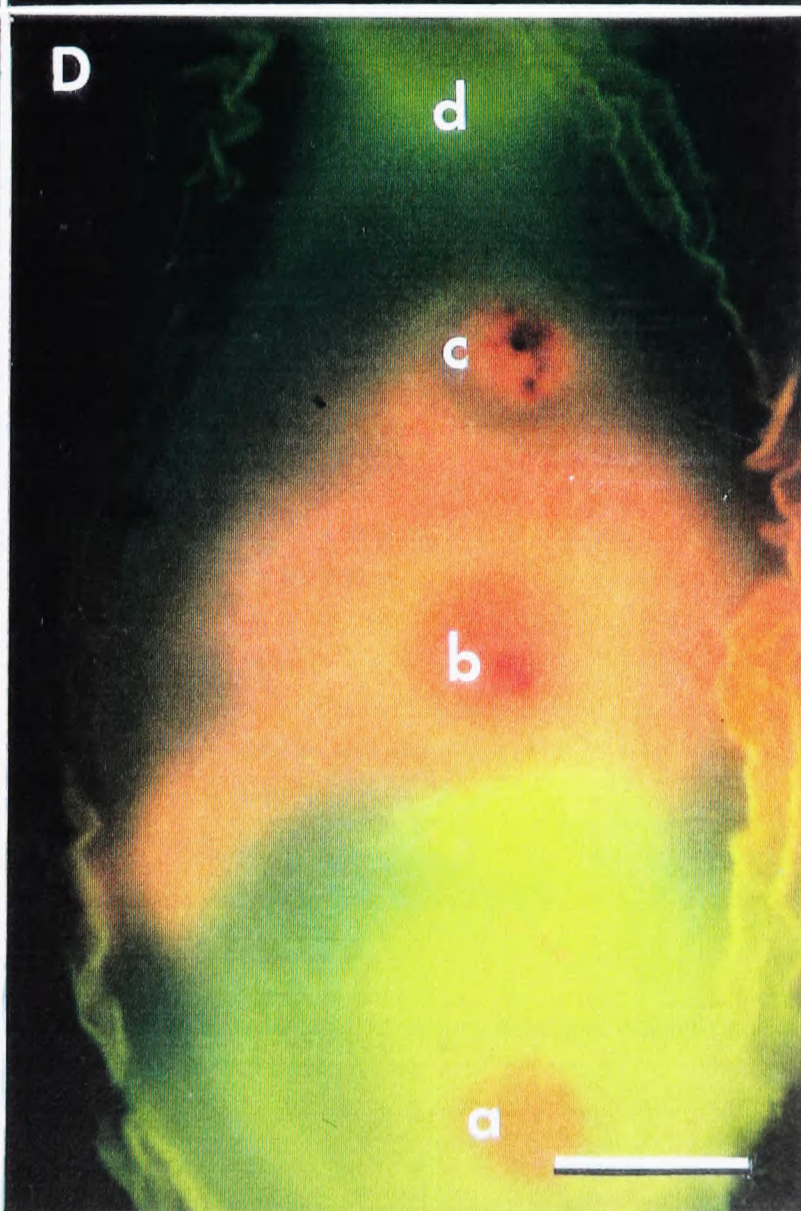
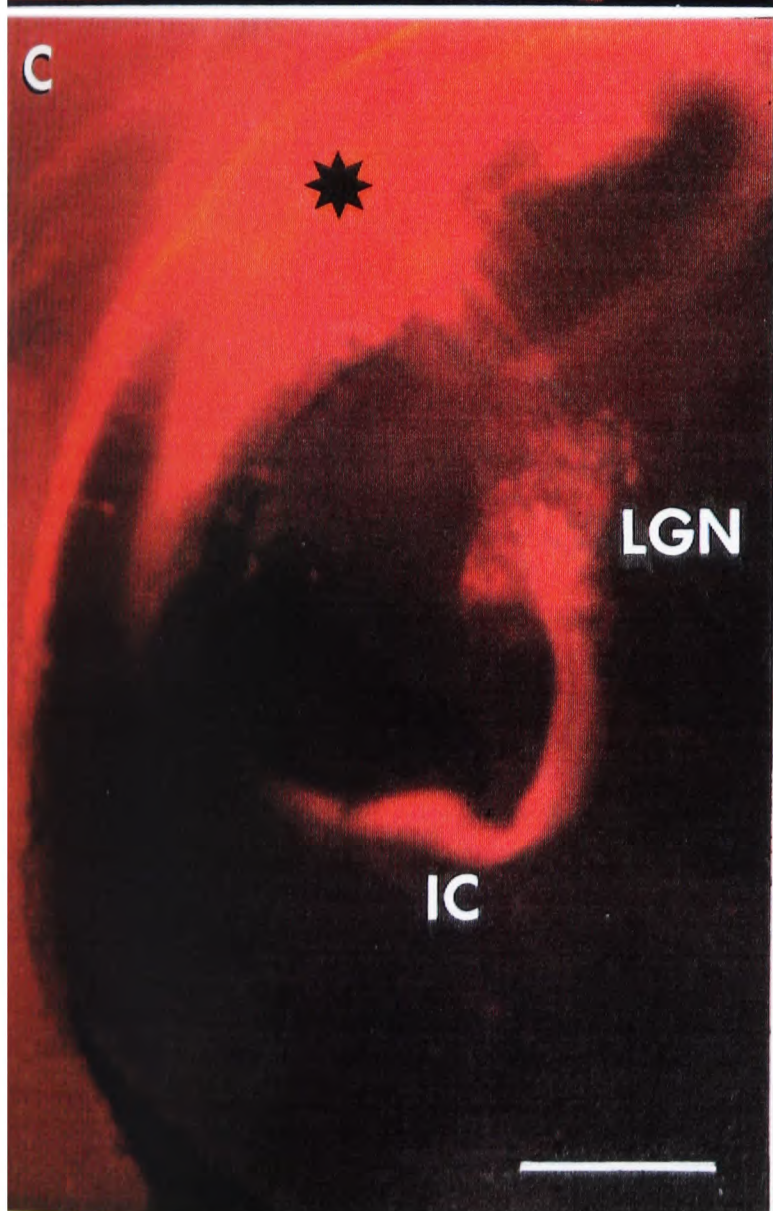


Fig. 3.19 (continued)

E: A crystal of DiI was inserted into the convexity of the left hemisphere of an E13.5 brain, then after 3 weeks incubation at room temperature it was coronally sectioned (75µm). The photomicrograph, which was taken (under epifluorescent illumination, viewed with rhodamine filter) from a region similar to that of D demonstrates axons arising from the thin layer of postmitotic cells between the pial surface and the ventricular and subventricular zones. The dense plexus and the intensely labelled cell bodies form a thin bright zone in which the cells and their processes are very difficult to resolve with ordinary fluorescent microscopy. The dye also revealed some of the ventricular and subventricular zone cells extending from the ventricular zone to the preplate, with small spindle shaped nuclei. The processes of the first postmitotic cells run within the preplate (primordial plexiform zone), and when the cortical plate splits the preplate into marginal zone and subplate, the initial portions of the fibres remain within their original zones. Since the division of the preplate is unequal, the subplate contains more cells than the marginal zone. Hence, the fibre layer is more apparent in the subplate, but nevertheless some cells with descending axons are present in the marginal zone.

F: By mid-late E14, the processes of the preplate cells leave the occipital cortex, run medially and turn under the developing striatum, heading towards the diencephalon. The fibres end in large growth-cones (arrowheads).

G: DiI diffusion from a crystal placed on the occipital cortex of a E16 fixed fetal brain to reveal interconnection with the thalamus. Low-power fluorescence micrograph of a coronal section showing the labelled bundle of fibres running in the intermediate zone (upper left), turning medially, narrowing as it passes through the internal capsule and then sweeping up towards the dorsal thalamus (lower right). Scale bars: A,B and F: 50µm, D and E: 100µm, G and C: 250µm

Fig. 3.19

A: High-power photomicrograph taken from a 10µm, cresyl-violet-stained coronal section of the occipital cortex of an E13 embryo. At this early stage the wall of the telencephalic vesicle consists of the ventricular and subventricular zones (VZ and SVZ), and a thin layer of postmitotic cells forming the primordial plexiform zone, or preplate (PP).

B: 50-µm-thick coronal section of an E13 cerebral cortical vesicle (similar stage to A), viewed under epifluorescent illumination with a rhodamine filter. A small crystal of DiI had been inserted into the cortex nearby, leading to intense local labelling of a few processes, presumably of dividing germinal cells and of radial glia, with spindle-shaped cell bodies at different depths within the VZ and SVZ. Most of these somata are presumably precursor cells producing the first postmitotic cells. No fibres are seen leaving these cells at this very early stage, but, a little later, a very dense plexus of axons is seen growing out of the preplate (E).

C: The ventral dorsal morphogenetic gradient is apparent in this (10 µm-thick, cresyl violet stained) coronal section of a E13.5 rat brain. The thalamus (th) is not differentiated into individual nuclei, the primitive internal capsule (ic) is an indistinct feature below the corpus striatum (st) at this early stage. The cortical plate has not appeared yet, not even at the ventralmost part of the cortex. The upper-medial segment of the cortex is delayed compared to the ventral-lateral parts in its development. The hippocampus (h) develops from the neuroepithelium of the medial wall of the telencephalon.

D: Taken from C with higher magnification showing the most developed lateral-ventral sector of the cortex.

(Please turn for E,F and G)

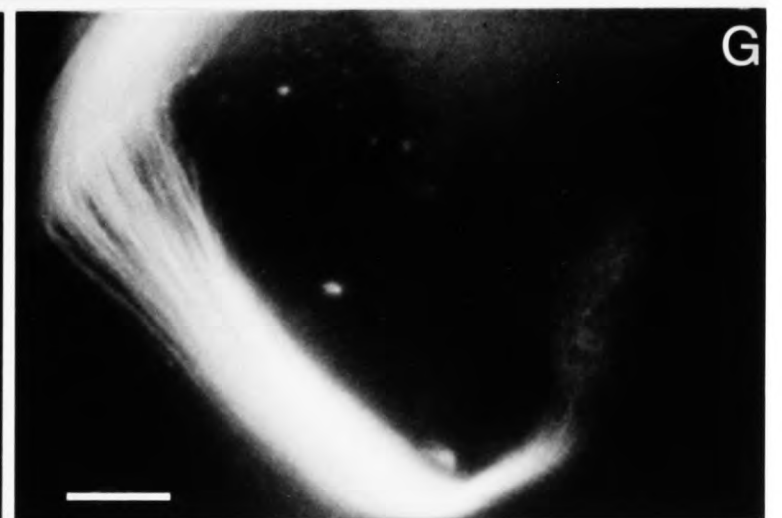
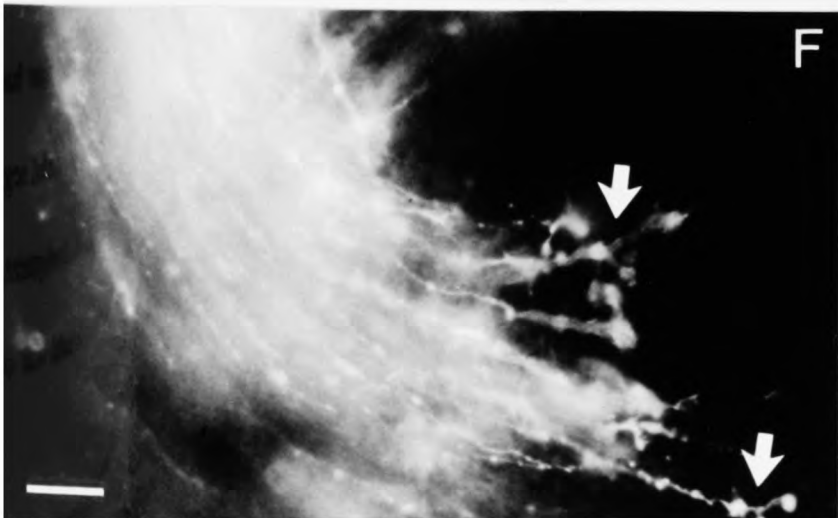
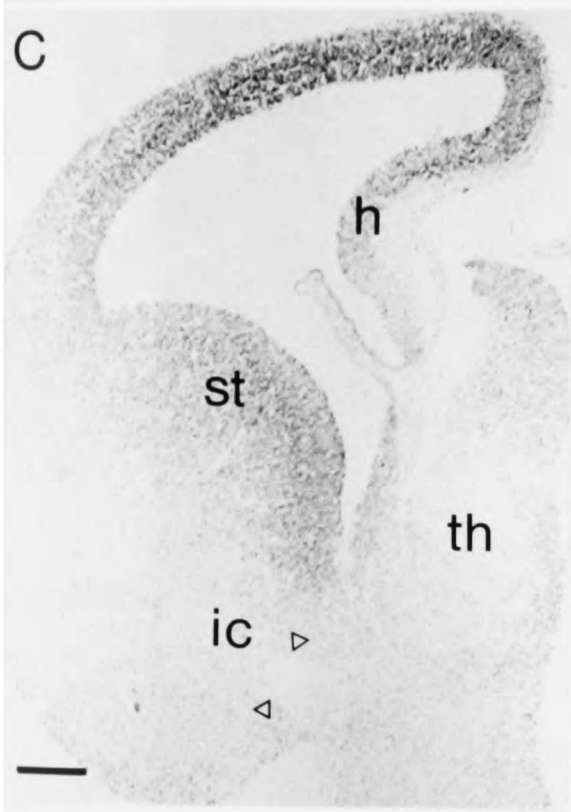
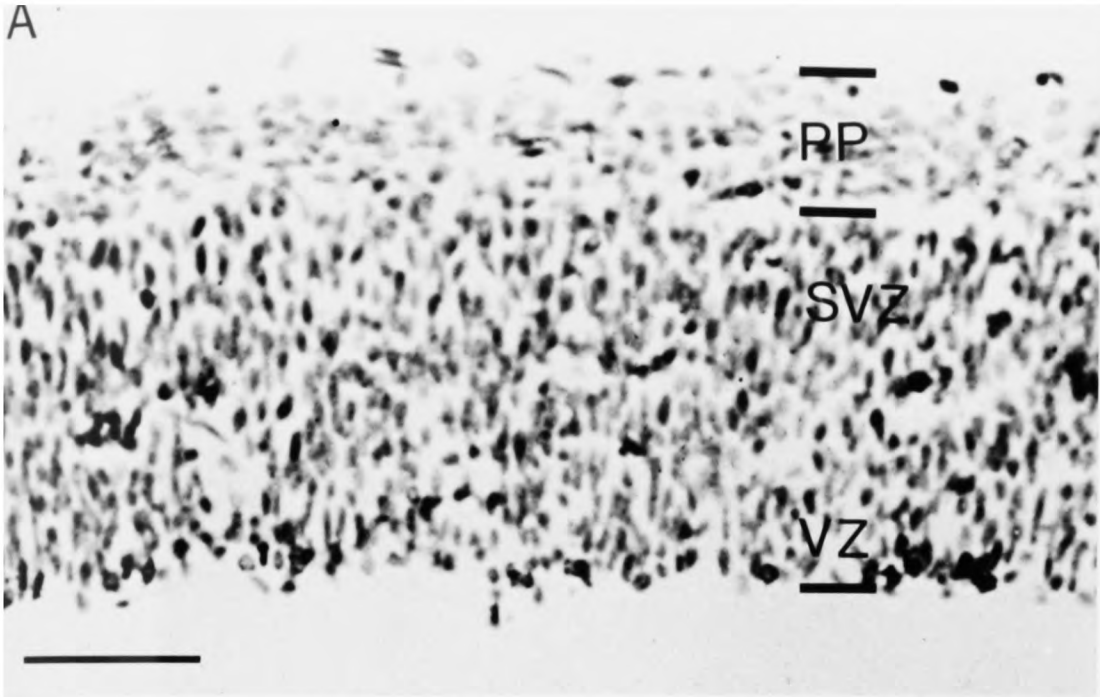


Fig. 3.20

Similar stage to Fig. 3.19G (E16). A single crystal in the occipital cortex labelled a single fibre bundle. B demonstrates the crystal placement site (*) in low-power (d-dorsal, l-lateral). To resolve fibres in the bundle the section was examined with a confocal microscope at higher magnification. High-resolution reconstructions were made from fine optical sections to create 3-D datasets. The datasets then were projected with 14 deg disparity to create left-right anaglyphs (Please view with the red/green glasses provided). The assembled fluorescent micrographs (A) demonstrate the labelled bundle of fibres running in the intermediate zone (*upper right*), turning medially, narrowing as it passes through the internal capsule and then sweeping up to the dorso-lateral part of the thalamus. The fibre bundle has two major turns, the first as it leaves the intermediate zone, where the bundle turns towards the internal capsule, and a second, sharper turn towards the diencephalon. (One back-labelled cell can be seen at this second turn, near the internal capsule.) With short (2 weeks) incubation at 37°C few thalamic cells were back-labelled (see Fig. 3.14G). But 3-4 weeks incubation, used for this section, revealed a small group of thalamic cells. This indicates that the labelled bundle contains both corticofugal and corticopetal fibres. The location of the back-labelled thalamic cells was highly predictable from the cortical crystal placement site. The thalamic cells appeared rather immature, with small dendritic arbors.

Scale bars: A: 100µm; B: 500µm.

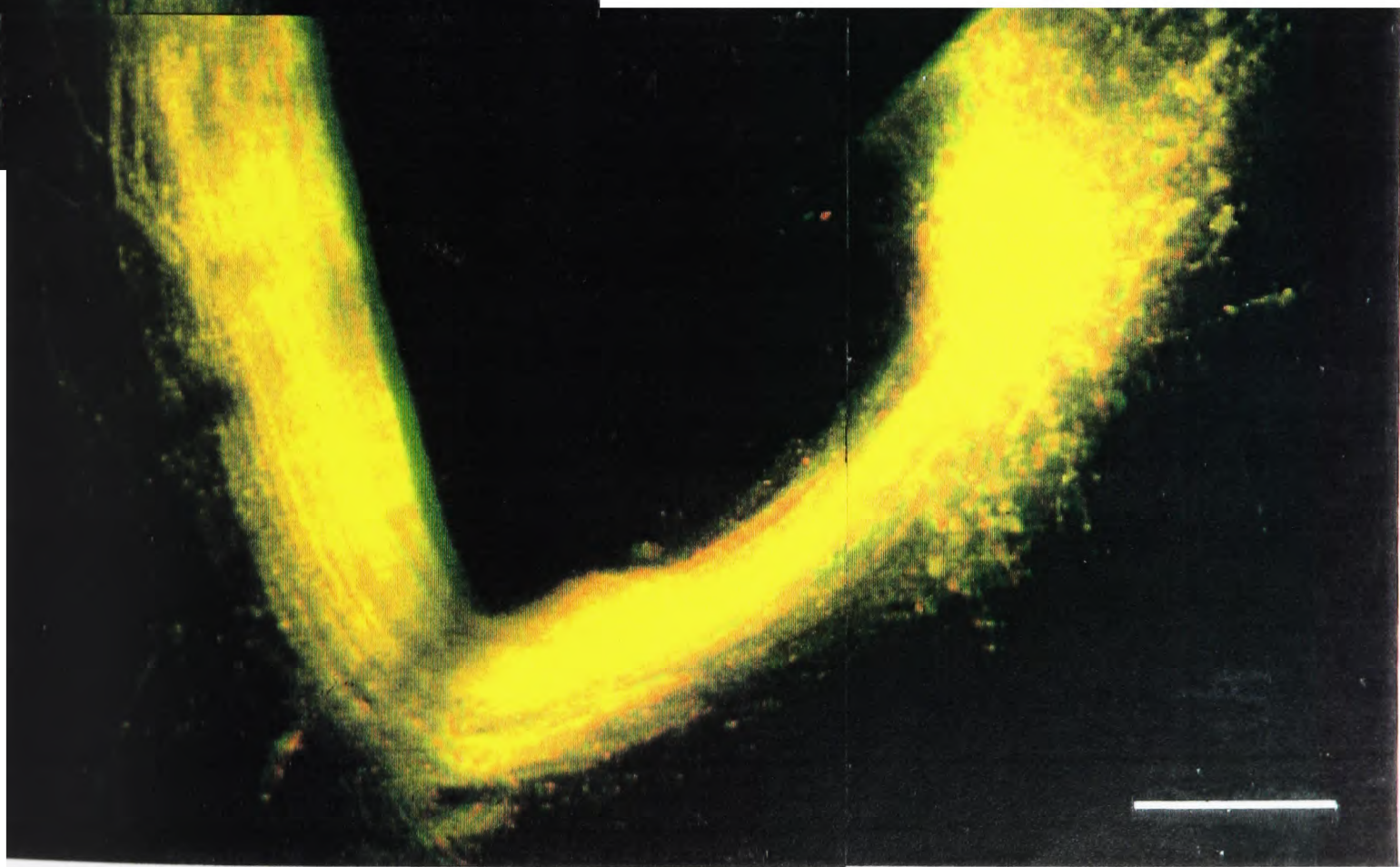
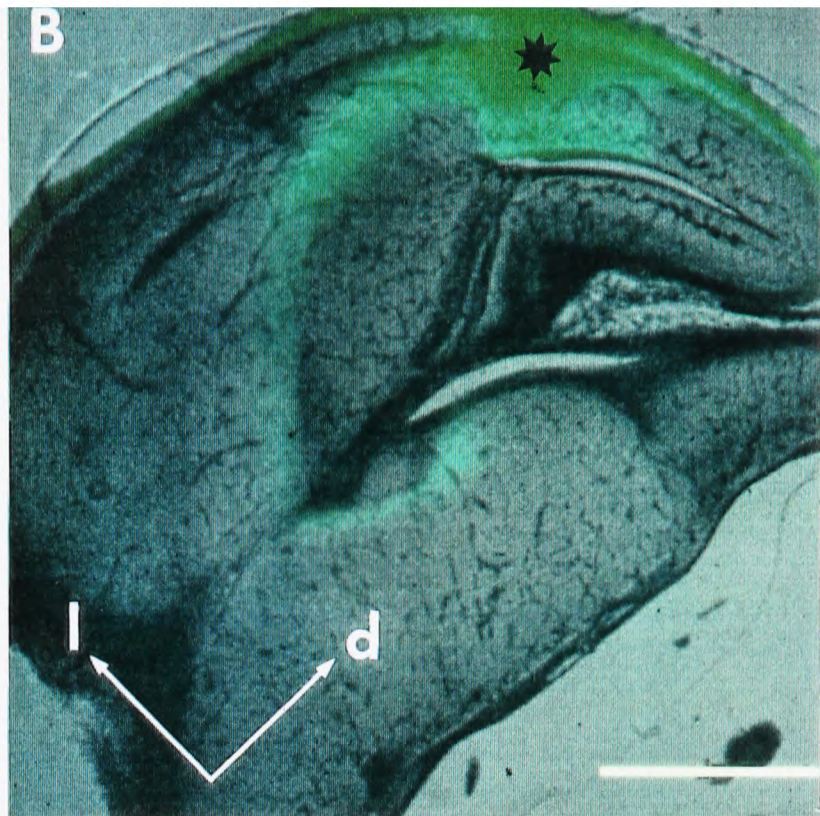
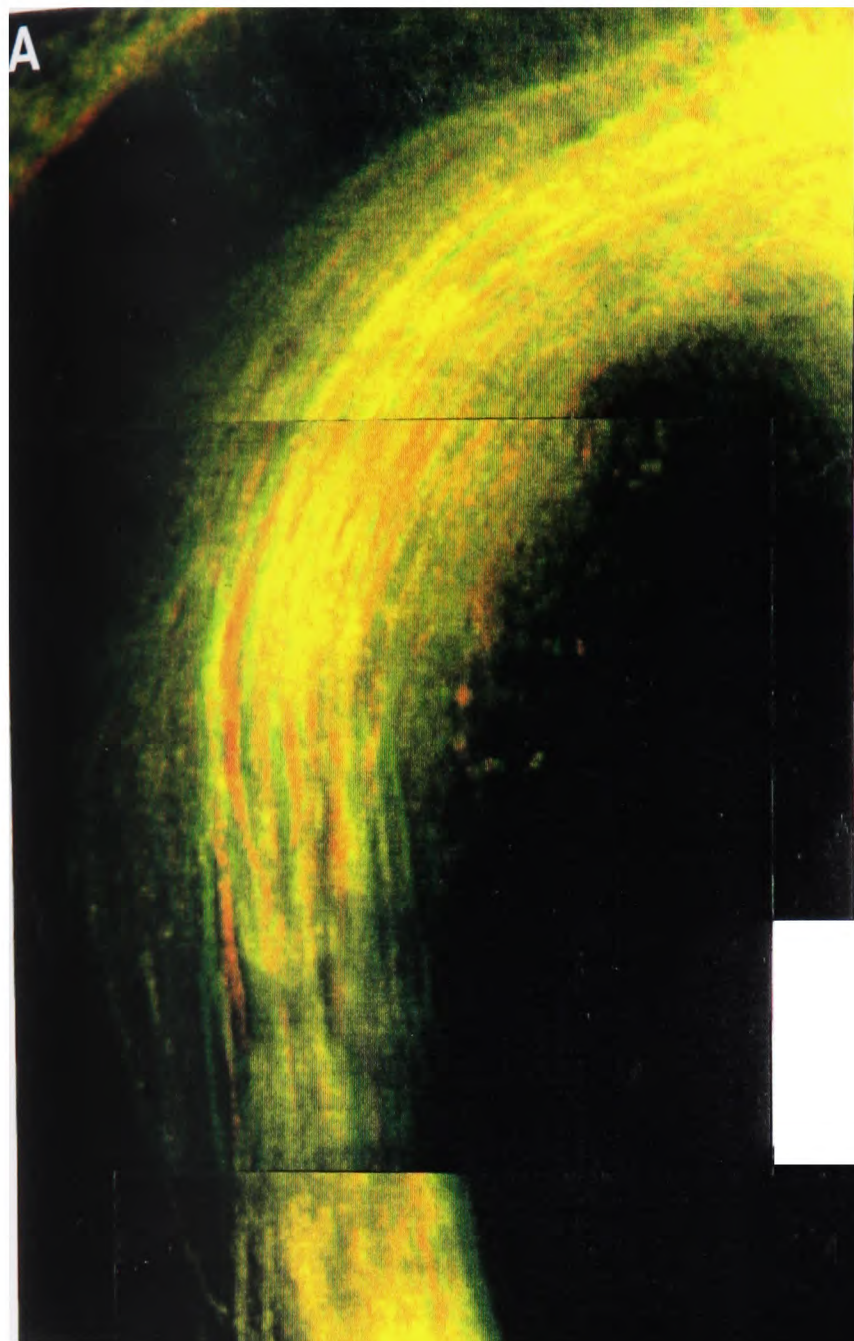


Fig. 3.21

Fast-DiI was dissolved in methanol and injected at multiple sites (6 injections 20 μ m apart from each other) along a coronal line along the dorsal surface of the left hemisphere of a E19 embryonic rat brain. After 4 weeks incubation at room temperature, 200 μ m horizontal sections were cut.

A: The small insert was taken with fluorescent and bright field, with low power to demonstrate the location and orientation of the labelled fibre bundle extending from the intermediate zone (IZ), through the striatum (ST) towards the internal capsule (IC).

Scale bar; 200 μ m

B: With confocal microscope a montage was constructed from overlapping 3-D datasets, collected along the labelled fibre bundle from the same 200 μ m thick brain section as demonstrated at A. Each image is a two-colour stereo anaglyph constructed from 32 optical sections imaged at 5 μ m spacing. The fibres (containing corticofugal and corticopetal fibres) have three different patterns of organization at different parts of the pathway. Axons in the white matter display an organized, parallel distribution with apparently little rearrangement. Within the striatum the array of axons reorganises into fascicles. There is some exchange of individual axons between adjacent fascicles. Entering the internal capsule the fascicles dissolve into a tight bundle of closely-associated, quite strictly parallel axons.

Each sub-image is 100 μ m across, the scale bar is 25 μ m.

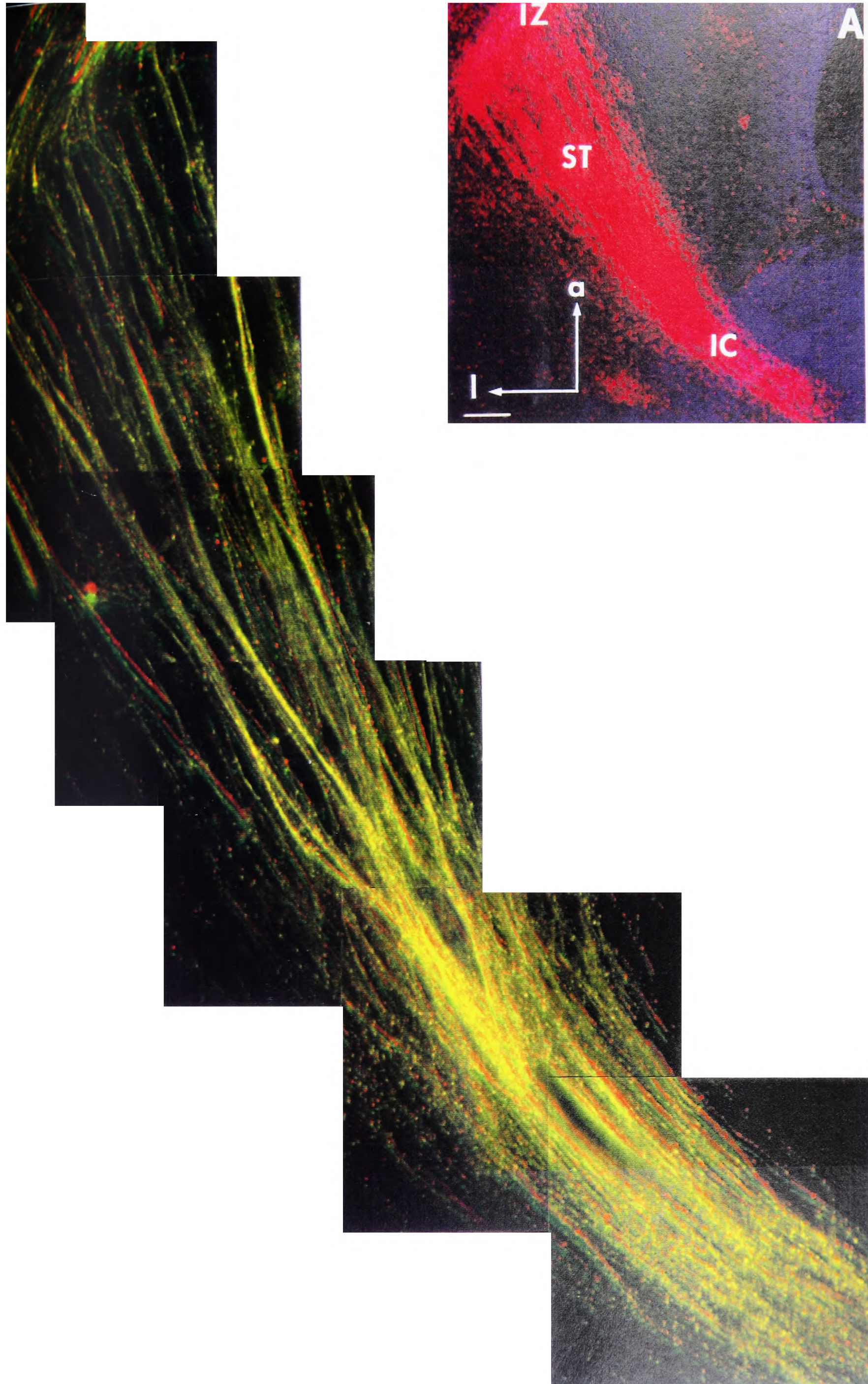
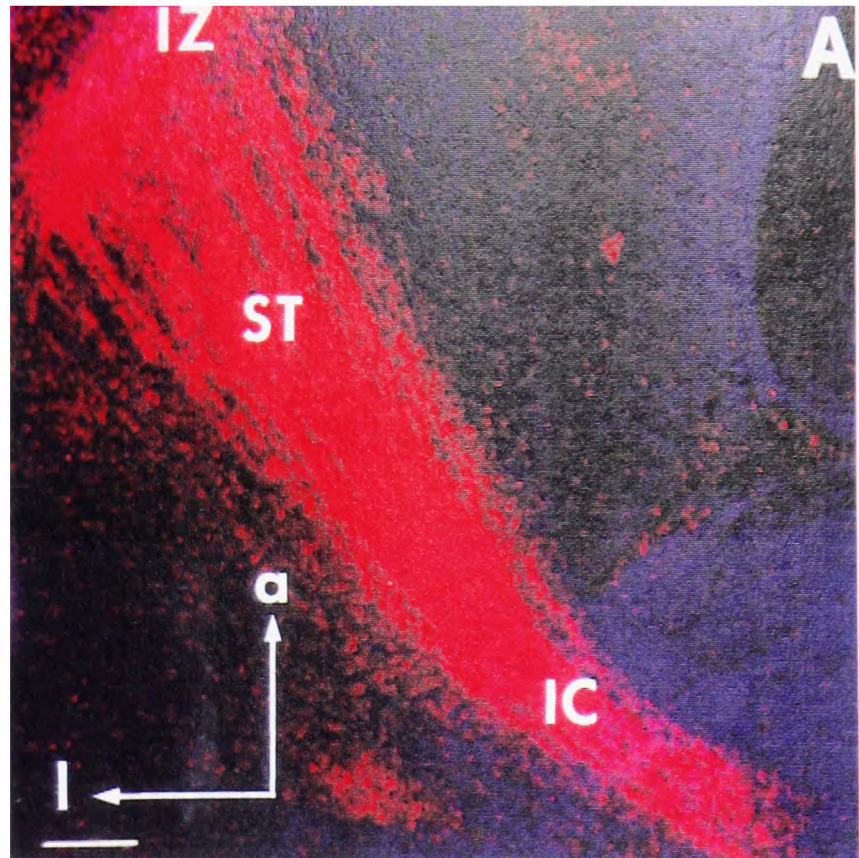


Fig. 3.22

Fluorescent photomicrograph taken from a 100µm thick horizontal section of an E16 brain by exposing the same film three times using rhodamine, fluorescein and ultraviolet filters (rostral, up), after crystal placements in the cortex. Since thalamic fibres have already arrived at the cortex at E16, the crystal placements revealed both thalamocortical and corticofugal axons linking cortex and diencephalon. 5 weeks incubation was used at room temperature to allow full anterograde and retrograde diffusion. Three labelled bundles (containing both corticofugal and thalamocortical axons) were revealed by application of three crystals of carbocyanine dyes (DiA, DiI and DiA) in a parasagittal row along the right hemisphere (A), and two bundles from two crystals placed coronally into the left hemisphere (DiA ventral to DiI) (B). A and B were taken with the same magnification from the different sides of the same horizontal section.

A: Three distinct bundles are clearly visible within the ventral telencephalon (a,b and c to the right) approaching the primitive internal capsule and running towards different thalamic nuclei. The labelled thalamic cells line up along antero-posterior lines forming slabs (a', b' and c' on the left) such that the more anterior cortical crystal placement (a) revealed a more medial thalamic region (a). More posterior cortical crystal placements (b and c) were connected to more lateral slabs (b and c). The antero-posterior sequence of the carbocyanine dye implantations in the cortex (a,b,c) is reflected in a medio-lateral sequence in the thalamic labelling (a',b' and c').

B: In the left hemisphere two crystals were placed along a coronal line (DiA ventral to DiI). The two back-labelled cell groups line up along a continuous anteroposterior slab in the thalamus, where the ventral crystal placement (DiA, yellow-green) labelled more anterior thalamic cells (a'). Ventro-dorsal sequence of the carbocyanine dye implantation along a coronal line on the cortical convexity seems to correspond to an antero-posterior sequence of thalamic labelling along a continuous slab.

Scale bar: 300µm

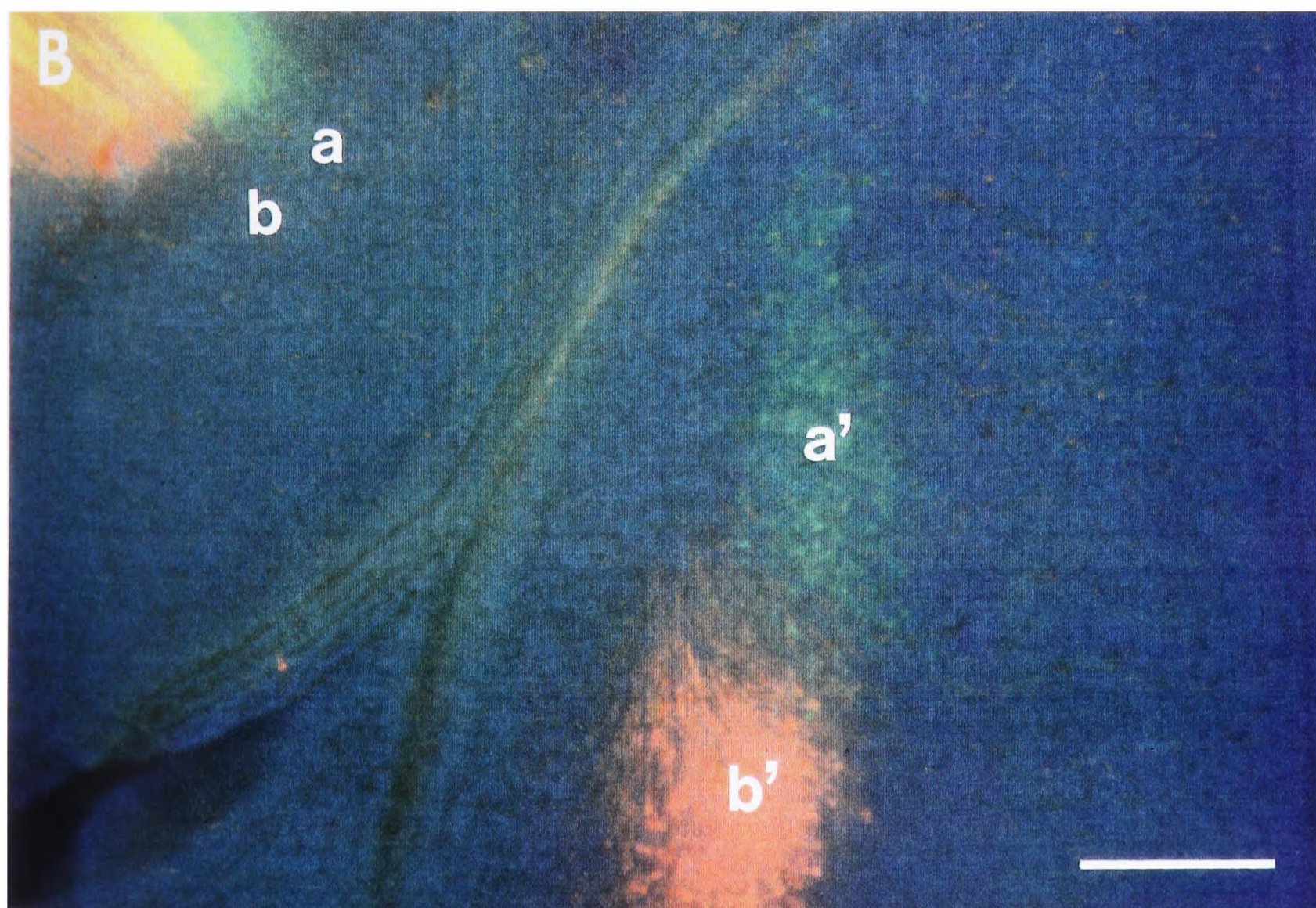
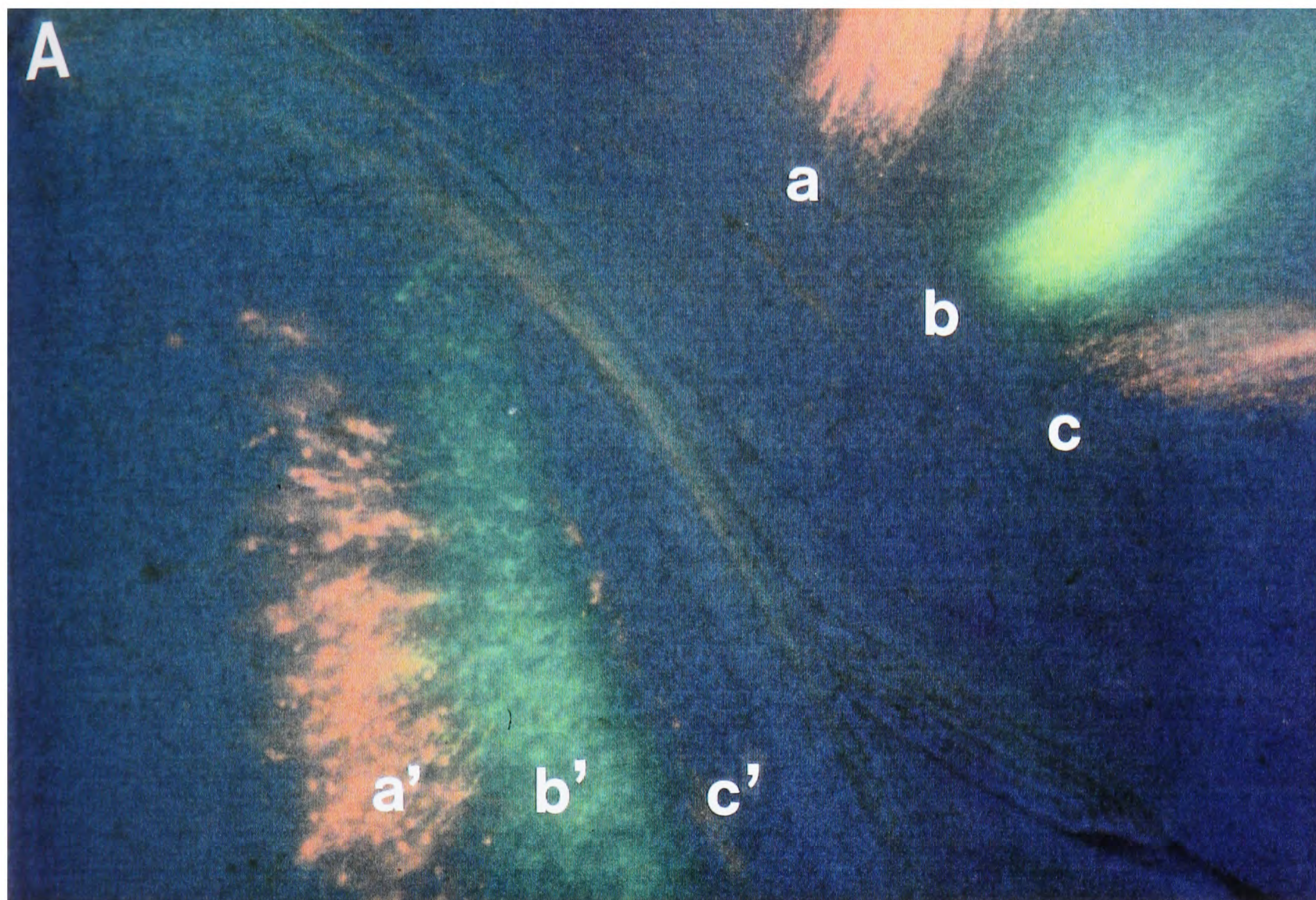


Fig. 3.23

Bundles of axons linking cortex (ctx) and thalamus (thal) at E20 (containing both corticofugal and thalamocortical axons) were revealed by application of three carbocyanine dyes (DiA, DiI and DiAsp) at points in a parasagittal row along the right hemisphere of a fixed E20 rat brain. After 6 weeks for anterograde and retrograde diffusion, 100µm-thick horizontal sections were examined with different filters in the fluorescence microscope to reveal the various dyes. Three distinct bundles were clearly visible passing through the primitive internal capsule without substantial mixing or crossing (B), and running to different thalamic nuclei (A). A and B are adjacent sections, A is superior to B. hip = hippocampus

Scale bar: 500µm

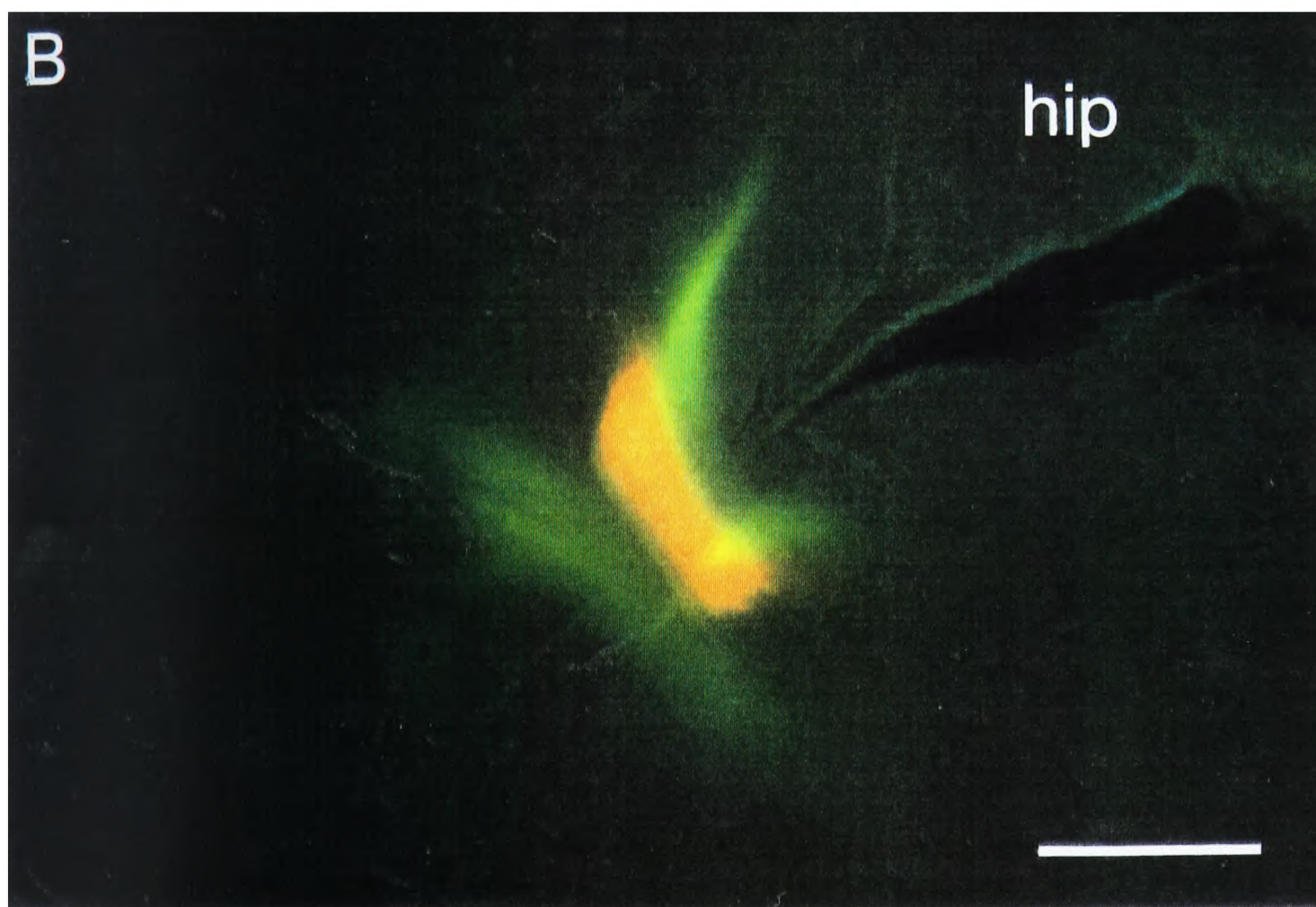
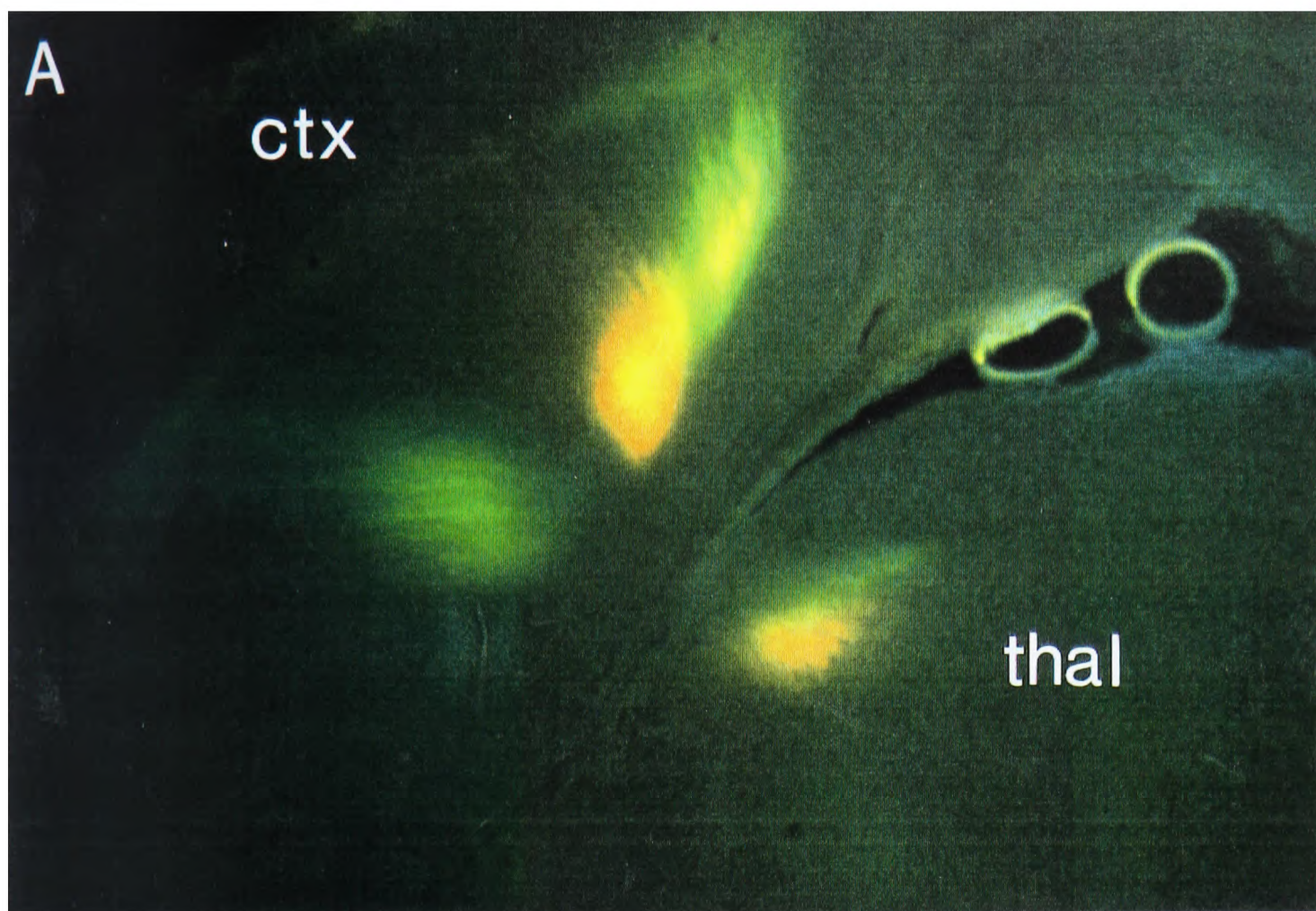


Fig. 3.24

Bundles of axons linking cortex and thalamus at E20 (containing both corticofugal and thalamocortical axons) were revealed by application of five discrete crystals of carbocyanine dye at points in a parasagittal row along the left hemisphere (indicated by arrows on inset diagram of surface view). After 4 weeks for anterograde and retrograde diffusion, 250µm-thick coronal sections were examined with different filters in the fluorescence microscope to reveal the various dyes. Five distinct bundles were clearly visible passing through the primitive internal capsule without obvious mixing or crossing, and running to different thalamic nuclei: the tip of each bundle is marked with an arrow. This coronal section was at the level of termination of the bundles from the two caudal crystal placements, and retrogradely labelled thalamic cells are visible at the end of those bundles. The tracts labelled from the more anterior crystals ended at more ventral and medial levels in the thalamus, labelling thalamic cells in different regions. Bar, 1mm.

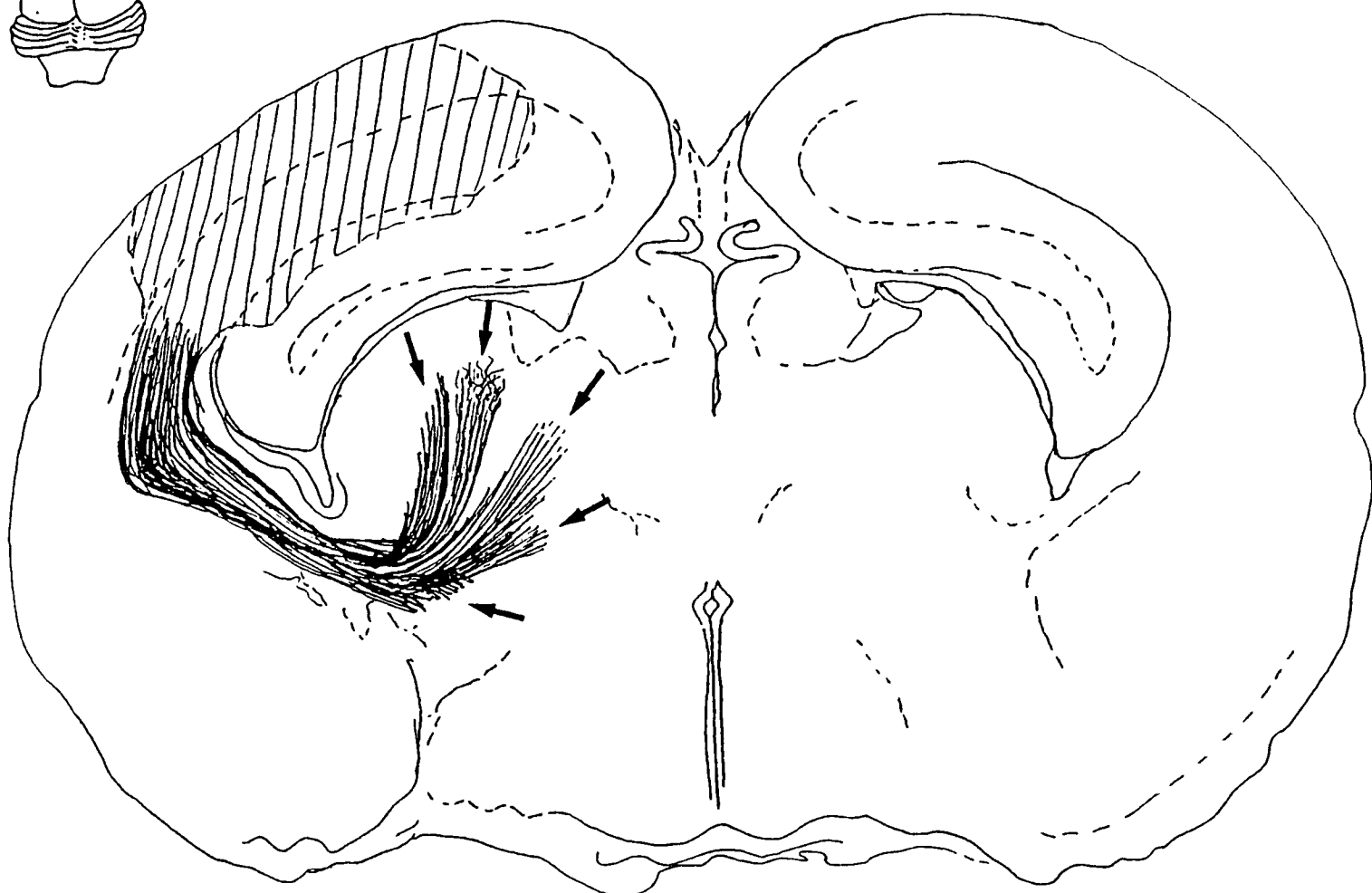
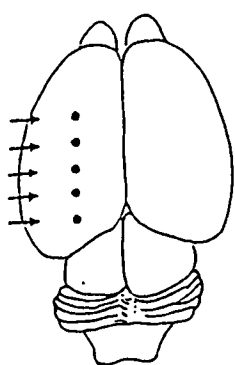


Fig. 3.25

Bundles of axons linking frontal cortex and thalamus at E16 (containing both corticofugal and thalamocortical axons) were revealed by a pair of DiI crystals placed parasagittally from each other in the right hemisphere and coronally in the left hemisphere. After 6 weeks incubation to allow anterograde and retrograde diffusion, 75µm-thick horizontal sections were cut and counterstained with acridine orange. From the series (of 43 sections) each section was imaged in a fluorescent microscope both with rhodamine and fluorescein filters and 3-D datasets were constructed from the images.

A: Viewing the superimposed sections from the side (rostral to the right), the contours of the sections outline the characteristic shape of an E16 brain (with its cortical surface, olfactory bulb, pons etc).

B: Viewing the reconstructions from above, two distinct bundles were noticeable on both sides passing through the primitive internal capsule without mixing, running to different thalamic regions. In the right hemisphere the two groups of back-labelled thalamic cells were situated side by side, forming two parallel slabs, whereas in the left hemisphere only one slab was observed, each half of which was labelled with a different dye: each slab is marked with a white arrow. The reconstructions demonstrate the angle (approximately 45°) of the oblique path of the thalamo-cortical connections linking frontal cortex with thalamus. Scale bar: 1mm

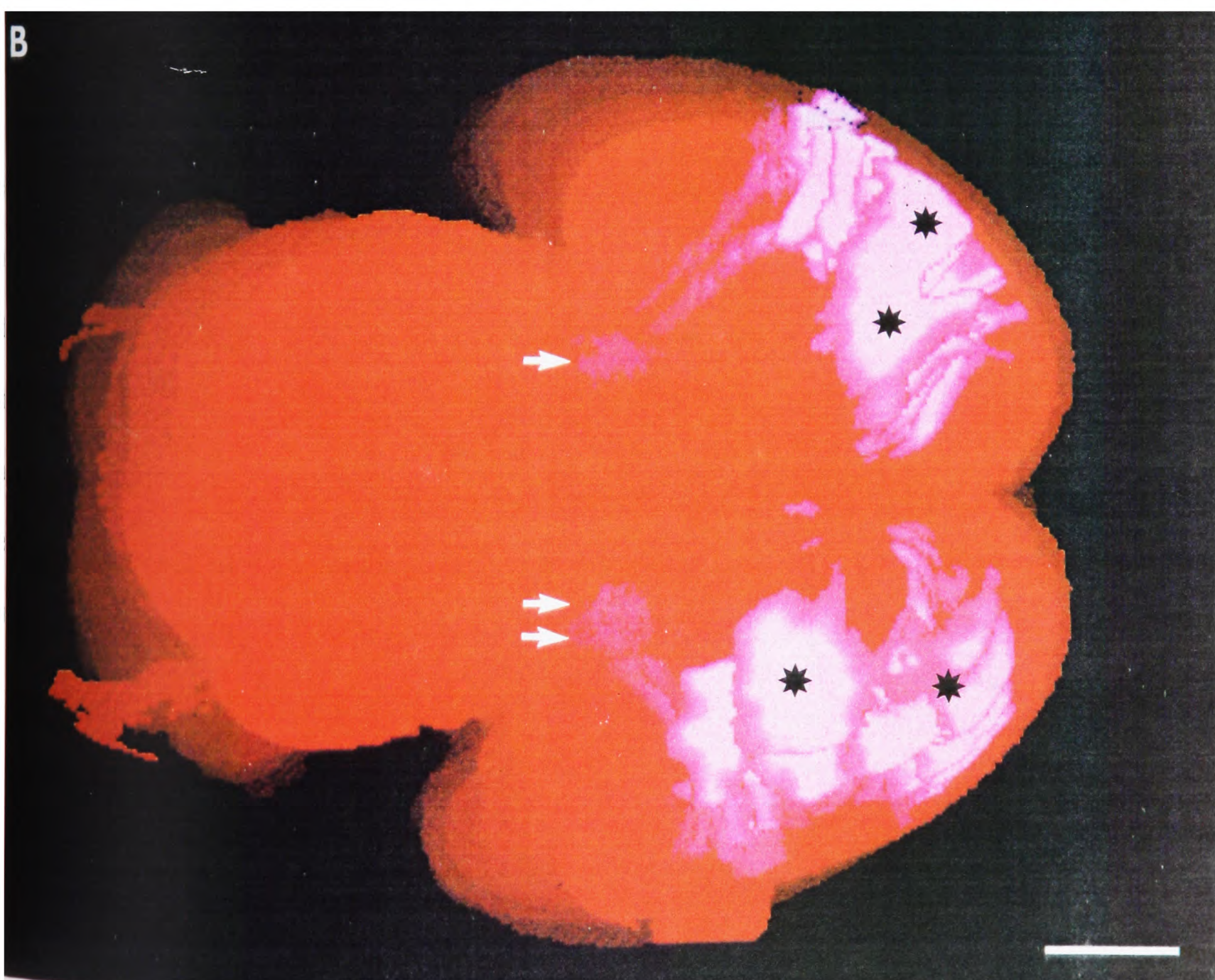
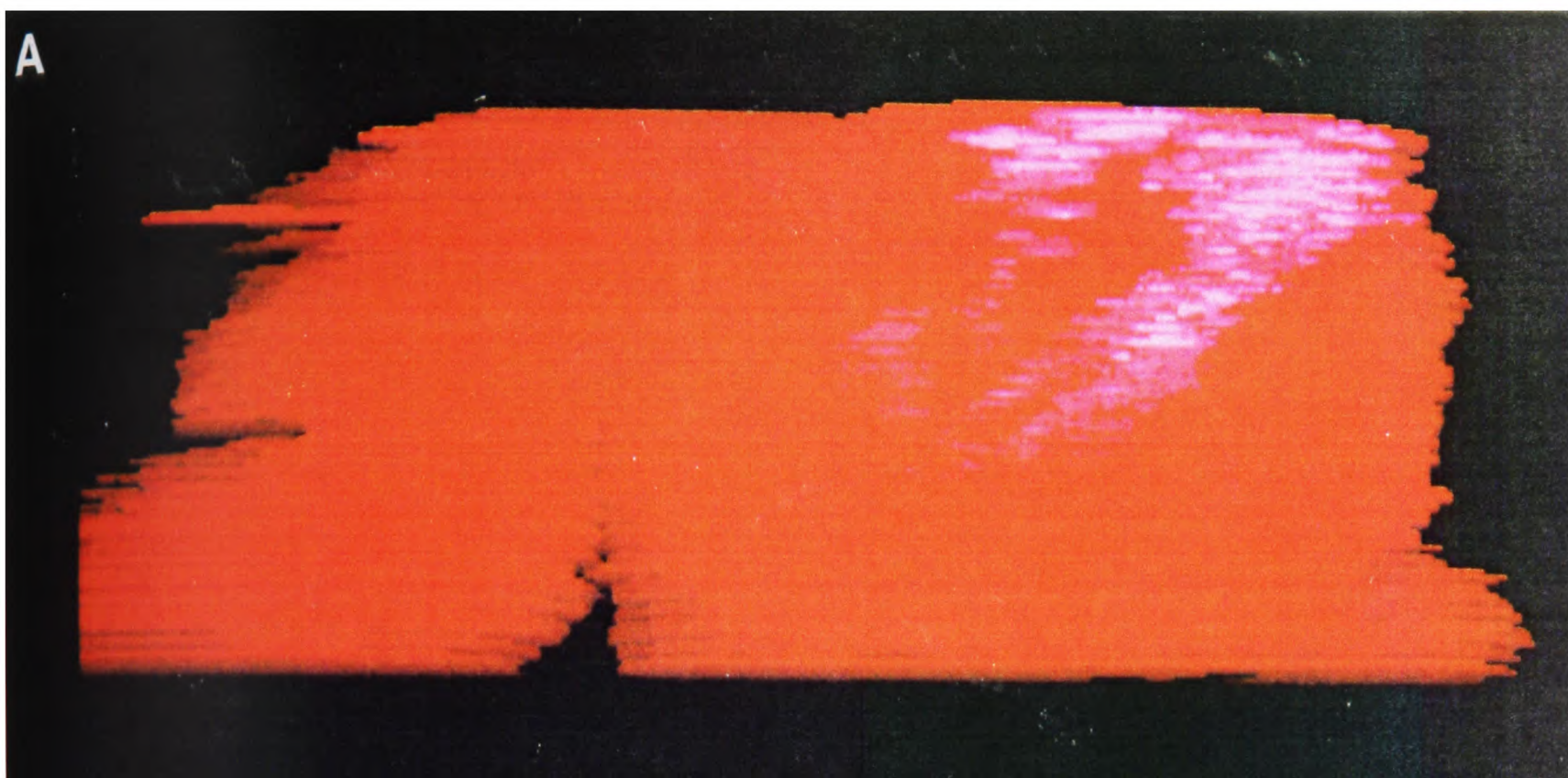


Fig. 3.26

Two different carbocyanine crystals were placed along a parasagittal axis in the convexity of the left cortex of a P2 rat to examine further the matching of the different cortical areas and corticopetal projections. A DiI crystal was placed approximately 3mm caudal to a DiAsp crystal.

Four of the serial (100µm, coronal) sections are shown in antero-posterior sequence (A-D). Each frame shows the ventral diencephalon, just medial to the internal capsule. As each labelled fibre bundle leaves the internal capsule it divides into two. The thalamic component (contains thalamocortical and corticothalamic fibres) turns dorsal (upwards), while the corticotectal, corticopontine and corticospinal fibres run ventral in the cerebral peduncle. The two crystal placements label two distinct cell groups in the thalamus and two distinct fibre bundles in the cerebral peduncle, suggesting fibre ordering in both systems. This dichotomy is noticeable as early as E18/19.

Bar 300µm.

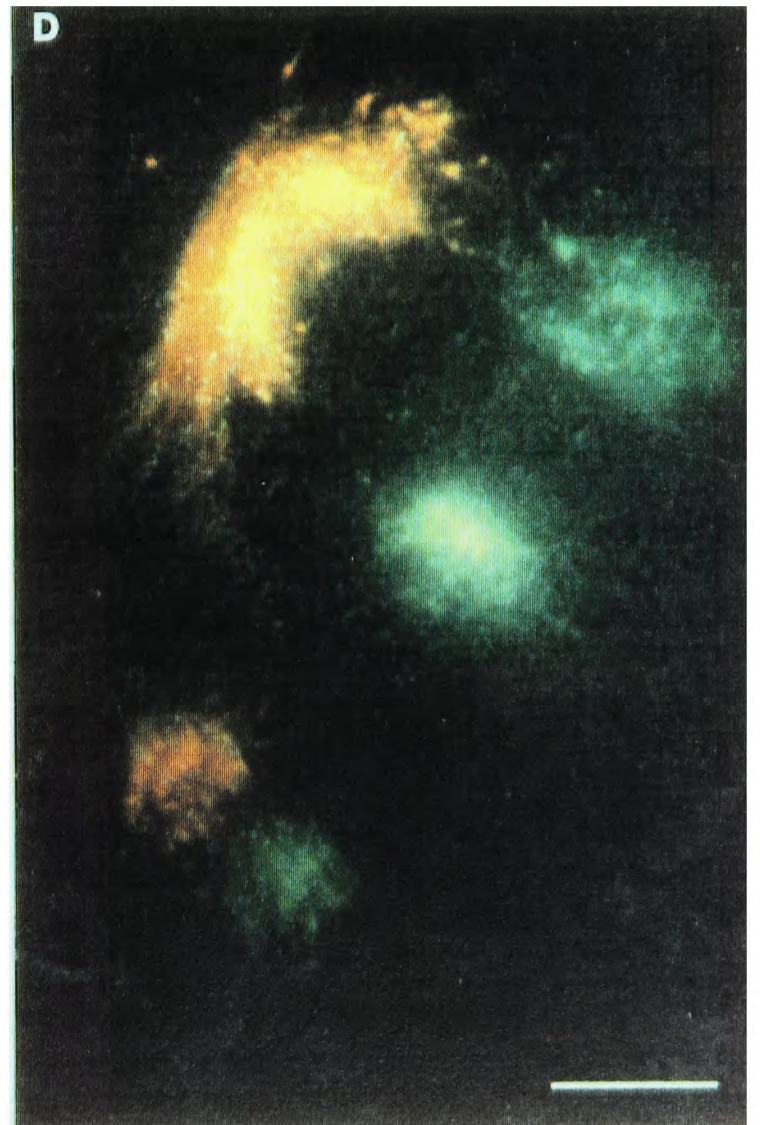
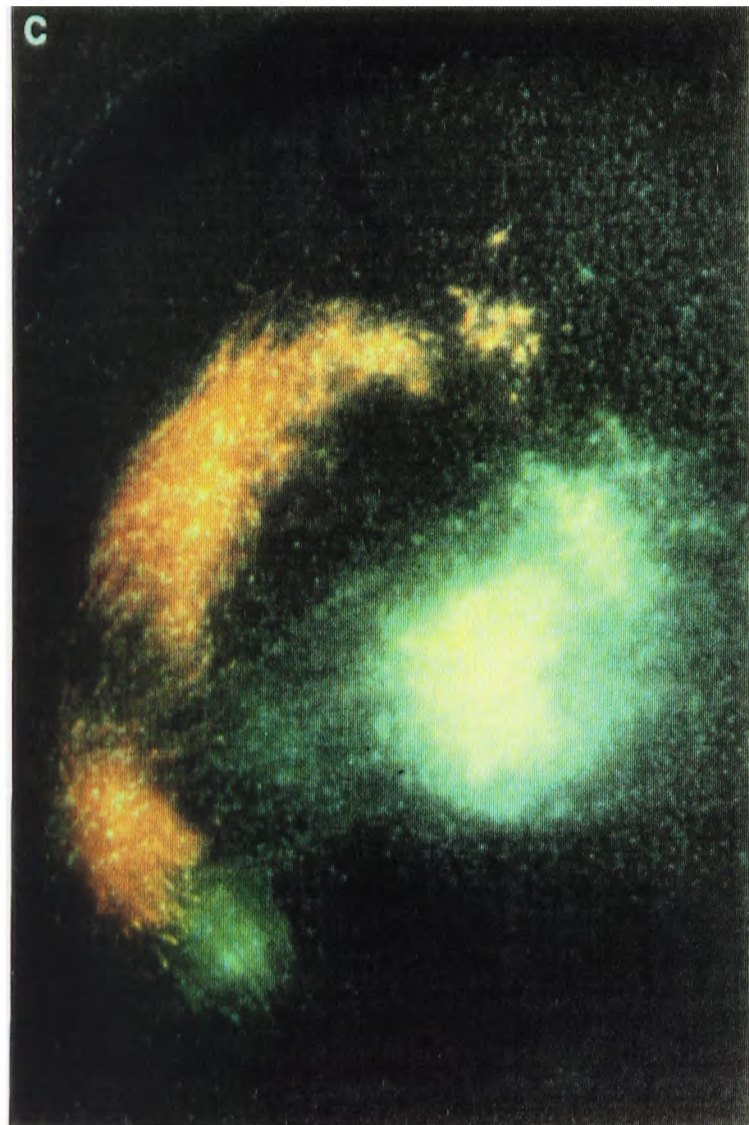
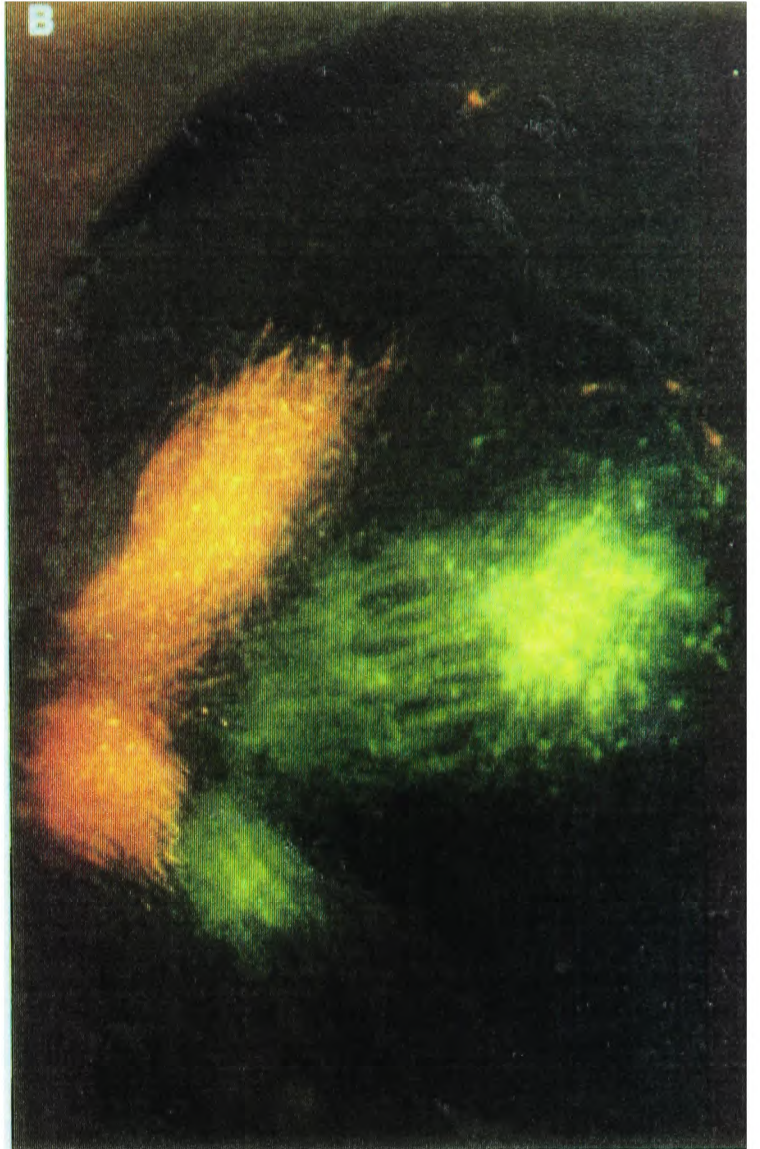
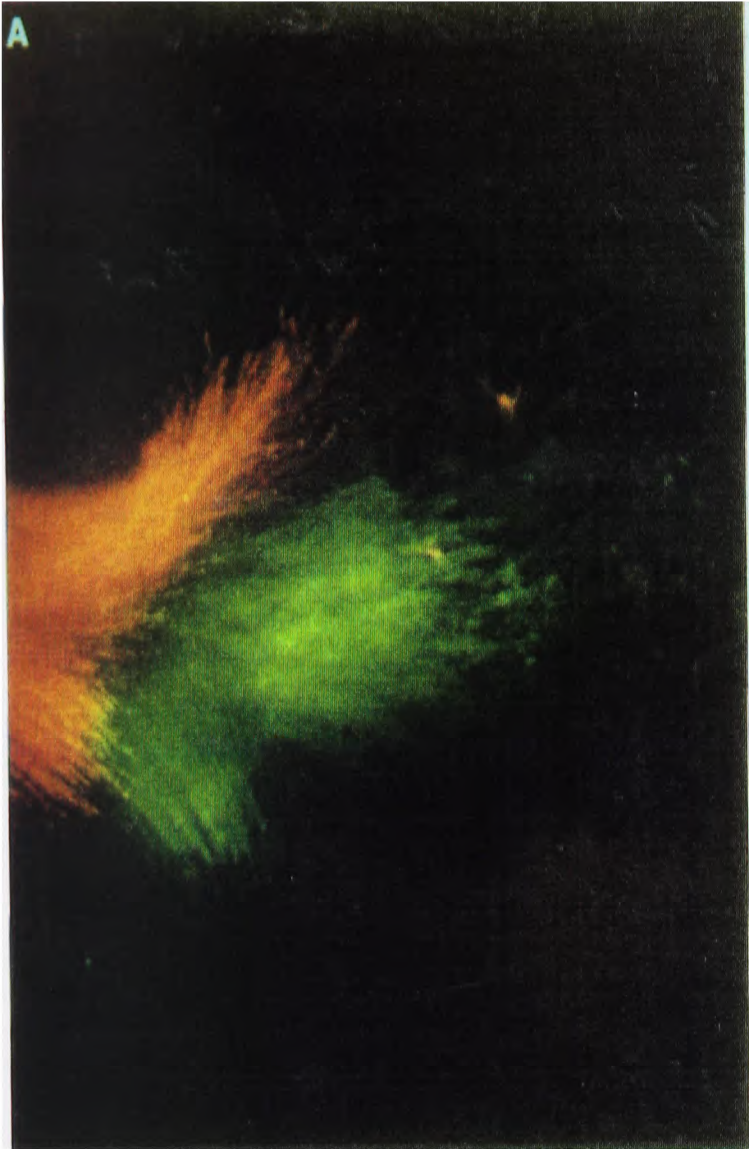


Fig. 3.27

A,B: A small crystal of DiI was inserted into the cortical plate of the somatosensory cortex in P2 brains. After 4 weeks of incubation at room temperature the brains were sectioned coronally, then examined under fluorescent and confocal microscopes.

A: Drawing made with the help of a *camera lucida* under epifluorescent illumination of a 100µm-thick section. The crystal labelled several connections of the small cortical sector (marked with triangles), which was also specifically examined by confocal microscopy (Biorad 600).

B: The crystal labelled (a) the thalamo-cortical connections, (b) the radial glia and the (c) callosal fibres. From B it is apparent that the callosal connections lie under the thalamic ones of the very same area. The lateromedial distortion of the trajectories of the radial glia was apparent (see A).

C: A DiI implantation into the ventrobasal thalamus of a P2 brain revealed the thalamic connections. These fibres apparently lie above the callosal fibres, the position of which is indicated by the two arrows.

D: The same as C, but under ultraviolet illumination. The corpus callosum (CC) and the lateral ventricle (LV) are revealed by the bisbenzimidazole counterstain.

Scale bar: 100µm.

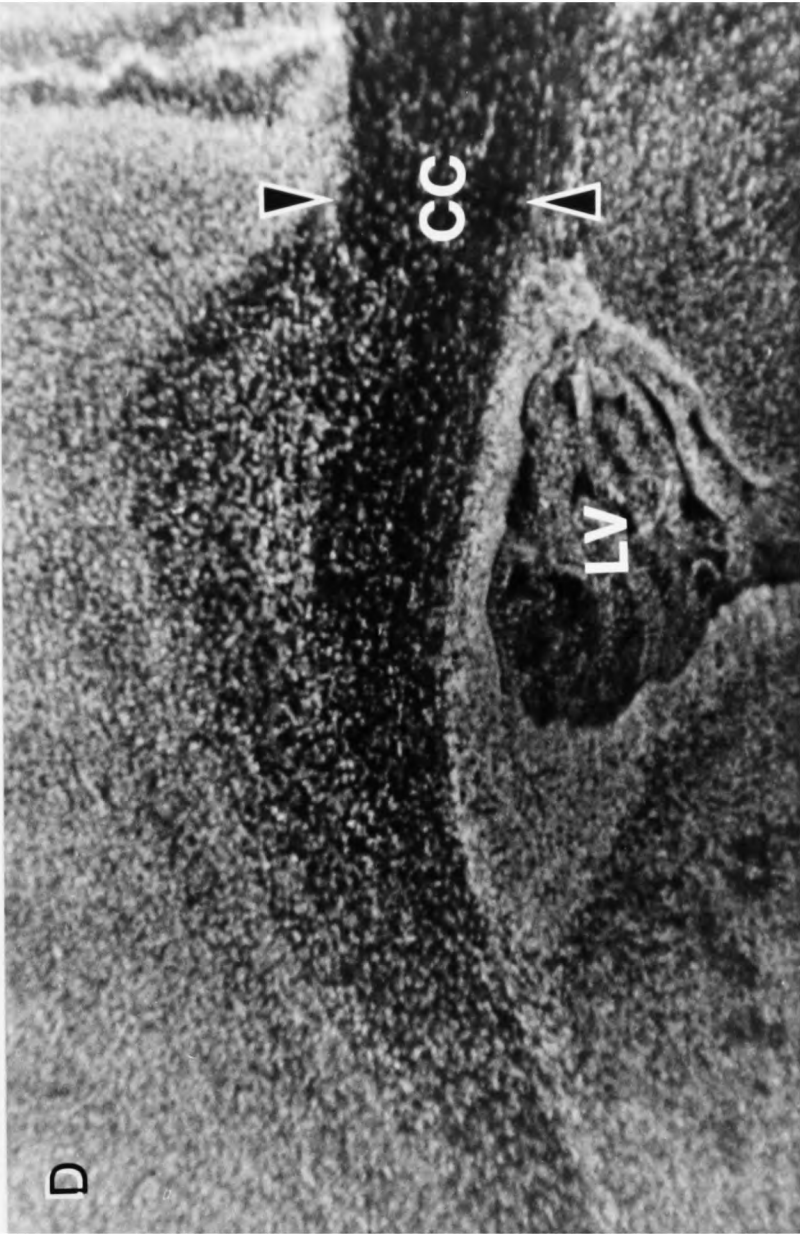
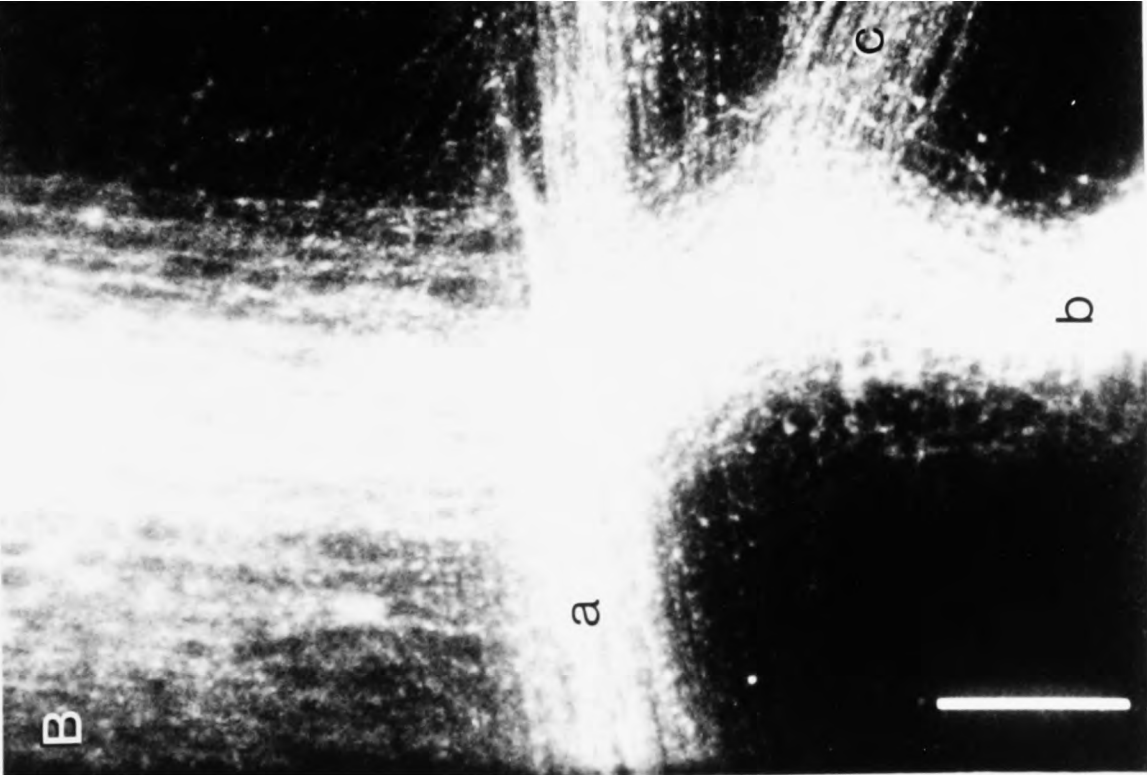


Fig. 3.28

To examine the developing thalamic projections in relation to the descending corticofugal projection, DiI crystals were inserted into the dorsal thalamus at various stages of development.

A: At mid-E14 the thalamic fibres have left the diencephalon and turned lateral to enter the region below the striatum (ST), forming the primitive internal capsule (PIC).

B: A few hours later (early E15) the front of the thalamic fibres (ending in growth cones) are halfway through the PIC, but they do not reach the frontal cortex until E15.5 (Fig. 3.3) and the occipital cortex at E16.

To examine the relationship of the thalamocortical and the earliest corticofugal projections two different carbocyanine dyes were implanted into the dorsal thalamus and the occipital cortex of the same hemisphere. The (75 μ m, coronal) sections were counterstained with bisbenzimidazole, and triple exposures were made.

C: At E14.5 the thalamic fibres (labelled with DiI, appearing red) have passed under the developing striatum, left the internal capsule and turned upwards towards the ventral part of the cortex. The earliest corticofugal fibres (labelled with DiA appearing yellow) run in the intermediate zone, and have yet to turn medially under the developing striatum (as demonstrated in Fig. 3.2). The two fibre systems have not yet reached each other.

D: At a slightly later stage (early E15) the two fibre bundles have reached each other and run in the same region under the striatum. To examine the relationship of the two fibre systems at this stage, the brain was sectioned, perpendicular to the trajectories of the thalamic fibres. Thalamic fibres appear red, corticofugals yellow. This section, which is from the region at the base of the striatum, demonstrates that it is possible to place the two crystals into 'matching' regions, so that the thalamocortical and early cortico-thalamic fibres are intermingled as they run in opposite directions. Many yellow fibres are associated with reds, indicating that the corticofugal and thalamocortical fibres are in intimate contact. It should be emphasized that at this early stage no thalamic fibre have reached the cortex (no yellow back-labelled cells are seen in the thalamus) and the DiI-labelled fibre population was also exclusively thalamo-cortical (no red back-labelled cortical cells were found).

E: Same experiment as described in D but section cut coronally. Since this brain was E16, somewhat older than D, although two different coloured fibres were found in the same fascicles crossing the developing striatum we cannot be sure that each colour represents a pure fibre system.

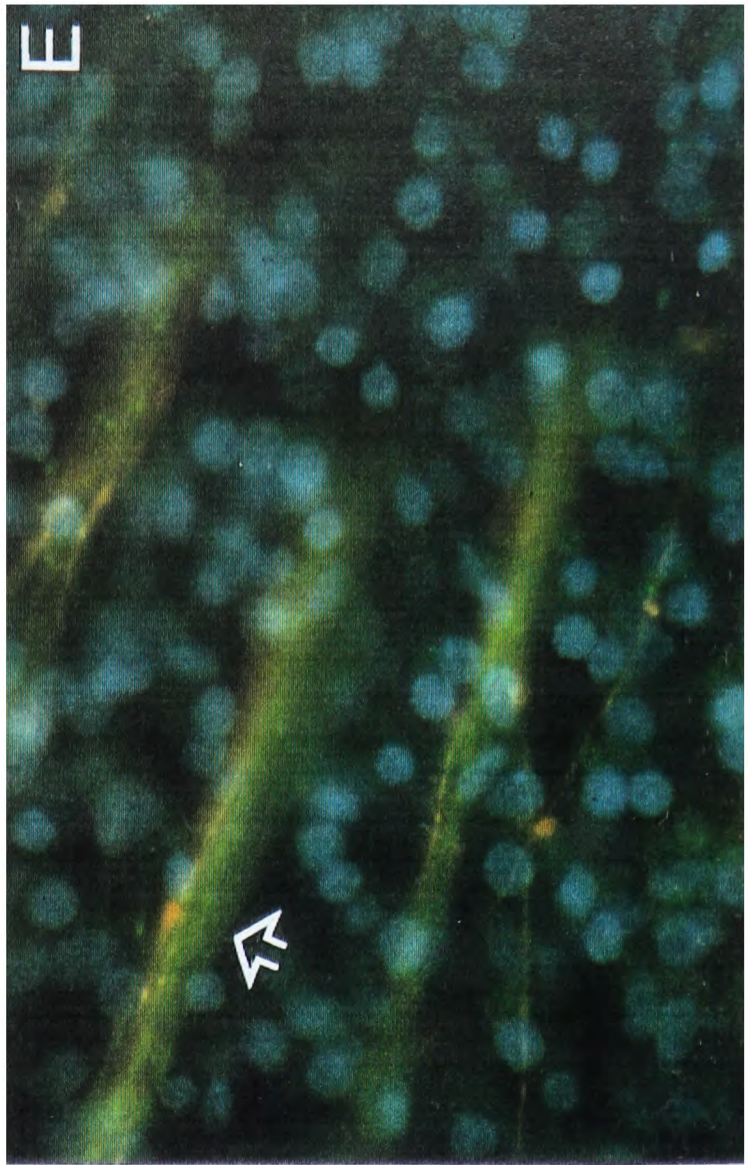
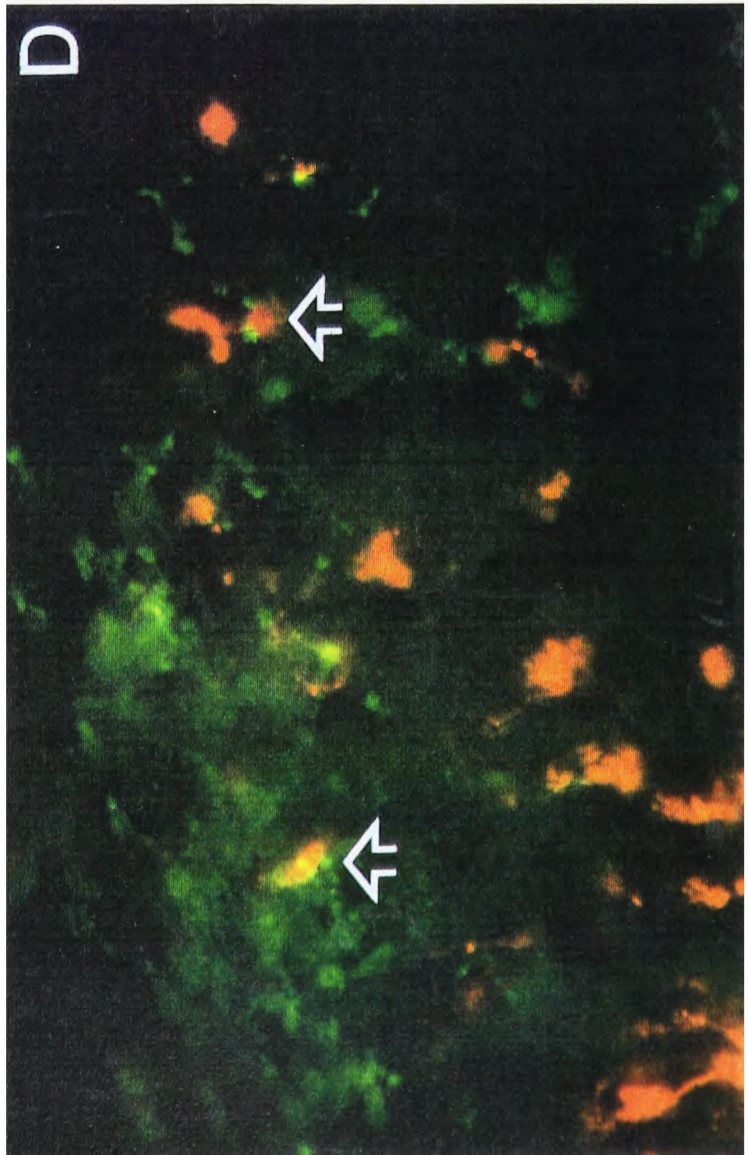
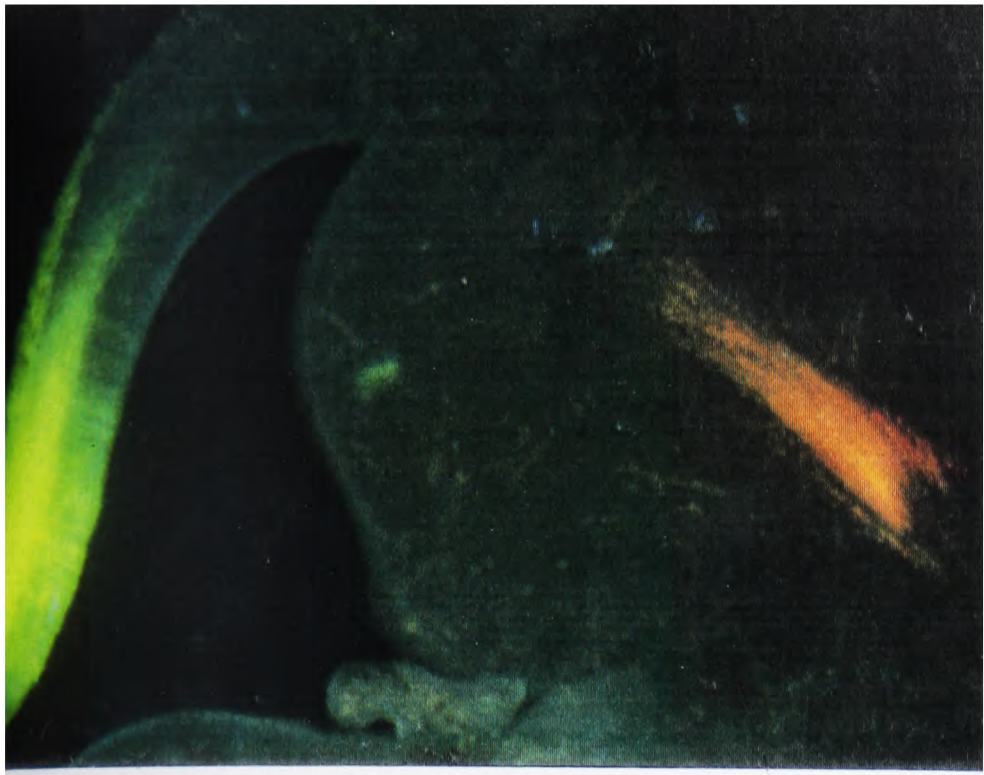
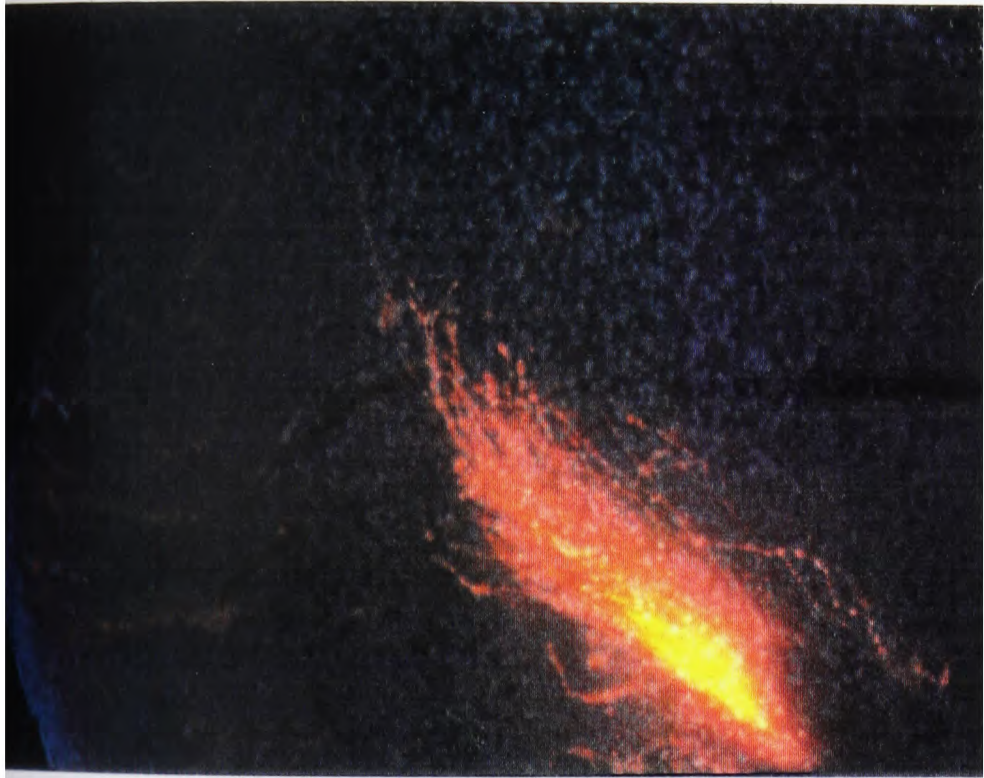
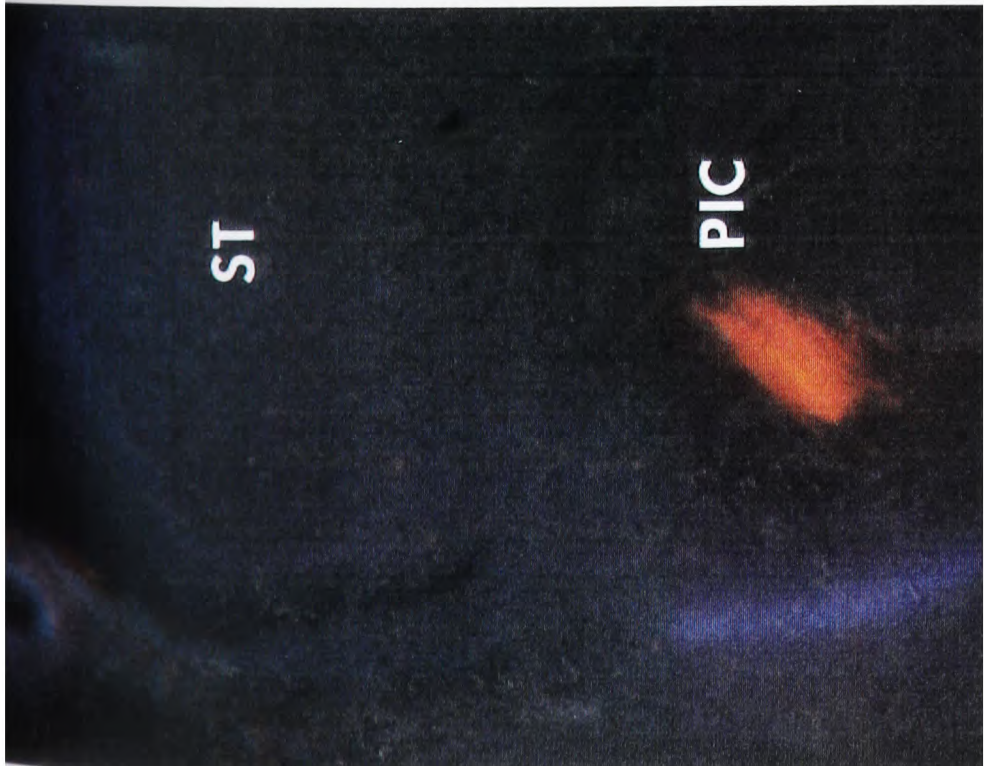


Fig. 3.29

At E15.5 I placed a DiI crystal into the dorsolateral thalamus (into the putative LGN) and a DiO crystal into what I presumed to be the 'matching' region of the occipital cortex (putative area 17), so that both thalamocortical and cortico-thalamic fibres were labelled. At this early stage very few if any thalamic fibres have reached the crystal placement in the cortex (no yellow backlabelled cells were seen in the thalamus) and the DiI-labelled fibre population was also exclusively thalamo-cortical (no red backlabelled cortical cells were found).

A: In this coronal section, many DiA-labelled (orange) fibres are closely associated with DiI-labelled ones (reds) in the same plane of focus, indicating that the corticofugal and thalamocortical fibres are in intimate contact, and are intermingled as they run in opposite directions. Scale bar 100µm.

B: To examine the precise 3-D relationship of the thalamocortical and early corticofugal fibre systems within the bundles, the same section was examined with confocal microscope. Thalamic fibres appear red, corticofugals blue in this confocal microscopic reconstruction. The section, which is from the region near the border between the white matter and the striatum (white arrow at A), demonstrates that the thalamic fibres are closely associated with the corticofugal fibre bundles.

Scale bar: 10µm.

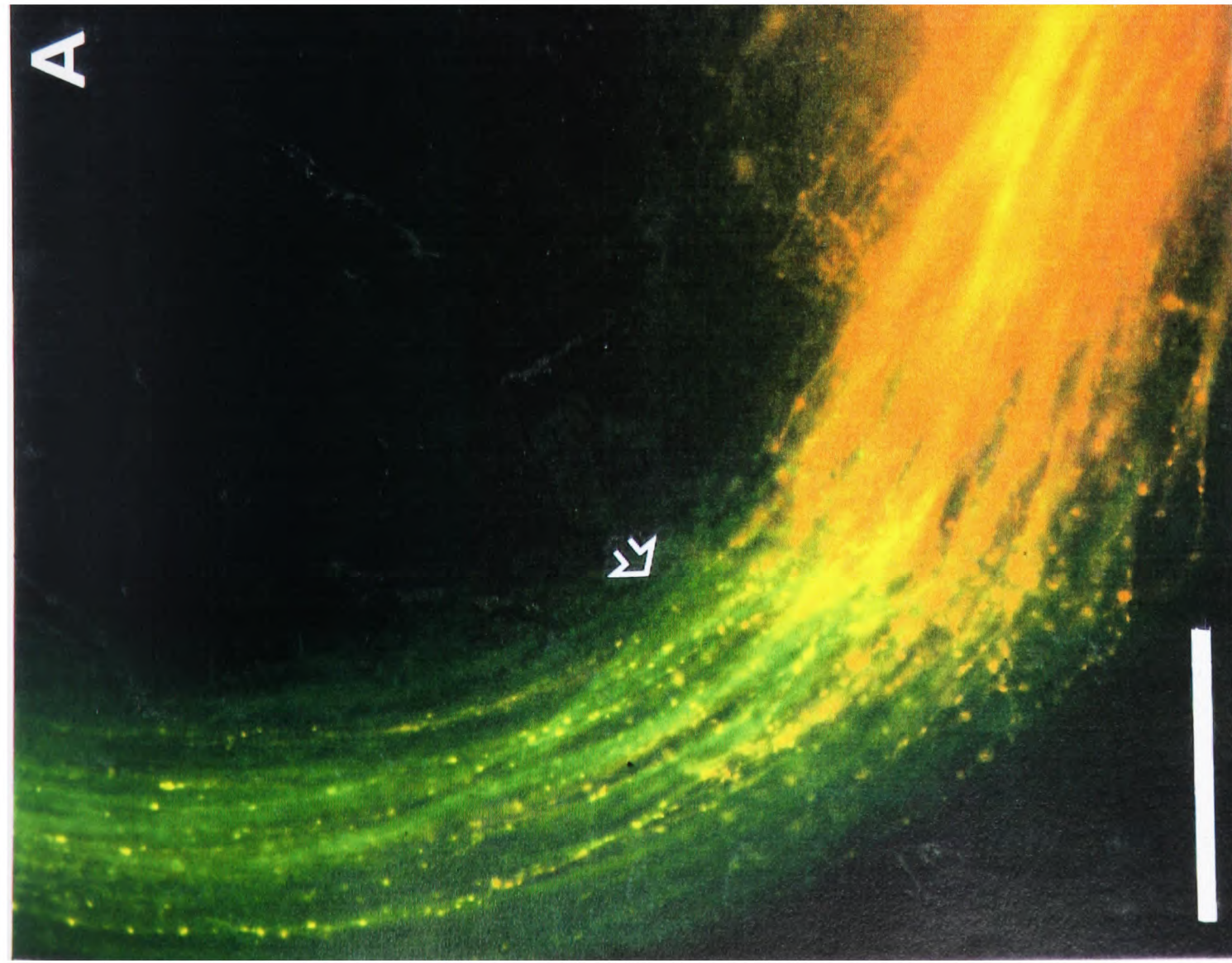


Fig. 3.30

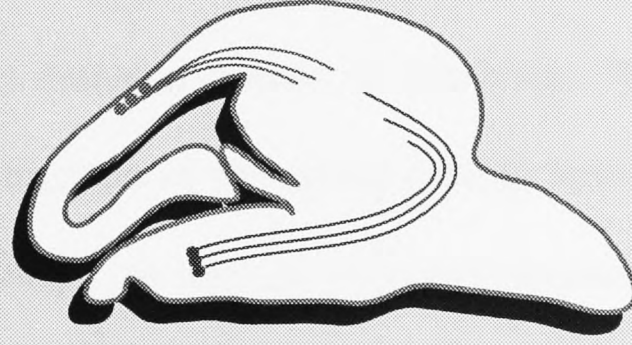
Summary diagram from the early development of the reciprocal thalamocortical and corticofugal projections.

E14: Synchronous outgrowth of the early thalamic and corticofugal fibres.

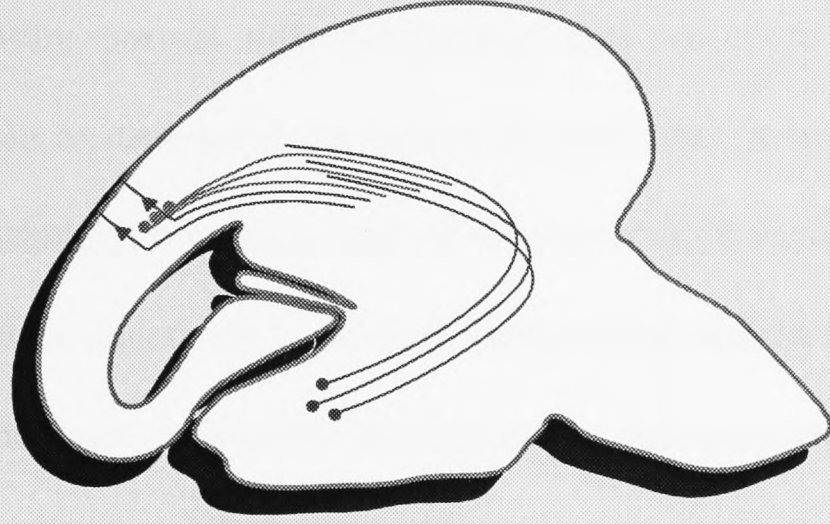
E15.5: The first encounter of the thalamo-cortical and the earliest corticofugal (preplate) projections outside the primitive internal capsule, under the striatum. The fibres from the corresponding regions of the cortex and the thalamus become closely associated with one another.

E19: The thalamic fibres establish a topographic map while 'waiting' in the subplate. The corticospinal (etc.) projections from layer 5 form the cerebral peduncle.

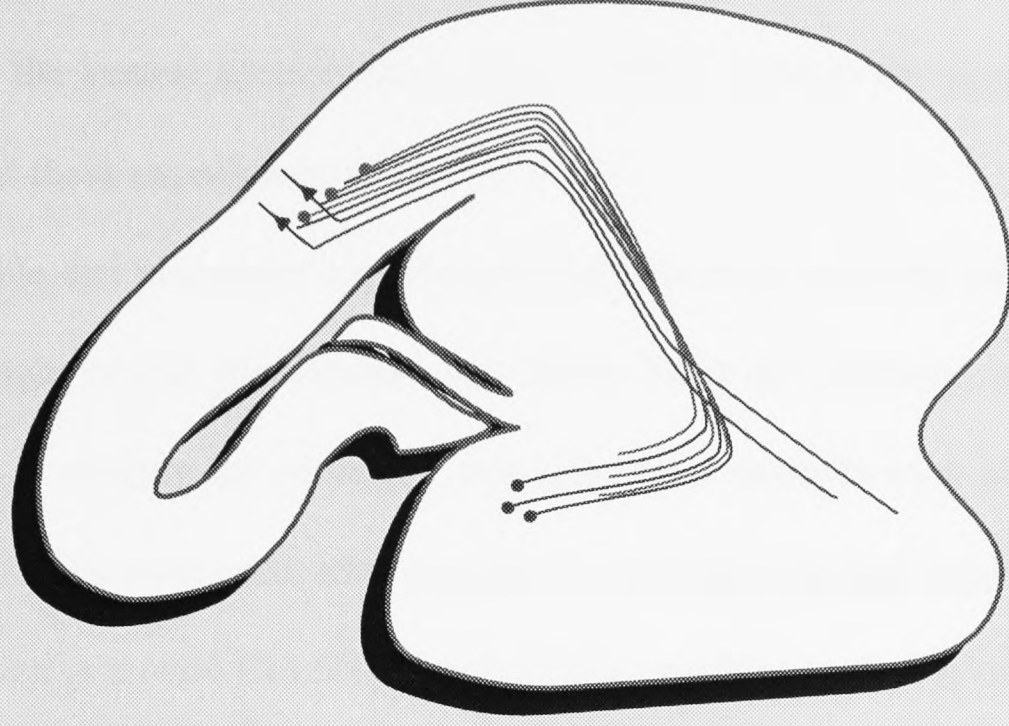
E14



E15.5



E19



definitive. I also found that a number of double placements (two third of all experiments, E15, n=10; E15.5, n=8; E16, n=8) in what I presumed to be corresponding thalamic and cortical sites labelled adjacent rather than intermingled fibre bundles. However, in 6 cases there was definite intermixing of labelled cortical (subplate) and thalamic axons in the ventral telencephalon (Figs. 3.28D; 3.29A,B are representative examples for all of these experiments).

These double dye placement experiments confirmed my previous observations (obtained with single crystal placements) that fibres from the thalamus and cortex appear to emerge at roughly the same age and they grow towards each other in an organized fashion. They meet each other outside the primitive internal capsule, in the ventral telencephalon at around E14.5/15 (for the LGN - occipital cortex system). After the two fibre systems confront each other, just outside the primitive internal capsule, they become mixed. With normal light microscopy and especially with confocal microscopy it is possible to distinguish the course of each fibre array independently (Fig. 3.29). After the thalamic fibres have reached the cortex (E16 for occipital cortex) it is not possible any longer selectively to label the corticofugal and the thalamocortical fibres because of the additional retrograde labelling of the thalamocortical fibres.

DISCUSSION

3.10 Cortical 'maps' and thalamic innervation

Topographic order is a very common feature of the brain. Information becomes represented *spatially* across structures of the central nervous system. This tendency of the brain to be organized in 'maps', as they are commonly known, is particularly clear in the major sensory and motor areas of the cerebral cortex. In visual, auditory and somatosensory cortical areas, the representation of the receptor surface is arranged

across the cortex in an *isomorphic* fashion. This is not to say that cortical maps are topologically simple, linear projections of receptor sheets: rather they are distorted and sometimes fragmented, in various interesting ways. In particular, a general feature of central sensory maps is that densely innervated regions of the receptor surface occupy a larger fraction of the map than regions that are more sparsely innervated. This anisotropy in central representation means that the local scale of the mapping is determined by the density of nerve fibres arriving from that particular region of the periphery. This in turn suggests that the scale of the cortical representation is related to the density and spatial arrangement of afferent fibres bringing information into the cortex from the periphery. In motor areas of the cortex too, regions devoted to the richly innervated musculature of the hands and face (in man) occupy larger regions than those concerned with the muscles of the trunk. This anisotropy obviously reflects the different volumes of neuronal machinery required for the control of richly, as opposed to sparsely innervated, muscle. The development of these maps is gradual and probably based on a sequence of mechanisms.

Since thalamic axons in all mammalian species reach the cortex at a very early stage in cortical development, there is the possibility that those axons not only determine the positions and boundaries of major cortical regions but even impose on sensory and motor areas their initial internal topography. The mapping within a cortical region may be fundamentally dependent on the array of thalamic axons that reach it. The aim of the work described in this Chapter is to understand more of the initial phases of the establishment of thalamocortical connectivity and to elucidate the way in which thalamic axons grow and are guided towards the cortex. If the distribution of thalamic axons does indeed influence the parcellation of the cortex and the formation

of its maps the guidance of thalamic axons is the key to the understanding of early cortical differentiation.

3.11 Chronological sequence of synchronized outgrowth

The appearance of the early fibres in the rat cortex was originally described by Hicks and D'Amato (1968). Using normal silver-stained material, they indicated that axons spread below the surface of the pallium on embryonic day 14 and that cells later move radially between these axons to form the cortical plate. They suspected that these fibres have thalamic origin. My observations (in agreement with those of DeCarlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992) have demonstrated that in the rat, as in the cat, ferret and mouse (Marin-Padilla 1971; McConnell et al. 1989; Molnár and Blakemore, 1992; Chapter 4), there is already outgrowth of axons from cells at the margin of the cerebral wall at a time when true cortical plate cells are only just beginning to be generated in the ventricular and subventricular zones. These early corticofugal projections originate from the first-generated cells lying in the preplate, or primordial plexiform zone. Preplate cells become separated by the arrival of cells of the cortical plate, to form the marginal zone and the subplate, which is displaced downwards. Both marginal zone and subplate cells of the occipital cortex can be back-labelled from the internal capsule as early as late E14, demonstrating that their projection extends to the internal capsule prior to the *arrival* of thalamocortical fibres at the cortex.

Corresponding thalamocortical and early corticofugal subplate projections start developing virtually synchronously in the two structures. The entire process is not however simultaneous everywhere across the cortex and the thalamus. It is well known that the generation, migration and layering of cortical neurons in the rostrolateral

portion, of the cerebral hemispheres of the rat is about 1.5-2 days ahead of that at the caudomedial portion throughout the latter half of gestation and the first postnatal week (Berry and Rogers, 1965; Lund and Mustari, 1977). At any particular coronal level, the contrast between the maturation of the ventral and the dorsal areas of the cortex is very striking, the ventral cortex appearing much thicker than the dorsal, and the pioneering fibres of its subplate layer reach the internal capsule earlier (as revealed by the anterograde and retrograde tracing).

3.12 Do subplate axons enter the thalamus?

Small dye crystal placements in the cortex, from E16 onwards, appear to label a continuous bundle of axons running down from cortex to thalamus. However, we cannot tell from such experiments whether the whole length of the bundle contains subplate as well as thalamic axons. Prior to the completion of the growth of axons into the thalamus from true layer 6 corticothalamic neurons (E19-see below), the diencephalic terminal portion of the bundle labelled from the cortex could consist entirely of thalamic fibres. Indeed, while I was studying the pattern of growth of thalamic axons in the rat, by using small dye placements within the thalamus itself, mainly in the region of the LGN between E14 and E18 I hardly ever observed any back-labelled cells in the cortex. Even when I increased the incubation periods by several weeks beyond those usually employed (Table 3.2) only the radial glia became more intensely labelled, but there were still very few labelled cortical cells even in the subplate and marginal zone. Carbocyanine dyes are supposed to label pathways retrogradely as well as anterogradely, and labelled cell bodies were indeed seen in both cortex and thalamus after crystal placement, after E14, in the internal capsule (Fig. 3.10). It thus seems that early corticofugal projections from subplate axons and marginal zone (at least for the

occipital cortex) actually stop short of thalamic relay nuclei.

Mitrofanis and Baker (1993) reported that cells of the thalamic reticular 'nucleus' send the first axons to the dorsal thalamus and suggested that these early projections may guide or escort the growing thalamocortical axons out of, and corticothalamic axons into, the thalamus. I obtained very similar results (both in rat and mice), but in addition to the cells of the thalamic reticular nucleus back-labelled from the thalamus I also found some back-labelled cells within the internal capsule at E14/14.5 (Fig. 3.1). Mitrofanis (1992) called these cells perireticular cells. My results indicate that the axons of some perireticular cells do reach the dorsal thalamus at E14, but later the outgrowing thalamic fibres overshadow this area and the connectivity is more difficult to resolve. At E14, crystal placements into the region of the reticular 'nucleus' itself reveal numerous cells within the neighbouring segment of the internal capsule. Adams N. et al., (1993) reported that after E16, some of the perireticular cells project to the cortex. In postnatal rats it is well documented that perireticular cells project to the thalamus (Mitrofanis and Baker, 1993; Mitrofanis and Guillery, 1993). Their sustained depolarization after thalamic stimulation can be observed with optical recording, studied from E19 to P7 (M. Crair, K. Toyama and Z. Molnár, unpublished observation).

In the developing cat and the ferret, Antonini and Shatz (1990) reported that subplate cells can be back-labelled by injecting tracers in the thalamus (as well as in the contralateral hemisphere and even the superior colliculus). However, in the opossum the first cells to be backlabelled from the thalamus are in layer 6, not in the subplate (Sheng et al., 1991). For the rat, corticothalamic connections from layer 6 can be traced to the ventral thalamic nuclei by E19, according to Schreyer and Jones, (1982) and

Jones et al. (1982). De Carlos and O'Leary (1992) have recently reported that, although tracer injections into the superior colliculus alone labelled only cells of layer 5 in the rat (and only after P1.5), they were able to back-label large numbers of subplate cells (as well as true layer 6 neurons) in the newborn rat with large injections of fast blue involving the thalamus. They concluded that 'subplate axons do not send axons into the superior colliculus, a layer 5 target, but that many do project to the thalamus, a layer 6 target.'

My own results and those of Miller et al. (1993) suggest an even more limited distribution of subplate axons in the rat. With very small crystal placements *within* the thalamus itself, I saw very few subplate cells back-labelled at any age. Such discrete thalamic placements produce clear anterograde labelling of thalamic axons all the way to the cortex (after about E16 for the LGN) but do not label substantial numbers of subplate cells nor even of true layer 6 pyramidal cells before about E20. Slightly larger crystals, intruding into the ventrolateral diencephalon, or small crystals placed directly in the region of the primitive internal capsule, produce considerable back-labelling of subplate cell bodies (as well as of layer 6 and 5 cells, depending on age and crystal position).

These results suggest that very few if any subplate axons actually enter the major thalamic nuclei, and I suspect that the majority of the subplate array ends its growth somewhere in the ventrolateral diencephalon. It remains to be seen whether subplate axons actually terminate on some target structure. Miller et al. (1993) made very similar observations at corresponding ages in the hamster. No subplate cells were back-labelled from thalamic carbocyanine crystal placements, and the true layer 6 cells became labelled relatively late (P6 in the hamster), but then quite suddenly and in large

numbers.

At E16 in the rat, after implanting carbocyanine dyes into the thalamic reticular nucleus (only a few hundred microns away from the dorsal thalamus), I could observe backlabelled layer 6 and subplate cells in the cortex, whereas a similar dye implantation into the dorsal thalamus (of the contralateral hemisphere of the same animal, therefore under the same conditions), did not label any cortical cells (Fig. 3.15). There are several possible explanations for this. The corticothalamic fibres might have reached the thalamus but not arborized extensively and therefore they did not take up the dye sufficiently to appear labelled. Crandall and Caviness (1984) injected HRP into the embryonic mouse cortex at E16 and commented on the very sparse density of corticofugal fibres within the thalamus. It is also conceivable that the corticofugal fibres 'wait' outside the thalamus, perhaps in the thalamic reticular nucleus and do not enter the dorsal thalamus. It would be essential to use purely anterograde tracing from the cortex to resolve this issue.

Shatz and Rakic (1981) used ^3H -proline (a purely anterograde tracer) to investigate the development of corticothalamic connections in the fetal rhesus monkey. They noted that the orthogradely transported label appears in the surroundings of the LGN weeks before it is detected within the LGN itself. In the opossum, where the thalamus is more accessible for direct tracer injections *in vivo*, HRP injection in the thalamus revealed that thalamic fibres reach the cortex long before the first cortical cells, in layer 6 and not in the subplate, become back-labelled (Sheng et al., 1991).

Mitrofanis and Guillery (1993) drew attention to the numerous similarities (early generation and maturity, antigenic expression and differential cell death) between the perireticular, reticular and subplate cells. It is plausible that these earliest generated cells

have a related evolutionary origin. These early generated cells may all play a part in the guidance of thalamocortical projections. The evolutionary origin of these cells is yet to be determined. They might form the bridge between the reptilian and mammalian organization of the brain. I shall speculate further about the logic of these early mechanisms and offer a possible evolutionary account in Chapter 7.

3.13 The autonomous nature of thalamocortical organization

Both anterograde and retrograde tracing experiments demonstrated that by E16 thalamic axons have arrived under the entire cortex. Back-labelling from various points on the cortex at this early stage revealed the spatially organized deployment of these thalamic fibres. The discrete fibre bundles, containing both corticofugal and thalamofugal axons, do not cross each other and they maintain their neighbourhood relationships along the entire path between thalamus and cortex. Unfortunately it is rather difficult to draw firm conclusions about the organization of individual fibres within each bundle, even by examining fine 3-D reconstructions of confocal microscopic sections. We shall need to develop new techniques (e.g. making even smaller multiple injection sites) to resolve individual fibres along the whole pathway. Nevertheless it is clear that thalamic fibres arrive in the subplate layer and (at least for the occipital cortex) wait under the cortical plate in a topographically organized fashion.

All this topography is accomplished as early as E16, before the arrival of the peripheral afferent input to the thalamus. For example the first axons of the optic tract reach the level of the LGN at around E16, but they seem to be destined for the superior colliculus (Lund and Bunt, 1976). There is general agreement that optic tract fibres do not invade the LGN substantially until about birth in the rat (Karlsson, 1966). This is well after the construction of the initial thalamocortical links. This implies that

development of the early pattern of connection between thalamus and cortex is not dependent on afferent input (from sensory pathways or other subcortical structures).

The idea that the periphery does not orchestrate the **initial** layout of thalamocortical projections is not new. Coggeshall (1964) noted that "obvious fascicles of fibres can be seen joining the dorsal thalamus to the telencephalon via the internal capsule" by at least the 15th day of gestation in the rat. By contrast, ascending afferent pathways could not be traced into the dorsal thalamus until rather later. Thus, he felt he could correlate the onset of nuclear differentiation in the dorsal thalamus with the arrival of subcortical afferents: *"This transforms the embryonic dorsal thalamus, a unit that is forming connections with the telencephalon alone, into the adult dorsal thalamus, a mosaic of units some of which connect the telencephalon synaptically with lower centres ... It seems likely that optic, tactile, and other incoming pathways plug into circuits that are, to some extent, already formed."* Before the advent of fluorescent carbocyanine tracers these ideas of Coggeshall were rejected, since the general opinion was that subcortical afferents arrive in the thalamus before connections are made to the cortex (Jones, 1985). My results support Coggeshall's observations and hypothesis.

The notion of the autonomy of thalamocortical development is supported by the observation that anophthalmic mice (Cullen and Kaiserman-Abramof, 1976; Godement et al., 1979; Kaiserman-Abramof, 1983), as well as bilaterally enucleated hamsters (Rhodes and Fish, 1983) and ferrets (Guillery et al., 1985), develop geniculocortical and corticogeniculate interconnections with an essentially normal topography (within the precision of tracing methods). If a monkey is binocularly enucleated at an early fetal stage, before full innervation of the LGN by the optic tract, about two-thirds of LGN cells degenerate and the total size of area 17 is proportionately reduced (Rakic, 1988).

This experiment is commonly cited as evidence that cortical areas develop without thalamic afferents. Unfortunately the study documents only the adult stage. It remains to be established whether the cell loss in the LGN occurs so rapidly that the whole topography of the *initial* outgrowth of thalamocortical axons is affected (unlikely, but cannot be excluded at present), or whether the projection forms normally and is subsequently reduced in areal extent as geniculate cells die. Without this no inferences can be made of the causal relationships.

Guillery et al. (1985) suggested that the "mapped projection that grows from the retina to the lateral geniculate nucleus must in normal development conform with the geniculocortical map in order to produce the normal pattern of retinogeniculocortical projections of the adult." Guillery (1986) also considered the possibility that early in development, the geniculocortical and retinogeniculate systems are initially 'two independent processes, each programmed to produce its portion of the final visual pathway'. My results strongly support this suggestion. The establishment of the early topographically organized geniculocortical projection, around the 16th day of gestation in the rat, cannot be dependent on the retinal input because the optic tract has not grown into the LGN at this time. This hypothesis of course does not exclude the possibility of a later modification. For instance in albino mutants and in monocularly enucleated animals, abnormal retinogeniculate input can cause secondary changes in the geniculate and in the optic radiation (for reviews see Guillery, 1986; Trevelyan and Thompson, 1992). It would be interesting to follow the initial phases of development in such animals. At present we know very little about the nature and mechanisms of these secondary changes. At what stage and how will the periphery influence thalamocortical topography?

Evidence for the early gross specification of thalamocortical topography and for the autonomy of early thalamocortical development also comes from the results of developmental manipulations that induce sensory afferents of one modality to project to central targets of a different modality (reviewed by Sur et al., 1990). In spite of such manipulations (including cortical lesions), there is very little change in the distribution of thalamocortical pathways, suggesting that these have already been specified at the time of these manipulations. After a lesion of the visual cortex, superior colliculus and LGN of a newborn ferret (corresponding in development to an E16 rat fetus) fibres of the optic tract terminate in the '*auditory*' thalamus and visual information is relayed to the region of temporal cortex (the original target of the 'auditory' thalamus) that would normally become primary auditory cortex. Similarly, in the naturally blind mole rat the auditory afferents project to the LGN and, through the otherwise normal geniculocortical projection, this auditory input is relayed to what would be the visual cortex (Heil et al 1991). In each case, the matching of a given thalamic area to its original cortical region is unchanged, indicating that early thalamocortical development is independent of the periphery, and is based on some kind of innate automatism.

3.14 The origin of fibre order: chronotopy and morphogenetics

Based on clinical observations it was widely believed that in most nerve tracts the fibres are, at least to some extent, ordered with respect to both their origin and their target: the location of any fibre in the tract correlates with the relative location of its cell of origin and the location of its target (see Polyak, 1957; Horder and Martin, 1977; Martin and Perry, 1983). One of the best known example of fibre ordering from the clinical medicine is the ascending (funiculus gracilis and funiculus cuneatus) pathways in the dorsal columns of the spinal cord (see e.g. Szentágothai, 1977). Because of its

importance in the differential diagnosis of spinal injury, every medical student has to know that the ascending fibres are grouped according to their origin and that their neighbourhood relationships are maintained along their ascent. Fibres from the cervical regions run lateral to those from the thoracic segments, and the thoracics run lateral to those from the lumbar region, etc. The retinotopic fibre ordering within the visual pathway and its diagnostic importance are emphasized in almost all textbooks of neurology and neurosurgery (e.g. see Lindsay, Bone and Callander, 1986). Lesion, compression or transection of the visual pathway at various locations results in various characteristic visual field defects.

How is such fibre order achieved?

Horder & Martin (1978) and Bayer & Altman (1987) pointed out that many of the major anatomical interconnections of the telencephalon can be related to the order of neurogenesis and to neurogenetic gradients of either the source neurons (entorhinal cortex, olfactory bulb mitral cells), receiving neurons (lateral septal nucleus, cortical nuclei of amygdala, anterior olfactory nucleus, primary olfactory cortex) or both (magnocellular basal nuclei, striatum) (Bayer 1985; 1986; 1980; Bayer and Altman 1987; 1991). The prerequisite for the translation of a temporal gradient into topographically ordered connectivity is that the outgoing fibres are organized and they preserve their position as they reach their targets and occupy a position on a first-come-first-served basis. This however has **not** been established for most of these systems. This possibility, that fibre ordering is dependent on the relative timing of their establishment, i.e. the translation of temporal events into spatial distributions, is termed *chronotopy*. Let us return to our original example in the spinal cord (ascending fibres of the dorsal columns) for illustration. The development of the spinal cord follows a

rostral-caudal gradient. The first established cervical fibres run deeper in the funiculus posterior (funiculus cuneatus and gracilis) than the later developing thoracic and lumbar fibres. The fibres originating from the sacral segment take the most superficial position. This arrangement is called Kahler's Rule (see Szentágothai, 1977). The tractus spinothalamicus has a very similar arrangement. This type of fibre arrangement reflects the timing of its establishment, i.e. it has chronotopic fibre ordering.

Based on these early observations Horder and Martin (1977) proposed that during development spatiotemporal patterns of outgrowth and ordered axon elongation can provide topography within the retino-tectal system. It was indeed demonstrated that the retinofugal fibres are arranged according to their time of outgrowth within the initial part of the optic nerve (Scholes, 1979; Walsh and Guillery, 1985; Colello and Guillery, 1992; Guillery and Walsh, 1987), but the degree of retinotopic order found behind the eye deteriorates along the course of the optic nerve and chronological ordering of axons is no longer obvious in the distal part of the optic nerve (Horton et al., 1979; Naito, 1986; 1989). Individual fibres become considerably rearranged and the retinofugal pathway no longer replicates the sensory surface along its full course (Colello and Guillery, 1992; Williams et al., 1986, 1991; Williams and Rakic, 1985). Moreover the developing optic fibres become rearranged at the chiasma by mechanisms that cannot be simply explained in terms of spatio-temporal patterns (Reese and Baker, 1992). This all suggests that fibre ordering in the retino-tectal system is not sufficiently precise to generate a specific map at the target region, that the initial outgrowth pattern is not maintained and therefore cannot establish precise maps by the simple translation of temporal events into a spatial distribution of fibres at the termination site (for review see Reese and Baker, 1992).

The degree of fibre order in the developing cerebral cortex has not been studied systematically. If one is interested in the role of early fibre ordering in establishing the topography of projections then one must examine them during their development, before subsequent changes might alter the early arrangement.

Hankin and Silver (1986) demonstrated that the gross topography of the cortical surface is maintained among the axons of the corpus callosum in the developing rodent. Using in vitro imaging techniques, Halloran and Kalil (1994) provided additional evidence for crude topographic organization that fits the time constraints. Although the callosal projections from neighbouring cortical regions maintain their neighbourhood relationships, individual fibres do cross each other while they navigate across the midline and establish connections with corresponding targets in the contralateral cerebral cortex. This suggests that although the completion of the fine callosal topography is based on additional remodelling mechanisms, the gross topographic order in the initial axon outgrowth might still play an important role in the layout of the map (Kind and Innocenti, 1990).

I propose that the arrangement of descending (subplate) and possible of ascending (thalamic) axons in the fetus is determined, at least in part, by the *chronology* of their outgrowth. The idea is that a spatiotemporal wave of cell generation and differentiation occurs across corresponding parts of the cortex and thalamus, and that this wave is converted into two arrays of axons.

My study clearly demonstrates that both the subplate projection and the thalamic outgrowth form ordered arrays. This is clearly revealed by the experiments in which several different dye crystals were placed in parasagittal or coronal rows along one hemisphere, labelling bundles containing both corticofugal and thalamocortical fibres

that are topographically organized. The timing of outgrowth of the pioneering subplate projection from different cortical regions follows the overall pattern of maturation of the cerebral hemispheres. Fibres leave the subplate of the rostral cortex 1.5-2 days before they grow out of the occipital cortex. They all stream towards the primitive internal capsule, creating a fan of axon bundles, preserving the front-to-back lateral to medial topography of their cortical origins. Thus the spatial arrangement of axons is partly related to the timing of their outgrowth.

However, time is unidimensional and the cortical surface is essentially two-dimensional: clearly chronotopy cannot account for everything. While the arrangement of the subplate scaffold along the anteroposterior axis certainly correlates with the time of outgrowth of the axons, I believe that chronotopy is much more important for the organization of the **mediolateral** axis of the subplate scaffold (and of the thalamocortical axon array within the telencephalon). Subplate fibres from regions at different anteroposterior levels do not share the same segment of the intermediate zone and therefore inevitably stay distinctly separate from each other if they simply grow towards the primitive internal capsule. But fibres from all points along any strip of cortex radiating out from the primitive internal capsule (coronal strips in the middle of the hemisphere) share the shallow depth of the intermediate zone and therefore have ample opportunity to mix and lose their order. This seems to be prevented by strict arrangement in the white matter according to the time of outgrowth of the axons. The one-dimensional time axis essentially determines the mapping of the mediolateral axis of the axon array. At any coronal level, axons first stream out from the more ventral and lateral parts, and fibres are subsequently extruded into the intermediate zone from progressively more medial cortical locations. The results of multiple labelling with dye

crystals placed in mediolateral rows clearly establish that the later generated subplate axons, from more dorsal and medial cortex, lie *deeper* in the white matter. Thus, the depth profile of subplate fibres in the intermediate zone of the ventral portion of the hemisphere is a quite precise reflection of their time of outgrowth, which itself depends on the mediolateral positions of their cells of origin across the cortex. Other corticofugal projections may follow the same chronotopic gradient; for instance Woodward et al. (1990) reported that, in the adult rat, fibres arising from lateral cortical neurons are superficial to those from more medially located cells. My own observations on the distribution of axons of pyramidal cells of layer 6 and 5 in the white matter, labelled from the internal capsule or cerebral peduncle after E16, also showed remarkable fibre ordering, following the same lateral-superficial / medial-deep sequence (see Fig. 3.17C and D). Chronotopic ordering of corticofugal fibres in the white matter might be a general principle.

The array of thalamic axons is also influenced and partly determined by the timing of their outgrowth. In particular, thalamic fibres destined for more ventral areas of cortex leave the thalamus before those that reach more dorsal parts at the same coronal level. This is reflected in their times of arrival under the cortical plate (ventral anterior areas first) along trajectories parallel to the highest differential of the neurogenetic and morphogenetic gradient (see Chapter 1; Catalano et al. 1991; Molnár and Blakemore 1990a,b). This arrangement of thalamocortical trajectories remains preserved in the adult (Caviness and Frost, 1980; Frost and Caviness, 1980).

I propose that both thalamocortical and early corticothalamic subplate fibre arrays are generated in a spatio-temporal sequence and they simply maintain their gross fibre-fibre relationships from thalamus to subplate and from subplate to the region of

the thalamic reticular nucleus (respectively). The timing of outgrowth will certainly determine which particular fibres reach the 'zone of interaction' below the corpus striatum at any particular time. If the timing of initial outgrowth from thalamus and cortex is roughly synchronized and the distance from each each to the zone of interaction is roughly the same, then when the first thalamic fibres reach the interaction zone, they presumably meet only the first-formed subplate axons (from rostromedial cortex). Then, over the following 1.5 days or so, successively generated thalamic fibres might reach the interaction zone just as their cortical subplate counterparts arrive. This sequential interaction of arriving fibres, on a first-come-first-served basis, might reduce the possibility of aberrant interaction between the two arrays.

Chronotopy in other white-matter systems

From examination of the corticospinal projection from layer 5 (Fig. 3.15C and D) it is apparent that its fibres are also arranged chronotopically, the fibres originating from ventral areas running superficial to the fibres from more dorsal areas. The more recently outgrowing fibres have to undercut and run deeper than the older ones.

Hankin and Silver (1986), studying the chronology of callosal development (in the rat), showed that callosal fibres are generated in a spatio-temporal sequence, those from more medial cortical areas (cingulum) crossing first, with those from more lateral areas reaching the midline later, coming to lie deeper in the callosum. The initial gross matching of the two hemispheres on to each other might be based on timing of the establishment of these connections. It is interesting to note that, in the rat, subplate axons pioneer the interhemispheric route, but only from the cingulate cortex (Koester and O'Leary, 1991). The initial layout of the callosal fibres is very clear in embryonic life, but their distribution across the hemisphere is substantially altered with subsequent

development.

Interestingly the order of fibres in the callosum itself preserves the simple juvenile pattern (i.e. the original chronotopic order) as demonstrated by Nakumara and Kanaseki (1989) in the adult cat. It is interesting to speculate whether the rule that earlier fibres lie superficial to the later ones in the white matter can be extended to the relationship between different fibre systems. Fig. 3.28 and Table 3.3 gives a summary of the timing of the establishment of the different fibre systems. Fig. 3.25 demonstrates that the callosal projections (from the very same cortical segment) lie under the thalamocortical ones in the white matter. It has previously been shown in the adult rat (Woodward and Coull, 1984) and cat (Nelson and LeVay, 1985), that geniculocortical fibres are localized most superficially in the white matter, whereas later developing corticofugal pathways occupy successively deeper strata. These studies were performed in the adult and do not reveal the earliest corticofugal projections from the subplate. Nevertheless they seem to support the notion that the earliest generated fibres occupy the most superficial position (closer to the cortical plate) than the later developing ones.

Observations of the arrangement of developing fibre bundles of different origin in the cortical white matter suggest general principles operating during the development of the extrinsic connectivity of the cortex: that early connections maintain their neighbourhood relationships during their growth; that they show gross ordering from the time of their establishment; and that they are chronotopically arranged. Individual fibres of each system and different fibre populations appear to occupy positions in the cortical white matter according to their time of establishment. During this developmental sequence, the chronology of the appearance of the different tracts is preserved in the ordering of the fibres in the adult white matter, but can be

Table 3.3 Timing of the establishment of cortical connections in the white matter

E14-15	subplate projections reach the internal capsule	DeCarlos O’Leary, 1992; Erzurumlu and Jhavery, 1992
E15.5-16	thalamic fibres reach cortex	Catalano et al., 1991 DeCarlos and O’Leary, 1992
E16	corticothalamic fibres reach TRN	Chapter 3
E19	corticospinal fibres reach cerebral peduncle	Chapter 3 Mitrofanis and Guillery, 1993
E19-20	callosal projections reach contralateral hemisphere	O’Leary et al. 1990

substantially modified at their termination or waiting points (see 3.15 below). In other words, *for two different (thalamo-cortical, subplate, corticospinal or callosal) fibre systems, the first developed will usually occupy a more superficial position under the grey matter.*

3.15 Contact guidance (and repulsion)

The morphology of subplate growth cones

O'Leary et al. (1990) in the rat and McConnell and her colleagues (1989) in cat and ferret, suggested that the growth cones of subplate axons, growing down in the nascent intermediate zone, are unusually large (as much as 10µm across), with complex morphology (having excessive numbers of florid filopodia). As the scaffold extends close to the primitive internal capsule, the growth cones become smaller and their morphology much simpler. Mason and Godement (1992), using time-lapse techniques to observe optic axons in the optic chiasm, suggested that the morphology of the growth cones (which were also large, with many filopodia) could reflect a stage of 'decision-making' (i.e. route selection) by the growing axons. Brown (1984), examining the formation of the neuromuscular junction, observed similar phenomena. As the developing axons of motor neurons grow rapidly, the structure of their growth cones is simple, but at choice points, their morphology changes, gaining a characteristic 'exploratory' appearance. In an *in vitro* assay, Stuermer et al (1992) observed the growth of axons into a 'dead-end' stripe consisting of a growth-permissive surface surrounded by a growth-inhibiting membrane preparation. When the axons reached the end of the stripe, time-lapse recording showed spectacular changes in the morphology of their growth cones. Numerous filopodia were extended over the entire distal 200-300µm of the fibre, before the axon pulled back or died.

This all implies that subplate axons are in an 'exploratory' mode of growth as they pioneer the pathway through the intermediate zone. They are presumably using some form of 'contact guidance' to determine their trajectories, i.e. they are influenced in their patterns of growth by the molecular (and/or mechanical) characteristics of the surfaces with which they come into contact. It is interesting to consider what surfaces are available at this stage with which the pioneering axons could interact. One obvious candidate is the pre-existing processes of other cells. Mitrofanis (1992a) recently described a transient cell population in the internal capsule, in the gateway to the thalamic nuclei. Although, these so-called perireticular cells are in a strategic position, they do not seem to develop projections to the cortex in large numbers until E16 (Adams N. et al, 1993). We are far from the understanding their significance. Radial glial cells, which are widely considered to be primarily responsible for neuronal migration, are generally distributed orthogonal to the routes taken by subplate axons. They might also influence their growth, with their roles in neuronal migration and axonal pathfinding possibly depending on entirely *different* surface molecules, thus avoiding 'crosstalk' between the two functions.

Not all influences on growing axons are attractive. Some molecules inhibit or discourage axon growth (Caroni and Schwab, 1988a,b; Schwab and Caroni, 1988) and it may be that the pioneering subplate axons are constrained within the routes that they take by the existence of hostile surfaces outside those routes. The behaviour of the advancing front of the axon bundle as it reaches the ventral telencephalon (late on E14 for the occipital cortex) is interesting in this respect. The leading axons diverge over a much wider territory than that occupied by the bundle behind them (Fig.3.16B, 3.17F). Could these very first axons be searching laterally and finding hostile

characteristics that force them back into the correct, tight trajectory?

On the other hand, one has to keep in mind the fact that the extracellular space surrounding the route taken by one group of subplate axons already is (on the superficial side) or will soon be (on the deep side) occupied by subplate axons from neighbouring regions of cortex. Thus, if growth-inhibiting factors do exist outside the preferred route of any subplate bundle, either they must be specific for each particular bundle (unlikely, because it would demand myriad different molecules to discipline every bundle), or they could exist on the surfaces of those neighbouring axons. Perhaps the first descending axons diverge from the advancing bundle until they contact neighbouring subplate axons. One principle underlying the maintenance of the ordered array could simply be that subplate axons tend to deposit themselves on pre-existing axons but cannot cross them.

Expression of cell adhesion molecules in the embryonic cortex

Godfraind et al. (1988) and Chung et al. (1991) have demonstrated complex and rapidly changing patterns of different antigen expression in the embryonic mouse cortex (from E12 on). Many of the observed antigens were associated with the subplate or subplate fibres. For instance, L1 was specifically expressed along the path of the first corticofugal projections. Chun and Shatz (1988) described fibronectin immunoreactivity in cat subplate, demonstrating that the subplate might play role in shaping the extracellular matrix. The subplate might establish a sheet or a three-dimensional compartment which is preferred only by ingrowing thalamic fibres, but not by cortical plate efferents or callosal axons. There is some evidence that specific cortical pathways can be marked with different molecules. Two chondroitin sulphate proteoglycan core proteins are apparently restricted to the afferent thalamocortical pathway and subplate

but not the corticothalamic pathway (Bicknese et al., 1991; Sheppard et al. 1991; Miller et al. 1992).

The various fibre tracts traversing the intermediate zone are segregated from each other (Fig. 3.25; radial glia, callosum, thalamic fibres) so each might conceivably have its own preferred growth-promoting surface expressed within the intermediate zone. Alternatively, since they are laid down successively, each underneath its predecessor, they might grow preferential on the surfaces of the pre-existing fibre bundles, which might, at that stage, be expressing growth-promoting surface molecules appropriate for the next set of axons. Obviously the whole system might operate with few classes of surface molecules along the organized trajectories of pre-laid fibres and radial glia. Contact guidance need not necessarily be limited to mechanical factors but might involve a range of different cell-cell interactions. The orderliness of the fibre paths may indicate the existence of either (1) precise chemoaffinity cues which are followed by the growing fibres along the entire pathway, or (2) local cues at each point along the route. These two mechanisms are not mutually exclusive - the first fibre path might be guided entirely by chemoaffinity cues along the extracellular pathway and the rest might be 'blindly' led by contact guidance over the surface of the pioneering fibres. This two-phase mechanism has been suggested to explain the development of the retinotectal system (Horder and Martin 1977) and the interhemispheric projections (Hankin and Silver 1985).

3.16 The role of the subplate scaffold

Are subplate axons true pioneers?

The presence of the transient subplate population and the early corticofugal scaffold that it constructs are distinctive features of the developing mammalian forebrain (McConnell

et al. 1989; DeCarlos and O'Leary, 1992; Blakemore and Molnár, 1990; Molnár and Blakemore, 1990a,b). McConnell et al. (1989) suggested that the pioneering subplate axons could establish a topographic scaffold towards the internal capsule, which could be used to guide corticofugal and thalamic axons over their route to their target areas (Shatz et al., 1990). But is this curious cell group really needed for the establishment of the subsequent cortical connections? Does the subplate projection actually fulfil all the following conventional criteria (see Chapter 1) for a pioneer projection?

1) *It extends axons into territory in which no other axons exist.*

Certainly this is true for the subplate array. From the primordial plexiform zone through the intermediate zone to the primitive internal capsule they are the first fibres in the area (McConnell et al. 1989; Blakemore and Molnár, 1990a,b; DeCarlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992). There are numerous cells present within the primitive internal capsule (in the perireticular 'nucleus', Mitrofanis, 1992), but they do not yet seem to project to the cortex at this early stage (Adams N. et al., 1993). I have established that the subplate fibres are indeed ordered and directed towards the different regions of the primitive internal capsule as they descend, but the majority probably do not reach the thalamus itself. And they most certainly do not have the chance to guide the thalamic axons over the proximal part of their journey, because the latter have passed into the telencephalon by the time the corresponding subplate axons arrive (this Chapter; Blakemore and Molnár, 1990). But until they encounter the thalamic array under the primordial striatum, the subplate axons are apparently the earliest fibres in the pathway.

2) *Later growing axons follow the same pathway.*

The hypothesis that the later developing ascending and descending projections follow

the trajectories of the first established subplate projections is rather attractive because it could provide (or at least contribute to) the initial topography without the need for any strict, inherent, area-specific chemical matching between the interconnecting cortical and subcortical regions. The idea that thalamocortical axons require subplate axons for their growth to their appropriate cortical target areas was stimulated by the observation that the first growth cones of LGN axons and occipital cortical subplate neurons are extending in opposite directions within the internal capsule at about the same time (E30 in the cat: McConnell et al. 1989; Ghosh and Shatz 1992b). In the rat similar observations were made at E14-15 for the putative visual cortex (Molnár and Blakemore 1990a,b; DeCarlos and O'Leary 1992) and for the putative somatosensory cortex (Erzurumlu and Jhaveri, 1992).

To test this idea numerous workers (see below) attempted to label the thalamocortical and corticothalamic axons with different dyes from corresponding regions of the thalamus and cortex at the same time. Since the carbocyanine dyes travel anterogradely as well as retrogradely this type of selective labelling is only possible before the thalamic fibres reach the cortex. However, before the thalamic fibres reach a particular cortical area it is very difficult to tell where exactly they are destined to go. Valid general predictions can be made (e.g. fibres from dorso-lateral thalamus reach the occipital cortex) but the finer matching (between a small cortical area and a small group of thalamic neurons) can be precisely defined after the arrival of the thalamic fibres at the subplate layer of the corresponding cortical area. At that point (E16 for occipital cortex), they can be back-labelled from the cortex (together with the corticofugal projections, within the same bundle). But from this moment, selective labelling of thalamic and corticofugal fibres is no longer possible.

Bicknese and Pearlman (1992), in the rat, and Miller and colleagues (1991, 1993), in the hamster, have tried to discover whether ascending thalamic axons grow along the same routes as those traversed by the groups of subplate axons from corresponding cortical areas, but their double-labelling experiments were performed well after the arrival of the thalamic fibres and after the establishment of layer 6 and 5 connections. They concluded that the labelled axon bundles from the cortex and from the thalamus run in separate fascicles, with the afferent thalamocortical axons travelling more superficially, in the subplate, and the efferent corticothalamic axons deeper in the intermediate zone. This is very similar to the adult pattern (in the rat: Woodward and Coull, 1984; and cat Nelson and LeVay, 1985).

Unfortunately the location of the subplate axons in relation to the afferent and efferent pathway was not determined in these studies. In earlier stages, however, similar double labelling experiments of Bicknese and Pearlman (1992) revealed that growing thalamocortical and early corticofugal axons were intermingled within the internal capsule as implied by the single labelling study of Blakemore and Molnár (1990).

In my own double-labelling experiments I also observed mingling within the intermediate zone in addition to the internal capsule (Fig. 3.27). The spatially distinct pathways of corticofugal and corticopetal pathways in the adult does not exclude the possibility that the earliest thalamic and subplate axons do selectively fasciculate on each other. 3-D confocal microscopic reconstructions with identified subplate axons at early stages would be required to resolve this question. It is obviously extremely difficult to be sure that two dye placements at early stages are indeed in precisely corresponding positions: the misalignment of the stained bundles in the experiments of Miller et al (1991, 1993) could simply reflect a failure to identify the exact relationships

between thalamus and cortex in the fetal brain. Since afferent and efferent projections co-exist from E16, single crystal placements on the cortex label *both* fibre systems. The results of such tracing of mixed fibre bundles suggests that they do follow very similar routes although individual fibres, especially within the depth of the intermediate zone, are difficult to resolve (this chapter; Molnár and Blakemore, 1990a,b; Blakemore and Molnár, 1990).

3) *Pioneers actually **guide** later axons, providing cues that they follow.*

This point is more difficult to establish, since it would require the demonstration that thalamic fibres are unable to reach their corresponding cortical target after the deletion of the subplate cells of the corresponding cortical region. Ghosh et al. (1990) have indeed lesioned the subplate in the cat by injections of kainic acid and this resulted in a bizarre behaviour of the corresponding thalamic axons, which grew under and past the cortical region and appeared to terminate nowhere. However, in this experiment, the lesion was made at a late stage, almost a week after the arrival of the thalamic fibres. The crucial, but technically difficult, experiment is selectively to delete the subplate system *before* the growth of thalamic fibres into the intermediate zone, without changing any other properties of the region. In the grasshopper embryo the role of the selective fasciculation of the anterior-growing axons on the posterior-growing longitudinal pathways was tested by specific laser deletion of the neurons of origin of the posterior growing-axons. These posterior growing pathways proved to be essential for the normal path and target finding of the anterior-growing axons (Bastiani et al. 1986).

Until similar clearcut lesion studies are performed in mammals, we have only indirect evidence, like the appearance of the growth cones (O'Leary et al., 1990; Ghosh

and Shatz, 1992b), the lack of regional specificity in co-culture experiments (Molnár and Blakemore, 1991; Yamamoto et al. 1992; Chapter 2), and the expression of surface molecules in the embryonic cortex (Godfraind et al., 1988; Chun and Shatz, 1988a; Chung et al., 1991), to support the idea that the subplate scaffold might indeed be used by the thalamocortical fibres for their normal development. If the subplate scaffold does guide the distal portion of the journey of the thalamic axons, why is this necessary?

The subplate scaffold compensates for the distorting effects of growth

At the time of initial outgrowth of subplate and thalamic axons, the forebrain is very small and very simple in its organization. The developing cerebrum is wrapped around the developing diencephalon, the shortest route between corresponding points being a smoothly curved trajectory. But even between mid E14 (when axons first begin to leave the LGN) and E16 (when they arrive at the occipital cortex) the cerebrum expands dramatically, leading to a substantial change in the interlinking trajectories between cortex and thalamus. This inevitably distorts the pathways taken by the pioneering subplate axons. Initially, at the time that they grow through the intermediate zone, the bundle of subplate axons from a single point on the cortex follows a simple, smoothly curved route towards the primitive internal capsule. But over the following day or two, when the thalamic axons grow through the same segment of the brain, the bundle of fibres labelled from a single cortical point becomes more complex in form, with sharp bends and twists appearing in it (see Figs. 3.20,25,28).

A possible functional reason for the early establishment of the subplate scaffold, and the early completion of thalamocortical fibre growth (E16 for occipital cortex) is to **preserve spatiotemporal precision** which would otherwise, because of the distorting effects of growth, become increasingly difficult to orchestrate. Interestingly this early

establishment of these trajectories seems to be true for many other gyrencephalic species at the equivalent stage. I have examined human fetal brains (see chapter 7) as well as rat and mouse, and although the timing of the outgrowth differs enormously, the *spatial relationships* between cortex and thalamus are remarkably similar in all species: only the overall size of the brain differs substantially. In higher mammals with large brains and gyrencephalic cortices, thalamocortical projections reach the cortex before the characteristic migration and rotation of the LGN (Clarck, 1932a,b; Rakic, 1976, 1977) and before the appearance of sulci and gyri.

However, inevitably, the formation of the preplate is followed by a period of rapid proliferation and migration of cortical neurons and hence thickening of the cortex and expansion of its surface area. The telencephalon grows much faster than diencephalic structures, because most thalamic cells have already all been generated. After E14, the whole cerebrum expands rapidly, pushing backwards and downwards, around the brainstem. The relative delay in the formation of the occipital cortex (Berry and Rogers 1965; Lund and Mustari 1977), the closure of the internal capsule and the relative rotation of the diencephalon (Kalil 1978) all contribute to the rapid structural complication of the trajectories between thalamus and cortex.

Thus the subplate axons establish their scaffold before the explosive growth of the cerebrum, and the structure of the pathway changes substantially by the time the thalamic axons traverse the same region. Perhaps one function of the subplate scaffold is to lay down a guidance structure at an early stage, when simple fibre-fibre ordering is sufficient to achieve topography, which can then be followed by thalamic axons as the route becomes too tortuous for simple, local interactions to preserve topographic order. The subplate array may be necessary to overcome structural complications in the

telencephalic part of the pathway caused by explosive growth.

The waiting period

Although there is pressure on thalamic axons to establish their projections to the cortex as early as possible (because the route between is increasing in structural complexity all the time), there is nowhere for them to go when they arrive. On E16, when LGN fibres reach the occipital cortex, only the lowest part of the true cortical plate is in place and the cortical plate is not yet permissive to axonal ingrowth (see Chapter 2). Moreover, most of the main target cells of the thalamic axons, the cells of layer 4, do not take up their position in the plate until after birth (Lund and Mustari, 1977). Wise and Jones (1976) first used the term 'waiting period' to describe the behaviour of commissural axons in the rat, which accumulate for a protracted period in the intermediate zone before they invade the cortical plate. A similar phenomenon has been reported for thalamocortical axons in the monkey (Rakic, 1976, 1977), cat (Shatz and Luskin, 1986) and rat (Lund and Mustari, 1977). Thalamic axons arrive below the correct regions of cortex before the cortical plate has fully formed and they accumulate and 'wait' over the subplate layer (for several weeks in cats and monkeys) before invading the plate proper. Catalano et al., (1991) have recently questioned the existence of a waiting period in the rat. Using DiI application in the ventral thalamus, they demonstrated that some thalamocortical afferents invade the deep part of the cortical plate of the somatosensory cortex on E18/19. They suggest that thalamic axons simply move up through the cortical plate progressively, as the layers differentiate, always extending up to the lower edge of the dense cortical plate of newly arrived, immature neurons. However, the data of others (Erzurumlu and Jhaveri, 1990; Reinoso and O'Leary, 1990; Molnár and Blakemore 1990a,b) indicate that, although thalamic fibres

indeed invade the cortex before birth, there is nevertheless a waiting period of a few days. In the occipital cortex, thalamic fibres arrive on E16, but do not invade the cortex significantly until around the 19th day of gestation.

The subplate engineers the 'waiting period'

Experiments with co-cultures (Bolz et al.1992; Chapter 2) demonstrate that the cortical plate is not strongly growth-permissive for thalamic axons until about E19-20. Using an in vitro quantitative growth assay Bolz and collaborators demonstrated the existence of a growth-permissive, membrane-bound factor (or factors) in postnatal cortex which is not present in embryonic cortex (Götz et al. 1991; Bolz and Götz, 1992). Moreover thalamic growth cones collapse on E16 cortical membrane preparations (Hübener et al. 1992).

Interestingly, I have been unable to reproduce the behaviour characteristic of the waiting period in co-culture. Axons from fetal LGN cultured with occipital cortex younger than E20 simply grow haphazardly around the cortical slice and certainly do not preferentially accumulate over or terminate in the subplate layer. The only similarity to the situation in vivo is that only very few axons enter the cortical plate. With cortex older than E20, they simply invade the cortical slice, without even hesitating in the subplate layer. This suggests that the waiting period, which is such a distinctive feature in vivo, depends on intimate relationships having been established between the subplate scaffold and the thalamic axons *before* the latter reach their target areas, in their natural environment in vivo. Thus, the growth of thalamic axons over the permissive subplate scaffold, in the otherwise 'hostile' embryonic extracellular environment, may be necessary to establish the waiting period, which is itself essential to avoid the kind of unterminated ingrowth by thalamic axons that is seen in co-culture with cortex between

E19 and about P2 in which layer 4 is not yet expressing its 'stop signal' (Chapter 2).

If each thalamic axon literally fasciculates on a subplate axon, it may inevitably end its growth in immediate proximity with the cell body of that subplate axon, and be compelled to terminate there. Miller et al. (1993) reported that occasionally few thalamic axons grow straight up to the marginal zone through the cortical plate, while the majority are restricted to the subplate. I demonstrated (Fig. 3.12) that initially both marginal zone and subplate cells can be backlabelled from the internal capsule (at E14.5) when the thalamic axons reach the same region. It is an interesting possibility that the early growth of a small proportion of thalamic fibres through the cortical plate and up to the marginal zone is related to the corticofugal projections from the marginal zone. These descending fibres stretch across the non-permissive territory of the cortical plate and may provide a pathway for some thalamic axons to pass through the hostile cortical plate (I shall discuss this idea further in the next chapter on the Reeler mouse).

Dynamic interactions during waiting period.

Naegele et al. (1988) in the hamster, and more recently, Ghosh and Shatz (1992b) in the cat have shown that during a limited period some thalamic fibres send transient side branches towards inappropriate regions of cortex on their way to their correct target area. Initially the growing thalamic fibres each consist of a single parent axon tipped with a growth cone. I looked for such side branches in the rat but saw relatively few of them at E16. They might still exist in the rat for a limited period but their detection may be more difficult due to the relative rapidity of cortical development in rodents.

Perhaps some of the first fibres entering the cortex at E18 are side branches of principal axons. Alternatively, if they are absent in the rat this might be an interesting difference between species with long and short waiting periods. As the thalamic fibres

arrive beneath the non-permissive cortical plate (Chapter 2) this might activate the appearance of lateral filopodia, from which these transient side branches can develop, as in the 'dead-end' street experiments of Stuermer (1990).

Williams, Davenport and Kater (1993) recently demonstrated that filopodial-like outgrowth can be induced along a developing neurite shaft in vitro by a locally applied electric field. The development and extension of side branches might conceivably be caused by electric fields set up by electrical activity in thalamic axons within the subplate region. Shatz and her colleagues (Herrmann et al., 1991; Friauf et al., 1990, Friauf and Shatz, 1991) have provided considerable evidence that at least some thalamic axons in the cat actually terminate and form functional synapses on subplate neurons and these connections might relay activity into the subplate sheet, which is in a strategic position (see Fig. 6.3) to influence further development of thalamocortical and intracortical circuitry.

3.17 Subsequent distortions of thalamocortical relations

So far, I have argued only that topographic ordering in the early thalamocortical projection establishes the *gross* distribution of input to cortical areas as early as the beginning of the waiting period (Fig. 3.20). However, it is tempting to extend this argument to the proposal that the ordering also creates the internal maps that are such a vivid feature of sensory and motor areas of the cortex. This attractive notion, that cortical mapping is initially determined by simple ordered projection from thalamus to cortex, has been challenged on the basis of the topography of the visual field representations in LGN and striate cortex. Conolly and Van Essen (1984), analysing the visual field representation in the dLGN and the visual cortex of the macaque monkey, found that the map is mediolaterally but not anteroposteriorly reversed between LGN

and cortex. They concluded that the geniculocortical fibres in the white matter must reverse positions along one major axis but not the orthogonal one.

Nelson and Le Vay (1985), using pairs of tracer injections to reveal fibre order in the adult cat, did indeed report crossing of thalamic axons in the mediolateral but not in the rostrocaudal axis of the optic radiation. This anisotropic decussation occurred, however, only a short distance (2-500 μ m) below the visual cortex, at a depth that might well have been in the transient subplate during development. Perhaps in the adult the decussation pattern seen in the adult represents a modification of the array of thalamocortical fibres that occurs during or at the end of the waiting period. Perhaps the transient side branches described by Naegele et al. (1988) and Ghosh and Shatz (1992b) form the anatomical substrate for this reversal. It would be interesting to follow up this question by examining the early ordering of thalamic fibres in the occipital cortex of albino or early monocularly enucleated animals.

The body surface is represented as a sensory homunculus in the primary somatosensory cortex. In humans this map is fragmented in an interesting way, differently from the motor homunculus (see Szentágothai, 1977). It would be interesting to examine the fibre ordering all the way from the somatosensory thalamus, as Nelson and Le Vay (1985) did for the visual projection. The initial layout of thalamic axons might be very clear and regular in embryonic life, but become substantially altered with further development near the termination site. However the fibre ordering within the rest of the pathway may preserve the simple juvenile topography. Direct injection of tracers at the internal capsule would be necessary to resolve this question, as was performed in the callosum by Nakumara and Kanaseki (1989) (see Chapter 7).

It is interesting to note that in the adult there is an other area within which there

is extensive crossing of fibres, namely the thalamic reticular nucleus (Bernardo and Woolsey, 1987; Mitrofanis and Guillery, 1993). Since both of these studies were performed with HRP (anterograde and retrograde tracer) we do not know whether these fibres had thalamic or cortical origin. I have speculated (see section 3.10) that corticothalamic fibres might wait in this area (based on my own retrograde labelling experiments at E16, and on the work and suggestions of Shatz and Rakic (1981), Sheng et al, (1991) and Miller et al, (1993). If there is indeed a waiting period for corticofugal fibres within the thalamic reticular nucleus, then it might be important in the rearrangement of the fibres. It seems to make sense for early connectivity to be established with gross topographic order, while the distances for growth are minimal, and then 'fine adjustment' of thalamocortical fibres and corticothalamic fibres to be performed in the subplate, and the thalamic reticular nucleus respectively. These two regions have numerous similarities (Mitrofanis and Guillery, 1993). The crossing of fibres seen in the adult within regions corresponding to these largely transient compartments might then reveal the fine modifications of the distribution of these fibres made during the early phase of development.

Invasion of the cortical plate and death of the subplate

The wholesale entry of thalamic fibres into the cortical plate occurs around the time that the cells of the subplate start to die (Ferrer et al, 1992). This opens the possibility that death of the subplate neurons is *necessary* for the release of the axons and their invasion of the cortex. If so, the subplate would play the additional role of determining when thalamic axons are able to invade the cortex. The obvious next question is: what causes subplate cell death? Is it simply innately 'programmed' to occur at the appropriate time in development, as is much of the apoptotic cell death that occurs in

the nervous system of both invertebrates and vertebrates (Driscoll and Chalfie, 1992). Or is it triggered and regulated by other events (see Purves, 1988; Oppenheim, 1989).

There is a considerable recent interest in the 'social' control of cell death patterns during development (Raff, 1992). One attractive possibility is that spontaneous impulse activity starts in thalamic axons around the time of birth, releasing glutamate from their terminals, which might then kill the temporary subplate target cells through excitotoxic effects. Certainly subplate cells express NMDA receptors (Herrmann and Shatz, 1992) and are particularly vulnerable to excitotoxins: injection of kainate into the cortex of the newborn cat selectively kills subplate cells and leaves the cortical plate apparently intact (Ghosh et al., 1990). I and my colleagues (Molnár, Yee, Lund and Blakemore, 1991) have tried to test this hypothesis by producing lesions of the primitive internal capsule at around E15, so as to interrupt the thalamic innervation of the posterior part of the hemisphere (and the subcortical projections from that region of cortex). We have preliminary evidence that subplate neurons are to some extent indeed 'rescued' in the deafferented region, but further quantification is needed to be sure of this.

Shatz and colleagues recently reported that, for isolated cultures of ferret cortex a larger fraction of subplate cells survive than in vivo (Hohn et al. 1993). This may support the view that external factors, including thalamic innervation, play a role in the initiation or regulation of subplate death *in vivo*, since in ferret cortex the majority of subplate cells disappear by adulthood (Shatz et al., 1990). However, more precise quantification will be needed to settle this point. Using a sensitive, quantitative assay for dying cells in the confocal microscope, Adams R. et al. (1993) did see a distinct layer of dead or dying subplate (and marginal zone) cells in postnatal rat cortical

cultures.

3.18 Growth and plasticity of thalamic axons in the cortical plate

The early morphology of thalamic fibres, before their entry into the cortex, has not been thoroughly studied. By filling early postnatal thalamocortical fibres with horseradish peroxidase in the hamster, Naegele et al. (1988) demonstrated that, just after birth (a period equivalent to E16/17 in the rat), as the geniculocortical fibres grow tangentially below the cortical plate, they extend one to four collaterals into the subplate and cortical plate, which survive only transiently. As I discussed above we do not yet understand the significance of these side branches.

The precise mechanisms regulating the entry of thalamic fibres into the cortical plate are not understood and deserve further study. In particular, the substantial transitions or partial reversal of parts of thalamocortical maps (see Guillery, 1986) might depend on the fate of the transient side-branches described by Naegele et al. (1988) and Ghosh and Shatz (1992b).

After thalamic fibres detach from the subplate layer, they turn upwards to enter the cortical plate, presumably responding to the local trophic and growth-permissive influences of the cortex revealed in co-culture experiments (Molnár and Blakemore, 1991; Bolz et al. 1992; Chapter 2). By comparison with the remarkably strict parallel arrangement of thalamic fibres seen in the white matter at early stages, they become more topographically disordered as they enter the cortical plate. Many axons grow up roughly radially but others grow more obliquely within the plate, following varied courses through the deeper layers. It almost looks as if the high degree of order, which may be created by fibre-fibre interactions during growth, is 'deliberately' slightly disturbed as it is transformed into the topography of projection into the cortex itself.

Perhaps such local mixing of the projection is advantageous in that it allows postsynaptic cells to have access to a scattered group of axons, and hence to have the opportunity to employ Hebbian synaptic plasticity to detect local correlations of activity. By P0-2 some axons appear already to have terminated in layer 6 and others run all the way up to the marginal zone; but the majority of the fibres grow upwards, then branch and lose their growth cones about 2-300µm below the pial surface. Each arbor extends laterally over about 300µm diameter and the zone of termination is not necessarily centred above the point of entry of the axon. Naegele et al. (1988) demonstrated that there is a slight reduction with age in the size of individual thalamic arbors relative to the total size of area 17 in the hamster. They suggested that changes in arbor size, due to collateral formation and withdrawal, contribute to fine map adjustment.

Although a rough thalamocortical map can form without a peripheral afferent projection or input (Kaiserman-Abramof, 1983; Rhodes and Fish, 1983; Guillery et al., 1985), there is a wealth of evidence that the physiological input from the periphery influences the local connectivity of thalamocortical axons in sensory areas of the cortex (for review see Shatz, 1990). In some areas, such as the barrel field of the mouse (Van der Loos and Woolsey, 1973) and layer 4 of the monkey and cat striate cortex (LeVay et al., 1980; Swindale et al. 1981; Stryker and Harris, 1986), these activity-dependent changes are particularly pronounced and rapid during a well-defined 'sensitive period' early in postnatal life, but even in adults, maps can apparently rapidly shift locally (within 300µm or so in a few days) in response to deafferentation or even local over- or under-stimulation (Merzenich et al., 1983, Gilbert and Wiesel, 1991). At least some of the remapping in these kinds of experiments is likely to be occurring at the cortical level, but it is important to note that, for the somatic sensory system, rapid changes in

response to deafferentation have been described at the level of the thalamus, the dorsal column nuclei and even the spinal cord (e.g.: Wall and Egger, 1972; Merrill and Wall, 1978). After peripheral nerve injury in the adult monkey, Garraghty and Kaas (1991) reported that functional reorganization occurred not only in the somatic sensory cortex but also, to some extent, in the ventrobasal thalamus.

The organization of immature cortical neuronal circuits is very different from that in the adult and involves the subplate as well as neurons of the cortical plate (Friauf and Shatz, 1991; see Chapter 6). Obviously this immature circuitry should be taken into account if we are to understand the changes in cortical representation that can occur during development and to contrast them with superficially similar plasticity in the adult.

It appears likely that the fine grain of cortical topography is adjusted locally on the basis of correlations in the patterns of firing of thalamic axons and the extent of such modification is relatively large in late embryonic and early postnatal life (Jensen and Killackey 1987b; Guillery, 1986).

The modification of arborization patterns of individual thalamocortical axons within the cortical plate has been demonstrated as a result of early sensory deprivation (e.g. Garraghty et al., 1986; Jensen and Killackey 1987 a,b; LeVay and Stryker 1978; Blakemore et al., 1981; Swindale et al., 1981; Friedlander et al., 1991). Although such redistributions of thalamic axons are clearly correlated with physiological changes, especially clear for the ocular dominance columns of the primary visual cortex (LeVay et al., 1981), it is not at all clear whether all arbors that survive are functional: some may have few or no synaptic connections. Antonini and Stryker (1993) very recently demonstrated that there was very little difference between the total length of

arborization and the total number of branch points of individual geniculocortical arbors after long-term (4 weeks) and short-term (6 to 7 days) deprivation during the critical period in the cat, but in both cases there was a dramatic reduction of complexity compared to the arbors from LGN cells driven by the non-deprived eye. This implies that both functional and anatomical changes are rather prompt and at least approximately matched in extent during this period of development.

We can consider, as a first approximation, that the basic initial topography of the thalamic projection is established during embryonic life. From different thalamic regions fibres arrive to their target cortical areas at different times along roughly parallel trajectories. Fibre bundles preserve their neighbourhood relationships as thalamic regions interlink with their appropriate cortical fields. But then different mechanisms presumably operate subsequently 1) to form branches within the subplate; 2) to permit individual axons to cross each other and disorganize slightly as they grow into the cortical plate; 3) to control the local 'sculpting' of axon arbors within the 200-300µm field of their initial arborization; and 4) to enable individual synapses to be adjusted in strength within their anatomical distribution. Even prenatally, multiple mechanisms are involved in determining thalamic innervation, but additional, activity-dependent processes appear to take over after the basic innervation is in place.

CHAPTER 4

THE ROLE OF THE FIRST POSTMITOTIC CELLS IN THE DEVELOPMENT OF THALAMOCORTICAL FIBRE ORDERING IN THE REELER MOUSE

INTRODUCTION

There is considerable evidence that the specific thalamic input to each field of the cerebral cortex plays a substantial role in promoting cortical differentiation of at least the primary sensory fields (Rakic, 1988; O'Leary, 1989; see Chapters 1, 2 and 3). The guidance of thalamic axons to their appropriate cortical areas might then constitute a crucial step in the development of the cortex. The mechanism underlying that guidance could be one of the fundamental processes that determine the regional fate of the cerebral cortex.

The first postmitotic cells in cortical neurogenesis form the subplate and the marginal zone (Marin-Padilla, 1971, Luskin and Shatz 1985a,b). These neurons are generated as a single population, before the formation of cells destined for the cortical plate itself, and they migrate to the outer edge of the cerebral wall to become the primordial plexiform zone (Marin-Padilla, 1971), or preplate (Rickmann et al., 1977; Stewart and Pearlman, 1987). The subsequently migrating neurons of the cortical plate split this preplate into two laminae, the marginal zone above (the future layer 1), and the subcortical plate or subplate, below the thickening cortical plate (Marin-Padilla, 1971; König et al., 1977 and Luskin and Shatz, 1985a,b).

Even before the intrusion of the first cells of the cortical plate, the neurons of the primordial plexiform zone send out pioneering efferent axons towards the primitive

internal capsule, the gateway between telencephalon and diencephalon (Marin-Padilla 1971, 1988; McConnell et al, 1989). Shatz et al. (1990) and Blakemore and Molnár (1990) suggested that this precocious projection towards the thalamus from the preplate, mainly the subplate, has an important role in the targeting of thalamocortical connections during development. The first established corticofugal pathway might be a crucial 3-dimensional guiding structure, 'scaffold' (McConnell et al., 1989) over which the thalamic afferents grow to reach their cortical destinations.

The strongest form of this hypothesis requires the subplate projection itself to be topographically organized and to connect each cortical region with its own particular thalamic nucleus before fibres start to leave that nucleus. I have indeed established that the subplate projection in the rat is ordered and that the array of axons in its growing wavefront probably constitutes a topographic representation of the surface of the cortex (Chapter 3). However, the outgrowth of thalamic axons is not delayed until this scaffold is fully established: as axons project downwards from the subplate, fibres start to leave the differentiating nuclei of the thalamus, also heading towards the primitive internal capsule and pioneering their section of the pathway within the diencephalon (Chapter 3; Blakemore and Molnár, 1990; Molnár and Blakemore, 1990; De Carlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992). The two sets of pioneers for posterior dorsal thalamus and occipital cortex meet each other late on E14 in the rat, somewhere below the corpus striatum, on the telencephalic side of the primitive internal capsule.

Although the array of subplate axons is therefore not available to guide the initial part of the thalamocortical projection, they could do so for the distal section of its growth, through the intermediate zone (Chapter 3). Subplate efferents and thalamic afferents in rodent certainly take such similar routes through this segment of the

pathway that thalamic axons may well be fasciculating on their subplate counterparts (Blakemore and Molnár, 1990; Bicknese et al., 1992; Molnár et al., 1993). A further possible important role for cells of the subplate is to act as a 'waiting station' for thalamic afferents after they reach their cortical territory. In monkey and cat a delay of several weeks occurs after the arrival of thalamic axons under the cortex, before they suddenly innervate the cortical plate proper (Rakic, 1976, 1977; Shatz and Luskin, 1986). In the rat thalamic axons arrive under the occipital cortex by E16 (Molnár and Blakemore, 1990a,b,c; DeCarlos and O'Leary, 1992) and although it probably does not last as long (until P0) as was originally suggested by Lund and Mustari (1977), there is a 'waiting period' of a few days before the substantial innervation of the cortical plate (Catalano et al., 1991; Kageyama and Robertson, 1993).

Earlier observations with carbocyanine dyes reported thalamic fibre ingrowth into the cortical plate surprisingly earlier than the previous studies, and the whole existence of the waiting period was questioned (Catalano et al., 1991). As in the rat, the initial studies in the cat based on less sensitive ³H-proline autoradiography (Shatz and Luskin, 1986) were also overtaken and qualified by the results obtained with carbocyanine dyes (Ghosh and Shatz, 1992b). According to the more recent study in the cat the waiting period lasts from E36 to E50, rather than E40 to E55. During this period, thalamic axons accumulate in the subplate and at least some of them form temporary synaptic contacts on subplate neurons (Herrmann et al., 1991). Their distribution within the subplate has been shown to be topographically quite precise in both mouse (Crandall and Caviness, 1984) and rat (Chapter 3, Blakemore and Molnár 1990), axons from each thalamic nucleus lying under their own cortical area. Only after a substantial fraction of the cells of the cortical plate proper have migrated into position

do thalamic axons detach from subplate cells and turn into the immediately adjacent region of cortex, through which they grow to reach layer 4 just as its neurons have arrived and started to express the stop signal that makes the thalamic axons arborize and terminate (Chapter 2).

The hypothesis developed in Chapter 3 suggests that the subplate projection is involved, then, in guiding thalamic axons over the distal part of their journey to the cortex and in holding them until the cortical plate is adequately constructed to accept them.

4.1 The problem of the Reeler mouse

The Reeler mouse has an autosomal recessive mutation that spectacularly disorganizes the genesis of the cerebral cortex (Caviness and Sidman, 1973; Caviness 1976, 1982; Caviness and Rakic 1978; Goffinet, 1979, 1992). Although the cause of the abnormality is still not fully understood, the consequential changes in cell migration, cytoarchitecture and connectivity have been described in great detail (for a review see Caviness et al., 1988). In Reeler, as in the normal mouse, the earliest postmitotic cells migrate to the outer edge of the cerebral wall to form the typical polymorphous neurons of the primordial plexiform layer or preplate (Pinto-Lord and Caviness, 1979; Goffinet and Lyon, 1979; Goffinet, 1979; 1980; Caviness 1982). However, rather than splitting the preplate and accumulating in an inside-out sequence between marginal zone and subplate, the neurons of the cortical plate then gather, in a somewhat irregular but basically outside-in sequence, entirely below the preplate (see Fig.1.4 in Chapter 1, taken from Caviness, 1982). Caviness (1981) has termed the abnormal unsplit preplate of Reeler the 'superplate'.

Despite this radical disturbance of the genesis and lamination of the cortex,

thalamic axons reach the appropriate cortical areas (Steindler and Colwell, 1976; Dräger, 1976; Caviness and Frost, 1980 and Simmons and Pearlman, 1982) and terminate principally in the middle of the cortical plate, corresponding to layer 4 (Frost and Caviness, 1980; Caviness et al., 1988). However, the precise distribution of thalamic axons within the cortex is highly abnormal in the adult animal: instead of running radially from the white matter to arborize in layer 4, they form long loops, running up through the entire thickness of the cortex in obliquely orientated fascicles and then defasciculating and plunging down to reach layer 4 from above rather than from below (Caviness, 1976).

The accurate overall topography and the correct laminar termination of thalamic innervation seen in the adult Reeler might be thought to cast doubt on the theory of thalamocortical development advanced in Chapter 3. If the guidance of thalamic axons and the waiting period essential for the establishment of thalamocortical connectivity depend on an intimate relationship between thalamic axons and the fibres and cells of the subplate, how can the surprisingly normal overall topography be produced in Reeler, in which the cells that should constitute the subplate lie above the inverted layers of the cortical plate? And how can the strange local trajectories of thalamic axons within the cortex be explained? These questions can be answered only by a detailed study of the maturation of thalamocortical interconnections at the earliest stages of development in Reeler.

Rapid Golgi, neurofilament and monoaminergic immunohistochemical techniques have been used to investigate some aspects of the development of the afferent connections in Reeler (Caviness and Korde 1981, Yamamoto et al 1986) but it has been described only in very general terms. Caviness et al. (1988) described the similar

primary targeting of the first generated cells in the subplate of normal mice and the 'superplate' in Reeler, but the key issue of the timing of the establishment of the early corticofugal pathway and the relationship to it of the thalamocortical projections has not yet been examined. The capacity of carbocyanine dyes to visualize axon pathways in fixed tissue through anterograde and retrograde passive diffusion (Godement et al., 1987) provides a direct way to explore the entire sequence of development of connections. I have used axon labelling to study the interactions between thalamic axons and precocious cortical neurons in Reeler and to examine the mechanisms by which normal regional and laminar targeting are achieved despite the gross disturbance of corticogenesis.

METHODS

The techniques employed for these experiments were essentially similar to those used in Chapter 3 to study the development of thalamocortical interconnections in normal rats. Carbocyanine dyes were used to trace axon pathways in fixed brains from normal and Reeler mice of various gestational ages. 10 litters (96 individuals all together) from time-mated pregnant female mice were kindly supplied from the colony of the Département de Physiologie Humaine, Facultés Universitaires Nôtre Dame de la Paix, Namur. Half of these animals (5 litters, 48 individuals) were born from heterozygous females (rl/+) that had been mated with homozygous (rl/rl) males and the other half (5 litters, 49 individuals) had heterozygous (rl/+) father and mother. The first 5 litters thus contained roughly 50% Reeler and 50% normal, whereas the second 5 litters consisted of approximately 25% Reeler and 75% normal, conveniently providing control animals at developmental stages exactly matched with the Reelers (see table 1. for the list of the numbers and gestational ages below). This breeding strategy (that the mother was

always heterozygous) also facilitated the successful rearing of the offspring, since homozygous Reeler female have difficulty doing so.

Fetuses were removed by caesarian section under pentobarbital anaesthesia (100mg/kg i.p. to the pregnant female) on post-conceptual days 13, 13.5, 14, 14.5, 15 and 18. They were immediately deeply anaesthetized by chilling on ice and were perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) delivered intracardially through a glass micropipette under a dissecting microscope. Postnatal animals (P1.5 and P3) were anaesthetized by chilling. The brains were removed and, after a few days postfixation in 4% buffered paraformaldehyde, single crystals (0.1-0.3 mm diameter) of fluorescent carbocyanine dye ("DiI"- C18 [D-307], "DiA" - 4-di-16-ASP [D-3883] or "DiASP" - 4Di10ASP [D-291] Molecular Probes, Inc., Eugene, OR) were inserted into the different parts of the diencephalon (after transsecting the brainstem rostral to the colliculi) or into the cerebral cortex, using a fine pair of forceps or a fine stainless steel wire, under an operating microscope. For these experiments I used both hemispheres. (Right column of Table 4.1 gives a summary of the crystal placements; see below). Since each litter contained both rl/rl and rl/+ genotypes (or even +/+, in the second five litters from rl/+ fathers), and it was not possible to distinguish between them on the basis of the macroscopic appearance of their brains, all members of each litter were used for the same procedure, on the assumption that roughly half of them were Reeler (or 25% in case of the litters from heterozygous fathers and heterozygous mothers). The fact that mutant individuals could be identified only after all the experimental procedures had been completed guaranteed objectivity in the performance of the experiments, which were thus essentially performed 'blind'.

Before E13.5 the cortices of the two genotypes looked identical (Fig. 4.1). The outermost layer is the preplate (or primordial plexiform zone) and the cortical plate itself has not yet begun to appear. Since there were no obvious differences in the appearance of the preplate, ventricular and subventricular zones in the two genotypes, embryos younger than E13.5 could not be classified (Pinto-Lord and Caviness, 1979; Goffinet, 1979, 1980; Stanfield and Cowan, 1979; Derer and Nakanishi, 1983). Thus, at E13 (n=6) no distinction between the two genotypes could be made with confidence. At E13.5 (n=24) differentiation was also usually impossible, although very occasionally (3 individuals), I could distinguish Reeler from normal by looking at the most anterior ventral sections, where the first signs of appearance of the cortical plate itself could be detected (Fig. 4.2.A and B). At each stage, the development of ventral-anterior parts precedes that of the dorsal-posterior regions by 1-1.5 days. (Considering the rapidity of cortical development in the mouse, this is a very significant difference). The development of embryos within the same horn of the uterus can vary somewhat according to the number and location of the embryos. For embryos from small litters or from close to the distal end of the uterine horn, development can be somewhat more advanced. This might explain why it was possible to detect differences in some but not all brains from the same E13.5 litter.

In stages older than E13.5 the two genotypes could be distinguished with great confidence after bisbenzimidazole counterstaining. In the normal (rl/+) the cell dense cortical plate is surrounded with two sparse cell layers, the marginal zone above and the subplate below, while in the Reeler (rl/rl), the absence of this variation in cell density is characteristic (see Results below and Figs. 4.2 and 4.3 for more details)

From E14 onwards, roughly as predicted from the classic Mendelian rules for

autosomal recessive genes, there were 58% Reeler, rl/rl (21 individuals) and 42% normal, $rl/+$ (15 individuals) in the litters from heterozygous females ($rl/+$) that had been mated with homozygous (rl/rl) males, and 74% (23 individuals) normal ($rl/+$ or $+/+$), and 26% (8 individuals) rl/rl from heterozygous ($rl/+$) fathers and heterozygous ($rl/+$) mothers.

Table 1. below lists the numbers and gestational ages, the distribution of the genotypes of all the mice in this study and the dye placements used.

Table 4.1

Number of animals studied

Age	Wild-type (rl/+)	Uncertain phenotype	Reeler (rl/rl)	Dye placements (both hemispheres were used)
5 litters from rl/+ X rl/rl parents				
E13.5	2	8	2	CTX, Thal
E14	3		5	CTX, Thal
E15	5		6	Thal, CTX
E18	4		6	Thal, CTX
P1.5	3		4	Thal
5 litters from rl/+ X rl/+ parents				
E13		6		IC, Thal
E13.5	1	8	3	IC, Thal + CTX
E14	6		5	IC
E14.5	12		2	Thal
P3	5		1	Thal

After the fluorescent carbocyanine dye placement the brains were stored in PBS (with Na Azide 0.1 % added to prevent contamination) at room temperature or at 37°C, to facilitate the diffusion of dye along axons. Dye diffusion required different incubation periods depending on the age of the specimen and the temperature. Table 4.2 below indicates the incubation time in weeks at different ages and different temperatures.

At the end of the incubation, the brains were embedded in 4% agar (made up in 0.9% saline) and cut by vibratome. The sections, 75-200 µm thick, were counterstained with bisbenzimidazole (Riedel-De Haën AG, Seelze-Hannover), 10 minutes in 2.5 µg/ml solution in phosphate buffer, and coverslipped in Hydromount (National Diagnostics, N.J.) or glycerol-phenylenediamine solution (0.1% p-phenylenediamine, 10% phosphate-buffered saline, 90% glycerol made up and stored according to Johnson et al, 1982).

Each series of sections was examined in the fluorescence microscope: double or triple exposure colour micrographs were taken under epifluorescent illumination using different filters to reveal the various dyes. The counterstaining with bisbenzimidazole revealed major anatomical features, such as the pial surface of the cortex, the bottom of layer 1 and the grey matter/white matter boundary. Some sections, selected in conventional fluorescence microscopy, were subsequently examined and photographed with a laser scanning confocal microscope (Biorad MRC-600 or Leica). Sections in which DiI had been used for axon tracing, destined for confocal microscopic examination, were counterstained with another chromatin stain (in addition to bisbenzimidazole), acridine orange (10µg/ml in PBS, Molecular Probes), staining the nuclei with a green emission on excitation with light of 488nm. This stain was not used when DiA or DiAsp had been employed. To obtain high-resolution reconstructions of the fibre

Table 4.2 Carbocyanine Dye Incubation Period in Weeks

Age of specimen	20°C	37°C
E13-15	3-4	1-2
E15-18	6	2
P1.5-3	6-8	3-5

pathways and back-labelled cells we used fine optical sections (1-4µm, interval, up to 32 sections) to construct 3-D datasets. From these 3-D datasets stereopairs were constructed (with +-7 deg disparity) and these are presented as red-green anaglyphs. These pictures should be viewed with the glasses enclosed at the back of the thesis.

RESULTS

The genesis of the cortex in normal mice is essentially similar to that in rats (see Chapter 3), except that each step occurs about 1 day earlier in the mouse, whose total gestation lasts 21 days, compared with 21/22 days in the rat. Thus, for the occipital cortex of the normal mouse, the first postmitotic neurons are born in the ventricular zone at E12 and they have migrated to the outer edge of the cerebral wall by E13, to form the preplate. The birth of neurons of the cortical plate proper starts around E13 and the first of them complete their migration around E13.5 (Sidman et al., 1959; Angevine and Sidman, 1961; Caviness, 1982).

The appearance of the material from the wild-type (rl/+ or +/+) mice in the present study conformed to the previous descriptions of corticogenesis in normal mice at every stage. Moreover, I saw no difference between the genotypes (+/+, rl/+ and rl/rl) until after the time of the arrival of the first true neurons of the cortical plate, at E13.5/14 (Pinto-Lord and Caviness, 1979; Goffinet, 1979, 1980; Stanfield and Cowan, 1979; Derer and Nakanishi, 1983).

4.2 E 13-E13.5

I examined three litters at this gestational age (total n=30: 12 E13.5 individuals from rl/+ mothers and rl/rl fathers; 6 E13 and 12 E13.5 individuals from rl/+ mothers and rl/+ fathers). Each litter presumably consisted of roughly 50% wild-type, 50% Reeler (for rl/+ x rl/rl matings) or 75% wild-type and 25% Reeler (for rl/+ x rl/+), as was seen

in all the older litters, where the two strains could easily be recognized from the appearance of the cortex. However, with rare exceptions (as in the examples in Fig. 4.2A and B, for which the true cortical plate was just beginning to form in the most ventral-anterior region of the cortex) it was impossible to distinguish between the two genotypes by any histological criterion in the bisbenzimidazole stained sections of cortex or thalamus. Since no obvious differences in the patterns of corticofugal and thalamofugal projections were seen between individuals in this age group, the description below is assumed to apply to both Reeler and normal, except for those individual cases (e.g. Fig. 4.5) in which the phenotype was confidently identified.

For all the E13.5 embryos, I placed crystals of dye into the cortex of one hemisphere only (n=12 from heterozygous fathers and n=12 from homozygous fathers: see Table 4.1) in the thalamus of the other hemisphere of these E13.5 embryos and also into one hemisphere of the E13 embryos (n=6) from heterozygous fathers.

In this early stage the ventricular germinal zone consists of spindle-shaped neuroblasts which are generating the earliest born population of polymorphic daughter cells. These postmitotic cells have already reached the outer edge of the cerebral wall, to form the preplate. The crystal placements on the occipital cortex revealed that some of these preplate neurons have already extended axonal processes, which run within, and then under the primordial plexiform layer, into the intermediate zone. Fig. 4.4 demonstrates the distinct bundle of axons, extending downwards towards the primitive internal capsule with growth cones at their tips. The pioneer fibres at the leading front of this projection diverge somewhat from the tight bundle of axons behind them, as if exploring before withdrawing into a narrower trajectory (see Figs. 4.9B for a 3-D reconstruction). Some of the growth cones were particularly large and had elaborate

morphology.

Each individual of one litter at E13 and another at E13.5 (n=6 and 12; both from heterozygous fathers) received a small DiI crystal implant into the primitive internal capsule to back-label the source of these early corticofugal fibres. These experiments demonstrated that the projections revealed with anterograde transport (see Figs 4.4 and 4.9) originated from primordial plexiform zone cells (Figs 4.5 and 4.6). To resolve individual fibre ordering and to examine further the morphology of these cells I examined these specimens with laser scanning confocal microscope (in addition to fluorescent microscopy). These experiments demonstrated that there is a distinct difference between the morphology of the spindle-shaped ventricular and subventricular zone cells (Fig. 4.4) and the polymorphous, polygonal preplate cells (Fig. 4.7). The preplate cells, have extensive arbors and their overall appearance is more mature than the cells in the ventricular and subventricular zones. Just as for the anterograde labelling from the cortex, no obvious differences in the distribution or appearance of the back-labelled cells and their processes could be distinguished between the individual members of each litter, although they should have contained 25% Reeler phenotype.

In 3 litters (30 individuals) at E13-13.5, crystals were placed in the dorsolateral aspect of the diencephalon (posterior thalamus) of one half of the brain, the other half being used for thalamic or internal capsule crystal placements.

In one litter (n=12) I placed different carbocyanine dyes in roughly corresponding positions in the same hemisphere to provide a precise comparison of the status of the corticofugal and corticopetal outgrowths. These experiments demonstrated that the afferent axons from the thalamus, with growth cones at their tips, are just passing through the primitive internal capsule (Fig. 4.8) and are very close to meeting

their corticofugal counterparts beneath the striatum (Fig. 4.9). A similar, roughly synchronized outgrowth of the early corticofugal and corticopetal projections was observed in every member of the litter (although 50% should have had Reeler phenotype). The thalamic tracing experiments revealed that around E13.5 the thalamic fibres pass through the narrow primitive internal capsule, then at around E14 they diverge slightly, and the bundle loosens, as they run through the striatum. By the time the fibres from dorsal thalamus arrive at the occipital cortex (E15/E15.5), the cortical plate is around 100-150µm thick (see below).

4.3 E14.5 - E18

Within 2 days of the arrival of the first postmitotic cells in the primordial plexiform zone, individual animals of these mixed litters were easily differentiated into two groups, corresponding to wild-type and Reeler, depending on whether the cells of the true cortical plate were coming to rest within or below the preplate, respectively (Fig.4.2C and D; see table 1 for classification). As in the mouse, just as in the rat (Chapter 3) a spatiotemporal wave of maturation sweeps across the hemisphere. At each stage, the development of ventral-anterior parts antecedes that of the dorsal-posterior regions by 1.5 - 2 days. In the occipital cortex, the first cortical plate cells arrive at about E14 and their ectopic placement below the preplate was clear to see in roughly half the individuals of each litter at E14 (or 25% in those cases in which both the mother and the father were also heterozygous); see Table 4.1. Bisbenzimidazole counterstaining alone was quite adequate, at this and later stages, to reveal the absence of a distinct lower boundary to layer 1 and the irregular cell sparse zones in the cortical plate typical of the Reeler (Fig. 4.3). In normal mice, the migration of cortical plate neurons through the lower part of the preplate creates a sharp cell-sparse/cell-dense

boundary under layer 1. The subplate is pushed down below the thickening cortical plate, whereas in the Reeler the first generated cell layer, the primordial plexiform zone, stays at the top to form the superplate.

To examine the subsequent changes in the early corticofugal fibre scaffold, DiI crystals were inserted into the internal capsule of two litters (n=11 at E14, and n=14 at E14.5). In normal animals the descending axons from subplate cells, which were sent out a day or two earlier, assume their characteristic position, running as a broad sheet directly under the subplate (Fig. 4.10A). The much smaller numbers of precocious efferent axons arising from the most superficial first-born cells, in the marginal zone, are now occasionally seen extending diagonally downwards, through the cortical plate itself, before joining the band of corticofugal axons below the subplate. In these early stages marginal zone cells can be backlabelled from the internal capsule, indicating that their projections, together with those of the subplate cells, reach at least the ventral telencephalon (Fig. 4.11). The separation of subplate and marginal zone cells and the patterns adopted by their axons are entirely explained by the intrusion of cortical plate cells between these two divisions of the original preplate.

In Reeler, the migratory arrest of true cortical plate neurons below the polymorphous neurons of the primordial plexiform zone leaves the precocious efferent axons of all these first-born cells crossing diagonally through the thickening cortical plate (Figs. 4.10B and 4.11B), just like those of the rare marginal zone cell axons seen in normal mice. These axons tend to form fascicles as they pass obliquely between the cells of the cortical plate. Except for the difference in their distribution through cortical plate itself, the trajectories of the early corticofugal axons is indistinguishable in Reeler and normal mice at this and subsequent stages.

I placed DiI crystals in the thalamus of each individual of four litters between E14 and E18 (see Table 1 for details). The thalamic fibres reach the occipital pole around E14.5-15 in both Reeler and normal phenotypes. The difference between the organization of the thalamocortical projections is clear only when we look at the cortex itself, the segment in the region of the striatum and the internal capsule being indistinguishable in normal and Reeler. On their arrival at their cortical target zones, however, the thalamic axons assume quite different patterns in the two strains (Figs. 4.12 and 4.14). In normal animals, just as in rats (Chapter 3), the thalamic fibres accumulate densely below the cortical plate, in the subplate layer, and very few of them penetrate the plate itself. By contrast, in Reeler, the thalamic axons run through the plate diagonally in fascicles and gather over the cells of the superplate, corresponding to subplate neurons in the normal animal.

It is important to emphasize that the earliest neurons of the cortical plate proper have completed their migration by the time thalamic axons arrive in the vicinity of the target region of the cortex. Therefore, in Reeler, these thalamic axons must grow through at least a thin layer (around 100-150µm thick) of cortical plate cells, without being 'distracted' by them, to reach the superplate layer above.

In all members of a E14.5 litter (n=14; 12 normal and 2 Reeler) both thalamic and internal capsule crystal placements were performed in different hemispheres of the same brains. In these experiments the early corticofugal projections and cells derived from the preplate could not be back-labelled from the dorsal thalamus, but could from the internal capsule (compare Figs. 4.10 and 4.12). The internal capsule crystal placements labelled both the thalamocortical and corticothalamic projections. With high-power confocal microscopic examination I occasionally saw individual back-labelled

subplate cells (or superplate cells in Reeler) together with closely associated growth cones (presumably of thalamic axons)(Fig. 4.13).

Figure 4.14 shows the general pattern of the thalamocortical projection in normal and Reeler mice at E18, when thalamic fibres have reached the corresponding cortical zones over the whole hemisphere. Single crystals placed in the dorsal thalamus at this age reveal distinct patterns of thalamocortical axon distribution in normal and Reeler, indistinguishable until they reach their target areas. In both genotypes, axons from one particular region of the thalamus form a tight bundle as they stream through the primitive internal capsule and up through the intermediate zone, running close to the base of the cortical grey matter. At E18 (Fig. 4.14B) the thalamic fibres have still not penetrated the cortical plate in normal animals (although they reach the occipital pole at around E14.5-15). But in Reeler, the thalamic fibres run in fascicles through the cortical plate, all the way up to the superplate, in fascicles and gather under the pial surface (Fig. 4.14D). The subplate and the superplate seem to play a similar role in accumulating the ingrowing thalamic axons. These observations emphasize the intimate relationship established between thalamic axons and preplate cells in both phenotypes, and suggest that some distinct mechanism must be involved in guiding thalamic axons towards the population of cortical neurons with precocious efferent axons.

At E15, in both phenotypes, a small DiI crystal implant into the occipital cortex leads to the labelling of a single, small bundle of axons, which can be traced down through the intermediate zone, through the internal capsule and up into the corresponding region of the diencephalon (Fig. 4.15). At the thalamic end of the bundle there is a back-labelled cluster of thalamic cells, providing additional evidence that thalamic axons have reached their cortical target zones by this stage. Therefore, the

labelled bundle within the intermediate zone must contain both early corticofugal axons, including axons deriving from preplate cells, and the corresponding thalamic afferents. The fact that only a single narrow bundle can be seen in each case strongly implies that the ascending axons are tightly associated with, perhaps fasciculated upon, the pioneer corticofugal fibres.

To compare the tangential targeting of the thalamic projections on the cortex in normal and Reeler, I implanted a number of different carbocyanine dyes in a row along the convexity of the cortex and examined the topography of the back-labelled cells in the diencephalon. These experiments demonstrated a close similarity in the distribution and topography of thalamocortical projections in the two phenotypes, best shown in Fig. 4.16. This compares horizontal sections through the hemisphere of E18 normal and Reeler mice after placement of three crystals of different dyes in a sagittal row along the cortex. The overall topography of the reciprocal projection system between preplate and thalamic axons is indistinguishable in normal and Reeler: in both strains, the bundles of axons labelled by the three different dyes have the same relationship to each other in both the telencephalic and the diencephalic portions of the pathway and stay separate from each other, even as they pass through the constriction of the primitive internal capsule. The antero-posterior sequence of crystal placement sites along a parasagittal line on the cortex corresponds to a medio-lateral sequence of parallel slabs of back-labelled cells in the thalamus, just as in the rat (Chapter 3).

4.4 The postnatal period

After the waiting period (which lasts from the arrival of thalamic fibres at the appropriate cortical regions (E14.5-15) until the entry of these fibres (not before E18) in both wild-type and Reeler mice, thalamic fibres leave the preplate cells with which

they have been associated and turn to grow into the cortical plate. From the material available in this study (E14, E14.5, E15, E18, P1.5 and P3), it seems that the timing and sequence of invasion are identical in the two genotypes; only the direction of axonal growth is different.

Postnatally I examined one litter at P1.5 (n=7) and another at P3 (n=6). Figs. 4.17 and 4.18 demonstrate the appearance of thalamic axons, labelled by DiI crystal implantation in the dorsal thalamus at P3. In the normal animal the thalamic fibres still lie in fascicles along their path through the intermediate zone to the subplate region. From there they turn sharply upwards and run individually through the cortical plate, following somewhat irregular paths, and arborizing broadly, some 200 μm below the pial surface among cells that have just completed their migration and will presumably become layer 4 of the mature cortex (Fig. 4.17B).

In the Reeler cortex, the thalamic fibres still lie in their characteristic diagonal fascicles in which they ascended from the intermediate zone to the superplate region (Fig. 4.17D and 4.18); but their distal ends have now detached from the superplate, defasciculated and turned sharply back down into the cortical plate as single fibres. This turning and defasciculation could be followed under high-power examination of 100 μm -thick horizontal (pia-parallel) sections from cortical flatmounts. After growing down through most of the depth of the grey matter in somewhat irregular patterns the thalamic fibres arborize broadly, much as in the normal animal, but towards the bottom of the cortical plate, where the cells with birthdates equivalent to those of layer 4 cells in normal animals have presumably just ended their migration (following the abnormal, outside-in pattern typical of Reeler; see Fig. 1.4 in Chapter 1).

**4.5 Subplate and marginal zone cells in normal and
superplate cells in Reeler can be backlabelled from the
internal capsule but not from the thalamus**

Placement of small dye crystals directly in parts of the thalamus, well away from the primitive internal capsule (at various ages from E14.5 to P3), almost never led to the retrograde labelling of subplate and marginal zone cells (derived from preplate) or superplate neurons (see figs.4.12, 4.14, 4.16 and 4.18), whereas such cells were profusely labelled if dye crystals were introduced into the primitive internal capsule itself at E13, E13.5, E14 and E14.5 (Fig. 4.10, 4.11 and 4.13). Increasing the incubation period for the dye transport by an extra few weeks did not lead to the appearance of label in preplate cells after crystal placement in the dorsal thalamus.

Fig. 4.1

100µm-thick, coronal sections through the most mature, ventral anterior part of the hemisphere of a E13 mouse embryo. The sections were stained with acridine orange and examined with confocal microscope.

A: The immature cortex consists of the preplate (or primordial plexiform zone), ventricular and subventricular zones. All members of the litter (6 individuals) from a rl/+ mother and a rl/+ father showed indistinguishable patterns of cortical development, even in the anteroventral segment of the hemisphere.

B: High-power view of the preplate (primordial plexiform zone, PPZ) and the ventricular (VZ) subventricular (SVZ) zones.

Scale bar in A 250µm; in B 50µm.

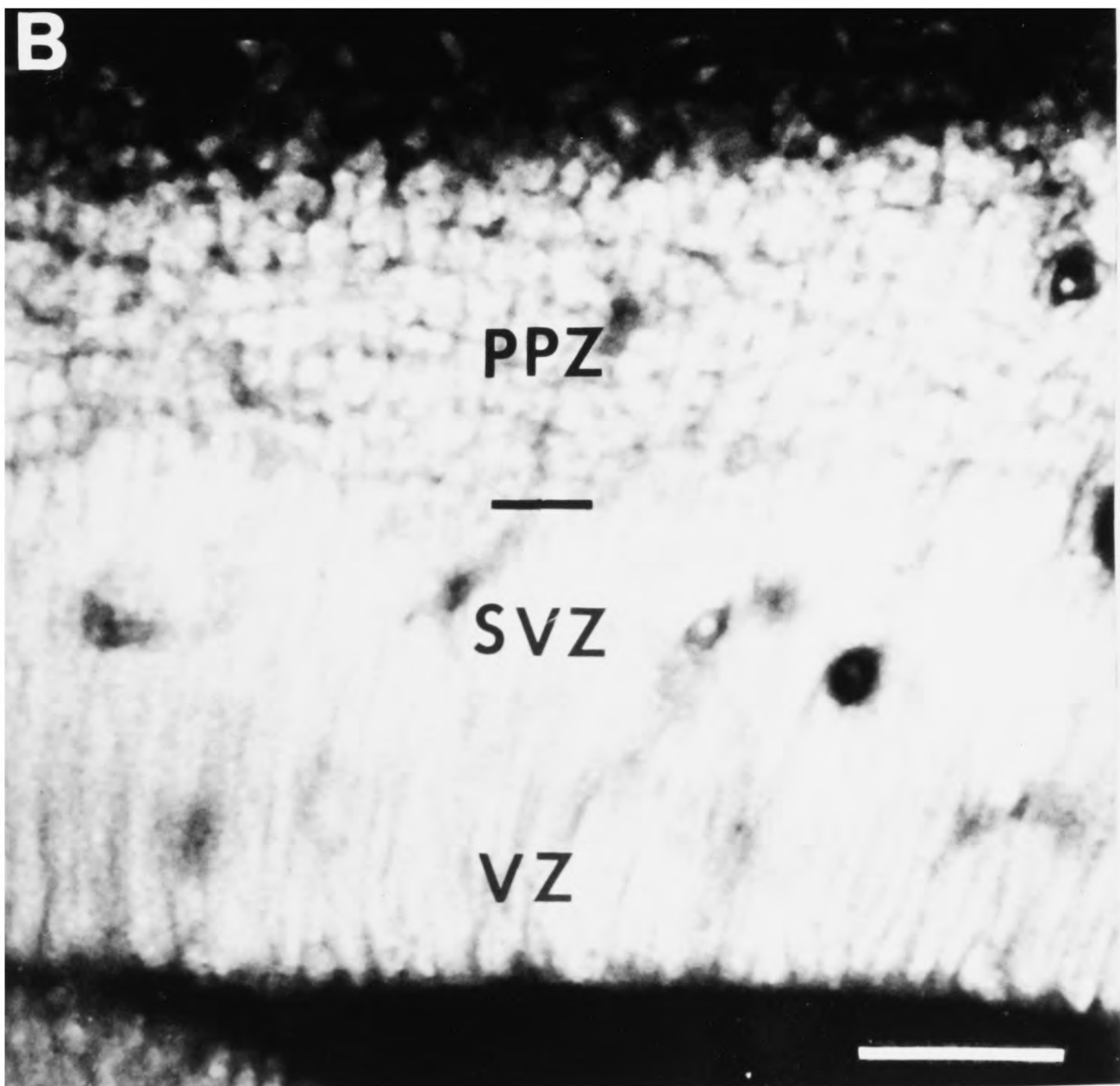
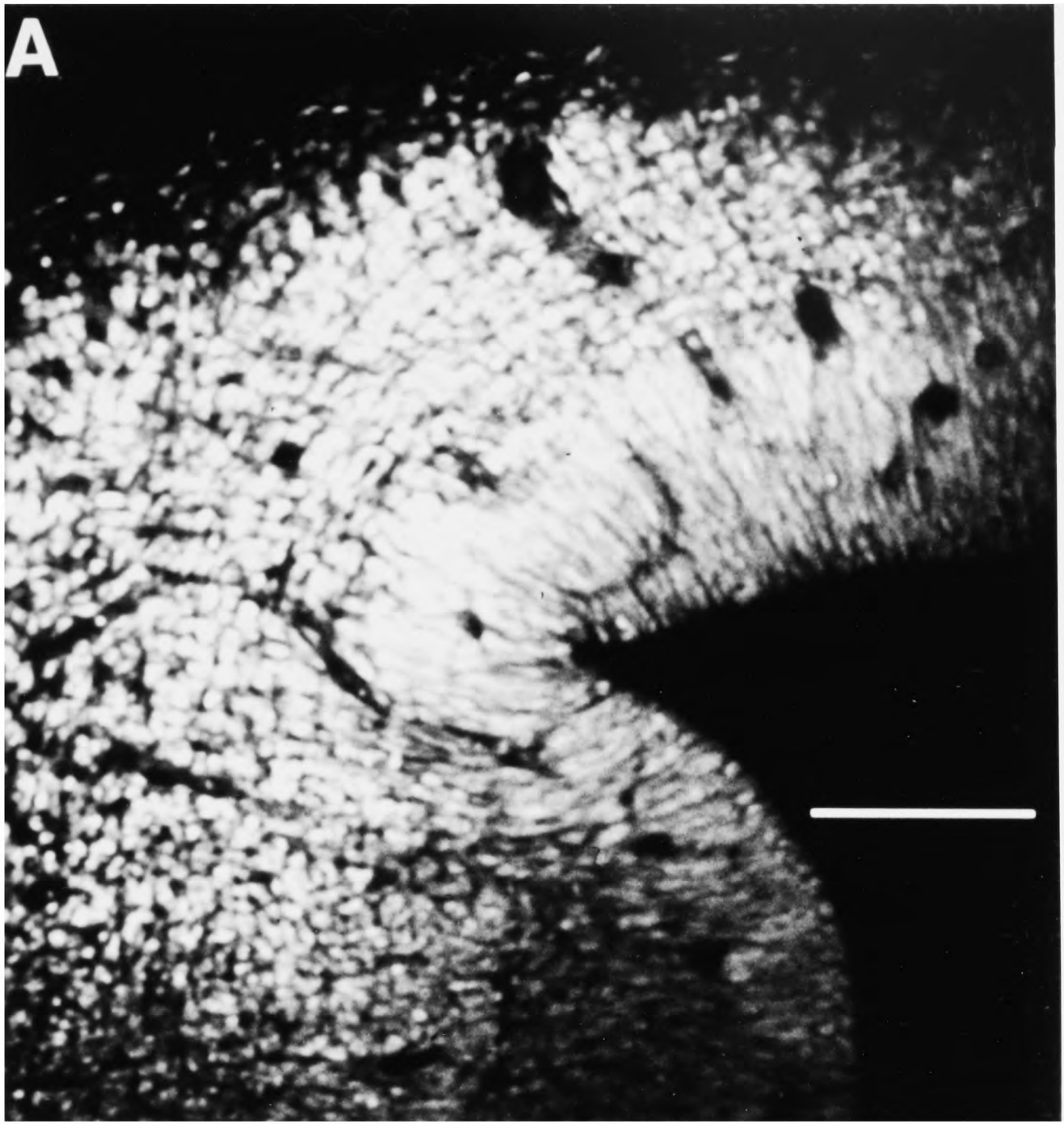


Fig. 4.2

100µm-thick, bisbenzimidazole-stained transverse sections through the most mature, ventral anterior part of the hemisphere of E13.5 (A and B) embryos and the occipital cortex of a pair of E15 embryos (C and D) where the two different genotypes can be already distinguished. The first noticeable difference is the appearance of the cortical plate (CP).

A,B (E13.5). In a section from the ventral anterior part of the hemisphere of the E13.5 cortex of a normal mouse (A) a dense layer, a few cells thick, appears below the more cell-sparse, thin marginal zone (MZ). In the Reeler (B), lamination is already slightly different in this part of the hemisphere: the cells lying above the intermediate zone (IZ) form a more homogeneous stratum, which contains both the first postmitotic cells (superplate, 'S'P) above and the true cortical plate below. No clear border is apparent between them at this early stage. The ventricular and subventricular zones (VZ, SVZ) appear identical in the two genotypes. Sections from more occipital and dorsal regions were indistinguishable in these two specimens at E13.5, since they lagged behind in the neurogenetic and morphogenetic gradients and the cortical plate itself was not yet detectable. Unfortunately the distinctions demonstrated in A and B could not be made with confidence in every member of the E13.5 litters (2 litters, 24 individuals). Therefore not all individuals could be classified as normal or Reeler at this age.

C,D (E15). By E15, even here in the occipital parts of the hemisphere the cortical plate (CP) is almost 200µm thick in the normal (C), and the cell-dense cortical plate is surrounded with two sparse cell layers - the marginal zone (MZ) above and the subplate (SP) below. In the Reeler the absence of this alternation of cell density is characteristic. By this stage the two genotypes can be distinguished with great confidence, even in sections of occipital cortex. Bar 200 µm.

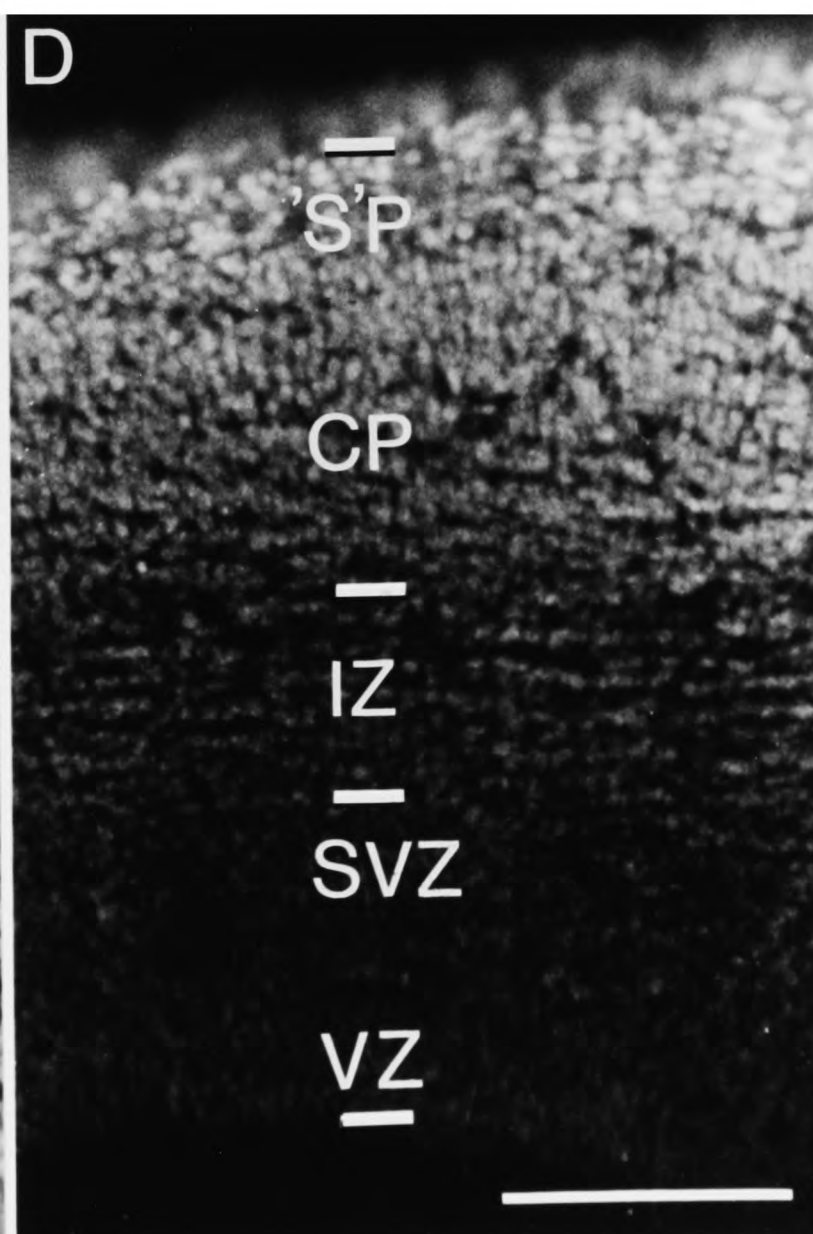
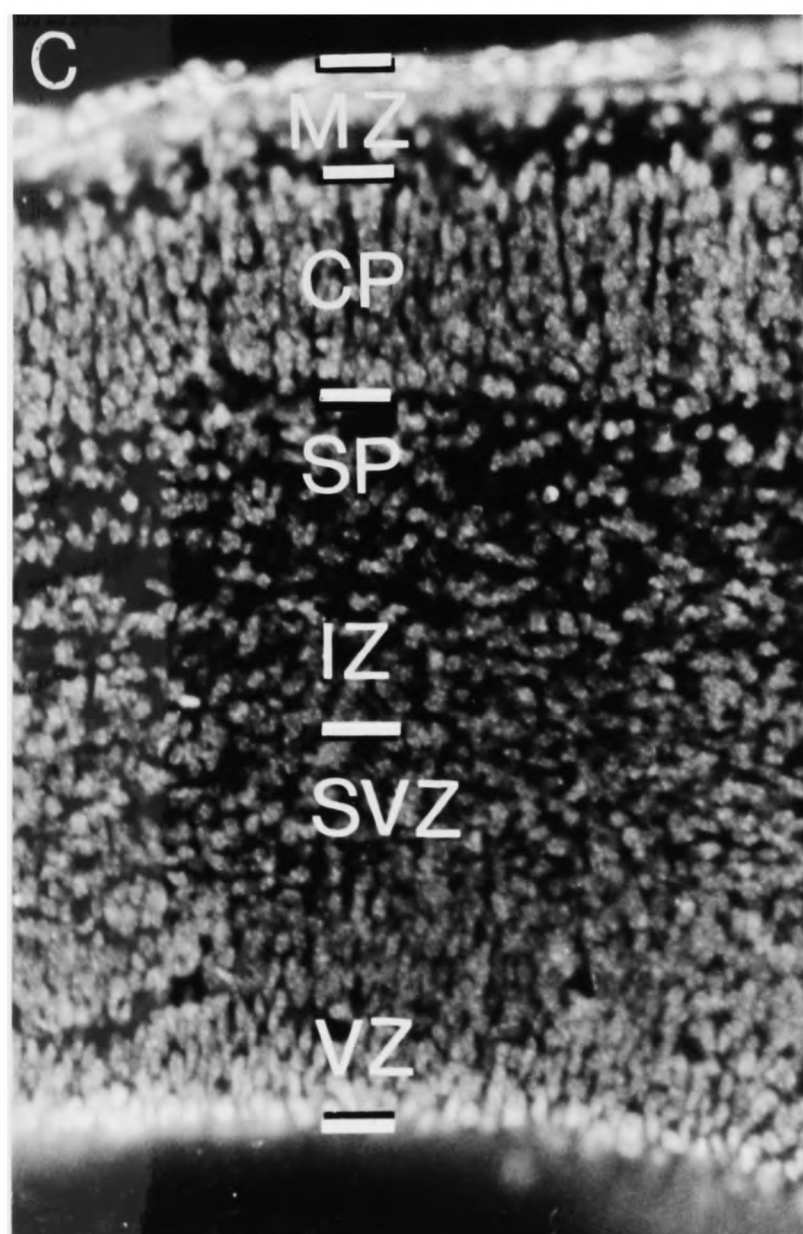
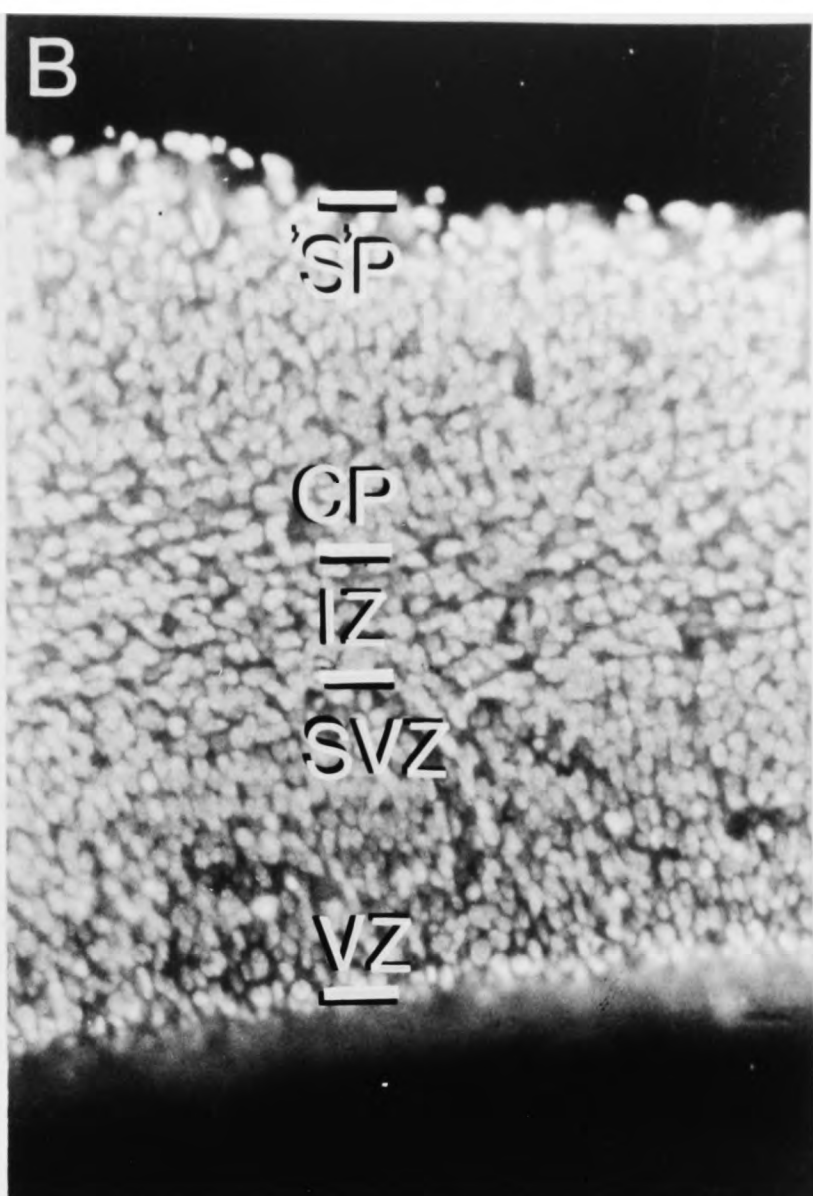
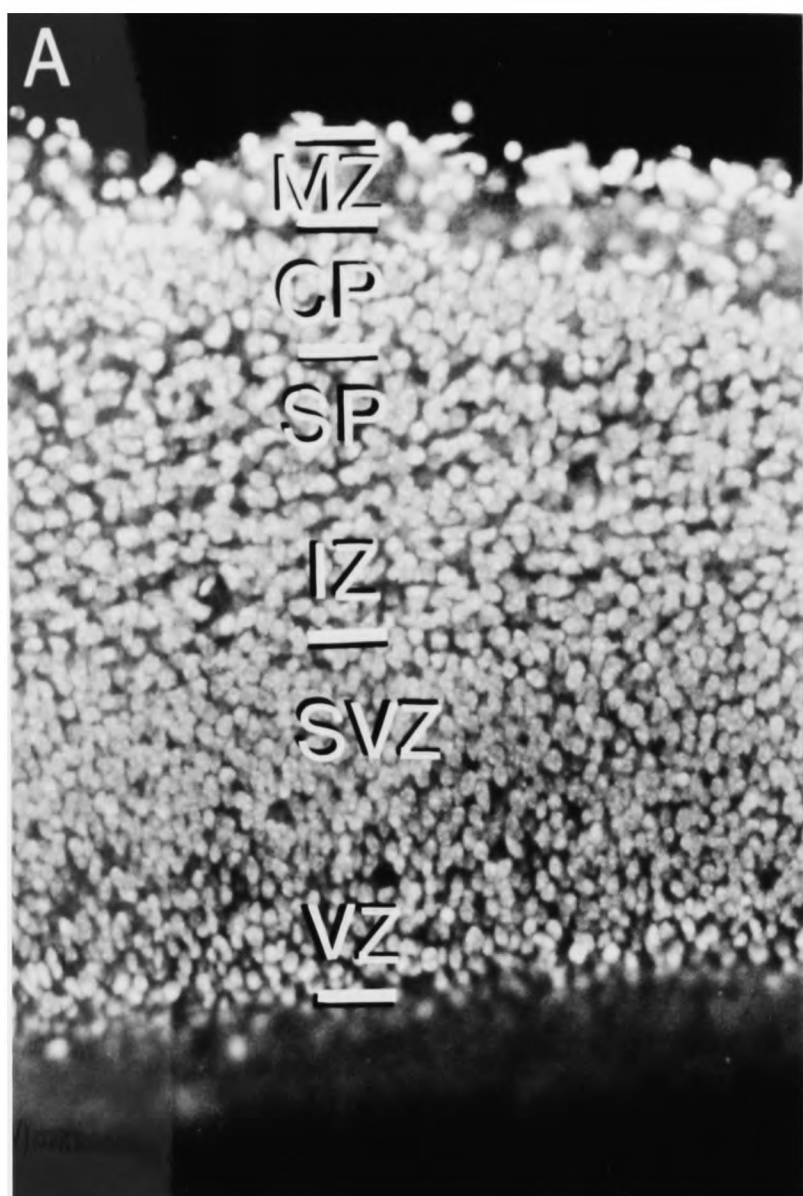


Fig. 4.3

A, B (E15). In these low-power views of the dorsolateral part of the right hemisphere (two brains, from the same litter at E15) the two genotypes can be clearly distinguished. In the normal (A) the cell-dense layer of the cortical plate (cp) is sandwiched between two cell-sparse zones of the subplate (sp) and the marginal zone (mz). In the Reeler (B) there is no separate marginal zone and the superplate ('s'p) possesses similar cell density to that of the cortical plate. The absence of a clear marginal zone and the less demarcated cortical plate, with oblique cell sparse lines, is a reliable indicator of the Reeler phenotype.

C, D (P1.5): Bisbenzimidide-stained, higher-power coronal sections from the brains of two postnatal (P1.5) animals differ most in the cell density of their upper layers. The Reeler (D) has a more dense superficial layer than the normal (C, rl/+), since the superplate ('S'P) contains cells equivalent to both the subplate (SP) and the marginal zone (MZ), whereas the normal has only a 'spotty', cell-sparse upper layer (MZ). Reeler's cortical plate (CP) looks less homogeneous and abuts directly the white matter. In the normal animal (C) only layers 6 and 5 are in position; the upper layers have not yet formed. The undifferentiated upper zone in the cortical plate, termed the dense cortical plate (DCP), stands out with its relatively higher cell density.

Scale bar at A and B: 500 μm , C and D: 100 μm .

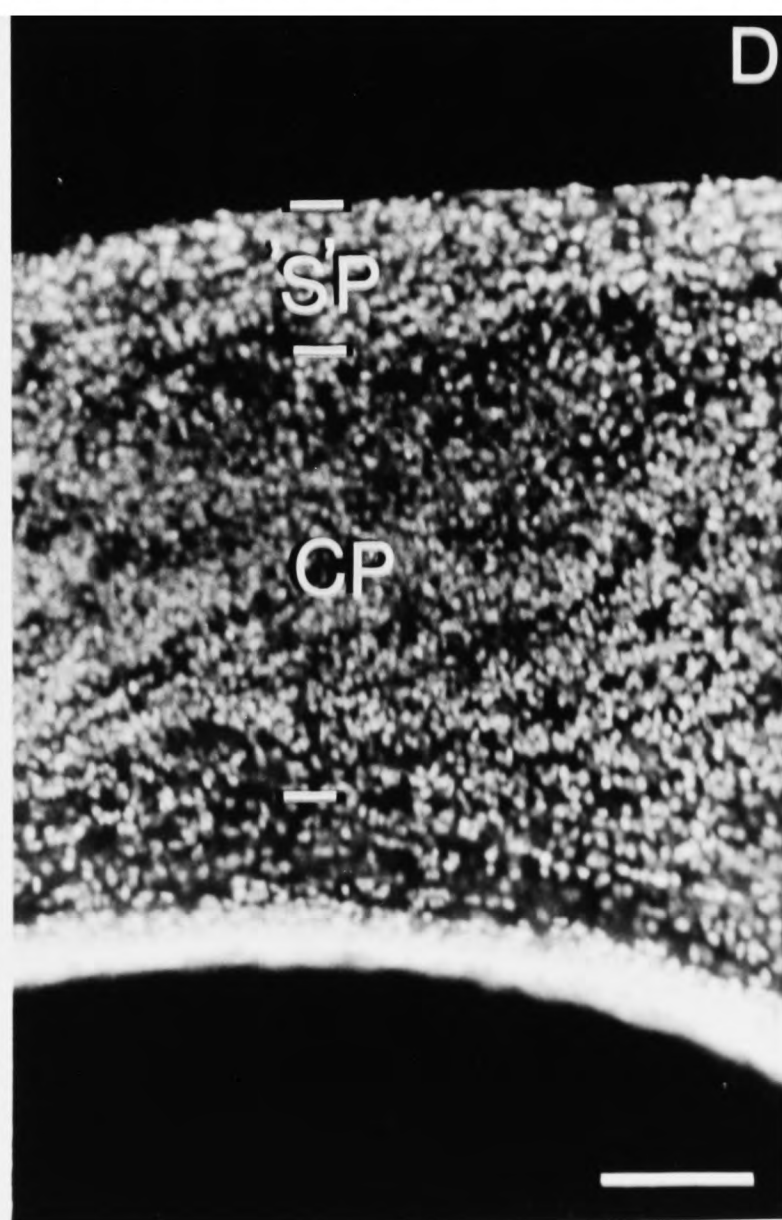
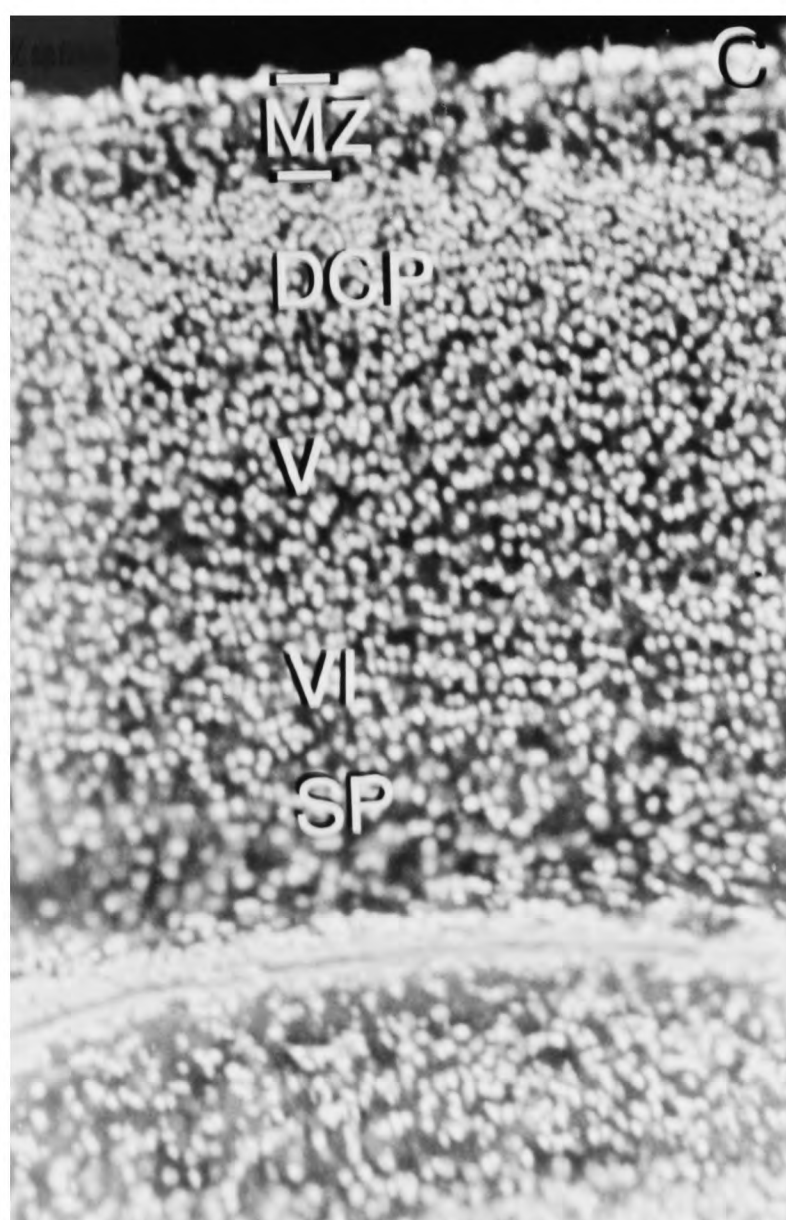
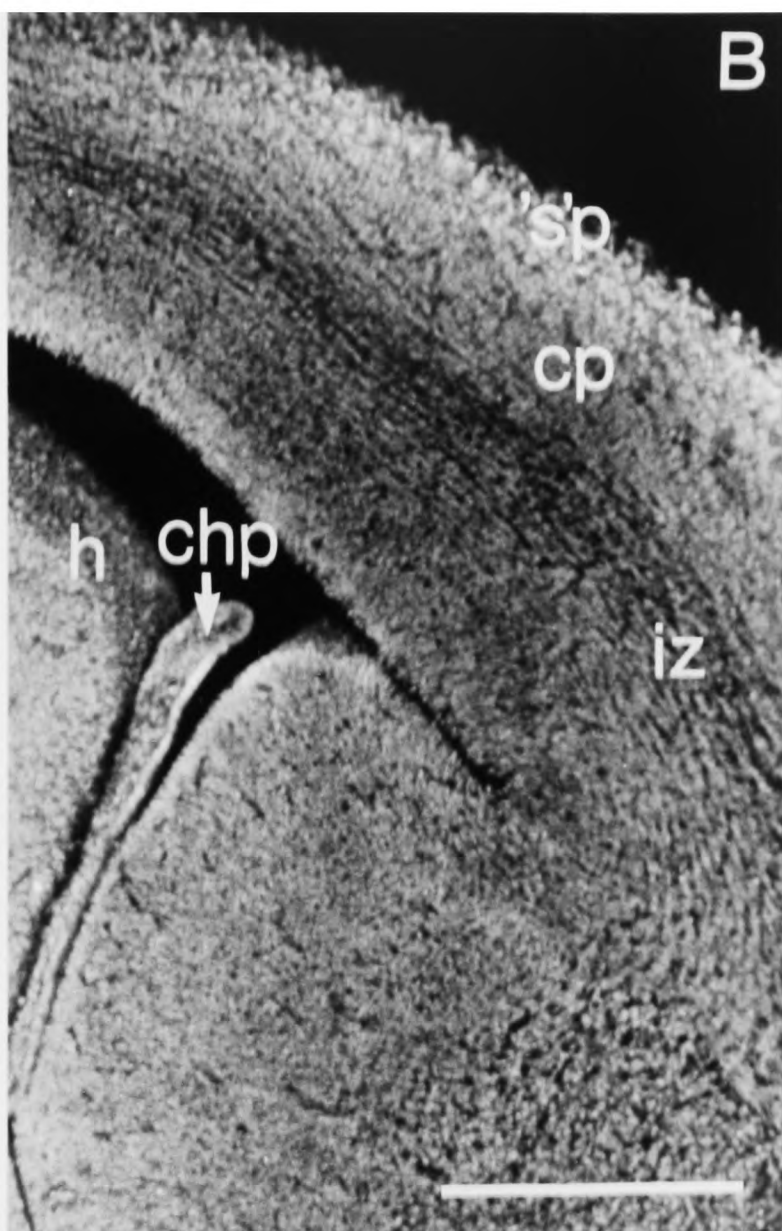
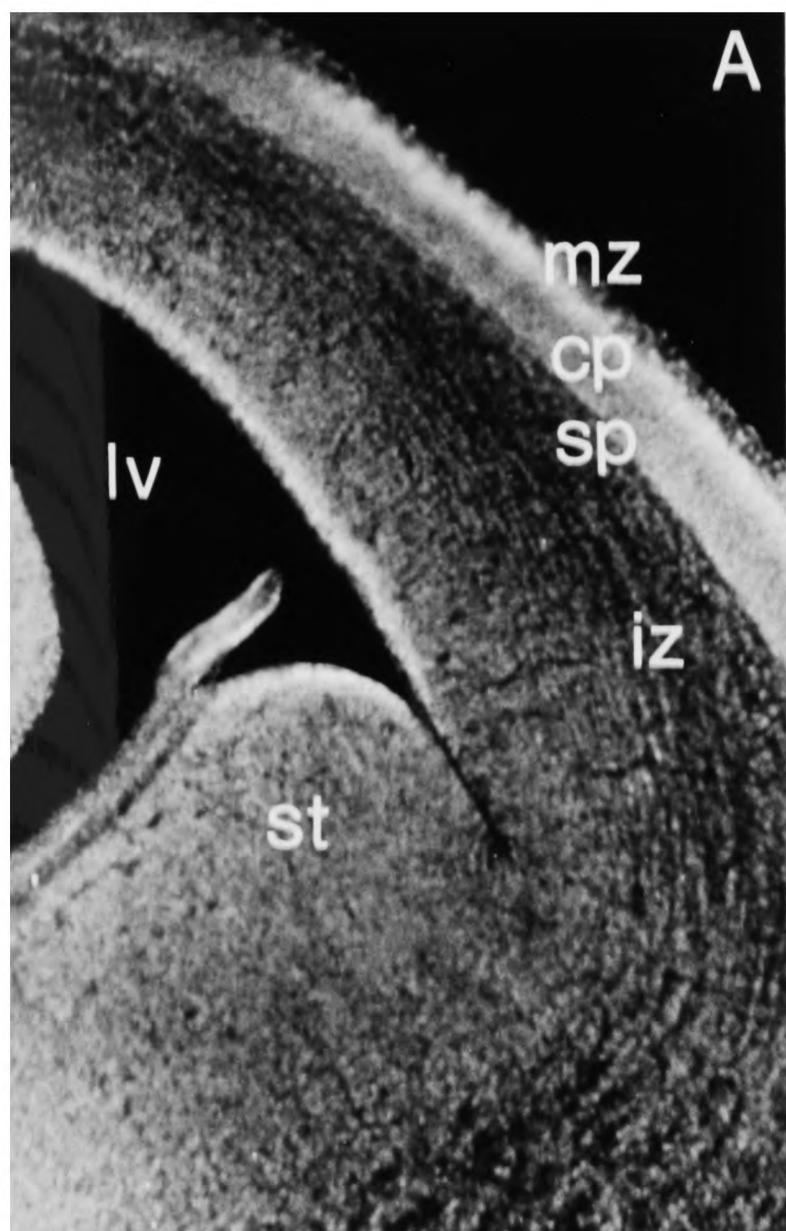
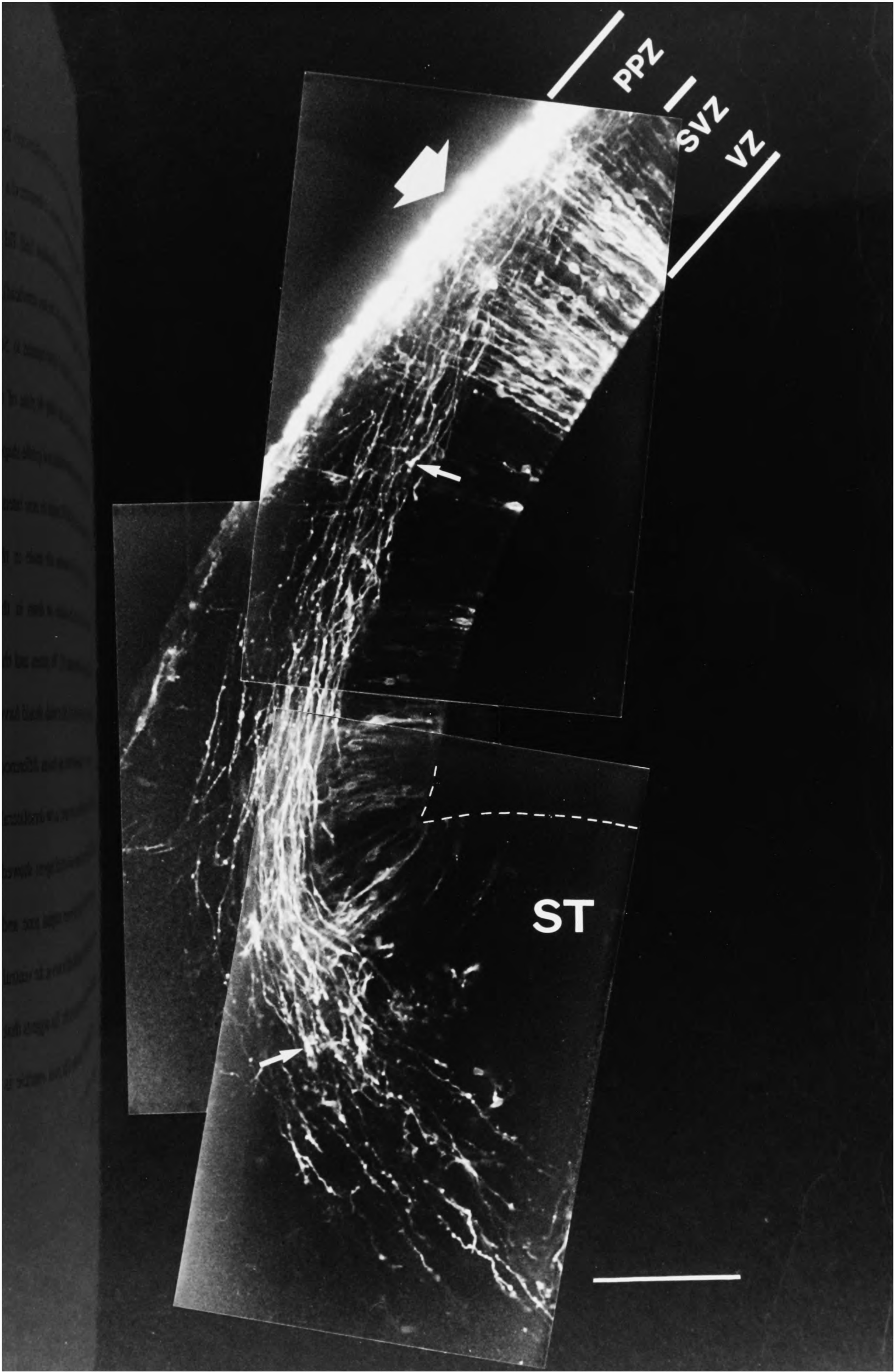


Fig. 4.4

These assembled pictures were taken (using a Biorad confocal microscope) from a coronal section of the dorso-lateral aspect of the cortex of the left hemisphere of a 13.5 days old mouse embryo (75µm-thick, coronal section paraformaldehyde fixed). DiI had been placed in a similar position, on the convexity of the cortex (large arrowhead), of all members of the litter (n=12) of a rl/rl male and rl/+ female (expected to 50% Reeler). The thickness of the optical sectioning was 2µm, avoiding the plane of the brightest part of the crystal placement, thus allowing the visualization of spindle-shaped cells in the ventricular and subventricular zone (VZ and SVZ) outside the most intense part of the halo around the crystal site. The first postmitotic cells reside in the primordial plexiform zone (PPZ) or preplate. Their projections run down in the intermediate zone then turn under the developing striatum (ST). The pattern and the extent of the projections in all members of the litter (n=12, half of which should have had Reeler genotype) were very similar, suggesting that there is no obvious difference between Reeler and normal genotype at this stage in this respect, in the dorsolateral region of the hemisphere. Examination of the anterior ventral cortical regions showed no obvious intervention of the emerging cortical plate between marginal zone and subplate, whereas this pattern, typical of normal animals, could be seen in the ventral anterior part of the hemisphere in some other individuals in the litter. This suggests that this embryo was of Reeler phenotype. The ventral boundary of the lateral ventricle is demarcated with an interrupted line.

Scale bar 100 µm.



PPZ

SVZ

VZ

ST

Fig. 4.5

Descending projections from preplate neurons in an E13.5 animal of presumed normal phenotype. Fluorescent photomicrographs were taken from a bisbenzimidazole stained, 100µm-thick, coronal section of this E13.5 brain, viewed by conventional fluorescence microscopy using ultraviolet (A) and rhodamine (B) filters.

A: Bisbenzimidazole staining reveals that at this stage, prior to the appearance of the cortical plate, at least in the posterior part of the hemisphere, the developing cortex consists of primordial plexiform zone (PPZ) and the subventricular and ventricular zones (SVZ and VZ respectively). Although it is rather difficult to state the genotype of the embryo with total confidence, in more anterior sections the first cells of the true cortical plate appeared to be accumulating within the preplate rather than below it, indicating that this particular embryo had normal phenotype.

B: A small DiI crystal was implanted just outside the primitive internal capsule, below the striatum (ST). After 3 weeks diffusion the dye back-labelled cells through the full thickness of the demonstrating preplate indicating that their axons had reached the crystal placement site in the internal capsule by E13.5.

Scale bar 200µm.

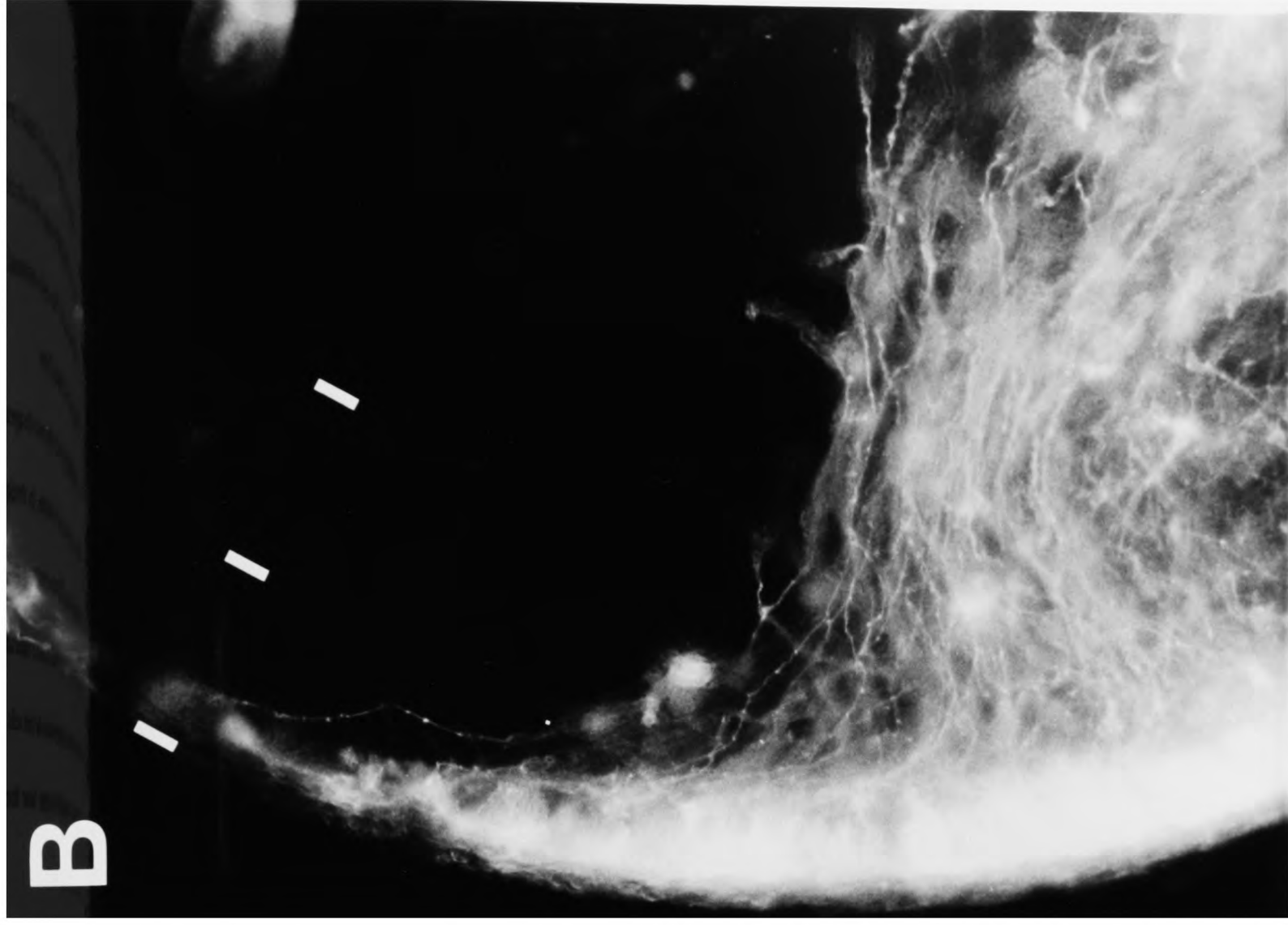
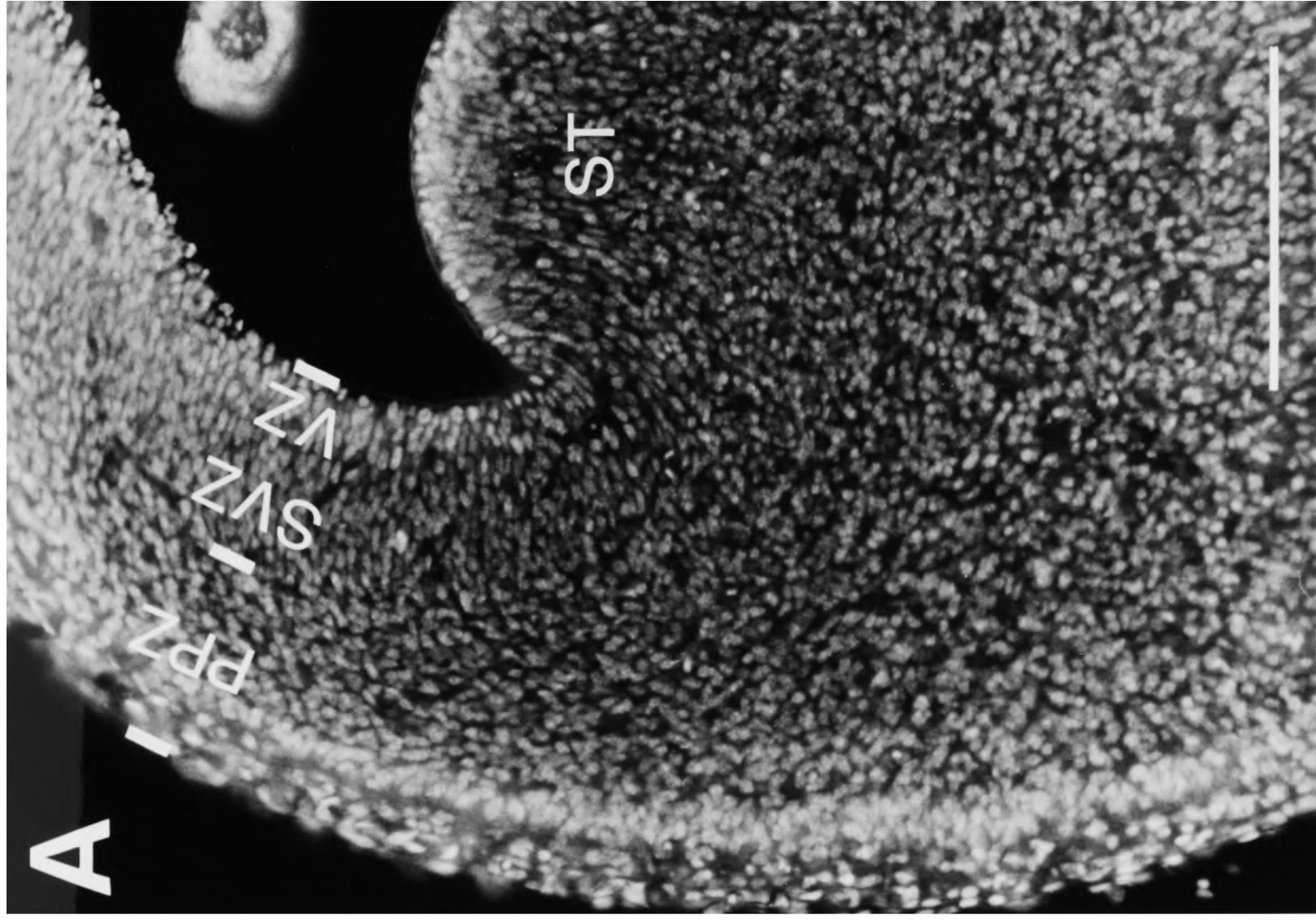


Fig. 4.6

A: Descending projections from preplate neurons in an E13.5 animal of presumed Reeler phenotype. As for the example in Fig. 4.5 a small DiI crystal was implanted just outside the primitive internal capsule of another E13.5 embryo. After 3 weeks diffusion the dye had again back-labelled preplate cells.

B: Same field as A but the photomicrograph was taken by double exposing the views using rhodamine and ultraviolet filters. Bisbenzimid staining reveals the primordial plexiform zone (PPZ) and the subventricular and ventricular zones (SVZ and VZ respectively) indistinguishable from the example in Fig. 4.5. However, in this embryo, bisbenzimid counterstaining of more anterior sections did not reveal splitting of the preplate by cells of the emerging cortical plate, suggesting that this particular embryo was of Reeler phenotype. Although classification of phenotype is difficult in the majority of embryos at this stage, the arrangement of back-labelled cells and their processes after crystal placement near the primitive internal capsule appeared very similar in all members of both E13.5 litters examined (6 and 12 embryos from two litters from a rl/+ mother and a rl/+ father). These litters should have contained approximately 25% Reeler phenotype. Scale bar 200µm.

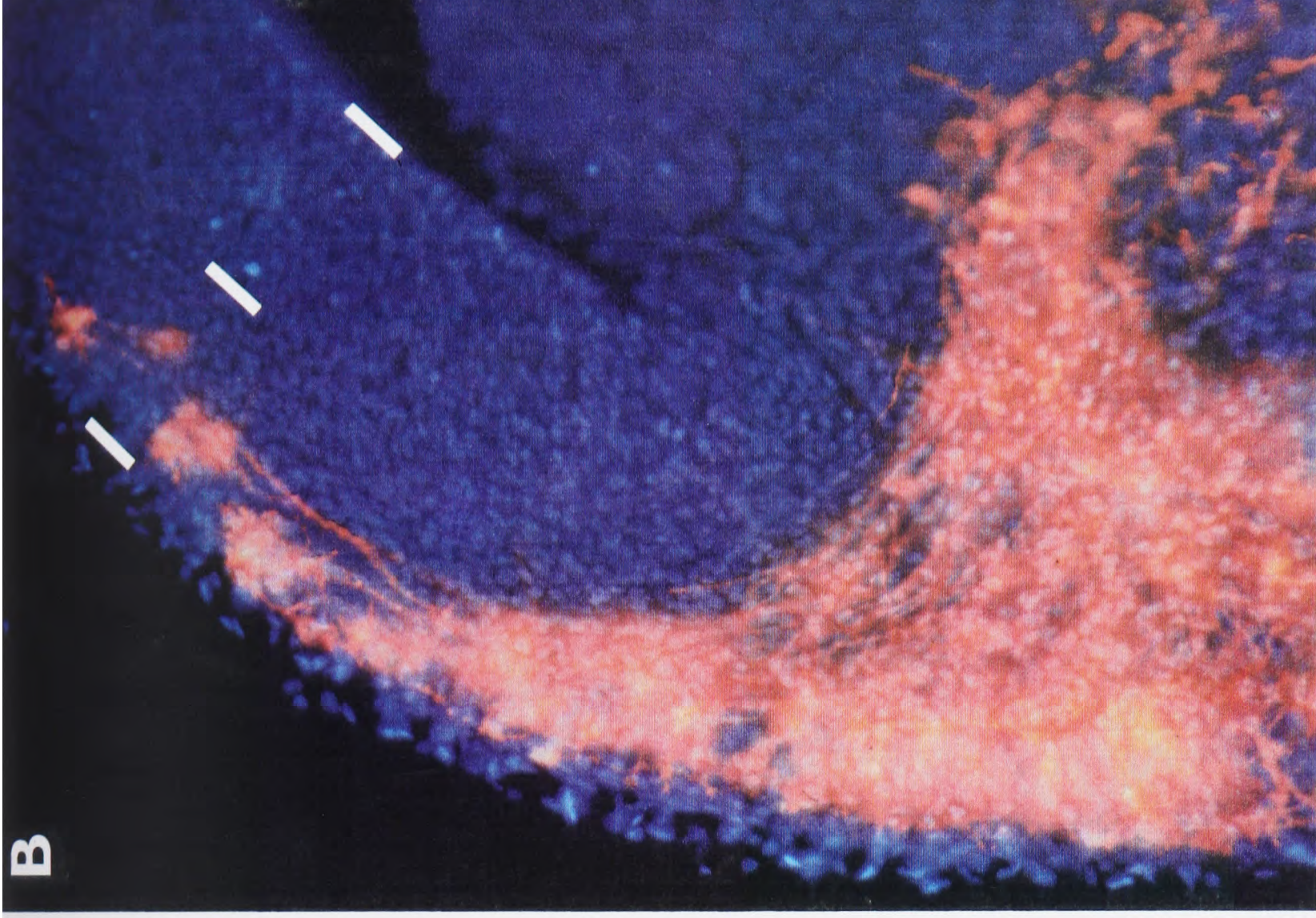
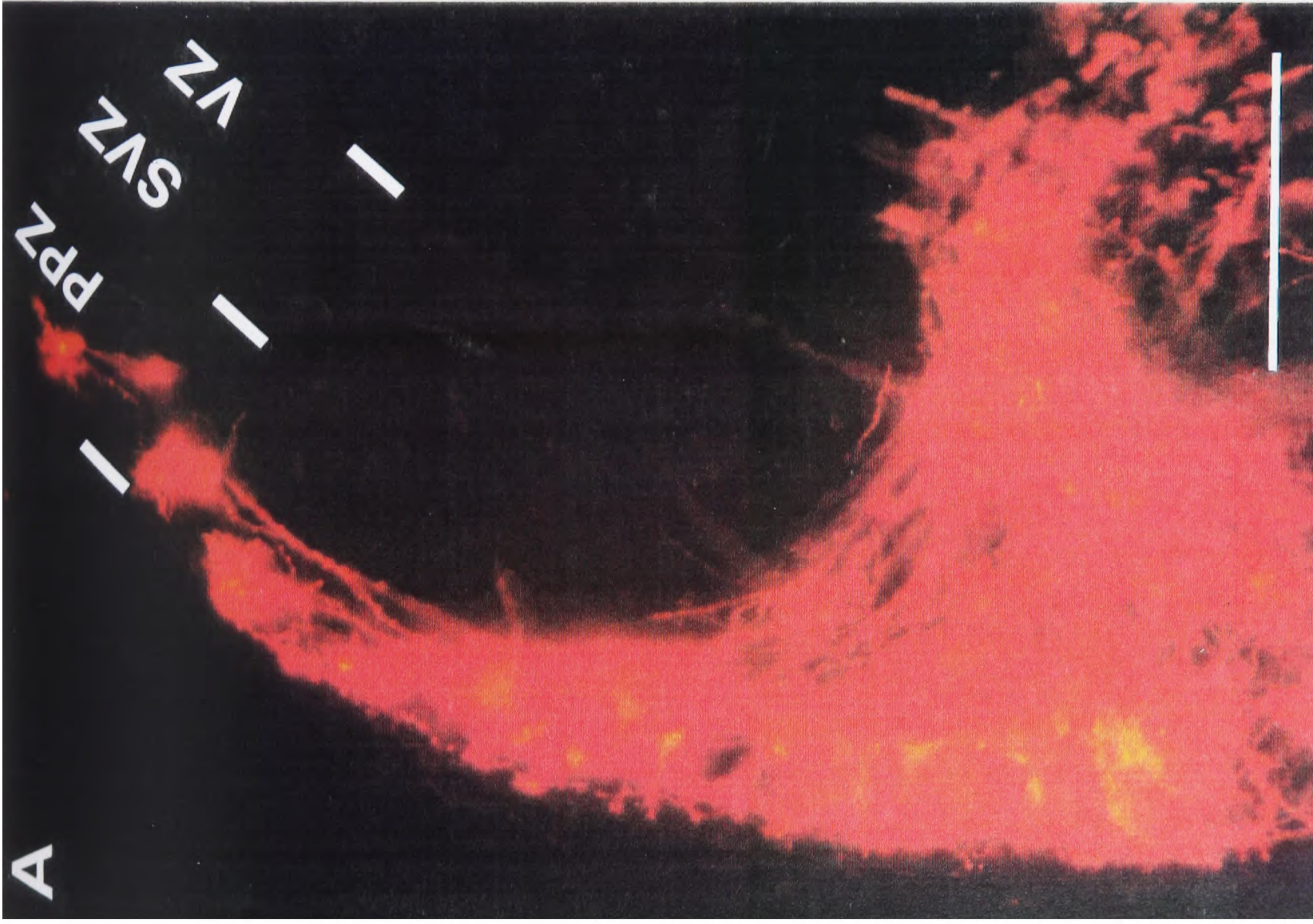


Fig. 4.7

To reveal the more detailed morphology of the cells in the primordial plexiform zone back-labelled (from internal capsule) and to examine the fibre ordering of the early corticofugal projections 3-D confocal microscopic reconstructions were made from the presumed Reeler specimen illustrated in Fig. 4.6. A Leica laser scanning confocal microscope was used to collect 2µm-thick optical sections, then 30 adjacent optical sections were superimposed.

A: Low-power view of the same segment as Fig. 4.6 but now the cells and individual fibres can be resolved.

B: High-power view from a segment indicated with a box in A. Note that the fibres originating from cells in more ventral parts of the section (to the left) take positions superficial to those from dorsal cortex.

Scale bar in A 150µm, in B 30µm.

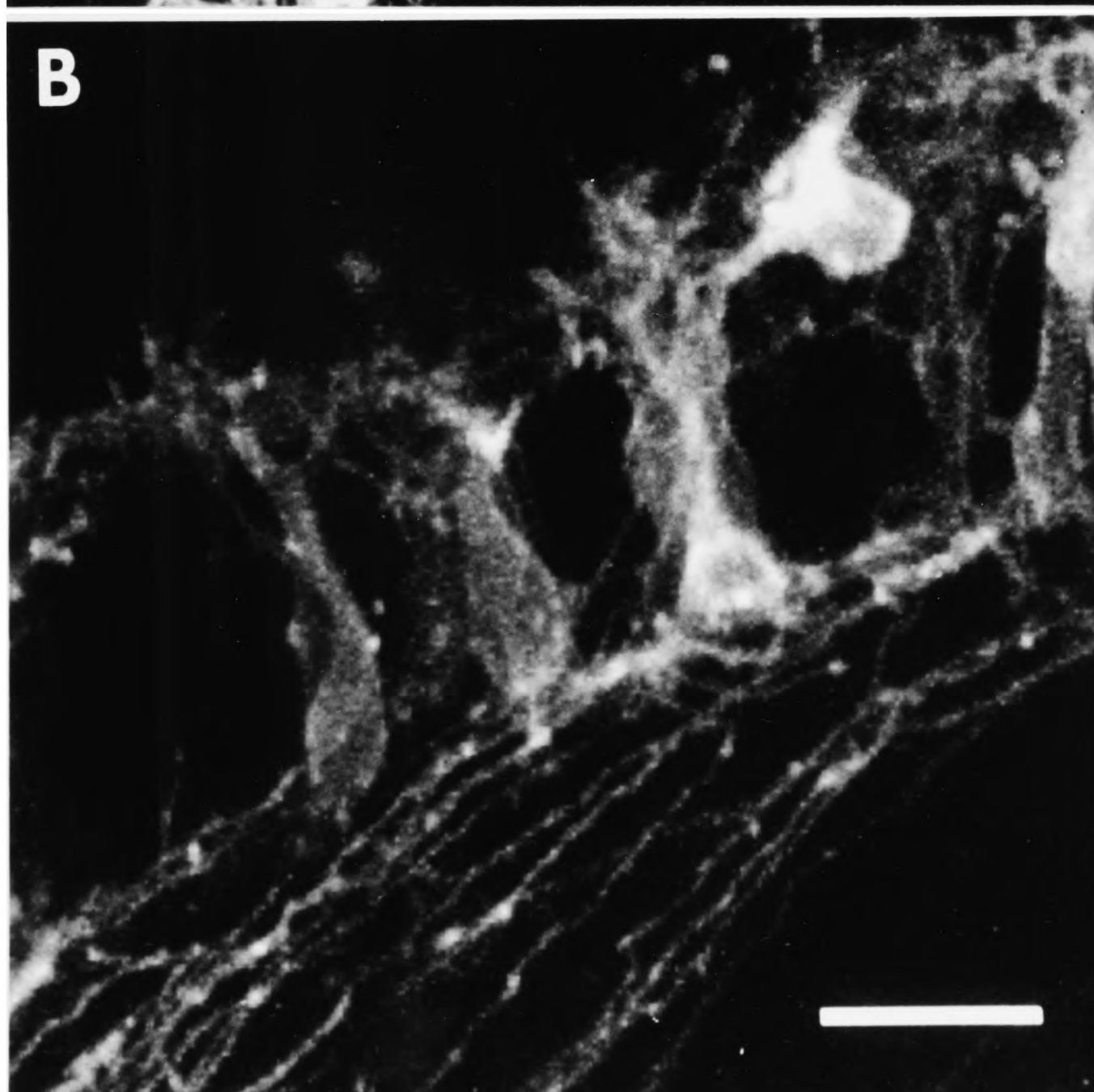
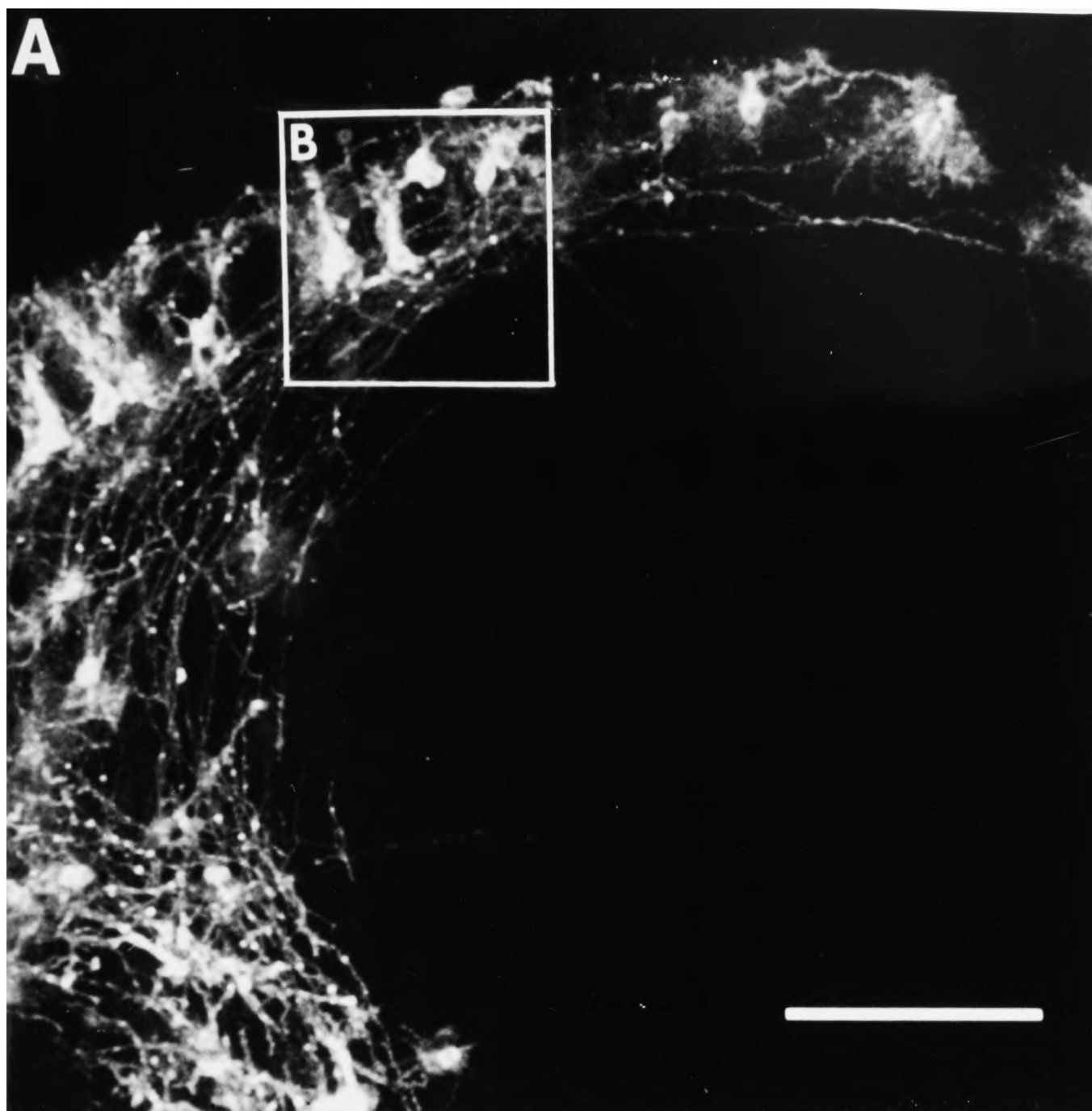


Fig. 4.7-II

From the same region of cortex in the presumed Reeler specimen of Fig. 4.7, 3-D datasets were constructed by confocal microscopy. The datasets were projected with 14 deg disparity to provide left-right anaglyphs, which should be viewed with the glasses enclosed (inside the back cover of the thesis) for the 3-D effect.

Scale bar 50µm at all parts.

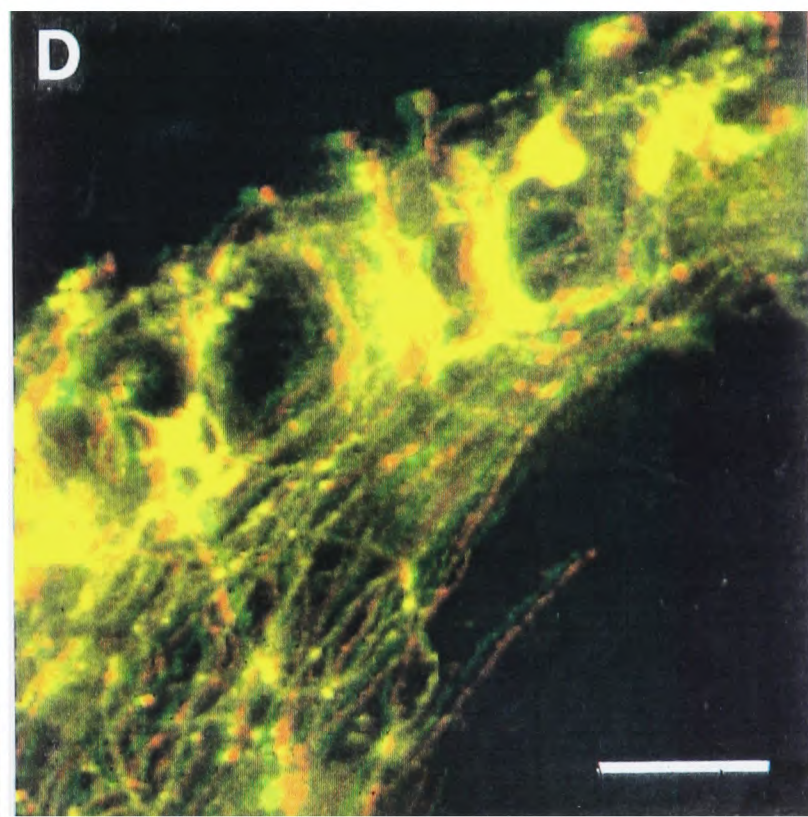
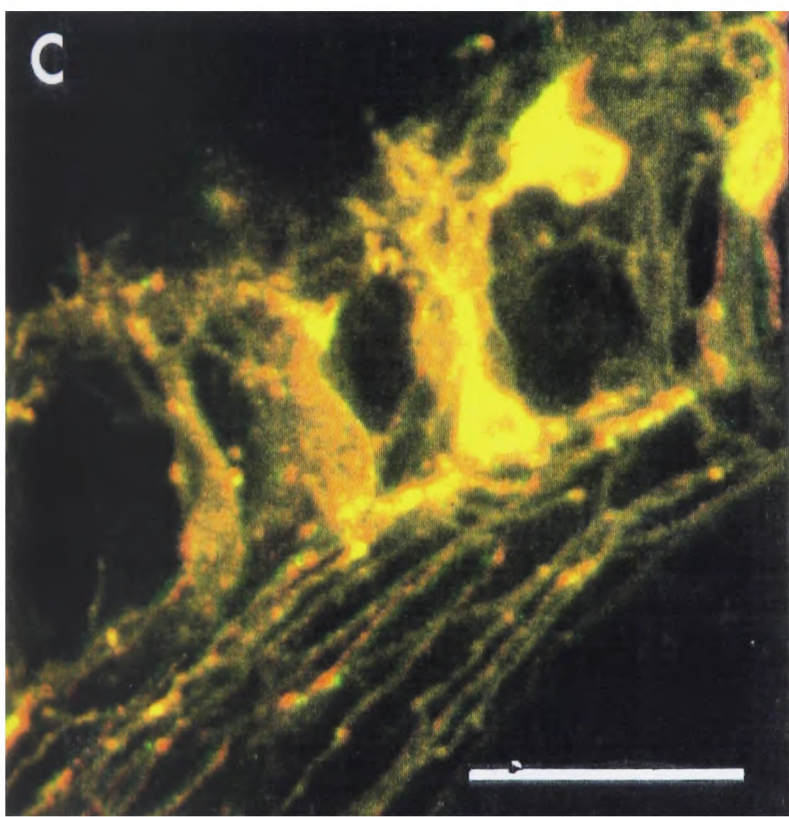
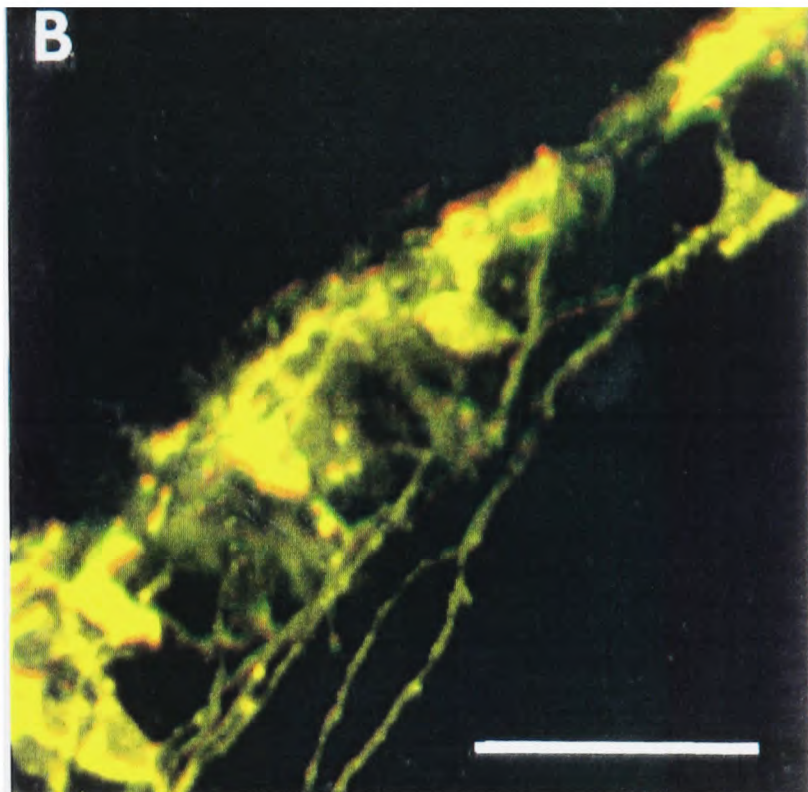
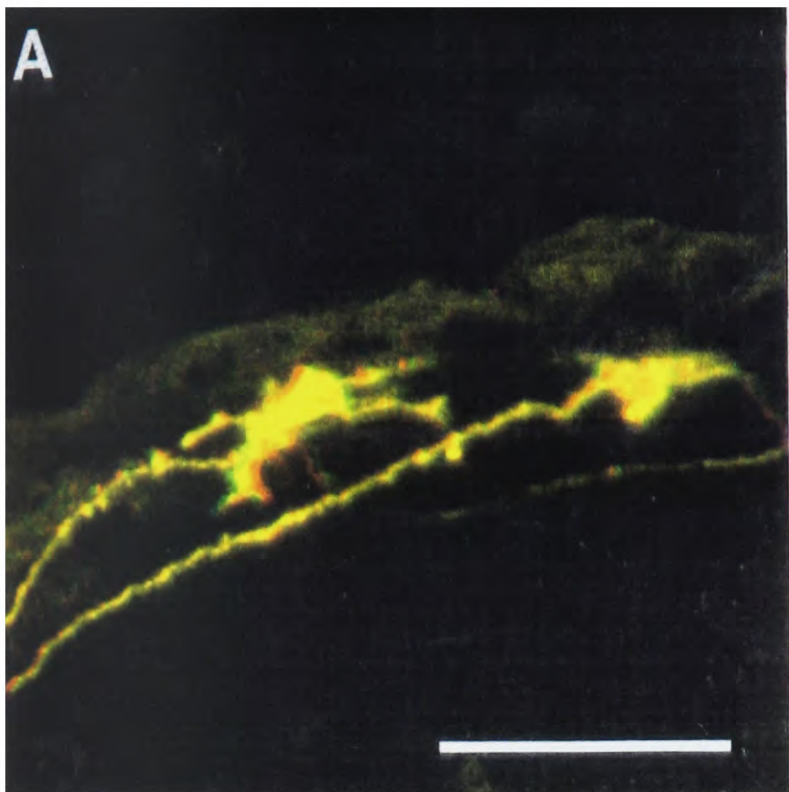


Fig. 4.8

Outflow of thalamic axons in an E13.5 embryo of probable Reeler phenotype.

A: Coronal section of the E13.5 brain, in which a single DiI crystal had been placed into the dorsal part of the thalamus (filled arrow), viewed by fluorescent microscopy using a rhodamine filter. (Dorsal is up and lateral to the right at all parts of the Figure.) The fibres originating from the area of the placement site run ventrally then turn laterally to pass through the primitive internal capsule (IC). The preparations were counterstained with bisbenzimidazole and the micrographs in B,C and D were taken using two different filters (rhodamine and UV) double-exposing the same field.

B: High-power view of the injection site. There are labelled cells in the epithalamus and in the ventral thalamus (labelled ET and VT).

C: Adjacent section to A, demonstrating the full extent of the thalamic fibres as they run under the striatum (ST), but not yet reaching the cerebral cortex.

D: High-power view of the labelled fibres passing through the internal capsule. A few cells were also back-labelled around the fibre bundle. Filled arrows indicate corresponding points in C and D.

There was no hint of an interposing cortical plate, even in the most anterior and ventral cortical regions, in this particular individual, suggesting that it was of Reeler phenotype. The result is, in any case, representative of all members (12) of the litter of a rl/rl male and a rl/+ female, roughly half of which should have been Reeler.

Scale bars A and C: 300µm, B and D: 100µm

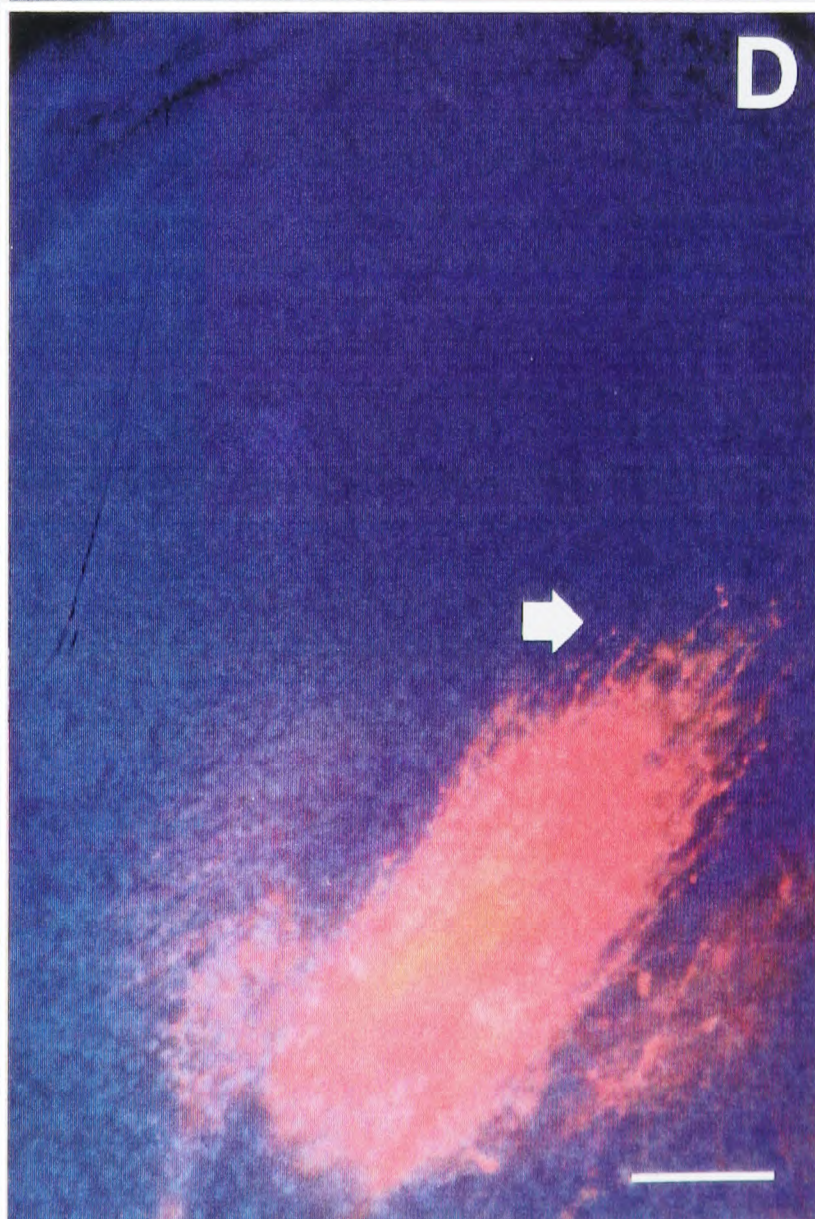
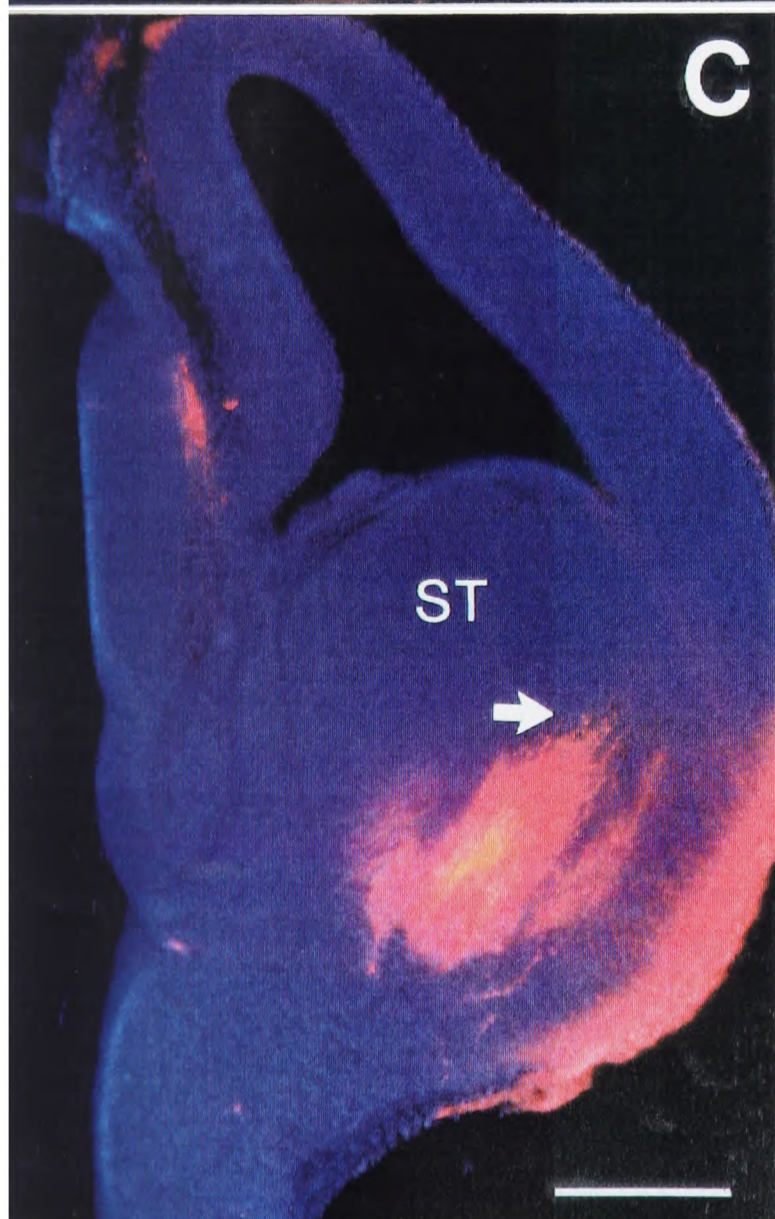
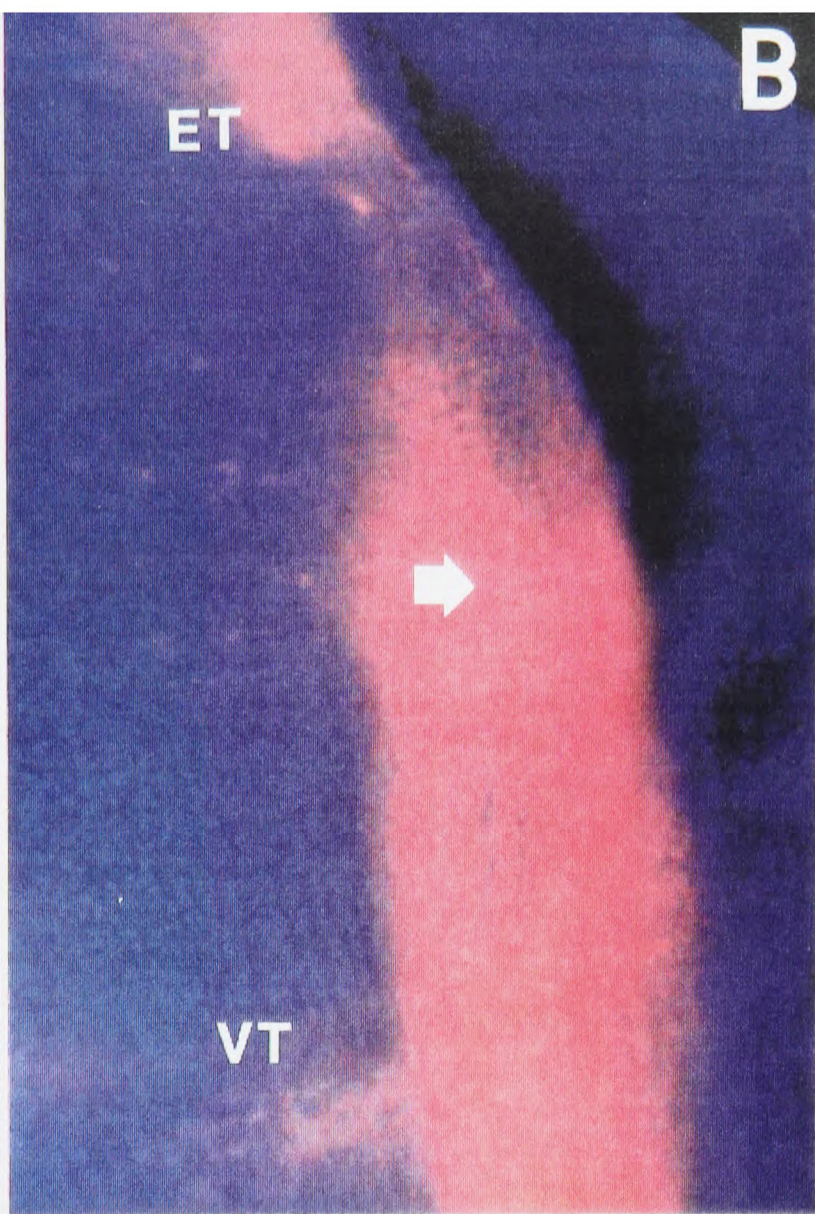
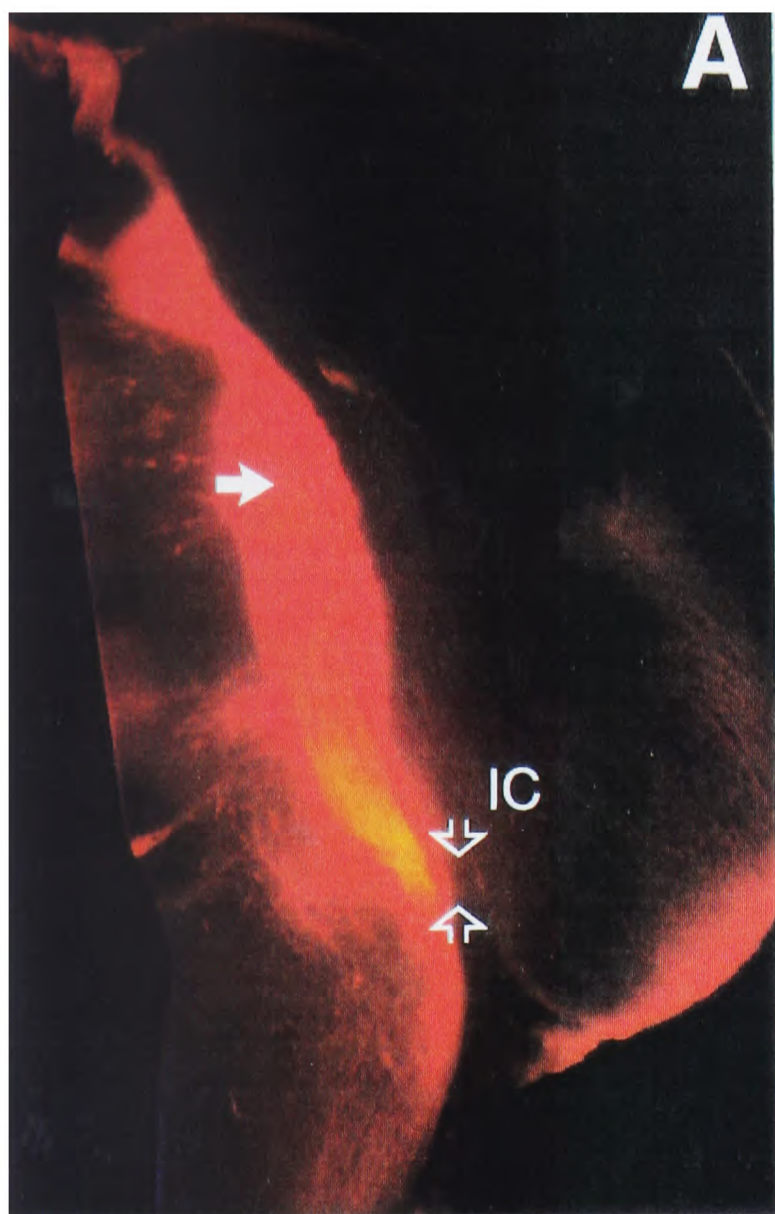


Fig. 4.9

A small crystal of DiI was placed in the convexity of the cortex and a small crystal of DiO in the dorsal thalamus of the same hemisphere of an E13.5 mouse (the phenotype of which would not be judged with confidence even from the most mature cortical regions). The labelled fibres were imaged separately with different photomultipliers using a Leica confocal microscope.

A, B: The earliest corticofugal projections (red) fan out from the intermediate zone and loosen up as they turn under the striatum. The fibres end in large growth cones. The thalamic fibres (green) heading towards the cortex, have just reached the same region.

C: To examine the topography of the DiI labelled earliest corticofugal projections, left-right anaglyphs were constructed from the collected 3-D datasets. Initially the fibres form a narrow array within the intermediate zone then loosen up as they reach the striatum. For viewing, glasses can be found inside the back cover of the thesis.

Scale bar; 250µm for A,B and 100µm for C.

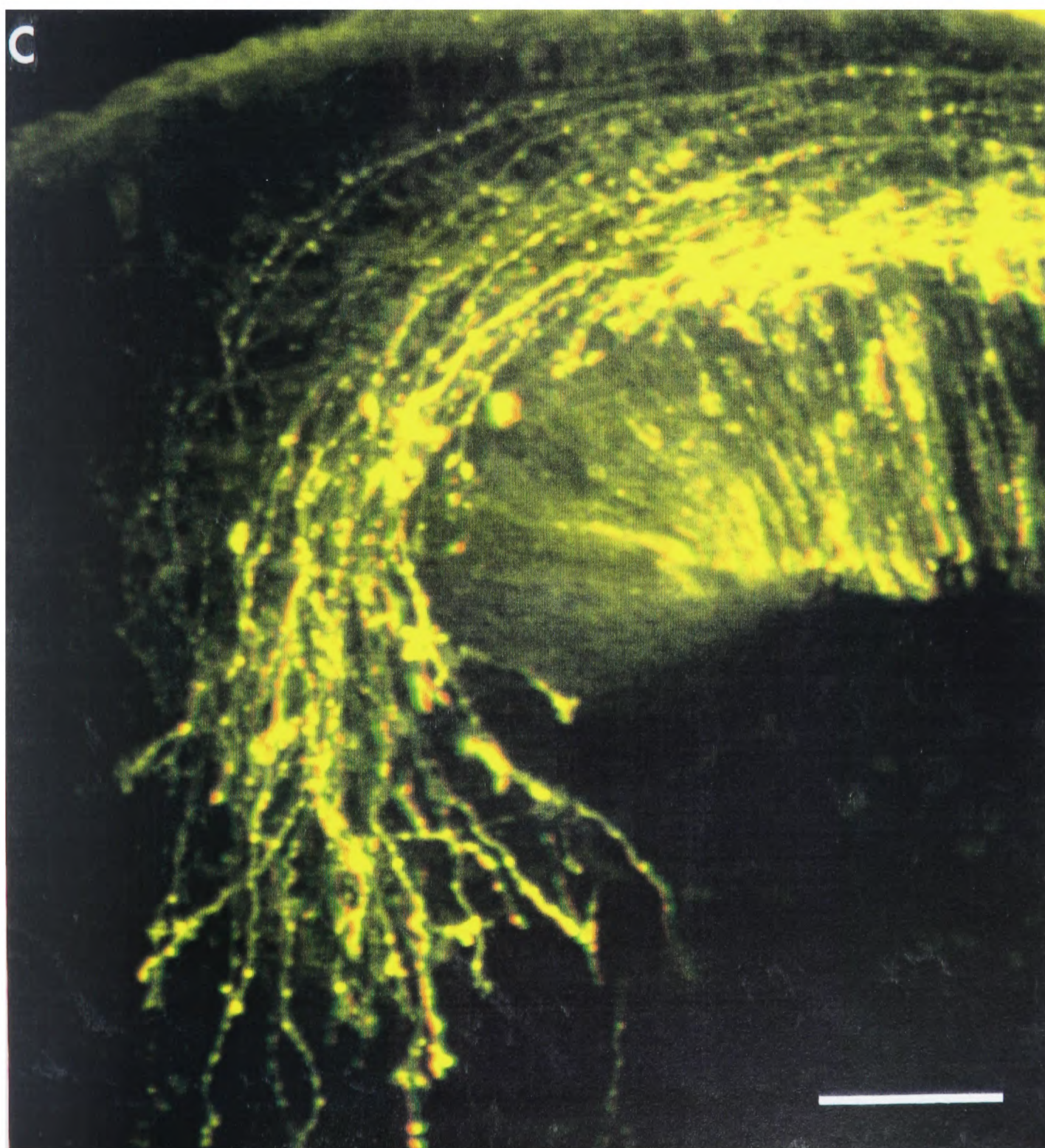
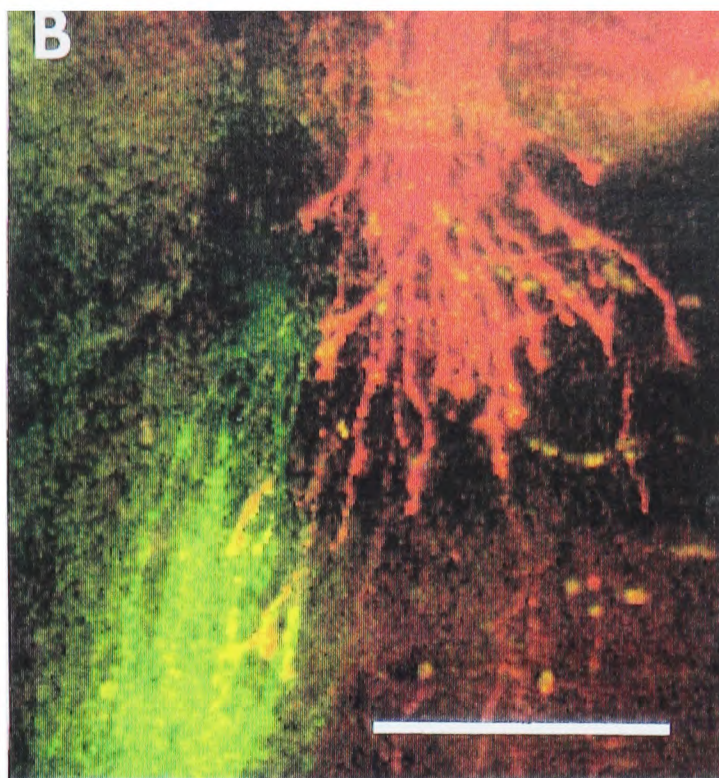
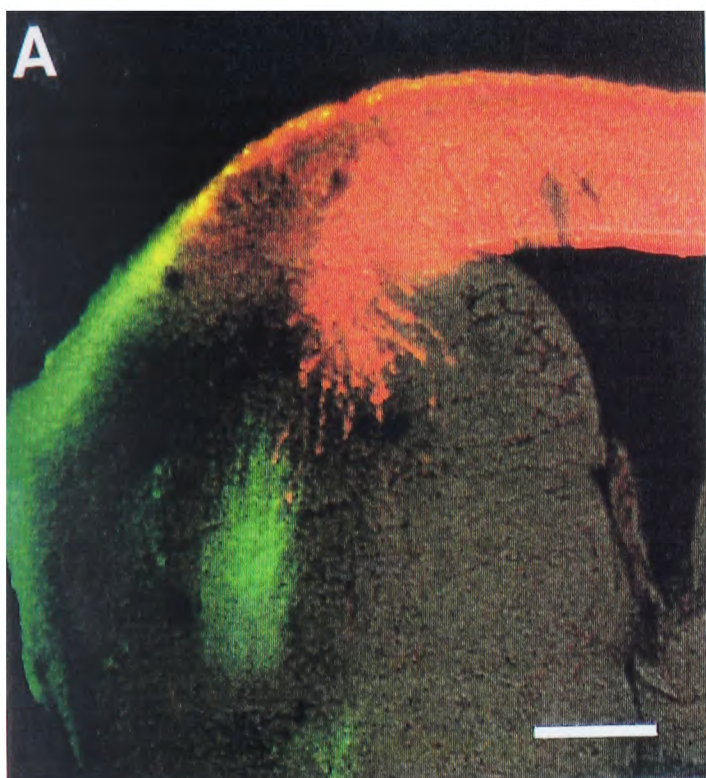


Fig. 4.10

Comparison between preplate projections in normal (A) and Reeler (B) at E14.5. In all members (n=14) of a E14.5 litter from a rl/+ mother and a rl/+ father, the early corticofugal projections were back-labelled from the internal capsule. After 4 weeks incubation at room temperature 100µm-thick coronal slices were cut and counterstained with 10µg/ml acridine orange. 32, 2µm-thick optical sections were collected in the confocal microscope and they were used to construct 3-D datasets. The datasets were projected with 14 deg disparity to provide left-right anaglyphs, which should be viewed with the glasses enclosed.

A: The major difference from the previous E13.5 stage (Figs 4.6-4.9) is that by E14.5, in normal mice, the true cortical plate has split the preplate in all regions of the cortex and forms a distinct layer, approximately 100µm thick, above the subplate. The back-labelled subplate cells are now seen below the cortical plate, with a very small number of back-labelled cells in the marginal zone and in the cortical plate itself. The fibres (probably thalamocortical as well as corticofugal at this stage) run in the intermediate zone and the subplate layer. Note one fibre passes obliquely through the cortical plate and reaches the marginal zone at the right-hand side of the picture and another at the right side. I found similar results in all 12 normal individuals in this litter.

B: In Reeler (2 individuals in this litter) the back-labelled polymorphous, polygonal cells were located immediately below the surface of the cortex in the 'superplate' and their axons were seen crossing the underlying cortical plate in oblique fascicles.

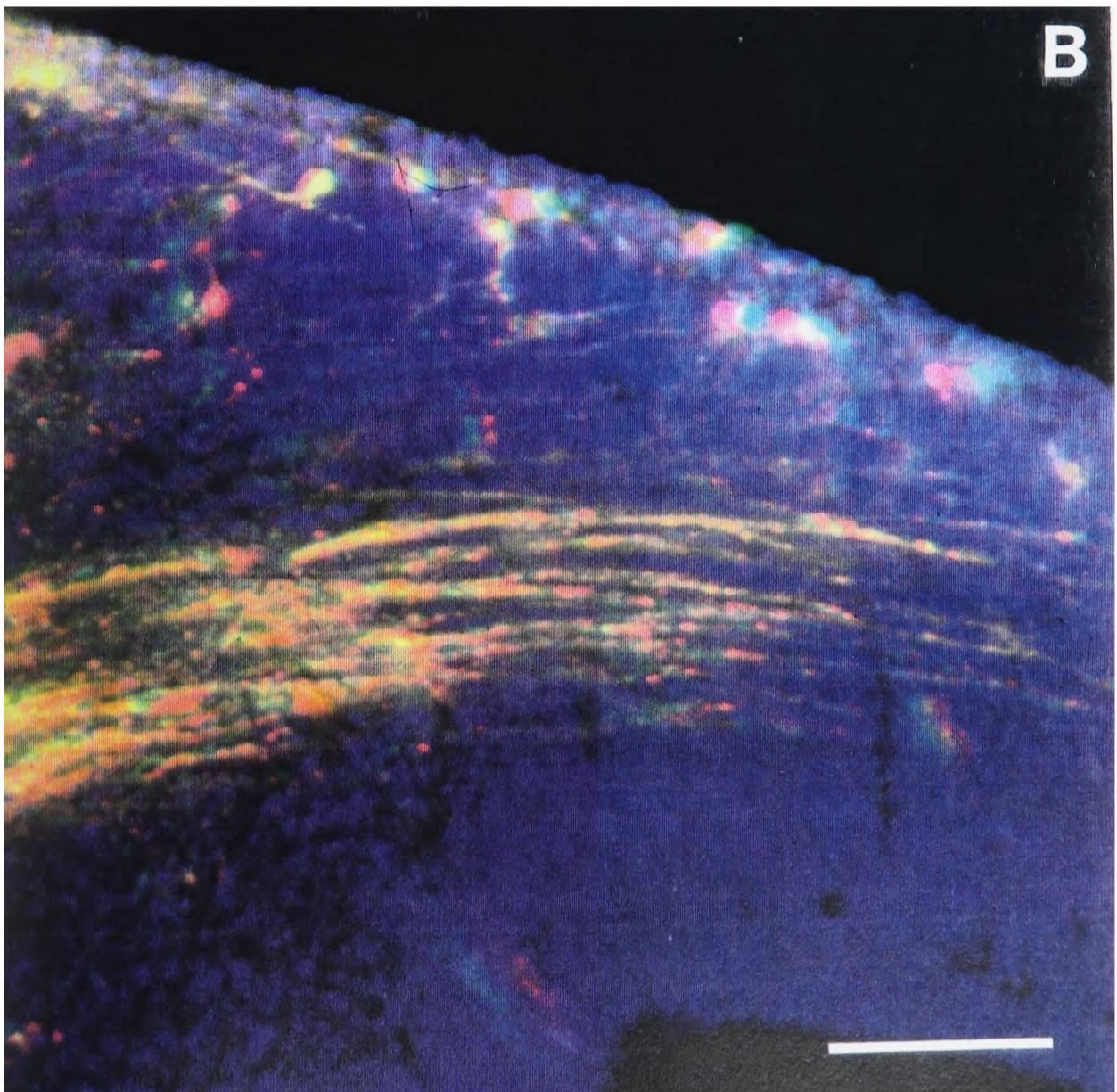
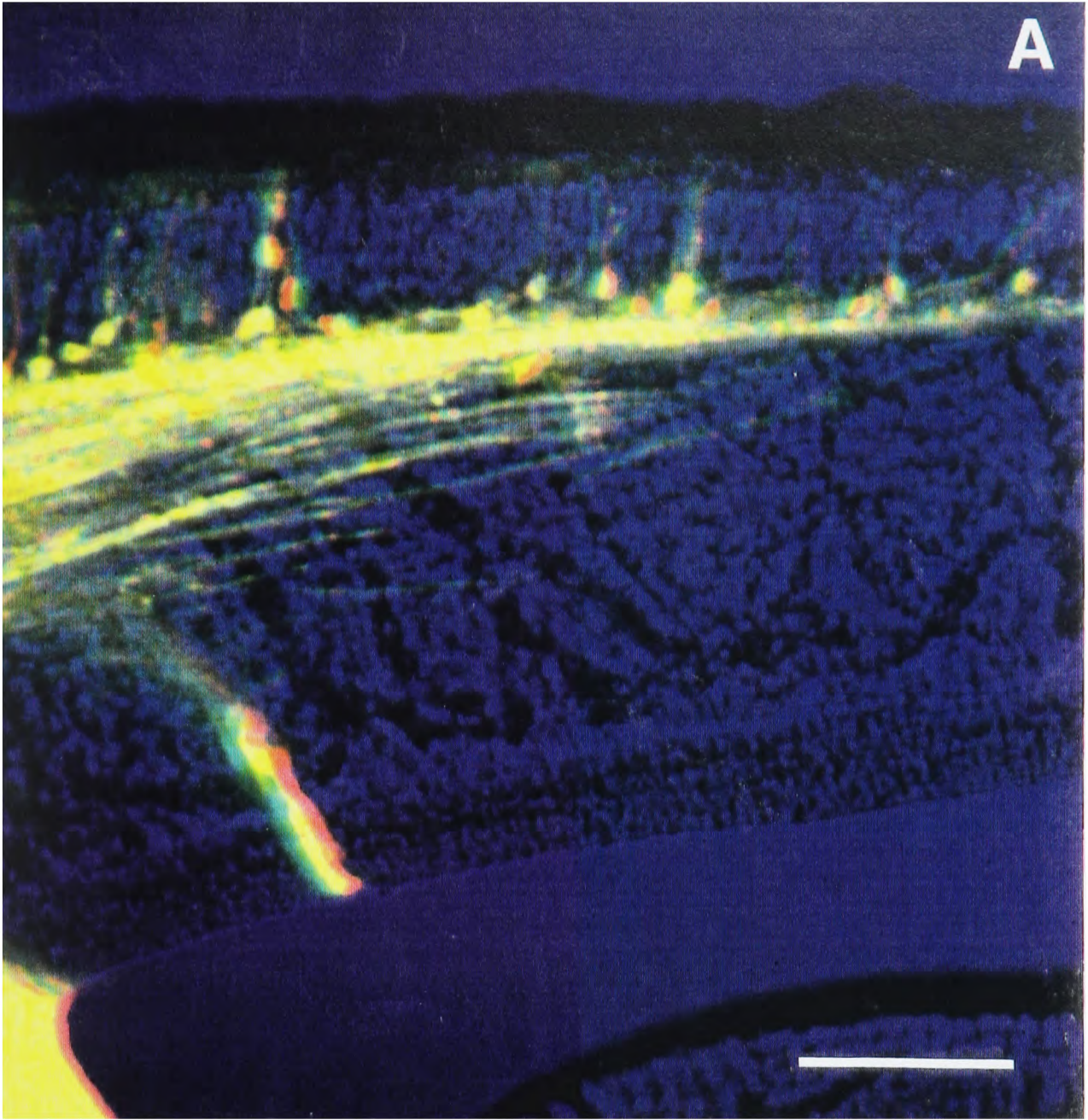


Fig. 4.11

A: Photomicrograph taken with double exposure of the cells back-labelled from the internal capsule in both the subplate **and** the marginal zone of the occipital cortex of an E14.5 normal mouse. The back-labelled cells are mainly situated below and above the plate but some lie within the cortical plate itself, which is only 3-4 cells thick. Scale bar: 200µm

B: High-power view of the polymorphous polygonal cells in the superplate of an E14.5 Reeler mouse (same specimen as in Fig. 4.10B).

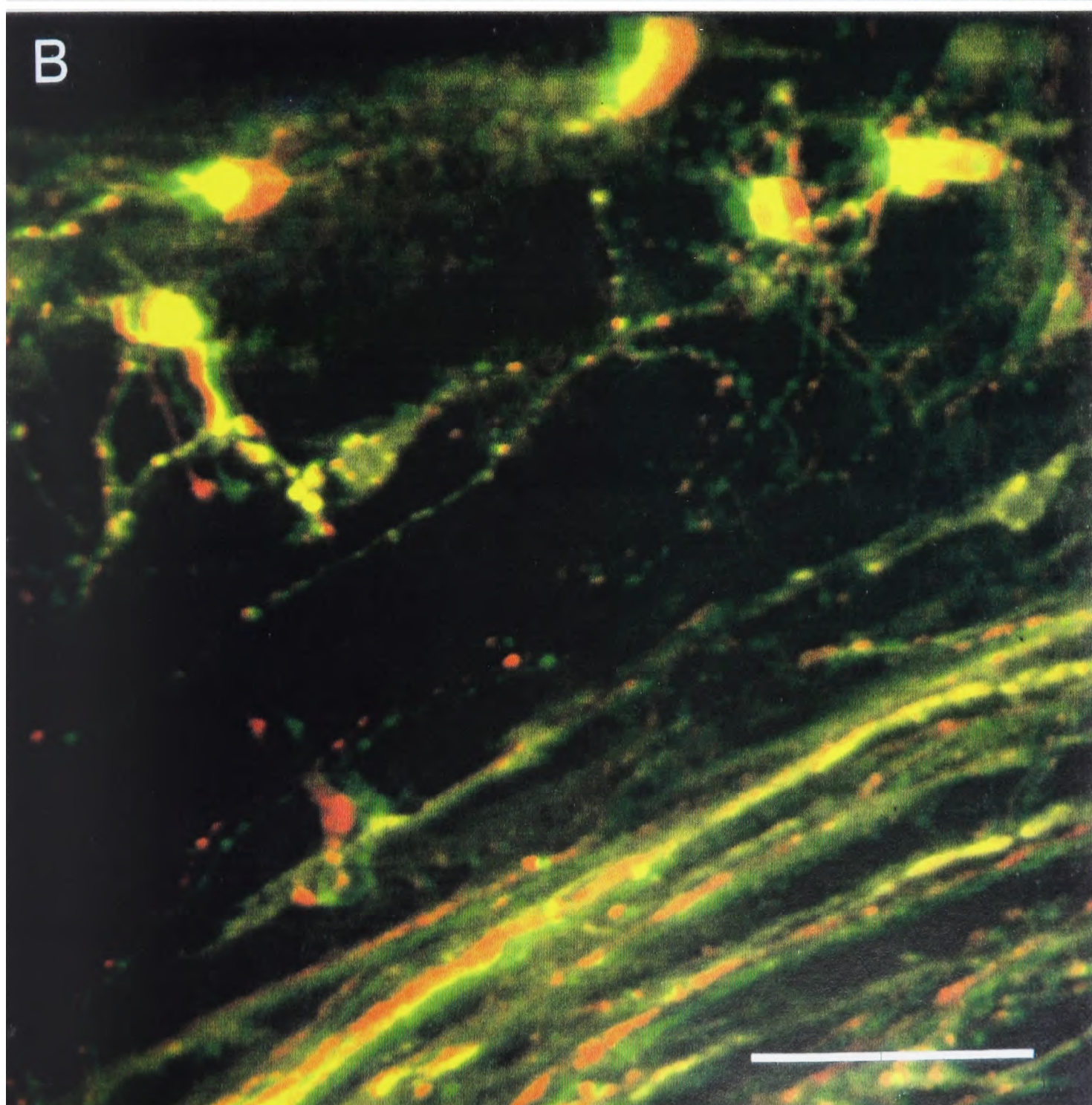
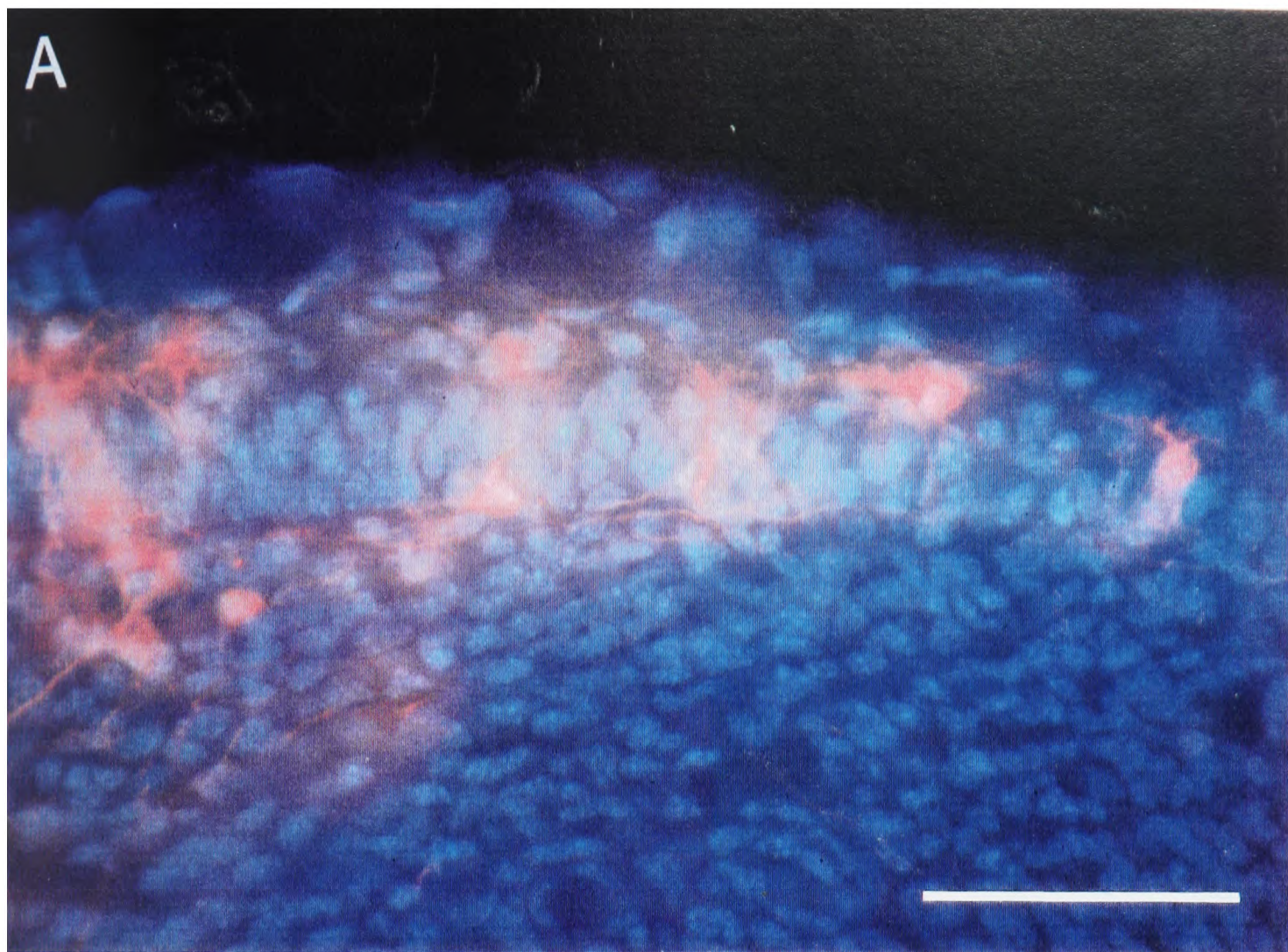


Fig. 4.12

Comparison of thalamic axon distribution at E14.5 in normal (A,B) and Reeler (C,D). A single DiI crystal placement into the dorsal thalamus at E14.5 reveals the thalamocortical fibres as they head towards the cortex in these coronal sections. These fluorescent micrographs were taken from similar sectors of the dorso-lateral aspect of the right hemisphere of the two different genotypes using ultraviolet (A,C) and rhodamine (B,D) filters (right is lateral and left is medial).

The upper two pictures (A,B) were taken from the normal genotype (rl/+), the lower ones from Reeler (rl/rl). The bisbenzimidazole counterstain reveals that in the Reeler the cortical plate (CP) does not split the primordial plexiform zone into marginal zone (MZ) and subplate (SP); instead, the entire population of first-generated cells stays together as the superplate ('S'P).

In the Reeler (D) the thalamic fibres grow all the way up to the superplate, whereas in the normal (B) they are restricted to the intermediate zone and in the subplate. Occasionally growth cones can be observed at the tips of the ingrowing thalamic fibres at this stage. The results demonstrate that the thalamic fibres arrive at the occipital pole after the appearance of the cortical plate.

Scale bar 200µm for all figures.

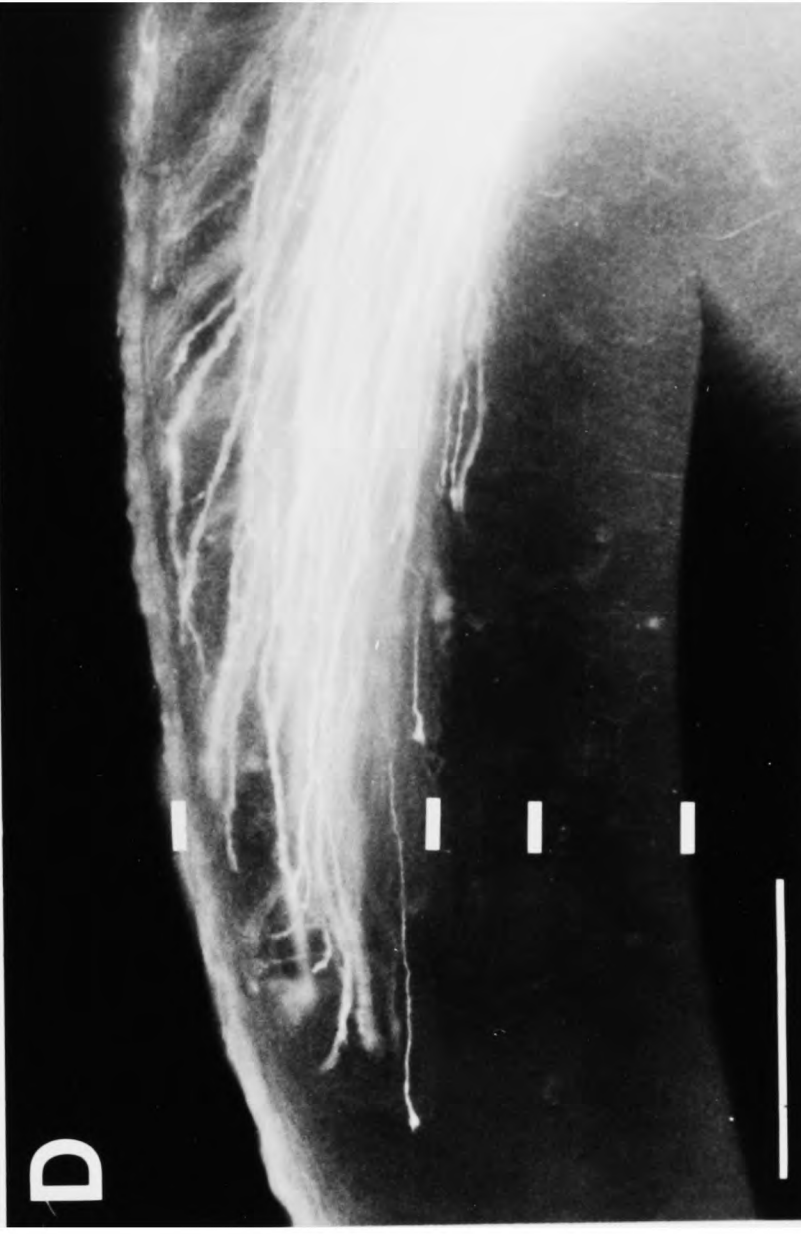
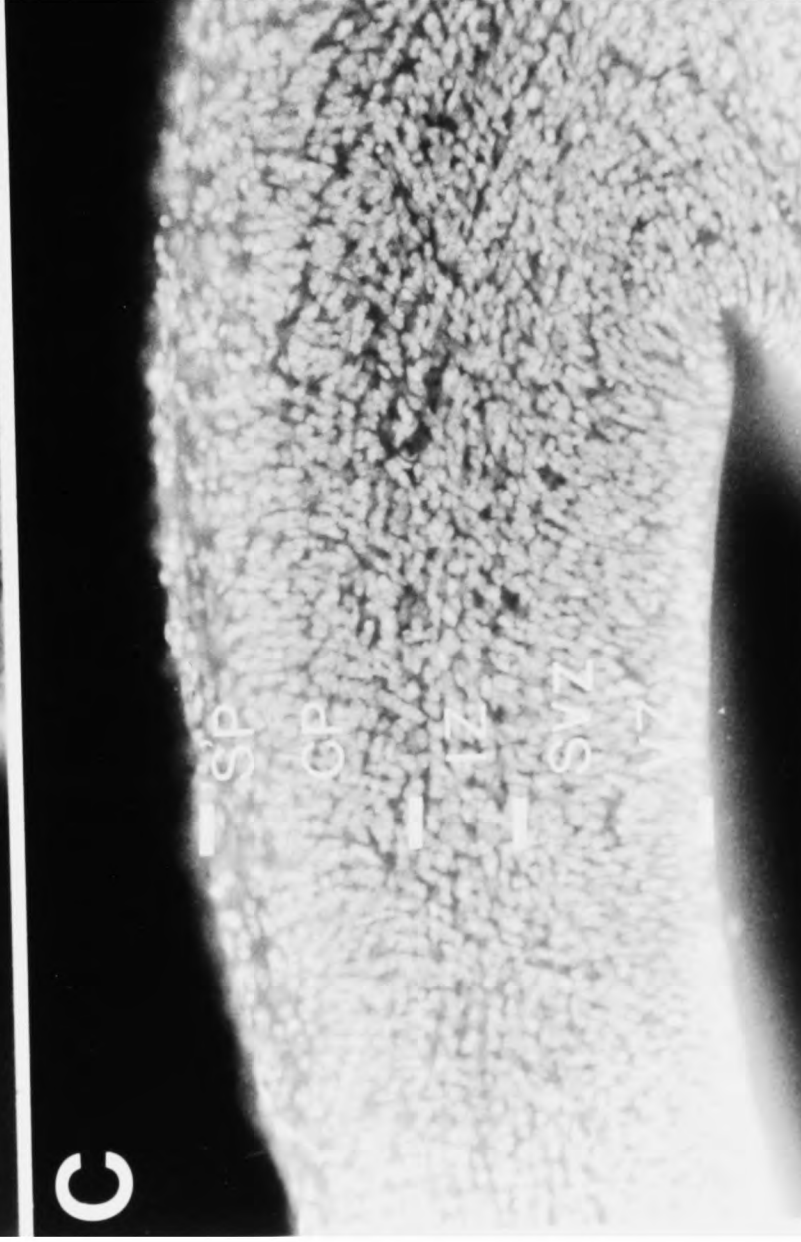
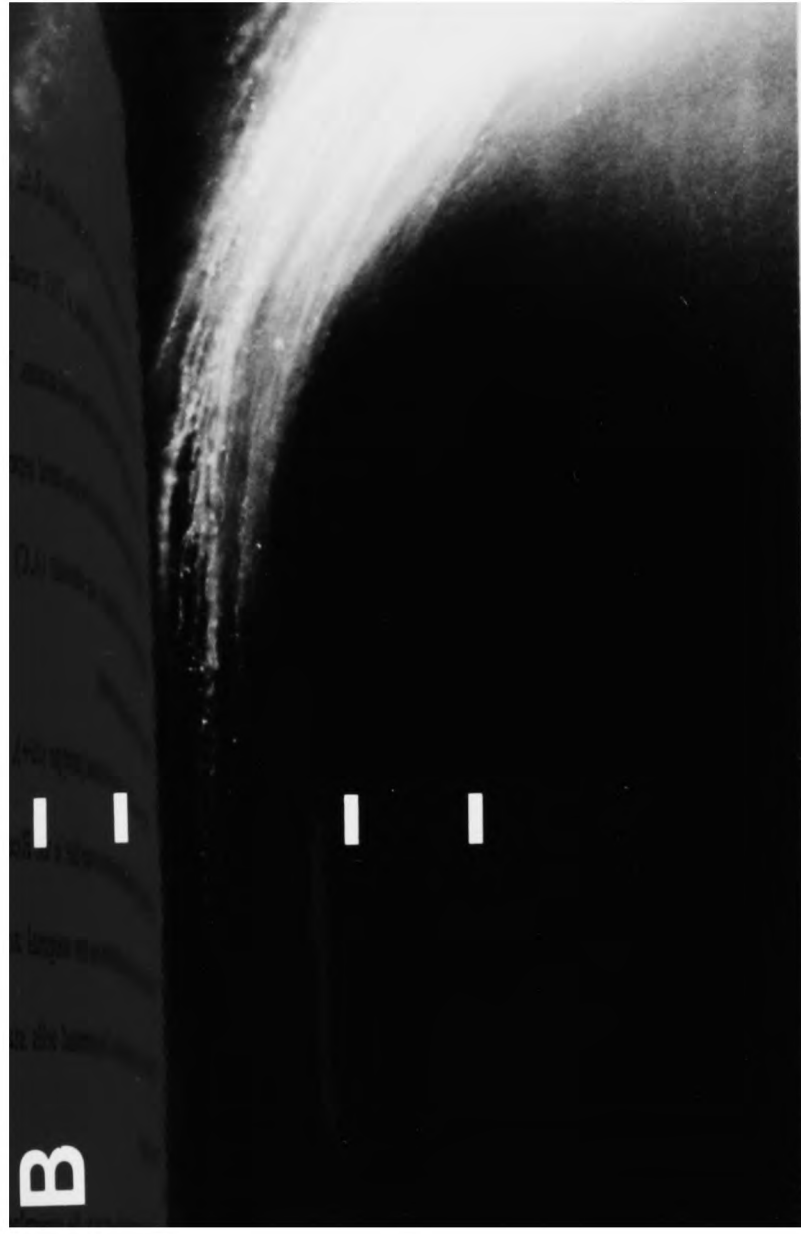
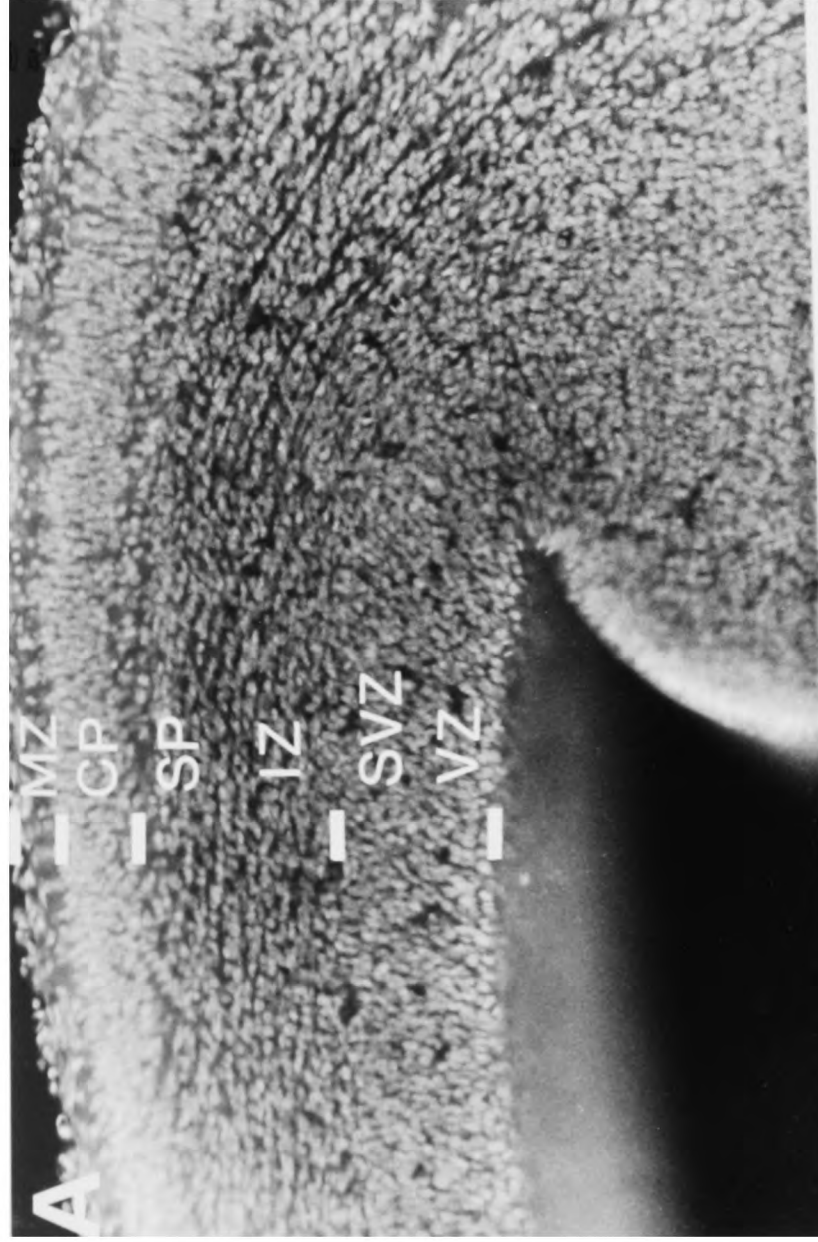


Fig. 4.13

A: A small crystal placement into the internal capsule of an embryonic Reeler (rl/rl) brain at E15 orthogradely labelled thalamocortical fibres and back-labelled the corticofugal projections. Examination of these sections with high power in the confocal microscope showed fascicles of labelled axons passing through the cortical plate to the superplate. Reconstruction of the upper end of one such fibre fascicle revealed a growth cone, presumably of a thalamic axon (large yellow profile) associated with a back-labelled subplate cell whose chromatin is revealed with acridine orange counterstaining (darker profile).

B: The acridine orange staining was imaged with a separate photomultiplier. Only the back-labelled cell contained stained chromatin. Scale bar: 10µm

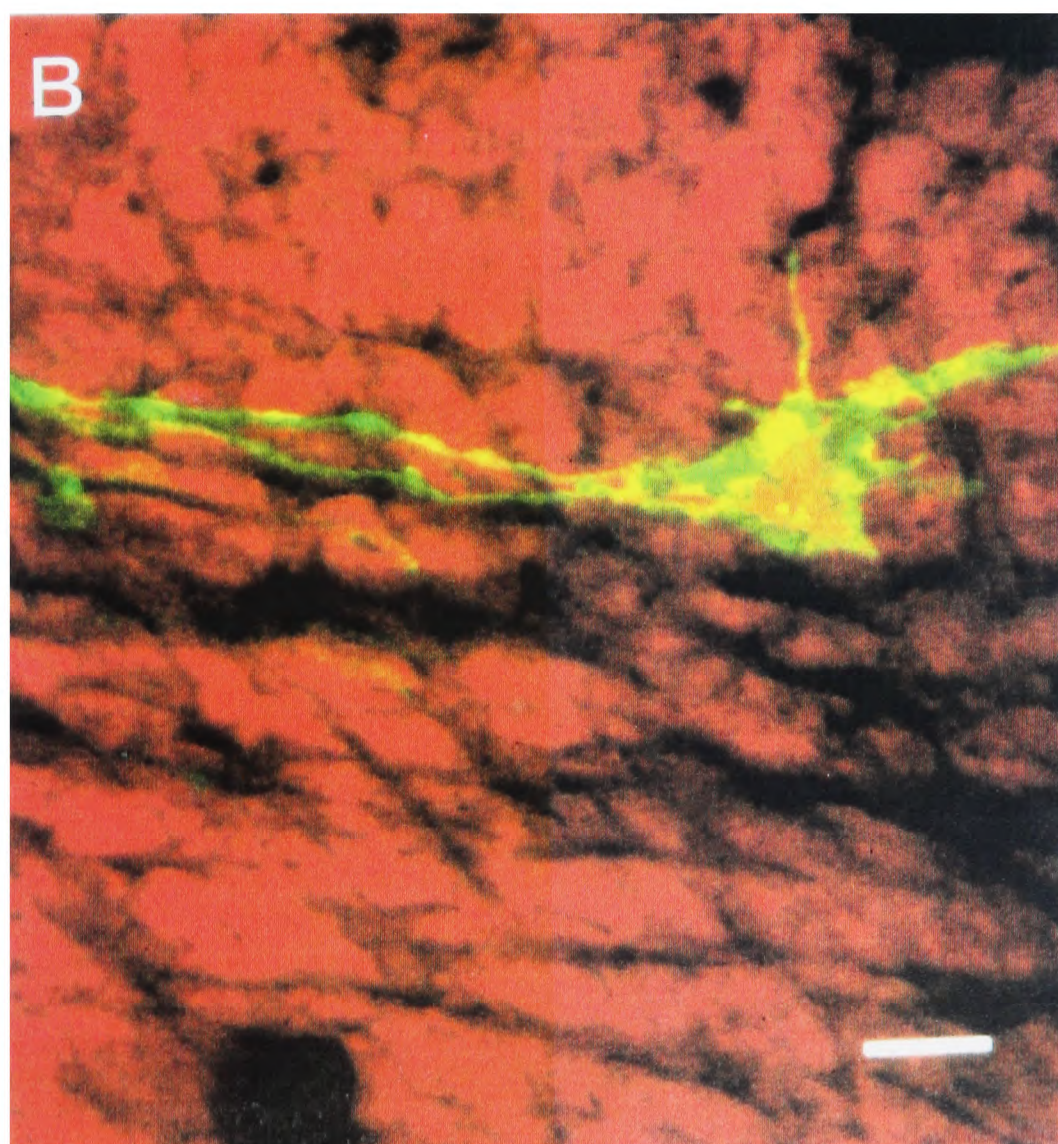
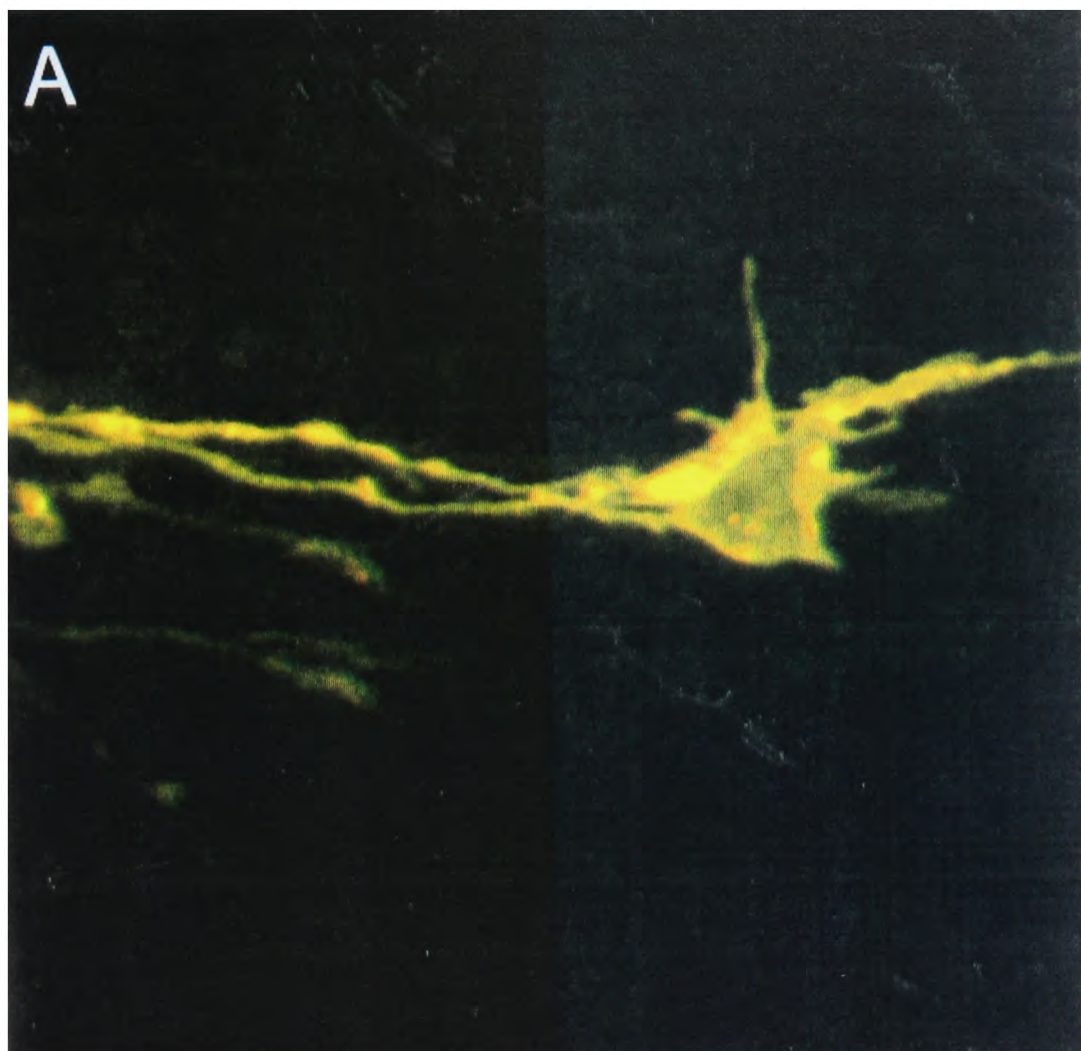


Fig. 4.14

The thalamocortical projection of each member of a E18 litter (n=10, 4 rl/+ normal and 6 rl/rl, Reeler) was revealed by a single DiI crystal placement into the dorsal thalamus (placement site and thalamus not seen). The two phenotypes differ only in the distribution of fibres distal to the striatum/intermediate zone border. In the Reeler (C and D) the fibres run all the way up to the superplate in oblique fascicles, whereas in the normal (A and B) they stay just above the intermediate zone and form localized endings within the subplate. In the Reeler this accumulation of thalamic axons occurs in the superplate, right under the pial surface (see D). Scale bars; 100 µm. B and D were taken from the sectors indicated by arrows in A and C.

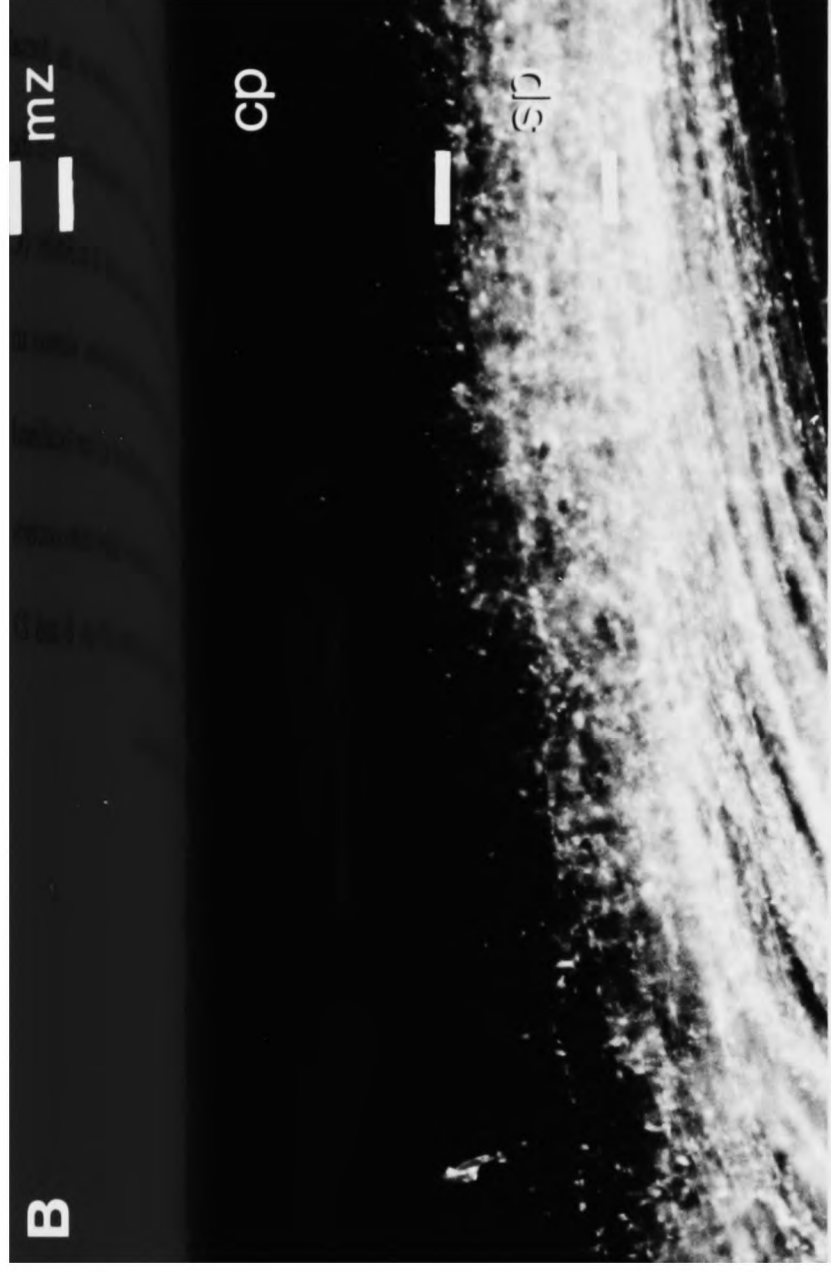
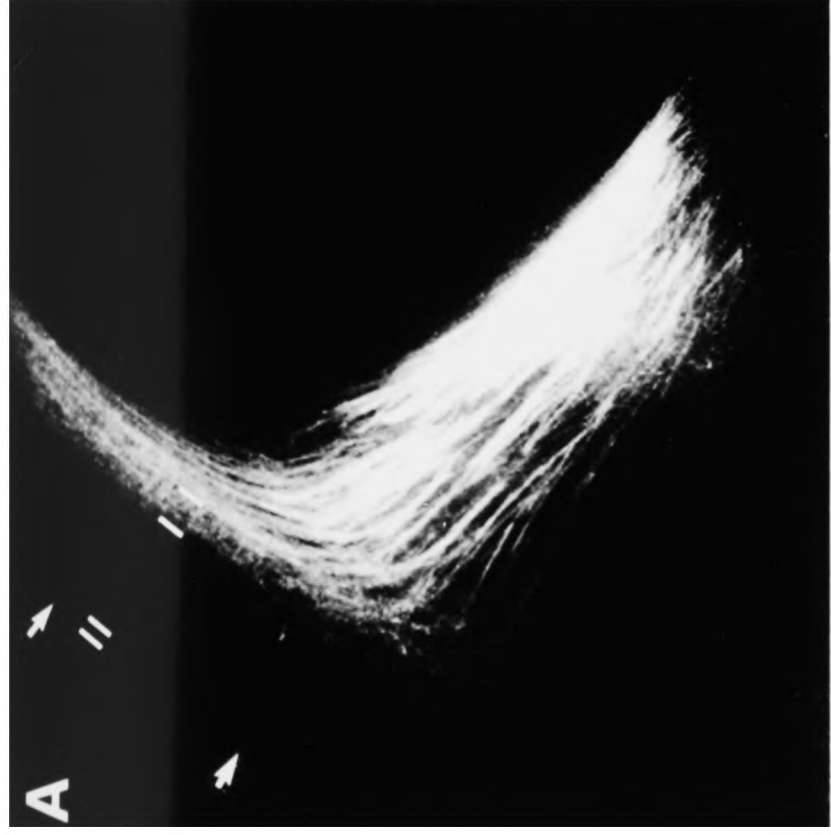


Fig. 4.15

Low-power fluorescent photomicrograph taken with double exposure (rhodamine and uv filters) from a bisbenzimidazole-stained section of an E15 Reeler (rl/rl) mouse. A small DiI crystal was placed in the occipital pole of the left hemisphere, and after 5 weeks incubation at room temperature, 200µm-thick coronal sections were cut. These photomicrographs demonstrate the single bundle of labelled axons which descends through the intermediate zone and internal capsule and then runs up to join a group of back-labelled cells in the dorsal thalamus. The bundle presumably contains both early corticofugal axons, almost exclusive from superplate cells (labelled anterogradely) and the corresponding thalamic afferents (labelled retrogradely). The oblique labelled fascicles running through the cortical plate lie in a different plane of section, but were obscured by the diffuse staining around the implanted crystal.

Scale bar 500µm.

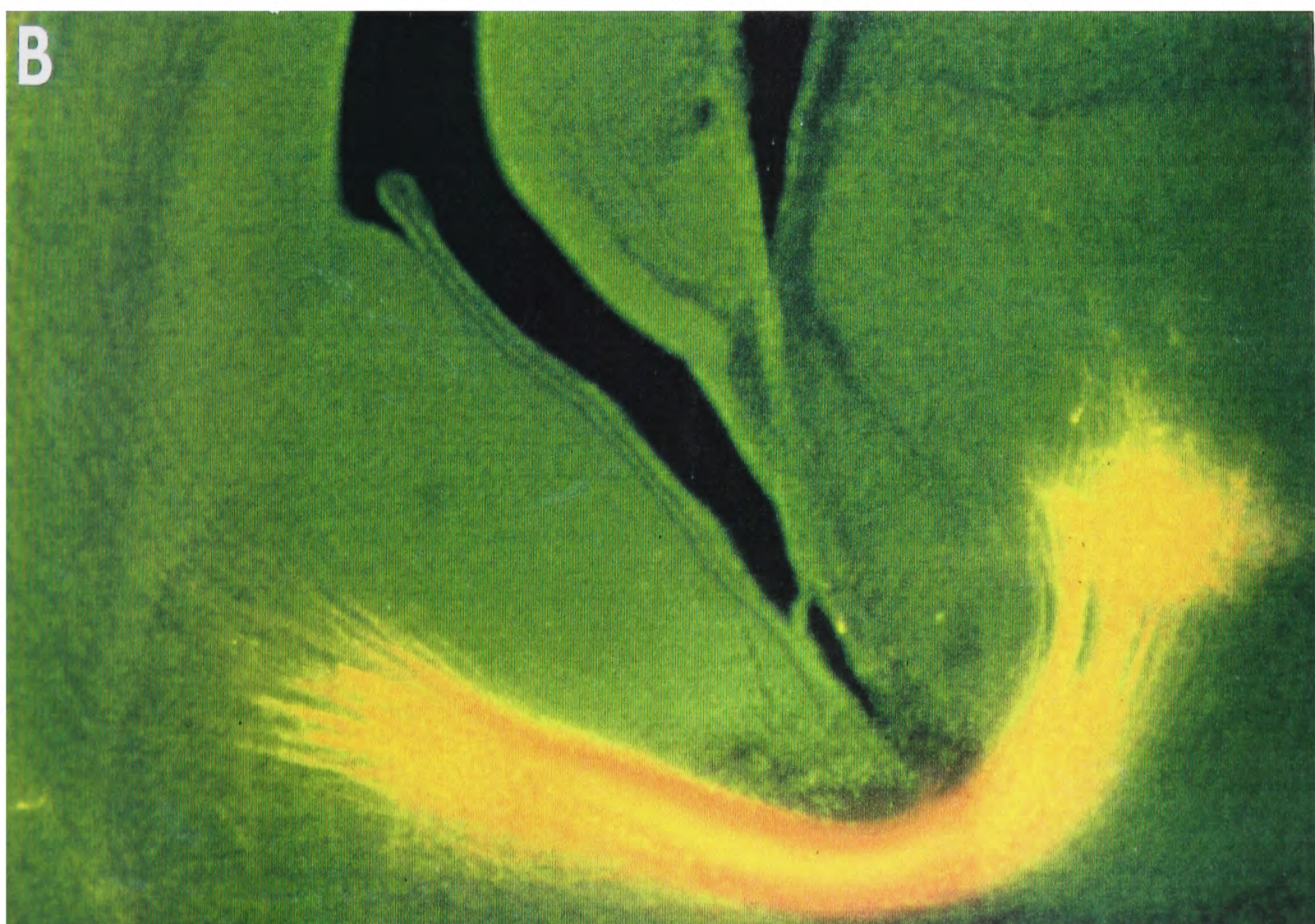
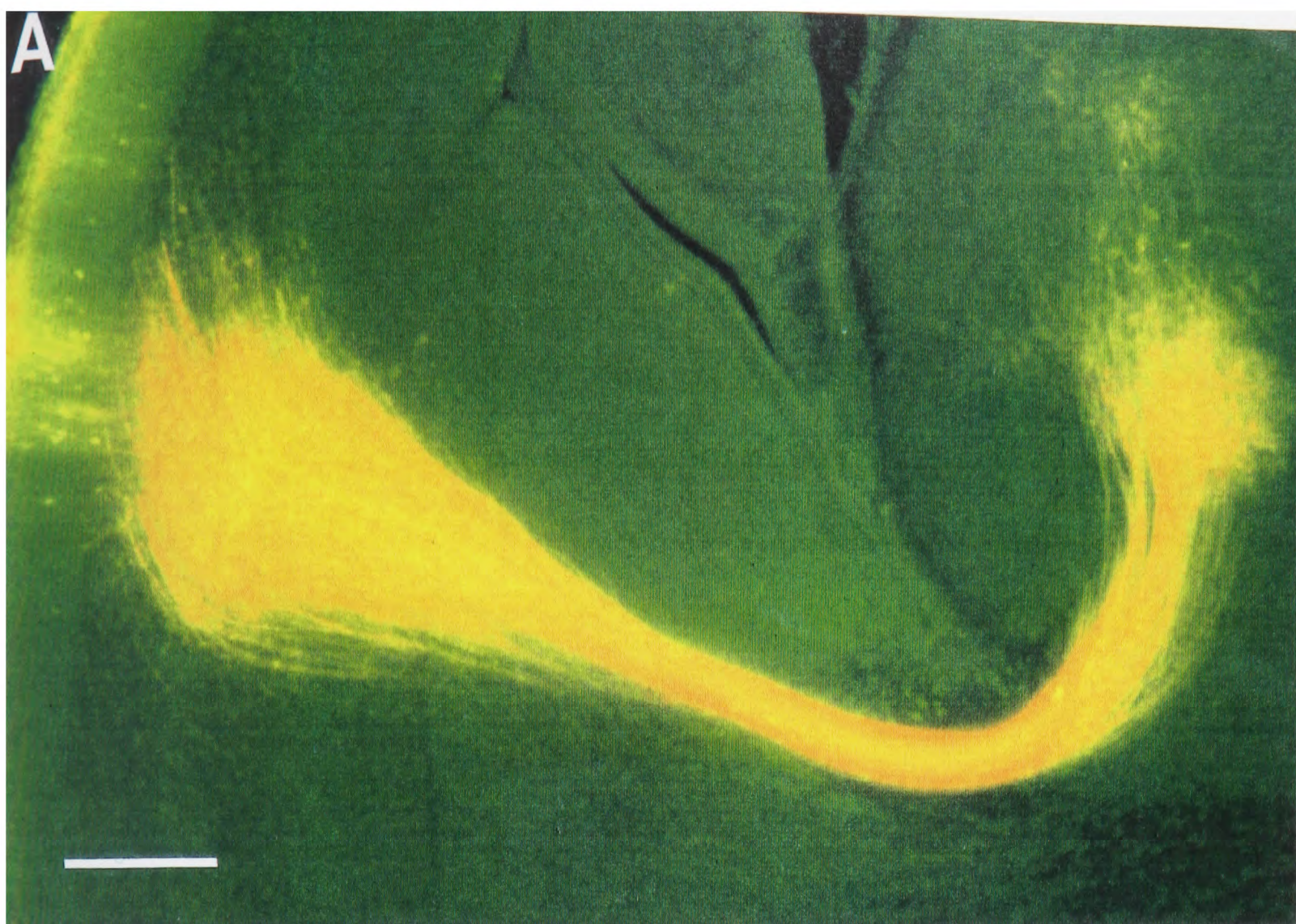


Fig. 4.16

From corresponding horizontal sections of an E18 normal (A) and a Reeler (B) brain (from the same litter of an *rl/rl* father and an *rl/+* mother) triple-exposure pictures were taken of the same field using 3 different filters. Three different carbocyanine dyes (DiAsp, DiI and DiA) had been placed in a parasagittal line along the convexity of the left hemisphere of all brains in this E18 litter (subsequent classification revealed that the litter contained 4 normal and 6 Reeler animals). The sections were counterstained with bisbenzimidazole to reveal the borders of the thalamus and to reveal the genotype of the animal. The crystals labelled fibre bundles (a,b and c) running down towards the primitive internal capsule (left side), and, at the tip of each bundle, back-labelled thalamic cells appeared in a stereotyped manner (a,b and c, right side). (DiAsp and DiA both appear as yellow). The three cortical points were matched with the same thalamic regions, in the two phenotypes, indicating that the thalamic projections had the same pattern of tangential targeting and the corticofugal projections were also indistinguishable in their topography. (Scale bar 300µm)

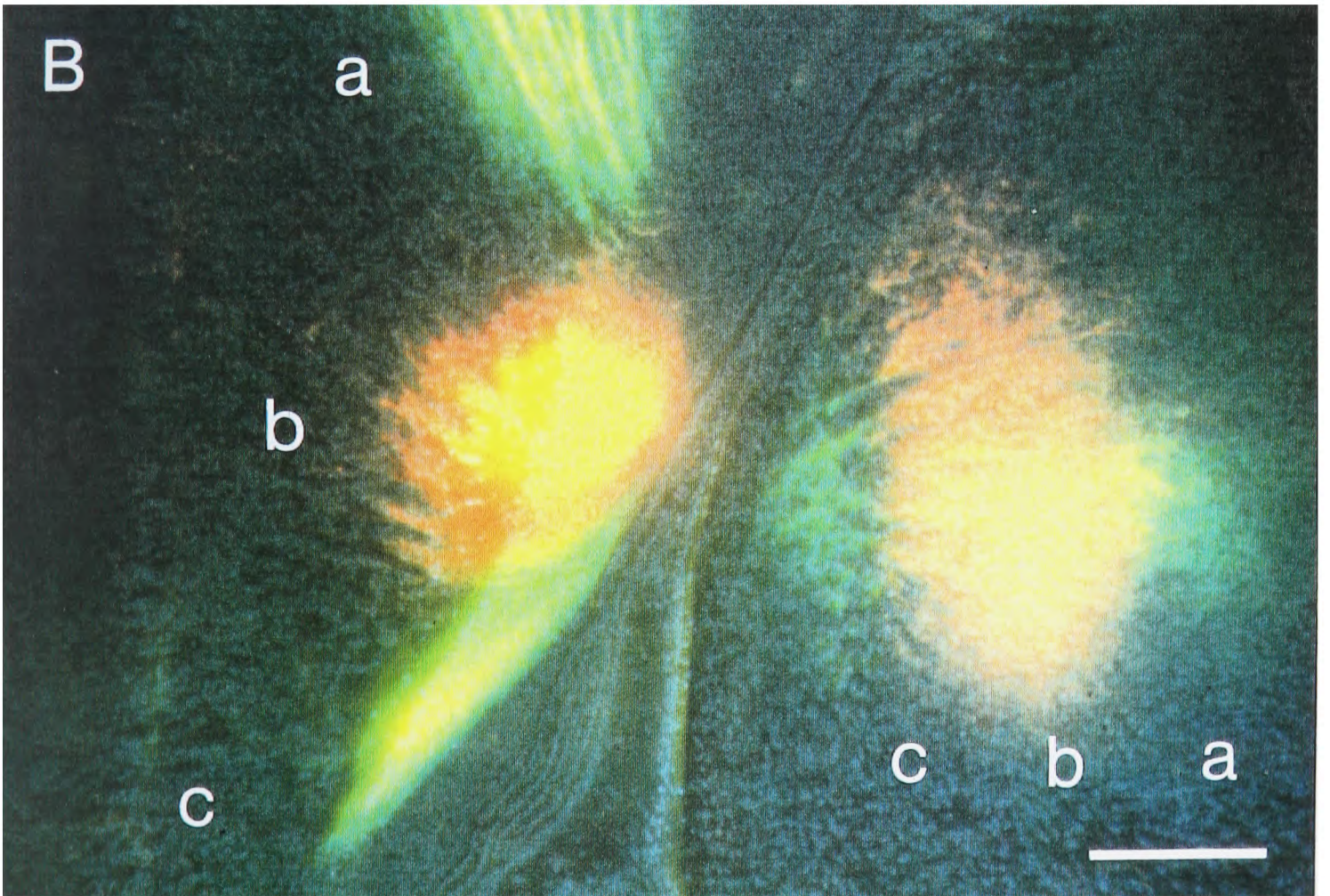
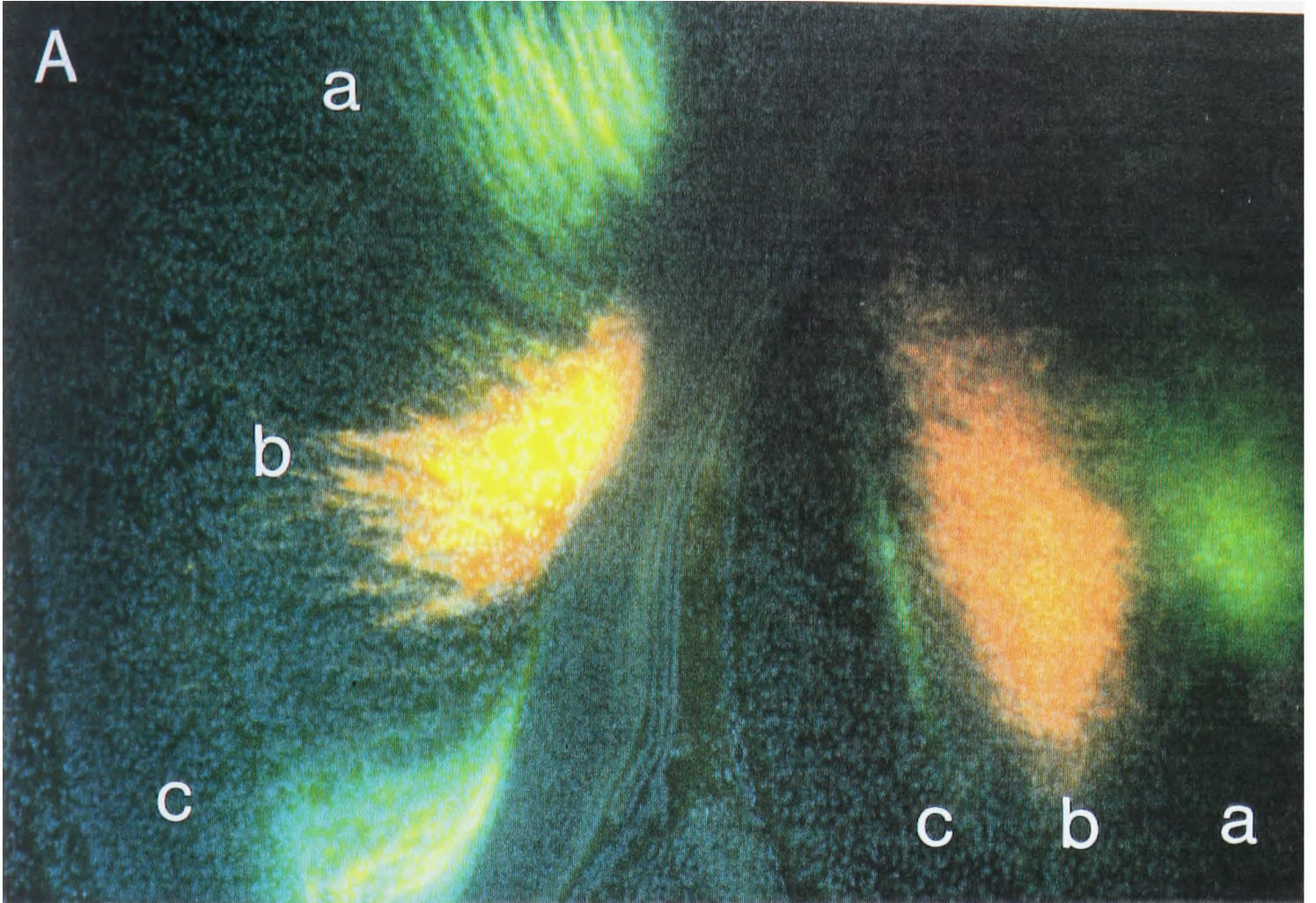


Fig. 4.17

This Figure demonstrates the distribution of thalamocortical fibres in a P3 normal (A,B) and Reeler cortex (C,D). LGN fibres were labelled bilaterally with thalamic DiI crystal placements in all the 6 brains obtained from a litter of an rl/+ mother and an rl/rl father. After 6 weeks diffusion time the brains were sectioned coronally (100µm thick) and counterstained with bisbenzimidazole. B (normal) and D (Reeler) show equivalent fields to reveal the thalamic axons. A and C show the right halves of each field under ultraviolet illumination to reveal lamination and hence the phenotypes.

In the normal animal (B) the thalamocortical fibres turn upwards into the cortical plate from the subplate layer (SP) and arborize below the dense cortical plate (DCP). In the Reeler the thalamocortical fibres form a very different characteristic pattern within the cortical plate. They run all the way up in fascicles to the superplate ('S'P), where they disperse into individual fibres, which then plunge down into the cortical plate below. With higher magnification a small fraction of the oblique ascending fibres are seen to give side branches into the cortical plate, but the vast majority of terminating fibres descend from the superplate. By comparing the images with the different filters it is apparent that the cell sparse regions visible in C and the fibre trajectories in D are in register. Very few (if any) back-labelled cells were observed in either phenotype with these restricted crystal placements in the dorsal thalamus.

Scale bar 200µm.

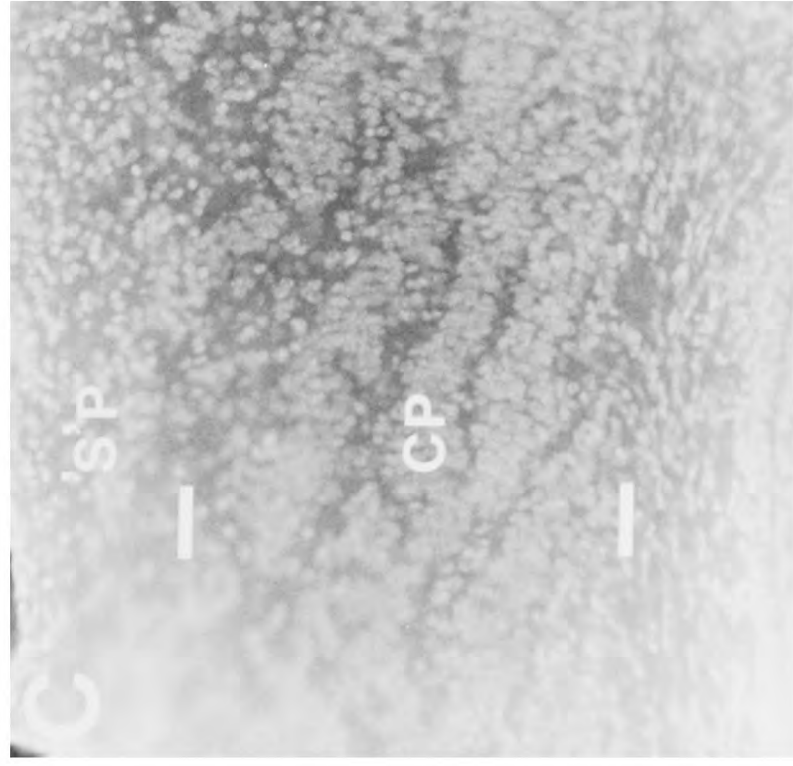
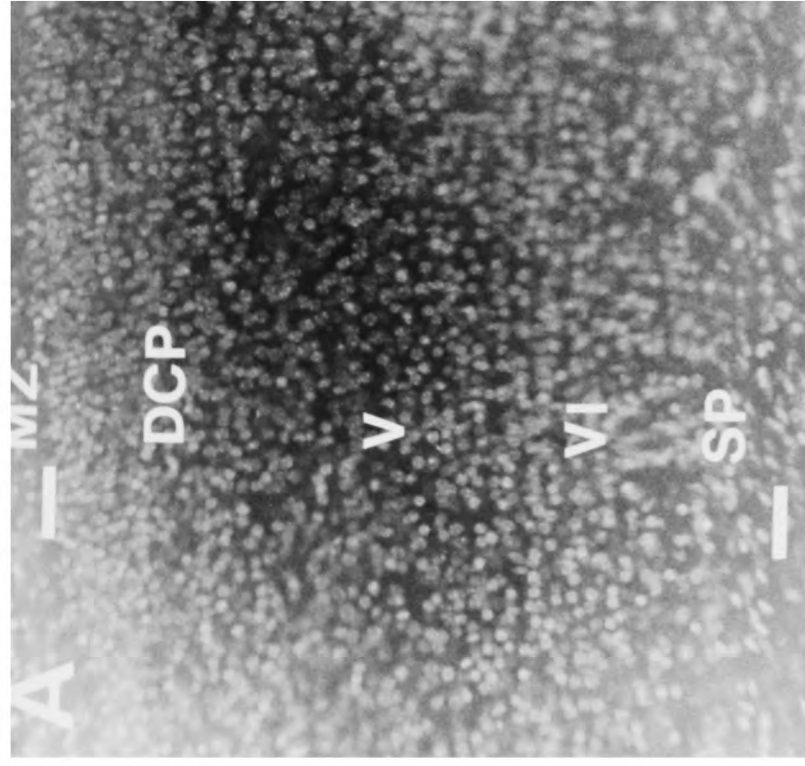


Fig. 4.18

Thalamic axons were labelled with a DiI crystal placed into the dorsal thalamus of a P3 Reeler brain. After 6 weeks diffusion the brain was sectioned coronally (100 μ m thick) and counterstained with bisbenzimidazole. A and B represent the same field under different filters, and C is a high-power view showing the segment demarcated by arrows in A.

Neighbouring thalamocortical axons show three distinct patterns of organization in different parts of the pathway. The white arrowheads mark the sites of transitions between the different segments. Within the diencephalon and throughout the internal capsule (IC), the adjacent axons remain parallel and closely associated. Upon entering the ventral striatum (left white arrowhead), the tightly packed homogeneous bundle breaks up and the fibres reorganize into small fascicles. Exchange of small numbers of axons between neighbouring fascicles can be observed in the high-power view of the striatal part of the pathway (segment between the two white arrowheads). At the lateral edge of the striatum (upper right arrowhead) the fibres do not defasciculate (as in the normal animal) but run all the way up in characteristic fascicles through the cortical plate (CP) to the superplate ('S'P). (H: hippocampus, CPU: Caudate Putamen, CP: cortical plate, VPL: Nucleus ventralis postero-lateralis, RT: Reticular Nucleus.)

Scale bars are 250 μ m at both A and C.

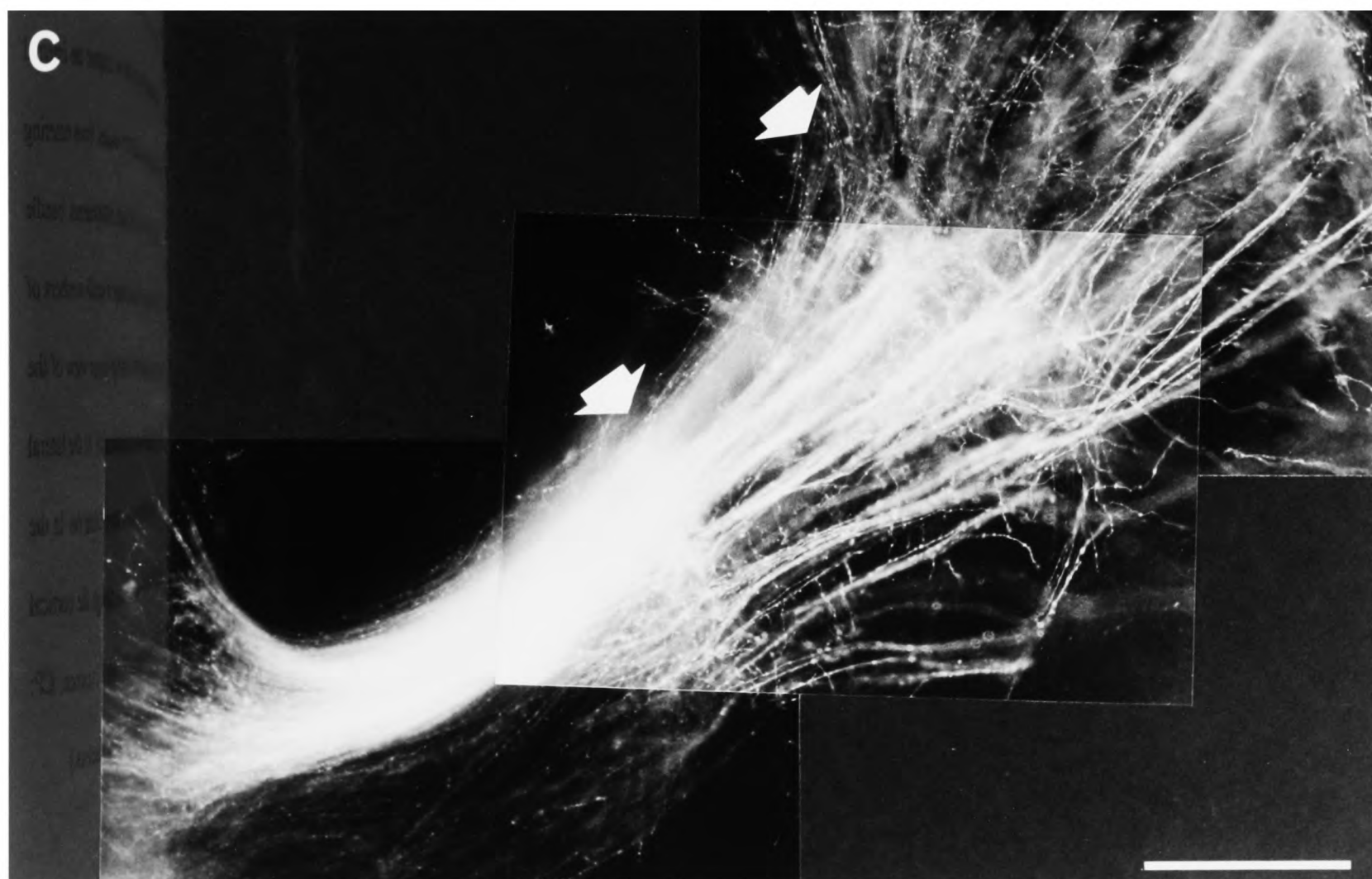
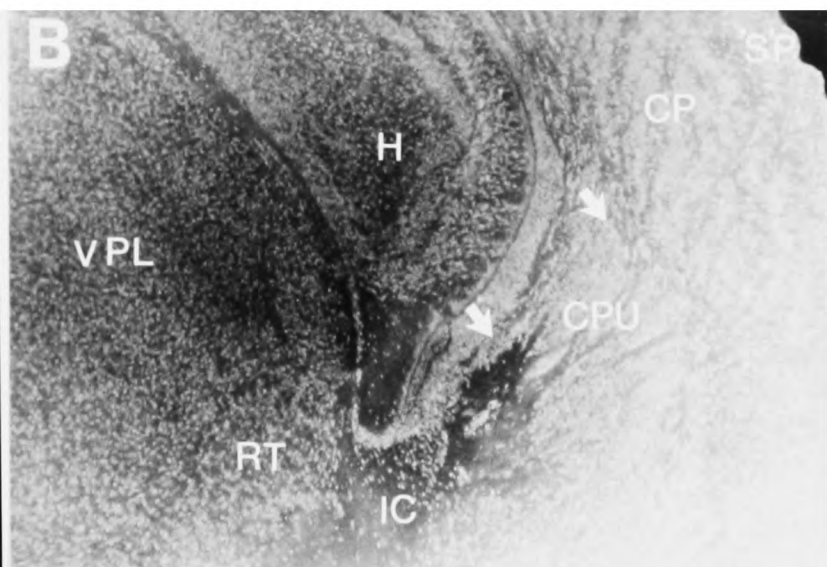
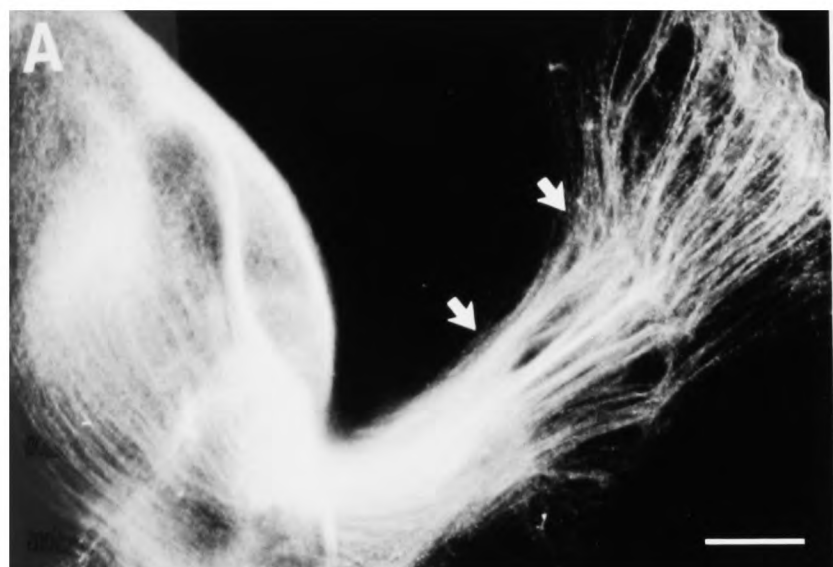


Fig. 4.19

These schematic diagrams represent equivalent sectors of the dorso-lateral aspect of the left hemisphere of the two different phenotypes at around E15, corresponding to the time of arrival of the thalamic fibres (not indicated) at the occipital pole. The upper is the normal animal (rl/+), the lower is Reeler (rl/rl). (Left is lateral and right is medial). In Reeler the cortical plate (CP; stippling) accumulates below the entire population of first-generated cells which stay together as the superplate ('S'P). The projections of preplate cells, which were laid down before the arrival of the cortical plate, run through the cortical plate in fascicles in Reeler. In the normal animals the cortical plate accumulates between the marginal zone (MZ) and subplate (SP).

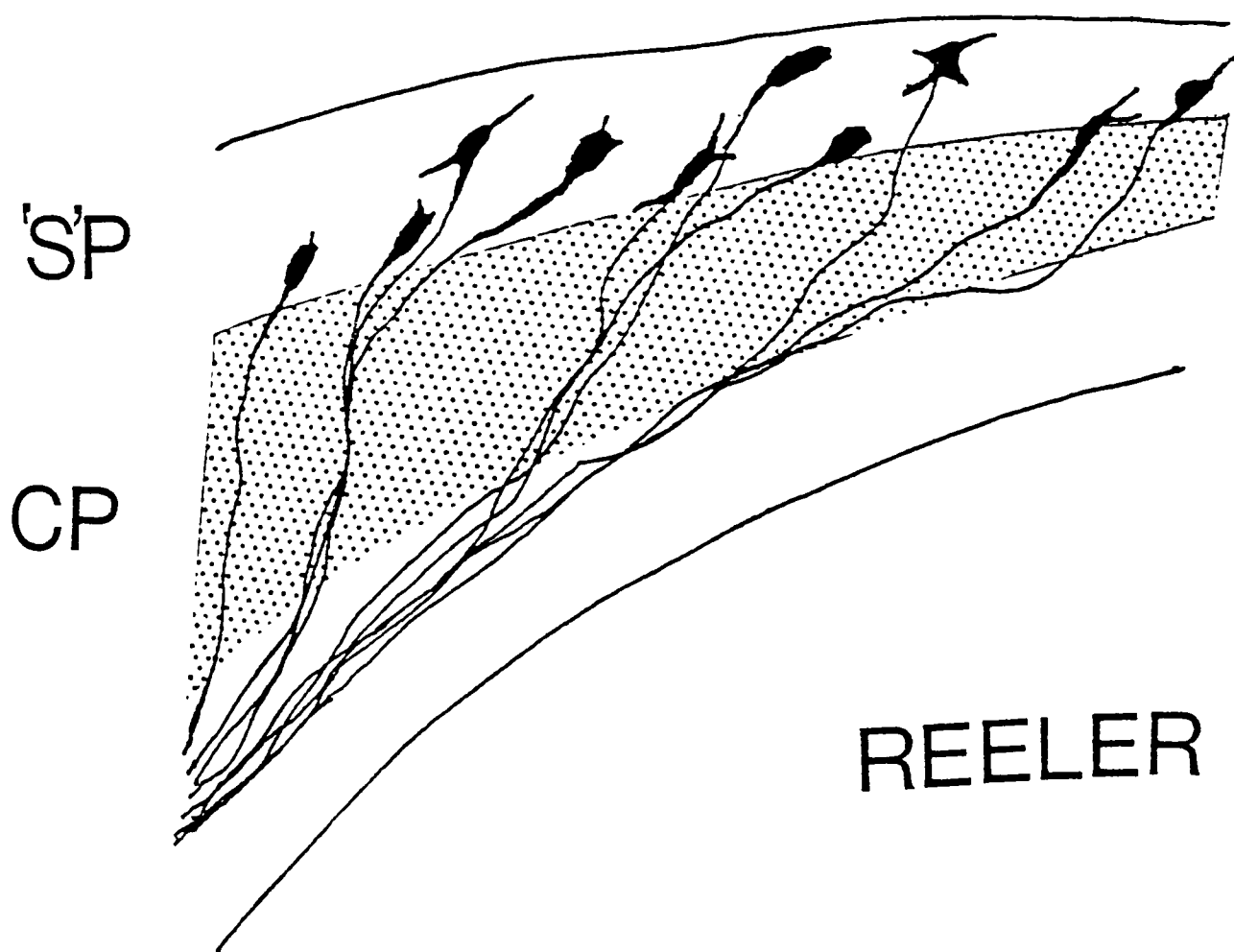
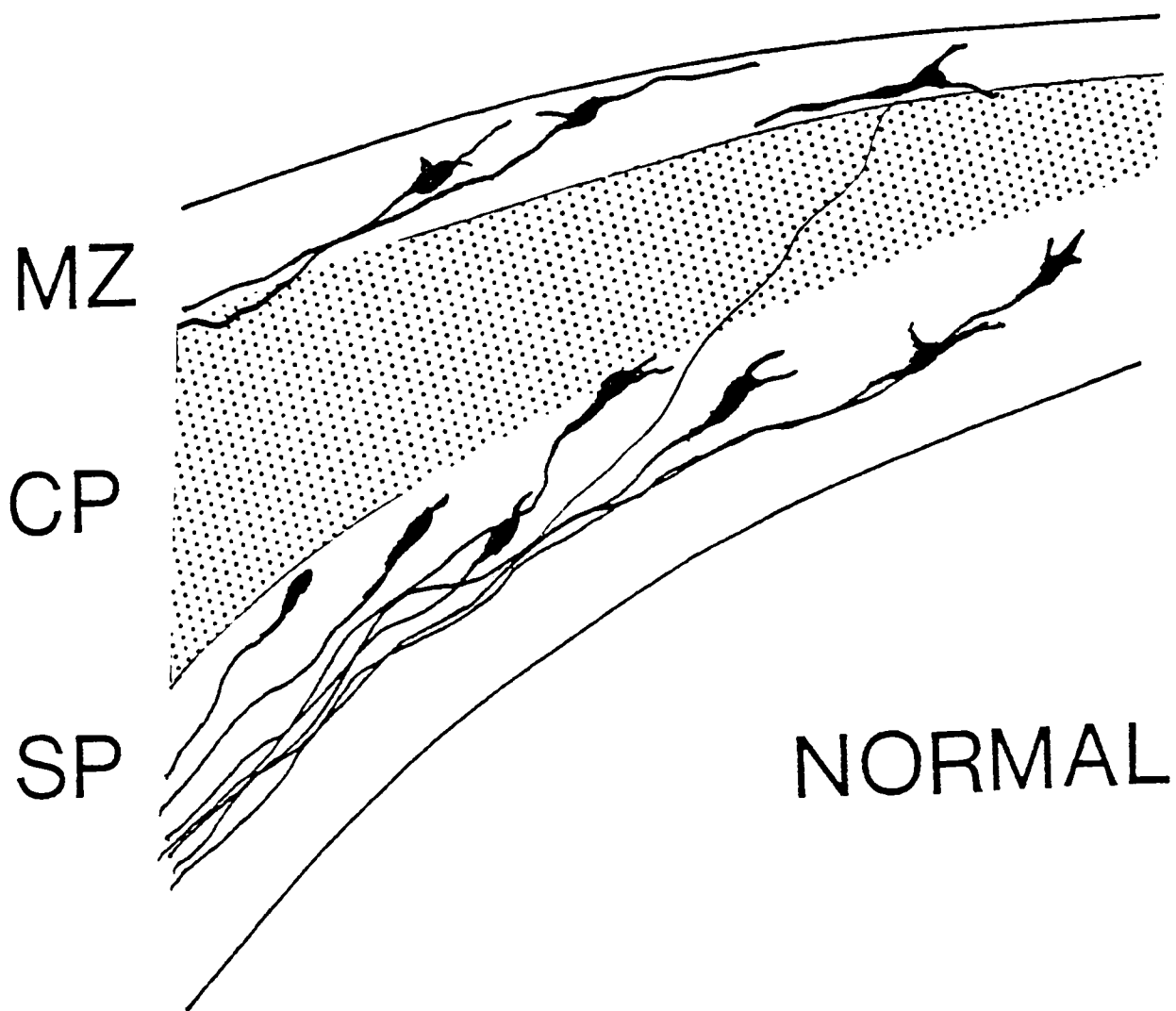
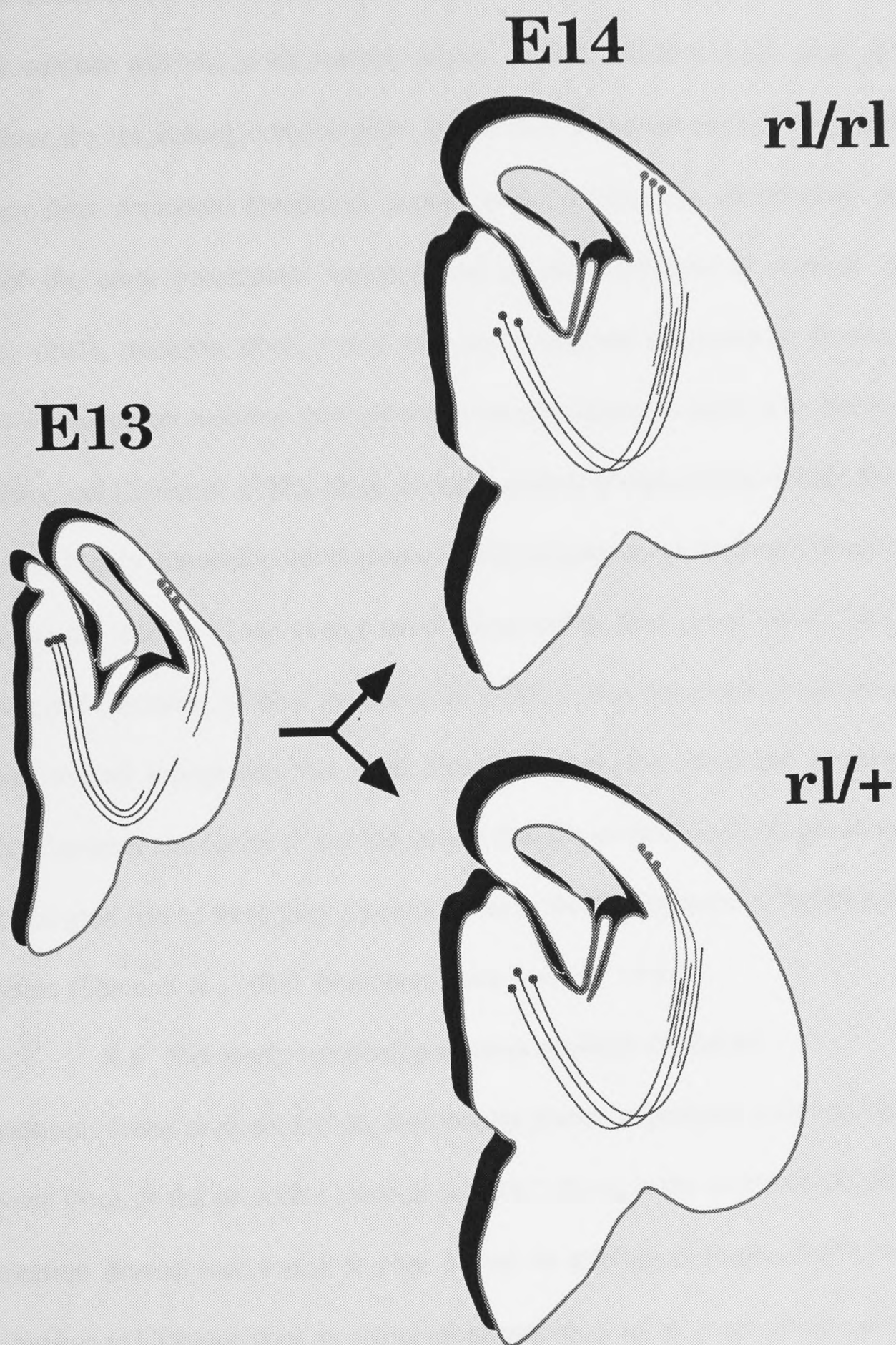


Fig. 4.20

These schematic diagrams summarise the establishment of the early corticofugal and corticopetal projections in the normal (rl/+ or +/+) and Reeler (rl/rl) phenotypes.

At E13 the formation of the preplate, outgrowth of an array of descending corticofugal axons and the roughly synchronized growth of pioneer thalamic axons through the primitive internal capsule all occur indistinguishably in Reeler and normal animals. By the time the thalamic axons arrive under their target areas (E14.5-15 for occipital cortex), the cortical plate itself has started to form. In Reeler the plate forms under the 'superplate' cells, whose pioneer axons come to lie in oblique fascicles, running through the thickening plate.

The selective fasciculation of thalamic axons on the axons of preplate cells established at very early stages might then explain both the normal accumulation of most thalamic axons in the subplate layer and, in Reeler, the fact that they run up diagonally in fascicles through the plate, to gather over the 'superplate' cells above.



DISCUSSION

The Reeler mouse is a remarkable 'experiment of Nature'. Those early postmitotic cells that lie towards the bottom of the preplate, which would be destined to become subplate neurons in the normal mouse, are left stranded in the superplate of Reeler, above the thickening cortical plate, which thus separates the arriving thalamic axons from their presumed temporary target cells. Despite this abnormality in the position of the early postmitotic neurons and in the formation of cortical layers (Caviness, 1982), thalamic fibres reach the correct regions of cortex in Reeler, and ultimately terminate on neurons that appear to be equivalent to layer 4 in the normal animal (Frost and Caviness, 1980). Only the local pattern of innervation within the grey matter seems highly abnormal, the thalamic fibres looping up to the top of the cortex, and reaching the middle of the cortex from above rather than from below (Caviness, 1976; Frost and Caviness, 1980; Caviness et al, 1988). I was interested to discover how the correct overall topography but local abnormality of the projection is generated during development and hence to test the notion that the earliest corticofugal axons and the cells that give rise to them play a guiding role in the deployment of thalamocortical afferentation (Shatz et al., 1990; Blakemore and Molnár, 1990).

4.6 The early corticofugal axon scaffold in Reeler

Many questions come to mind. Do the abnormally placed superplate cells send pioneer axons down towards the primitive internal capsule? If so, is the overall topography of this projection normal and could it play a part in guiding thalamic axons to their cortical territories? The answers to those questions seem to be emphatically positive.

The results described here suggest that the timing and the overall topography of both the corticofugal and thalamofugal projections are essentially identical in the two

genotypes. I could discern no differences between individuals in the appearance of cortex, thalamus or their axons in the large litters examined at E13 and 13.5 (which surely contained both genotypes) even for those few individuals for which the phenotype could be distinguished at the early stage by examining of the more mature rostroventral parts of the hemisphere. The polymorphous cells of the preplate spin out their pioneering axons, in both strains, at E13 and E13.5, before the arrival of any cells of the cortical plate proper, and before the arrival of thalamic or brainstem (Crandall et al., 1992) afferents (Fig. 4.19). These descending axons approach the primitive internal capsule, with a similar degree of fibre order in the two strains, at about E13.5 (for the occipital cortex). At roughly the same stage, axons from the dorsal thalamus have grown in quite strict order through the primitive internal capsule and are approaching their corticofugal counterparts. In both wild-type and Reeler, the thalamic fibres distribute in topographic order to the appropriate cortical regions, closely intermixed with, and conceivably fasciculated upon, the pioneer corticofugal axons from those same cortical areas.

4.7 Do preplate axons reach their corresponding thalamic nuclei?

Just as for the rat (Chapter 3), there is evidence that the pioneering axons of preplate cells in both normal and Reeler mouse do not actually reach the thalamic nuclei to which they correspond. Although DeCarlos and O'Leary (1992) did observe backlabelled subplate cells from thalamic carbocyanine crystal placement sites at E17-18 in the rat, in my experience very few if any cells in the cortex are back-labelled from discrete, localized dorsal thalamic DiI placements in which there is no involvement of the ventral diencephalon. Recently Miller et al. (1993) published a very

similar observations to mine in the hamster. The study reports few back-labelled cortical cells from thalamus until postnatal day 6, when there is a sudden increase in the number of layer 6 projections to the thalamus (revealed with localized thalamic DiI placements). There are several explanations for this, the most obvious being that corticofugal fibres from preplate cells never penetrate deep into the thalamus itself, and even the corticothalamic axons from layer 6 do not reach their thalamic nuclei until long after they grow out from the cortex. An alternative explanation is that the access of carbocyanine dyes to a particular fibre bundle might depend on the abundance of the branches and/or terminals near the placement site. The absence of clear back-labelling does not necessarily prove the lack of a connection. Corticofugal fibres might have already invaded the thalamus at early stages but might not have formed sufficient branches and terminals to take up the dye adequately to cause clear back-labelling. This argument, often advanced as an explanation for the failure of retrogradely transported markers (such as horseradish peroxidase) to back-label sparse projections seems less convincing for carbocyanine dyes which do not depend on membrane recycling for their uptake.

It cannot be excluded, but it is rather unlikely, that there is a different velocity and/or efficiency of carbocyanine dye transport orthogradely or retrogradely in fixed tissue. Although these dyes were introduced several years ago (Honig and Hume, 1986; Godement et al., 1987) and their use is extremely widespread, certain aspects of their behaviour in fixed embryonic pathways is still not fully understood; therefore alternative techniques should be used for further examination.

Indeed, there are also hints from studies using more conventional tracing techniques, that subplate fibres do not penetrate the thalamus and that layer 6

corticofugal fibres have a relatively delayed thalamic invasion. These studies were conducted in rather different species, so the comparison with the rodents should be made with caution. Nevertheless the similarity in findings to my own in rat and mouse is gratifying. Sheng et al. (1991), for instance, performed a detailed developmental study in the wallaby (Macropus eugenii). Using horseradish peroxidase conjugated to wheatgerm agglutinin for direct thalamic injections the authors demonstrated that the thalamic fibres arrive at the occipital cortex around 22 days post-conception, whereas back-labelled layer 6 and 5 cells were not observed until 40 days post-conception onwards. In the wallaby no back-labelled subplate cells were seen with this technique at any age.

In fetal rhesus monkey, injections of ^3H -proline (a purely orthogradely transported tracer) into the embryonic putative visual cortex labels terminal axons surrounding the LGN at E72, two weeks before the labelled endings spread (at E84-86) into the actual LGN itself (Shatz and Rakic, 1981), suggesting that there is a 'waiting period' for the corticothalamic innervation. The early development of the corticothalamic projections (from layer 6 as well as preplate cells) deserves further study.

The failure of preplate axons to pass far into the diencephalon (or perhaps to take up dye) enabled me to label thalamic axons selectively, even after E15 (when the two fibre systems were thoroughly intermingled in the intermediate zone), by placing the dye into the dorsal thalamus. This differential labelling of corticofugal and corticopetal fibres might be extremely useful for future 3-D confocal microscopic studies on the mutual fasciculation of thalamocortical and corticofugal axons.

4.8 Superplate cells as temporary targets in Reeler

The differences in cortical structure between Reeler and normal mouse start to become evident immediately after the arrival of the first wave of migrating cortical plate cells, which stop below the preplate in Reeler rather than penetrating and splitting it (Goffinet, 1979; Pinto-Lord and Caviness, 1979). The abnormal placement of the gathering plate cells in Reeler forces a change in the appearance of the pre-existing early corticofugal axons, leading them to form oblique bundles, running from the superplate, through the cortical plate below (Fig. 4.19). Nevertheless this does not change the paths of the distal parts of these axons, which have already turned through and under the developing striatum towards the primitive internal capsule, and possibly made contact with the array of thalamic axons advancing towards the cortex, just as in the normal animal (Fig. 4.20).

Superficially, the behaviour of thalamic axons as they reach their cortical field is very different in the two strains. In normal mice, as in rats (Chapter 3), with the exception of a few thalamic fibres that penetrate the cortical plate and reach the marginal zone very soon after their arrival, the vast majority of axons accumulate below the cortical plate and do not enter it for several days. But in Reeler, the thalamic fibres immediately penetrate the cortical plate and run up diagonally to the preplate layer at the top of the cortex, where they then wait for roughly the same period of time before turning down again into the plate itself. However, these two patterns are both compatible with the hypothesis that thalamic axons are specifically guided towards the preplate cells by running in close association with the axons that grew down earlier to establish the corticofugal scaffold.

4.9 Mechanisms of guidance and target finding

The general topographic order of the developing thalamocortical projections in Reeler has been described by Caviness and Korde (1981) and by Yamamoto (1986), based on immunohistochemical techniques. Caviness and Frost (1980) had already suggested that there is no essential difference in the tangential distribution of thalamic projections to cortical areas in newborn Reeler and normal mice, and Crandall and Caviness (1984) suspected that the gross organization of the thalamocortical system in Reeler is already topologically equivalent to that of the normal at about E15 and E17. Indeed, Caviness has even proposed that the regional deployment of the afferent system within the cortex is mediated through the polymorphic and large pyramidal cells of the superplate (Caviness et al. 1988). What was uncertain was the mechanism by which the distribution of thalamic axons became regionally correct but radially aberrant.

The Reeler mouse was often presented as an argument against, rather than for pre-laid guiding trajectories and contact guidance. It was proposed that the entire guidance of thalamic axons to the cortex might be regulated by diffusible substances originating from target cells, rather than by contact cues along the trajectories of pioneer axons (Caviness and Rakic, 1978; Caviness et al. 1988). The operation of such a chemotropic mechanism was suggested to explain both the correct tangential distribution of thalamic axons throughout the cerebrum in Reeler and their unusual local behaviour.

On the other hand, my results in co-culture (now broadly confirmed by others: Yamamoto et al, 1991, 1992; Caric et al., 1991; Bolz et al., 1992) suggest that any early influences of cortex on thalamus in vitro are not regionally selective and are therefore unlikely to be directly responsible for the correct topographic distribution of thalamic axons (Chapter 2). They also imply that the subplate is not strongly and selectively

attractive to thalamic axons at any stage in culture, which one would expect if subplate cells in vivo attract and hold thalamic axons by remote trophic or tropic signals. Rather, the intimate relationship between thalamic fibres and subplate cells, and the waiting period in the subplate layer, appear to occur only if the thalamic axons approach the subplate by their normal route - over the scaffold of pioneer corticofugal fibres after interaction within the environment of the striatum and intermediate zone (Chapters 2 and 3).

Remember that the cortex does not become growth-permissive for thalamic axons in the rat (see Chapter 2, Götz et al. 1992) until about E20 (presumably equivalent to about E19 in the mouse), long after the arrival of thalamic axons at the cortex in vivo. Yet here, in Reeler, thalamic axons grow through the plate cell layer at a much earlier age to reach the superplate above. Interestingly a small number of thalamocortical axons also reach the marginal zone by growing obliquely through the cortical plate, even in normal animals. The mouse does not seem to be an exception, since early ingrowth of a small fraction of thalamocortical fibres to the marginal zone has also been reported in neonatal cat (up to 2 weeks) and E14 hamster (Kato et al., 1984; Miller et al., 1993). It is conceivable that preplate cells release a diffusible agent which then attracts the thalamic fibres to the displaced preplate cells in the superplate through the cortical plate in Reeler (Caviness and Rakic, 1978; Caviness et al., 1988): the same mechanism could account the few fibres in normal animals that reach the marginal zone at early stages. However axons would be expected to take the shortest available route (i.e. radial) if they were responding to the gradient of a diffusible agent, whereas in Reeler their trajectory is clearly oblique - identical to that of the descending corticofugal bundles laid down earlier.

I suggest, then, that the characteristic pattern of thalamocortical fibre ingrowth in the Reeler might be due to their close relationship with the early corticofugal axons. The appearance of the thalamic axons, streaming in oblique, fasciculated bundles through the plate is remarkably similar to the appearance of the outflowing axons of preplate cells seen before the thalamic axons arrive (for a schematic summary see Fig. 4.19). This picture is entirely compatible with the thalamic axons actually being fasciculated on the corresponding preplate pioneer axons as proposed by Shatz et al., (1990) and Blakemore and Molnár (1990). The latter would then provide the attractive surface over which thalamic axons could negotiate their way to the superplate cells in Reeler, despite their having to pass through the non-permissive cortical plate layer.

In normal animals the primordial plexiform zone splits into subplate and marginal zone. Even after this splitting of the preplate, some marginal zone cells can be back-labelled from the internal capsule (together with numerous subplate cells) indicating that they too initially maintain their projections through the thickening cortical plate, similarly to the superplate cells in the Reeler. At present we have insufficient information about how long these sparse connections from marginal zone cells exist in the rat. McConnell et al., (1989) reported no back-labelled marginal zone cells from DiI crystal placements into the embryonic internal capsule of the cat. In cat these connections might disappear earlier or perhaps they are simply more difficult to detect because there are fewer marginal zone cells in carnivores than in the rat.

From my own birthdating studies in the rat (Molnár et al., 1991) the rough estimate for the marginal zone/subplate cell ratio is 1:4 at P5 and 1:7 at P10 (³H-thymidine injection at E12). This ratio in cat, as I estimated from Fig. 1.2 in Chapter 1 (taken from Luskin and Shatz, 1985), is 1:50 at P0 and 1:25 at P78 (³H-thymidine

injection at E27, E28 respectively). The primordial plexiform zone is itself generated in an outside-first/inside-last fashion, therefore the degree of labelling in future marginal zone and subplate might depend critically on the time of the ^3H -thymidine injection (Shoukimas and Hinds, 1978; Raedler and Raedler, 1978; Rickmann et al., 1977; König and Marty, 1981; Bayer and Altman, 1990), which could then affect the ratio of labelled marginal zone/subplate cells. It would be interesting to study this ratio at an earlier time, corresponding to the arrival of the thalamic axons, and see whether these numbers correlate with 1) the abundance thalamic axons reaching layer 1 versus the subplate and 2) the number of marginal zone cells back-labelled from the internal capsule in the different species. In any case, it certainly seems possible that the sparse early corticofugal projections from layer 1 and the small number of thalamic fibres reaching layer 1 at early stages depend on mechanisms similar to those operating for the whole of the superplate in Reeler.

The intimate relation of thalamic axons to preplate axons as they approach the cortex may also explain why they come to rest in the subplate (or, for Reeler, superplate) layer and probably form synapses on the cells there (Herrmann et al., 1991). If each thalamic axon does grow along a particular preplate axons, it will inevitably reach the cell body of that axon (subplate and marginal zones in normal or superplate in Reeler) and may be stimulated to terminate there, since other cell surfaces in the cortical plate are not growth-permissive at that stage. This particular trajectory of approach may be an essential prerequisite for the formation of temporary synapses and hence the establishment of a waiting period. In Reeler mice, the thalamic afferents arrive in the superplate at 14.5/15 (Fig. 4.12) and gather for a period of some days, at least until E18 (Fig. 4.14), just as they do in the subplate of normal mice. Thus a

‘waiting period’, in association with the appropriate target cells, occurs in both strains, even though those target cells lie in quite different locations in the two cases.

I have argued in Chapter 3 that the topography of both the pioneering corticofugal and thalamofugal projections is laid down at least in part by chronotopic ordering of outgrowth patterns, and maintained by fibre-fibre interaction. Within each coronal segment the earliest generated corticofugal projections take the most superficial position in the white matter and the later generated ones (from more dorsal regions) come to lie deeper in the white matter. This type of fibre ordering could be generated by the morphogenetic gradient (for review see Horder and Martin, 1978), the fibres becoming ordered below the subplate layer on a first-come first-served basis. The basic direction of growth of the early corticofugal projections towards the primitive internal capsule might be determined by trophic or tropic influences from key structures; and the distal phase of growth of the thalamic afferents may take place over, and be guided by, the pioneer scaffold of subplate pioneer axons (see Chapters 2 and 3). The thalamic fibres grow towards the developing cerebral cortical sheet in the direction along which the cortical morphogenetic gradient has its highest differential (see Fig. 1.1C in Chapter 1). All these mechanisms could be available and entirely normal in Reeler since they do not depend on the subsequent organization of the cortical plate. In particular, the early outgrowth of axons from preplate cells is achieved before the arrival of the ectopically placed cortical plate (Fig.4.20). Moreover, the scaffold established by preplate axons, and the relationship of thalamic axons to it, are indistinguishable from normal.

In Reeler, the sequence of behaviour of thalamic axons, after they reach their cortical fields, which leads to the peculiar local pattern of innervation seen in the adult,

is also entirely compatible with, and provides good evidence for, the theory of axon guidance developed in Chapter 3. Observations in vitro strongly suggest that the cortical plate does not become permissive for invasion of thalamic axons until several days after the time of arrival of those axons in vivo (Chapter 2; Bolz, et al., 1992; Hübner et al, 1992; Götz et al, 1992). This presumably explains, in part, why those axons do not extensively invade the cortical plate when they arrive, but gather below it. However, at this early stage even the subplate does not offer an attractive environment for thalamic axon ingrowth in vitro, unassisted by the intimate relationship with subplate axons that appears to be established in the ventral telencephalon in vivo. The subplate layer is no more attractive to thalamic axons than the cortical plate itself in the rat, in culture (Chapter 2). Therefore, the fact that fibres do enter and wait in the subplate layer in vivo, and even form temporary synapses on subplate cells (Herrmann et al., 1991), strongly suggests that the particular, natural trajectory of approach of thalamic axons, over the subplate axon scaffold is essential for this bond to be formed (rather than simply a chemotropic or trophic attractiveness of subplate or superplate cells, as previously proposed by Caviness et al., 1988 and Price, 1992). My hypothesis does not, of course, exclude the possibility that there are cortical trophic effects on the thalamus, nor that they play some part in the process of guidance towards the cortex.

The axons of subplate neurons are known to express immunoreactivity to the surface molecules L1 (Godfraind et al, 1988; Chung et al, 1991) and fibronectin (Chun and Shatz, 1988) which could provide highly attractive surfaces for the growth of thalamic axons in an otherwise relatively non-permissive environment. The selective expression of glycosaminoglycans (Derer and Nakanishi, 1983), chondroitin sulphate core proteins (Miller et al., 1992) and specific peanut agglutinin lectin binding (Götz

et al, 1992) has also been demonstrated in the subplate region in corresponding ages. It is yet to be determined whether thalamic fibres require more than one of these extracellular cues (sequentially or simultaneously) or whether there is a specific requirement for only one or a subset of them.

I have suggested (Chapter 3) that thalamic axons fasciculate on their subplate axon counterparts, and hence are provided with precise pathways by which they reach the somata of the appropriate neurons (subplate cells in the normal animal) and are encouraged to form temporary synapses. The existence of these privileged pathways for axon growth could explain how thalamic axons in Reeler are able to penetrate the cortical plate and stream straight up to reach the equivalent cells in the superplate, while ignoring the hostile territory of cortical plate cells around them. In the Reeler mutant various adhesion molecules (L1, NCAM, and fibronectin), and glycosaminoglycans were found to be similarly expressed by early-born cortical neurons, despite their different dispositions (Godfraind et al., 1988; Derer and Nakanishi, 1983). Shatz and her colleagues (Ghosh et al, 1990; Ghosh and Shatz 1993) have attempted to test the hypothesis that subplate neurons are crucial for the thalamic innervation of the cortex by making kainic acid lesions of the subplate in cats. However, since this was done a week after the arrival of thalamic axons and, given the possibility that the chemically lesioned area might undergo pathological changes other than the elimination of subplate neurons, the results are not easy to interpret. It would be interesting to perform similar experiments before the arrival of thalamic axons and to do them in Reeler where the thalamic fibres could approach the cortical region without travelling through a damaged region of destroyed subplate cells.

In conclusion, the development of thalamocortical connectivity in Reeler

supports the hypothesis that the preplate scaffold serves a guiding role for thalamic axons. The similarity of appearance of the fascicles of preplate axons, running obliquely through the thickening cortical plate in the Reeler (Fig. 4.6), and of the subsequent bundles of thalamic axons passing upwards through it (Fig. 4.8) again strongly suggests that the thalamic fibres grow in intimate contact with the early corticofugal scaffold. Caviness et al. (1988) suggested that the oblique fascicles of axons that they saw crossing the thickness of the developing cortical plate in Reeler (labelled with the rapid Golgi technique) might consist only of thalamic fibres, but when similar preparations were stained with neurofilament immunohistochemistry, the same bundles appeared larger. Caviness et al (1988) therefore suggested that the fascicles, which he called "plexiform tributaries", include the fibres of superplate cells, running together with the thalamic fibres.

At present it is rather difficult selectively and exclusively to label both thalamocortical and early corticofugal fibre populations at the same time so close to the cortical surface. Nevertheless, over a brief period of development this is possible. One can label the two set of fibres with different carbocyanine dyes when the two fibre systems have already met but the thalamic fibres have not reached the cortex (over a narrow time frame, around E14.5-15.5 for occipital cortex in the rat). 3-D confocal microscopic reconstructions obtained from rat brains labelled with different carbocyanine dyes from the cortex and thalamus at this stage indeed provide evidence for fibre-fibre interaction (Molnár et al., 1993). The selective fasciculation of growing axons on pioneer axon scaffolds appears to operate, in at least this situation, in vertebrates, as it does in insects (Goodman et al., 1984)

CHAPTER 5

NEURONS PRODUCING NITRIC OXIDE

IN THE DEVELOPING CORTEX OF THE RAT

INTRODUCTION

5.1 The nitric oxide system

Thomas and Pearse (1961, 1964) first described a sparse population of 'solitary active cells' in the central nervous system, especially the cerebral cortex and striatum, containing the oxidative enzyme *NADPH-diaphorase* (NADPH-d). This enzyme transfers electrons from nicotinamide adenine dinucleotide phosphate to electron acceptors; the histochemical reaction used by Thomas and Pearse involves the NADPH-dependent reduction of a tetrazolium salt, generating a blue formazan reaction product. Since Scherer-Singler et al. (1983) introduced a modification of the method to allow its use in aldehyde-fixed tissue, the distribution of NADPH-d-positive cells has been widely studied. They remained a functional mystery until Hope et al. (1991) discovered that NADPH-d is a Ca^{2+} -activated *nitric oxide synthase* (NO synthase): it catalyses the production of NO from L-arginine via a Ca^{2+} /calmodulin dependent process.

NADPH-d (NO synthase) activity has been described in such diverse locations as interneurons of rat and primate cerebral cortex (Scherer-Singler et al., 1983; Vincent, 1986; Sandell, 1986) and hippocampus (Vincent and Kimura, 1992), neurons in cat and monkey cerebral white matter (Sandell, 1986; Mizukawa et al, 1988), cells of the monkey amygdala (Pitkänen and Amaral, 1991), basket and granule cells of the cerebellum (Vincent and Kimura, 1992), magnocellular neurosecretory cells of the hypothalamus (Bredt et al., 1990) and cholinergic neurons of the septohippocampal

system (Pasqualotto and Vincent, 1991) and the mesopontine tegmentum (Vincent et al., 1983). In both striatum and cerebral cortex, NO synthase is co-localized with somatostatin (SOM), avian pancreatic polypeptide (APP) and neuropeptide Y (NPY) (Vincent, 1983; Kowall et al., 1987). Since gamma-aminobutyric acid (GABA) is known also to be co-localized with these neuropeptides in the cerebral cortex, it seems likely that NADPH-d-reactive cells in the cortex are GABAergic, presumably inhibitory, interneurons. The complete distribution of NO synthase-containing neurons in the rat brain has recently been reviewed by Vincent and Kimura (1992).

5.2 NADPH-diaphorase activity in the visual system

NO synthase is found in cells at all levels of the adult visual pathway. In many species, various classes of retinal amacrine cells are NADPH-d-positive (Sandell, 1985; Sagar, 1986; Wässle et al, 1987; Vaney and Young, 1988; Cobcroft et al., 1989; Provis and Mitrofanis, 1990; Vaccaro et al., 1991) and many cones are positive in the human retina (Provis and Mitrofanis, 1990). A striking pattern of positive cells is seen in the rat superior colliculus (Vincent and Kimura, 1992) and a subset of small GABA-immunoreactive cells in the rat dorsal lateral geniculate nucleus are lightly positive (Mitrofanis, 1992b). In the monkey striate cortex, not only are certain aspinous cells, mainly in layers 2 and 3, intensely positive but the neuropil is also reactive, with a pattern of labelling identical to that of cytochrome oxidase (Sandell, 1986). In the rat, the fibre plexus of layer 3 and some cells, mainly in that layer and in layer 6, are positive (Scherer-Singler et al., 1983; Vincent and Kimura, 1992). Intensely stained cells have also been reported in the corpus callosum of the rat (Vincent and Kimura, 1992) and in the white matter of monkeys (Sandell, 1986) and cats (Mizukawa et al., 1988): these may be survivors of the subplate population.

5.3 Functions of nitric oxide

NO is thought to act as an intercellular messenger (see Vincent and Hope, 1992). It is produced in cells that contain NADPH-d when intracellular Ca^{2+} rises (as a result of activation of ligand-gated or voltage-gated Ca^{2+} channels, or the mobilization of intracellular Ca^{2+} stores). It was recently demonstrated in the rat brain that the nitric oxide synthase (NOS) neurons express NMDA receptor mRNA (Price et al., 1993). NO is lipid soluble, and therefore crosses the cell membrane. Although it has a very short half-life, only 0.3 sec, its small size allows it to diffuse very rapidly through aqueous and lipid phases to penetrate other neurons (no surface receptor system is needed) within a 3-4 cell radius. It can then act by stimulating the haem-containing heterodimer, *soluble guanylyl cyclase*, to produce cyclic GMP (Arnold et al, 1977; Miki et al., 1977; Ignarro, 1990), which then regulates protein kinases, phosphodiesterases and ion channels (see Vincent and Hope, 1992).

Soluble guanylyl cyclase can be revealed immunohistochemically (Ariano et al., 1982). Significantly, it occurs in different neuronal types from those containing NO synthase. For instance, soluble guanylyl cyclase is localized in the efferent neurons of many structures (pyramidal cells of cerebral cortex, Purkinje cells of the cerebellum, medium spiny neurons of the striatum), while NO synthase occurs in smaller interneurons (see Vincent and Hope, 1992). Indeed, the very different optimal Ca^{2+} concentrations required for the two enzymes makes it unlikely that they can act in the same cell. This implies that there are separate donor and recipient cells in the NO system.

NO is a transmitter in the enteric nervous system and is known to act as a vascular relaxing factor by stimulating guanylyl cyclase in blood vessel walls.

NADPH-d-positive, non-adrenergic non-cholinergic autonomic axons are seen in the brain (Bredt et al., 1990); they appear to play an important role in the regulation of cerebral circulation and could control the permeability of the blood-brain barrier (Iadecola, 1993). But the most exciting possible role of NO in the central nervous system is in interneuronal signalling. The stimulation of cGMP production and hence of protein kinases and phosphodiesterase could enable NO to act during development and in the mature animal to regulate a variety of interactions between neurons (Vincent and Hope, 1992), including the control of their growth and differentiation (Mattson, 1988).

In the rat and cat retina, NO synthase-containing, presumed amacrine cells are most numerous and most prominently labelled during the period of synaptogenesis in the inner plexiform layer (Mitrofanis, 1989; Vaccaro, 1991): these cells may be playing a part in promoting synapse formation. The NO-stimulated synthesis of cGMP may regulate gap junction conductivity in retinal interneurons (Miyachi et al., 1990). There is evidence that NO produced in response to Ca^{2+} entry through NMDA receptors (activated by glutamate) may play a part in precipitating cell death (Dawson et al., 1991). However, chronic NO synthase inhibition do not protect hippocampal neurones from NMDA induced lesions (Lerner-Natoli et al., 1992).

The possible role of the NO signal transduction pathway in regulating synaptic modification is a topic of intense current interest. In the cerebellum, activation of either climbing fibres or mossy fibres elevates cGMP levels in Purkinje cells (Wood and Rao, 1991), probably via their actions on basket cells and granule cells respectively, both of which can release NO. Correlated activation of climbing and parallel fibres produces long-term depression (LTD) at the synapses of parallel fibres on to Purkinje cells and

this has been reported to be blocked by the NO synthesis inhibitor L-N^G-monomethyl-arginine (Ito and Karachot, 1990; Shibuki and Okada, 1991). Thus NO released from basket cells during climbing fibre activity might mobilize cGMP in Purkinje cells and this in turn might activate a Ca²⁺-dependent process in synapses receiving coincident input along parallel fibres. There is also weaker evidence that NO might act as a retrograde messenger in hippocampal long-term potentiation (LTP), communicating between activated postsynaptic elements and the simultaneously activated presynaptic terminals so as to produced changes in transmitter release (see Vincent and Hope, 1992; Fazeli, 1992).

NADPH-d activity in the visual system might then depend on visual stimulation and its expression might change in various deprivation paradigms. Indeed, Sandell (1986) has shown that monocular deprivation in the adult monkey, which is known to produce changes in the activity of cytochrome oxidase in the neuropil of layer 4 of the striate cortex affects NADPH-d activity in just the same way: both enzymes are reduced in activity in a pattern of stripes presumed to correspond to the ocular dominance stripes of the deprived eye.

In view of the potential power of NO to regulate the growth of cellular processes, the conductivity of gap junctions and cell death, and both to control synaptogenesis and to modulate existing synapses, I was interested in the possibility that NO producing neurons might play an important role in regulating the formation of cortical circuitry, neuronal excitability and local differentiation of cortical fields. In this Chapter, I document the distribution of NADPH-d-positive neurons in the developing rat cortex.

METHODS

Fetal Hooded Lister rats were obtained by time-mated pregnancies (in the Department of Human Anatomy, Oxford). Female rats in oestrus were exposed to a male for approximately 24 hours and the following day was designated as day E0 of the resulting pregnancy. Pregnant rats were anaesthetized with sodium pentobarbitone (60 mg/ml i.p.) and the fetuses were removed by Caesarian section at E15 (2 fetuses), E17 (4), E19 (1). For the postnatal period (in which the day of birth was counted as P0) the following ages were examined: P0 (6 animals), P1 (2), P2 (2), P6 (1), P7 (1), P8 (3), P9 (4), P10 (4), P13 (7), P24 (5) and adult (1).

For Nissl and histochemical staining of brain sections, rats were anaesthetized (chilling till P4, sodium pentobarbital thereafter) and perfused transcardially with phosphate-buffered saline (pH 7.4) followed by 4% formaldehyde with the addition of 0.125% glutaraldehyde at room temperature (200 ml for adults and 10-50 ml for young rats). After perfusion, brains were postfixed in fresh fixative for 1-2 h, then immersed in 20-30% sucrose in phosphate-buffered saline for about 4 days. The brains were then frozen on dry ice, and sectioned coronally or horizontally on a microtome at thicknesses ranging from 50-100 μm .

Sections were processed for NADPH-d (nicotinamide adenine dinucleotide phosphate-diaphorase) histochemistry according to the method of Mitrofanis (1989), as follows. The sections were incubated (at 37°C, for a period ranging from 2-12 h) in phosphate-buffered saline containing 0.2% Triton, 15 mM malic acid, 1 mM cobalt chloride, 0.5 mM NADP and 0.2 mM nitroblue tetrazolium (all chemicals from Sigma). Sections were mounted on dried slides; some were counterstained with Neutral Red and the rest were simply washed with distilled water (to remove the phosphate crystals). All

were then dried, cleared in Xylene and coverslipped with DPX.

I examined and photographed the sections under brightfield illumination. To document the areal and laminar distribution of NADPH-d-reactive cells, I plotted the characteristic blue-stained cells in selected sections from the different ages using a *camera lucida*. Drawings were made from a coronal section of the cerebral hemispheres including a sector from the putative somatosensory cortex.

RESULTS

NADPH-d-positive cells were easily recognized by the dense blue reaction product in their cytoplasm (the nucleus was unlabelled), often extending into the dendritic processes. Labelling was also seen as background staining and as stained fibrous processes in the neuropil, but it was impossible to identify the stained elements as axons or dendrites except where they could be seen to be in continuity with stained cell bodies. None of the NADPH-d-positive cells had the morphological appearance of glia.

5.4 The prenatal period: E15, 17 and 19

By E15, the earliest postmitotic cells have migrated into position at the margin of the cerebral wall to form the preplate and have sent their pioneering projection down towards the primitive internal capsule (Chapters 1 and 3). At E17, the first neurons of the cortical plate proper have arrived and lodged within the preplate to split it into the marginal zone above and subplate below, and thalamocortical axons are accumulating over subplate cells, under the cortical plate. By E19, further arrival of neurons has thickened the cortical plate to about half its ultimate thickness (see Chapter 1 and 3) and the whole of the cortex is just about to start expressing the membrane-bound permissive factor that encourages invasion by thalamic axons (Chapter 2).

No obvious NADPH-d reactivity is seen in the E15 cortex. Even at E17 and 19,

no labelled cells are present in either the cortex or the underlying white matter; however, there is diffuse reactivity over the neuropil of the subplate and the marginal zone, but not in the emerging cortical plate (Fig. 5.1A). The blood vessels are surrounded by background labelling and some profiles assumed to be endothelial cells are stained. This is particularly apparent in the ventricular and the subventricular zones, but even the cortical plate, which shows no diffuse staining of neuropil, also contains labelling associated with blood vessels (Fig. 5.1A). One very rarely come across with labelled cells in the putative somatosensory or visual cortical areas whereas the entorhinal cortex contains numerous NADPH-d positive cells at E20.

5.5 Early postnatal development: P0-7

At birth (P0), heavily stained cells appear, scattered sparsely within the subplate and in the immediately adjacent putative layer 6 of the cortical plate (Fig. 5.1B, 5.2B). Reactive cells are occasionally seen in the middle of the cortical plate, probably equivalent to the ultimate layer 5 (Figs. 5.1B and 5.2A,B). Few stained cells are seen initially in the white matter (Fig. 5.3A).

Over the first postnatal week the appearance of NADPH-d reactivity generally follows the known temporal patterns of maturation of the cortex. Labelled cells tend to appear first in the subplate and the lowest cortical plate layers, and later in the more superficial layers, following the general 'inside-out' pattern of laminar development (Chapter 1). Also, the whole process of emergence of NADPH-d reactivity is more advanced in rostrolateral than caudomedial cortex, reflecting the temporal gradient of maturation across the hemisphere (Chapter 1). At E20 and at birth, the entorhinal cortex already contains substantial numbers of reactive cells and their density is generally higher in the lateral and ventral cortical areas, around the entorhinal sulcus, then in the

more dorsal parts (Fig. 5.4). At later stages (P6-P8), when cortical maturation is advanced and the temporal gradient across the cortex is generally no longer evident, the pattern of NADPH-d staining is also more uniform.

Morphology of NADPH-d-reactive cells in the newborn rat

Reactive cells are generally intensely stained to give a Golgi-like appearance, revealing a great deal of their dendritic morphology. Most dendritic arbors can be followed over long distances, often to what appear to be their terminal processes. The axons, however are not well labelled. The majority of the stained cells are multiform or polymorph, showing the characteristics of mature neurons with elaborate dendritic morphology. Interestingly, although a fair proportion of subplate cells are stained, no labelled cells were seen among the co-generated cells of the marginal zone. The stained cells in the cortical plate are bipolar or bistellate interneurons, with no evidence of spines on their dendrites (Figures 5.6B and 5.7A,B). No pyramidal cells are labelled at any age.

Some of the endothelial cells of cerebral blood vessels coursing through the cortex are reactive, and in addition some of the stained processes of presumed neuronal cells were closely associated with blood vessels.

5.6 Later postnatal development: P8 onwards

Just after the end of the first postnatal week, when cortical lamination is virtually mature, stained multiform cells reach peak numbers (roughly 250-350 cells per mm³). They are distributed in all layers and are now more common in the white matter, but are still very rare in the marginal zone. Thereafter, the density of labelled cells begins to *decline*. By P13 the overall density of stained cells (200-250 per mm³) is clearly somewhat reduced. This seems true for all layers (Fig. 5.4) and the effect is

probably partly due to the expansion of volume of the cortex. Among the positive cells, small bipolar and bistellate interneurons dominate in the supragranular layers (Figs. 5.5A,B and 5.7A,B), while in lower layers most are polymorphous cells, with fewer bipolar and bistellate neurons (Figs. 5.3B and 5.5B,C). No pyramidal cells are labelled. Some labelled polymorphous cells are seen below the cortex and their processes extend widely within the white matter. Although the density of labelled cells declines slightly after the first postnatal week, the meshwork of NADPH-d-positive fibres increases in clarity (Figs. 5.5D, 5.6A and 5.7C,D). This fibre labelling is very widespread: by P7 it is present in all cortical areas and in all layers, although earlier in the first postnatal week the staining is more intense in the infragranular layers of the cortex, with only diffuse staining above layer 4 (Fig.5.1D). Gradually, after the first week of postnatal life, the NADPH-d-positive processes become more dense and elaborate, forming a meshwork that covering the entire cortex.

At later ages (P24-adult) there are no further striking changes in the pattern of labelled cells and the fibre network remains dense. In addition to the neuronal and fibre staining, a diffuse blue staining of neuropil is also observed, most commonly in layer 4, and it tends to change in density and/or laminar distribution at cytoarchitectonic boundaries. In the somatosensory cortex this diffuse NADPH-d staining reveals the vibrissal 'barrels' (Van der Loos and Woolsey, 1973), the barrel-centre being more intensively stained, and in area 17 layer 4 is particularly densely stained.

Fig. 5.1

A: Photomicrograph of a coronal section of the putative visual cortex of an E17 brain. The NADPH-diaphorase reaction reveals the developing blood vessels and presumed endothelial cells (arrows) in the ventricular and the subventricular zone (vz, svz). There is a diffuse immunoreactivity in the intermediate zone and subplate (i and sp) region but there are no reactive neuronal cell bodies within either subplate of cortical plate (cp). (Scale bar: 100µm)

B: This coronal section from a P2 brain shows the first polymorphous NADPH-d-positive cells in the subplate and the bottom of the cortical plate. The neutral red counterstaining reveals the border of the white matter (interrupted line). (Scale bar: 100µm)

C: Horizontal section of the right somatosensory cortex at P1. Labelled cells are already abundant in the striatum (s) below the cortex. The small numbers of NADPH-d-reactive cortical cells are mainly located in the lower half of the cortical plate. (Scale bar: 300µm)

D: Horizontal section through the dorsal part of the right somatosensory cortex at P7. Cells of various shapes (polymorphous or bitufted) are now sparsely but regularly distributed in the white matter (W) and most layers of the cortex with the exception of the uppermost layers. The white arrow indicates the diffusely stained layer 4. (Scale bar: 300µm).

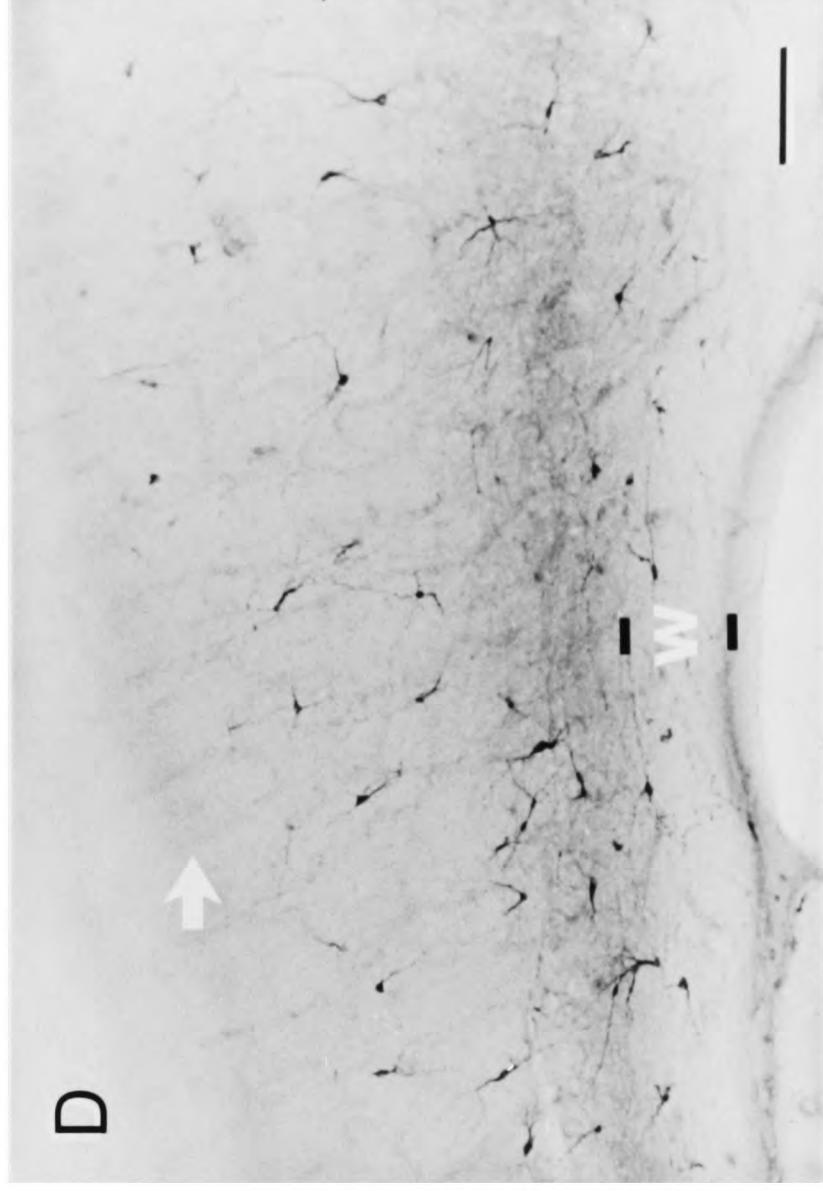
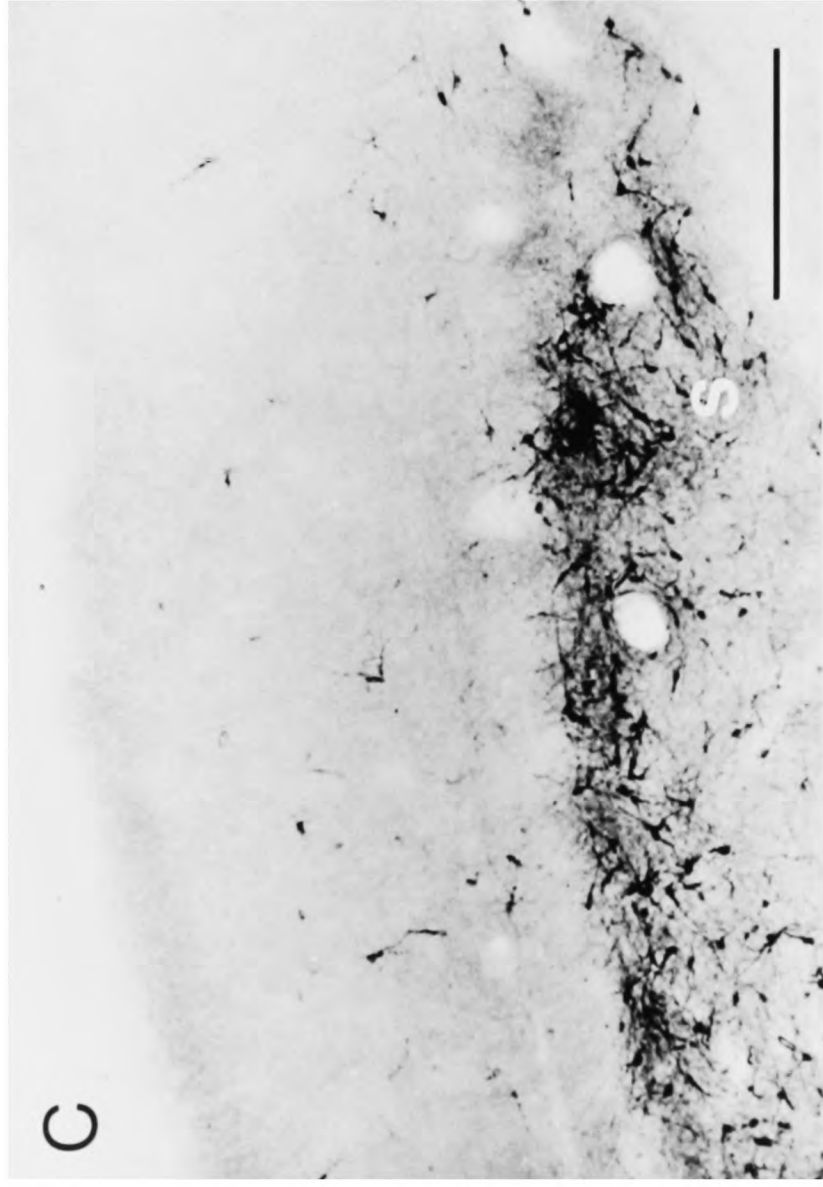
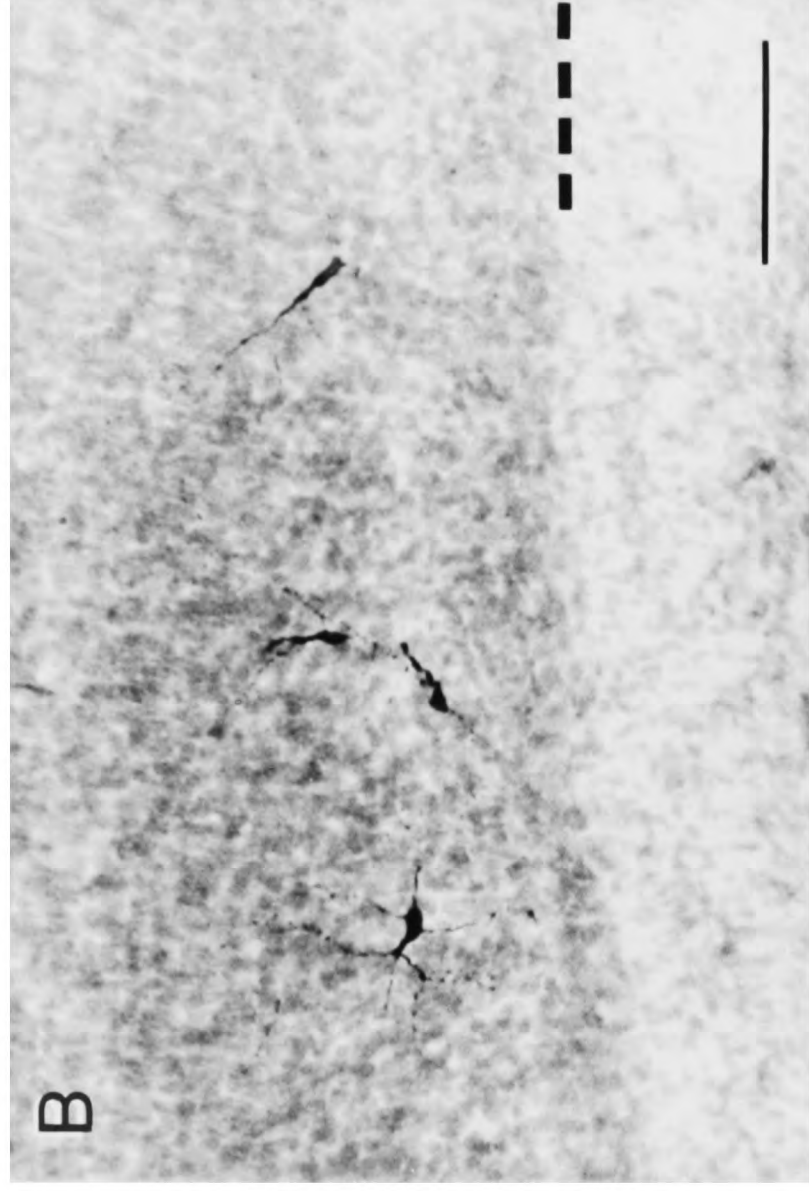


Fig. 5.2

A.: Coronal section through the putative visual cortex at P0. A few labelled cells are seen at the bottom of the cortical plate and in the subplate beneath it. The neutral red counterstaining reveals the white matter (wm) subplate (sp) border. No labelled cells are seen in the marginal zone (mz), the upper part of the cortical plate or the dense cortical plate (dcp), which contains migrating and newly-arrived, immature neurons. (Scale bar: 100µm).

B: Horizontal section through the ventral part of the somatosensory cortex of a newborn (P0) rat, stained for NADPH-d, then counterstained with neutral red (which intensely stains the dense cortical plate - dcp). NADPH-d cells with various shapes and orientations are scattered in the lower, postmigratory part of the cortical plate and subplate. (mz - marginal zone) (Scale bar: 100µm).

C: The laminar distribution of NADPH-d-reactive cells in a coronal section of the P8 visual cortex. The layering (bars) was revealed by counterstaining with neutral red. With the exception of layer 1 all layers now contain NADPH-d-reactive cells with highly variable morphology. The bottom layers and the white matter contain more polymorphous cells, whereas layers 2-4 have more bipolar and bitufted neurons which tend to be orientated vertically. No pyramidal neurons are stained. (Scale bar: 300µm)

D: Horizontal (i.e. transverse) section through the somatosensory cortex at P24. The laminar distribution of labelled cells does not change drastically after the first postnatal week, but their dendritic arbors seem more elaborate, though they are probably smaller in number. Around layer 4 the diffuse NADPH-d reactivity is particularly dense. The section was not counterstained. The dark tone is due to the extremely rich NADPH-d-positive fibre meshwork: see also figures 5.5, 5.6 and 5.7. (Scale bar: 300µm).

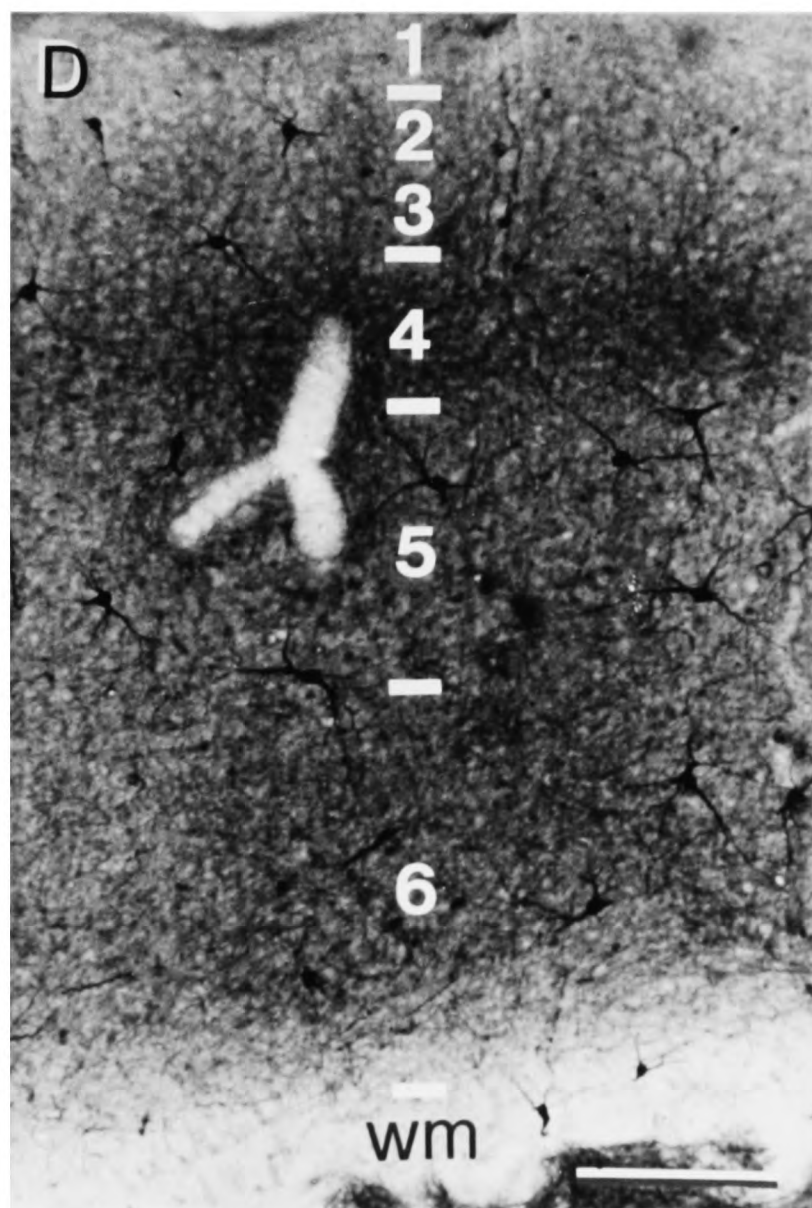
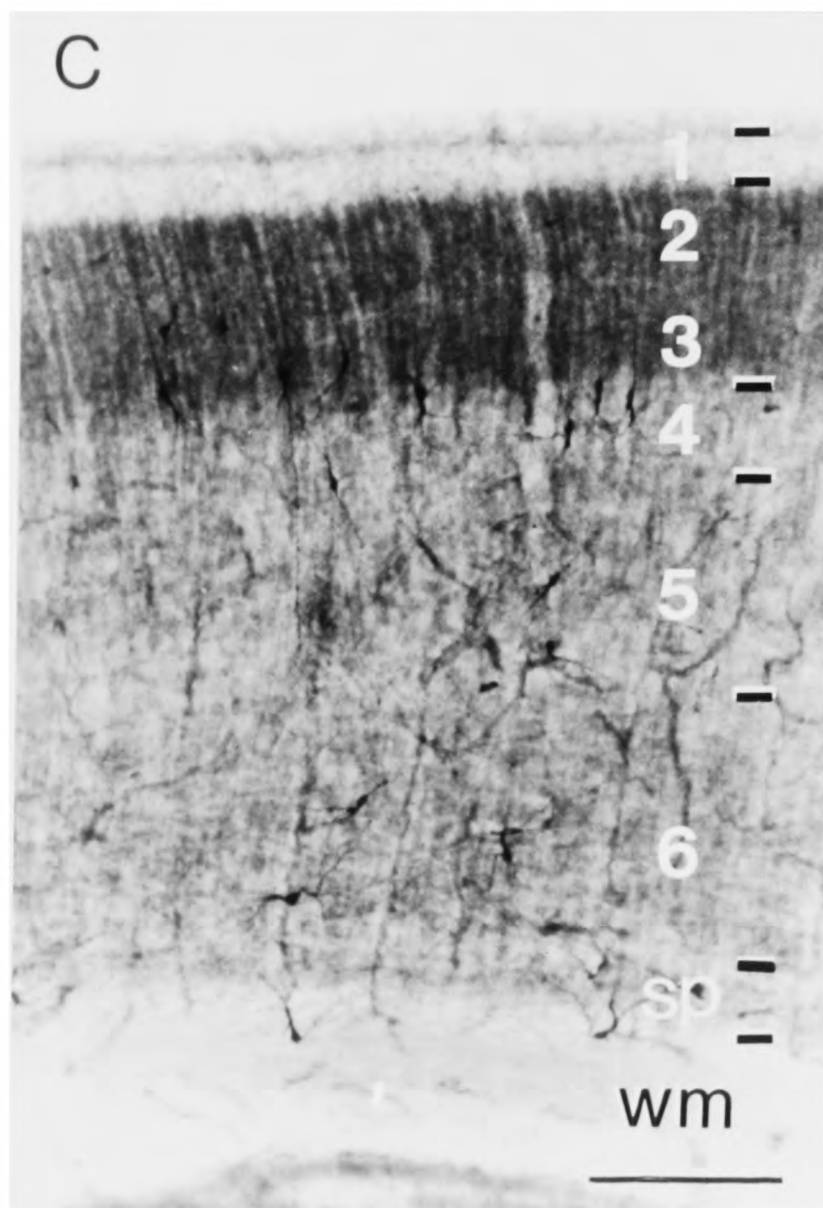
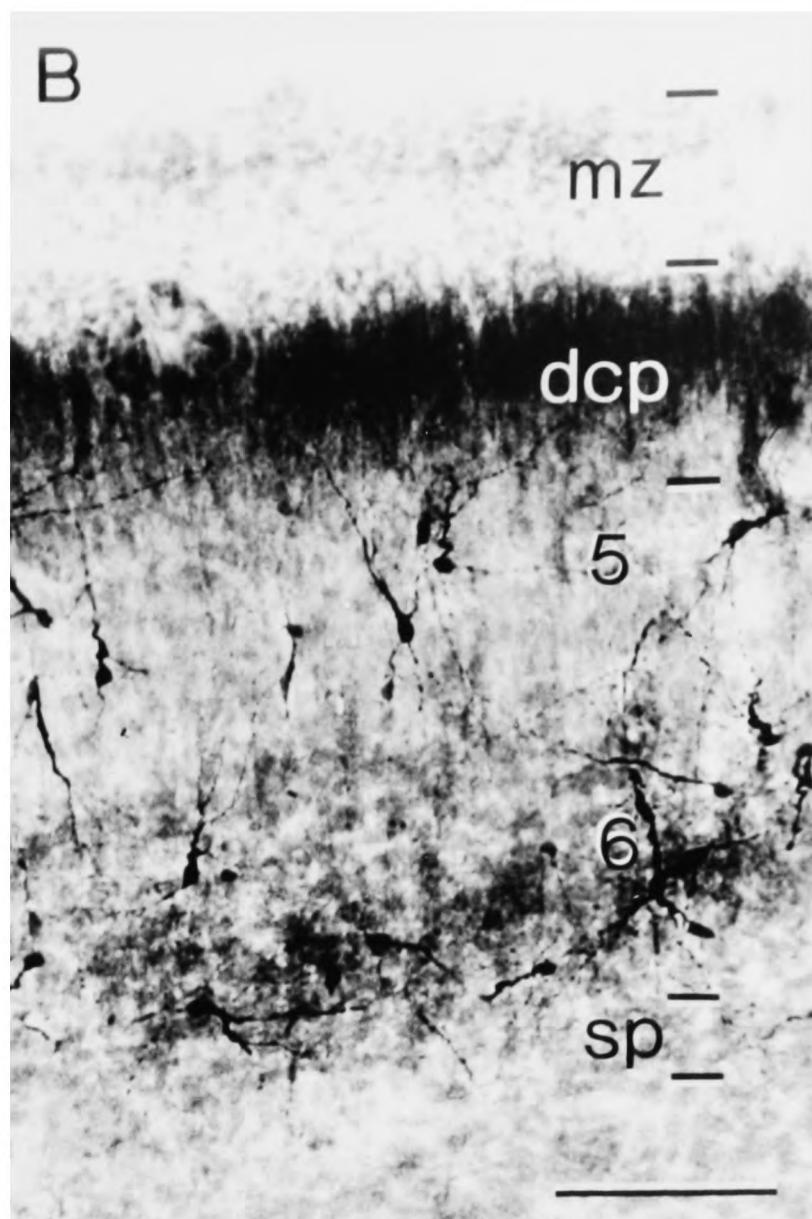
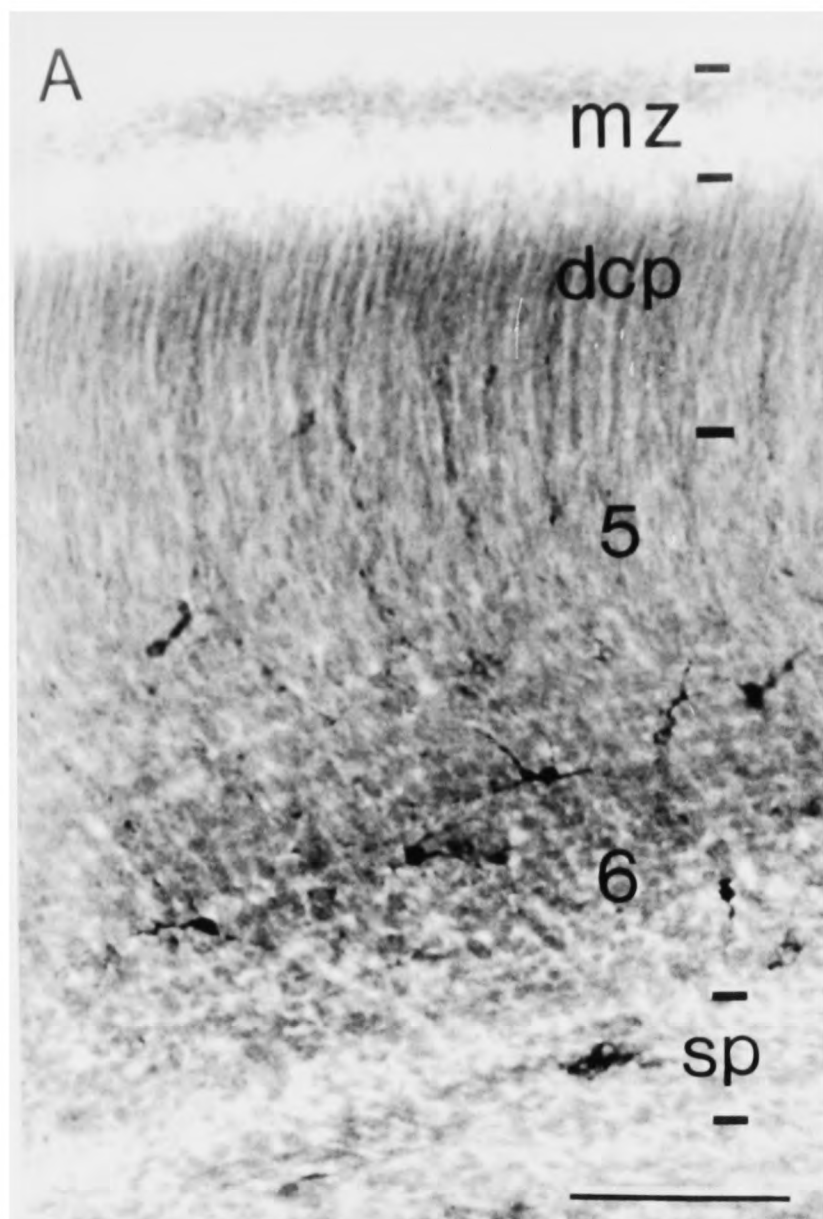


Fig. 5.3

A: High power photo-micrograph of a coronal section through the putative visual cortex at P2. Few dark-blue labelled cells are seen at the bottom of the cortical plate and in the subplate beneath it. The neutral red counterstaining reveals the white matter (WM)/subplate (SP) border.

B: The heavily stained polymorphous cells at the white matter/layer 6b border appear to have more elaborate processes at P24.

(Scale bar 100µm on both photomicrographs)

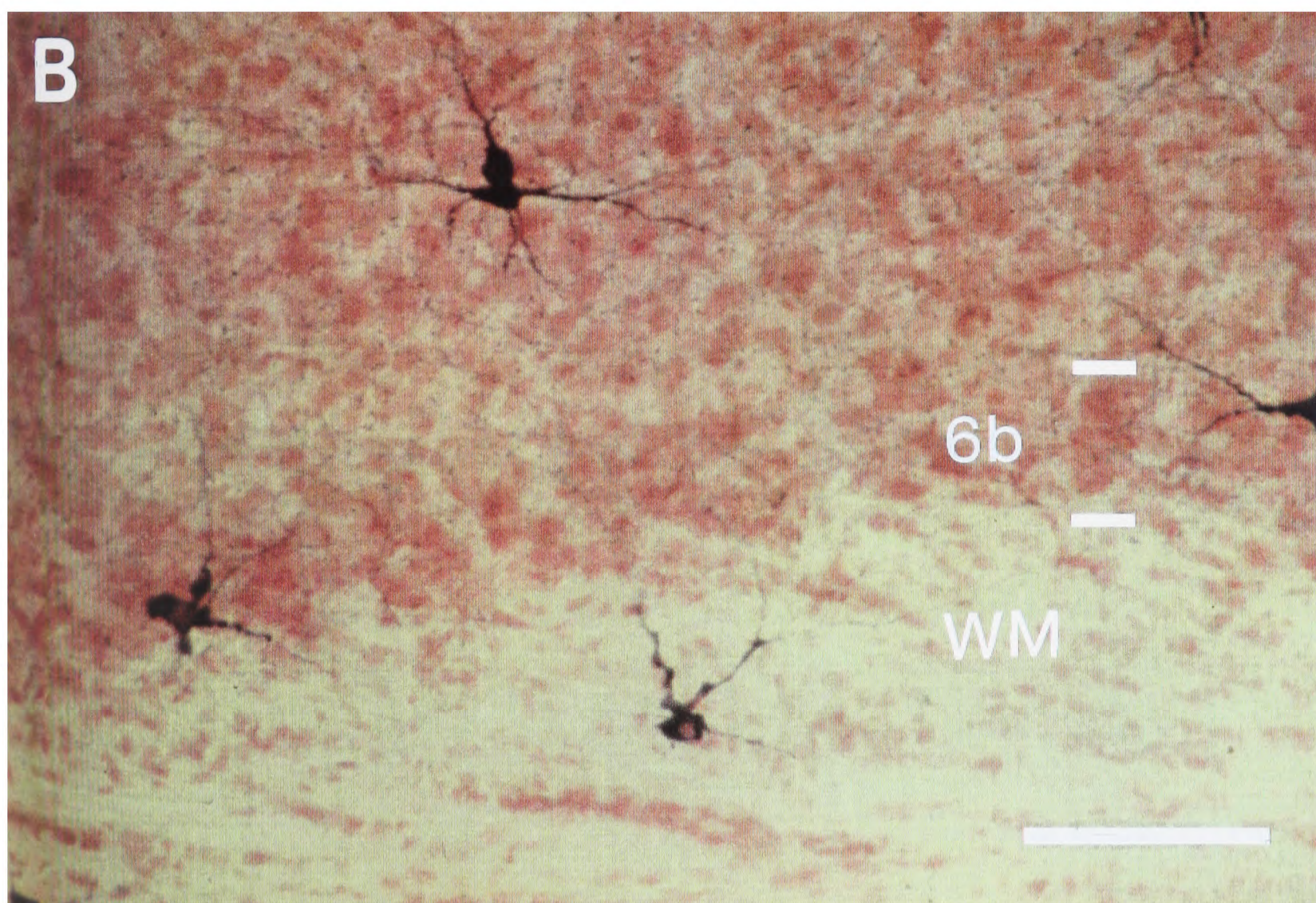
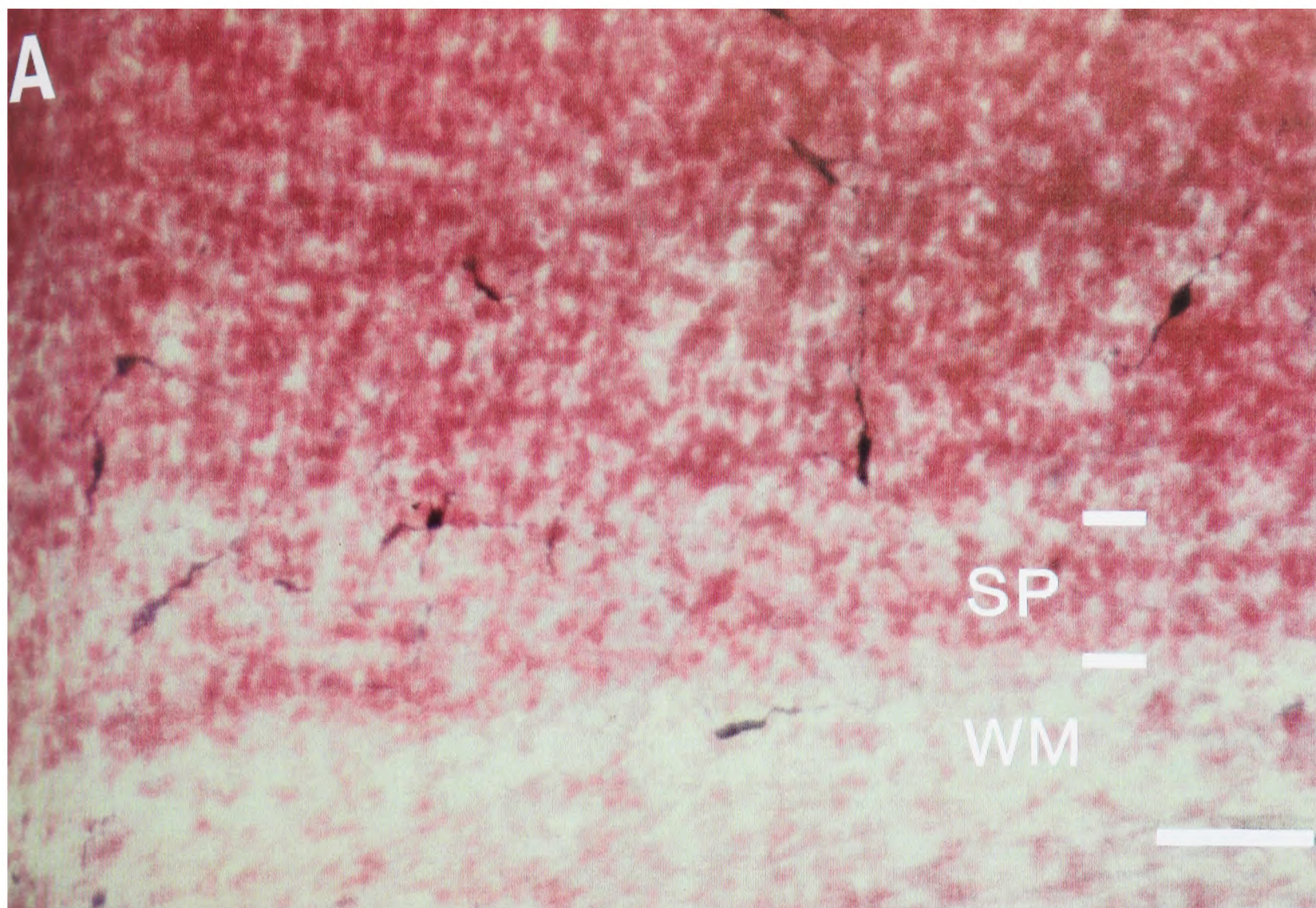
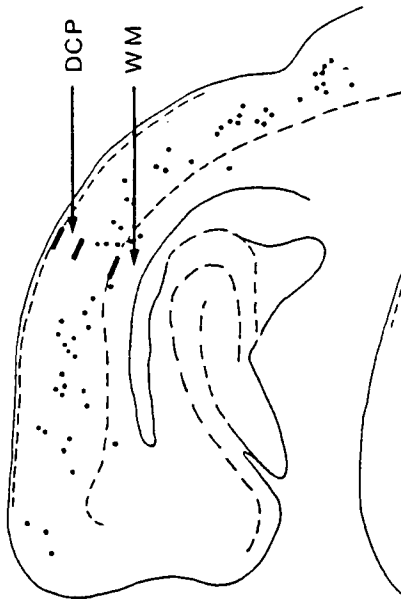
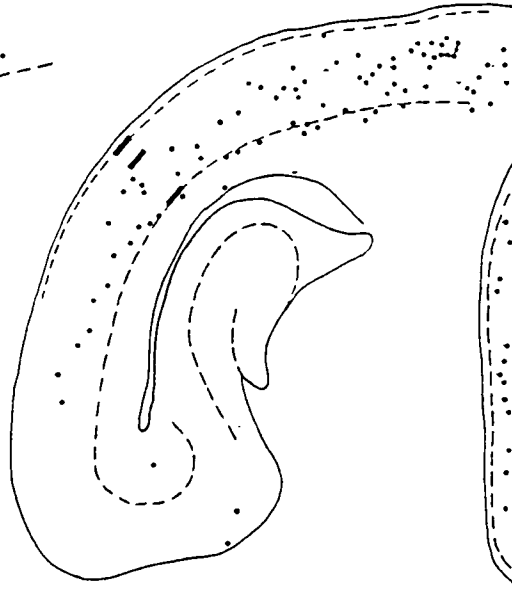


Fig. 5.4

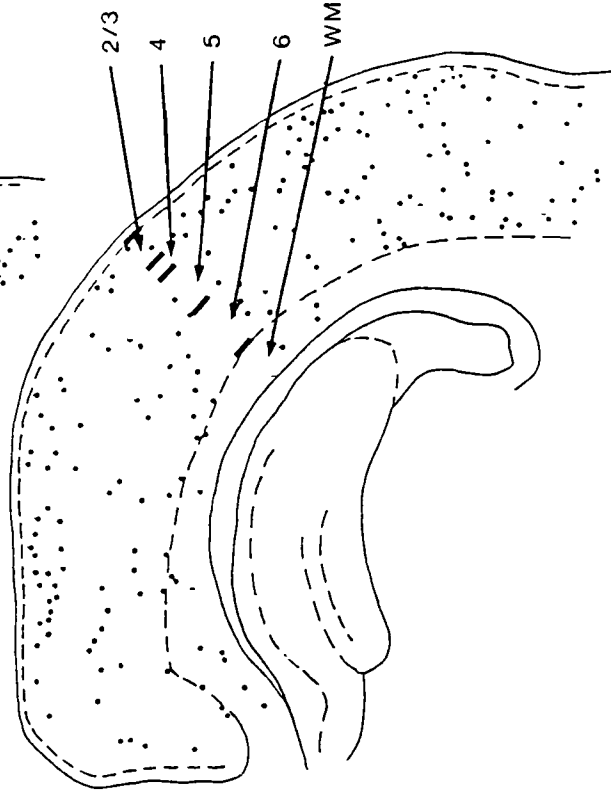
The areal and laminar distribution of NADPH-d cells at different ages (P0, P2, P8, P13, P24) illustrated in *camera lucida* drawings of coronal sections through comparable sectors of the somatosensory cortex. Each dot represents a stained cell body (but labelled cells in the hippocampus were not drawn). Over the first week, NADPH-d cells gradually appear in all layers (following the general cortical neurogenetic and morphogenetic inside-out gradient) except in layer 1. In the second and third week there is a slight decline in the cell number per unit area. The white matter also contained labelled cells. At P0 and P2, the superficial two bars indicate the borders of the dense cortical plate and the lower two the white matter: at all the other ages, all layers are indicated, the superficial pair of bars indicating the borders of layers 2 and 3 combined. (DCP: dense cortical plate, WM: white matter) (Scale bar: 1mm).



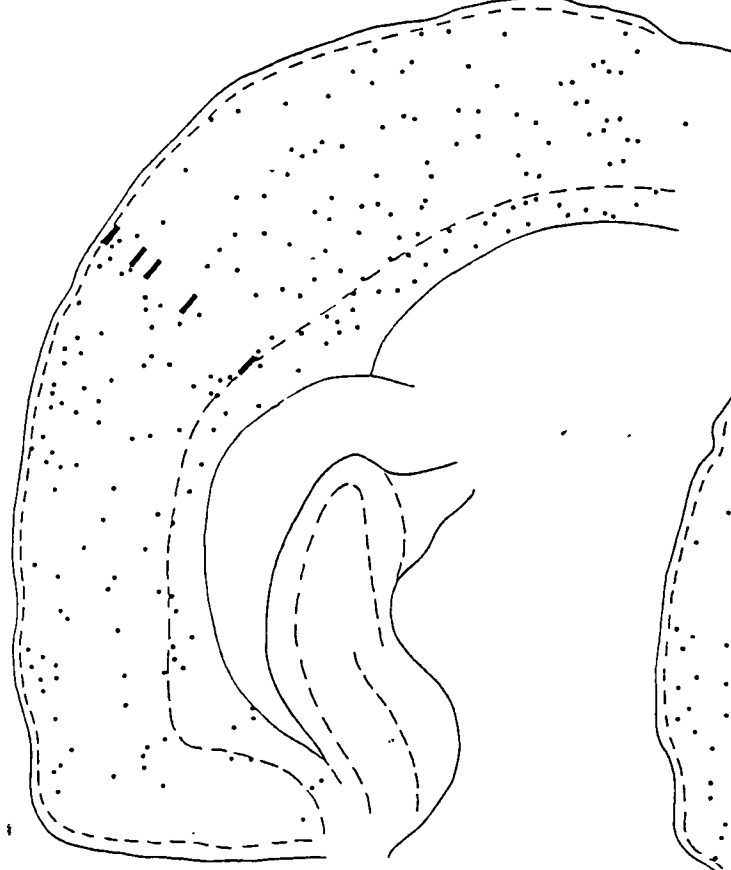
P0



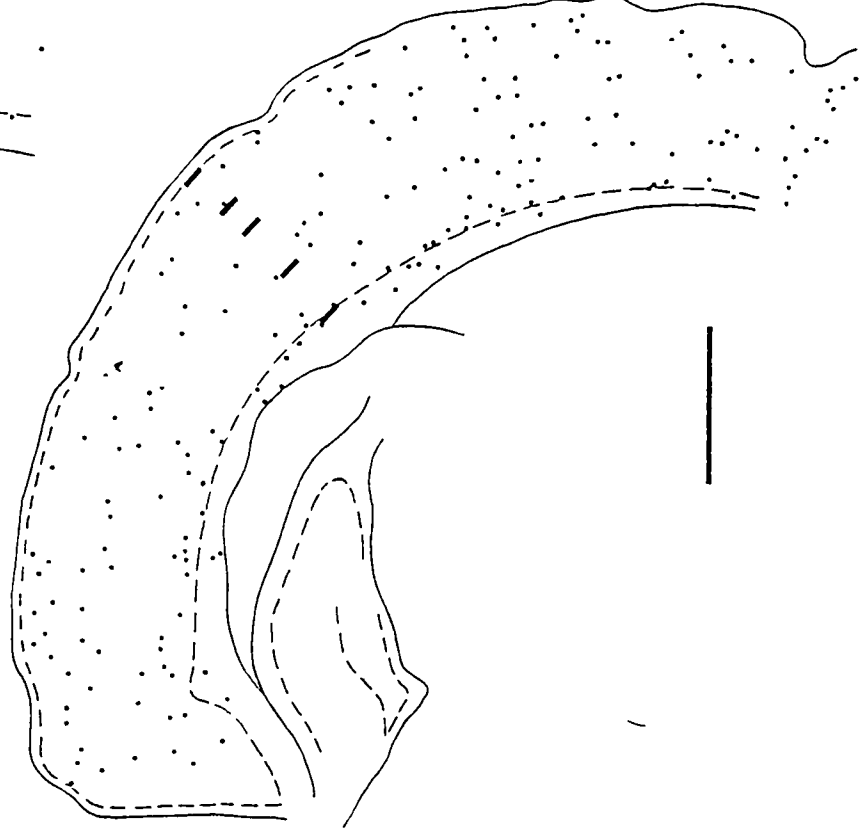
P2



P8



P13



P24

Fig. 5.5

Photomicrographs of NADPH-d-reactive cells in coronal sections from P7 (A and B), P10 (C), and in P24 (D) somatosensory cortex. (Scale bar: 100µm for all). In general, the cells show highly variable morphology, do not have dendritic spines (they are smooth), and are most probably interneurons.

A: This shows the upper layers of the P7 cortex, where most of the labelled neurons are bipolar or bitufted, oriented vertically. The staining in this particular preparation is less intense, and the nuclei appear unstained. The layer 1/2 border is indicated with an interrupted line.

B: The lower part of the P7 cortex (including sublayer 6b; presumed vestige of part of the subplate) contains bipolar and multipolar cells.

C: The white matter (W) and layer 6 and 5 of P10 somatosensory cortex. Polymorphous cells are very intensely labelled, revealing Golgi-like staining.

D: Polymorphous cells at the layer 5/6 border of P24 cortex. The cells now show clearer processes, which can be traced into the dense meshwork between the labelled cell bodies.

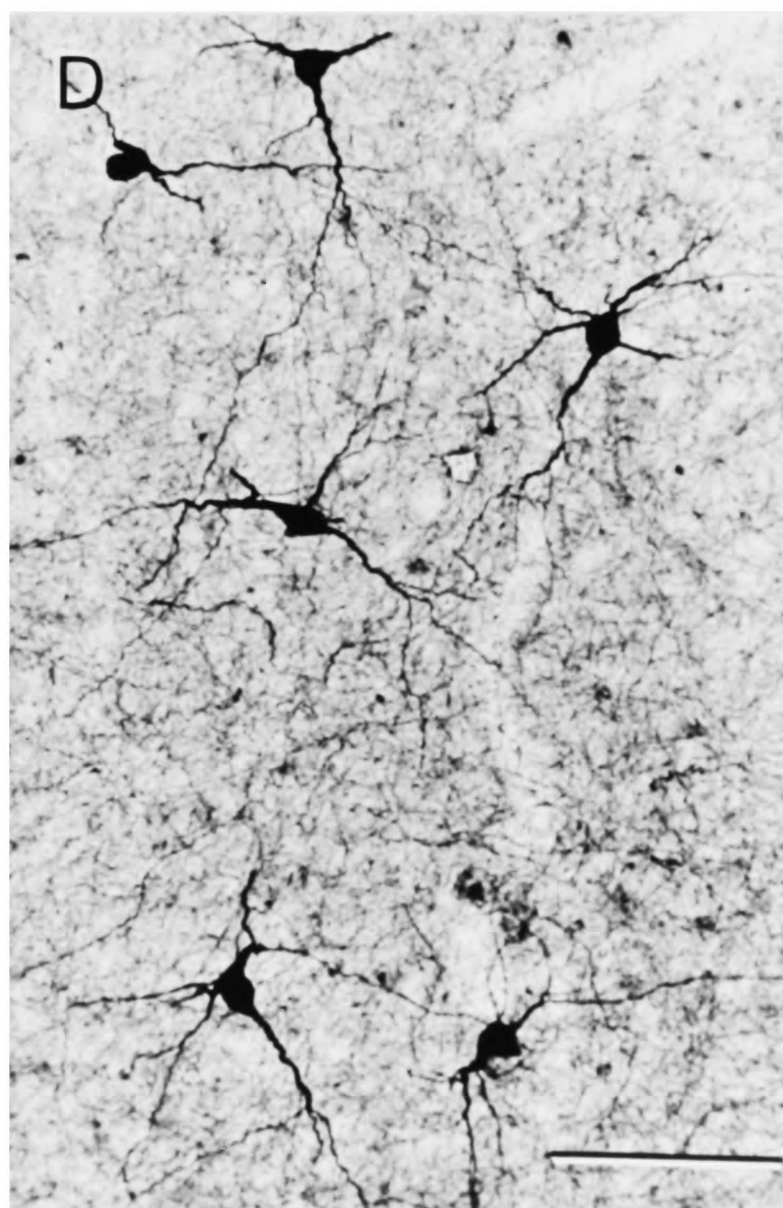
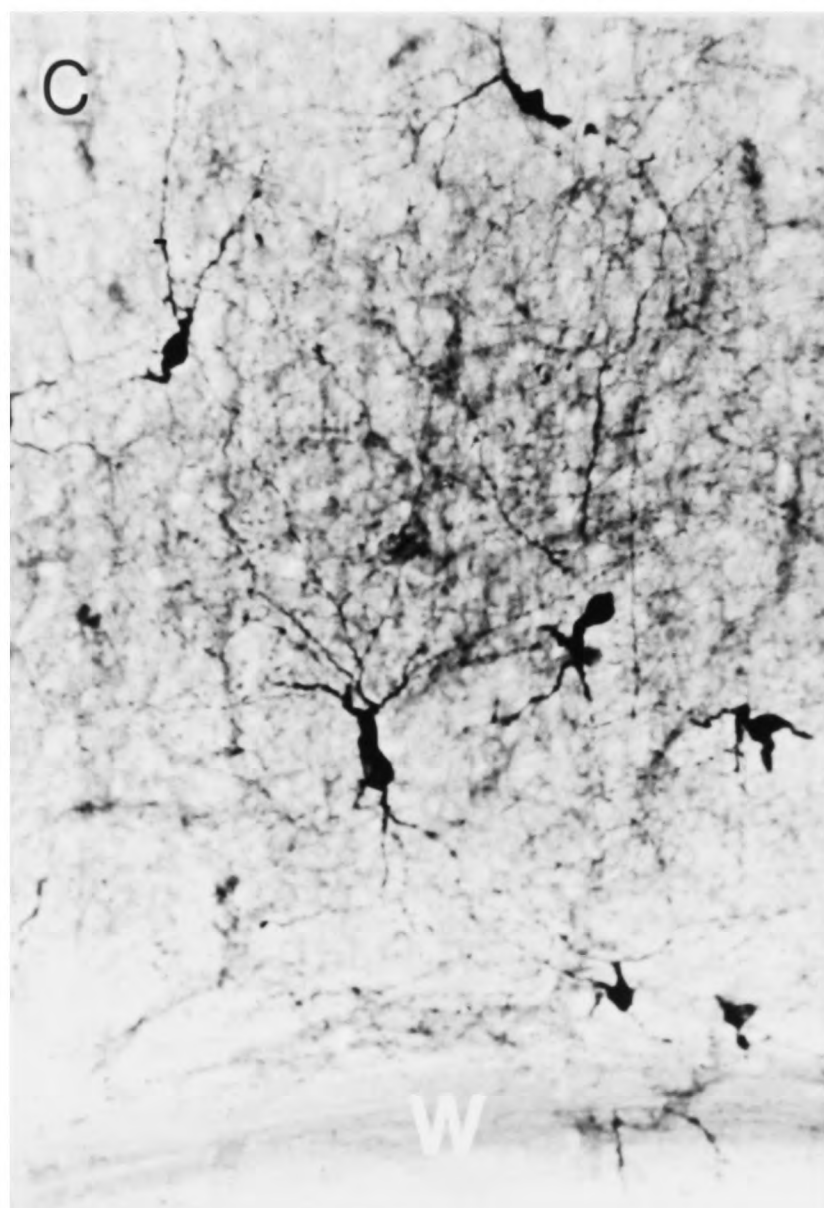
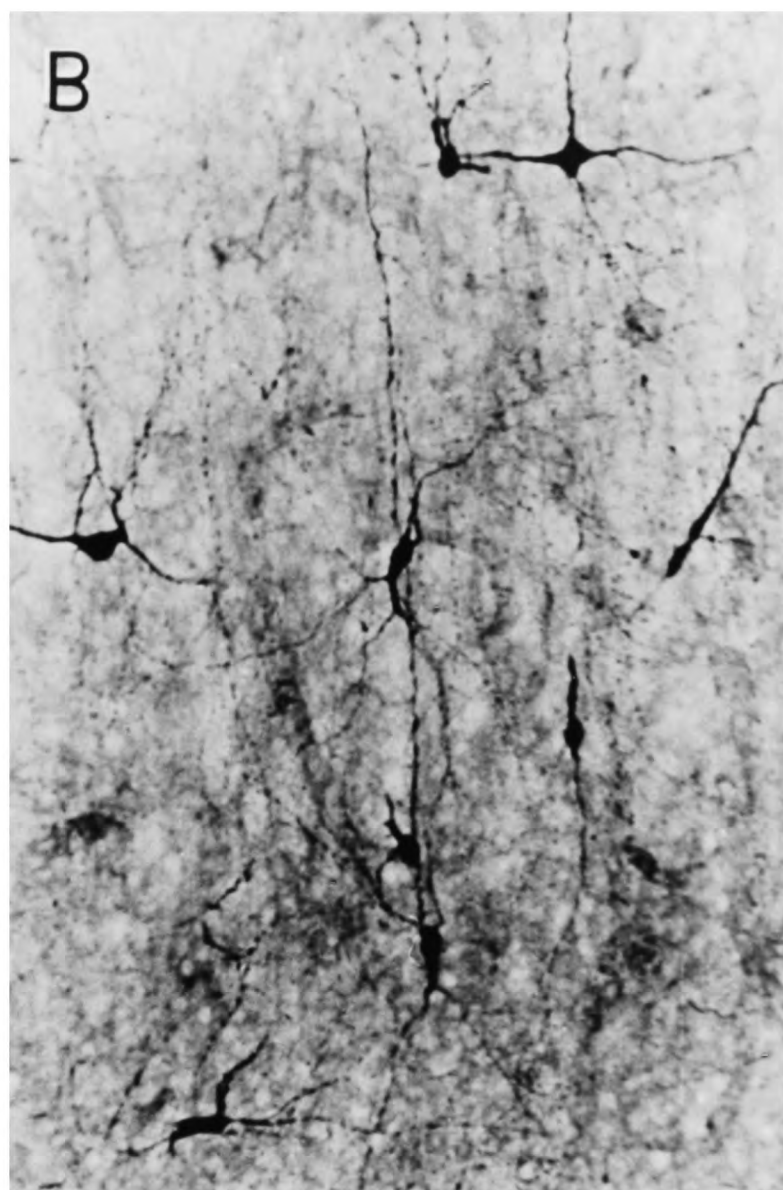
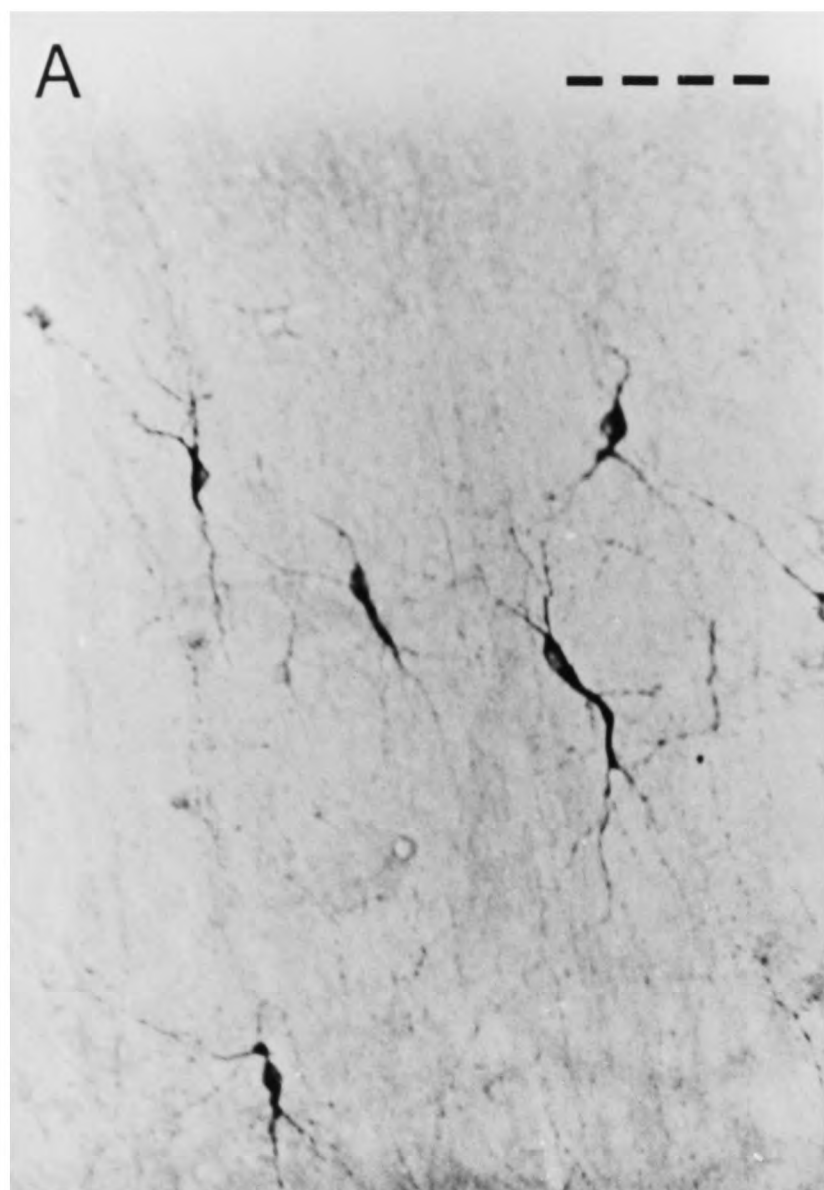


Fig. 5.6

A: Dense network of dark blue labelled processes in coronal section through layer 2/3 of P13 visual cortex.

B: Smooth bitufted neurons in layer 2/3 of P13 visual cortex.

(Scale bar: 50µm)

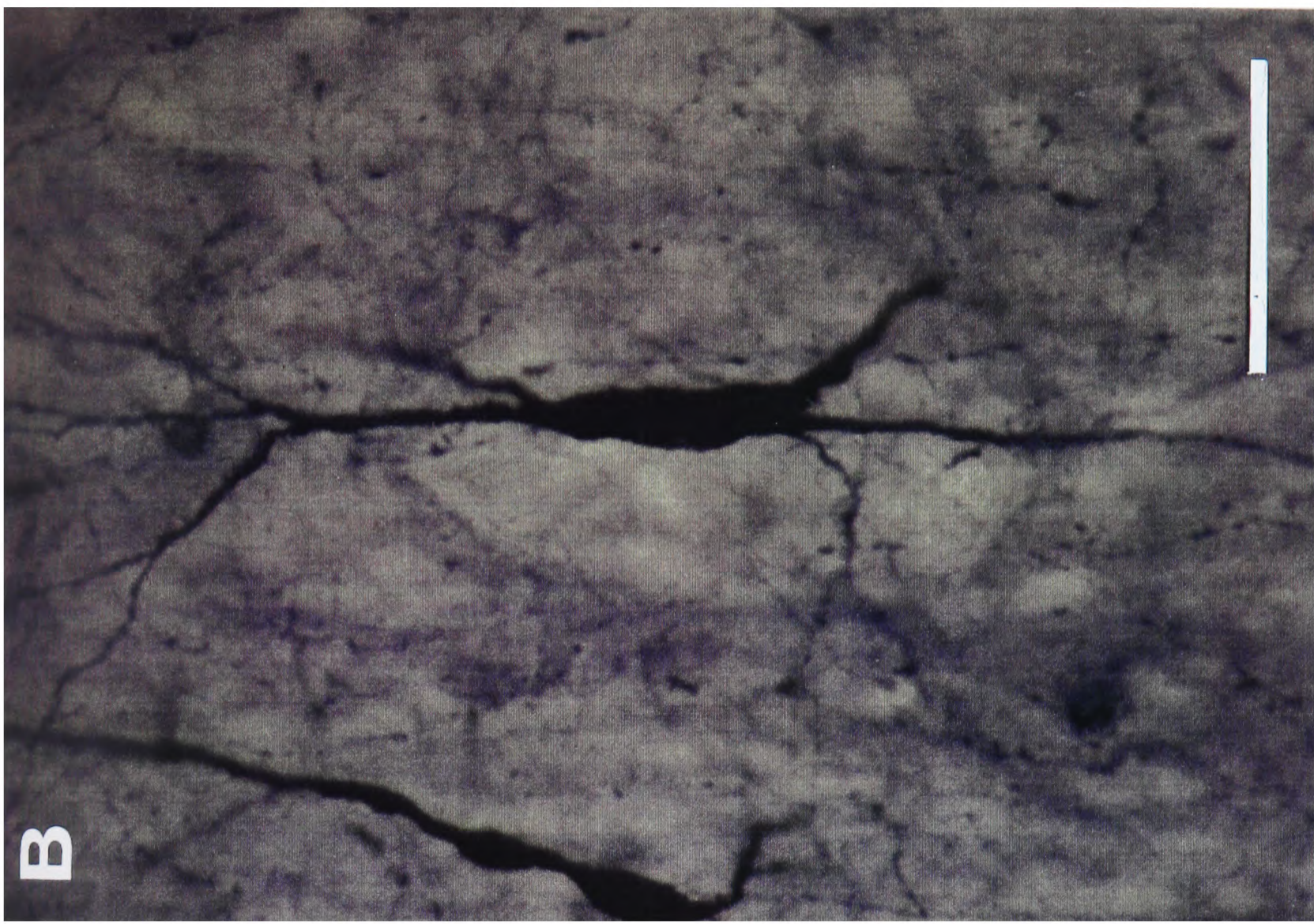
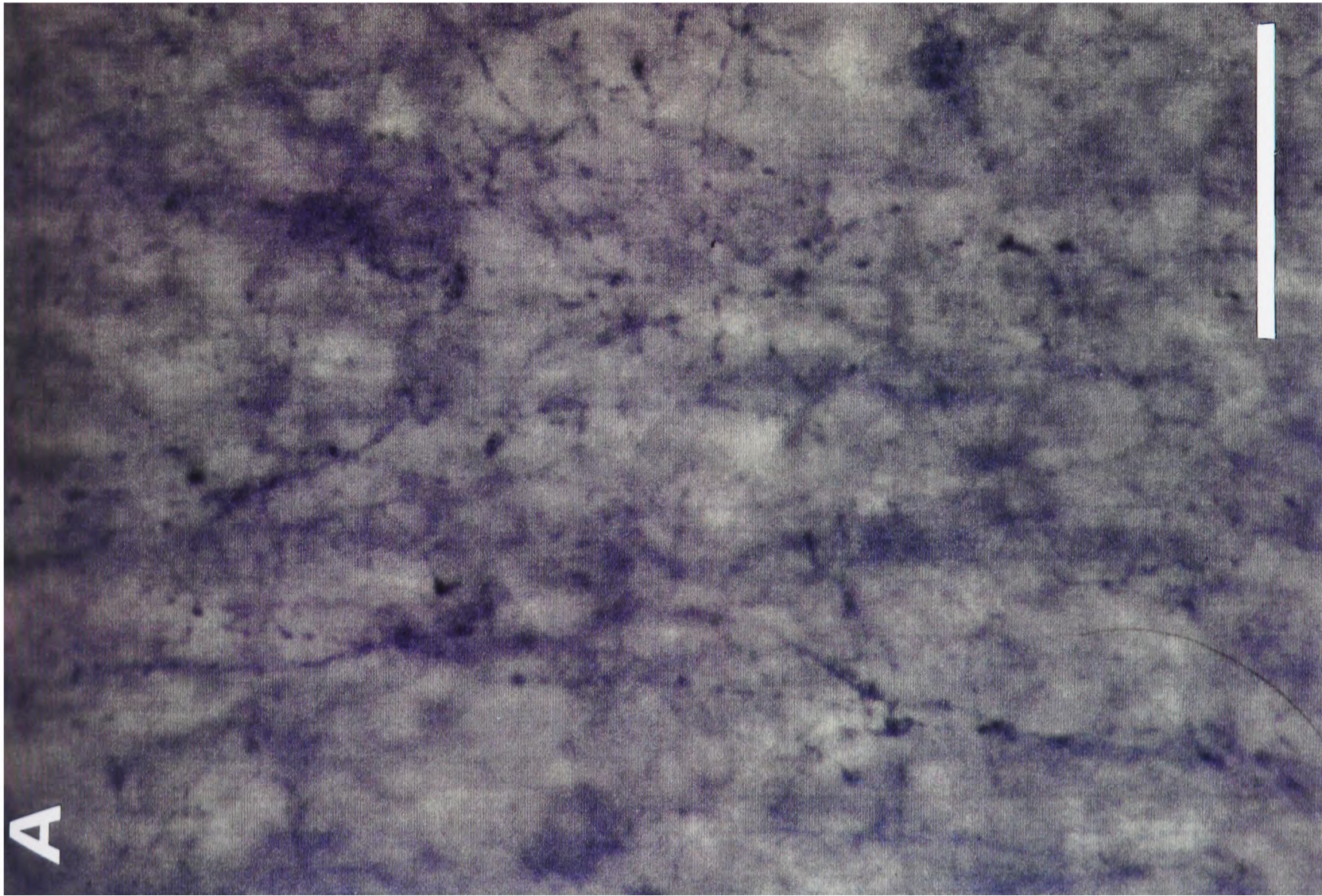


Fig. 5.7

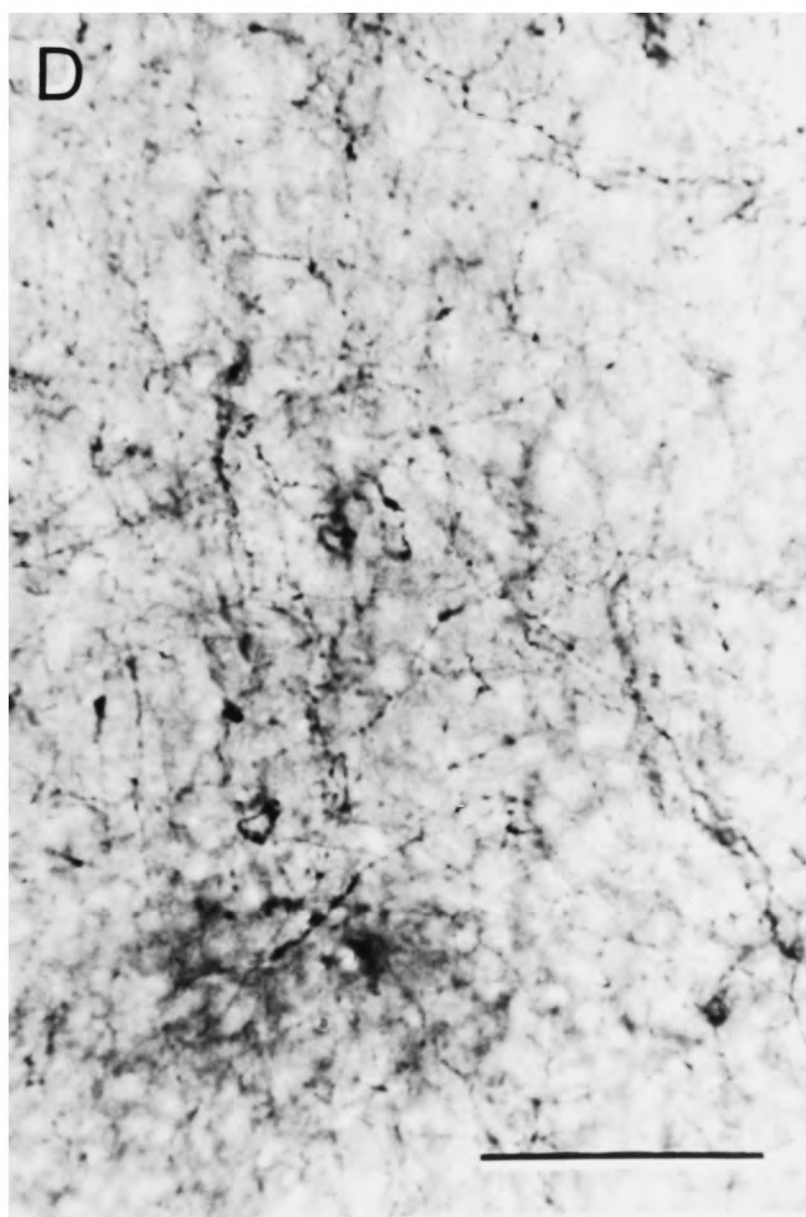
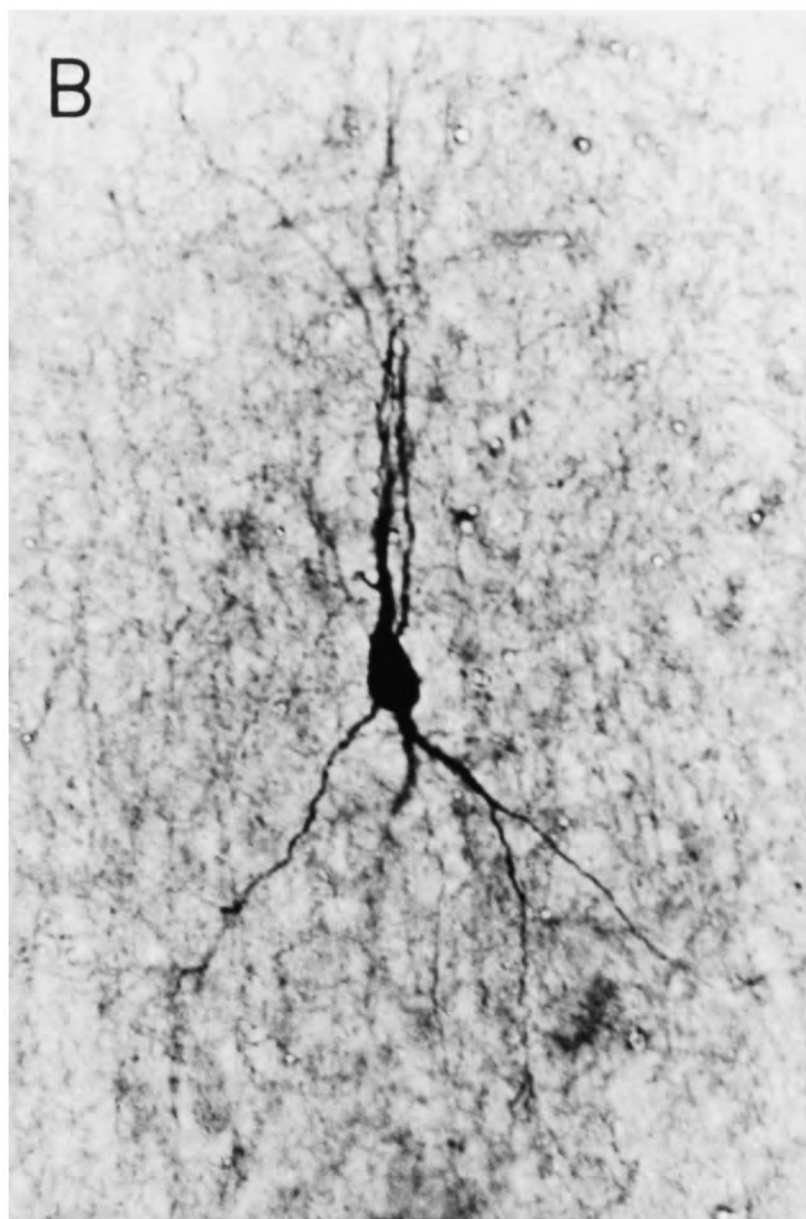
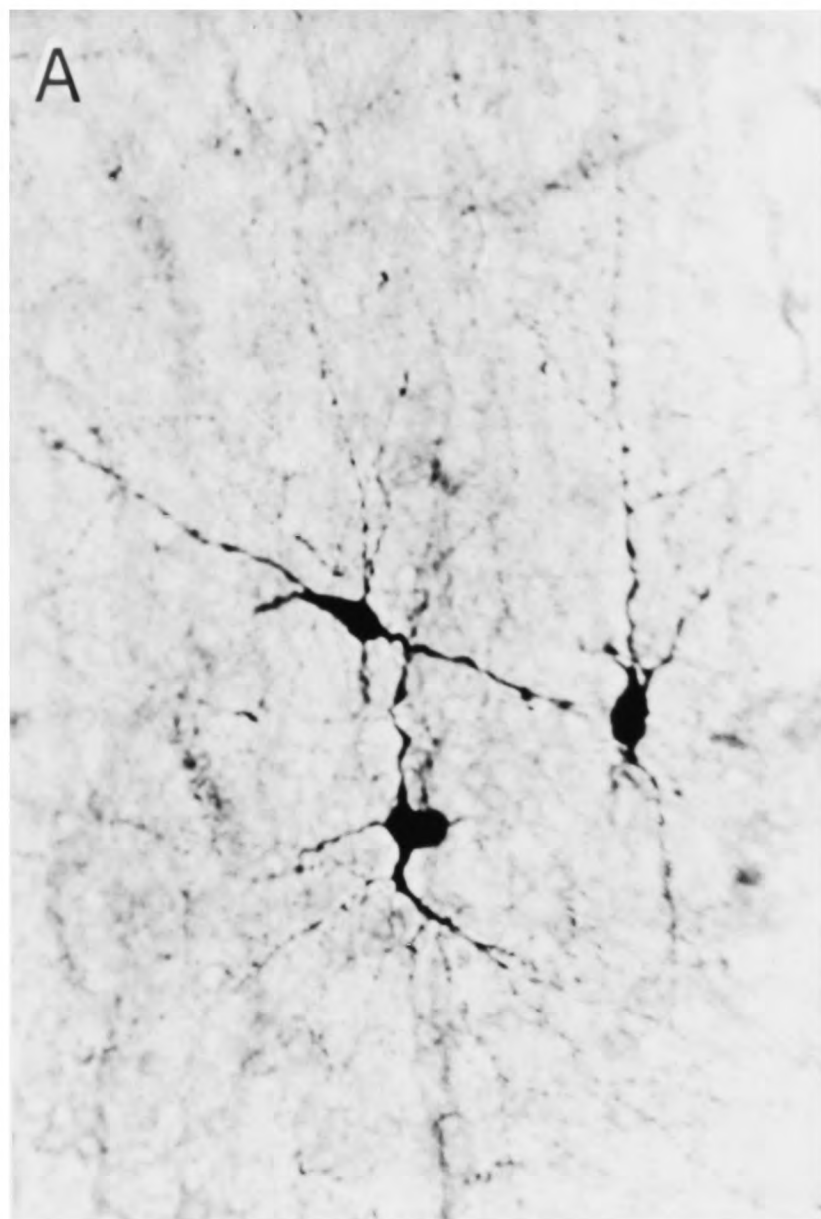
A: Smooth multipolar and bitufted neurons in the supragranular layers of P8 somatosensory cortex. Note the beaded (but not spiny) appearance of some of the dendrites.

B: Bitufted neuron from layer 2/3 of P10 somatosensory cortex.

C: Dense network of labelled processes in layer 2/3 of P10 somatosensory cortex.

D: Elaborate meshwork of labelled processes in layer 5/6 of P13 somatosensory cortex.

(Scale bar 100µm for all parts).



DISCUSSION

The present study shows that NADPH-d-reactive cells, which synthesise NO (Hope et al., 1991), first appear around the time of birth (P0) in the subplate and the lower layers of the cortical plate, but not in the remainder of the first-generated cell population located in the marginal zone, suggesting that subplate and marginal cells are phenotypically distinct. With increased maturity, NADPH-d-positive bipolar and bistellate cells gradually appear in increasing numbers in the upper layers, while NADPH-d polymorphous cells remain present in the lower layers, including the subplate, and in the white matter (beginning around P9). With further development, the density of all these cells generally declines (starting about P13), reaching approximate adult levels about three weeks postnatal. The processes of these cells establish an ubiquitous, dense network, which infiltrates all areas and all layers of the developing cortex.

5.7 Cell types producing nitric oxide

In all cases in which they have been studied, NADPH-d-positive cells, presumed to be generating NO, are a distinctive sub-population of neurons. They are usually small interneurons rather than larger principal cells and their axons distribute locally.

In the adult monkey, individual intensely labelled NADPH-d cells are seen in all layers of striate cortex and in the white matter (Sandell, 1986). They are most abundant (though they constitute only a small fraction of the entire population) in layers 2, 3 and 6, and least common in layers 4 and 5. The white matter is also especially rich in NADPH-d cells (Sandell, 1986, Mizukawa et al., 1988). Vincent and Kimura (1992) and Druga and Syka (1993) obtained similar results in adult rats, except that the NADPH-d cells were distributed with equal density from the white matter to layer 2,

a finding confirmed here.

My results show that NADPH-d-positive cells in the rat cortex are of the same morphological classes throughout development: in other words, there does not appear to be transient expression of NADPH-d in an unusual population in which it is not found in the adult (unlike the situation for parvalbumin; see Chapter 6). Only certain bipolar, bistellate and multiform neurons ever express NADPH-d. No pyramidal cell, judged on morphological grounds, was labelled at any of stage examined.

Labelled cells deep in the white matter, whose individual processes could be followed more easily than those of cells in the grey matter, have a tremendous lateral spread, as described by Sandell (1986) and Mizukawa et al. (1988). In the cortical layers, the arbors of the major dendritic branches can usually be followed and they are clearly very extensive. It was usually impossible to follow the fine axons of reactive cells far from the cell body, but it seems likely that they also contribute enormously to the widespread dense meshwork of fine NADPH-d-positive processes that covers the entire cortex.

Similarities to GABA, Somatostatin, APP and NPY interneurons

It has been previously reported that in the adult rat cerebral cortex SOM, APP and NPY are co-localized with NADPH-d in the same cortical neurons (Vincent et al., 1983). It is thus not surprising, then, that the majority of NADPH-d reactive cells, at all ages, have morphological appearance very similar to that described for interneurons containing the neuropeptides SOM and NPY (Parnavelas et al., 1988; Hendrickson, 1983; Hendry et al., 1984a,b). Indeed, the timing and laminar sequence of expression of NADPH-d reactivity closely resembles those described for the appearance of NPY and SOM in the rat cortex (Parnavelas et al., 1988, Hendrickson, 1983; Hendry et al.,

1984a).

Hendry et al. (1984a) reported that the SOM and NPY immunoreactive cells in the monkey cortex have no visible axon. This obviously contrasts with the NADPH-d cells described here. However, the labelling of axons is notoriously capricious and there may be insufficient peptide transmitter in the axons of such cells for them to stain clearly with immunohistochemical methods.

Laminar distribution during development

At P0, NADPH-d neurons are present only in the subplate and the lower part of the cortical plate, and they gradually appear in progressively more superficial layers over the following days. At birth the cells that will form the upper layers (2,3,4) are still migratory and the cortex is quite immature. NADPH-d expression, just like the expression of putative neurotransmitters (Parnavelas et al., 1988) seems to be a postmigratory phenomena. However, the inside-out appearance of NADPH-d-positivity is not simply an inevitable temporal sequence, following the postmitotic ages of the cells, since the neurons of the marginal zone are not NADPH-d-reactive at birth (nor indeed at any stage) despite the fact that they are co-generated with those of the subplate (Luskin and Shatz, 1985a,b).

Areal distribution of NADPH-d reactivity

I was interested in the possibility that NADPH-d expression might vary between cortical areas in a way that might correlate with the occurrence of synaptic plasticity or other phenomena in which the NO system might be involved. However, with the exception of the first postnatal week (when the neurogenetic and morphogenetic gradients across the cerebral hemisphere are reflected in the areal distribution of NADPH-d maturation) I did not observe obvious regional differences in the distribution of reactive cells or of

the fibre plexus correlated with cytoarchitectonic boundaries (revealed, for instance, by cytochrome oxidase histochemistry; Sandell, 1986). On the other hand, the diffuse background staining of the neuropil, especially of layer 4, showed clear variation in density, demarcating, for instance, area 17 (where this staining was particularly intense) and the centres of the barrels in the somatosensory barrel field.

5.8 The fibrous meshwork

One of the most interesting features of NADPH-d reactivity revealed by this study is its distribution in a dense, ubiquitous plexus of fine processes. Although, even at the peak of expression, around P10, only a tiny proportion of cortical neurons stain for NADPH-d, the stained processes, assumed to be the dendrites and intrinsic axons of these cells form a very widespread meshwork. This plexus extends from the white matter to the pial surface in mature animals (Figs. 5.5C and D) but during the first week of life, there is clearly more fibre staining in the infragranular layers, surrounding the labelled cell bodies.

Since the vast majority of cortical neurons in the adult are immunoreactive for soluble guanylyl cyclase (in the cell body and the projections), they are presumably capable of responding to NO (Ariano et al., 1982; Nakane et al., 1983). This means that the system of NO-synthesizing neurons, though relatively small in number, is, through its extensive fibre meshwork, potentially capable of influencing all those cells that contain guanylyl cyclase. It would be interesting to know how the distribution of guanylyl cyclase matures in the developing cortex and to see whether its appearance correlates with the distribution of NADPH-d-reactive cells and their processes.

5.9 Possible functions of the NO system

Ca²⁺ influx (perhaps particularly through the ionophore of the NMDA receptor) is

needed for NO production from arginine in the presence of calmodulin and NO synthase. NO needs no release mechanism or surface receptor system: it is membrane soluble and diffuses rapidly in both lipid and aqueous domains. So, although its half life is only 0.3 secs, it is capable of influencing several cells surrounding its site of production. Since the fibre meshwork is very densely distributed, it is likely that all cortical neurons are effectively within reach of a site of NO production.

NO appears to act by stimulating soluble guanylyl cyclase (Arnold et al., 1977; Miki et al., 1977) and may directly activate a cytoplasmic protein ADP-ribosyltransferase (Brune and Lapetina, 1989). It may also play a part in regulating cell death (Wallis et al., 1992). The cGMP produced by activated guanylyl cyclase could have a variety of intracellular effects via its influence on protein kinases and phosphodiesterase. These include influences on the cytoskeleton (and hence growth) and the regulation of gap junction conductivity. The most exciting possible role for the NO system is as an intercellular messenger mediating synaptic modification (see Vincent and Hope, 1992; Izumi and Zorumski, 1993; Chapman et al, 1992). It seems likely that NO released by donor interneurons in the cerebellum triggers long-term depression of parallel fibre/Purkinje cell synapses (Ito and Karachot, 1990; Shibuki and Okada, 1991); and in the hippocampus too there is evidence that NO, again produced by interneurons, might be responsible for mediating changes in transmitter release contributing to long-term potentiation in the hippocampus (see Vincent and Hope, 1992). Since similar long-term changes in synaptic efficacy are known to occur in certain cortical areas, it is possible that the NO system revealed here plays a crucial role in such synaptic plasticity. Although plasticity seems to persist, at least in some cortical areas, throughout life, there is no doubt that cortical circuitry is generally

especially modifiable during an early postnatal period. It may then be significant that the density of the NO system appears to reach a peak just after the first postnatal week and then to decline somewhat to adult values.

As well as its quite likely role in mediating synaptic modification, NO could contribute to many other processes specifically during development. It is interesting to note that there is a similar age-related pattern of NADPH-d reactivity in the rat retina, where NADPH-d cells reside in the inner nuclear layer (INL) and their diffusely labelled dendrites spread in the inner plexiform layer (IPL). The soma sizes and dendritic fields of NADPH-d cells are largest and most prominent between P11 and P15, and reduce with further development (Mitrofanis, 1989). The period of their maximum size coincides closely with the first appearance of amacrine and bipolar cell synapses in the IPL (Horsburgh and Sefton, 1986) and with the beginning of electrical activity (Weidman and Kuwabara, 1968). It seems possible that NO plays a role in the synaptogenesis in the inner plexiform layer. The possible modulatory effect transmitted by NO, might be responsible for the synchronization of activity patterns in the developing retina (Meister et al., 1991; Wong, 1993).

The demonstration of NADPH-d-positive interneurons in the developing cerebral cortex raises the possibility that NO diffusion could supplement gap junction and synaptic transmission as a form of cell-cell communication during development. NADPH-d cells appear in the cortex when coupling of the immature neurons through gap junctions is rapidly declining (Yuste et al., 1992). The NADPH-d fibre plexus could synchronize activity patterns at a stage when the majority of the cortico-cortical synapses have not yet formed, but the gap-junction coupling of developing neurons has declined. Its unique characteristics suggest that NO could play an essential role in the

establishment of the precise cortical microcircuitry. The 'functional crystallization' of cortical circuitry might need the local 'binding' between neighbouring neurons that NO could provide, after the loss of gap junctions.

CHAPTER 6

EXPRESSION OF PARVALBUMIN IN THE DEVELOPING RAT CEREBRAL CORTEX

INTRODUCTION

6.1 Chemical differentiation in the cortex

With the development of various new anatomical tracing methods (carbocyanine dyes, intracellular dye injection, confocal microscopy) and *in vitro* recording techniques, our understanding of the pattern of development of the functional connections of cortical neurons has dramatically increased. But neurons are specialized not only according to their morphology and the connections that they form; they also differentiate according to their transmitter chemistry. To understand more fully the modelling of cortical circuitry during development more fully we need to know about the maturation of neurotransmitters and their receptor systems.

The differentiation of transmitter systems in cortical neurons is considered to be a postmigratory phenomenon; neurochemical maturation, revealed by immunocytochemical techniques, takes place only after the migrating neuron has reached its final destination (Parnavelas et al., 1978, 1988). Recent imaging techniques have been used to demonstrate the selective effects of various transmitters on migrating neurons (Komuro and Rakic, 1992; 1993; Yuste et al, 1992). There are characteristic, age-related and species-specific patterns of appearance of different transmitters in the cortical plate. In the rat, for instance, the expression of neuropeptides (Somatostatin (SOM), Neuropeptide Y (NPY), Vasoactive Intestinal Polypeptide (VIP), Cholecystokinin (CCK) in interneurons mainly occurs after birth, appearing in

progressively more superficial cortical layers over the first two postnatal weeks, in an inside-out sequence (McDonald et al., 1982a,b). SOM, NPY, CCK and/or VIP usually co-exist with Gamma-aminobutyric acid (GABA) in the same neurons (Jones and Hendry, 1986). In such cells, the expression of the various transmitters tends to occur in the sequence GABA, NPY, SOM then CCK (Wolff et al., 1984; Parnavelas et al., 1988).

Celio (1986) and Hendry et al. (1989) recently demonstrated that *parvalbumin*, a 20-30KD molecular weight calcium-binding protein, also co-exists with GABA and the neuropeptides in the same interneurons. Ca^{2+} is thought to play a key role in cellular signal transduction. Parvalbumin and the other intracellular Ca^{2+} -binding proteins, calbindin and calretinin (Celio, 1990), are believed to play a passive buffering role in the regulation of intracellular Ca^{2+} . The concentration of calcium-binding proteins has been demonstrated to be dependent not only on growth factors but also on neuronal activity (Lowenstein et al., 1991; Masiakowski and Shooter, 1988), and it therefore might be expected to reflect the changing pattern of activation during the rearrangement of the cortical neural circuitry recently described by Friauf and Shatz (1991).

6.2 Changing neuronal circuitry during cortical development

In the rat, substantial remodelling of cortical circuitry occurs around birth, when the accumulating thalamic fibres leave the subplate and enter the cortex (Lund and Mustari, 1977; Wise and Jones, 1978; Catalano et al., 1991; Kageyama and Robertson, 1993). This sudden transition in circuitry seems to occur generally in mammalian cortical development (Rakic, 1976; Wise et al., 1977; Shatz and Luskin, 1986; Chapter 3). In the rat, thalamic fibres arrive under the occipital cortex around E16 (Chapter 3; Molnár and Blakemore, 1990a,b; Reinoso and O'Leary, 1990; Erzurumlu and Jhaveri, 1990) but

only very few of them enter the cortex before about E19/20 (Chapter 3; Blakemore and Molnár, 1990; Catalano et al., 1991; Kageyama and Robertson, 1993). During the 'waiting period' (Lund and Mustari, 1977; Rakic, 1976) at least some thalamic fibres form synapses with subplate cells (Kostovic and Rakic, 1990; Herrmann et al, 1991) and at least some of these connections are clearly functional (Friauf et al, 1990; Friauf and Shatz, 1991). Some subplate cells receive terminals *en passant* from collaterals of the corticofugal axons of layer 5 and 6 cells (Lowenstein and Shering, 1991; Chapter 3). Many also send an axon into the developing cortical plate, which terminates in the marginal zone and gives collaterals into the middle of the cortical plate (Friauf et al, 1990). In return, spiny stellate cells of layer 4 (in the cat) send their axons into the subplate at an early postnatal age (Callaway and Katz, 1992). At this phase, then, cortical microcircuit is very different from in the adult and involves **both** the subplate and neurons of the cortical plate (Friauf and Shatz, 1991).

In the rat, thalamocortical fibres invade the cortical plate just before (Catalano et al, 1991; Chapter 3; Blakemore and Molnár, 1990) or around the time of birth (Lund and Mustari, 1977), but the laminar-specific innervation pattern becomes apparent only at the end of the first postnatal week (Lund and Mustari, 1977; Wise and Jones, 1978). It is probable that synapses start to form as soon as thalamic fibres invade the cortex (Lund and Mustari, 1977; Jones, 1981) but synaptogenesis appears to continue for many days and possibly weeks afterwards (Cragg, 1975; Kristt, 1978; Wise et al., 1979), perhaps especially for intrinsic connections.

Electrophysiological changes are well correlated with changes in the anatomical circuitry (Armstrong-James and Johnson, 1970; Verly and Axelrod, 1975). Synchronously with the establishment of the ultimate thalamic innervation pattern the

subplate cells **stop** participating functionally in the circuitry (Friauf and Shatz, 1991) and many of them start to die (Luskin and Shatz, 1985b). In contrast with the initial gross distribution of thalamocortical and corticothalamic connections, which might be due to a cascade of a few, relatively simple mechanisms (Chapters 2 and 3; Blakemore and Molnár, 1990), the remodelling of cortical circuitry during thalamic fibre invasion is probably a more complex process in which patterns of afferent and local activity, expression of surface molecules and growth factors, and cell death all play crucial roles. In turn the remodelling leads to the formation of new synapses and therefore to new distributions of activity. It is rather difficult to get access to cortical neurons in order to record their activity in situ during early phases of cortical development. During embryonic and early postnatal life, cortical neurons are locally linked into coupled domains via gap junctions (LoTurco and Krigstein, 1991; Yuste et al., 1992). Intracellular injections of neurobiotin, a derivative of biotin, revealed that neurons in the cortical plate of neonatal rats are extensively coupled and that this coupling allows the neurons to establish coordinated activity patterns, revealed by Ca^{2+} -imaging in acute cortical slices, before the establishment of synaptical connectivity (Katz, 1993). The interplay and transition between these two phases of intercellular communication is uncharted territory.

Ca^{2+} acts ubiquitously as an intracellular messenger and trigger. It plays a part in the generation of the action potential and in transmitter release; it activates a variety of intracellular pathways, including nitric oxide production; it could play a part in growth regulation through its influence on the cytoskeleton; its entry through the NMDA receptor has been implicated in activity-dependent long-term potentiation (Kandel, 1981; Eccles, 1983; Lynch and Baudry, 1984). In view of the many roles that

Ca²⁺ could play in local developmental processes in the cortex, I became interested in the distribution of the Ca²⁺ binding protein, parvalbumin, at different embryonic and early postnatal phases of cerebral cortical development. In this chapter I correlate the age-related patterns of parvalbumin immunoreactivity with the different steps in cortical circuit formation in the rat.

METHODS

Fetal Hooded Lister rats were obtained from time-mated pregnancies (Department of Human Anatomy, University of Oxford). A female in oestrus was exposed to a male for approximately 24 hours and the following day was designated embryonic day zero (E0) of the resulting pregnancy. Pregnant rats were anaesthetized with sodium pentobarbitone (60mg/ml I.P.) and the fetuses were removed by caesarian section at E15 (n=4), E17 (n=4) and E20 (n=4). The day of birth was counted as P0 and the following series of postnatal animals was studied: P0 (n=7), P2 (n=4), P5 (n=4), P6 (n=6), P7 (n=7), P8 (n=8), P9 (n=1), P10 (n=6), P12 (n=4), P13 (n=4), P17 (n=4), P=20 (n=2), P24 (n=3), P25 (n=2) and adult (n=5).

For Nissl and immunocytochemical staining of brain sections, rats were anaesthetized (by chilling before P5, by I.P. sodium pentobarbitone at older ages) and perfused transcardially with phosphate-buffered saline (pH 7.4) followed by 4% formaldehyde plus 0.125% glutaraldehyde at room temperature (200ml total fixative for adults and 10-50ml for fetal rats). After perfusion, the brain was removed, postfixed in fresh fixative for 1-2 hours then immersed in 20-30% sucrose in phosphate-buffered saline for about 4 days. It was then frozen on dry ice and sectioned coronally or horizontally on a microtome at thicknesses ranging from 50-100µm. Although sections thicker than 75µm may prevent antibody penetration through the entire thickness of the

section, it was essential to cut at this thickness for satisfactory preservation of the younger material during the immunocytochemical procedure.

For Nissl staining, sections were collected into 10% formalin in phosphate-buffered saline, mounted on gelatinized slides, air-dried, rinsed in distilled water, then dried again, cleared in HistoClear, hydrated with descending alcohols and immersed in 0.3% cresyl violet (pH 3.5). Sections were then dehydrated in ascending alcohols, cleared and coverslipped with DPX.

For the immunohistochemistry, monoclonal mouse anti-parvalbumin primary antibody (Sigma, 1:1000) was used. (In a parallel study, staining for mouse anti-calbindin antibody (Sigma, 1:200) was examined.) The bound primary antibody was further reacted with the avidin-biotin-peroxidase complex (Vectastatin) and visualized with a Nickel-enhanced 3,3-diaminobenzidine (Sigma) method. Sections were then dehydrated in ascending alcohols, cleared in HistoClear and coverslipped with DPX. Some sections were counterstained with Neutral Red and dehydrated as above. For control experiments, the primary antibody was replaced by phosphate-buffered saline with the addition of 0.5% bovine serum albumin (Sigma) and then reacted as above. These control sections showed no positive immunoreactivity for any of the antibodies used. In addition, when reacting fetal or early postnatal brains, sections of adult or older postnatal brains were reacted simultaneously for comparison, to ensure the effectiveness of the technique.

Unless otherwise stated, all the descriptions below are of the somatosensory cortex.

RESULTS

Before birth there is very little staining if any by parvalbumin antibody in the cortical plate or the marginal zone. In the same specimens the cortical subplate, Perireticular Nucleus and the Thalamic Reticular Nucleus are heavily stained (Mitrofanis and Guillery, 1991; Mitrofanis, 1992a), indicating that the antigen is already being expressed in other structures and the lack of immunoreactivity in the cortical plate is not the result of methodological problems.

Fig 6.1 is a series of coronal sections through the somatosensory region of the cortex showing the changing patterns of immunoreactivity from E20 to adulthood. Parvalbumin expression in the cortex appears in two distinct waves during the period of remodelling of cortical circuitry, first transiently in the subplate (E16-P10), and later in the cortical plate itself (P13-20).

6.3 P0-10: transient expression in the subplate

Parvalbumin first appears in the cells of the subplate before birth, around the sixteenth embryonic day in the somatosensory cortex (Fig. 6.2A). The first immunostained cells show pyramidal, inverted pyramidal or polymorphous morphology and are distributed exclusively in sublayer 6b, the postnatal location of the vast majority of subplate cells in the rat (Fig. 6.2A,B). The cortical plate itself and the marginal zone do not express any immunoreactivity at this stage. If the pia is not removed during histological preparation, it appears heavily stained. However, this pial staining proved to be non-specific since it was still present after the primary antibody was replaced by phosphate-buffered saline with the addition of 0.5% bovine serum albumin.) Labelled cells become more common and their staining is more intense at P5, P8 and P10. More and more pyramidal cells, some of them inverted pyramids, are stained in sublayer 6b

with a few appearing in the lower part of layer 6 proper. Subplate labelling reaches its peak density about the end of the first postnatal week (Fig.6.1 P7). But at this stage no stained cells are seen more superficially in the cortical plate, except for the small number in layer 6a.

6.4 P13-adulthood: expression in the cortical plate

After P10 the density of staining and the number of stained cells rapidly declines in the subplate (Fig. 6.1 P10, P13), although background staining of the neuropil of layer 6b persists somewhat longer.

At the end of the second postnatal week, stained bipolar, bitufted and polymorphous cells (probably interneurons) suddenly start to appear in the middle of the cortical plate, but only in a narrow band around the border of layers 4 and 5. The neuropil also exhibits immunoreactivity in this region (Figs. 6.1, 6.2C). Then gradually, starting from this band in the middle of the cortical plate and spreading up and down through the plate, more and more stained interneurons appear in all layers of the cortex (Fig. 6.1 P17 and P25). Around P20 the adult-like distribution of parvalbumin immunoreactive interneurons is achieved (Figs. 6.1 P25 and 6.2D).

There is slight variation between different cortical regions: the somatosensory cortex, for instance, appears relatively more mature at each age than the visual. By the end of the first postnatal week, the neuropil shows patchy staining in layer 4 in the primary somatosensory cortex. The lighter stained zones most probably correspond to individual whisker barrels.

Fig. 6.1

Photomicrographs, taken from parvalbumin-immunostained coronal sections of the rat somatosensory cortex at various ages as indicated (E20, P0, P7, P10, P13, P17, P25, A: Adult). From E20-P10, subplate cells are virtually exclusively labelled, with a few positive cells of pyramidal morphology in layer 6a (arrows indicate the subplate layer). The stained subplate cells gradually become more numerous and more intensely labelled, with a peak at around P7, but they then decline in number. By P13 only a few subplate cells are labelled, but the neuropil still shows diffuse immunoreactivity at the bottom of the cortex and in the white matter (lower arrow at P13). At the same time, small immunostained cells, most probably interneurons, appear in the mid-thickness of the cortical plate (upper arrow at P13). These immunoreactive cells are accompanied by diffuse neuropil staining around the layer 4/5 border (also see Fig. 6.2C for higher power view). During the third postnatal week stained cells with interneuronal morphology gradually appear in the supra- and infra-granular layers and by P25, parvalbumin-positive cells are distributed in an adult like (A) pattern. The photomicrographs were taken with 20.25 (at E20), 10.25 (at P0) and with 4.5 times magnification (from P7 to adult).

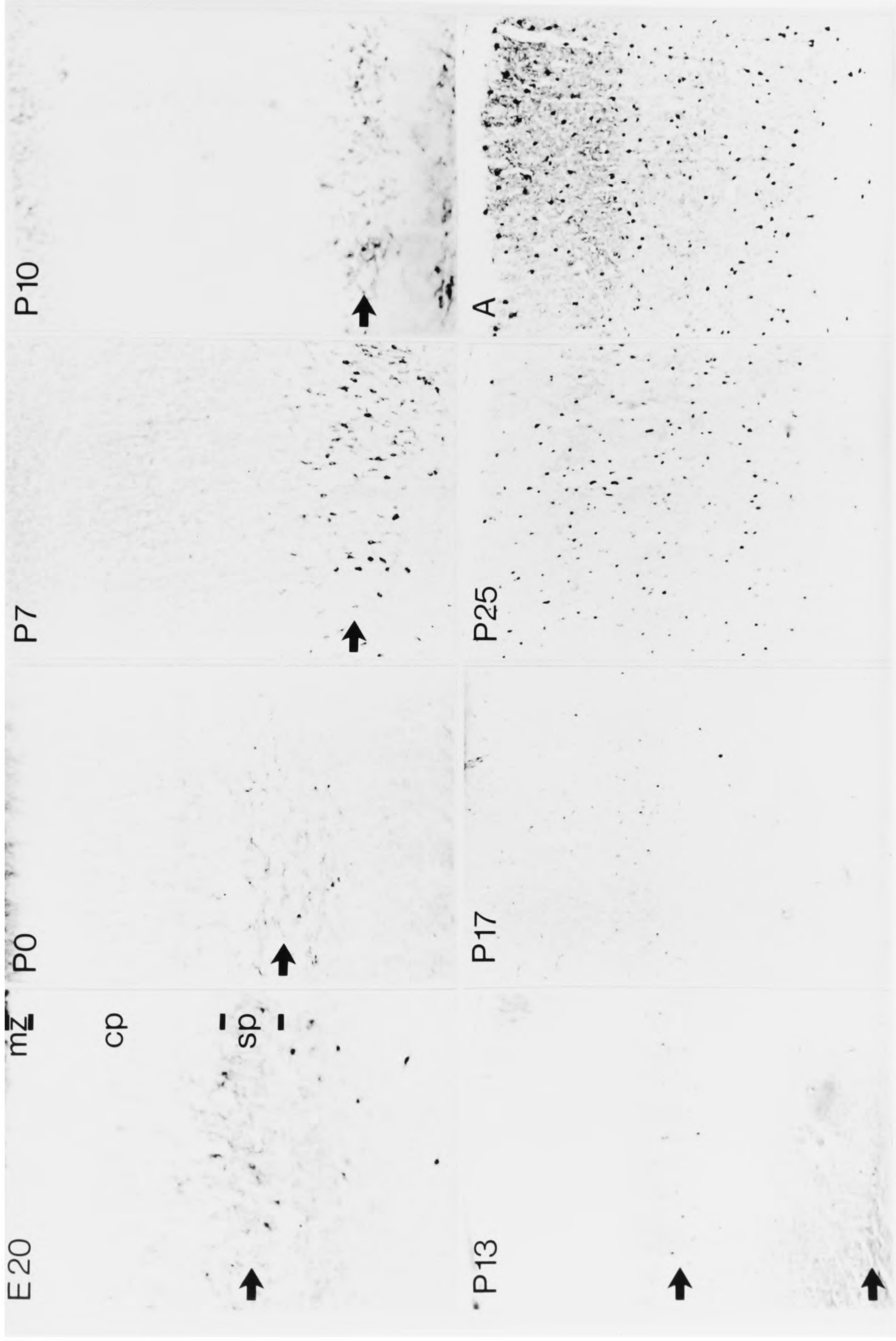


Fig. 6.2

A,B: Parvalbumin stained cells from the base of layer 6 of the somatosensory cortex at E16 (a) and P8 (b). The immunostaining reveals some cells with pyramidal and inverted pyramid morphology. In A, pial surface to the left, ventricular surface to the right, at B, C and D: pial surface up, ventricular surface down.

C: Higher-power view, centred on the layer 4/5 border region at P13. Parvalbumin-positive cells first appear here at this age in the somatosensory cortex. They are bitufted and bipolar cells (most likely interneurons). Also note the diffuse neuropil staining.

D: Labelled bipolar and multipolar cells from the layer 4/5 border of the somatosensory cortex of a P24 rat.

All photomicrographs were taken with 20.5 times magnification.

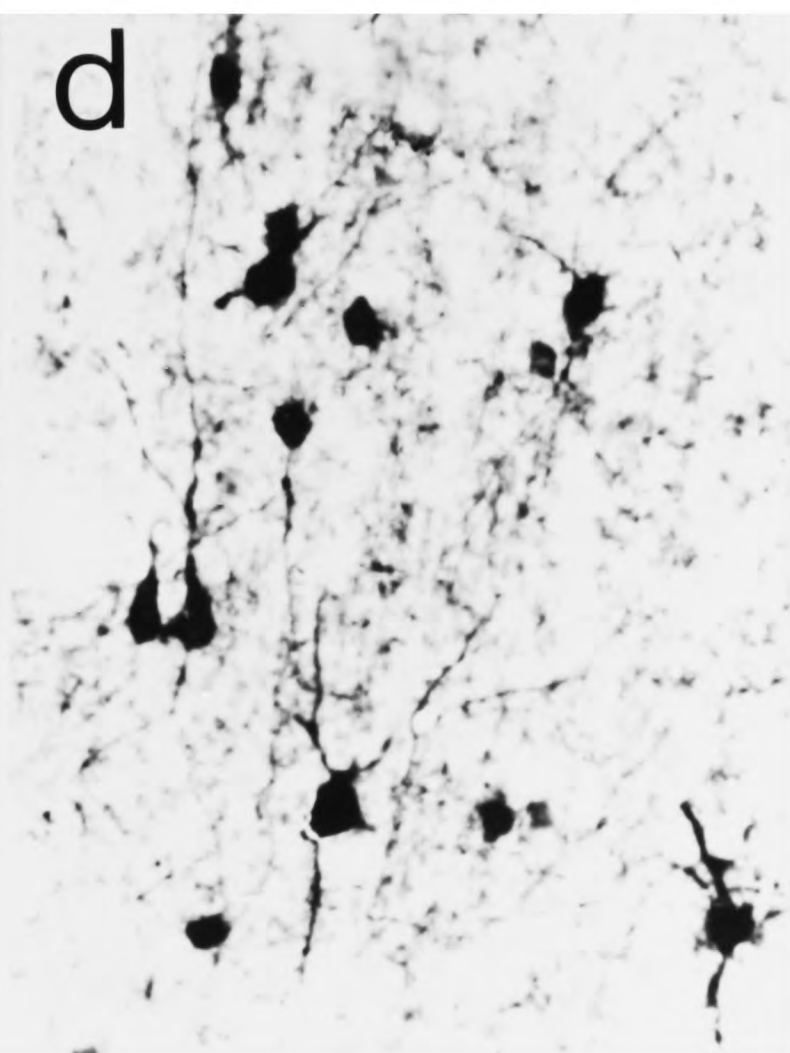
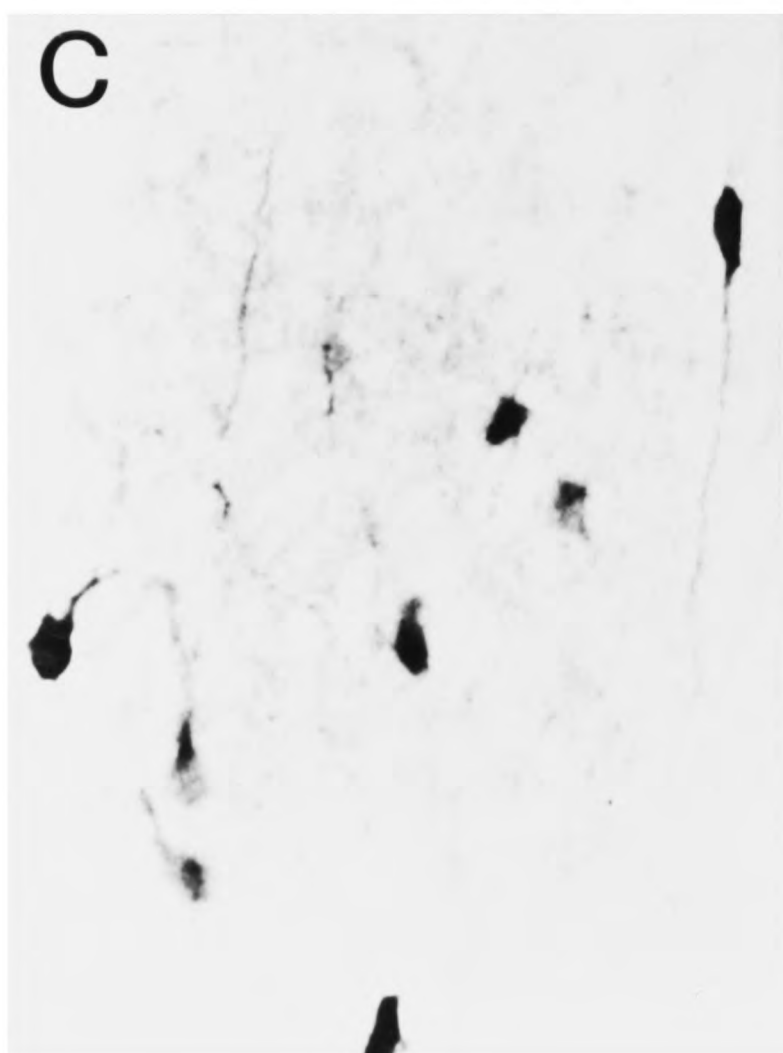
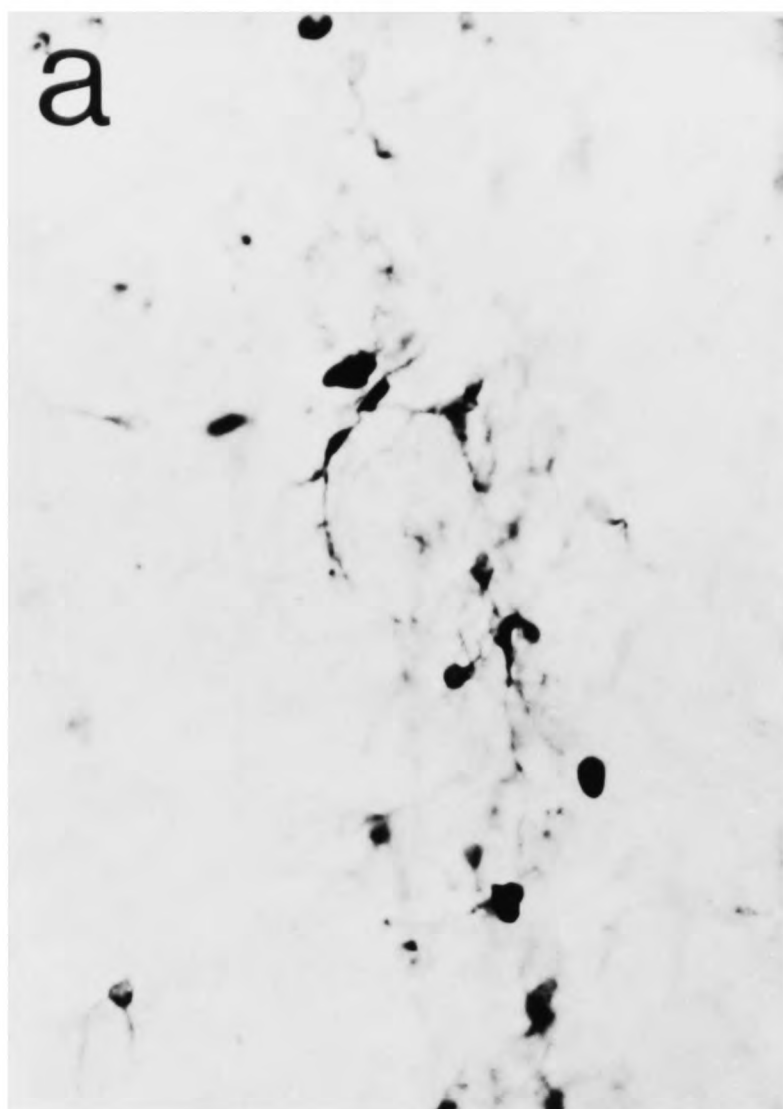
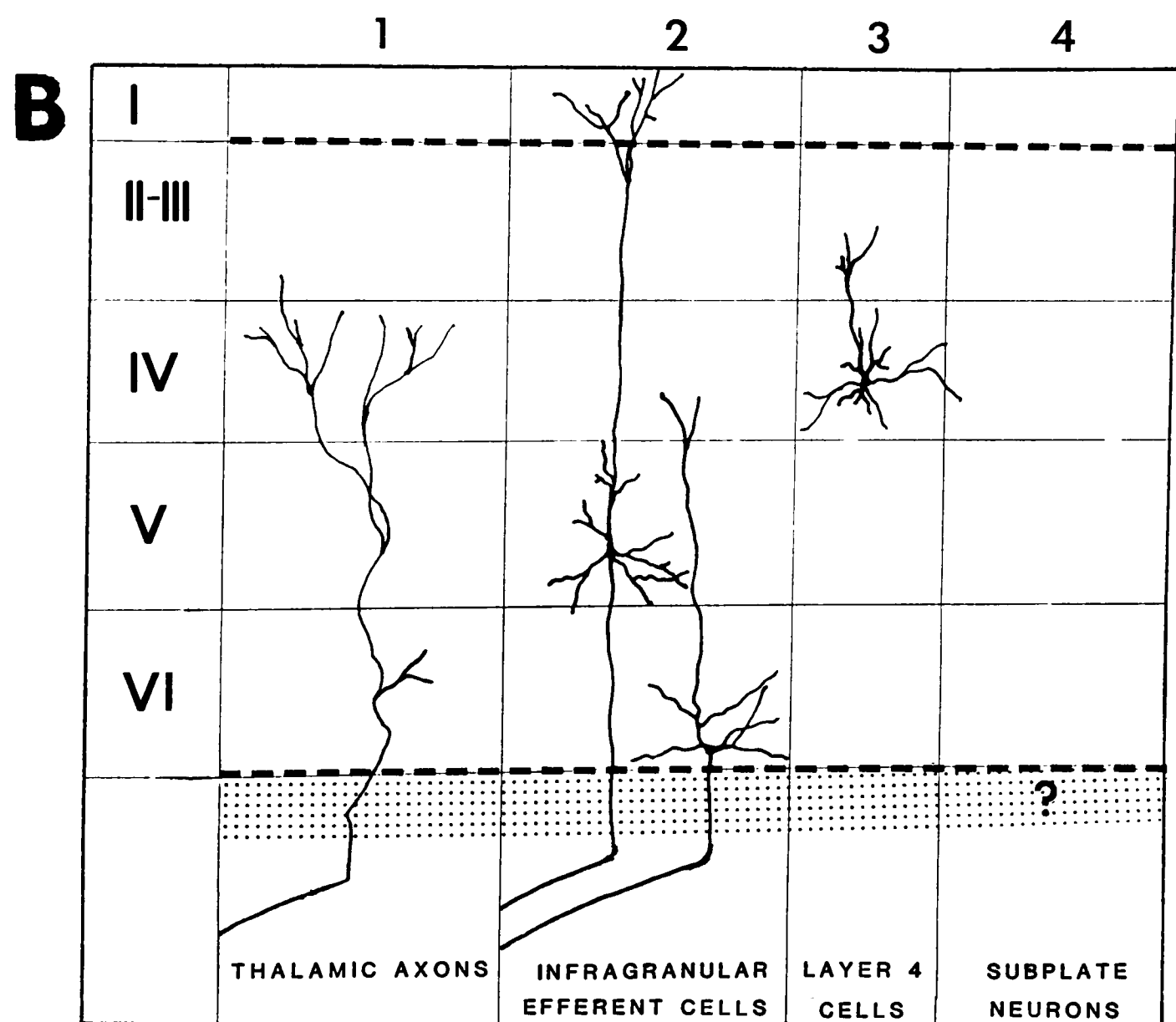
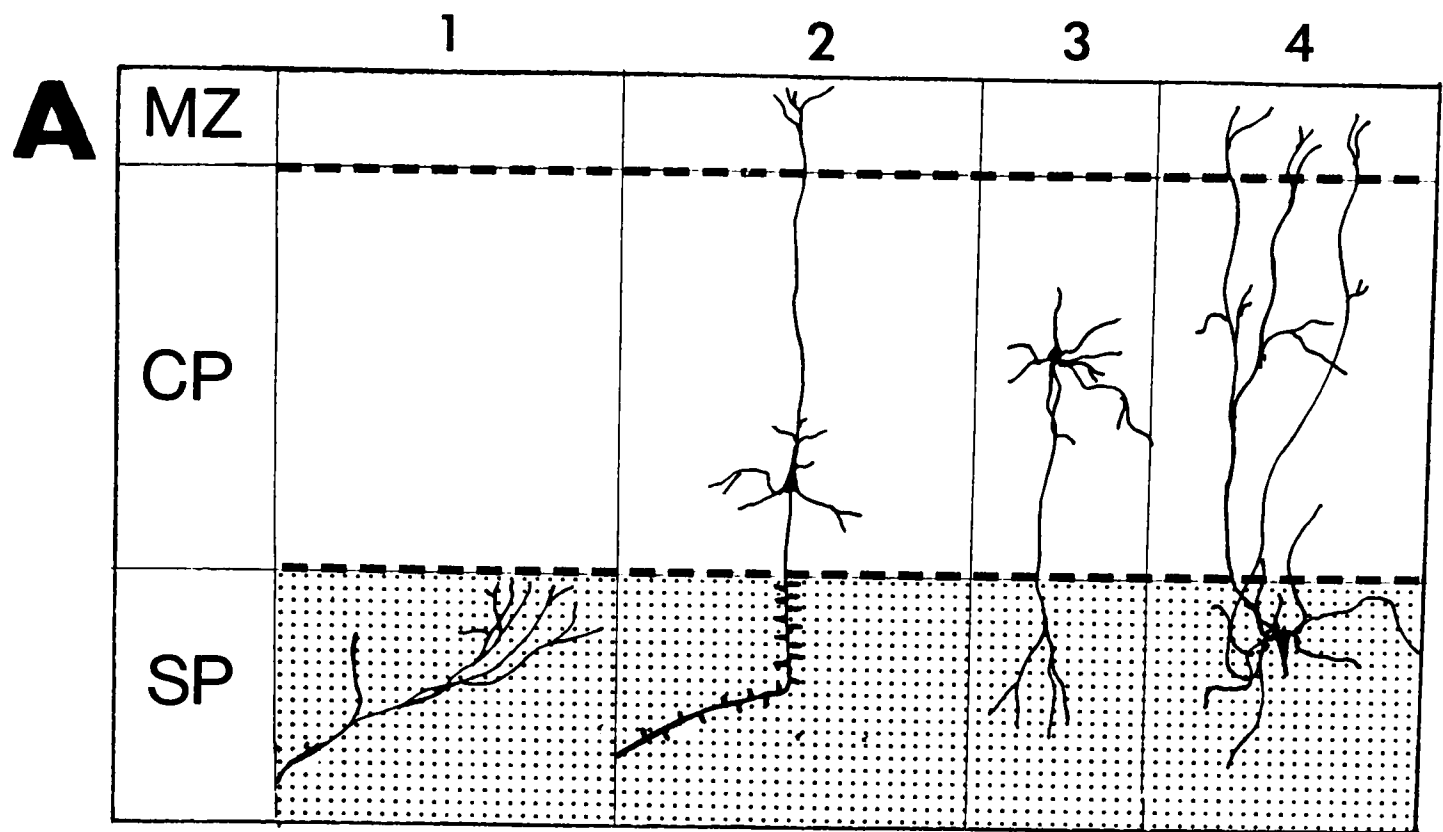


Fig. 6.3

Summary diagram (inspired by Friauf and Shatz, 1991) showing the changes in thalamocortical fibre arrangement and the morphology of granular and infragranular cells as well as the subplate during the re-assembly of thalamocortical circuitry. The vertical panels represent the 1: Thalamic axons, 2: Infragranular efferent cells, 3: Layer 4 neurons, 4: Subplate cells.

A: In the rat, during the period E16-20, when the first parvalbumin stained cells appear in the subplate (SP), thalamic fibres accumulate below the cortical plate (CP) and form synapses in the subplate (panel 1) (Rakic, 1976; Herrmann et al, 1991). Some subplate cells send their axons to the marginal zone (panel 4) and on the way the ascending axons collateralize in the middle of the cortical plate (Friauf et al, 1991; Friauf and Shatz, 1992). Corticofugal fibres from layers 5 and 6 form boutons *en passant* on subplate cells (panel 2) (Lowenstein and Shering, 1991; Chapter 3). Spiny stellate cells of layer 4 send axons that form contacts with subplate cells in this early period (panel 3) (Callaway and Katz, 1992).

B: Just before birth (E20-21 in the rat) *thalamic fibres (panel 1) leave the subplate* and (in addition to layer 6) arrive principally in layers 4 and, to a lesser extent, layer 1, which also represent the major targets of subplate cells. The peak of parvalbumin expression in the subplate (P8) occurs after the start of the invasion of the cortical plate by thalamic fibres (E20), but it probably coincides with the peak of their *synapse formation*. At P13 the first parvalbumin immunoreactive interneurons appear at the layer 4/5 border. This might be a sign of the establishment of new cortico-cortical connectivity. Speculations about the possible activity patterns in the developing cortex would have to take into account other projections (including the corticothalamic, corticotectal and callosal) which also have contacts with the subplate.



DISCUSSION

The present results show that parvalbumin expression in the developing rat cortex occurs in two quite distinct phases, which cannot be viewed as one continuous wave moving upwards through the layers of the cortex (unlike, for instance, the inside-out sequence of expression of NADPH-diaphorase; see Chapter 5). To the best of my knowledge, the spatiotemporal pattern for parvalbumin is unique for molecular expression in the developing rat cortex. Even the expression of calbindin, another Ca^{2+} -binding protein, follows the usual inside-out sequence and is therefore quite different from parvalbumin (Ferrer et al., 1992; J. Mitrofanis and Z. Molnár, unpublished observations). Although many examples urge caution in making generalizations about the occurrence of parvalbumin in the central nervous system of different species, the mammalian cerebral cortex seems to be an exception. There is striking similarity in the distribution of various calcium binding proteins in the cortex of different mammals (Baimbridge et al., 1992) and preliminary studies on the developmental expression of parvarbumin in ferret have revealed a pattern very similar to that in the rat (J. Mitrofanis and Z. Molnár, unpublished observations).

(i) In the rat, the first stage occurs perinatally, between E16 and P10 and is virtually entirely restricted to the subplate cells of layer 6b. The cortical plate and even the early-postmitotic cells of the marginal zone are almost completely parvalbumin negative during this phase. The phase starts with a slow increase of parvalbumin expression in the subplate starting before birth, and reaching a peak around P7. After P10, expression almost completely disappears from below the cortical plate.

(ii) The second phase starts after P10 in non-pyramidal (bistellate, bipolar and polymorphous) cells, presumed interneurons, in the middle of the cortical plate, around

the layer 4/5 border. Two weeks after birth the band of parvalbumin immunostained interneurons (and neuropil) is strictly localized to the middle of the cortical plate. Then gradually, spreading up and down from the middle of the cortical plate, more and more reactive interneurons appear in all layers of the cortex. After P20 the distribution of parvalbumin immunoreactive interneurons stabilizes and persists apparently unchanged into adulthood.

6.5 Comparison with expression of GABA and neuropeptides

Although all cells expressing parvalbumin in the adult rat cortex appear also to contain GABA (Celio, 1986) and a variety of neuropeptides (Somogyi et al., 1984; Hendry et al., 1984; Jones and Hendry, 1989; Hendry et al., 1989), the sequence of appearance of these putative transmitters is quite different from that described here for parvalbumin. This is not to say that all these transmitter molecules appear absolutely simultaneously. In general GABA appears before the peptides, which are expressed in the following order: NPY, SOM then CCK (Wolff et al., 1984; Parnavelas et al., 1988). However, in each case, expression starts in the deep layers of the cortex and progressively moves up through the cortex in an inside-out sequence (McDonald et al., 1982a,b; Parnavelas et al., 1988).

Parvalbumin expression is not only distinctly different in its laminar sequence (not following a single wave through the cortex) but it also occurs very late compared with its principal companion GABA. GABA expression begins around birth and most GABA-containing neurons appear to have revealed that identity by the end of the first postnatal week (Parnavelas et al., 1988); but parvalbumin does not even appear in the cortical plate itself until the end of the second week.

Double labelling studies revealed that the physiological properties of the cells

are the determinant factors in the expression of parvalbumin and other calcium binding proteins rather than their neurotransmitter content (Baimbridge et al., 1992). Furthermore Mione et al., (1992) recently demonstrated that in contrast with the uniform expression of principal neurotransmitters (Glu, Asp, GABA), the expression of the different calcium binding proteins (parvalbumin and calbindin) is heterogeneous in clonally related neurons in the rat cerebral cortex. These observations are strongly suggestive that epigenetic factors regulate the expression of calcium binding proteins in the cortical neurons.

In view of the various roles of Ca^{2+} in cell function, it is interesting to speculate on the possible significance of the two phases of parvalbumin expression.

6.6 Possible functions of parvalbumin during the subplate phase

Association with cell death

The expression of parvalbumin in the subplate may be related to the probable period of cell death in that layer. In cats and monkeys, the majority of subplate neurons die during the period that thalamic axons are detaching from the subplate layer, at the end of the waiting period, and moving up to form their adult pattern of connections in the cortical plate (Luskin and Shatz, 1986; Friauf and Shatz, 1991). Although there is cell death in the rodent subplate (Ferrer et al., 1992; Wood et al., 1992), it is not yet clear exactly what proportion of subplate cells die (though it seems certain that it is a smaller fraction than in higher mammals). However, my own collaborative thymidine autoradiographic studies (Molnár et al., 1991) show that the decline in the number of first post-mitotic cells (heavily labelled by thymidine injection at E12) occurs mainly around the end of the first postnatal week, when parvalbumin immunoreactive cells are also disappearing from the subplate region. It is not clear to what extent the loss of

immunoreactivity is due to down-regulation of parvalbumin in surviving neurons, or due to the death of cells expressing parvalbumin.

High levels of free intracellular Ca^{2+} entering the cell, for instance, through the NMDA-receptor ionophore in response to extracellular glutamate, can trigger neuronal cell death (Garthwaite et al., 1991; Choi, 1991; Schousboe et al., 1991). Selective kainate sensitivity was first described in the developing retina (Catsicas and Clarke, 1987). In cat cortex, subplate neurons appear to be especially sensitive to the excitotoxic effects of amino acids, since injections of ibotenate into the cortex around the time of birth selectively destroy the subplate, apparently leaving neurons of the cortical plate unaffected (Ghosh et al., 1990; Ghosh and Shatz, 1992). Both parvalbumin and calbindin are believed to be capable of playing a passive 'excitoprotective' role by serving as buffer proteins and reducing the concentration of free intracellular Ca^{2+} .

It has also been demonstrated that chronic depolarization suppresses 'programmed' neuronal death produced by moderate sustained elevation of cytoplasmic calcium (Nishi and Berg, 1981). Franklin and Johnson (1992) proposed a ' Ca^{2+} set-point hypothesis' of neuronal survival and dependence on neurotrophic factors. According to this hypothesis, dependence on trophic factors is determined by the cytoplasmic calcium concentration. Unfortunately both variables (trophic factor availability and cytoplasmic calcium) are unknown in the case of the subplate neurons. It is not known whether physiological activity during the different phases of cortical development causes intracellular Ca^{2+} to rise to levels that tend to promote survival or to even higher levels. It is possible that at certain phases of cortical development the sustained depolarization mediated by the thalamic fibres is lower or higher than is optimal for the survival of subplate neurons. I suggest that a rise in extracellular glutamate levels, perhaps

associated with increased spontaneous and driven activity in thalamic neurons around the time of birth, might swamp the regulatory mechanisms and have a powerful excitotoxic effect on subplate neurons (on which those thalamic fibres have temporarily terminated). The activity level might increase considerably during the course of the waiting period, which might, in part, be terminated by the onset of lethal changes in the temporary subplate target cells. Since very little is known of the relevant activity patterns, released transmitters, available trophic factors, receptors and channels, these ideas are highly speculative at present.

The expression of calcium binding proteins might indicate the level of cytosolic calcium and serve as important markers during cortical development. Parvalbumin expression might be upregulated in response to an increase in activity (and hence in Ca^{2+} entry) as a feedback protective reaction. The precise mechanism that triggers the increase or decrease of parvalbumin immunoreactivity in these cells is not known, but it is probably complex since activity and growth factors both affect the levels of parvalbumin and calbindin (Lowenstein et al., 1991; Masiakowski and Shooter, 1988).

It is surely significant that a very similar pattern of parvalbumin expression has been observed in another recently described transient cell population, in the Perireticular and the Thalamic Reticular Nuclei of the developing diencephalon (Mitrofanis and Guillery, 1991; Mitrofanis, 1992a; Mitrofanis and Guillery, 1993).

The disappearance of parvalbumin in the subplate could have a variety of explanations. Strong expression of parvalbumin might simply delay but not prevent the death of the cells involved, in which case the disappearance of parvalbumin from the subplate would simply be due to the loss of those cells. On the other hand, parvalbumin expression might be actively down-regulated as soon as the thalamic axons

have detached and exposure to glutamate is reduced: in that case the cells previously expressing parvalbumin might still be alive. Finally, despite a continuing excitotoxic risk, some intrinsic mechanism might down-regulate parvalbumin and hence trigger cell death. Such internally regulated cell-death could be important in controlling the timing of the detachment of thalamic axons and their displacement into the cortical plate. It would have major importance to reveal the precise timing of parvalbumin- (and other calcium binding protein) gene expression in relation to the pattern of activity in thalamic axons and to subplate cell death.

Remodelling of subplate projections

The time of parvalbumin expression in the subplate is not only a period of cell death, it is also a stage at which the afferent and probably the efferent connections of subplate cells are changing.

Between E16 and E20 thalamic fibres arrive and accumulate in the subplate region beneath the rat occipital cortex. In the cat, intracellular recordings indicate that during the corresponding period (E30 and E50; Shatz and Luskin, 1986) subplate neurons receive some of their input from identified thalamic fibres (Herrmann et al., 1991) and electrical stimulation of the optic radiation can induce excitatory postsynaptic potentials in these subplate cells (Friauf et al. 1990). Axons of corticofugal layer 5 and 6 cells also form boutons *en passant* as they leave the cortex (Chapter 3; Lowenstein and Shering, 1991); and the axon collaterals of subplate neurons themselves also contact other subplate cells.

The axons of some subplate neurons in the cat enter the cortical plate, give collaterals mostly within cortical layer 4, and terminate in the marginal zone (Friauf et al. 1990). In this respect the cat and the rat seems to be quite similar (J. Lübke;

unpublished observation of Lucifer Yellow-filled subplate cells in rat and cat). It is interesting to note that the marginal zone and layer 4 are ultimately also targets of thalamic fibres. The peak of parvalbumin expression in the subplate closely correlates with the invasion of the cortical plate and synaptogenesis by thalamic fibres (Lund and Mustari, 1977; Chapter 3). Since parvalbumin can regulate free Ca^{2+} , which in turn can influence diverse intracellular mechanisms, including transmitter production and growth, it is possible that the changing pattern of parvalbumin expression in the subplate is partly associated with the remodelling of subplate axonal circuitry (Fig. 6.3).

It is possible that the redistribution of thalamic fibres, into the very layers occupied by the axons of subplate cells (Friauf et al, 1990; Friauf and Shatz, 1991) causes the withdrawal of the axons of those subplate neurons. Preliminary results of Molnár, Yee, Lund and Blakemore (1991) support this hypothesis: prevention of thalamic innervation of an area of cortex apparently causes subplate axons in that region to terminate more extensively and persistently in the cortical plate as judged by the extent to which they become labelled by application of DiI crystals to the pial surface. A detailed morphological study (similar to that of Friauf et al, 1990) is needed, however, to confirm the implied changes of subplate morphology after thalamic deafferentation.

6.6 Possible functions of parvalbumin in the cortical plate

Two weeks after birth, the second phase of expression starts in a distinct band of presumed interneurons (and neuropil) in the middle of the cortical plate. Thalamic terminals have presumably formed by this stage, mainly on cells of layer 4, and this phase of parvalbumin expression probably coincides with the establishment of multi-synaptic activation outside layer 4 through the development of horizontal cortico-

cortical axon collaterals (Katz 1992; Katz and Callaway, 1992; Toyama et al., 1991) and the dramatic decline of gap junctions (Yuste et al., 1992; Katz, 1993). The decline of gap junctions and the increasing number of synapses may significantly alter the overall activity patterns of the cortex. But this transition, from a coupled state into a synaptically linked assembly, surely has a strong effect on the cytosolic calcium transients of individual cells and this change might be reflected in calcium binding protein expression. In return, the expression of calcium binding proteins might change the electrical properties of the cytosole, altering the duration of action potentials, and might modify the Ca^{2+} dependent conductances (by inhibiting Ca^{2+} dependent K^{+} currents and inhibiting the Ca^{2+} dependent inactivation of voltage-dependent Ca^{2+} channels) (for review see Baimbridge et al., 1992). Until we get a better understanding of the interplay between the shift from gap junction-coupling to a synaptic coupling these ideas remain rather speculative.

The function of parvabumin in the adult, and the reason for its co-localization with GABA and neuropeptides, are uncertain. However, it seems likely that it plays an important part in the control of intracellular signalling through its buffering of free Ca^{2+} . Perhaps the appearance of parvalbumin in these presumed inhibitory interneurons excludes such cells from synaptic modification mediated via raised intracellular Ca^{2+} , associated with activation of the NMDA receptor (long-term potentiation). Calcium binding protein expression certainly seems to depend on afferent activity since it is reduced in the visual cortex after enucleation (Omidi et al., 1988; Tigges and Tigges, 1991). Very recently Cellerino et al. (1992) described that monocular deprivation in the rat results in a dramatic reduction of parvalbumin immunoreactivity in the visual cortex contralateral to the deprived eye. Interestingly this reduction is restricted to the

binocular portion of the visual cortex and can be elicited only during a restricted time window (sensitive period). This phenomenon might be related to binocular competitive effects, since **binocular** deprivation and dark rearing fail to induce it (Cellerino et al., 1992).

6.7 Different forms of parvalbumin in the two phases?

Using immunoblotting methods, Hogan and Berman (1991) demonstrated that parvalbumin in brain homogenates from newborn cats differs from that in adult homogenates (Hogan personal communication). They suggested that parvalbumin exists in two distinct forms, of different molecular weight, in young and old animals. Presumably, the antibody that I and others (Hogan and Berman, 1991; Berman and Hogan, 1991) have used might recognize an epitope shared by the two forms and therefore not distinguish between them.

It seems very likely that the parvalbumin expressed in the subplate is of the juvenile form detected biochemically by Hogan and Berman (1991). In that case, this particular form may play a special role in those neurons, perhaps intimately connected with the control of cell death. The labelling in the cortical plate itself might be entirely due to the presence of the 'mature' form of parvalbumin. Perhaps only this form is systematically co-localized with neuropeptides and GABA.

The role and regulation of parvalbumin expression in the developing cerebral cortex is far from fully understood, but it seems likely that it could have a variety of important functional roles.

CHAPTER 7

THE ORIGIN OF PREPLATE CELLS:

PHYLOGENY COMPARED WITH ONTOGENY

János Szentágothai, the celebrated neuroanatomist, once told the following story in Budapest to illustrate our way of imagining the higher cognitive functions of the mind:

*Last century there was a scientist called Farkas Kempelen who claimed that he had constructed a machine which could play chess. He travelled around the World and the machine scored victory after victory. Everybody admired the clever machine. On one occasion, however, someone in the audience stood up, pointed to the machine and said: "There must be a man in this machine". Everybody laughed at the suspicious person as he went to the machine and stabbed the model figure with his knife. The laughter became stronger, because the machine could still play. However, it turned out that in Farkas Kempelen's machine there was indeed a retired, legless Polish cavalry officer, a chess-playing expert, hidden **under** the chess board. A similar story was published in English by Edgar Allen Poe (see *Tales of Mystery and Imagination*, Chancellor Press, 1988 edition).*

The moral of this story is that we are all victims of the limitations of our ways of thinking, which is reflected in our behavioural repertoire, emotions, culture, history, art and even our science. Our instinctive ideas about the universe, evolution and even the structure of the brain are inevitably rather anthropomorphic.

7.1 Subplate cells in cortical development

During the development of the mammalian cerebral cortex there is a 'mysterious'

transient cell population, the subplate. These fascinating cells are believed to assist in the development of the external cortical circuitry by forming an early scaffold followed by corticopetal and possibly by corticofugal projections (McConnell et al., 1989; Blakemore and Molnár, 1990; Ghosh et al., 1990). Besides their long range connections, these cells integrate into the early cortical circuitry, by establishing numerous local connections (Friauf et al., 1990; see fig. 6.3 for details). Studies in this field have increased dramatically in the last 5 years and we are beginning to understand the strategic position these cells have during cortical circuit formation. After apparently performing numerous 'tasks' they largely disappear some time after birth. We now take these cells to be responsible for an increasing number of steps during the assembly of cortical circuitry. Is this perceived responsibility simply our own illusion, a convenience for explaining phases in mammalian cerebral cortical development? Has our teleological way of thinking simply created the 'man in the machine'?

In the first part of this final chapter I shall give a historical overview of the different studies on the subplate, examine how the whole field began, and argue that the description of the various roles subplate cells perform is the natural consequence of the amazing recent progress made in developmental neurobiology. In the second part I shall try to examine the possible origin of this group of cells. I shall compare ontogeny and phylogeny and examine whether the preplate is a structure homologous to the reptilian cortex and whether, therefore, the subplate is part of the 'reptilian framework' of Marin-Padilla (1971). Although the relevance of the various forms of cell-cell interactions in which the subplate participates is yet to be fully established, I argue that these transient cells were present before the mammalian neocortex evolved and are essential for its development.

7.2 Historic overview of studies on the subplate

First description of the subplate zone (1974)

It is rather surprising that the subplate zone was described as a separate entity only relatively recently (Molliver et al., 1973; Kostovic and Molliver, 1974). It was known long ago that the cells situated in both the marginal zone and the layer below the cortical plate are different from those in the cortical plate itself. These cells tend to have large somata and extensive processes (Cajal, 1911; Astrom, 1967; Marin-Padilla, 1971, 1972). The first analysis of human fetal cerebrum and subsequent experimental study of the distribution of thalamocortical axons in the fetal monkey (Rakic, 1977) indicated the existence of a prominent but transient layer situated below the cortical plate. This layer between the cortical plate and the intermediate zone was termed the 'subplate zone' (Rakic, 1982). Nissl, Golgi, electron microscopic, and histochemical investigation showed this transient zone to consist of migratory and postmigratory neurons, growth cones, loosely arranged axons, dendrites, synapses, and glial cells (Kostovic and Rakic, 1990).

Subplate and marginal zone cells are co-generated as the primordial plexiform zone (1977, 1985)

In the embryonic cortex of mammals, the subplate forms a substantial layer. In the adult, however, layer 1 and the white matter under the cortex contain few cells (Ramon y Cajal, 1911). Interestingly, most of Cajal's work was on young animals, and he and Retzius made substantial observations on the unusual transient cell population (see DeFelipe and Jones, 1988). However, the fact that the vast majority of them disappear in the adult is surely one of the reasons why the subplate, and subplate cells, were largely ignored until 1974. In the sixties, thymidine autoradiographic studies were

developed to examine the distribution of cortical cells generated at various gestational ages. The analysis of this kind of material was initially performed in adults (Sidman et al., 1959; Berry and Rogers, 1965; Rakic, 1974) in which the transient population was not prominent. The emphasis of interpretation was therefore on the inside-out sequence of cortical neurogenesis, which characterizes the cortical plate itself.

It was Marin-Padilla (1971) who first suggested that migrating neurons of the cortical plate split the first postmitotic cell layer, the primordial plexiform zone, into two. He based his observations on Golgi preparations. It was only few years ago that Luskin and Shatz (1985) confirmed with precise birthdating techniques that the subplate cells and the marginal zone cells are co-generated as the primordial plexiform zone. This was the first study to demonstrate that cells of the subplate and marginal zone have a common origin and that they both decline dramatically in number after birth in the cat compared with cortical plate cells (Luskin and Shatz, 1985; see fig. 1.2). Injections of [³H]thymidine between E24 and E30 in the cat yielded large numbers of labelled cells in the white matter and layer 1 at birth, but very few when examined 2 months postnatal. Injections made at E33, only a few days later, resulted in large numbers of labelled cells in cortical layer 6, not only at birth but also in adulthood (Luskin and Shatz, 1985). The disappearance of many of the first generated cells could not be explained by differential growth of the brain or volume 'dilution' due to growth and the expansion of the cortical surface, as previously suggested (Rickmann et al., 1977).

Numerous more recent birthdating studies (Valverde et al. 1989; Bayer and Altman, 1990; Wood et al., 1992) have demonstrated that there is also a transient subplate component in rodents. According to Wood et al's (1990) work in the mouse,

a substantial fraction of the cells generated at E12-13 and seen below the cortical plate or in the marginal zone at E17-20 die between birth and P21. Unfortunately there is no study at present which address the disappearance of these cells with higher time resolution.

In the mouse Derer and Derer (1992) quantified the density of acetylcholinesterase-positive marginal zone cells (Cajal-Retzius cells), and measured the total area of the cortex. From these two measurements they estimated the total number of these cells at various developmental ages (E16, E19, P0, P2, P4, P5, P8, P10). The study demonstrated that the density of Cajal-Retzius cells declined linearly from E16 onward, with the exception of the period between P2 and P5, where the loss speeded up dramatically, whereas the cortical surface increased linearly during the whole period (Derer and Derer, 1992). This implies that there is a distinct phase of death of Cajal-Retzius cells shortly after birth.

It is now established that the first generated cells undergo excessive cell death in many species, e.g. cat (Luskin and Shatz, 1985b; Chun and Shatz, 1988b), ferret (Allendoerfer et al., 1990), hamster (Woo et al., 1990), mouse (Wood et al. 1990; Derer and Derer, 1992) and rat (Valverde et al., 1989; Al-Ghoul and Miller, 1989).

The waiting period (1976)

Molliver et al. (1973) first established, in human embryonic cortex, that the earliest synapses are formed above and below, but not in the cortical plate and therefore proposed that the initial absence of synapses within the cortical plate may reflect a lack of axons traversing it. A few years later the so-called waiting period was explicitly described in the primate and the rat (Rakic, 1977; Lund and Mustari, 1977; Wise and Jones, 1978). The first thalamocortical fibres accumulate and wait in the subplate layer

before entering the cortical plate (Rakic, 1977; Chun et al., 1987) even after their target cells have arrived in the cortical plate (Lund and Mustari, 1977; Shatz and Luskin 1986; Ghosh and Shatz, 1992b; Chapter 3).

Recently Catalano et al. (1991) and Miller et al. (1993) questioned the existence of the waiting period because their carbocyanine dye studies revealed some thalamic fibre invasion much earlier as it was originally described by Lund and Mustari (1977). Now there is a concensus that there is a waiting period even in rodents (certainly in the occipital cortex), but it is much shorter, perhaps only 3 days (E16 to E19), than it was originally described (E18 to P1) by Lund and Mustari (1977). The new techniques confirmed the existence of the waiting period in the cat (Ghosh and Shatz, 1992b).

Such a waiting period is not specifically characteristic of thalamocortical fibres, since a similar phenomenon has been demonstrated for the callosal (Wise and Jones, 1977; Goldman-Rakic, 1982), cholinergic basal forebrain (Kostovic, 1986) and monoaminergic brain stem projections (Molliver and Kristt, 1975; Lidov et al., 1978). As embryonic tracing (Godement et al., 1987) and various *in vitro* techniques developed, understanding the nature of the transient circuitry (Friauf and Shatz, 1991) and the molecular mechanisms underlying this puzzling period became possible (Chapter 2; Götz et al., 1991 and 1992; Bolz and Götz, 1992). It was suggested that, during formation of thalamocortical projections, the subplate provides a temporary target for the arriving fibres, while they wait for the cortex to mature (Molliver et al., 1973; Shatz and Luskin, 1986). In 1991, Herrmann, Antonini and Shatz performed ultrastructural analysis of PHA-L (*Phaseolus Vulgaris Leucoagglutinin*) labelled thalamic fibres in neonatal ferrets and established that, during the waiting period, thalamic fibres form many of the synapses present in the subplate. Indeed, it is now

clear that the integration of subplate cells into cortical circuitry is much more elaborate (see Chapters 3 and 6 for details). For instance, the overlying cortical neurons (of layers 5 and 6) also form monosynaptic input on subplate cells (Chapter 3; Rakic, 1976; Lowenstein and Shering, 1991). These first-generated cells integrate into the developing cortical plate, and this integration might be essential for the developing connections of the cortical plate (Ghosh and Shatz, 1992a). Subplate cells might provide a framework for the development of the cortical plate of all mammalian cerebral cortices (Marin-Padilla, 1988) and have widespread influence on the establishment of cortical circuitry.

Transient subplate cells possess several characteristics of mature neurons (transmitters, local and long range projections) (1989)

In early embryonic cortex the subplate is quite a thick layer, but after a few weeks postnatal, it partly or largely disappears: 60-95% of the subplate cells die in cat (Luskin and Shatz 1985b, Chun and Shatz 1989b). But during their short life they show several characteristics of mature neurons. They precociously mature morphologically, they express neuronal markers, produce transmitters, and develop local and distant projections (Chun and Shatz, 1989a,b; Chun et al., 1987; Whale and Meyer, 1987; Marin-Padilla, 1971; McConnell et al., 1989; Antonini and Shatz, 1990).

Although most of these studies were performed in the cat and ferret, studies on the rat suggest that, although its subplate system is not exactly the same, in many respects it is very similar. Subplate cells in the rat also have long and short range connections (see Chapters 3 and 5). Their descending projections are quite similar in arrangement (DeCarlos and O'Leary, 1992; Blakemore and Molnár, 1990). It is possible that their transmitters are also CCK, GABA, Substance P and Somatostatin, as in cat and ferret (Antonini and Shatz, 1990).

The primordial plexiform zone cells show great maturity at early stages, when compared to the immature cortical plate. It is interesting that the subplate and the marginal zone form the first synapses (Molliver et al., 1973) express a variety of transmitters (Chun and Shatz 1989) and generate receptors (beta-adrenergic, nicotinic cholinergic, benzodiazepine and NMDA receptors: W. Lichtensteiger, personal communication) prematurely. It seems likely that second messenger systems are also coupled to the receptors earlier than in neurons of the cortical plate. The precocious and excessive expression of the NMDA receptor appears to render subplate cells hypersensitive to excitotoxic lesions before cells of the cortical plate (Ghosh et al., 1990; Ghosh and Shatz, 1992a). The relevance of these connections and transmitters is far from understood, but it is difficult to believe that they are not functional in some way.

Subplate pioneers (1989)

The telencephalon lies rostrolateral to the diencephalon in the embryonic prosencephalon. A direct route exists for ascending axons from the thalamus through the ganglionic eminence to the outermost zone of the hemisphere wall (His, 1904; Hochstetter, 1909; Windle, 1935; Marin-Padilla, 1971, 1988; Molliver et al., 1973). Molliver, Kostovic and van der Loos (1973) described, first in human, the way in which the first fibres traversing this pathway travel via the subplate, which forms a separate sheet in the developing human brain. The origin of these first fibres, before the appearance of the cortical plate, was not clear and they were considered to be thalamocorticals (Hicks and D'Amato, 1968). The new tracing techniques provided the first evidence that the axons of subplate cells themselves pioneer the pathway through the intermediate zone (Marin-Padilla, 1971; McConnell et al., 1989). These fibres pass

through and under the developing striatum and they express fibronectin (Chun and Shatz, 1988) and L1 (Godfraind et al., 1988; Chung et al., 1991).

The subplate fibres are the first to grow through the telencephalic part of the path and thalamocortical fibres then follow similar routes. Thalamocortical fibres themselves also run through the striatum in fascicles. These fascicles contain both corticofugal and corticopetal projections (Chapters 3 and 4). The subplate not only provides the first corticofugal projections (Shatz et al., 1990), but also sets up an organized scaffold over which the thalamic fibres appear to grow to reach their cortical areas (Chapter 3; Blakemore and Molnár, 1990). This allows the formation of the first external cortical connections while the brain is still small, the thalamus is relatively undifferentiated and the cortex is lissencephalic.

In the adult the thalamocortical and corticothalamic projections (from layer 6) take different routes, in some species at least. The thalamocorticals run lateral and superficial to the corticofugals (see Chapter 3). But during development the thalamocortical fibres follow trajectories very similar to those of the earliest projections from the subplate. The rest of the developing cortex and the body of the striatum seem to be hostile environments for thalamic ingrowth at early stages. This is very likely due to the lack of a membrane-bound growth-permissive factor on immature neurons (Götz et al. 1992; Bolz and Götz, 1992; Chapter 2). It could however be due to the presence of *growth-inhibiting* surface molecules, which have been recently demonstrated during the development of the spinal cord and in the early phase of retinal ganglion cell elongation (Caroni and Schwab, 1988a,b; Schnell and Schwab, 1990; Snow et al., 1991; Oohira et al., 1991; Cole and McCabe, 1991), or even diffusible inhibitory factors, such as those acting during the establishment of the olfactory afferent pathway (Pini, 1993).

Thalamic fibres seem to have no other choice than to follow obediently the subplate efferents while travelling through the otherwise inhospitable environment of the striatum and even the subplate layer itself. Given the present evidence this seems to be the case in the rat (Chapter 3; Molnár and Blakemore, 1990a,b), and findings in the Reeler mutant, where the thalamic fibres negotiate themselves through the entire thickness of the non-permissive environment of the embryonic cortical plate further substantiate this notion (see Chapter 5; Molnár and Blakemore, 1992).

Subplate integrates into the neuronal circuitry (1973-92)

While the subplate is serving as a guidance system and waiting compartment for the ingrowing thalamic fibres it also sends projections up into the cortical plate (mostly to layer 4 and layer 1: Friauf et al., 1990; Friauf and Shatz, 1991). The subplate also receives input from the efferent axons of layers 5 and 6 as they pass through it (Chapter 3; Lowenstein and Shering, 1991). At least some components of the local and long-range transient neural circuitry of the subplate are functional, in the sense that they are capable of mediating postsynaptic responses (Friauf and Shatz, 1991), although their relevance is yet to be determined (Ghosh et al, 1990; Ghosh and Shatz, 1992a). Certainly subplate cells, through their local and long-range projections, occupy a strategic position.

There seem to be species differences, however, in the extent of subplate connectivity. In the cat and ferret, subplate cells probably extend to the thalamus, contralateral hemisphere and some even to the superior colliculus (Antonini and Shatz, 1990), whereas in the rat none of these targets seem to be properly innervated (Chapter 3; Blakemore and Molnár, 1990; DeCarlos and O'Leary, 1990; 1992). The opossum may also be different from the cat and ferret in this respect, since no subplate cells were

back-labelled from the thalamus at various ages (Sheng et al., 1991).

Recently Ghosh and Shatz (1992a) proposed that impulse activity delivered to the cortical plate by the subplate projections during the waiting period is essential for the maturation of the future connectivity of the cortical plate. They suggested that, without this transient circuit, the thalamic fibres that grow in later cannot establish normal ocular dominance columns through competitive interaction. This might sound strange at first but, as we know more about the various subplate interactions during the development of the cerebral cortex, we have to consider the subplate as an extra factor in establishing dynamic circuitry. Maybe the transient feature of the subplate cells is important in the establishment of cortical 'maps'. Maybe the 'freezing' of other aspects of cortical plasticity also depends on the disappearance of the subplate at the right time. We know very little about the 'programming' of the death of subplate cells. My suspicion is that this is regulated to some degree epigenetically (Molnár et al., 1991). This question clearly deserve further study.

7.3 Other transient cells in the developing mammalian central nervous system.

Most knowledge of transient neurons and the mechanisms of their death comes from studies in invertebrates (Driscoll and Chalfie, 1992), where there is good evidence that their death is 'programmed', in the sense that it is determined by innate mechanisms . Although subplate death in mammals is also generally considered to be programmed (Shatz et al., 1990), Molnár et al. (1991) have provided evidence that it is partly regulated by thalamic innervation.

The subplate was not the first transient cell population to be described in the mammalian central nervous system, and is certainly not the last. Another example of

a transient neuronal population is the so-called border cells of the primate spinal cord. They are present for a defined but limited period during embryonic development (Knyihár et al., 1978).

The reticular and the perireticular nuclei in rats are surprisingly large relative to the thalamus at early stages, but with increasing maturity these nuclei are dramatically reduced in size (Mitrofanis, 1992a; Mitrofanis and Guillery, 1993). Their cells have a distinct migratory pattern, they disappear a few weeks after birth, and their antigenic properties are very similar to those of the subplate: they also express parvalbumin, calbindin, NADPH-diaphorase, somatostatin and GABA (Mitrofanis and Guillery 1991, Mitrofanis, 1992a). Using birthdating, in the same way that Luskin and Shatz (1985a,b) did to define the origin of the subplate in the cat, J. Mitrofanis (personal communication) has established that perireticular cells are also an early-born, transient population. How do these cells integrate into the developing brain and what is their significance? After the first corticofugal fibres have passed through the internal capsule, they take distinct paths (Chapter 3). Mitrofanis has proposed that the perireticular cells might be responsible for the guidance of cortical efferents from different layers to their various destinations. He is now testing the possibility that corticothalamic fibres from layer 6 find these cells attractive but layer 5 cells do not.

Sretavan and his colleagues very recently described a population of neurons in the future region of the optic chiasma, prior to the arrival of retinal axons, which are also among the earliest generated cells in the mammalian forebrain (Feng and Sretavan, 1991; Sretavan and Reichardt, 1992; Sretavan, 1993). Sretavan suspects that the incoming retinal axons grow and turn among these early chiasmatic neurons. A selective ablation of the chiasmatic neurons at E11.5 (with complement and a specific antibody,

anti-CD44) demonstrated that the incoming retinal axons, upon reaching the future region of the optic chiasma, stall and do not project further into the ventral diencephalon (Sretavan, 1993).

The idea that there are many such transient cell populations, playing important roles in the developing brain is attractive and topical. But we may just be creating "a man in the machine"! On the other hand, even without speculating about the function of these transient populations, we can certainly ask whether they are a new development in phylogeny or whether they are a vestige of earlier organization.

7.4 Evolutionary origin of the preplate

The problem of selection of appropriate living representatives of a genealogical series

The ultimate ideal in phylogenetics would be to reconstruct all the branches of an evolutionary process (Fig. 7.1) in detail, as it actually happened. Now, when the ancestors have perished, we can only speculate about what might have happened: fossils give very limited information about the connectivity of brains! The examination of existing species can, of course, provide some interesting clues. But we have to avoid simply looking at the end branches of the evolutionary tree, setting up a morphological sequence and fooling ourselves into believing that this was the historical sequence. If we are constantly aware of this danger, the comparative approach can provide important information. Such a comparison shows that many strategies in development are well conserved (or evolved in parallel) across numerous different phylogenetic levels. Sometimes the repetition of certain stereotyped developmental steps might not seem be the most economical way of making of a nervous system. But we have to keep in mind that these steps evolved over millions of years and each new development had to be

based, to a certain extent, on existing structures (Fig. 7.1). That makes it difficult for us to understand the logic of development, even of the youngest phylogenetic structure in the brain, the cerebral cortex (see Introduction).

The problem of homology

In comparative neuroanatomy, the term homology can be used in a phylogenetic or a structural sense (Campbell and Hodos, 1970). The *structuralist* interpretation of the term homology refers to similarity in the morphology, topological relationships and connections of a given structure. In contrast the *phylogenetic* meaning is based on the assumed evolutionary origin. Which groups of cells in modern species are derived from a single group of cells in a common ancestor? To avoid confusion I shall use the term homology in this phylogenetic sense.

Structures and other entities are homologous when they could, in principle, be traced back through a genealogical series to a stipulated common ancestral precursor irrespective of morphological similarity (the quotation is from Campbell and Hodos, 1970; based on Ghiselin, 1966, 1969; Bock, 1969).

If one keeps this definition in mind we are less likely to get misled by superficial similarities. Nevertheless, similarity is a very important basis for the recognition of homology: the more similarities we find, from various different aspects, the greater the probability of homology. It was Marin-Padilla (1971) who pointed out (based on rapid Golgi preparations) that the mammalian neocortex develops on a reptilian framework. Nowadays we have much more information about the ontogenetic steps during the development of the mammalian cortex and comparative studies on non-mammals have also progressed enormously (Peters and Jones: *Cerebral Cortex*, Vols 8a and 8b).

Fig. 7.1

Evolutionary filiations of upper vertebrates. Time in millions of years since the beginning of the geological period (from Goffinet 1990).

Fig. 7.2

Comparison of pallial organization in reptiles and mammals (taken from Ulinski, 1990). This figure shows transverse sections through the telencephalon of (A) a tegu lizard, **Tupinambis**, and (B) an opossum, **Didelphis**. Unlike placental mammals, marsupials (and monotremes) lack a corpus callosum, thereby simplifying comparisons with the lizard brain.

Both telencephalons have a lateral ventricle and are divided into three major parts. The septum (S) makes up the ventromedial face of the telencephalon. Olfactory structures such as the diagonal band (DB) or olfactory tubercle (OTu) are present on the ventral surface. The forebrain bundles run rostrally into the telencephalon. They consist of the medial forebrain bundle (MFB) and the lateral forebrain bundle (LFB) or internal capsule (IC), passing below the corpus striatum (STR). The pallium or roof of the telencephalon has two components in reptiles: a cortex and an anterior dorsal ventricular ridge (ADVR). The pallium in mammals has only a single component: the cortex. In both groups, the pallium can be divided into three functional segments. The olfactory cortex (Olf) is situated laterally. The intermediate segment (Int) is present dorsally. It includes part of dorsal cortex and ADVR in reptiles and the cortex between the rhinal fissure (RF) and the hippocampal formation (HF) in mammals. The limbic cortex (Lim) occupies the medial wall of the pallium in both groups. It is involuted by the hippocampal fissure in mammals. Note that the two sections are drawn to different scales.

Fig. 7.1

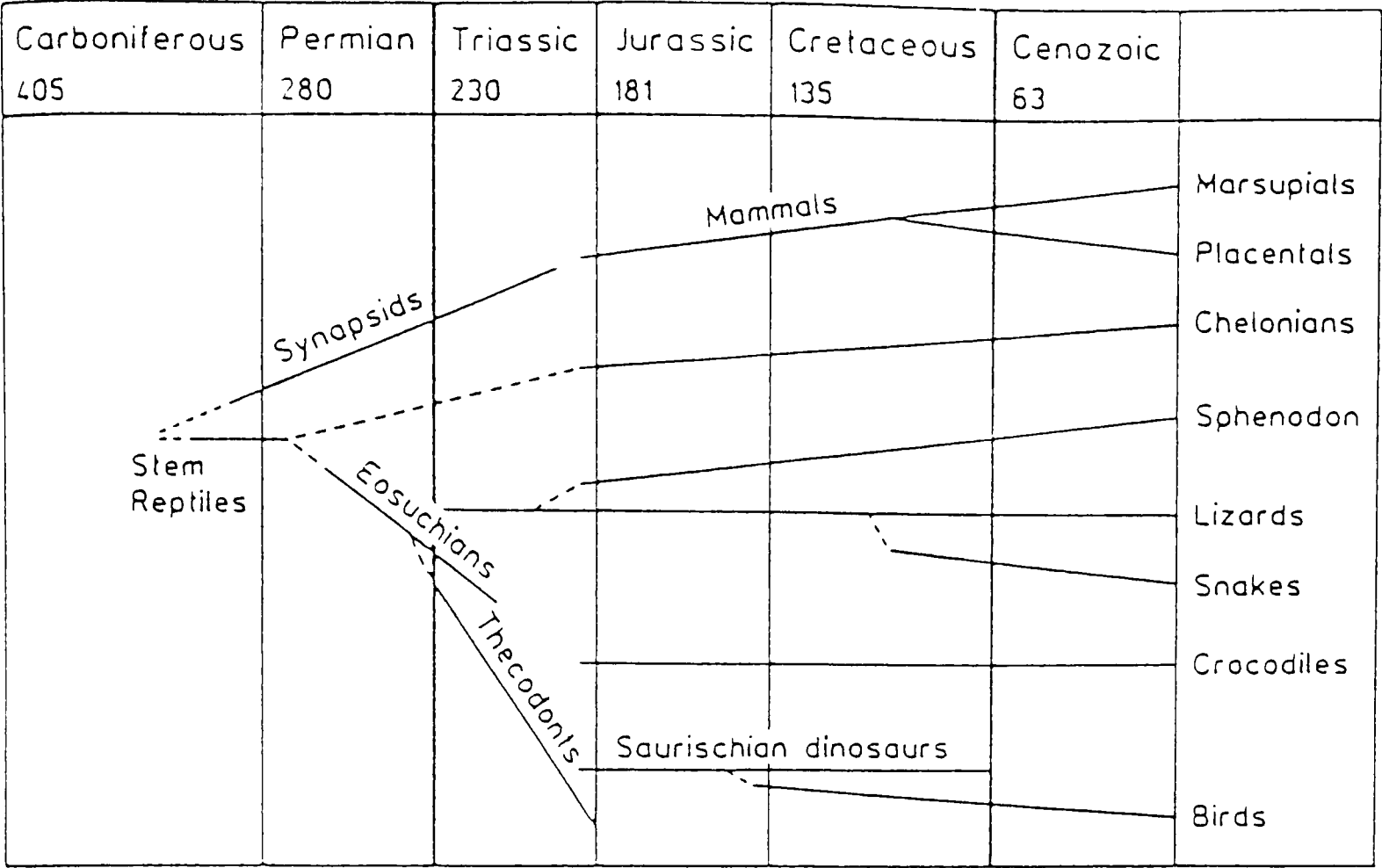


Fig. 7.2

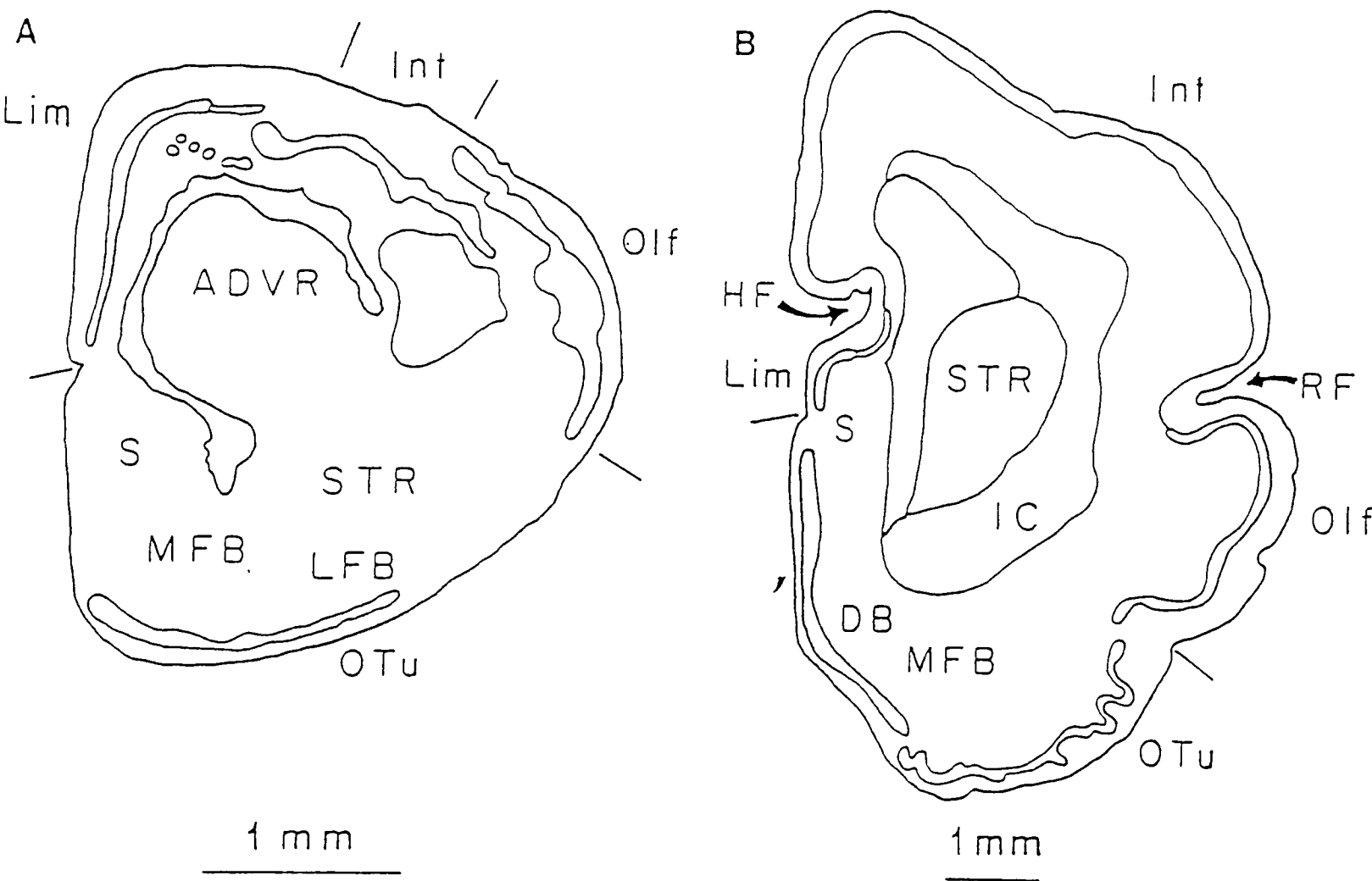


Fig. 7.3

The appearance of vertebrate embryos at various stages of development (from Romanes, 1901). The striking similarity of the different embryos during early development made Haeckel suggest that the key to the ontogeny lies in phylogeny. Although he put far too much emphasis on phylogeny as an explanation of ontogeny, it is indeed rather suggestive that these different animals share both a common ancestor and the same basic mechanisms of development.

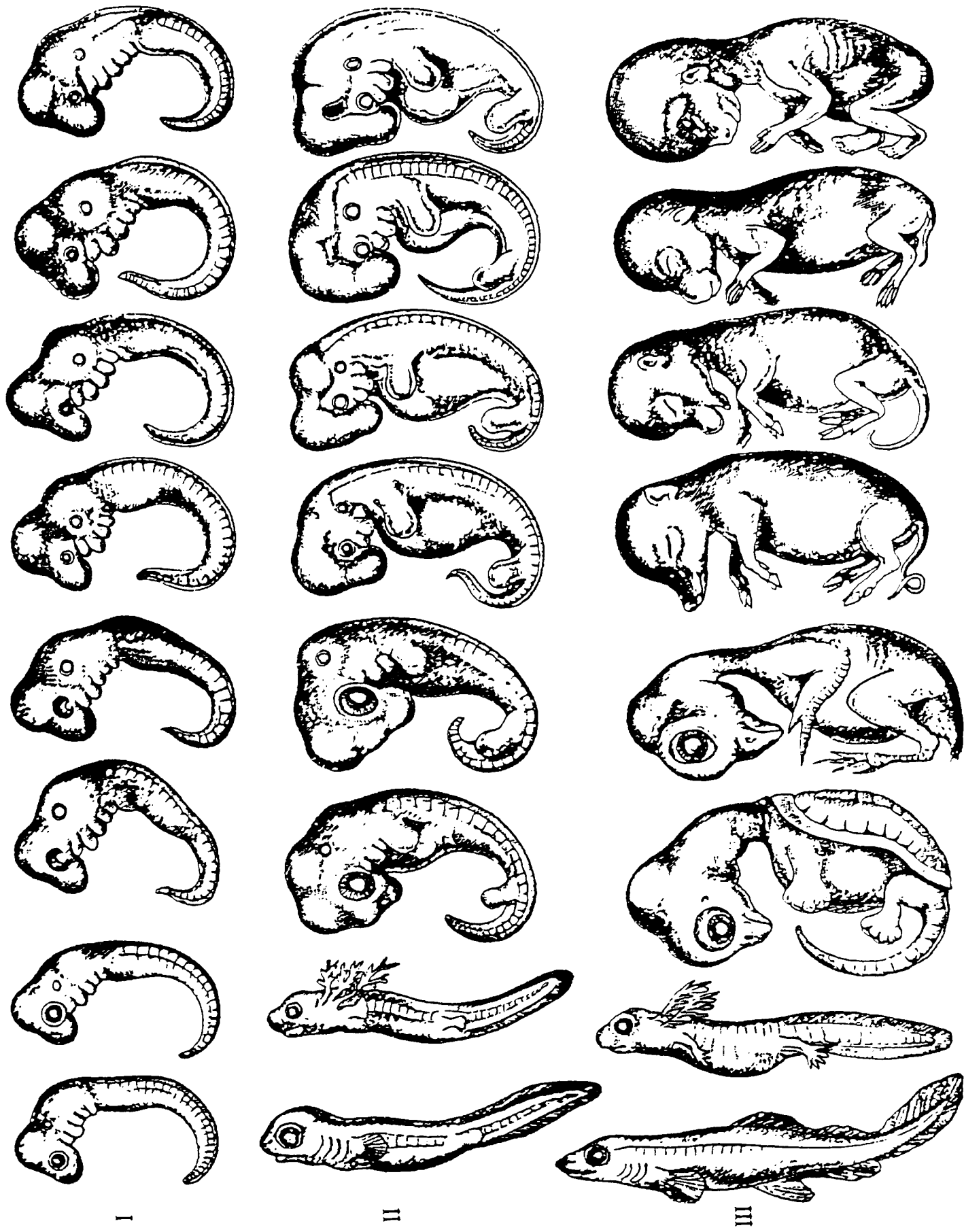


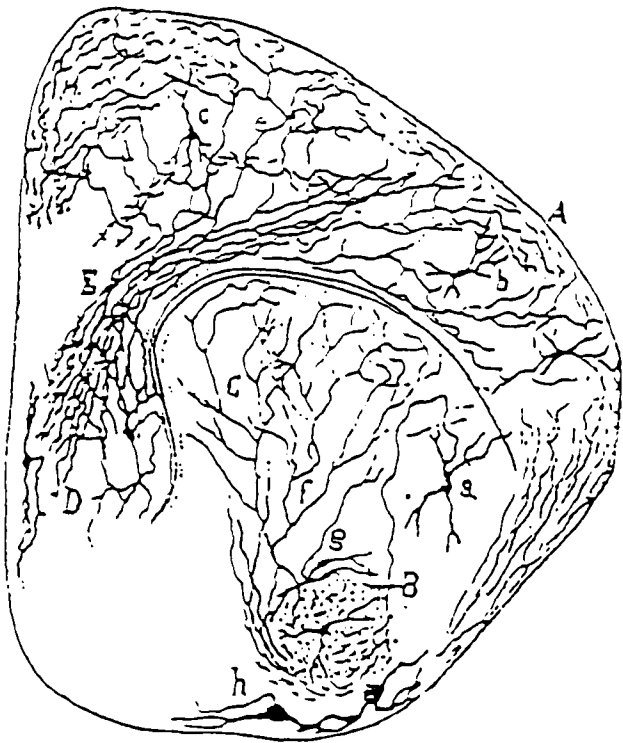
Fig. 7.3

Fig. 7.4

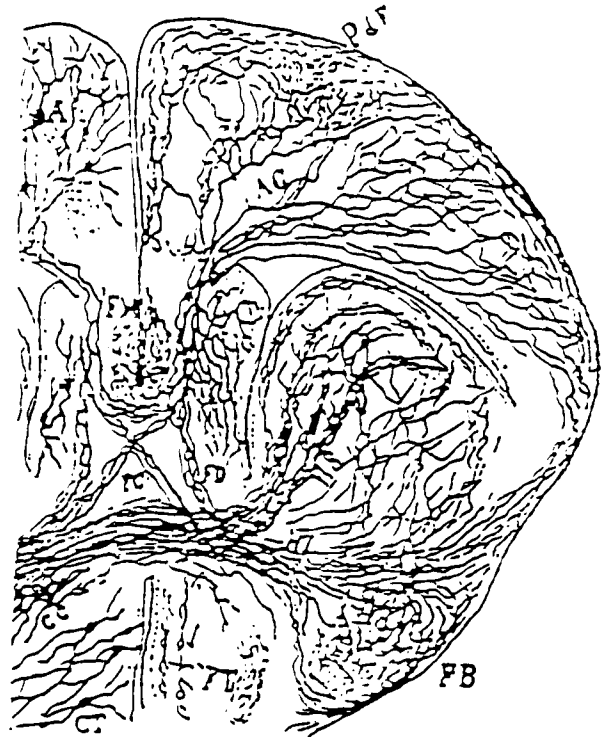
Cajal's comparison of the telencephalon of different vertebrates (adapted from *Cajal on the Cerebral Cortex* by DeFilipe and Jones, 1988).

With the exception of the mammals (marsupial and placental) the intermediate cortex (dorsal pallium) is just a layer of a few pyramidal cells organized into 1 or 2 plexiform zones depending on the species (examples are adapted from *Cajal on the Cerebral Cortex* by DeFelipe and Jones, 1988).

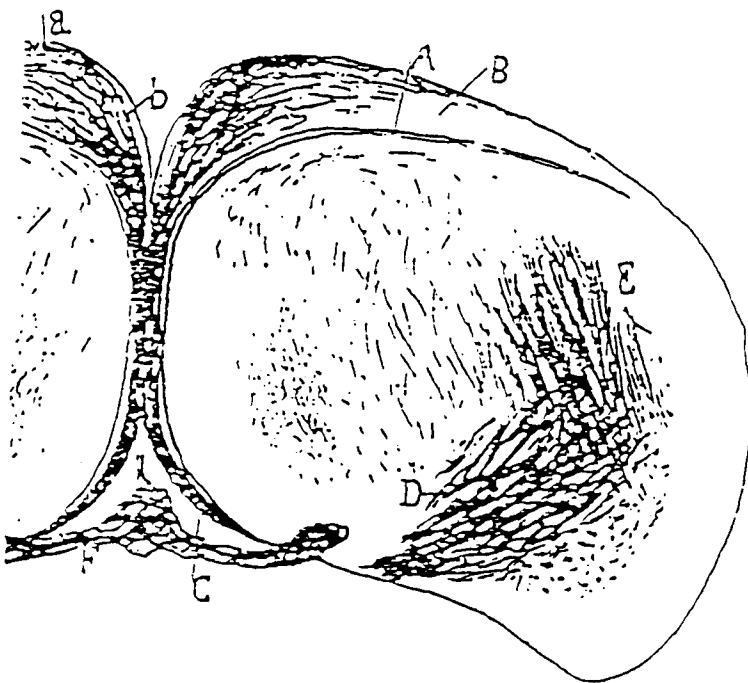
Fig. 7.4



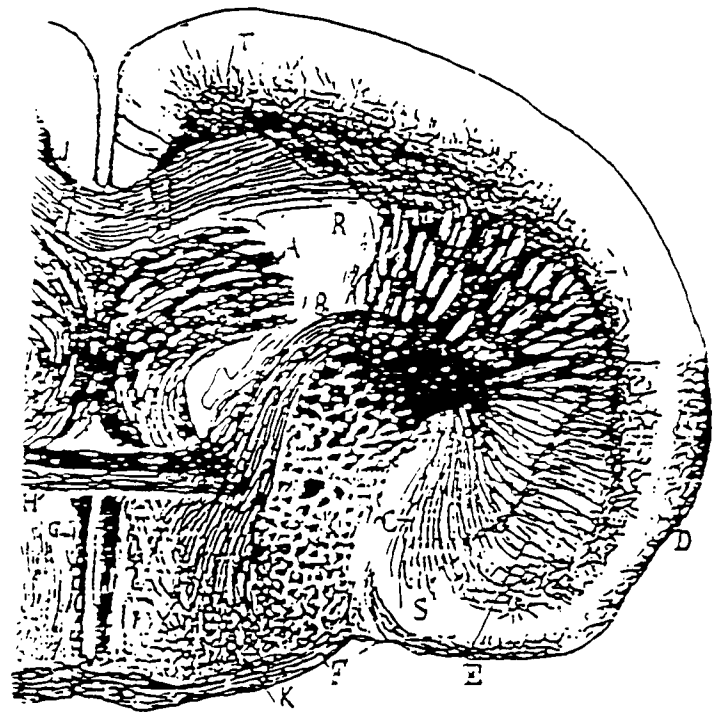
CHAMELEON



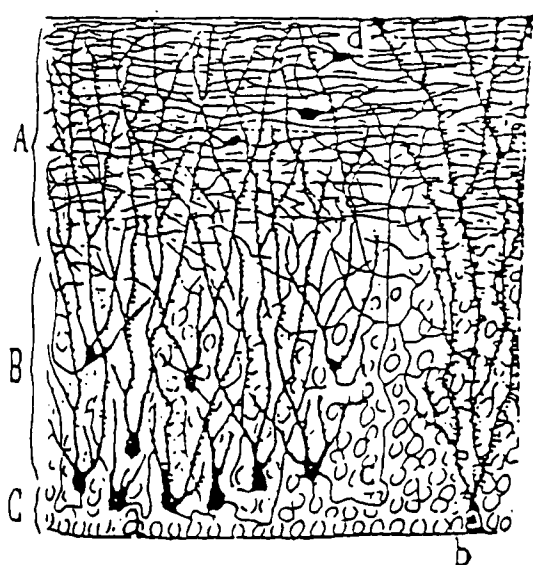
LIZARD



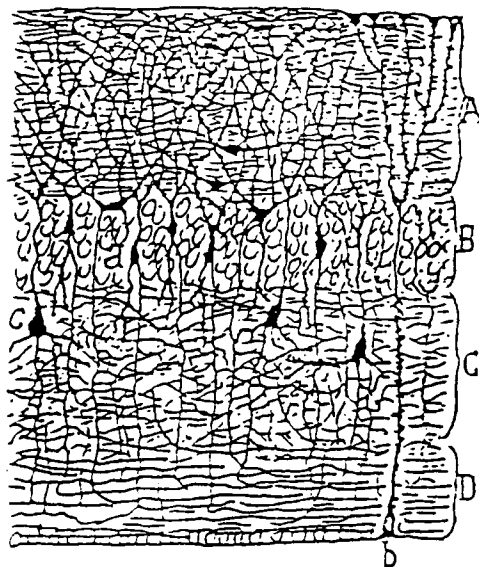
CHICKEN



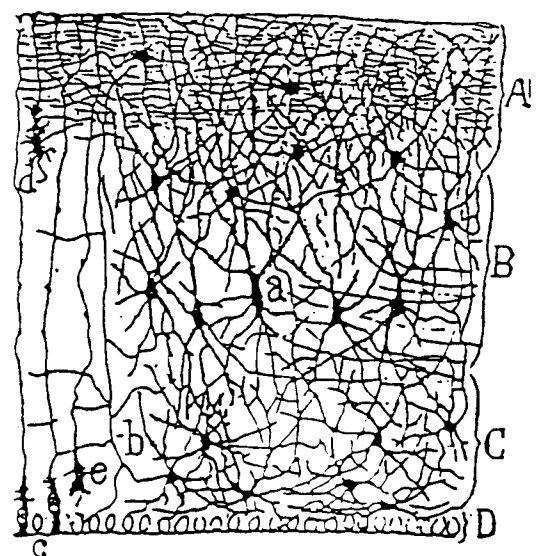
MOUSE



FROG



LIZARD



CHICKEN

Fig. 7.5 Thalamocortical projections revealed *post mortem* with carbocyanine fluorescent dye, DiI, in a E14-week human brain (from an aborted fetus).

A single crystal of DiI was placed into the dorsal thalamus (* indicates the crystal placement site) of a *post mortem* immersion-fixed brain. (Ethical permission for the use of human fetal material was given from the Research Ethical Committee of the Albert Szent-Györgyi Medical University.) After 12 months incubation at 37°C, the whole hemisphere was coronally sectioned (200µm) and every third section was drawn under epifluorescent illumination using a *camera lucida*. The serial sections are in a rostro-caudal order (left is rostral, right is caudal). The other hemisphere was embedded in paraffin and 10µm cresyl violet stained reference sections were prepared to reveal the state of thalamic and cortical development. The figure demonstrates that the overall topography of the thalamic projections bears a strong resemblance to that in the E15 rat (Chapter 3). Some of the thalamic fibres have already left the diencephalon through the primitive internal capsule (PIC). The fibres form fascicles in the striatum (ST), but defasciculate in the intermediate zone and the subplate (area outlined with dotted lines). The fibres are not labelled throughout their full length, nevertheless the results already demonstrate that thalamic fibres reach the subplate layer of the human cortex before the characteristic migration of the LGN and before the gyrencephalic transformation of the cerebral cortex. The study suggests an analogy between the early phases of ontogeny in rodents and in primates. In these early stages, the LGN of primates is situated at the lateral aspect of the thalamus as it is in fetal adult rodents (Clark, 1932; Rakic, 1974). Subsequently, due to the expansive growth of the pulvinar, the LGN is simultaneously rotated upon its transverse and sagittal axes and becomes displaced to the inferior aspect of the mature thalamus (Rakic, 1977). Note that the DiI crystal also retrogradely labels the optic tract; arrowhead). Scale bar: 10mm

14WS

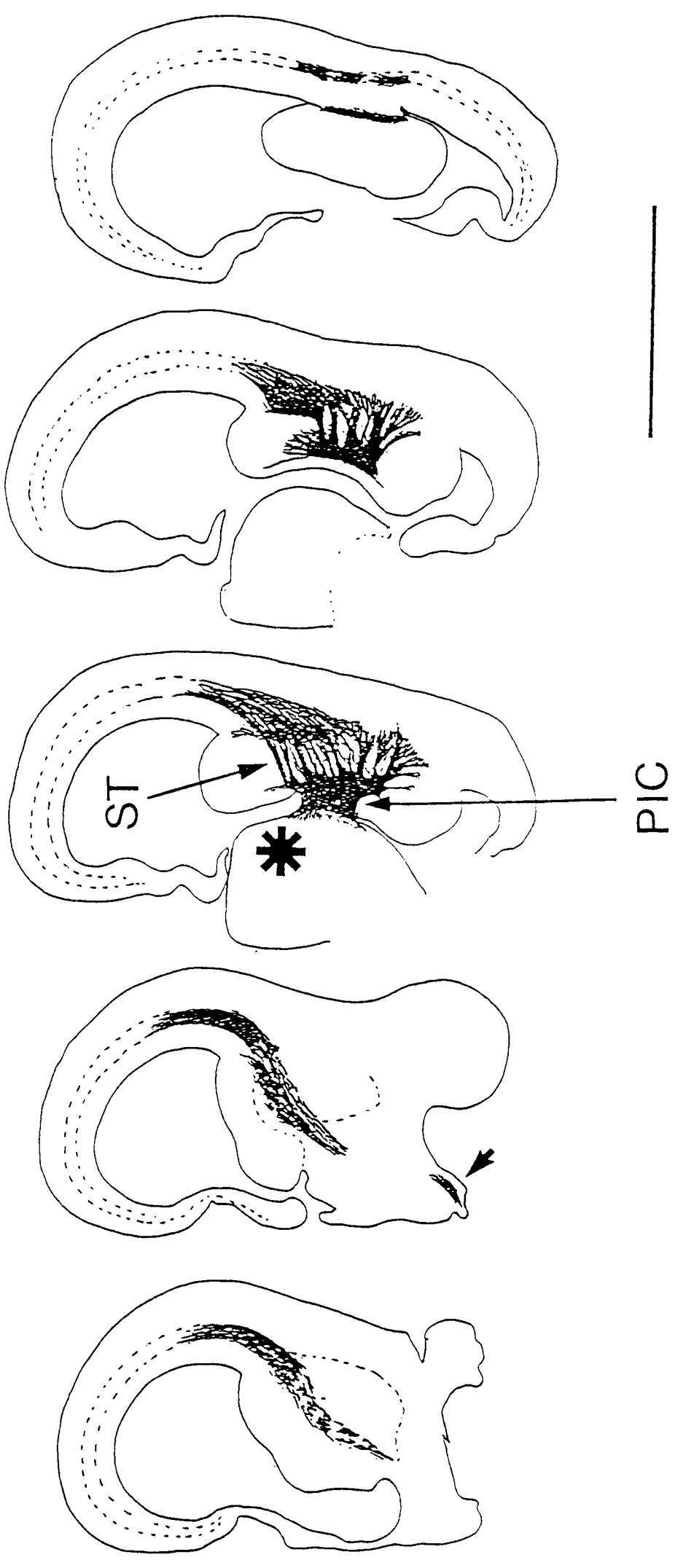


Fig. 7.5

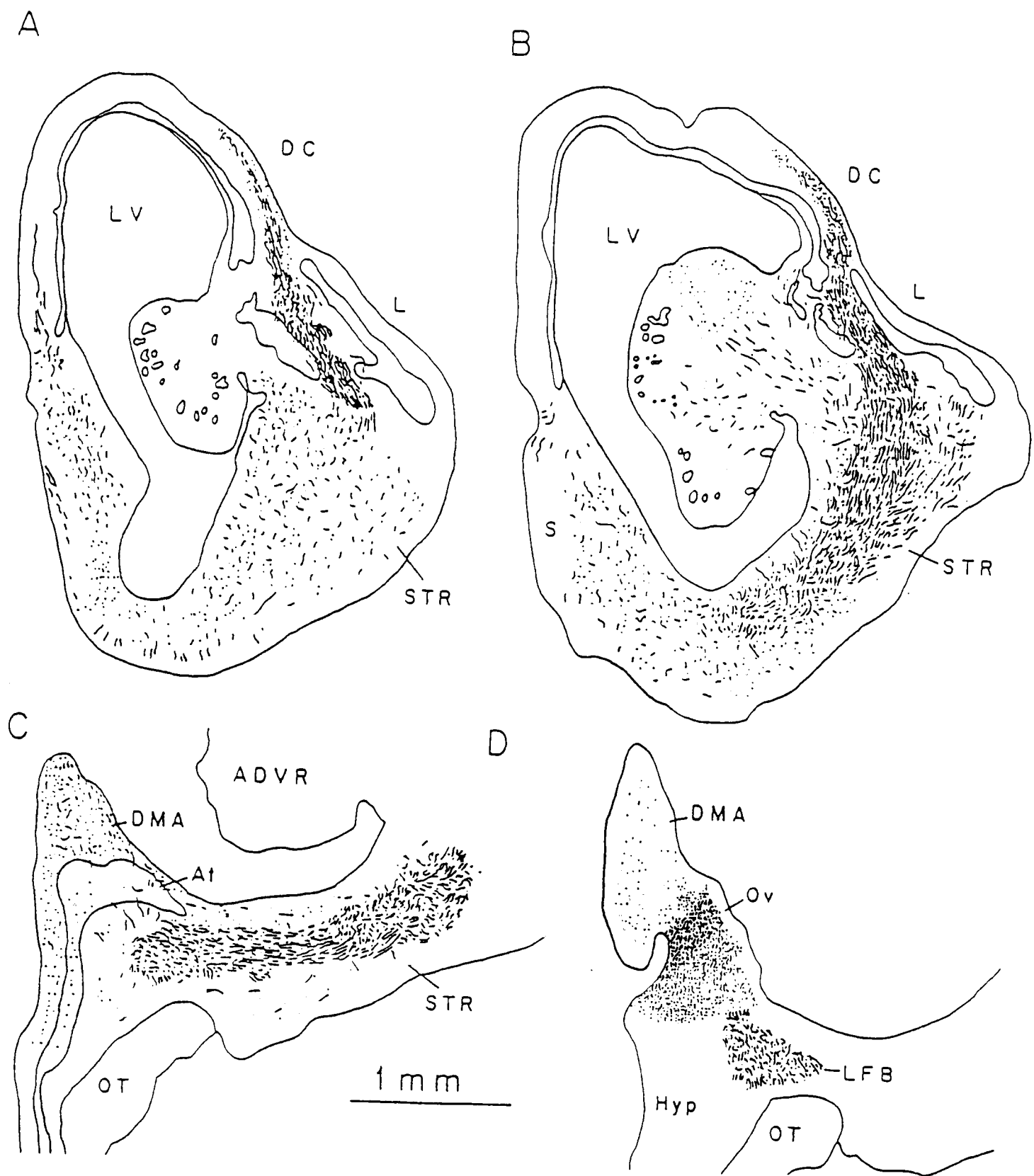
Fig. 7.6

Thalamotelencephalic projections in turtle revealed by anterograde transport of HRP (Heller and Ulinski, 1987).

The injection site involved the subnucleus ovalis of the geniculate complex (Ov in section D). Most of the labelled axons leave the diencephalon by taking an anterolateral course, run through the striatum (STR in sections C and B) and *reach the dorsal cortex* (DC in sections A and B). A few of the axons terminate in the striatum (S). Others, after having pass through the striatum, turn medial and terminate in the anterior dorsal ventricular ridge (ADVR, section C and B), instead of entering the pallial thickening. The serial sections are in a rostro-caudal order; section A is the most rostral; D is the most caudal.

At, area triangularis of the ventral thalamus; DMA, dorsomedial anterior nucleus; Hyp, hypothalamus; L, lateral cortex; LFB, lateral forebrain bundle; LV, lateral ventricle; OT, optic tract; S, septum

Fig. 7.6

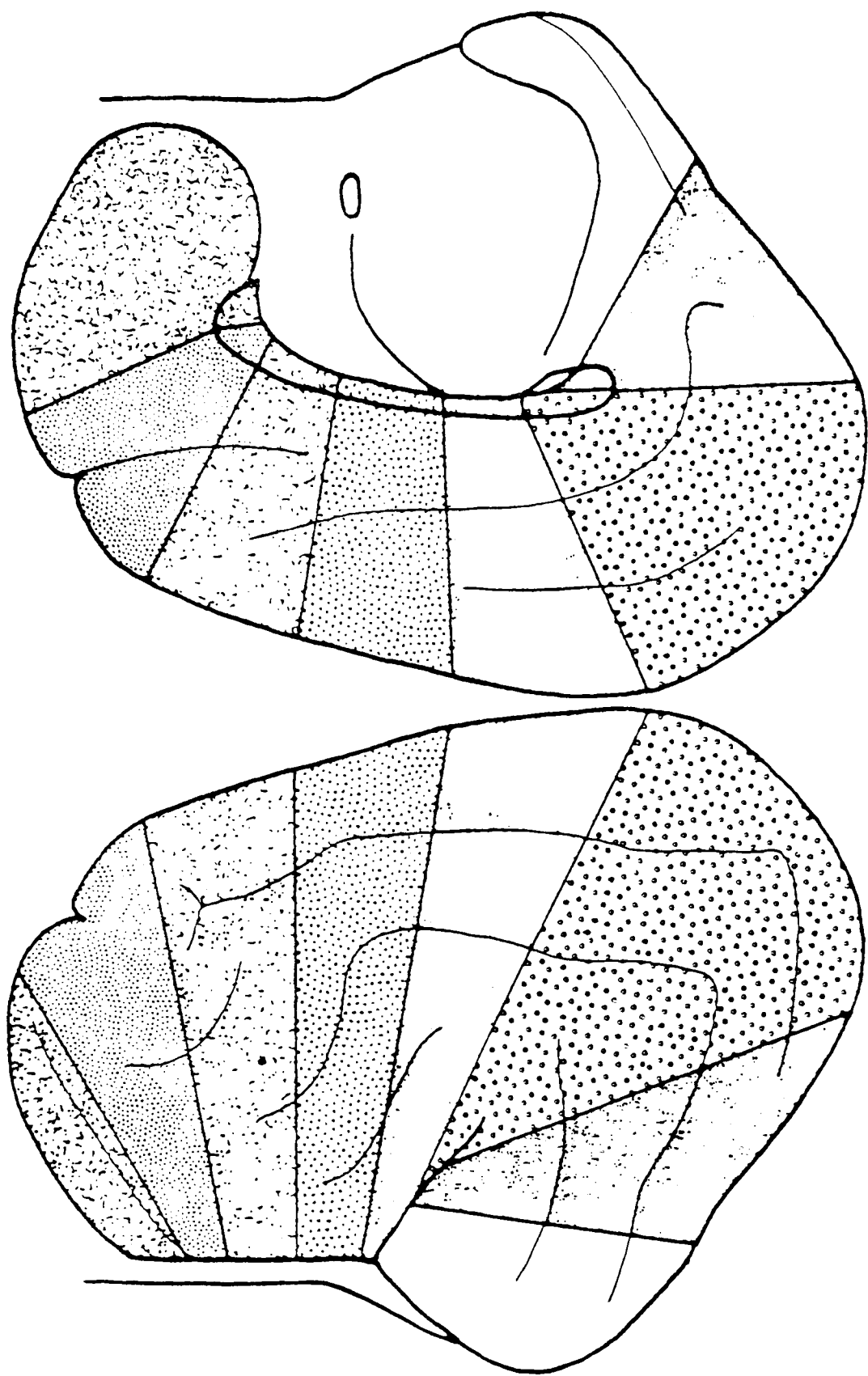


THALAMO-TELENCEPHALIC PROJECTIONS IN TURTLE (Heller and Ułinski 1987)

Fig. 7.7

A diagram (taken from Nakamura and Tanaseki, 1989) showing the sectorial topographical correspondence between the corpus callosum and the cortex in the cat. The lower drawing shows the lateral view, the upper one shows a medial view (inverted). The rostral side of the hemisphere is to the left; the dorsal side is at the junction of the two drawings.

Fig. 7.7



Nakamura & Tanaseki (1989)

Fig. 7.8

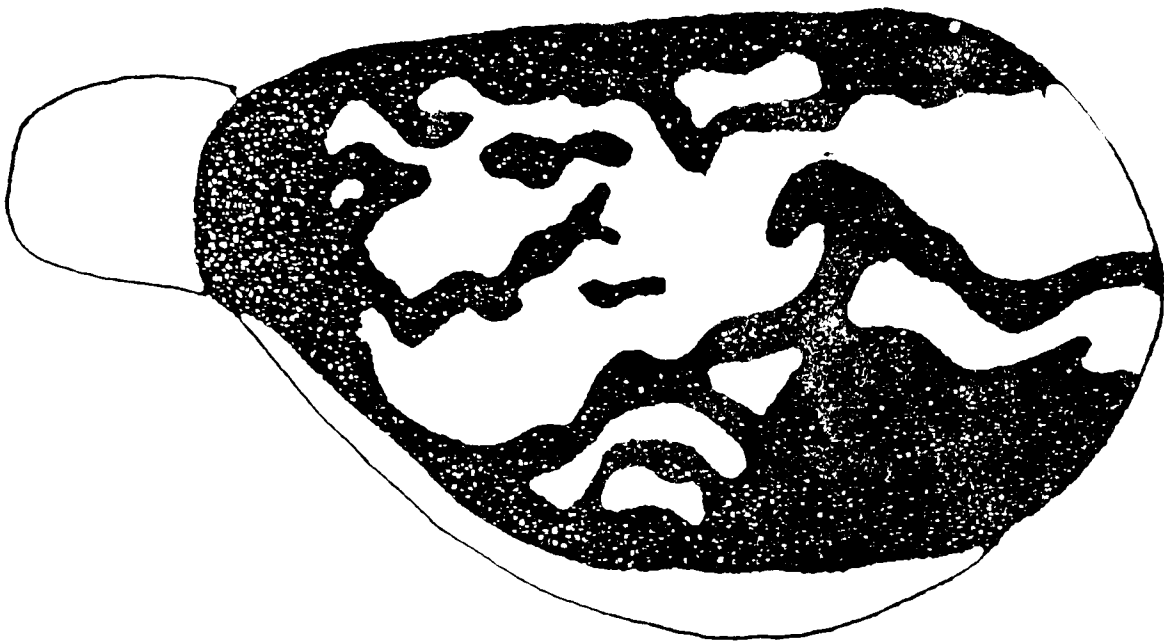
A: Summary of the organization of the callosal connections, on a dorso-lateral view of the rat brain (from Akers and Killackey, 1978). The filled regions indicate areas of callosal projection (revealed by degeneration studies).

B: Comparing the above diagram with cyto- and myeloarchitectonical maps (taken from Zilles et al. 1980; lateral, dorsal and medial views respectively) suggests that callosal connectivity does not include primary visual cortex or certain regions of the motor and somatosensory cortex.

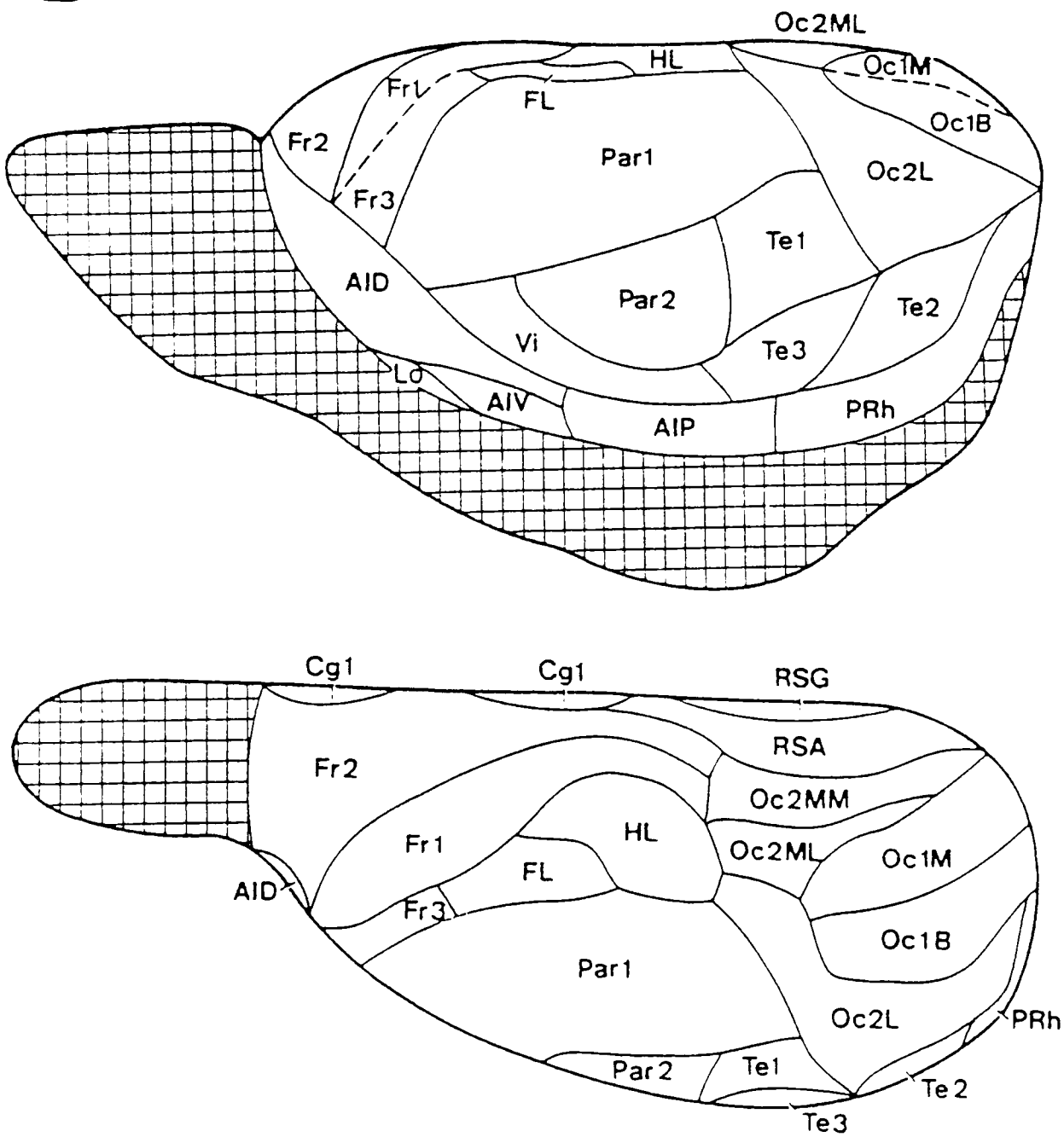
Fr1-Fr3-frontal areas; Par1-Par2, Fl, HL-parietal areas; Te1-Te3-temporal areas; Oc1B, Oc1M, Oc2L, Oc2MM, Oc2ML-occipital areas; Cg-Cg3-medial prefrontal cortex; IL-infralimbic area; MO, LO, VO-orbital cortex; AID, AIP, AIV-insular cortex; PRh-perirhinal cortex; RSA, RSG-retrosplenial cortex. The allocortex and parts of the periallocortex are cross-hatched.

Fig. 7.8

A



B



I wish to examine the possibility that the *mammalian preplate is homologous to the neurons of the reptilian cortex*. Since it is not possible to examine this particular question from fossil records, I shall concentrate on developmental and structural similarities.

Comparison of pallial organization in reptiles and mammals

According to Herrick (1933), the reptilian cortex is characterized by superficial laminae of grey substance in the pallium of the cerebral hemispheres, divided into 3 longitudinal stripes: a mediodorsal cortex, a dorsal cortex and a lateral cortex. By tracing their connections, hodological arguments suggest that the lateral cortex is homologous to the pyriform cortex and the mediodorsal to the hippocampus (reviewed by Ulinski, 1983; see Fig. 7.2). The mammalian cortex is most probably related to the medial pallium (Goffinet, 1983, 1984). The DVR (Dorsal Ventricular Ridge) is included among cortical areas because it contains several sensory fields and is presumably phylogenetically related to pallial structures (Ulinski, 1983).

Evidence for the homology

(i) *Ontogenetic similarity between reptilian and mammalian cortices* It was Haeckel (1866) who stated that 'ontogenesis is the recapitulation of phylogenesis'. In this crude form, his statement is surely incorrect, but it is indeed very striking that most early developmental steps are highly conserved in all vertebrates (Fig. 7.3). There is no reason to imagine that neural development is fundamentally different from the generation of other organ systems. Since most if not all of these developmental patterns are inherited, they strongly support the notion of homology. In the medial, dorsal and lateral supraventricular cortical zones of reptiles, neurogenesis follows a lateral to medial, and an anterior to posterior gradient (Goffinet, 1990). These tangential gradients

correlate with the pattern of development seen in mammalian embryos (reviewed by Bayer and Altman, 1991).

Pre-mammalian cortices develop in an outside-first, inside-last fashion (Goffinet 1990). Only in mammals is the cortical plate assembled as a 'sandwich', with the cortical plate itself splitting the primordial plexiform zone into two and developing in an inside-first, outside-last pattern (Marin-Padilla, 1971, Luskin and Shatz, 1985a,b). It is interesting to point out here that the primordial plexiform zone itself (before it is split into subplate and marginal zone by the cortical plate) is generated in the 'reptilian' outside-first, inside-last fashion (Bayer and Altman, 1991). In reptiles an outside-first, inside-last morphogenetic gradient has been demonstrated at the ultrastructural level (Goffinet, 1990): synapses appear first in the molecular layer, then in the subcortex, and finally within the cortical ribbon. All these similarities strongly suggest homology with the mammalian preplate.

(ii) *Minuteness and multiplicity of similarities between reptilian cortex and primordial plexiform zone cells (similarities in morphology and histochemical properties)*

Cajal already noticed the similarities between the polymorph cells of layer 1, and cells of the lizard cortex (Fig. 7.4). The work of Marin-Padilla suggested that these marginal zone cells are indeed descendants of the primordial plexiform zone. He described the splitting of the first generated, polymorphous cell layer (the primordial plexiform zone or preplate) into the marginal zone and the subplate (Marin-Padilla, 1971, 1972, 1988) and he called these premature zones the 'the reptilian framework' of the developing mammalian brain (Fig. 1.1B).

The subplate cells have surprisingly similar transmitter systems to those of reptilian cortical neurons. Neuropeptide Y (NPY), cholecystokinin (CCK), somatostatin

(SOM), glutamic acid decarboxylase (GAD) and GABA (Somogyi et al., 1984; Reiner et al., 1984; Chun et al., 1987; Chun and Shatz, 1989; Antonini and Shatz, 1990 have all been demonstrated in mammalian preplate/subplate cells). Davila et al., (1991) recently demonstrated that cells of the cortical regions of turtles, lizards and mammals universally contain NPY, SOM and GABA. Surprisingly, these neurons are only found at telencephalic levels, not in any other cerebral regions. Davila et al., (1991) take this as evidence for the conservative nature of evolution, and believe that these similarities support the view that these cortical neurons were present in stem reptiles.

Principle of radial organization

If the preplate is the equivalent of the reptilian cortex, where do the extra cells of the cortical plate come from? Shatz et al. (1990) proposed that a mutation in the cell cycle produced extra cells and that this is responsible for the emergence of the cortical plate. The idea that preplate and plate cells come from the same ventricular and subventricular stem-cells (same cell lineage) could now be tested directly with the retrovirus gene transfer technique (Price et al., 1987; Cepko et al., 1990). Do preplate cells and the cortical plate cells belong to a common or separate clones?

My own collaborative autoradiographic studies suggest that radioactive thymidine administered at E12 (hence producing strong labelling of preplate cells) also produces dilute label in the later generated cells of the cortical plate (Molnár et al., 1991), although the thymidine disappears from the circulation within 2 hours (Luskin and Shatz, 1985a,b). This could indicate that the same progenitors generate firstly the subplate neurons, which become postmitotic at E12-14 (thus they have a high concentration of [³H]thymidine), and then cortical cells proper through several mitotic divisions (therefore with lower [³H]thymidine content). However it is equally possible

that the progenitors of subplate neurons and cortical cells represent two distinct populations, one of which, i.e. the progenitors of the cortical neurons generate only other progenitor cells and not postmitotic cells at E12. The diluted [³H]thymidine label would then appear in the cortical cell progeny of the late-generated progenitor cells. The investigation of the precise lineage relationships with recombinant retrovirus will be important in deciding this issue and might also cast light on the control of subplate death.

To accommodate the extra cells of the mammalian cortex, there must have been some principle of vertical organization. This raises another mystery, namely the transition from the outside-first, inside-last reptilian pattern of cell accumulation (still seen for the mammalian preplate) into the inside-first outside-last sequence of the mammalian plate. Teleologically, simple continuation of the outside-in arrangement (as is found in Reeler; Chapter 4) would seem to be a more straightforward way to have accommodated the extra cortical plate. Why is the primordial plexiform zone split into two and the settling pattern reversed? Is this the result of another mutation?

Goffinet (1990) argues that the radial sequence in which the cortical plate is laid down is not an all-or-none property. There are various degrees of organization in the different segments of the embryonic pallium in different reptiles. On the basis of examination of the various descendants of the stem reptiles he suggests that radial cytoarchitectonics must be regarded as a "property which has been acquired gradually, independently and to variable extents after phylogenetic divergence" (Goffinet, 1990). He also noticed a rough negative correlation between laminar organization and the presence of an outside to inside gradient. The inside-first, outside-last pattern, together with the prelaying of 'highways' of subplate efferents, may provide certain advantages.

Perhaps cortico-cortical connectivity would be physically constrained in certain ways if outside-in accumulation of neurons were continued. Maybe Reeler is a step-back mutation in the sequence of change: Reeler is certainly disadvantaged in that its survival is poor (though this may be due to disorganization elsewhere in the brain than in the cerebral cortex). Also, in some of the different Reeler strains there is no corpus callosum (A. Goffinet and V. Caviness, personal communications).

Highly advantageous mechanisms are usually discovered early and well conserved throughout the different branches of the evolutionary tree (or they develop separately and convergently). In all mammals, for instance, there is remarkable uniformity in the number of cortical neurons in a given columnar volume (with the major exception of the primate striate cortex; Rockel, Hiorns and Powell, 1980). The radial organizing principles are probably rather universal among mammals.

Perhaps the emergence of the various forms of interaction with preplate neurons was the prerequisite that made it possible for a bigger and more powerful cerebral cortex to emerge (Fig. 7.4). Thalamic fibres might not be able to reach the appropriate cortical areas without the pre-laid trajectories of preplate axons and without the subplate compartment to wait on. This stage of thalamic axon guidance all happens when the embryonic cerebrum is still lissencephalic (in all mammalian species), and the morphological context is therefore simple and the distances are small. The waiting period (Rakic, 1977; Lund and Mustari, 1977) may be essential in the development of bigger brains, since if the thalamic fibres did not reach the appropriate cortical area early enough, the pathways might become too tortuous for them ever to arrive in accurate order.

In the early stages in the human (12-16 weeks), the LGN is situated at the lateral

aspect of the thalamus, much as it is in fetal (and adult) rodents (Clark, 1932a,b; Rakic, 1974). Subsequently, due to the expansive growth of the pulvinar, the LGN is simultaneously rotated in its transverse and sagittal axes and becomes displaced to the inferior aspect of the mature thalamus (Rakic, 1977). My own preliminary results, tracing projections (with DiI) in postmortem human embryonic material from the first trimester, suggest that thalamic fibres grow out before this characteristic migration and rotation of the LGN (Fig. 7.5). The interconnections are laid down when the cortex is at a lissencephalic phase and when the morphology of primate and rodent forebrains is very similar.

Although birthdating studies demonstrate a distinct population of first-generated cells (Luskin and Shatz, 1985a,b), the subplate region itself is a dynamic structure. Its borders are constantly changing. This suggests intensive, constant interactions between subplate and its neighbours (Chapter 4). Kostovic and Rakic (1990) noted that "The subplate may not be a vestige of the phylogenetically old network but a transient embryonic structure that expanded during evolution to subserve the increasing number of its connections." A study similar to that of Rockel, Hiorns and Powell (1980) is needed to establish the ratio of cortical plate cells to subplate cells in each area of developing mammalian brains before the stage of subplate death. However, even if the relative subplate cell number increases in primates this would not exclude the possibility that the subplate is derived from a previously existing cell group with an earlier evolutionary origin.

The higher vertebrates all evolved from 'stem-reptiles' (Fig. 7.1). The products of the different branches of this evolutionary tree found different ways to cope with the environment. It is meaningless to ask which strategy is best, since there are survivors

in each environmental niche. Mackintosh (1992) blames our teleological (and anthropocentric) views: "we are unable to rid ourselves of the misconception that there has been a linear increase in intelligence as we progress up the phylogenetic scale". However the anagenetic approach is losing ground and at last we are beginning to accept the Darwinian idea that our brain is the end product of undirected mutations and accidents of selection (the first critics were Hodos and Campbell, 1969; Campbell and Hodos, 1993).

7.5 Emergence of afferentation of the cortex

Finally, I would like to examine the development of the different sensory inputs to the cortex in various vertebrates. At which phylogenetic stage did the cortex begin to receive sensory afferentation? How was the cortical representation of the external world achieved? What do we know about the nature of this representation in the brain?

The cerebral cortices of fish, birds, lizards, and frogs all receive inputs from sensory systems. The vast majority of the cortical input in these Classes is from the olfactory system. Other modalities (vision, etc.) are also represented, but most of those fibres terminate in the anterior dorsal ventricular ridge (Ulinsky, 1983). Most of the information other than olfactory seems to be processed subcortically in the colliculi or the striatum. In these species the intermediate cortex (dorsal pallium) is just a layer of a few pyramidal cells organized into 1 or 2 plexiform zones depending on the species (Fig. 7.4). However, even this intermediate cortex receives input from the thalamus (Fig. 7.6.: from Heller and Ulinski, 1987).

When and how did the first afferents, carrying sensory information other than olfactory, reach the cortex? Did corticothalamic projections precede thalamocortical projections in phylogeny? How did the cortex process the first thalamic input? Did

cortical differentiation progress as a result of this new afferentation, or did the two processes proceed in parallel?

7.6 Thalamus as a developmental device for the expanding cortex

The answers to these questions have yet to emerge from comparative studies, but I suspect that the expansion of cortical areas required an increase in the matching thalamic regions, and an increase in their interconnection. The two probably appeared in parallel on the evolutionary time scale. During the ontogenesis of the visual pathways, the different levels of the pathways interact horizontally and vertically. Dynamic interactions can result from changes at different levels. For instance, Guillery (1972) demonstrated that cells of the dLGN, in the region corresponding to an 'artificial monocular segment' made by destroying a small area of temporal retina in the non-deprived eye shortly after birth, are protected from the normal failure of growth caused by monocular deprivation. Because 'competitive' interactions between dLGN cells of different laminae almost certainly occur at their axon terminations in the visual cortex, the shrinkage of dLGN cell bodies during monocular deprivation is probably a retrograde effect, originating in competitive interactions in the striate cortex (Guillery, 1972). Maybe similar dynamic interactions between cortex and thalamus occurred during phylogenesis, with thalamus and cortex developing together. Among living mammalian species there seems to be a close correlation between the cortical surface area and the thalamic volume. When the so-called association cortices increased in size, so too did the 'non-primary' and 'non-specific' thalamic regions.

The neuronal and synaptic morphology of the pulvinar and the mediodorsal nucleus closely resembles that of other 'specific' sensory and motor relay nuclei (Mathers, 1972; Schwartz et al, 1991). The most distinct difference, however, is that the

synaptic sites in the glomeruli, generally reserved for ascending sensory afferents in major relay nuclei, are occupied by corticothalamic terminals in the non-primary nuclei (Mathers, 1972; Schwartz et al, 1991). Different cortical regions in the peristriate, posterior temporal or posterior parietal cortex converge on the same regions in the pulvinar and the outputs of different corticofugal layers (5,6) might terminate on different regions of the dendritic tree of cells in the pulvinar and mediodorsal nucleus (Mathers, 1972; Schwartz et al, 1991; Kuroda and Price, 1991). It is an interesting possibility that different cortical regions interact through these nuclei (Robinson and Petersen, 1992; Mitrofanis and Guillery, 1993).

The thalamus, structurally and functionally, is more closely associated with the phylogenetic expansion of the cerebral cortex than was previously thought. All this suggests that "the thalamus is not the intellectual desert of neurobiology" (R.W. Guillery, personal communication).

7.7 Multiple constraints during map formation in the 'protocortex'

Thalamic connections seem to be laid out in an orderly fashion on the basis of simple spatial and partly chronological algorithms (Chapter 3; Blakemore and Molnár, 1990; Shatz, 1992a,b). The topography of the initial intercortical connectivity is also rather simple and may depend on very similar mechanisms (Hankin and Silver, 1986). Fibre ordering preserves the chronological order of its establishment. Since the final representation is dependent on and complicated by many other factors, these initial clear-cut basic principles have gone unnoticed.

If we could examine the fibre ordering in the adult internal capsule or in the adult corpus callosum (away from the target area) the fibres might still show their basic topographic arrangement. Interestingly there has been such study on the adult callosum

in the cat (but not yet on the internal capsule). Nakamura and Tanakesi (1989) made small, discrete injections of HRP into different parts of the callosum of adult cats. Although there was no correlation between functional areas, there was a strict sectorial topographical correspondence between the corpus callosum and the cortical areas from which the cells were sending their projections (Fig. 7.7). Small injections into the adult cat presplenium labelled a zone of cortex very similar to that resulting from a neonatal injection into areas 17 and 18. Kind and Innocenti (1990) note that the topography of the juvenile callosal projection neurons may be related to that of their axons in the corpus callosum. I would rather say that the initial fibre ordering of the intercortical connections is preserved in the callosum, but substantially modified at the target fields (Figs. 7.8A and B).

Such experiments, examining fibre arrangements in tracts distant from origins and targets, would be extremely interesting in developing and adult albinos or after early enucleation. I suspect that the initial connectivity between the different cortical areas might also be explained with few very simple principles. Interestingly, the corpus callosum is a development of placental mammals: their close relatives, the marsupials, use only the anterior commissure for inter-hemispheric connection, even though the general construction of the neocortex is very similar (see Mark and Marotte, 1992).

Killackey (1990) argues that there was evolutionary pressure for the ‘uncoupling’ of a portion of the vertebrate telencephalon, the neocortex, from strict genetic determination. The very high degree of plasticity that seems to be such an important feature of this uncoupling of the maturing cortex, may tend to blur the degree of simplicity and order present at earlier stages especially for intrinsic and long-range cortico-cortical connections.

Young and Scannell (1993) have recently demonstrated with quantitative topological analysis that the vast majority of corticocortical connections within the same hemisphere in adult mammals are formed between neighbouring areas, and follow an anteroposterior sequence. Excluding the cingulate and the entorhinal cortex from this analysis, the correlation is even more apparent. The developing forward and backward cortico-cortical projections may be laid down through simple algorithms similar to those postulated for thalamocortical (Chapter 3; Blakemore and Molnár, 1990) and intercortical connectivity (Hankin and Silver, 1986). However the final pattern that is carved out from a simpler initial organization is complicated by morphological change, selective elimination and activity-dependent plasticity. Fortunately the degree of fibre order still present in the major pathways of the adult can stand as a witness to its earlier simplicity (Nakamura and Kanaseki, 1989).

7.8 Anatomical and functional units in the cortex

There is now a good deal of evidence, at least for lower mammals, that the various areas of the cerebral cortex are not entirely pre-determined for any particular input and connectivity, cytoarchitectonic appearance or function (reviewed by O'Leary, 1989). Neuronal self-organization and the influence of the environment play an important part in carving out the connectivity and internal organization of areas, and hence their functions.

Functional specialization becomes expressed in the emerging cytoarchitecture of the cortex (Brodmann, 1909), although the correlation between anatomical and functional subdivisions may be less precise than is sometimes imagined. For instance, area 18 of the primate extrastriate field is now known to contain a substantial number of separate representations of the visual field, at least some of which are highly

specialized for particular functions (see Zeki, 1990). In the opossum, the primary somatosensory cortex and motor cortex are not separate entities: instead they form a 'somatosensory-motor' amalgam, the two areas entirely overlap, and they perform the same functions as the two separate regions in the rat (Lende, 1963). This overlap of the sensory and motor systems in the opossum is restricted to the cortical level. VP and VL receive separate, non-overlapping inputs, but both project to the same cortical region.

Why does Nature sometimes employ different and separate neuronal representations and sometimes not? In the primate striate cortex the representation of the two eyes, different orientations, colour, motion, etc. use the same basic mapping in a single cortical field. Mitchison (1992) argues that this results in a reduction in the length of wiring of the brain. However, beyond a certain limit of overlapping or closely interdigitated function, the brain appears to add extra functionally and morphologically discrete areas to carry out particular functions within the framework of a large functional system. Keeping several functions in a single area reduces cortico-cortical connectivity; dividing them between neighbouring areas reduces local connectivity within each. New areas appear when it becomes more economical to process newly-discovered properties of the sensory input in a different network (perhaps because that processing requires the proximity of cells with particular properties not close to each other in the primary area). Usually this 'analytical delegation' does not spread far away in the hemisphere. It stays in the closest available target space, often with reversals of mapping at borders, and mirror-image topography in neighbouring areas.

The streams within the cortical representation of vision can be largely explained by neighbourhood relations; these streams are highly interconnected and might even reconverge (Young, 1992). However, one must also keep in mind the rich back-

projections between visual areas, informing V1 of the primate cortex of the 'higher' functions analysed in the extrastriate belt. Perhaps V1 (and the other primary sensory areas) should not be regarded as the most 'simple' and peripheral sensory areas just because they receive the primary thalamic input. Perhaps we should turn around the concept of higher and lower levels of visual processing and consider the primary areas to be both the major input to and the major output of each sensory field (C. Blakemore, personal communication).

The work in this thesis has revealed some of the mechanisms involved in the earliest phases of thalamocortical development. But thalamocortical connectivity itself provides only an initial framework for further organization of the neocortex. Although the thalamic fibres are undoubtedly the key factors in the delivery of information to the cortex, the final cortical representation of the external world is dependent on many other additional factors. The initial topography of the projection to the cortex may be explained by a cascade of relatively few, individually simple mechanisms (Chapters 2 and 3; Blakemore and Molnár, 1990). But the number of additional constraints rapidly increases during subsequent maturation. Our present knowledge of the interactions between these numerous constraints is far from complete. Undoubtedly there are many more '*men in the machine*' waiting to be discovered.

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