THE FORMATION OF CORTICOCORTICAL CONNECTIONS IN THE CAT'S VISUAL CORTEX

Damira Carić

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Edinburgh.

January, 1995

DECLARATION

I declare that this thesis was composed by myself. Contributions of others to the work are clearly indicated.

15. January 1995

Edinburgh, 15 January 1995 Damira Carić

ACKNOWLEDGEMENTS

I am very grateful to David Price for being an excellent supervisor, and I would particularly like to thank him for his endless encouragement and support

I would like to thank to my second supervisor Richard Ribchester for his instructive discussions.

I am particularly grateful to Katy Gillies and Jean Hunter for taking great care of the animals that I used in this work.

I would also like to thank Beau Lotto for his collaboration in culture experiments described in Chapter 6, Grace Grant for her assistance with the histology at the beginning of the study and Brendan McGrory for his assistance with some of the photography in this thesis.

I thank the Faculty of Medicine for awarding me a Fellowship to carry out the project. The experiments were funded by the Wellcome trust and the Medical Research Council.

Finally, I am very grateful to my parents and Toni for their constant support and encouragement

This thesis is dedicated to my friend Tihomila.

ABSTRACT

The aim of this study was to investigate the postnatal development of association projections in the kitten's visual cortex. New sensitive fluorescent lipophilic tract-tracers were placed side-by-side in area 17 of fixed brains of various ages, from postnatal day 1 to 30. These tracers showed that afferents from area 17 had just reached the deep layers of areas 18 and 19 at birth. A few had entered the cortex, mostly at points that corresponded retinotopically with those of the injection sites. These points were linked by distinct axonal tracts from postnatal day 1 on, suggesting that many corticocortical axons had grown in an ordered fashion between specific points in striate and extrastriate cortex. During the second and third postnatal weeks, corticocortical fibres became increasingly densely distributed in layers 5 and 6 of extrastriate areas. At the same time, more and more axons penetrated the superficial layers, but their tangential distribution was narrower than that of the projections to the deep layers. Confirmation of this more restricted penetration of upper layers came from in vivo injections of retrogradely transported tracers: injections restricted to the superficial layers of area 18 labelled a relatively narrower region of area 17 than injections in deeper layers. By the fourth postnatal week, corticocortical afferents in areas 18 and 19 were mainly restricted in patches. At all ages, injections of lipophilic tracers in area 17 retrogradely labelled cells in areas 18 and 19; from birth on, most of these cells were in the same regions where axons from area 17 were penetrating the cortex, indicating reciprocal topography.

In further neuroanatomical experiments I considered the role of layer 1 in the formation of corticocortical connections. From the earliest postnatal ages, apical dendrites of corticocortical cells project to layer 1, where they connect with long horizontal fibres that cross several cortical areas. I lesioned layer 1 in area 18 near the area 17/18 border in newborn kittens, let the kittens develop to one month of age and examined cortical projections after labelling them with lipophilic dyes in fixed brains. In lesioned animals, very few projections interconnected areas 17 and 18 although in Nissl-stained sections the cortex appeared normal below the lesions in layer 1. Thus, these findings suggest that the lesions produced a disproportionate loss of corticocortical connections, that is not easily explained on the basis of generalised cortical damage.

I examined the consequences of the neonatal thalamic destruction on the cortical cytoarchitecture and the maturation of corticocortical connections. Previous studies

suggest that visual deprivation does not abolish the formation of cortical connections. After thalamic destruction, the clustering in visual area 18 was more crude and overall projections were decreased. The most obvious changes were in area 18, where layer 4 was narrower and had a higher cell density than in the contralateral non-lesioned side.

The present study revealed that the initial postnatal growth of association axons from area 17 was more directed than had been described previously and that the process of postnatal refinement of corticortical pathways is influenced by intercellular signaling via layer 1 and thalamic afferents.

ABBREVIATIONS

A - anteroposterior coordinates

AP - anteroposterior axis

AP0 - anteroposterior level zero

BD - binocular visual deprivation

CP - cortical plate

DiA - 4-(4-dihexadecylaminostyryl)-N-methylpyridinum iodide

DiI - 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

DY - diamidino yellow

H^D- vertical coordinates indicating the depth from the surface of the cortex

HM - horizontal meridian

IBO - ibotenic acid

ILN - intralaminar nuclei

L - lateral coordinates

LGN - lateral geniculate nucleus

LC - locus coeruleus

LS - lateral suprasylvian area

MD - monocular visual deprivation

ML - mediolateral axis

MZ - marginal zone

P - postnatal day

PMLS - posteromedial lateral suprasylvian cortex

SP - subplate zone

SP_U - upper portion of the subplate

VM - vertical meridian

TTX - tetrodotoxin

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

The brain has been the subject of intense scientific inquiry since the early part of the twentieth century and the pioneering work by Ramón y Cajal (1911). However, it was only since the middle of this century that quick progress in understanding the function and the organization of the brain started. This progress was possible due to advances in neurobiological methods, for example, axonal transport of macromolecules for tracing pathways in the central nervous system and refinement of electrophysiological recording methods. Over the past few decades, there has been a gradual emergence of a more coherent picture of the structure and the function of the brain. A particularly interesting example can be seen in studies of the visual system. The visual system was always interesting to study, because it is relatively easy to control visual stimuli and observe physiological responses. Furthermore, most of what has been discovered about the visual system is similar to discoveries in other systems, for example somatosensory system. Thus, the similarities between different systems allow us to identify general principles of brain organization.

At present, despite our knowledge about the anatomical and the functional organization of the brain, it is still unclear how the complex connectivity in the brain is achieved. During the development of the brain, the neurones must be generated in the right quantity, they must migrate to the right location and their axons must select correct pathways and make the right connections. It seems that the early events in the development of the brain are under genetic control. On the other hand, the precision in the connectivity within the brain seems to be influenced by external factors, for example stimulation. Yet further studies are needed to provide clearer evidence of how stimulation is needed for the immature brain to complete its development. In recent years, new approaches have been used, such as finding the molecular mechanisms underlying the development of the connectivity of the brain

(reviewed by Daw and Fox, 1991). This modern approach is starting to provide a new insight into brain development at the synaptic and the cellular level.

In the following pages I will describe the basic principals of the organization of the visual system, the organization and connections in the visual cortex of the cat and the present understanding how these connections might develop. I will state the aims of may research on the development of corticocortical connections in the young kitten, describe my experiments and a hypothesis of how corticocortical connections might form.

1.2 THE ORGANIZATION OF THE VISUAL SYSTEM

1.2.1 Visual stimulus from the retina to the cortex

The visual response begins with the rods and cones of the retina, which transform light into neuronal signals. These cells send the signals via the retinal interneurones to the retinal ganglion cells. The axons of the ganglion cells form the optic nerve, which projects to the thalamic nucleus called the lateral geniculate nucleus (LGN). The cells of the LGN send the visual information to the primary visual cortex (Brodmann's area 17, 1909). In the cat, the LGN also projects to other visual areas. The transformation of the visual field into neural information is achieved by the nature of each cell's *receptive field*. This term refers to the restricted area on the retinal surface that influences, upon illumination, the signalling of an individual neuron in the visual system. The term receptive field was originally introduced by Sherrington (1933) in relation to reflex action and was applied to the visual system by Hartline (1940). In the retina, there are two basic receptive field types: the on-centre and the off-centre (i.e. ganglion cells that have on-centres respond best to a spot of light shone onto the central part of their receptive fields; illumination of the surrounding area reduces the discharges and causes responses when the light is turned off; Kuffler, 1953). The size of the receptive field of a ganglion cell depends on its location in the retina (Barlow et al., 1964). Ganglion

cells situated in the central areas of the retina have much smaller receptive fields than those at the periphery and thus a higher acuity of vision. The functional importance of receptive fields of various sizes was found in other systems; for example, cells innervating the skin of the fingertip have smaller receptive fields than those on the skin of the upper arm.

1.2.2 Topography in the visual system

From the retina to the LGN and the visual cortex the visual field is represented in a strict topographic order. Neighbouring neurones in the visual cortex receive information from neighbouring retinal ganglion cells. The topographic order in the visual system was first shown by Talbot (1940). Talbot used an evoked response method: after shining light onto a small part of the retina he recorded the evoked potentials on the surface of the cortex. Similar topographic representations were found in other sensory systems (e.g. somatosensory cortex, Marshall et al., 1941).

1.2.3 The vertical columnar organization of the thalamic input to the visual cortex

Lorente de Nó (1938) first suggested that there is a vertical columnar organization of the cortex, in addition to the more obvious layering pattern. Current understanding of the columnar organization of the cortex is based on observations by Mountcastle (1957) on the somatosensory cortex. He discovered that sensory neurones, such as those serving light touch of the skin, are grouped together and segregated from those neurones that respond to other stimuli, for example deep pressure. These neurones are grouped in columns that run radially from the cortical surface to the white matter and have receptive fields in more or less the same part of the sensory periphery.

The columnar organization of the thalamic input to the visual cortex was

discovered by Hubel and Wiesel (1962). Hubel and Wiesel (1959) first discovered that cortical neurones have receptive fields that are different from those of retinal or geniculate cells; thus, spots of light have little or no effect on cortical neurones as these cells recognize visual stimuli such as a narrow bar. The receptive fields of cells in the visual cortex have been divided into two classes, simple and complex. The receptive field of simple cortical cells (cells in cortical layers 4, 6 and lower part of layer 3) is elaborated by convergence of many geniculate neurones; the receptive field of complex cortical cells (cells in cortical layers 2, 3 and 5) is elaborated by convergence of simple and complex cells (Hubel and Wiesel, 1962). Hubel and Wiesel (1962) found that neurones in area 17 with similar axes of orientation in their receptive fields are neatly stacked on top of each other in discrete columns. Cells in each column receive their input from largely overlapping receptive fields on the retinal surface. Each orientation column is about 0.2 to 0.5 mm wide. In addition to these columns devoted to axis of orientation, Hubel and Wiesel (1962) found another pattern of alternating columns in the visual cortex, the columns for eye preference or ocular dominance columns. Within each column, cortical neurones are more strongly influenced by one or the other eye. The width of the ocular dominance columns is about 0.25 to 0.5 mm. Ocular dominance columns were also demonstrated in the monkey and the cat by anatomical methods, e.g. intraocular injection of the anterograde tracer, tritiated proline, which is transported transneuronally to the visual cortex and labels geniculocortical axons in an alternating pattern (Hubel and Wiesel, 1977; Shatz et al., 1977).

Soon, it became clear that there are other systems (related to thalamic inputs) where cortical cells are grouped in discrete clusters, e.g. the blob-like structures that were first seen in the monkey in cortical tisue that had been stained for the mitochondrial enzyme cytochrome oxidase (an enzyme indicative of high metabolic activity; Horton and Hubel, 1981). Intraocular injections of either horseradish

peroxidase (HRP) or tritiated proline indicated that clusters of geniculocortical axons end in close association with the blobs (Livingstone and Hubel, 1982).

1.2.4 The tangentially organized extrathalamic input to the visual cortex

Apart from the columnar organization of the neocortex, there is also a tangentially organized extrathalamic input to the neocortex. The tangential afferents have only recently begun to be understood (reviewed by Foote and Morrison, 1987). There are at least four extrathalamic projections to the cortex: i) the noradrenergic afferents arising from the pontine nucleus locus coeruleus (LC); ii) the serotoninergic afferents arising from the mesencephalic raphe nuclei; iii) the dopaminergic afferents arising from the substantia nigra-ventral tegmental area and iv) the acetylcholine afferents arising from the nucleus basalis of the substantia inominata (Foote and Morrison, 1987). These afferents seem to be more organized than initially thought; they show regional and laminar specialisation and it is clear that there are qualitative differences between species e.g. in primates and rodents. For example the distribution of noradrenergic afferents in primate cortex (not in the rodent cortex) shows a distinct pattern of regional specialisation (Foote and Morrison, 1987).

The exact function of extrathalamic innervation is not known. One of the hypotheses is that i.e. noradrenergic afferents have a "modulatory" influence on specific thalamic afferents as coactivity of noradrenergic fibres may enable other systems converging on the same target neurones to transmit more effectively during the period of simultaneous activity (Bloom, 1979). Another role (indicated by differences in regional specialisation in the primates) may suggest that during evolution, phylogenetic changes of the brain go in parallel with changes of the extrathalamic inputs. This is best illustrated in the dopaminergic innervation in the primate cortex which, unlike in rats, coincides with cytoarchitetonic and functional boundaries (see in Foote and Morrison, 1987). It even seems that the laminar

distribution of dopamine (most dense in layer 1) is in a position to influence the activity of corticocortical projections rather than thalamocortical. Extrathalamic fibres have been implicated also in the development of the cortex, as these fibres are among the first to innervate the cortex; e.g. in the rat, noradrenergic afferents innervate the developing visual cortex by E18, thus before specific thalamic afferents (Levitt and Moore, 1979) and the ablation of the origin of noradrenergic afferents (the LC) interferes with the differentiation of dendrites (Felten et al., 1982). Furthermore, the noradrenergic innervation has been implicated in structural and functional plasticity of the visual cortex (Singer and Rauschecker, 1982; Kasamatsu, 1987), and it seems that this is achieved by joint action of noradrenaline and acetylcholine (Bear and Singer, 1986; Greuel et al., 1988)

1.3 THE ORGANIZATION OF THE VISUAL CORTEX IN THE CAT

1.3.1 Multiple visual cortical areas

The visual cortex of the cat is divided into several distinct visual areas. In the past several approaches have been used to subdivide the visual cortex (and other regions of the cortex) into different areas. The traditional method, to compare the cellular morphology and lamination of different cortical regions (Brodmann, 1909), has endured despite some later modification. In 1971, Allman and Kaas used microelectrodes to electrophysiologically map the representation of the contralateral visual hemifield onto the cortex in the owl monkey. The topography of the visual field representation was determined by relating the positions of receptive fields for neurones in the retina and the locations of the recording sites in the visual cortex. A few years later, electrophysiological mapping of the cat's visual cortex largely confirmed the validity of old divisions of the visual cortex into over a dozen visual areas (Palmer et al., 1978; Tusa et al., 1978; 1979; Tusa and Palmer, 1980). These studies also showed that visual areas differ from one another in the portions of the

visual field they represent and the relative amounts of cortical tissue devoted to different parts of the visual field (called "magnification factor").

1.3.2 Area 17 in the cat's visual cortex

In the cat, the visual cortex occupies the most caudal regions of the brain. Area 17 lies on the crown and along the medial bank of the lateral gyrus (see Fig. 1a,b in Chapter 5) (Tusa et al., 1978). The entire contralateral visual hemifield is represented in area 17 (see Fig. 1c,d in Chapter 5). The receptive fields extend away from vertical meridian to 90^{o} along the horizontal meridian and to 50^{o} above and below the horizontal meridian (Tusa et al., 1978). A disproportionate amount of area 17 is devoted to central vision, i.e. the central 20^{o} of vision is represented on 50% of the surface of area 17. Area 17 contains a first-order transformation of the visual field, where adjacent points in the visual field are represented as adjacent points in the cortex (Allman and Kaas, 1974; Tusa et al., 1978).

1.3.3 Area 18 in the cat's visual cortex

Lateral to area 17, on the crown of lateral gyrus, lies area 18 (Tusa et al. 1979). Area 19 lies yet further lateral and occupies the rest of the lateral gyrus to the lateral sulcus (Tusa et al. 1979). Area 18 has a representation of only the central 50^{o} of the contralateral visual hemifild (see Fig. 1e,f in Chapter 5; Tusa et al. 1979). Area 18 has a second-order transformation of the visual field in which adjacent points in the visual field are not always represented as adjacent points in the cortex (Allman and Kaas, 1974; Tusa et al. 1979).

1.3.4 Thalamic projections to visual cortical areas in the cat

Visual cortical areas are connected with a number of subcortical regions, of which the most important are the thalamic nuclei, e.g. the LGN, the lateral posterior (LP)-pulvinar complex and intralaminar nuclei (ILN) of the thalamus (Garey and

Powell, 1968; Symonds et al., 1981; Raczkowski and Rosenquist, 1983; Kaufman and Rosenquist, 1985a,b).

In the cat, the LGN is subdivided into laminae A, A1 and C (C lamina includes C1, C2 and C3) (Guillery, 1970). Laminae A and C receive input from the contralateral eye, while lamina A1 receives input from the ipsilateral eye. Cells in laminae A and A1 project only to areas 17 and 18, whereas laminae C projects to area 17, 18, 19 and to a large number of other cortical visual areas (Garey and Powell, 1968; Raczkowski and Rosenquist, 1983). These afferents are retinotopographically ordered (Sanderson, 1971). For example the border between areas 17 and 18, where the vertical meridian of the visual field is represented, receives axons from the medial boundary of the LGN (Sanderson, 1971). More peripheral regions in area 17 and 18 receive inputs from the lateral parts of the LGN (Sanderson, 1971).

In contrast to retinotopic afferents from the LGN (and other specific thalamic nuclei e.g. the lateral posterior (LP)-pulvinar complex), afferents from the ILN of the thalamus are not retinotopographically organized. The ILN are non-specific thalamic nuclei which include the central-medial, paracentral and central-lateral nuclei. These nuclei have widespread afferents (e.g. from pulvinar, the pretectum, the superior colliculus and some visual areas) and widespread efferents to all visual cortical areas (Kaufman and Rosenquist, 1985a,b; Cunningham and LeVay, 1986; Kato, 1990). Thus, it has been hypothesized that these nuclei play a role in attention to visual stimuli (Kaufman and Rosenquist, 1985a).

1.3.5 Laminar arrangement of LGN and ILN afferents in the visual cortex

The neocortex is usually divided into six major layers, numbered 1 (the most superficial cortical layer) to 6 (the deepest cortical layer). The LGN cells in the A laminae project mainly to layer 4, and to a lesser extent to layer 6 and the lower part of layer 3 (LeVay and Gilbert, 1976). The C laminae project to layer 1, and along the upper and lower borders of layer 4 (LeVay and Gilbert, 1976). Fibres from the ILN

nuclei terminate in cortical layers 1 and 6 and at the layer 3/4 border (Kaufman and Rosenquist, 1985a,b; Cunningham and LeVay, 1986; Kato, 1990).

1.4 INTERCONNECTIONS AMONG VISUAL CORTICAL AREAS

Knowledge of the multiple representations of the visual areas within the cortex has led to basic questions of how they relate to each other and how exactly they analyse visual information. The turning point was the realisation that clustering of cortical cells into columns is not only important as an organizational model for thalamocortical inputs (see earlier in 1.2.3), but that cortical cells are organized in clusters as they form connections between different areas within each hemisphere (ipsilateral corticocortical connections) and between the two hemispheres (callosal connections). Furthermore, in primates, clustered distributions of corticocortical neurones were correlated with their function. For example, Livingstone and Hubel (1983, 1984) showed that, in the monkey, visual cortical cells clustered in blobs and in the interblobs regions (see 1.2.3) play a role in the analysis of colour and movement.

1.4.1 Intrahemispheric (or ipsilateral corticocortical) and interhemispheric (or callosal) connections in the cat

Symonds and Rosenquist (1984a,b) were two of the first researchers to investigate comprehensively intrahemispheric corticocortical connections in the cat's visual cortex. They made injections of an HRP/radioactively labelled proline mixture into the visual cortex. They found that these connections are organised in a highly specific and a reciprocal manner and that they link retinotopographically corresponding regions. They also found that corticocortical cells originate and terminate in clusters, mainly in superficial layers 2 and 3 and deep layers 5 and 6, thus often outside layer 4 which is the main recipient of geniculocortical axons. Interhemispheric or callosal connections in the cat were found to lie along the area

17/18 border and along the lateral border of area 19, and the two hemispheres exchange information only from the part of the visual field near the vertical meridian (Hubel and Wiesel, 1965; Shatz, 1977; Innocenti, 1980).

1.4.2 Functional significance of corticocortical connections

As mentioned earlier, in primates, the clustered distribution of corticocortical connection was correlated with the functional properties of these cells and it was suggested that each visual area is specialised to play a different role in vision (reviewed by Zeki, 1992). In the cat, the functional significance of the clustered organization of corticocortical cells is harder to clarify, perhaps because the range of possible functional properties is narrowed by the absence of a well-developed colour system in this species. A recent study suggests that, at least in the area 17-18 projection, the corticocortical cells of area 17 lie predominantly in regions that receive inputs from both eyes and have a tendency to avoid zones that are strongly dominated by stimulation through only one eye (Price et al., 1994a). This was confirmed by neuroanatomical studies; for example, injections of retrograde tracers in area 18 (to label corticocortical cells in area 17) were made in animals that had previously received an intraocular injection of the anterograde transneuronal tracer, tritiated proline, to reveal cortical inputs from that eye (Shatz et al., 1977). In addition, it was found that cells recorded within clusters in area 17 have a full range of orientation preferences. Overall, it seems that corticocortical projections are positioned to provide a representative and strongly binocular sample of the activity of area 17 for transfer to area 18 (Price et al., 1994a).

1.5 THE DEVELOPMENT OF THE VISUAL CORTEX IN THE CAT

1.5.1 Neurogenesis and cell migration

The cells of the cerebral cortex develop from precursor cells in the ventricular zone which migrate towards the outer surface of the developing hemispheres (Sauer,

1935; Rakic, 1974). Postmitotic cells migrate in the order in which they are generated (Rakic, 1974) and in close apposition to the radial processes of glial cells (Rakic, 1972, 1978).

The generation and migration of cells in the cat's visual cortex have been studied using radioactively labelled thymidine (Luskin and Shatz, 1985a,b; Shatz and Luskin, 1986). The first cortical cells are born between embryonic day 24 (E24) and E30 and form a transient embryonic zone called the preplate (Luskin and Shatz, 1985b). After arrival of later-born cells, the preplate zone splits into two zones: the marginal zone (later develops into cortical layer 1) and the subplate zone (the transient zone later incorporated into the white matter). It seems that the majority of these early born cells die in the postnatal period (Luskin and Shatz, 1985b). Cells of future layers 2-6 are generated between E31 and E56 (Luskin and Shatz, 1985a). In the newborn kitten, it is possible to identify cortical layers 1, 4, 5 and 6 while the superficial layers 2 and 3 are still immature; cells that form layers 2 and 3 complete their migration about 3 weeks after birth (Shatz and Luskin, 1986).

The functional properties of cortical cells in the kitten were investigated for the first time by Hubel and Wiesel (1963). They found that immature neurones have characteristic receptive field orientations, but the ocular dominance columns are not fully formed until about 6 weeks. Their most important finding was that during early postnatal life the connections of neurones in the visual cortex are susceptible to modification and can be irreversibly affected by inappropriate use.

1.5.2 The development of callosal connections in the cat's visual cortex

One of the most interesting features of the developing cortex which distinguishes it from the adult cortex, is the presence of exuberant and transitory projecting fibres. The first evidence of exuberance among developing cortical axons came from studies of interhemispheric (callosal) connections (Innocenti et al., 1977). In the adult cat, callosal connections link the borders of visual areas 17, 18 and 19

(Hubel and Wiesel, 1965; Shatz, 1977; Innocenti, 1980). In the newborn kitten, neurones project through the callosum from the entire extent of each visual area (Innocenti et al., 1977). During the first postnatal week, the axon terminals penetrate the grey matter of the contralateral hemisphere and form persistent connections only at the areal borders (Innocenti, 1981). Under other regions of the visual cortex, outside the boundaries, callosal axons reach the lowest part of layer 6, but most do not penetrate into the cortex. Experiments with long-lasting retrograde tracers have demonstrated that many initially "inappropriately" positioned callosal neurones survive, and that the postnatal refinement of the callosal pathways is achieved by the elimination of callosal axons (Innocenti, 1981).

1.5.3 The development of ipsilateral corticocortical connections in the cat's visual cortex

The early studies on the development of ipsilateral corticocortical connections showed that there are some striking similarities between the development of callosal and ipsilateral corticocortical connections (Price and Blakemore, 1985a,b; Price and Zumbroich, 1989). Retrograde tract-tracers have been used to demonstrate that the patchy distribution of area 17-18 projecting corticocortical cells emerges from initially continuous distribution of cells in deep and superficial layers in area 17 (Price Blakemore, 1985a,b; Price et al., 1994b). Patchiness in the distribution of these cells appears during the second and third postnatal weeks (Price and Blakemore, 1985a,b; Price and Ferrer, 1993; Price et al., 1994b). The use of long-lasting retrograde tracers has demonstrated that this change is brought about by axonal retraction, although it has been suggested that the loss of deep layer connections is achieved by cell death (Price and Blakemore, 1985b).

Experiments with the anterograde tracer, tritiated proline, have demonstrated that ipsilateral corticocortical axons from area 17 grow in an apparently undirected fashion through the white matter towards the grey/white matter border of the

extrastriate cortical areas (Price and Zumbroich, 1989). The developing axons that end up under appropriate regions penetrate the superficial cortical layers, while those that terminate under topographically incorrect regions are eliminated (Price and Zumbroich, 1989). Corticocortical axons from area 17 enter the adjacent area 18 during the first postnatal week, and a few days later they penetrate more distant area 19. This cortical penetration is patchy from the earliest postnatal age, as is the case for developing callosal connections. It is possible that very similar mechanisms are involved in controlling the development of callosal and corticocortical pathways (Price and Zumbroich, 1989). These authors hypothesised that, at least for the earliest corticocortical axons, interaction around the layer 6/white matter border may be crucial in the elimination of inappropriate connections.

Studies on subcortical and callosal pathways in rats give some evidence that selection of an appropriate target is determined very early in development; for example, when the generation and migration of cells in the ventricular zone is disrupted chemically or by irradiation, cells, although unable to migrate (they stay in ectopic position), still form connections with appropriate targets (Jensen and Killacky, 1984; Yurkewicz et al. 1984). These experiments are supported by other evidence from "nature" in the mutant *reeler* mice. In this mutation, the order of cortical layers is reversed (Caviness and Sidman, 1973). The earlier generated layers are positioned more superficial than those generated later. However, the cortical projections and afferents still follow the same reversed-layer order (Caviness and Yorke, 1976).

1.5.4 The effects of visual deprivation on the development of corticocortical connections in the cat's visual cortex

Many of the features of the organization of the corticocortical and callosal pathways, (topography, tangential and laminar distribution) begin to appear around the time of the birth, before the kitten has had visual experience. However, the subsequent refinement of the more detailed aspects of these pathways, such as the

numbers of projecting neurones or their precise locations within the overall organization, does require a normal visual input (Lund et al., 1978; Innocenti and Frost, 1980; Price and Blakemore, 1985a; Price et al, 1994b). For example, in cats that have been monocularly deprived from birth, the appearance of a patchy distribution of the area 17-18 projections is not prevented (Price et al, 1994b). However, within these patches the density of association cells remains abnormally high throughout development and into adulthood. Similarly as after monocular deprivation, the appearance of patchy distribution of association cells in area 17 is not prevented by binocular deprivation (Price and Blakemore, 1985a). However, measurements of cell density in binocularly deprived kittens at 1 month of age suggest that the number of area 17-18 projection neurons is lower than normal. In general, monocular deprivation can rescue normally transient connections that would otherwise disappear; following binocular deprivation, abnormally large numbers of connections are lost. The mechanisms by which environmental deprivation produces these changes are not understood, and Price et al. (1994b) suggested that the extent to which early projections are lost is related to the amount of neural activity in the cortex; monocular deprivation may raise this level, whereas binocular deprivation may suppress it. When an animal views the visual world, at any one moment only some of the disparity-selective cells in its cortex will be maximally activated, i.e. those where the binocular stimulus falls on points within each receptive field that have the disparity required to produce a maximal response. Any cells stimulated at positions not appropriate to produce a maximal response, presumably the majority of neurones, will be inhibited and respond at a level lower than resulting from monocular activation (Ferster, 1981). Thus the effect of monocular closure will be to remove this inhibition and raise cortical activity levels. Further clarification of these issues is possible from better understanding of the role of neural activity in the development of the area 17-18 connections and this was one of the main aims in my study.

1.6 AIMS OF MY RESEARCH

1.6.1 Chapter 2: The formation of corticocortical connections; the development of topography and laminar distribution

In this chapter, I describe experiments in which my main aim was to use neuroanatomical methods to examine the postnatal development of area 17-18 and 17-19 corticocortical connections in young kittens.

1.6.2 Chapter 3: Effect of neonatal ablation of layer 1 on corticocortical projections from area 17 to areas 18 and 19

I investigated whether cortical layer 1 is involved in the formation of corticocortical connections in the visual cortex of newborn kitten.

1. 6.3 Chapter 4: Ibotenic acid induced lesions of the lateral geniculate nucleus

The main objectives of these experiments were to lesion the LGN in the newborn kitten with an excitotoxic compound, ibotenic acid, make reconstructions of the lesion in each animal at the age of one month and examine the overall changes in the visual cortex.

1. 6.4 Chapter 5: LGN lesion and corticocortical connections

I examined whether the LGN is required for the maturation of corticocortical connections in the visual cortex of young kittens.

1. 6.5 Chapter 6:. Prospects for in vitro studies of corticocortical connections in the cat's visual cortex

My main aim was to see whether slices of the cat's visual cortex can be cultured and whether the presence of other explants would promote cortical growth.

CHAPTER 2

THE FORMATION OF CORTICOCORTICAL CONNECTIONS IN THE VISUAL CORTEX; THE DEVELOPMENT OF TOPOGRAPHY AND LAMINAR DISTRIBUTION

2.1 ABSTRACT

The aim of this study was to investigate the early postnatal development of association projections in the kitten's visual cortex with sensitive fluorescent lipophilic tract-tracers. I injected small quantities of different coloured anterogradely and retrogradely transported tracers side-by-side in area 17 of fixed brains of various ages, from postnatal day 1 to 30. Axons from area 17 had just reached the deep layers of areas 18 and 19 at birth. A few had entered the cortex, mostly at points that corresponded retinotopically with those of the injection sites. These points were linked by distinct axonal tracts from postnatal day 1 on, suggesting that many corticocortical axons had grown in an ordered fashion between specific points in striate and extrastriate cortex. Over the next few weeks, corticocortical fibres became increasingly densely distributed in layers 5 and 6 of extrastriate areas. At the same time, more and more axons penetrated the superficial layers, but their tangential distribution was narrower than that of the projections to the deep layers. Confirmation of this more restricted penetration of upper layers came from in vivo injections of retrogradely transported tracers: injections restricted to the superficial layers of area 18 labelled a relatively narrower region of area 17 than injections in deeper layers. At all ages, injections of lipophilic tracers in area 17 of fixed brains retrogradely labelled cells in areas 18 and 19; from birth on, most of these cells were in the same regions where axons from area 17 were penetrating the cortex, indicating reciprocal topography. Previous studies of the development of corticocortical connections in the visual cortex of the cat had stressed the importance of axonal elimination and cell death in the sculpting of the adult organization from initially exuberant population of projections. Present study revealed considerable topography among visual corticocortical projections as they developed. Although there was a degree of postnatal refinement, the initial growth of association axons was more highly directed than had been described in previous reports. New findings are compatible with the hypothesis (suggested by previous in vitro experiments) that both

the chemotropic attraction of at least a large proportion of corticocortical axons to specific target areas and the later loss or remodelling of exuberant projections are important mechanisms in the development of these pathways.

2.2 INTRODUCTION

Area 17 (striate cortex) is retinotopically and reciprocally connected with other visual areas (Symonds and Rosenquist, 1984a; Rosenquist, 1985). In the adult cat, the corticocortical projections from visual area 17 originate from pyramidal cells that are clustered in layers 2, 3 and upper 4 and, in lower numbers, in layers 5 and 6 (Gilbert and Kelly, 1975; Bullier et al., 1984; Symonds and Rosenquist, 1984b; Ferrer et al., 1988, 1992; Price and Ferrer, 1993; Price et al., 1994a). In areas 18 and 19, they terminate most densely in patches in layers 2 and 3, with fewer projections to layers 5 and 6, while in posteromedial lateral suprasylvian cortex (PMLS) they terminate mainly in layer 4 (Price and Zumbroich, 1989; Sherk, 1986). The reciprocal projections from area 18 originate mostly in layers 2 and 3, although a few originate in layers 4, 5 and 6 (Bullier et al., 1984; Symonds and Rosenquist, 1984b; Henry et al., 1991). Projections from area 19 originate equally in superficial and deep layers and those from PMLS come mainly from deep layers (Bullier et al., 1984; Symonds and Rosenquist, 1984b). Fibres from area 18 terminate in all layers of area 17 except lower layer 4 and those from area 19 terminate mainly in layers 5 and 6 (Henry et al., 1991). In the adult cat, regions of striate and extrastriate cortex that are interconnected represent regions of visual space that overlap (i.e. retinotopically corresponding points in the cortex are linked; Ferrer et al., 1988); although this general principal is well-established, the exact extent of overlap between the representations remains controversial (Price et al., 1994a; Salin et al., 1989).

Over the last few years, the postnatal development of ipsilateral corticocortical projections from area 17 has been studied using either anterogradely or retrogradely transported tracers in vivo. These studies showed that the clustered distributions of

association neurones in the adult cat emerge during the second and third postnatal weeks from initially continuous, dense distributions of cells in both the superficial and the deep cortical layers (Price and Blakemore, 1985a; Price and Ferrer, 1993; Price et al., 1994b). Experiments with long-lasting tracers suggested that the patchiness in the superficial layers is achieved by the selective elimination of inappropriate axons, without cell death, whereas the decrease in the density of cells in the deep layers is more likely caused by cell death (Price and Blakemore, 1985b). However, it is less clear how the beautifully precise retinotopic organisation of these connections becomes established. Studies using the anterogradely transported tracer, tritiated proline, suggested that in newborn kittens, association cells in area 17 send projections in only the general direction of the other ipsilateral visual areas and that those fibres that chance to arrive below retinotopically appropriate regions penetrate the cortex and grow towards their target neurones in superficial layers (Price and Zumbroich, 1989); a similar hypothesis had previously been proposed for the developing callosal connections (Innocenti and Clarke, 1984). Thus, it was suggested that corticocortical development involves an early period of *non-directed* growth, followed by a period of selective ingrowth at *specific zones* in the cortex. In this study, one of the aims was to re-examine the development of topography in the corticocortical pathways from area 17.

I examined the organization of the projections from area 17 in kittens of various ages by *anterograde* labelling with the fluorescent lipophilic tracers 4-(4-dihexadecylaminostyryl)-N-methylpyridinum iodide (DiA) and 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). Both dyes proved more sensitive than the previously used anterograde tracer, tritiated proline (Price and Zumbroich, 1989), and allowed me to follow and examine the morphology of labelled axons more readily. Because the two dyes have different colours, and can be differentiated, I could place them side-by-side in area 17 and examine more precisely the topography of fibres terminating in other areas. Another advantage with DiA and

DiI is that they are used in fixed brains and so injections could be placed with much more accuracy, transport was not affected by in vivo factors that may differ at different ages, and brains were examined at a *specific* age rather than after the prolonged survival periods needed for transport of tracers in vivo (these can complicate the interpretation of results in developing brains). The new tracers also *retrogradely* label cell bodies, which allowed me to examine the relationship between fibres that project from area 17 to other areas and the reciprocal connections. During this study, I carried out a few experiments with exclusively retrogradely transported tracers injected in vivo, to support some of my findings with DiI and DiA.

2.3 MATERIALS AND METHODS

2.3.1 Animals

All kittens were from an isolated laboratory colony. DiI and DiA were applied to 23 fixed hemispheres from 16 normal kittens aged postnatal day 1 (P1) (6 hemispheres), P6 (1 hemisphere), P9 (1 hemisphere), P10 (2 hemispheres), P16 (3 hemispheres), P28 (5 hemispheres), P29 (2 hemispheres) and P30 (3 hemispheres). In one of the hemispheres, from a kitten aged P29, I applied only a DiI crystal. Successful in vivo injections of DiI and diamidino yellow (DY) were carried out on 3 kittens aged P1, P3 and P4 (2 injections, one in each hemisphere).

2.3.2 Application of DiA and DiI in fixed brains

Kittens were given a lethal overdose of sodium pentobarbitone (20 mg, i.p.) and, immediately respiration had ceased, they were perfused transcardially with isotonic saline followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer at pH 7.4. After the perfusion, the brain was removed from the skull and the hemispheres were separated and stored in 4% paraformaldehyde in the same buffer. Small crystals, one of DiA (Molecular Probes, D-3883) and the other of DiI (Molecular Probes, D-282), were placed side-by-side in the mediolateral plane in

area 17 in each hemisphere. These pairs of injections were made on the medial side of the brain in the rostral or caudal part of area 17. According to Anker and Cragg (1974), Henderson (1982), Price (1985), Price and Blakemore (1985a, b), Blakemore and Price (1987), areas 17-19 occupy the same relative positions in kittens as in adult cats, and I positioned the injections in area 17 around the splenial sulcus, which was readily seen. Caudal injections were near anteroposterior level zero (AP0), the coronal plane through the external auditory meatuses, where area 17 represents the region of visual field from zero to five degrees above the horizontal meridian (Tusa et al., 1978; Rosenquist, 1985). Rostral injections were made 3 to 5 mm further rostral, where area 17 represents the region of visual field five to ten degrees below the horizontal meridian (Tusa et al., 1978; Rosenquist, 1985).

To inject tracer, single crystals of DiI or DiA (300-500 μm in diameter) were gently pushed under the surface of the brain with a glass micropipette (tip diameter 150 μm). Distances were marked on the micropipette so that I could control the depth to which crystals were pushed; I aimed to centre them at depths equal to approximately half the thickness of the cortex (i.e. from about 0.5 mm in the youngest brains to about 0.8 mm in the 28 and 30-day-old brains). Injected brains were stored at room temperature for up to 16 months to allow the dyes to diffuse.

2.3.3 Surgical procedures and application of DY and DiI in vivo

In each of three kittens, area 18 in one hemisphere was injected with DY, and in one of these animals (aged P4) area 18 in the other hemisphere was injected with DiI (DY and DiI tracers have indistinguishable uptake properties in kittens in vivo, see Price and Ferrer, 1993). Tracers were administrated as crystals, rather than being dissolved, since I found that this produced more localised injection sites and reduced diffusion. Anaesthesia was induced with ketamine hydrochloride (20 mg kg^{-1}, i.m.) and maintained with an intravenous infusion of alphaxalone-alphadolone (Saffan, Glaxo), diluted with isotonic saline to 15% of its original concentration for

newborn kittens and by 50% for older kittens. The rate of infusion was approximately 0.003 ml min^{-1}, adjusted as necessary to maintain full surgical anaesthesia throughout. The ventilation rate and rectal temperature were continuously monitored and body temperature was maintained at 37-38°C with a thermal blanket. The anaesthetized animal was placed in a stereotaxic frame and a small craniotomy was made over the lateral gyrus near to AP0, about 10 mm posterior to bregma and 2 mm lateral to the midline, over area 18. After incising the dura, small crystals (about 300 μm in diameter) of DY or DiI were inserted at different depths with a micropipette (tip diameter 150 μm). After injection, the wound in the scalp was sutured and kittens were allowed to recover and returned to their mothers. After survival for 3 days, kittens were anaesthetized with a lethal overdose of sodium pentobarbitone (20 mg, i.p.) and immediately perfused transcardially, as already described. I was very confident that 3 days survival time was adequate to allow labelling of all the area 17-to-18 projections in these young kittens, since other experiments revealed that labelling of these immature pathways occurs after as little as 1 day and survival times of longer than 3 days do not alter the patterns of labelling.

2.3.4 Histological preparation

Fixed brains were blocked and placed in a solution of 20% sucrose in phosphate buffer to equilibrate for 1-2 days. Some brains were snap-frozen with dry ice and cut coronally at a thickness of 50 μm. This method was used for brains injected with DY and DiI in vivo, and also proved highly effective in the young brains that had been fixed and then injected with DiA and DiI. Fixed material from older kittens, that had been injected with DiA and DiI, was better cut on a vibroslice at a thickness of 200 μm. Sections were mounted on gelatine coated slides. A 1-in-3 series of sections labelled with DiA and DiI (postmortem) was immediately coverslipped with Aqua-poly-mount (Polysciences, Inc.). A 1-in-3 series of sections

labelled with DY or DiI in vivo was air dried and coverslipped with p-xylene-bis-pyridinum bromide. For both experiments, a second 1-in-3 series was examined without coverslipping and later counterstained with cresyl fast violet. For brains injected with DY and DiI in vivo, a third series (1-in-9) was reacted histochemically to reveal cytochrome oxidase activity. Both cresyl fast violet and cytochrome oxidase staining enabled me to examine cortical layers and to locate the boundaries between visual areas at all ages (Garey, 1971; Anker and Cragg, 1974; Price, 1985; Price and Zumbroich, 1989). In the in vivo experiments, where DY or DiI had been injected into area 18, I took 50 μm sections (1-in-3 series) through the ipsilateral lateral geniculate nucleus (LGN). Examination of the pattern of labelling in the LGN provided additional information on the location of injection sites (Price and Blakemore, 1985, a, b).

2.3.5 Analysis

Sections were examined with a Leica fluorescence microscope equipped with four filter blocks D, I2/3, L3 and N2, or with a Zeiss confocal microscope. DiI was viewed with filter N2 (530-560 nm excitation), DiA with filters I2/3 (450-490 nm, long-pass suppression filter at 515 nm) and L3 (450-490 nm, band pass filter 525/20 nm) and DY with filter D (355-425 nm). Sections of interest were drawn with a camera lucida, using a 10x objective: the positions of injection sites, labelled fibres and cells were marked. Sections were later counterstained and the cortical laminae indicated on the drawings.

After injections of DY or DiI in area 18, I estimated both the degree of convergence from area 17 to 18 (in the rostrocaudal plane) and the density of labelled cells in area 17. The convergence was calculated using the method described in Ferrer et al. (1988 and 1992) and Price et al. (1994a, b). This involved subtracting the rostrocaudal diameter of the dense core of the injection site from the rostrocaudal extent of the region of labelled cells in area 17. If viewed from the surface of the

brain, the labelled regions in area 17 would have been roughly oval, and their full rostrocaudal diameters, including even those peripheral regions where the density of label was low, were used for calculations of convergence. Such a simplified calculation was possible because the retinotopic representation of the visual field in both areas is similar in the rostrocaudal direction, where it represents the upper and lower 50 degrees of elevation in the visual field (Tusa et al., 1978, 1979). As was done by Price et al. (1994b), I obtained a profile of the density of labelled cells in a rostrocaudal strip along area 17 by measuring this density in 0.7 mm^2 areas from the superficial layers in a series of 1-in-3 sections. These areas were in the mediolateral centre of the labelled zone (this region was near the suprasplenial sulcus in all animals). I used the sulcus as a landmark and, in each section, photographed this region on Ilford black and white film (ASA 400) with a 10x objective. Later, labelled cells in layers 2/3 and upper 4 in area 17 were easily counted from the photographs.

2.4 *RESULTS*

2.4.1 Evaluation of injection sites

Injections in area 17 in fixed brain

Injection sites of DiA and DiI contained a dense central zone surrounded by a narrow halo of labelled fibres; examples are shown in Figs. 1 and 3. The central zone contained the crystal. Its position was checked in Nissl-stained sections, in which layers 1, 5 and 6 were easily recognised even in newborn animals (Shatz and Luskin, 1986; Price and Zumbroich, 1989). At birth, cells of presumptive layers 2 and 3 are still migrating and only a small fraction are present within the narrow zone of compact cells below layer 1; it is only around the third postnatal week that these cells achieve their final position and the compact zone disappears (Shatz and Luskin, 1986). Interestingly, the laminar structure of the newborn visual cortex, in particular layer 4, is more readily seen in more lateral areas (Fig. 2), presumably because of the

lateromedial gradient in development of the cortical plate. In older kittens, from P6, layer 4 was also more distinct in area 17. Normally crystals were centred in layer 5 or above and the dense core of the injection sites covered all cortical layers in area 17 without involving the white matter. In two newborn and two 28-day-old kittens, crystals reached layer 6 and the injection sites spread slightly in the subplate/white matter, but labelling in extrastriate areas was similar to that in the other animals of the same age where injections were restricted to the cortex. It is important to appreciate that DiI and DiA are so intensely fluorescent that the injection sites sometimes appear more extensive than they really were in photographs taken with long exposures or, in the case of confocal microscopy, with the instrument's sensitivity high; such photographs were used to reveal the full extent of the less intense, nearby corticocortical labelling (an example of artefactual enlargement of the injection sites due to this saturation is seen in Fig. 4). The distance of each injection site from the area 17/18 border was estimated. The distances between DiI and DiA crystals did not exceed 0.5 mm. In both mediolateral and rostrocaudal planes, injection sites measured between 1-1.8 mm in diameter in younger animals and was slightly wider in the 28-day-old kittens.

Injections in area 18 in vivo

Injection sites of DY and DiI in area 18 in vivo again contained the crystal surrounded by a narrow zone of labelled cells; examples are seen in Fig. 8. The uptake of tracers by cells in area 17 and the LGN was similar to that seen when they were dissolved and pressure injected (Price and Blakemore, 1985b, Ferrer et al., 1988 and 1992; Price and Ferrer, 1993). One injection site was restricted to the superficial cortical layers in area 18. It comprised a crystal in layer 1 and the upper part of the cortical plate (CP) surrounded by a narrow zone of densely labelled cells. A second injection involved the CP above layer 5 (Fig. 8a) and a third and fourth

involved layers 5 and 6 (Fig. 8c). The widths of the injection sites were 1.15 to 1.35 mm in the rostrocaudal plane and 0.5-1.0 mm in the mediolateral plane.

Areas 17 and 18 were distinguished by examining layer 5, which is about 25% thicker in area 18 than in area 17 (Garey, 1971; Price, 1985; Price and Zumbroich, 1989). Areas 18 and 19 were distinguished by examining layer 6, which is thicker in area 19 than in area 18 (Garey, 1971). Cortical laminae and boundaries between visual areas were also distinguished in cytochrome oxidase-stained sections (Price, 1985). To confirm that injection sites in area 18 (i.e. those made in vivo) did not involved areal borders, I examined LGN labelling; as shown in Fig. 8b, labelling was confined to its central region without involving medial or lateral borders (Sanderson, 1971; Price and Blakemore, 1985 a, b; Geiseret, 1980).

2.4.2 Labelling with DiI and DiA in fixed brains

Although the wavelengths at which DiI and DiA emit their maximum intensity of light differ, these compounds emit appreciable intensities over broad, overlapping ranges. Furthermore, the styryl dye DiA is solvatochromatic and its emission spectrum shifts when it binds membrane, by an amount that depends on conditions such as the age of the tissue (Molecular Probes, Inc., personal communication). Filter N2 (the "rhodamine" filter) reveals DiI's fluorescence much more intensely and in greater detail than DiA's. Filter I2/3 (the "fluorescein" filter) reveals DiA's fluorescence much more strongly than DiI's. I found that high concentrations of DiA are faintly visible with filter N2 and high concentrations of DiI are visible with the I2/3 filter, although with this filter they appeared orange-yellow rather than the green-yellow colour of DiA. When I inspected two areas with equal concentrations of DiI and DiA with filter L3, only the DiA was visible, although the overall intensity of fluorescence was lower with this filter and photography was correspondingly harder. Thus, filter L3 was useful for resolving any ambiguities that arose through the use of filter I2/3.

These principles are illustrated in Fig. 3. Figure 3 d and e show high magnification photomicrographs of two retrogradely labelled cells and their processes, one of which contained DiI (that on the right), the other of which contained DiA (on the left). Fig. 3d was taken through the I2/3 filter, and Fig 3e was of the same field of view through the N2 filter. With filter L3, the cell on the left was seen (although it was less intense) and that on the right was not visible, i.e. it contained no detectable DiA (not shown). The cell on the left was barely visible with filter N2 (Fig. 3e); I was unable to rule out the possibility that this cell was double-labelled with a small amount of DiI. Overall, the use of DiI and DiA proved very satisfactory for the purposes of this study, as it was relatively easy to decide whether cells and their processes were labelled either *mainly* with DiI or *mainly* with DiA. Furthermore, it was possible to identify cells that were clearly double labelled with roughly equal amounts of DiI and DiA; it should be noted that, had I been primarily interested in a study of the incidence of double-labelling, this technique would not have been as sensitive as other previously described methods (e.g. Price and Ferrer, 1993) for detecting double-labelled cells that contained only very small quantities of one of the two dyes.

Efferent projections from area 17

Fig. 1 (a and b) illustrates the results of injections into area 17 in 1-day-old kittens; these photomicrographs are of the DiI labelling (N2 filter). The strongest labelling is of the pathways that connect the cortex to its contralateral counterpart via the callosum and to subcortical structures. These projections are seen crossing the lower edge of Fig. 1b. Labelled fibres project laterally to areas 18 and 19 from P1 on, although at P1 they are still very sparse, comprising relatively few thin and weakly-stained axons. The early area 17-to-18 corticocortical connections can be seen in Fig. 1a (between the double arrowheads), and although they are faintly labelled, it is clear that some of them have already penetrated the deep layers of area 18. They enter area 18 at a point that is retinotopically related to the position of the

injection site in area 17; that is, if the injection is nearer to the area 17/18 border, the point of penetration of area 18 is correspondingly nearer to this border, and vice-versa. Most interesting is the observation that the injection sites in area 17 and the regions of innervation in area 18 are linked by fibre-tracts (Fig. 1a), rather than there being a general, diffuse labelling of the white matter beneath area 18. Connections from area 17 to area 19 are present but less well developed at P1, and very few are penetrating area 19. These projections are passing laterally and rostrally from the injection sites through the white matter towards area 19, and are revealed in Fig. 1b: again, the labelling, although clearly present, is very sparse. The first reason to think that the developing area 17-to-19 projections are organized in a retinotopic fashion comes from my observation that most run in a laterorostral direction. This is the appropriate direction, since the retinotopic map in the part of area 19 studied here is staggered relatively rostrally to that in area 17 (Tusa et al., 1979). The second indication comes from a count of the numbers of labelled area 17-to-19 fibres in the white matter, as shown in Fig. 2. To generate these data, I count at high magnification the numbers of labelled fibres crossing lines drawn obliquely through the white matter, in the orientation indicated by the two arrowheads in Fig. 1b, in a series of progressively more rostral sections. These counts are superimposed on a photomicrograph of one of the sections that has been Nissl-stained (in Fig. 2, the data plotted on the line indicated by the arrowheads are derived from Fig. 1b). The results indicate that the fibres are not uniformly distributed through the white matter, but are clustered into tracts directed at area 19 and more lateral cortex (probably area 21a or the lateral suprasylvian area).

It is quite clear that, from P1 on, the connections linking area 17 with contralateral cortical areas and subcortical structures are highly ordered as they traverse the white matter. Figure 3 a and b shows, with the I2/3 and N2 filters, an example of two injection sites, one of DiA (positioned medially in area 17, Fig. 3a)

Fi*gure 1.* Fluorescence photomicrographs of coronal sections through visual areas 17, 18 and 19 in a 1-day-old kitten; all are taken with the N2 (530-560 nm) filter, which detects preferentially DiI. (*a*) A section 1 mm caudal to the cores of the DiI and DiA injections in area 17. (*b*) A section 1 mm rostral to the injections of DiI and DiA. In *a* and *b*, the areal borders are indicated by *small arrows*; cortical laminae are marked by *broken lines* in area 17 and by *short lines* in areas 18 and 19; since layers 2 and 3 are still forming at birth and can not be distinguished from layer 4, the cortex between the upper border of layer 5 and the lower edge of layer 1 is referred to as the cortical plate (CP) (Shatz and Luskin, 1986); cells in the upper portion of the subplate (SP_U) can be distinguished from the overlying cells in layer 6 as they are more loosely packed (Luskin and Shatz, 1985). The majority of the fibres labelled from the injections project contralaterally or subcortically (e.g. the labelled fibres crossing the lower edge of the photomicrograph in *b*). Nevertheless, ipsilateral corticocortical projections are seen. In *a*, *double arrowheads* indicate the edges of a faint but clearly present tract of fine, lightly labelled fibres projecting from area 17 to area 18. Such fibres are also seen growing towards area 19 in b; two arrowheads indicate the position of a line drawn obliquely through the white matter along which the numbers of labelled fibres crossing this line are counted to generate data shown in Fig. 2. In area 18, the majority of the labelled cells are distributed in continuous bands from the upper border of layer 5 through the cortical plate (an example of one such cell is shown at higher magnification in *c*), although smaller numbers are present in deep layers. In area 19, labelled cells are only in layers 5 and 6 (e.g. to the left of the *asterisk* in *b* or, at higher magnification, in *d*). All photomicrographs show labelling after a diffusion time of 6 months. Sections in *a* and *b* are at the same magnification (scale bar, 1 mm), as those in *c* and *d* (scale bar, 0.2 mm).

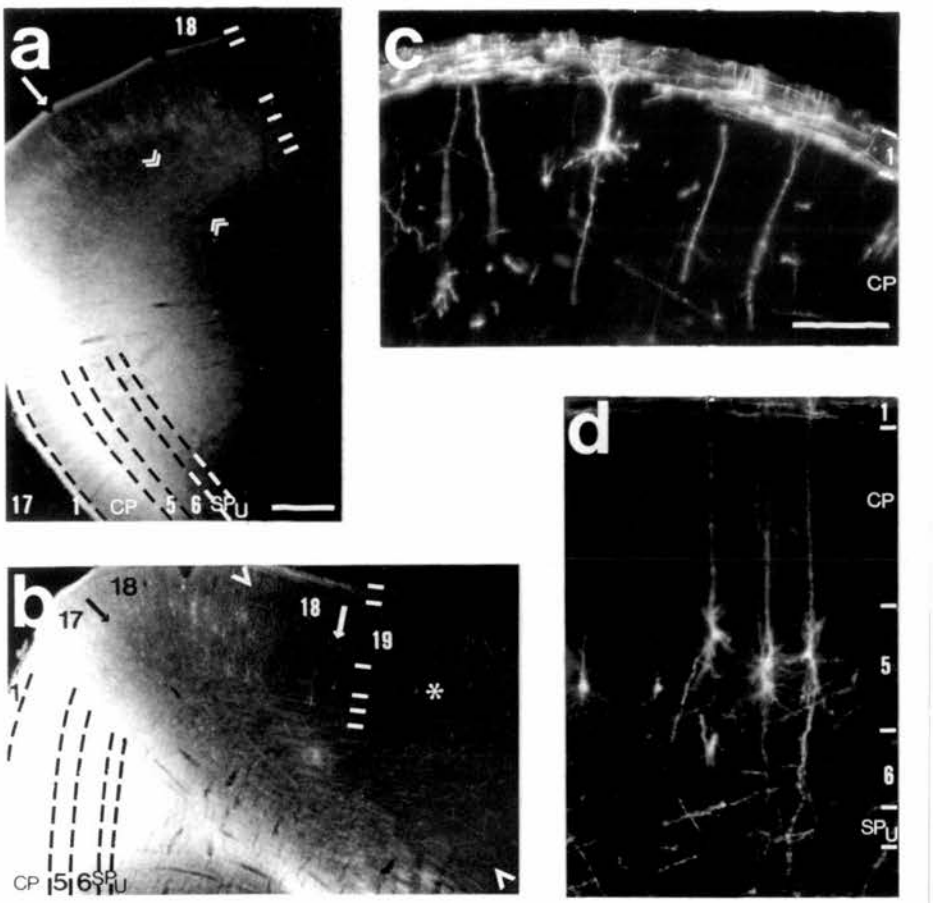
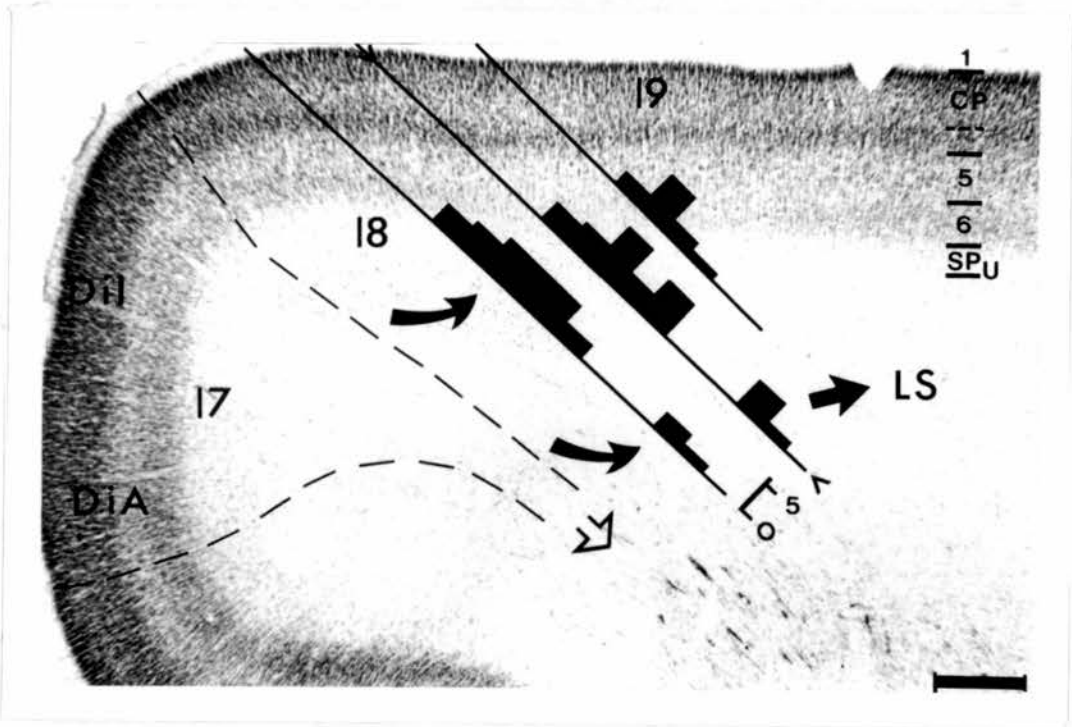

a
18
17
1
CP
5
6
SP
U
b
18
17
18
19
*
1
CP
5
6
SP
U
c
1
CP
d
1
CP
5
6
SP
U

Figure 2. Bright-field photomicrograph of a cresyl violet-stained coronal section through areas 17, 18 and 19 from a region just caudal to that shown in Fig. 1*b*. The positions of the injection sites in area 17 are outlined by a *broken line*, and the *open arrow* indicates the direction of the corticofugal fibres. The numbers of DiI labelled fibres crossing oblique lines drawn through the white matter are counted (in 0.2 mm bins) and are shown as histograms superimposed on the photomicrograph. Counts are shown along three different parallel lines (each drawn in a slightly different coronal plane, covering 0.5 mm rostrocaudally). The line that corresponds to the example indicated in Fig. 1*b* is marked with *arrowheads*. *Arrows* indicate the directions of two bundles of labelled fibres; some project to area 19 while others travel further laterally, presumably to the lateral suprasylvian (LS) area. Scale bar, 0.5 mm.


19
18
17
DiI
DiA
LS
1
CP
5
6
SPU

the other of DiI (more lateral in area 17, Fig. 3b) in a newborn kitten. Two intensely labelled bundles of fibres are seen in the white matter immediately beneath the injection sites. These bundles are very tight and well segregated at this level; the different elements within them (i.e. projections to the callosum and between cortex and subcortical nuclei) separate out further along their lengths (similar to the picture seen in P6 kittens, see Fig. 4a). These results show that, from birth on, topography is present in these projections as they traverse the white matter below area 17.

Corticocortical connections are much stronger in kittens that are just a few days older. As shown in Fig. 4 (a and d), cortical labelling of both areas 18 and 19 is increased in kittens aged 6 days or more compared to that in P1 kittens, although area 19 is still less intensely labelled than area 18. In Fig. 4 a and d, DiI labelled fibres are seen entering areas 18 and 19 from the region of an injection site in area 17. Most of these projections are clearly retinotopically organized. For example, the DiI injection site in Fig. 4a is closer to the area 17/18 border than to the medial border of area 17, and the bulk of the anterograde label in area 18 (that penetrating relatively superficial cortex) is correspondingly closer to the area 17/18 border than to the area 18/19 border (in agreement with the known mirror-reversal of the representation of visual space at the area 17/18 border, Tusa et al., 1979). This retinotopography of the majority of fibres is preserved to a very fine degree at this early age, as shown in Fig. 3c: this confocal photomicrograph demonstrates that most fibres penetrating area 18 from two adjacent points in area 17 (one injected with DiI, the other more lateral point with DiA) inervate adjacent points in area 18 in mirror-reversed order (i.e. the DiI is more lateral in area 18). Note that the fibres tracts linking the DiI injection site and innervation zones in extrastriate cortex are not seen along their full lengths in Fig. 4a since, as discussed above, the label near to the injection site is saturating the confocal microscope, whose sensitivity is set purposely high so as to enhance the visibility of the corticocortical connections.

Figure 3. Fluorescence photomicrographs of coronal sections of the visual cortex in (*a,b*) a 1-day-old kitten, (*c*) a 6-day-old kitten, (*d,e*) a 29-day-old kitten and (*f,g*) a 28-day-old kitten. (*a*) Section through the centres of the injections of DiA (on the *left*) and DiI (on the *right*) in area 17, viewed with the I2/3 ("fluorescein" filter). (*b*) The same section as in a viewed with the N2 ("rhodamine" filter). Note the fibre pathways below the injection sites, connecting these regions with the contralateral cortex and subcortical structures. In *a* and *b*, the grey/white matter (WM) border is marked by *broken lines*. (*c*) Section viewed with the confocal microscope, first through a fluorescein and then a rhodamine filter; the two images are then superimposed by the computer to generate the image shown here. In this case, the DiA was placed lateral to the DiI in area 17, and the DiA labelling of area 18 is correspondingly medial (on the *left*, in yellow) while the DiI labelling is lateral (on the *right*, in orange). (*d*) Two retrogradely labelled cells in layer 3 of area 17, viewed through a fluorescein filter: the cell on the *left* is retrogradely labelled with DiA from tracer placed in area 17; the cell on the *right* is labelled with DiI from a more lateral injection in the same area. (*e*) The same cells as in d are viewed through a rhodamine filter. With this filter, the cell labelled with DiA (on the *left*) is seen only faintly; the DiI labelled cell (on the *right*) is now visible in much greater detail than in d. (*f*) Labelling in area 18 viewed through a fluorescein filter; the DiI crystal was placed lateral to the DiA crystal in area 17. The majority of the DiA labelled cells (bright dots in the diffuse fibre background) are correspondingly lateral in area 18 (intensely labelled cells mostly on the right), while the DiI labelled cells are more medial (these cells are seen in g, and their positions are marked with *open circles*, mainly on the left here). (*g*) The same section as in *f*, viewed through a rhodamine filter, where the majority of the DiI labelled cells are on the left. In *c*, *f* and *g*, cortical laminae are indicated (CP, cortical plate). Scale bar in *a* and *b*, 1 mm; scale bars in *c*, *d* and *e*, 0.1 mm; scale bar in *f* and *g*, 0.2 mm

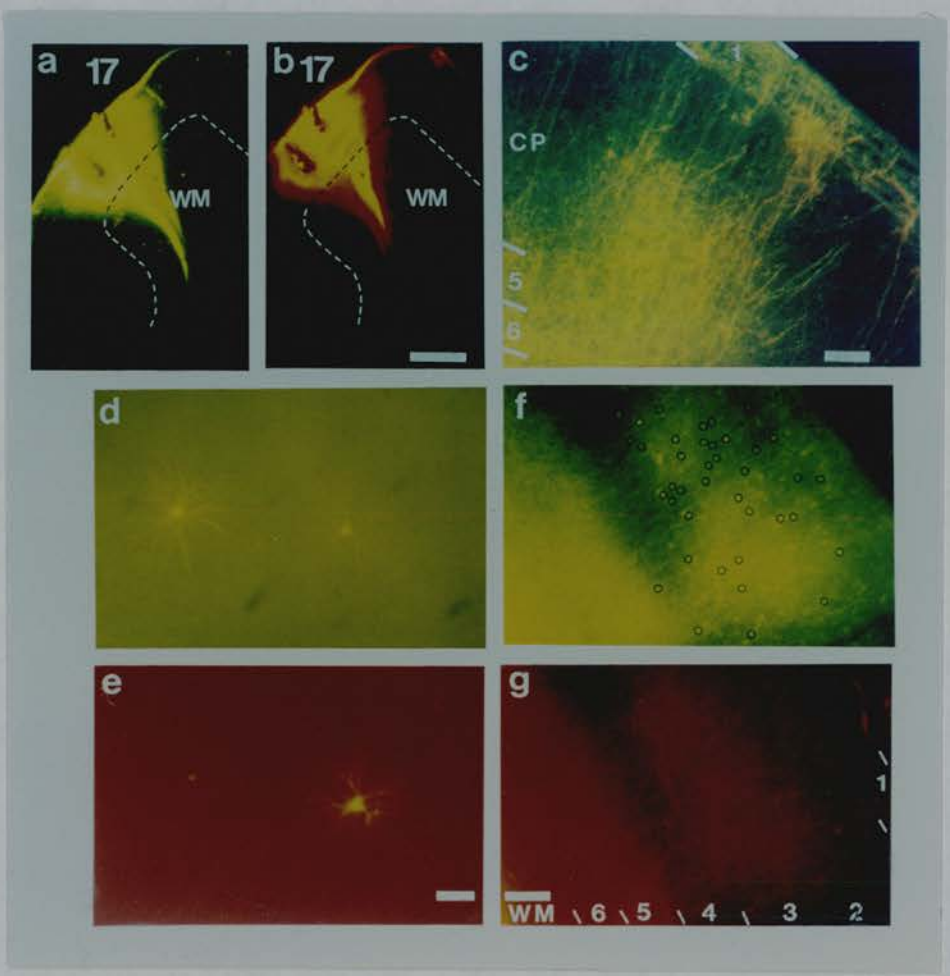
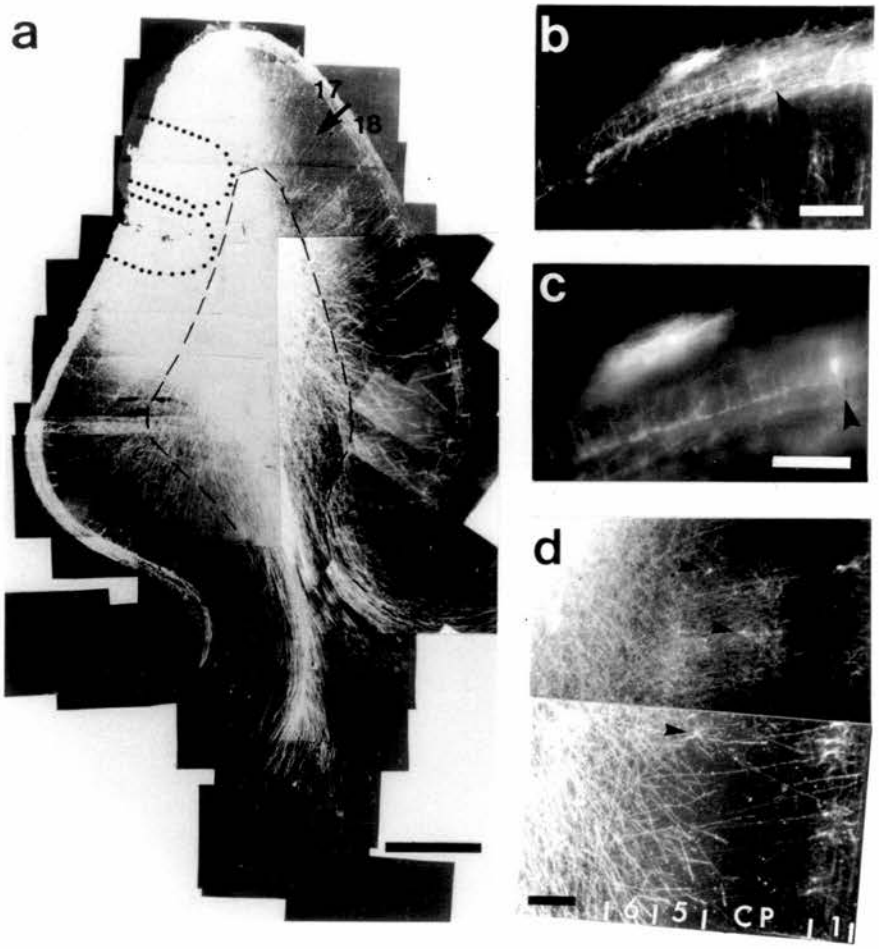

a
17
WM
b
17
WM
c
CP
1
5
6
d
f
e
g
1
WM
6
5
4
3
2

Figure 4. Fluorescence photomicrographs of coronal sections of the visual cortex in (*a, d*) a 6-day-old kitten and (*b, c*) a 1-day-old kitten, all viewed through a rhodamine filter. (*a*) A montage of fluorescence photomicrographs taken with the confocal microscope. The grey/white matter border is marked by a *broken line* and areal borders are indicated. The positions of the injection sites in area 17 are outlined by *dotted lines*; note that the sensitivity of the confocal microscope is high, to allow optimal detection of the relatively weaker label of the cortical connections, and so the DiI near to the injection site is saturating the detector and making the injection sites appear larger than they really are. Small regions of dense ingrowth are seen in both areas 18 and 19, although in area 19 these fibres appear less mature. Scale bar, 1 mm. (*b,c*) Labelling in layer 1 in area 17. Note the Cajal-Retzius cell on the right, which is shown at higher magnification in *c* (*arrows*). The bright staining above the cortical surface is artefactual (small fragments of the crystal of DiI are occasionally generated during sectioning, and they sometimes adhere at ectopic sites). Scale bar in *b*, 0.2 mm; scale bar in *c*, 0.1 mm. (*d*) Labelling in area 18 at higher magnification. Cortical laminae are indicated (CP, cortical plate). All photomicrographs show labelling after a diffusion time of 6 months. Scale bar, 0.2 mm.


a
17
18
b
c
d
CP

At P6, the density of labelled corticocortical afferents is still greatest in the deep layers. In those regions where the bulk of the area 17-to-18 and the area 17-to-19 projections are entering the cortex, some fibres are now penetrating the superficial layers, particularly in area 18 (the innervation of area 19 lags behind that of area 18). These fibres appear to enter the cortex in patches; this can be seen in Fig. 4d, where the fibres innervating the developing superficial layers (the cortical plate) of area 18 have a tendency to be clustered in two groups (seen in the upper half of the photomicrograph). Outside these regions where the *majority* of the corticocortical fibres are penetrating extrastriate cortex, smaller numbers of axons penetrate only deep layers (5 and 6). These axons run with scattered orientations and cross each other, as shown in the lower half of Fig. 4d. Fibres from the injection sites also penetrate the deep layers of area 17 itself (examples are seen in the medial aspect of area 17 in Fig. 4a). Furthermore, horizontal fibres labelled from the injections in area 17 run for considerable distances through layer 1, and extend to just beyond the medial border of area 17 and to area 19 laterally. These layer 1 fibres are also seen in neonatal kittens (e.g. Fig. 1c). The longest projections are confined to the lower third of the lamina (Fig. 4a).

Between P6 and P16, the corticocortical efferents from area 17, as well as the intrinsic connections within area 17 itself, continue to mature rapidly, with an increasing proportion projecting to superficial layers. The patchiness in the distributions of innervating axons, that is apparent at P6, becomes much more obvious during this time. By P16, the distributions of terminals of these corticocortical efferents are essentially similar to those seen at P30 (the oldest kittens studied here), indicating that, at the morphological level analysed in this study, their development slows beyond P16. Figures 5-7 serve to illustrate the main features of the projections seen between P16 and P30. I found that when the carbocyanine tracers are allowed to diffuse for only relatively short times (less than about 90 days), the cell bodies and the dendritic processes of reciprocal corticocortical connections are retrogradely labelled

Figure 5. Montages of fluorescence photomicrographs of coronal sections through visual area 18 after injection of DiI in area 17 in (*a*) a 28-day-old kitten and (*b*) a 29-day-old kitten. (*a*) Labelling of mainly fibres after a diffusion time of 3.5 months; (*b*) labelling of cell bodies after a diffusion time of 24 months. Scale bar, 1 mm

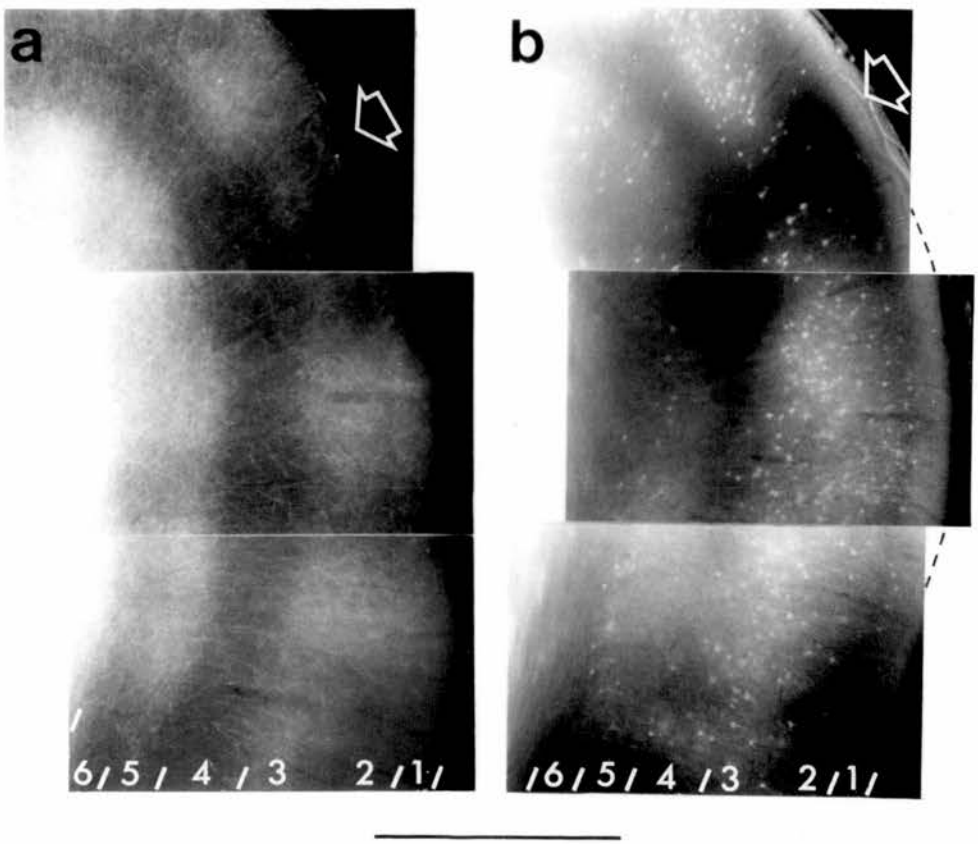

a
b
6 5 4 3 2 1
6 5 4 3 2 1

Figure 6. Fluorescence photomicrographs of a coronal section through visual area 17 in a 30-day-old kitten after injection of DiI and DiA in this area. Patches in the same region of area 17 are viewed through (*a*) a rhodamine filter and (*b*) a fluorescein filter; in b, the positions of the patches in a are outlined by *broken lines*. Cortical laminae are indicated. Scale bar, 1 mm

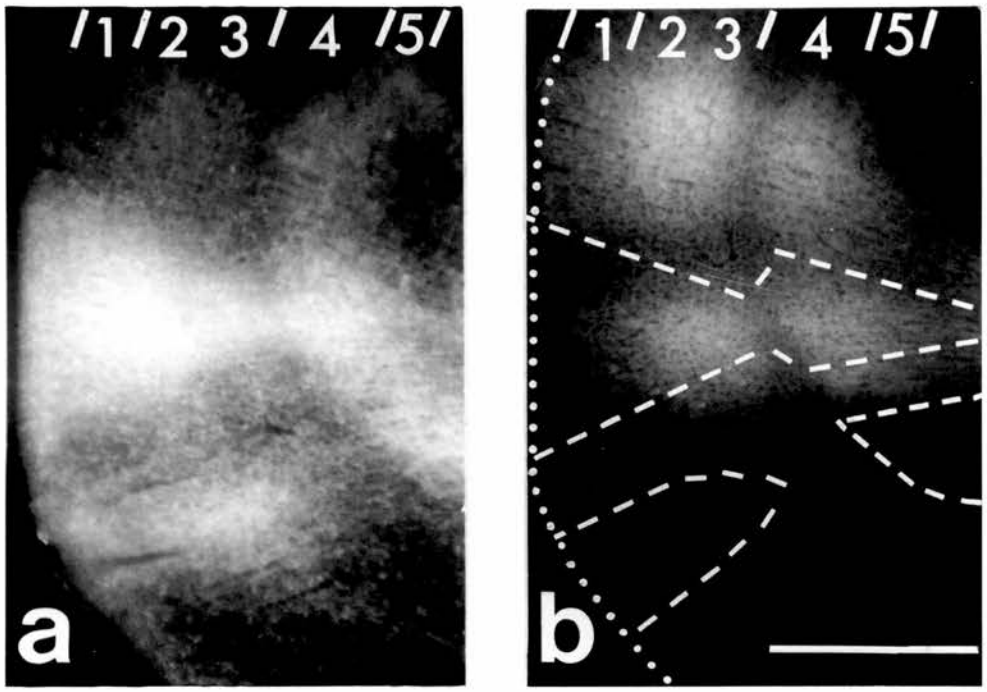
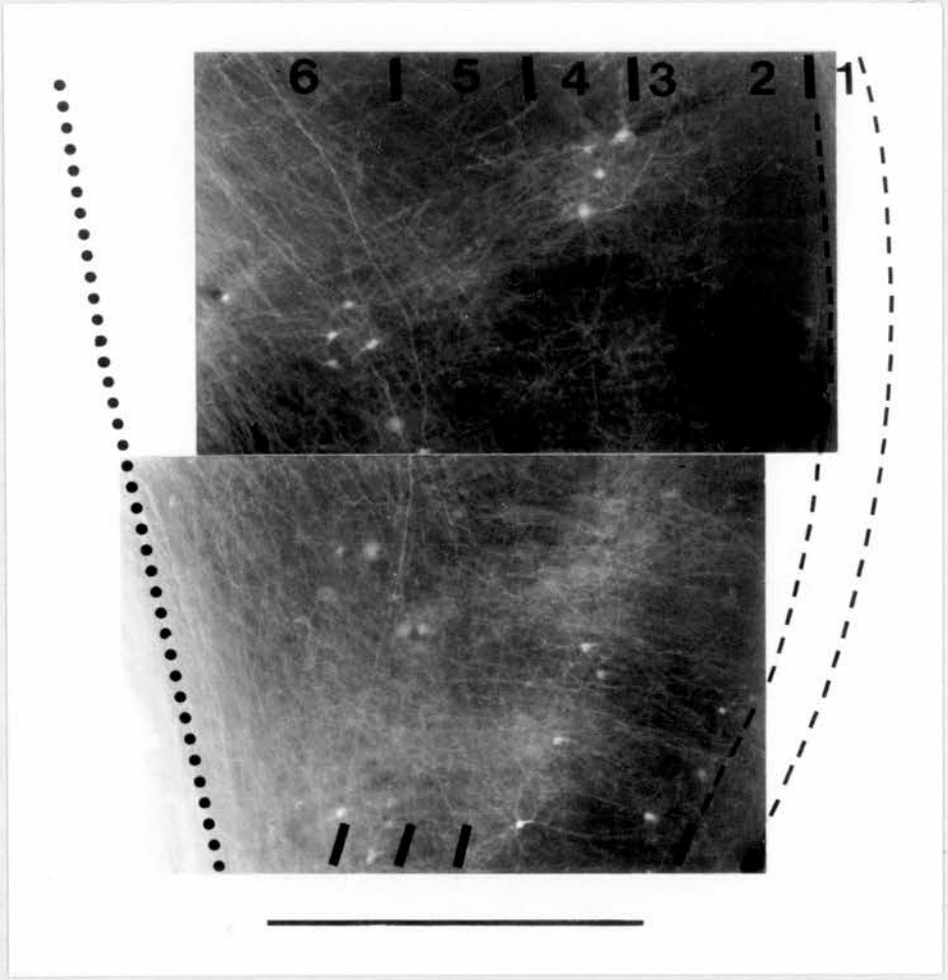

1 2 3 4 5
a
1 2 3 4 5
b

Figure 7. A montage of fluorescence photomicrographs of a coronal section through visual area 19 showing labelling with DiI from area 17 in a 29-day-old kittens. The grey/white matter border is marked by a *dotted line*, and cortical layer 1 with a broken line. Cortical laminae are indicated. Photomicrograph shows labelling after a diffusion time of 24 months. Scale bar, 1 mm.


6
5
4
3
2
1

only very faintly, allowing fibres to be seen easily. In these older kittens, it is sometimes impossible to decide whether these axons are of anterograde or retrograde projections; this is less of a problem in younger animals, where the ends of anterogradely labelled axons are more readily seen (for example, see the lower half of Fig. 4d). However, the results obtained here are very similar to those described previously with the exclusively anterogradely transported tracer, tritiated proline (Price and Zumbroich, 1989), indicating that at least a substantial amount of the label in areas 17, 18 and 19 in Figs. 5a, 6 and 7 is the result of anterograde transport. Figure 5a shows that, in older kittens, this label is distributed in clusters in area 18, and the density in the superficial layers is now closer to that in the deep layers than it is in younger animals. There is relatively little label in layer 4, although corticocortical fibres are seen crossing this layer to arborize in layers 2 and 3. Figure 6 shows a lower magnification view of label in area 17 itself in a P30 kitten: labelled patches such as those shown are grouped around the injection sites, in both the mediolateral and rostrocaudal directions. The density in these patches is highest in layers 2/3, lower 4 and 5. Figure 6a shows DiI label (with the N2 filter) and Fig. 6b shows DiA label (with the L3 filter) in the same field of view, with the outline of the DiI label superimposed. The injection sites are further rostral in area 17, and the DiI injection site is the more medial, as is the zone of DiI label. Although the two injection sites do not overlap, the labelled zones do, illustrating the convergence that is present among corticocortical connections (e.g. Ferrer et al., 1988 and 1992). Figure 7 shows DiI label in area 19 in a P29 kitten. Because of the longer diffusion time, retrogradely labelled cells are seen, but many of the labelled fibres are certainly area 17-to-19 projections. They are arranged in clusters with their densest arborizations in lower layer 3, upper layer 4, and layers 5 and 6 (Fig. 7). These arborizations do not extend as far superficially in area 19 as they do in area 18 (compare Fig. 7 with Fig. 5a). My overall impression is that, as late as P30, the connections from area 17 to area 19 remain somewhat more disorganized than those to area 18, in that the clusters are less

distinct in area 19 and there are many fibres that course horizontally or obliquely through the deep layers towards more lateral areas (examples are seen in Fig. 7). These fibres are not present in area 18 at this age, and are similar to those in extrastriate areas in younger kittens.

Reciprocal projections to area 17:

From birth on, there are reciprocal projections from areas 18 and 19 to area 17. Figure 1 a and b illustrates, at low magnification, the overall distributions of cells that are retrogradely labelled from a crystal of DiI placed in area 17. In area 18, these cells are mainly in the superficial part of the developing cortex, i.e. from the upper border of layer 5 through the cortical plate to the lower edge of layer 1 (examples are seen above the upper double arrowhead in Fig. 1a and below and to the left of the open arrowhead in Fig. 1b). Some of these cells are very superficial, lying just below layer 1 (an example is shown at higher magnification in Fig. 1c). Apart from these superficial cells, a second group comprising fewer labelled cells is scattered deep in layer 6 in area 18 (Fig. 1b). In area 19, retrogradely labelled cells are exclusively in deep layers (5 and 6) at P1; examples are shown in Fig. 1b and d. At P1, there is some evidence of retinotopography in the organization of the reciprocal projections to area 17; for example, the injection site in Fig. 1b, which is close to the area 17/18 border, has labelled many cells in area 18 that are correspondingly close to that border, although a few cells are scattered more laterally (two are seen at the area 18/19 border in Fig. 1b). Furthermore, I have the impression that the reciprocal projections to a small zone in area 17 may be more widely distributed than the afferents from that small zone (e.g. see Fig. 1a).

From P1, retrogradely labelled cells in the cortical plate and layers 5 and 6 are easily recognised as pyramidal because of their triangularly shaped cell bodies and distinct apical dendrites that reach to layer 1 (Fig. 1c and d). Another distinctive cell

type is occasionally labelled in layer 1 (Fig. 4b and c); these cells have small branches on their processes and have the typical appearance of Cajal-Retzius cells (Marin-Padilla, 1984).

In kittens aged between P6 and P16, the majority of reciprocal projections from both areas 18 and 19 to area 17 originate from pyramidal cells in the upper part of the cortical plate. Many of these cells are immediately below layer 1, where they are in a band over the regions of areas 18 and 19 that are being penetrated by the afferents from area 17, as is shown in Figs. 3c and 4a and d. These bands are broader in the horizontal direction than the region of afferent innervation. Thus, as suggested for the younger kittens, the regions of the extrastriate cortex that project back to a particular part of area 17 are wider than the extrastriate regions that receive afferent innervation from that same part of area 17. Although a comparison of the positions of DiI versus DiA labelled cells in the same brain gives evidence for a degree of topography among the reciprocal connections to area 17 (Fig. 3c), the generally high convergence of these projections can make it difficult to discern this organizational feature. Retrogradely labelled cells are also seen immediately below layer 1 in regions of area 17 itself, often at considerable distance from the injection site; examples are seen in Fig. 4a.

Not all the retrogradely labelled cells in areas 18 and 19 in P6 to P16 kittens are in the most superficial part of the cortical plate; a smaller proportion are in the lower part of the cortical plate, where layers 3 and 4 are forming, and in deeper layers, particularly layer 5. Examples of these labelled cells are shown in Fig. 4d, where three are highlighted with arrowheads. These cells are limited to the same regions of the extrastriate cortex that receive the bulk of the innervation from the injected zone in area 17, i.e. they do not extend beyond these regions, unlike the projections from the most superficial group of cells.

By P30, cells in area 18 that are retrogradely labelled from area 17 are mainly in superficial layers 2, 3 and upper layer 4, with fewer in the lower part of layer 5 and

upper layer 6. This is illustrated in Fig. 5b (retrogradely labelled cells are seen since the tracers have been allowed to diffuse for long periods); the clustering of these cells is less obvious than the clustering of the labelled fibres (described above, compare Fig. 5a). At this age, very few of these cells are immediately below layer 1 and they do not extend beyond the regions that contain labelled fibres. Furthermore, the topography of these reciprocal connections is much clearer by around P30. Figure 3 f and g show the same region of area 18 photographed through filter I2/3 (Fig. 3f) and filter N2 (Fig. 3g). In area 17, the injection site of DiI is lateral to that of DiA, and in area 18, the distribution of DiI labelled cells is shifted correspondingly medial to the distribution of DiA labelled cells. This is emphasized in Fig. 3f, where the positions of the cells that are intensely labelled with DiI (from Fig. 3g) are marked on the photomicrograph of the DiA labelled cells. The pattern of retrograde labelling of area 19 is similar to that in area 18, although there are generally fewer labelled cells and they are deeper, mainly in lower layer 3, upper layer 4 and layer 6 (Fig. 7).

2.4.3 Labelling with DY and DiI in vivo

The results of the anterograde tracing in the fixed brains of neonatal kittens suggests that the penetration of the superficial layers is more restricted than that of the deep layers (e.g. Fig. 4d). Injections of the retrograde tracers DY and DiI at different depths in area 18 in P1-4 kittens in vivo provide further evidence for this conclusion. Examples of the injection sites are shown in Fig. 8 a and c; that in Fig. 8a involves the cortical plate (i.e. developing layer 4 and above), while that in Fig. 8c involves lower cortical plate and layers 5 and 6. All injections retrogradely label cell bodies in retinotopically related regions of area 17 (e.g. between open arrows in Fig. 8a). These cells are distributed in a continuous band of almost uniform density, mainly in the superficial layers although smaller numbers of cells are labelled in deep layers. Figure 9 shows the profiles of density of labelled cells in the rostrocaudal direction through the superficial layers of area 17 (derived as described in Methods)

Figure 8. (*a*) A montage of fluorescence photomicrographs of a coronal section of visual cortex after an in vivo injection of DiI that involved the cortical plate (CP; future layers 2-4) and layer 1 in area 18 in a 4-d-old kitten. Areal borders are indicated. *Open arrows* indicate the extent of the retrograde labelling in area 17. PMLS, posteromedial lateral suprasylvian cortex. (*b*) Fluorescence photomicrograph of the ipsilateral lateral geniculate nucleus from the same animal as shown in a; note the DiI retrogradely labelled cells in the central region of the nucleus. (*c*) Fluorescence photomicrograph of a coronal section through an in vivo injection of DY centred in the lower CP/layer 5 in area 18 in a 3-day-old kittens. In *a* and *c*, cortical laminae are indicated. Scale bar in *a*, 1 mm; scale bar in *b*, 0.5 mm; scale bar in *c*, 0.25 mm.

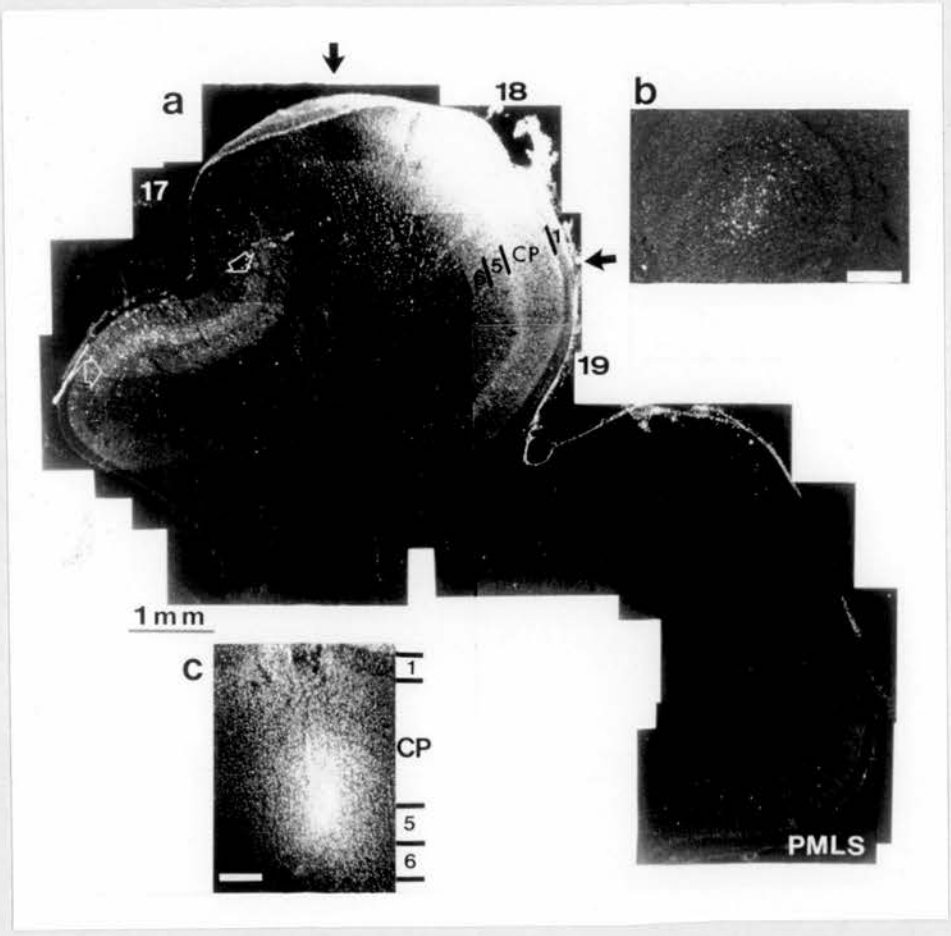
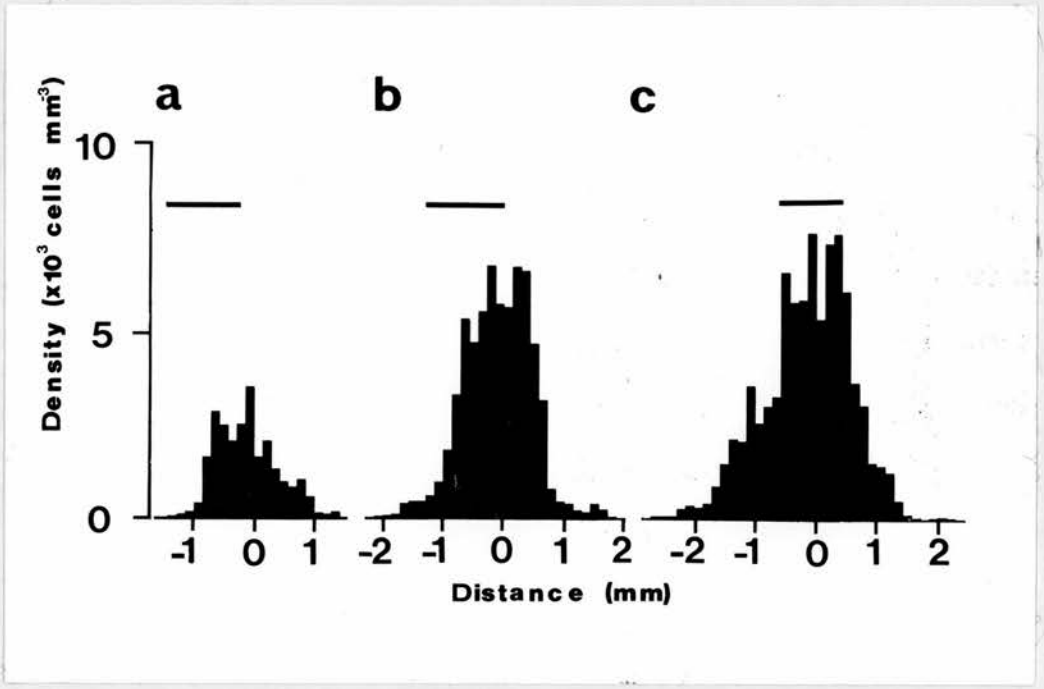

a
18
17
19
CP
1 mm
b
c
1
CP
5
6
PMLS

Figure 9. The profiles of density of retrogradely labelled cells in area 17, after injections of tracers into area 18, measured along the rostrocaudal direction: distances are from the centre of the labelled area; density is in the superficial layers (see Materials and Methods for a full explanation of the technique). The solid horizontal bars indicate the rostrocaudal diameters of the injection sites and their rostrocaudal positions relative to the labelled areas. Results are from injections of (*a*) DY restricted to the superficial part of the cortical plate and layer 1 in a P4 kitten, (*b*) DiI restricted to the cortical plate (i.e. above layer 5) and layer 1 in a P4 kitten, and (*c*) DY in the lower part of the cortical plate, layer 5 and layer 6 in a P3 kitten.


a
b
c
Density (x10^3 cells mm^{-3})
10
5
0
-1
0
1
-2
-1
0
1
2
-2
-1
0
1
2
Distance (mm)

for three injection sites, one in the upper part of the cortical plate at P4 (Fig. 9a), one spanning the cortical plate at P4 (Fig. 9b) and one involving layers 5 and 6 at P3 (Fig. 9c). The values for the convergence of projections from area 17 to area 18, calculated using the entire extent of the labelled region (i.e. including even those regions with a very low density of labelled cells; see Methods), are 1.7 mm to the most superficial injection site, 2.9 mm to the deeper injection that spanned the cortical plate and 4.1 mm to the deepest injection. As is evident from Fig. 9, deeper injections in area 18 label denser distributions of cells in the superficial layers of area 17. The overall conclusion is that, in agreement with the study with DiI and DiA in fixed brains, the projections from area 17 are more sparsely and narrowly distributed in more superficial layers of area 18 in the few days after birth.

2.5 DISCUSSION

Much of the current research on the development of the cerebral cortex is focusing on the molecular and cellular mechanisms that control neuronal differentiation and axonal growth. Clearly, before specific hypotheses regarding mechanisms can be raised and tested, it is essential to have accurate observations of these processes. While there have been numerous observational studies of the development of connections between the cortex and subcortical structures (Tsumoto et al., 1983; O'Leary and Koester, 1993), information on the development of the corticocortical pathways (particularly those that run ipsilaterally) is much sparser. These connections form a vast and complex network, and despite the fact that their functions are largely unknown, it is clear that an overall view of the factors that control the development of the cerebral cortex, and its connections with other structures, will be impossible to achieve without information on the formation of connections that are intrinsic to the cortex itself.

In the past, observational studies of corticocortical development have used either retrograde or anterograde tracers applied to living brains. There are several

potential problems with this approach. First, it is clear that these connections are extremely immature and very sparse at birth in species such as the cat, and so they are hard to label in the immediate postnatal period. Second, it is all too easy to generate injection sites that are too large or in the wrong place during surgery in very young animals. Third, some of the tracers used in the past have transport times of several days, and the fact that the system is still developing rapidly while the tracer molecules are diffusing makes for problems of interpretation. Fourth, quantitative aspects of the transport of tracers applied in living brains may depend on cellular factors that vary during development. Despite these possible difficulties, previous studies with these methods have raised important hypotheses regarding the events that occur as the corticocortical connections develop. For example, it has been suggested that, as corticocortical axons grow through the white matter, they have a very widespread distribution and that the specificity of innervation that generates topographic order occurs only when these fibres reach their cortical targets (Innocenti and Clarke, 1984; Price and Zumbroich, 1989). This has lead to the hypothesis that cues around the layer 6/white matter border are important for the selective validation of appropriately directed connections, while others are withdrawn (Price and Zumbroich, 1989). In this study, I have re-evaluated this and other hypotheses using fluorescent lipophilic tracers that are very sensitive and, because they can be injected postmortem and are transported along cell membranes in fixed tissues, avoid the possible difficulties mentioned above. My main conclusions are:

1. At birth, corticocortical connections are very sparse and links between the major visual areas are only just forming. The feedforward and feedback pathways interconnecting areas 17 and 18 have matured further towards their adult morphology than those interconnecting areas 17 and 19, suggesting that the relative times at which these areas become interlinked depends on how far apart they are. Despite the weakness of the interconnections at birth, it is clear that small regions in area 17 are

becoming connected by discrete fibre-tracts with topographically related regions in ipsilateral extrastriate visual areas.

2. During the first postnatal weeks, the development of corticocortical connections proceeds extremely rapidly. More and more fibres from area 17 penetrate the deep layers of extrastriate regions, some begin to enter more superficial cortical layers, and feedback projections develop from the superficial layers of area 19. The majority of this ever more prolific innervation links retinotopically related regions of the striate and extrastriate cortex. Smaller number of corticocortical axons grow in a more scattered fashion, outwith these retinotopic links, although they do not penetrate further than the deep layers of the extrastriate cortex.

3. The penetration of the superficial layers is narrower, in the tangential direction, than that of the deep layers (i.e. convergence of inputs to the superficial layers is less than that to the deep layers), implying that retinotopic organization becomes increasingly precise as the corticocortical afferents enter the superficial layers of the extrastriate cortex.

4. During the first 2-3 postnatal weeks, the convergence of the reciprocal projections from extrastriate cortex to area 17, particularly those arising from the most superficial parts of the cortical plate, appears greater than that of the afferents from area 17 to the superficial layers of the extrastriate cortex. As development proceeds, the spatial match between the feedforward and feedback projections becomes more precise, and by P30 their convergences match more closely.

Earlier observations on the development of ipsilateral corticocortical projections show that at least some aspects of the adult system, in which retinotopically organized connections originate and terminate in discrete clusters in specific layers, develop by the selective elimination of inappropriately directed axons from an initially exuberant and widely scattered population of fibres (Dehay et al., 1984; Price and Blakemore, 1985a,b; Clarke and Innocenti, 1986; Price and Zumbroich, 1989; Price and Ferrer, 1993; Price et al., 1994b). Similar processes

occur during the postnatal maturation of the callosal pathways in the cat (Innocenti, 1981; Innocenti and Clarke, 1984). Although it is widely accepted that such a mechanism is important for the emergence of clusters among the cells of origin of the ipsilateral pathways (Price and Blakemore, 1985a,b; Price and Ferrer, 1993; Price et al., 1994b), the extent of its involvement in the maturation of other features of these projections, particularly topography and laminar distribution, is less clear. For example, findings by Katz (1991) on the development of intrinsic horizontal connections (i.e. those confined to one cortical area) suggest that, although the elimination of inappropriate fibres is again important for the emergence of *clusters* of projections (Price, 1986; Callaway and Katz, 1990), *interlaminar* connections are highly specific from the outset. A recent study by Price et al. (1994b) suggests that the elimination of misdirected axons is not the major factor responsible for the development of topography among corticocortical connections, since the majority of these fibres interconnect retinotopically related zones with considerable precision from very early ages, and those axons that are eliminated after growing to retinotopically inappropriate regions are relatively sparse.

In the present study I have concentrated mainly on the development of topography and laminar distribution, and my overall conclusion is that the organization of these two features shows considerable specificity from the time when the corticocortical connections are first forming. It is clear that these connections are starting to be made at the time of birth, since they are still very sparse and, in the case of the area 17-to-19 projection, are only just entering the cortical grey matter. Although topography is much easier to detect a few days later, when it is already very precise, the fact that retinotopically corresponding points in the striate and extrastriate cortex are interconnected by fibre-tracts at birth implies that at least a large number of the corticocortical axons grow directly to appropriate points in other areas. This conclusion contrasts with that of Price and Zumbroich (1989), who suggested that the initial outgrowth of corticocortical fibres through the white matter

lacks specificity. This previous study used the anterograde tracer, tritiated proline, to label efferents from area 17 in young kittens aged P4 or more, and widespread labelling of the white matter after single small injections in area 17 was reported. Certainly, my present study reveals that, during the first postnatal week, some axons do grow in an apparently misdirected fashion through the white matter and penetrate the deep cortical layers of regions of the extrastriate cortex that do not correspond retinotopically with the region from which the connections originate. Furthermore, there is a refinement in the precision of the topography as the projections penetrate the superficial cortical layers. But, by examining the connections at an earlier time, my new study suggests that a large number of at least the earliest projections grow with greater specificity between retinotopically corresponding regions; it may be that Price and Zumbroich (1989) failed to recognize this specificity because they did not look early enough and, because of the extremely rapid development of the connections after birth, fibre-tracts through the white matter may have been obscured at P4 by the prolific growth of connections that criss-cross as they form the complex network of connections between the multiple visual areas. Indeed, my study shows intense labelling of the white matter in P6 kittens after injections into area 17, and had I not observed the faint but clearly present fibre-tracts at P1, I might have interpreted my data in a similar fashion to Price and Zumbroich (1989). Overall, my conclusion is that many, perhaps the majority, of axons from area 17 grow to specific sites in extrastriate cortex, but that some are misguided and may be retracted later. At present, it is impossible to decide whether the axons that project inappropriately have simply made errors in interpreting whatever cues direct axons to specific regions of the extrastriate cortex, or whether they represent a subpopulation of corticocortical axons that carry out some presumably transient function during the development of the large body of connections that persist in the adult.

The fact that there is a population of early corticocortical fibres that appears to be directed to specific locations alters the emphasis placed on the various

hypotheses that could explain the development of highly topographic projections. The previous report of non-specific growth (Price and Zumbroich, 1989) suggested a system that, although wasteful, required that a minimum amount of information be specified to the growing projections; essentially, this theory proposed that axons grow initially randomly (i.e. detailed guidance cues are not required) and that only those that chance to arrive at appropriate positions persist, through some interaction with their targets. My new findings suggest that guidance cues are provided to at least a large proportion of the growing corticocortical axons. It can not be that the topography of the corticocortical axons traversing the white matter results simply from the maintenance of fibre order among a homogeneous population of axons emerging from the striate cortex, since those projections directed to the more lateral cortical areas must cross those directed to the more medial areas, and their order must be reversed. It is more likely that there is chemotropic guidance of corticocortical axons between specific points in the striate and extrastriate cortices by one or more diffusible molecules distributed in gradients through the developing cortex (Tessier-Lavigne and Placzek, 1991; Heffner et al., 1990). This suggestion is supported by the evidence of Bolz et al. (1990) that cortical axons from superficial layers may be attracted to their cortical targets in organotypic co-cultures. It is possible that any chemotropic guidance is restricted for either the feedback or the feedforward projections, since axons growing in one of these two directions may grow over axons that have already linked specific points by coming from the other direction. For example, the axons of the feedforward projections from each point in striate cortex may fasciculate selectively on those axons reaching them from retinotopically corresponding points in extrastriate cortex. This suggestion is similar to that made previously by Molnar and Blakemore (1990) regarding possible fasciculation of the thalamic fibres on the descending axons of the subplate during the development of thalamocortical connections. If such a mechanism does play a role in the development of corticocortical fibres then it is likely that the first links would be

established by the feedback projections, which seem more mature than the feedforward projections at the time of birth, presumably because of the lateral to medial gradient of maturation of the cortex.

There is evidence of more precise retinotopography among the early feedback connections that arise from the deep layers than among those from the superficial layers, i.e. the latter project to the striate cortex with a much higher convergence. Similarly, Price et al. (1994b) report that cells projecting from area 17 to a small region of area 18 are distributed more widely in the superficial than in the deep layers. This is also apparent in Fig 8a. Immediately postnatally, the cells at the top of the cortical plate are the most recent immigrants (Luskin and Shatz, 1986) and the relative imprecision of their projections may reflect their immaturity. Coogan and Burkhalter (1988) show that, in the rat, area 17-to-18 corticocortical connections develop in a laminar sequence, with those from deep layers (5 and 6) being present at P1 and those from the superficial layers (2/3 and 4) developing from P5 on. Thus, it seems that cells form connections in the order that they arrive in the cortical plate. Overall, I suggest that there is much greater retinotopic precision among the early feedforward and feedback projections that originate in the deep cortical layers, and that the cues that guide the formation of the early specific links may be associated with these cells. As mentioned earlier, there is a degree of refinement of the retinotopography of corticocortical connections postnatally, and this may result from the loss of inappropriate connections from mainly superficial layer cells, perhaps under the influence of cues from the axons interlinking specific points in the deep layers.

Both this and previous studies (Price and Blakemore, 1985a,b; Price and Zumbroich, 1989) show that the development of the laminar distributions of corticocortical cell bodies and terminals is highly specific from an early age. From birth, the cell bodies of both the feedforward and feedback projections are in two groups, one superficial the other deep. As the feedforward projections penetrate

extrastriate cortical areas 18 and 19 they form a plexus in first deep and later superficial cortical layers. Although the probability that a proportion of these connections initially link inappropriate cortical laminae is not excluded, my results show that the cells of origin and the terminals of the projections are unevenly distributed among the cortical layers from birth and they are compatible with the suggestion of Bolz et al. (1990) and Katz (1991) that intracortical connections grow between specific laminae from the outset.

Two processes that appear to be widely used for the creation of specific circuitry in the developing nervous system are the guidance of growing axons directly to specific targets, and the operation of axonal withdrawal and cell death on initially exuberant and relatively undirected projections. It appears that the developing corticocortical connections use both mechanisms for the development of different aspects of their organization. My study indicates that many projections grow in a directed manner towards specific retinotopically related sites in other areas, although axonal withdrawal may refine the topography as the connections continue to develop. At least the majority of the corticocortical connections interconnect specific cortical layers from the outset. On the other hand, the emergence of patches of association cells from an initially continuous distribution is achieved through the selective withdrawal of inappropriate axons (Price and Blakemore, 1985a,b). These observations give important clues to the mechanisms that may underlie the development of the different aspects of the corticocortical pathways.

CHAPTER 3

EFFECT OF NEONATAL ABLATION OF LAYER 1 ON CORTICOCORTICAL PROJECTIONS FROM AREA 17 TO AREAS 18 AND 19

3.1 ABSTRACT

In this study I examined the role of layer 1 in the process of refinement of corticocortical connections between visual area 17 and visual areas 18 and 19. From the earliest postnatal ages, apical dendrites of corticocortical cells project to layer 1, where they connect with long horizontal fibres that cross several cortical areas. I lesioned layer 1 in the medial part of area 18 and the lateral part of area 17 in newborn kittens, let the kittens develop to one month of age and examined cortical projections after labelling them with lipophilic dyes in fixed brains. In normal kittens, by the end of the first postnatal month, fibres projecting from area 17 to area 18 terminate in patches in the cortex of area 18 and cells projecting back from area 18 are grouped in clusters, mainly in superficial layers with a few in deep layers. In lesioned animals, very few projections interconnected areas 17 and 18 although in Nissl-stained sections the cortex appeared normal below the lesions in layer 1. In the normal kittens about 8.2 % of cells in superficial layers were corticocortical cells projecting from area 18 to a specific point in area 17 and in lesioned animals the number of projecting cells was 0.7 %, which was significantly lower than in the normal kittens. Thus, these finding suggest that the lesions produced a disproportionate loss of corticocortical connections, that is not easily explained on the basis of generalised cortical damage. I postulate that intercellular signaling via layer 1 is important for the development of corticocortical connections in the visual cortex.

3.2 INTRODUCTION

The formation of the neocortex starts with the appearance of the phylogenetically older structure called the preplate (Marin-Padilla, 1978; Luskin and Shatz, 1985b). As corticogenesis progresses, new cells arrive and form the cortical plate (the future cortical layers 2 to 6) and the preplate splits into the superficial marginal zone (the future layer 1) and the subplate zone (the future white matter) (Kostovic and Molliver, 1974; Marin Padilla, 1978; Luskin and Shatz, 1985b). The

marginal zone (MZ) and the subplate zone (SP) seem to mature ahead of the other cortical layers and play an important part in the development of the rest of the cortical plate and its connections (Friauf et al., 1990; Ghosh and Shatz, 1992a; Meyer and Gonzalez-Hernandez, 1993); for example, it has been suggested that axons of the subplate cells pioneer the first axonal pathways from the cortex to the thalamus (McConnell et al., 1989; De Carlos and O'Leary, 1992) and are important in the formation of thalamocortical projections (Blakemore and Molnar, 1990; Ghosh et al., 1990). The role of the MZ (later referred to as layer 1) is understood less. Early in the maturation of the neocortex, layer 1 contains the apical dendrites of all cortical cells (Marin-Padilla, 1992). Only pyramidal neurones retain their apical dendrites in this zone, i.e. stellate cells in layer 4 withdraw their apical dendrite after the first postnatal week (Peinado and Katz, 1990). There are also some exceptions among pyramidal cells, i.e. callosal neurones lose their apical dendrite in layer 1, while corticocortical neurones retain theirs (Koester and O'Leary, 1992; Kasper et al., 1994). Taken together, this evidence indicates that layer 1 may have an important transitory role early in neocortical maturation and that pyramidal cells seem to be the most dependent, either transiently or permanently (Marin-Padilla, 1992). Despite this evidence, little attention has been focused on the role of layer 1 in the development of corticocortical connections, although from the earliest postnatal ages pyramidal corticocortical cells project to this layer (Koester and O'Leary, 1992; Kasper et al., 1994). Corticocortical cells connecting areas 17 and 18 are initially uniformly distributed in a band below layer 1 and, as the formation of the cortex progresses, these cells become positioned relatively deeper and become grouped in clusters (Price and Blakemore, 1985a,b; Price et al, 1994b). It seems that the refinement of these projections occurs after migration is completed and cells have contacted layer 1. Therefore, my hypothesis is that signaling via layer 1 is involved in the development of corticocortical connections. In the present study I lesioned layer 1 in the medial part of area 18 and the lateral part of area 17 in newborn kittens

and at one month of age we examined cortical projections after labelling them with the fluorescent lipophilic tracers 4-(4-dihexadecylaminostyryl)-N-methylpyridinum iodide (DiA) and 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) in fixed brains.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Successful experiments were carried out on 5 kittens obtained from an isolated laboratory colony. In 3 of them, I made a unilateral lesion of layer 1 in the medial part of area 18 and the lateral part of area 17 at postnatal day (P1) (1 animal) and P3 (2 animals) and perfused the kittens on P32. Other non-lesioned kittens were perfused on P28 (1 animal) and P29 (1 animal). DiI and DiA were applied to 6 hemispheres, 3 lesioned and 3 non-lesioned.

3.3.2 Surgical procedures and lesioning of layer 1

Anaesthesia was induced with ketamine hydrochloride (20 mg kg^{-1}, i.m.) and maintained with an intravenous infusion of alphaxalone-alphadolone (Saffan, Glaxo), diluted with isotonic saline to 15% of its original concentration. The rate of infusion was approximately 0.003 ml min^{-1}, adjusted as necessary to maintain full surgical anaesthesia throughout. The ventilation rate and rectal temperature were continuously monitored and body temperature was maintained at 37-38°C with a thermal blanket. The anaesthetized animal was placed in a stereotaxic frame and a large craniotomy and durotomy were made over the crown of the lateral gyrus of the left hemisphere. Each lesion was made in the middle of the mediolateral extent of the lateral gyrus (presumed medial part of area 18 and the area 17/18 border). Rostrocaudally, lesion was located from 3-4 mm anterior to 5-6 mm posterior to anteroposterior zero (AP0), where areas 17 and 18 represents the region of visual field below the horizontal meridian (Tusa et al., 1978, 1979; Rosenquist, 1985).

Layer 1 was ablated by suction using a metal needle with a flat tip (needle diameter 0.8 mm). Negative pressure was applied for a brief period as the needle was moved along the surface of the lateral gyrus (from the front to the back of durotomy). On the surface of the brain the ablation had the shape of a narrow rostrocaudal strip 8-10 mm long. After lesioning, the wound in the scalp was sutured, kittens were allowed to recover and returned to their mothers. At one month of age, kittens were anaesthetized and perfused transcardially as described below.

3.3.3 Application of DiA and DiI tracers

Lesioned and normal kittens were anaesthetized with a lethal overdose of sodium pentobarbitone (20 mg, i.p.). Once they had stopped breathing, they were perfused transcardially with isotonic saline followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer at pH 7.4. After perfusion, the brains were removed from the skull and stored in the same fixative. DiA (Molecular Probes, D-3883) and DiI (Molecular Probes, D-282) were applied as a single small crystal (approximate 300 μm in diameter). Two crystals, one of each tracer, were inserted under the surface of the brain side-by-side in the mediolateral plane in area 17 in the medial bank of the lateral gyrus and near AP0 (Fig. 1b,e). Crystals were pushed under the surface of the brain with a glass micropipette (tip diameter 150 μm). The thickness of the visual cortex in the 1 month old kitten is about 1.5 mm. I aimed to centre the injections at a depth equal to approximately half the thickness of the cortex. As a guide, a distance of 0.5 mm from the tip was marked on the micropipette and crystals were pushed in the cortex to this depth. Injected brains were stored in the dark at room temperature for 11 months.

3.3.4 Histological preparation

Fixed brains were blocked and placed in a solution of 20% sucrose in phosphate buffer to equilibrate for 1-2 days. Brains were cut on a vibroslice at a

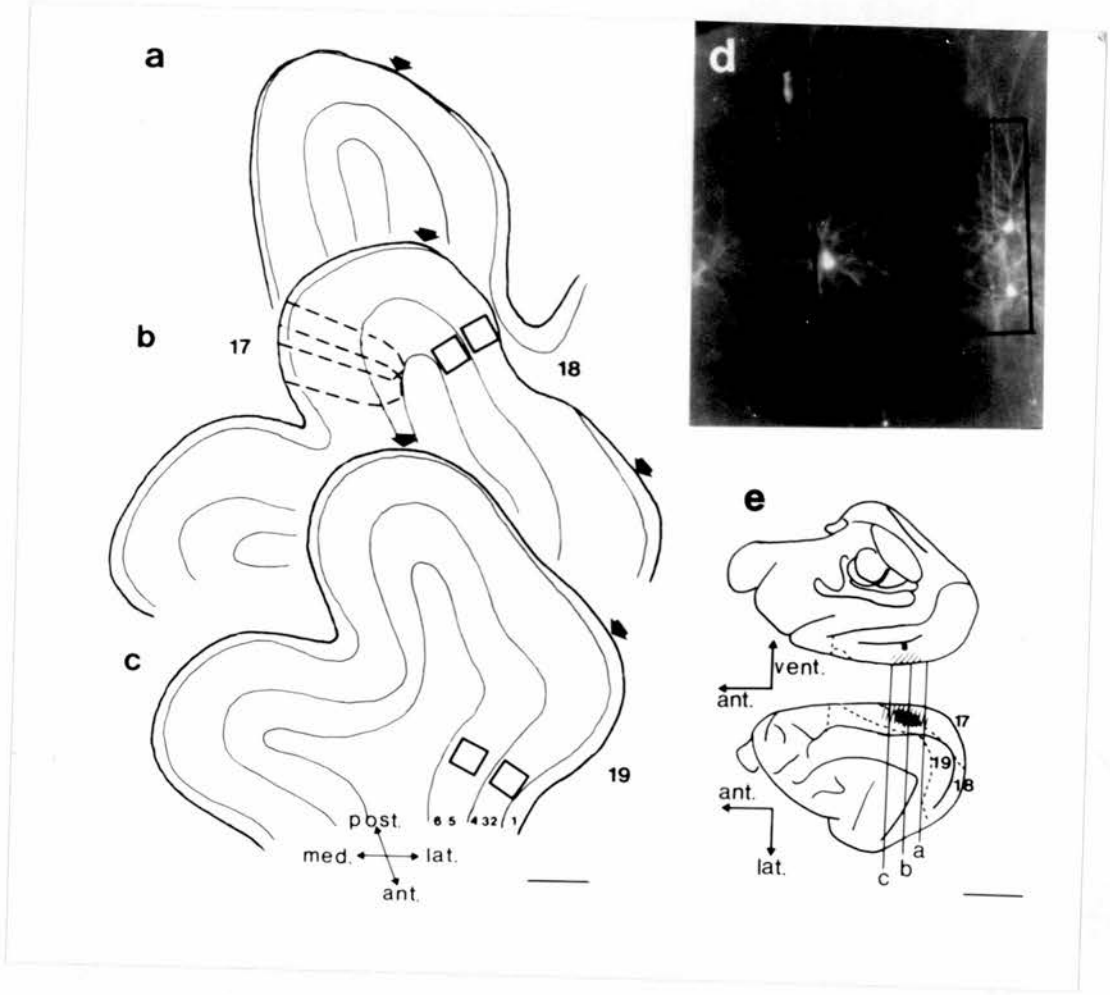
thickness of 200 μm. I mounted two series of the sections (each 1-in-3) on gelatine coated slides. One series was immediately coverslipped with 0.1M sodium phosphate buffer at pH 7.4 and examined with a fluorescence microscope and photographed. A second series was counterstained with cresyl fast violet to examine the cortical layers, the extent of the lesion and the position of the area 17/18 and 18/19 borders (Garey, 1971; Anker and Cragg, 1974; Price, 1985; Price and Zumbroich, 1989).

3.3.5 Analysis

The depth and the extent of the lesion observed in Nissl-stained sections (1-in-3 series) were drawn with a cameral lucida using a 1.6x objective (Fig. 1a-c). The topographic maps of the lesions were then reconstructed by superimposing the extent of the lesion at 600 μm intervals on the standardised dorsolateral view of the hemisphere (see Fig. 1e).

Fluorescent labelling was viewed with a Leica fluorescence microscope. DiI was viewed with filter N2 (530-560 nm excitation), DiA with filters I2/3 (450-490 nm, long-pass suppression filter at 515 nm) and L3 (450-490 nm, band pass filter 525/20 nm). After examining the overall labelling, quantitative analyses were carried on the peak density of corticocortical projecting cells from areas 18 and 19 to area 17. Analyses were carried out on sections taken from each of 3 lesioned hemispheres and 3 non-lesioned hemisphere (2 from normal animals and 1 from contralateral non-lesioned side). In lesioned hemispheres, counts were made medial in area 18 under the layer 1 lesion and lateral in area 19. In non-lesioned hemispheres, counts were made medial in area 18 and lateral in area 19. Corticocortical cells were counted in sections (one per hemisphere) that contained the centre of the labelling in area 18 or 19. Cells were counted in clusters in superficial layers (2, 3 and 4) and in deep layers 5 and 6 (Fig 1b-d) using a 10x objective with a grid in the eye piece. Labelled cells were counted in a surface of 0.1 μm^2 (25 grid squares). Numbers were divided by the volume of the cortex that

Figure 1. (*a-c*) Camera lucida drawings of 3 representative coronal sections through the visual cortex in a 1-month-old kitten illustrating the depth and the extent of the layer 1 lesion. Injection sites in area 17 in *b* are indicated with *broken lines; small squares* in areas 18 and 19 in *b* and *c* indicate sampling zones for quantitative analyses. (*d*) Fluorescence photomicrograph of coronal section of the cortex in area 18 below the layer 1 lesion in a 1-month-old kitten. A sampling zone for quantitative analyses is indicated with the *square*. (*e*) A surface reconstruction of the layer 1 lesion made from camera lucida drawings of a 1-in-3 series of Nissl-stained sections. Sections shown in a-c are taken at three different levels: *a*, caudal; *b*, medial; *c* rostral. The regions shown in *black* indicate complete destruction of layer 1 and the *hatched* region indicates margins of the lesion in which layer 1 was identifiable but attenuated and gliotic; the positions of a DiI and a DiA crystal are marked in area 17. Scale bar in *a, b,* and *c*, 1 mm; scale bar in *d*, 0.25 mm; scale bar in *e*, 10 mm.


a
b
17
18
c
19
post.
6 5 4 3 2 1
med.
lat.
ant.
d
e
vent.
ant.
17
19
18
ant.
lat.
c
b
a

contained these cells to calculate the peak density of corticocortical projecting cells (in cells mm^{-3}).

After quantifying the peak density of labelled corticocortical cells, I used the adjacent Nissl-stained sections to quantify the percentages of corticocortical labelled neurones in areas 18 and 19. First, I selected similar regions to the sampling zones already described. As the sections were 200 μm thick, cell counts in the Nissl-stained sections were made using a 40x objective with a grid in the eye piece. Cells were counted in a surface of 0.024 μm^2 (25 grid squares). The total cell number was divided by the volume of the cortex containing these cells to calculate the total cell density in each sampling zone. Finally, from the densities of corticocortical labelled cells and the total cell densities the percentages of corticocortical labelled neurones projecting from areas 18 and 19 to area 17 were calculated.

Calculated total cell densities were also compared in lesioned and non-lesioned hemisphere to estimate whether there were significant changes after the lesion. I also measured the thickness of superficial and the deep layers in the same sections and compared data in the normal and non-lesioned hemispheres.

3.3.6 Statistics

Data were statistically analysed using Student's *t*-test, or alternatively Welch's *t*-test in the cases where the differences between two standard deviations were significant. Differences were regarded as statistically significant at $P \leq 0.05$.

3.4 *RESULTS*

3.4.1 Evaluation of layer 1 lesions

Surface reconstructions of the position, the extent and the depth of the lesion were made for each animal. An example from one animal is shown in Fig. 1. In the centre of the lesion layer 1 was completely removed (Figs. 1b) and at the margins of the lesion, layer 1 appeared gliotic and narrow (Fig. 1a,c). When margins were

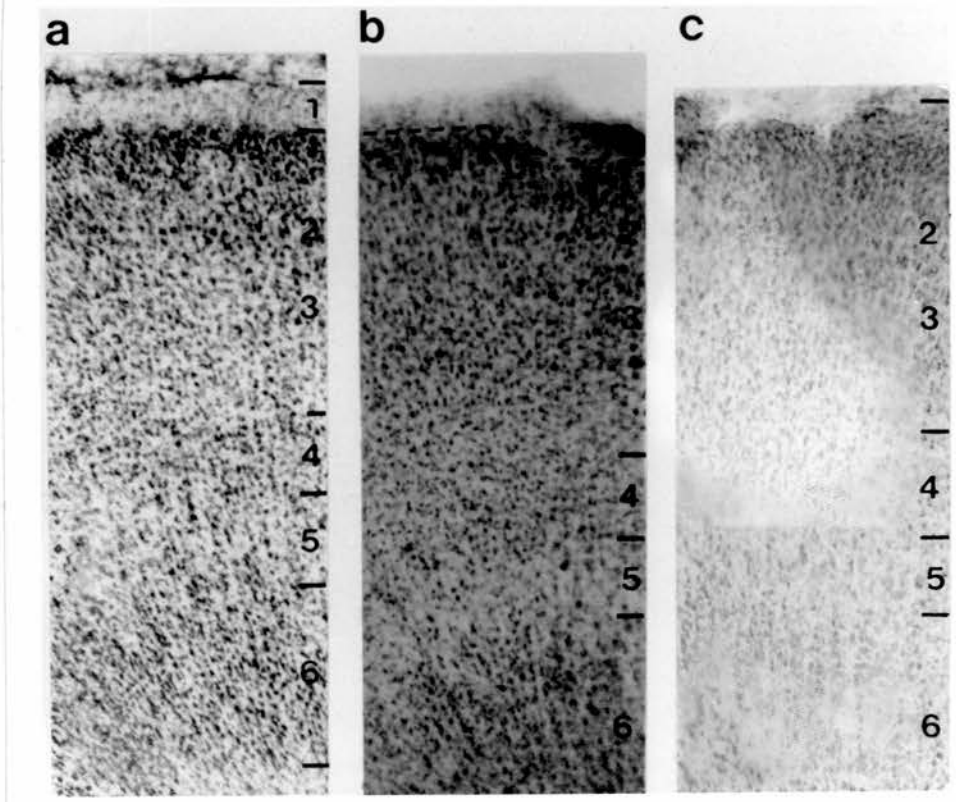
included, the total rostrocaudal length of the lesion was about 8-10 mm and mediolateral width 4-5 mm. In all experiments, lesions were medial in area 18 and lateral in area 17, across the area 17/18 border.

The superficial cortical layers in area 18 below the layer 1 lesion looked fused, whereas the deep layers looked similar to normal (Fig. 2b,c). In the superficial layers, the upper border of layer 4 was difficult to distinguish from layers 2 and 3. My impression was that this was caused by the absence of the small stellate cells that normally comprise layer 4. The thickness and cell densities looked unchanged; I found that the thickness of the upper cortical layers (layers 2, 3 and 4) was not significantly greater (Student's *t*-test; $P<0.81$) in non-lesioned hemispheres (mean $\pm$ S.E.M.; 0.76 $\pm$ 0.07 mm) than in the lesioned hemispheres (0.74 $\pm$ 0.02 mm). Similarly, the values for the thickness of the deep cortical layers were not significantly different (Welch's *t*-test; $P<0.23$) in the non-lesioned (0.59 $\pm$ 0.01 mm) and lesioned hemispheres (0.68 $\pm$ 0.05 mm). The mean values for cell densities in the same region in the superficial layers were 17269 $\pm$ 4618 cells mm^{-3} in the control and 12370 $\pm$ 1252 cells mm^{-3} in the lesioned hemisphere; these were not significantly different (Student's *t*-test; $P<0.36$). The cell density in deep layers in lesioned hemisphere seemed lower (14941 $\pm$ 2308 cells mm^{-3}) than in normal (21436 $\pm$ 2135 cells mm^{-3}) but the difference was not significant (Student's *t*-test; $P<0.10$). These data and the morphological appearances indicated that the ablation of layer 1 did not caused generalised cortical damage in area 18.

3.4.2 Injection sites

The sizes and positions of injection sites were checked in Nissl-stained sections. All injections were placed in the rostrocaudal plane near to the centre of the lesion. Injection sites were composed of two crystals, one of DiA and another of DiI, placed at the same rostrocaudal level and separated mediolaterally by centre-to-centre

Figure 2. Bright-field photomicrographs of cresyl violet-stained coronal sections through area 18 in a 1-month-old kitten. (*a*) Cortical layers in area 18 in a non-lesioned hemisphere. (*b*) After a lesion, only the deep part of layer 1 was present in the centre of the lesion (on the *left*), outside this region layer 1 was reduced in its thickness (on the *right*). (*c*) Layer 1 was totally removed. Scale bar, 0.25 mm.


a
b
c
1
2
3
4
5
6

distances between 0.5 and 0.8 mm (Fig. 1b,e). Each crystal produced an injection site containing a dense central zone and a narrow halo of diffuse staining. The two injection sites largely overlapped and the total dimensions of the two deposits of tracer in the mediolateral plane were 2-2.5 mm, and in the rostrocaudal plane 1.2-1.8 mm. Crystals were placed deep in layer 3 or in layer 4. The dense core of the injection sites covered all cortical layers.

All injections were made in fixed brains allowing us to position injections in area 17 (in the medial bank of the lateral gyrus) avoiding the 17/18 border along the dorsal surface of the lateral gyrus.

3.4.3 Topography

In the non-lesioned hemispheres, the positions of the injection sites in area 17 and the labelled parts of areas 18 and 19 always corresponded topographically (Fig. 3). The centre of labelling in area 18 lay at the same rostrocaudal level as the injection sites (Fig. 3a,b). Labelling in area 19 was slightly further rostral from the injection sites (Fig. 3c), as the retinotopic map is located slightly more rostral in area 19 than in areas 17 and 18 (Tusa et al., 1979). In the mediolateral plane, all injections lay lateral in area 17 and produced labelling in a correspondingly medial part of area 18 and lateral part of area 19.

In the lesioned hemispheres, overall labelling in area 18 was reduced compared to that in the controls (Fig. 4a). Most of the fluorescently labelled cells were seen with both rhodamine and fluorescein filters indicating that these cells were probably double labelled with the same amount of DiI and DiA (Fig. 4b). DiI and DiA labelling in area 19 largely overlapped; for example, after injections in area 17 with DiA lateral to DiI, only a few DiA labelled cells (Fig. 4c) were position slightly more lateral to DiI labelled cells in area 19 (Fig. 4d).

Figure 3. Fluorescence photomicrographs of coronal sections of the visual cortex in a normal 29-day-old kitten taken with the N2 (530-560 nm) filter. (*a*) A section caudal to the core of a DiA injection site (on the *left*) and a DiI injection site (on the *right*) in area 17. In area 18 retrogradely labelled cells were grouped mainly in two clusters, with DiI label mainly medial to DiA. (*b*) High power view of a cluster of mainly DiI labelled cells in area 18 shown in a. The majority of labelled cells were in superficial layers (2, 3 and upper 4) with a few in deep layers (5 and 6). (*c*) Labelling with DiI in area 19 from a region rostral to that shown in *a* and *b*. Note there are very few labelled cells in area 19. Scale bars, 0.5 mm.

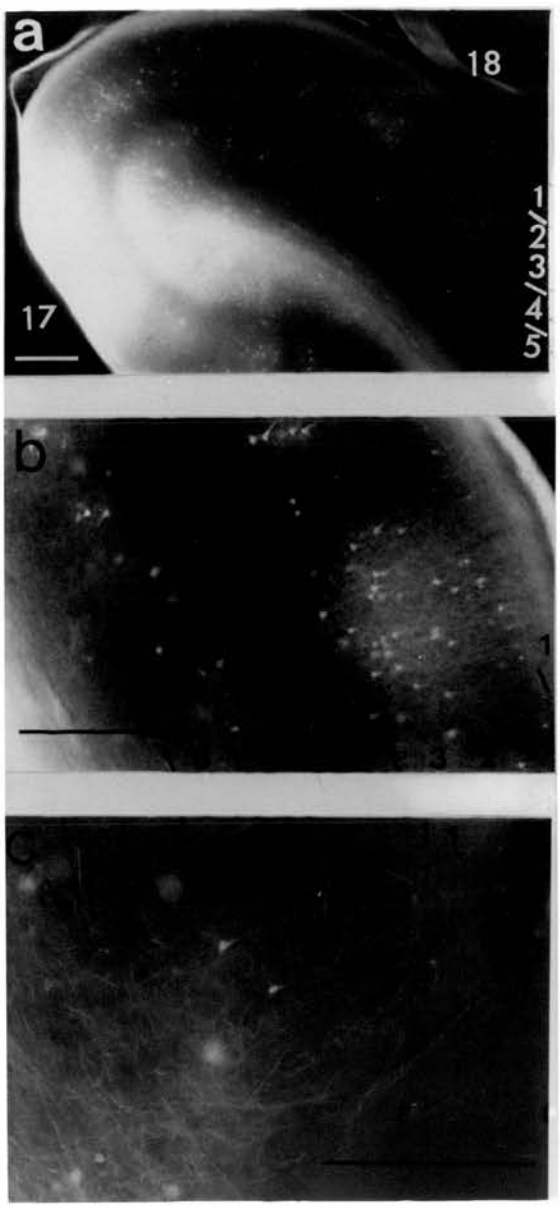
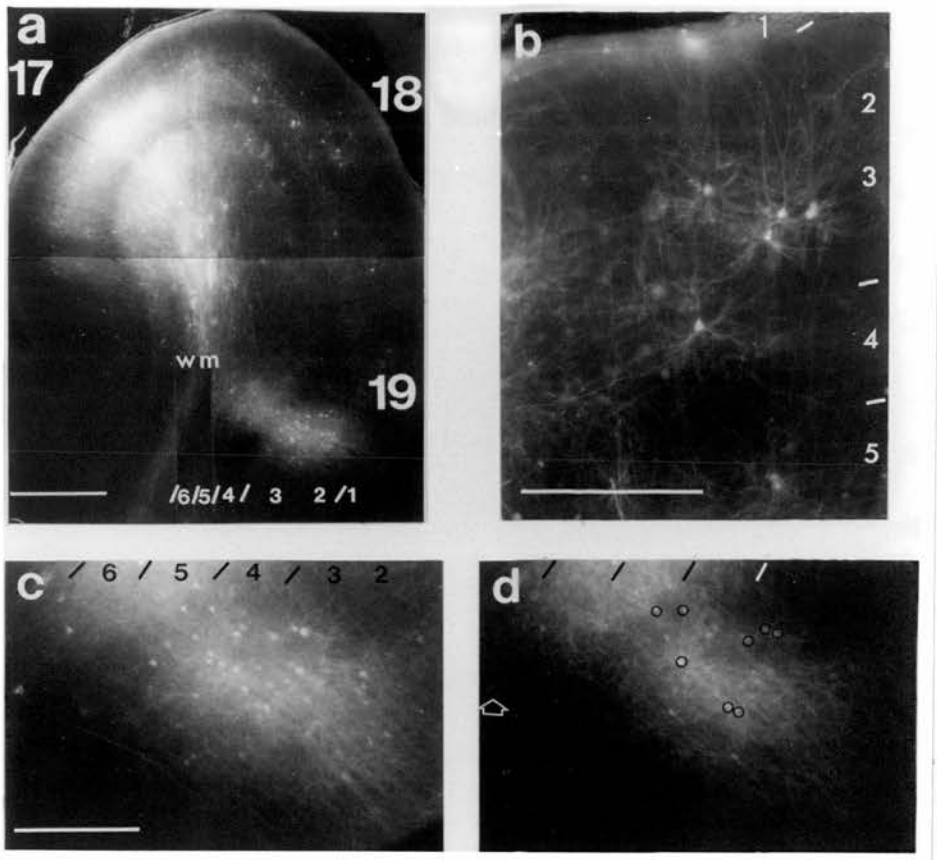

a
18
17
1
2
3
4
5
b
1
3
c

Figure 4. Fluorescence photomicrographs of coronal sections of the visual cortex in a lesioned 1-month-old kitten; (*a* and *c*) photomicrographs are taken with an I2/3 "fluorescein" filter; (*b* and *d*) are taken with the N2 "rhodamine" filter. (*a*) A section caudal to the core of the DiI injection site (on the *right*) and the DiA injection site more lateral (on the *left*) in area 17 illustrating reduced labelling in area 18 and increased labelling in area 19 after the lesion. (*b*) High power view of labelled cells in area 18 below the layer 1 lesion. The majority of cells were double labelled as their fluorescence was coming through under fluorescein and rhodamine filters. (*c-d*) High power view of a labelled cluster in area 19 viewed through a fluorescein filter (in *c*) and a rhodamine filter (in *d*). With a rhodamine filter, DiA labelled cells are visible only faintly (indicated by the *open arrow* in *d*); double labelled cells are intensely labelled (indicated by the *open circles* in *d*); others are DiI labelled cells. Scale bar in *a*, 1 mm; scale bar in *b*, 0.5 mm; scale bar in *c* refers also to *d*, 0.5 mm.


a
17
18
wm
19
/6/5/4/ 3 2/1
b
1
2
3
4
5
c
/ 6 / 5 / 4 / 3 2
d

3.4.4 Labelling in area 18

In normal kittens aged one month, fibres projecting from area 17 to area 18 terminated in patches in area 18 and cells projecting back to area 17 were arranged mostly in clusters although there were a few projecting cells between the clusters (Fig. 3a,b). The majority of cells and fibres were in superficial layers but a smaller number were also deep in layer 6 (Fig. 3a,b).

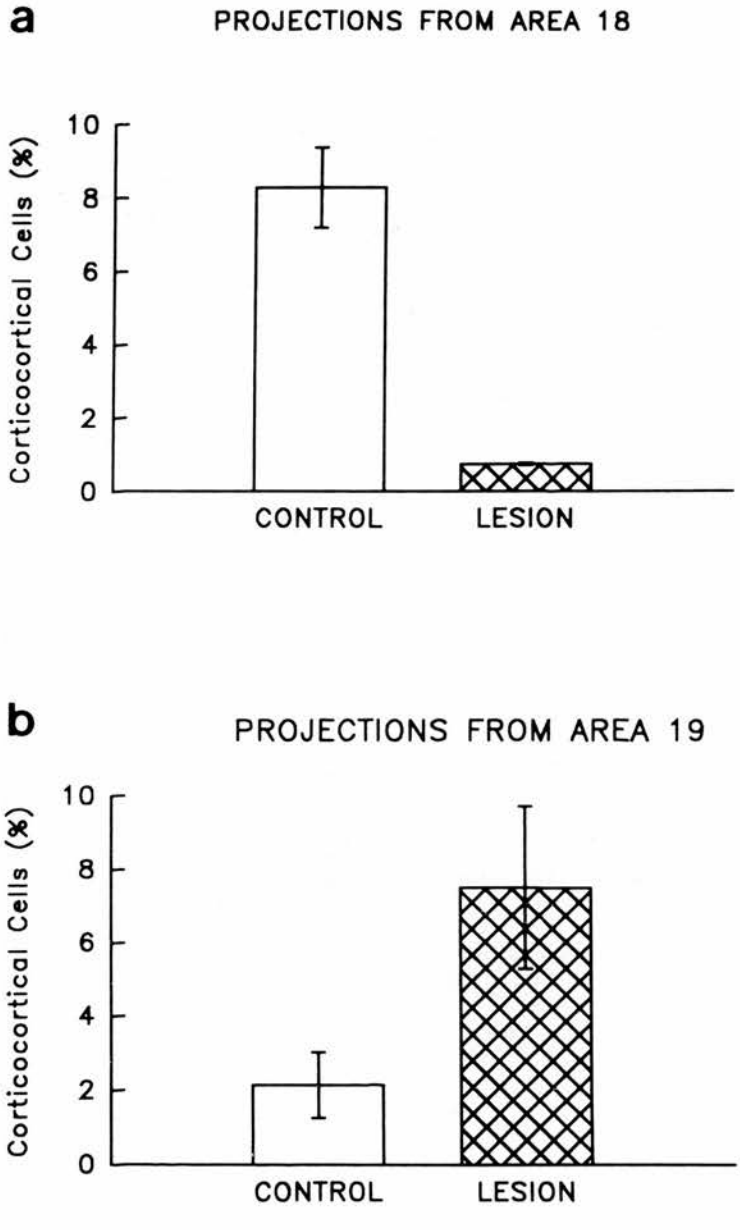
In all lesioned hemispheres, projections to and from the portion of area 18 below the layer 1 lesion were reduced in density compared to normal. Fig. 4a shows the cortex of area 18 below the lesion with a sparse continuous distribution of axonal arbors and cells in the deep layers (5 and 6) and the superficial layers (upper 4, deep 3 and 2). In the superficial layers, I often found small groups of 4 to 5 cells per section and in the deep layers there were a few scattered cells (Fig. 4a,b).

In each lesioned hemisphere and the control I calculated densities of fluorescently labelled association cells in superficial and deep layers in area 18 (see Materials and Methods). I found the densities of cells in the superficial layers significantly lower (Welch's test; $P<0.015$) in lesioned (94 ± 11 cells mm^{-3}) than in normal animals (1330 ± 156 cells mm^{-3}). Using these values and values for the total cell densities I calculated the percentages of corticocortical projecting cells in normal and lesioned animals. Fig. 5a shows changes in the percentages of labelled corticocortical cells within the superficial layers in area 18. In the superficial layers of area 18 in normal animals there were 8.26 ± 1.09 % cells projecting to area 17. After the layer 1 lesion the mean value was 0.75 ± 0.02 %, which was significantly different from normal values (Welch's test; $P<0.02$).

3.4.5 Labelling in area 19

In the normal kittens at P30, fewer cells projected from area 19 to 17 than from area 18 (Figs. 3c). In the lesioned hemisphere, the density of labelled cells,

Figure 5. The percentages of corticocortical cells in area 18 (*a*) and in area 19 (b) in lesioned and control hemispheres calculated from the peak density of labelled cells in these areas. In the lesioned kittens the percentages of cells in superficial layers projecting from area 18 to a specific point in area 17 were significantly lower (Welch's test; $P<0.02$) in lesioned ($0.75 \pm 0.02\%$; mean $\pm$ S.E.M.) than in normal ($8.26 \pm 1.09\%$) animals; projections from area 19 to area 17 seemed higher in lesioned ($7.5 \pm 2.2\%$) than in non-lesioned ($2.14 \pm 0.88\%$) animals but these values were not significantly different.


a
PROJECTIONS FROM AREA 18
Corticocortical Cells (%)
10
8
6
4
2
0
CONTROL
LESION
b
PROJECTIONS FROM AREA 19
Corticocortical Cells (%)
10
8
6
4
2
0
CONTROL
LESION

particularly those in superficial layers, appeared higher than in the normal (Fig. 4a,c,d). In the normal animals, the percentage of corticocortical cells projecting to area 17 ranged from 1.09 % to 3.9 % (mean was 2.14 $\pm$ 0.88 %) (Fig. 5b). After the lesion, the percentage ranged from 6.6 % to 11.7 % (7.5 $\pm$ 2.2 %). However these values were not significantly different from normal values (Welch's test; $P<0.15$).

3.5 *DISCUSSION*

The present findings indicate that layer 1 is involved in the development of corticocortical connections in the visual cortex. My main conclusions are:

1. Layer 1 lesions in the region of the area 17/18 border at the time of birth significantly decrease connections between areas 17 and 18. After the lesion, the incidence of double labelled cells in area 18 was high, suggesting that remaining projecting cells in area 18 have wider uptake sites in area 17 that is probably due to the larger terminal arborizations. The decrease in the projections between areas 17 and 18 does not seem to be caused by generalised damage in the cortex, since I found that the thickness of the cortical layers and the cell density below the lesions were similar to normal.

2. In lesioned animals, projections between areas 17 and 19 appeared increased which may suggests that a decrease in the corticocortical projection in one region could be compensated by their increase in a different region.

3. I postulate that intercellular signaling via layer 1 is important for the development of corticocortical connections in the visual cortex.

I suggest two mechanisms by which fibres in layer 1 could be involved in the formation of cortical connections: i) by general effects on the immature cortical neurones or ii) by correlation of activity which strengthens appropriate connections.

i) One possibility is as that layer 1 has some transitory effects on the immature neurones. Marin-Padilla (1984, 1992) proposed that layer 1 transmits information

that is necessary for the function of all pyramidal cells. In his view, the Cajal-Retzius cells (the first mature cells in layer 1) serves as a link between primitive corticopetal afferent projections and cortical cells. Since layer 1 contains numerous afferents from various sources from the early embryonic stages, it could be that this layer acts as the transitory innervation network until other, i.e. thalamocortical afferents, can take their role (Meyer and Gonzalez-Hernandez, 1993). It has been shown that after a neonatal lesion of the optic nerve in the rat, many cortical stellate cells in layer 4 do not retract their transitory projection from layer 1 (Peinado and Katz, 1990). This experiment indicates the necessity for layer 4 cells to retain projections to layer 1 in the absence of thalamic afferents. On the other hand, it seems that postnatal activity in thalamocortical afferents provides a critical cue for these cells to acquire their specific phenotypic and functional characteristics (Peinado and Katz, 1990). Similarly, Windrem and Finlay (1991) found in the hamster, that after a neonatal thalamic ablation layer 4 stellate cells were absent; these authors hypothesized that cells initially destined for layer 4 remain or become specified as the pyramidal cells of layers 2 and 3.

ii) Other possibility is that signaling via layer 1 to pyramidal cells could be a result of the correlated activity transmitted via long horizontal fibres in layer 1. In the cat's visual cortex, LGN afferents to layer 1 are dense at birth and then decrease in density within the first postnatal month (Kato et al., 1984). In the cat, after reverse suture at P30 (opening of the sutured eyelid and closing the other lid), the density of afferents to layer 1 is much higher than in normal animals (Kato, 1986). This experiment suggests involvement of layer 1 in the plasticity of the LGN axons to the visual cortex, which could be by the correlation of activity in layer 1 and 4 after opening of the eyes.

LGN axons project to layer 1 very early and innervate the MZ at about embryonic day 40 (Ghosh and Shatz, 1992b). They run for long distances forming small branches (Ghosh and Shatz, 1992b). It is possible that the early activity of

afferents, i.e. the geniculocortical input to layer 1, is forwarded to the apical dendrites of corticocortical cells in the visual cortex where it is matched with similar information briefly held in cells in other visual areas. This may influence the shaping of their connections. This hypothesis is similar to that proposed for layer 1 in the adult brain, in the processes of learning and memory (Vogt, 1991). According to Vogt's hypothesis (1991), layer 1 is involved by a process called event holding. The event holding begins with sensory activation of thalamic, cortical and cholinergic projections to the apical dendritic bouquets of pyramidal neurones in layer 1. The sensory event is sampled in the sensory cortex and then briefly held in the dendritic bouquets to integrate with similar events held in other cortical areas. Once sensory events are matched they are either stored in the short-term memory, cause a motor response, and/or a new stimulus occurs. The event holding in layer 1 allows parallel processing of sensory information in different cortical areas. For example, visual sensory inputs are decomposed in different systems in the cerebral cortex for movement, colour, stereopsis and orientation. A unified visual perception could result from the inputs being held in each of these systems and processed in a parallel way. Thus, if layer 1 is involved in matching sensory inputs between cortical areas, could this also be the key to the development of corticocortical connections in particular processes such as the refinement of cortical connections, i.e. in strengthening the appropriate and eliminating the inappropriate connections. The refinement of corticocortical connections could be the result of a process similar to event holding, as suggested by Vogt. During the formation of corticocortical connections (i) afferent inputs to layer 1 (i.e. thalamic) to the apical dendrites of pyramidal corticocortical cells that are intense, large, novel or long/lasting (as suggested by Vogt) would activate groups of cells in striate and extrastriate cortex located in similar retinotopographic regions, and (ii) simultaneous activity of particular corticocortical cells may increase synaptic strength in that pathway, whereas others may be eliminated.

CHAPTER 4

IBOTENIC ACID INDUCED LESIONS OF THE LATERAL GENICULATE NUCLEUS

4.1 ABSTRACT

The main objectives of these experiments were to lesion the lateral geniculate nucleus (LGN) in the neonatal kitten, make reconstructions of the lesions in each animal at the age of one month and examine the changes in the visual cortex. The LGN was lesioned with ibotenic acid (IBO), an excitotoxic compound. LGN neurones are spontaneously active at birth and thus can be destroyed with IBO. The amount of IBO needed to cause complete or large ablation of the LGN was determined. In each lesioned animal I reconstructed the position of the lesion and estimated the retinotopographic projections of the remaining LGN. After a complete or large LGN lesion, there was a significant decrease in the overall cortical thickness and the thickness of layer 4 in the lateral part of area 17 and in area 18, but not in the medial part of area 17. The decrease in the thickness of layer 4 was accompanied by a significant increase in cell density in layer 4. It seems that the changes in the cortex observed in Nissl-stained sections are the result of removal of large numbers of afferents to the visual cortex. Other methods are necessary to examine other anatomical and functional cortical changes (see Chapter 5).

4.2 INTRODUCTION

In the adult cat, LGN axons project topographically to visual cortical areas i.e. areas 17 and 18 (Garey and Powell, 1968) and terminate in cortical layers 1, 4 and 6 (LeVay and Gilbert, 1976). Geniculocortical axons are present in the cortex before corticocortical connections are established while corticocortical cells are still migrating to superficial layers 2 and 3 (Henderson, 1982; Shatz and Luskin, 1986; Ghosh and Shatz, 1992b). The first LGN axons arrive in the subplate (SP) of the future visual cortex by embryonic day 36 (E36) (Ghosh and Shatz, 1992b). Only the future layer 1 (called at this stage the marginal zone) is innervated as early as E40 by a few LGN axons that pass unbranched through the cortical plate and run tangentially within the marginal zone for long distances forming small branches (Ghosh and Shatz,

1992b). Layer 6 is innervated by E50 and layer 4 by E60 (Ghosh and Shatz, 1992b). This spatiotemporal relationship between the ingrowing LGN axons and immature corticocortical cells raises the question of a possible role for thalamocortical axons in the formation of corticocortical connections. In order to examine the relationship between the LGN axons and corticocortical connections I studied the consequences of early postnatal geniculocortical denervation on the visual cortex. Lesions of the LGN were made in the neonate, when corticocortical connections are starting to form. The LGN is not fully developed at this age (Kalil, 1978), but it is spontaneously active (Adrien and Roffwarg, 1974); thus it can be lesioned with an excitotoxic compound. In the present study, the LGN was lesioned with intracerebral injections of ibotenic acid (IBO) that can produce axon-sparing local neural degeneration, similar to kainic acid (Schwarcz et al., 1979; Kohler and Schwarcz, 1983). After the lesion, animals were allowed to develop until the end of the first postnatal month. Here I present: (a) a quantitative analysis and a reconstruction of the LGN lesion with IBO and (b) evidence for changes in the visual cortex after geniculocortical denervation; in the next chapter I present changes in corticocortical connections after the LGN lesion.

4.3 MATERIALS AND METHODS

4.3.1 Animals

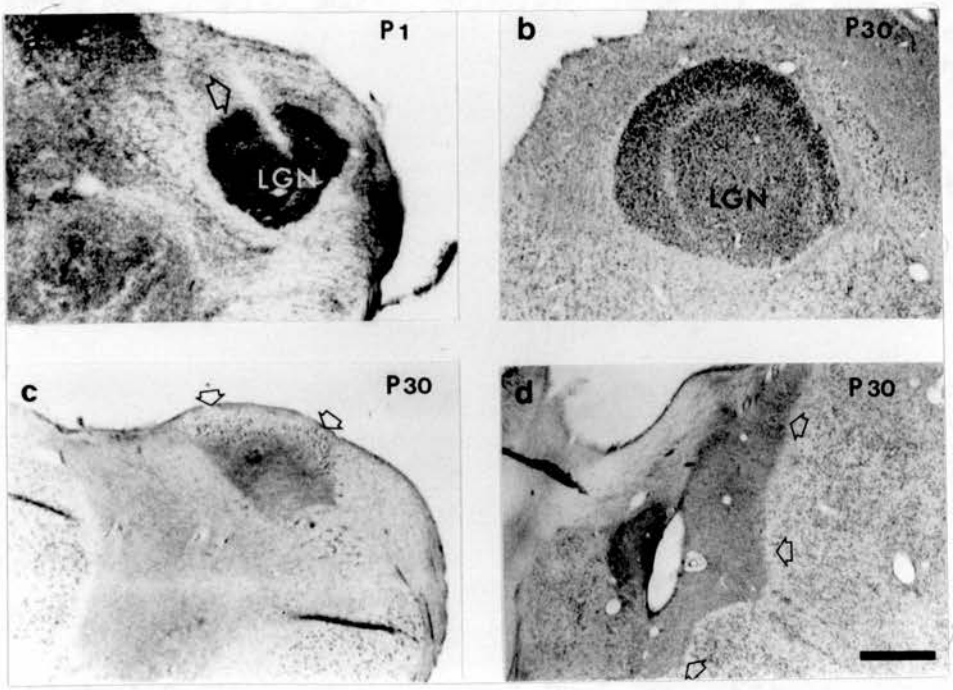
A total of 31 kittens obtained from an isolated laboratory colony were used in the present study. The stereotaxic coordinates to locate the LGN in the newborn kitten (based on the Rose and Goodfellow atlas (1973)) were confirmed in one of the newborn kittens, by injecting it in the left and right LGNs with black ink and sacrificing it immediately after injection. Other animals were given a single unilateral injection of IBO (Research Biomedical Inc.) in the LGN. Injections were made at P1 (7 animals), P2 (6 animals), P3 (8 animals), P4 (2 animals), P5 (5 animals) and P8 (2 animals). Five of these animals died shortly after the injections of IBO. Twenty

five kittens that recovered were perfused transcardially at end of the first postnatal month. The reconstruction of the lesion was made in each animal and is described in this Chapter. Seventeen of the lesioned animals were injected with tracers *in vivo* or in fixed brain and labelling is described in Chapter 5. Five days before they were perfused, 5 animals received two injections, one of DiI and one of DY, in the cortex of area 18 in the lesioned side. Injections were made at P28 (2 animals) and P29 (3 animals). In twelve of the remaining animals, the lipophilic tracer DiI (alone or with DiA) was applied in fixed brains to cortical area 17 in the lesioned and contralateral non-lesioned hemispheres. These animals were perfused at P28 (2 animals), P29 (5 animals) and P30 (5 animals).

4.3.2 Ibotenic acid lesion

Anaesthesia was induced and maintained following the procedure described in Chapter 2 (see Materials and Methods). The anaesthetized animal was placed in a stereotaxic frame and a small unilateral craniotomy was made over the suprasylvian gyrus. The stereotaxic coordinates based on the Rose and Goodfellow atlas (1973) were used to locate the LGN. These were confirmed in one of the newborn animals that had black ink injected in the left and right LGNs. Injections were performed with a Hamilton syringe (capacity 5 μl) held by a micromanipulator. The needle was positioned under a dissection microscope near the surface of the cortex and then lowered by motor under the surface. The stereotaxic coordinates used were: A 2 mm anterior to anteroposterior level zero (AP0); L 5 mm lateral to the midline; H^D 9.5 mm below the surface of the cortex. Immediately after ink was injected the animal was perfused transcardially, the brain was cut coronally and counterstained with cresyl fast violet. Examination of the needle tracks (easily visible with black ink) indicated the correct position of the needle within the LGN (Fig. 1a). The same stereotactic coordinates were used in other littermates to inject the IBO solution. IBO was dissolved in phosphate buffered saline pH 7.4 (4 μg μl^{-1}). The amount of

Figure 1. *(a)* Bright-field photomicrograph of a cresyl violet-stained coronal section of the normal LGN in a 1-day-old kitten injected with black ink. The location of the needle track is marked with the *open arrow*. (*b*) Bright-field photomicrograph of a cresyl violet-stained coronal section of the LGN in the non-lesioned side in a 1-month-old kitten. (*c,d*) Bright-field photomicrographs of cresyl violet-stained coronal sections of the lesioned region in a 1-month-old kitten that had unilateral lesions of the LGN on the day of birth. In *c*, the location of the LGN residua is marked with the *open arrow*. In *d*, the LGN was lesioned completely; the glial scar is marked with the *open arrow*. Scale bar in *d* refers all photographs, 1 mm.


P1
LGN
b
P30
LGN
c
P30
d
P30

the IBO solution given was varied (volume: 1.2-5 μl); I found that the optimal dose was 3-4.3 μl (4 μg μl^{-1}), as it caused severe or complete LGN degeneration. Larger amounts of the IBO solution (volume: 4.9-5 μl) often caused death soon after the injections, probably due to the leakage of the IBO to the respiratory and cardiovascular centres in the brain stem. Each animal was given a single injection of IBO in the LGN in one of the two sides over a period of 10 min After the injection, the needle was left in the place for an additional 10 min to avoid backflow along the injection track. The wound in the scalp was sutured and the kitten was allowed to recover and returned to its mother. Each animal was perfused transcardially at 1 month of age or injected *in vivo* with tracers DY and DiI and perfused 5 days later.

4.3.3 Histological preparation

After fixation, lesioned and contralateral non-lesioned LGNs were dissected together from each brain. LGNs were placed in a solution of 20% sucrose in phosphate buffer to equilibrate for 1-2 days and than cut coronally at a thickness of 50 μm. A 1-in-3 series of sections was mounted on gelatine coated slides and counterstained with cresyl fast violet.

4.3.4 Analysis

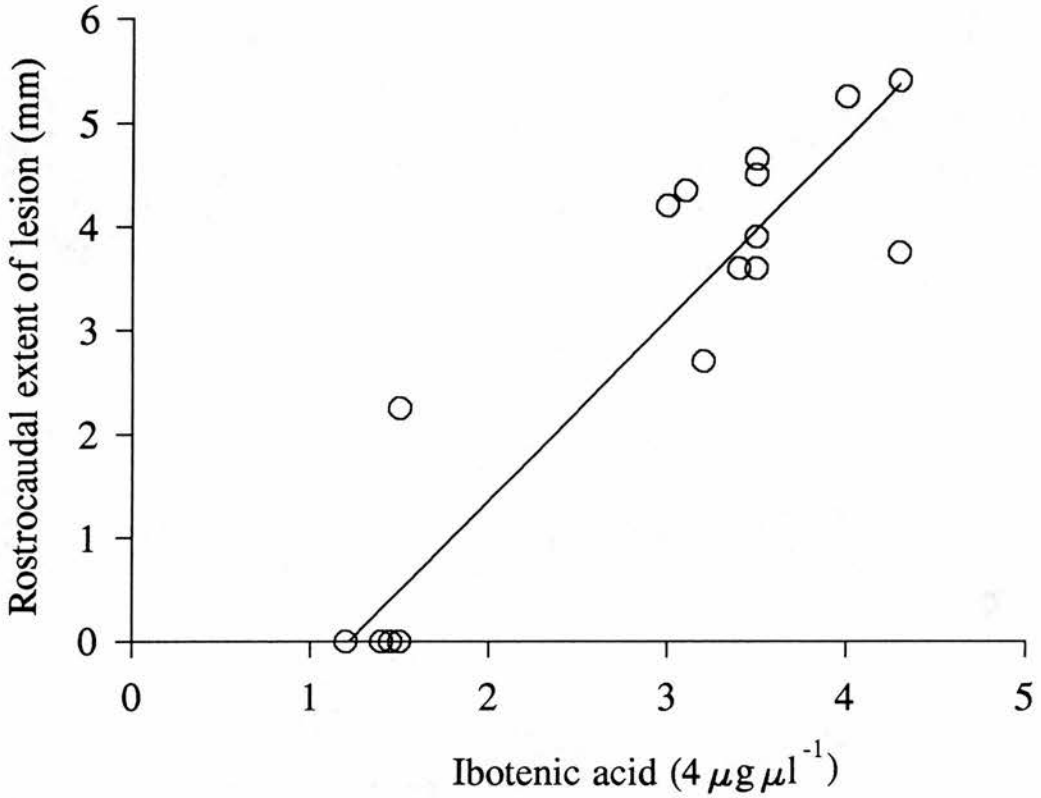
The reconstruction of the LGN lesions

A reconstruction of the position and the extent of each lesion was made by examining a 1-in-3 series of Nissl-stained sections.

1. The relationship between the overall size of the lesion and the amount of the IBO given was tested with a regression analysis (Fig. 2). The size of the lesion was determined by measuring its rostrocaudal extent.

2. I measured the rostrocaudal sizes of the LGNs in the non-lesioned contralateral

Figure 2. Graph plots the rostrocaudal extent of the lesion against the amount of ibotenic acid given. The regression line was calculated and plotted. The correlation coefficient was high ($r=0.936$, $p<0.0001$).


Rostrocaudal extent of lesion (mm)
6
5
4
3
2
1
0
0
1
2
3
4
5
Ibotenic acid (4 μg μl-1)

hemispheres (n=19) and in those lesioned hemispheres where there appeared to be no signs of a lesion (n=9). Data were compared using Student's *t*-test to see whether there was any decrease in the size of the LGN in the lesioned hemispheres.

3. Each lesion was reconstructed and the remaining LGNs were located along the anteroposterior (AP) and mediolateral (ML) axes. In cases where LGN lesions were not complete, I first calculated the rostrocaudal extent of the remaining nuclei. This was a valuable indicator to see whether the lesion was small or large (cases when more than half of the LGN was lesioned). The AP and ML positions of each LGN residua were estimated by comparing the location of the residua with the appearance of the LGN in the contralateral non-lesioned side as guided by Rose and Goodfellow's kitten atlas (1973) and Reinoso-Suarez's adult atlas (1961). The position of the lesion was drawn on the standardised coronal sections and dorsolateral view of the LGN (Fig. 3) and compared with the representation of the retinotopographic maps of the visual field in the LGN (Fig. 4; Sanderson, 1971).

Effects of the LGN lesion on the visual cortex

The effects of the destruction of the LGN on the visual cortex were examined in 3 brains, two of which had 100% of the LGN destroyed and one with a large lesion where a very few neurones remained. Cortical measurements were taken in the lesioned and the non-lesioned contralateral hemisphere at the same position in the retinotopic map near AP0. In each animal, five Nissl-stained coronal sections were chosen (600 μm apart). A total of four sampling sites were selected per section (Fig. 5c). Three of these were in the cortex of area 17, one was medial to the suprasplenial sulcus, the second was lateral to the same sulcus and the third was just before the apex of the lateral gyrus. The fourth location was in the cortex of area 18 in the region within the middle of the lateral gyrus. In each selected location, I

Figure 3. The site and the extent of the remaining parts of the LGNs were marked on standardised drawings of the LGN. Drawings on the *right* are standardised representative coronal sections from the LGN at three different levels: *1* rostral; *2*, medial; *3*, caudal. The drawing on the *left* is a standardised dorsal view of the LGN; the location of the sections on the *right* are indicated by *broken lines*. The overview of the remaining LGN was produced by aligning the drawings shown on the *right;* for example, the *stippled region* represents the remaining LGN in one of the kittens that had a severe unilateral lesion of the LGN on the day of birth. Laminae A, A_1 and C of the LGN are marked in *1*; abbreviations: ANT., anterior; POST., posterior; LAT., lateral; MED., medial.

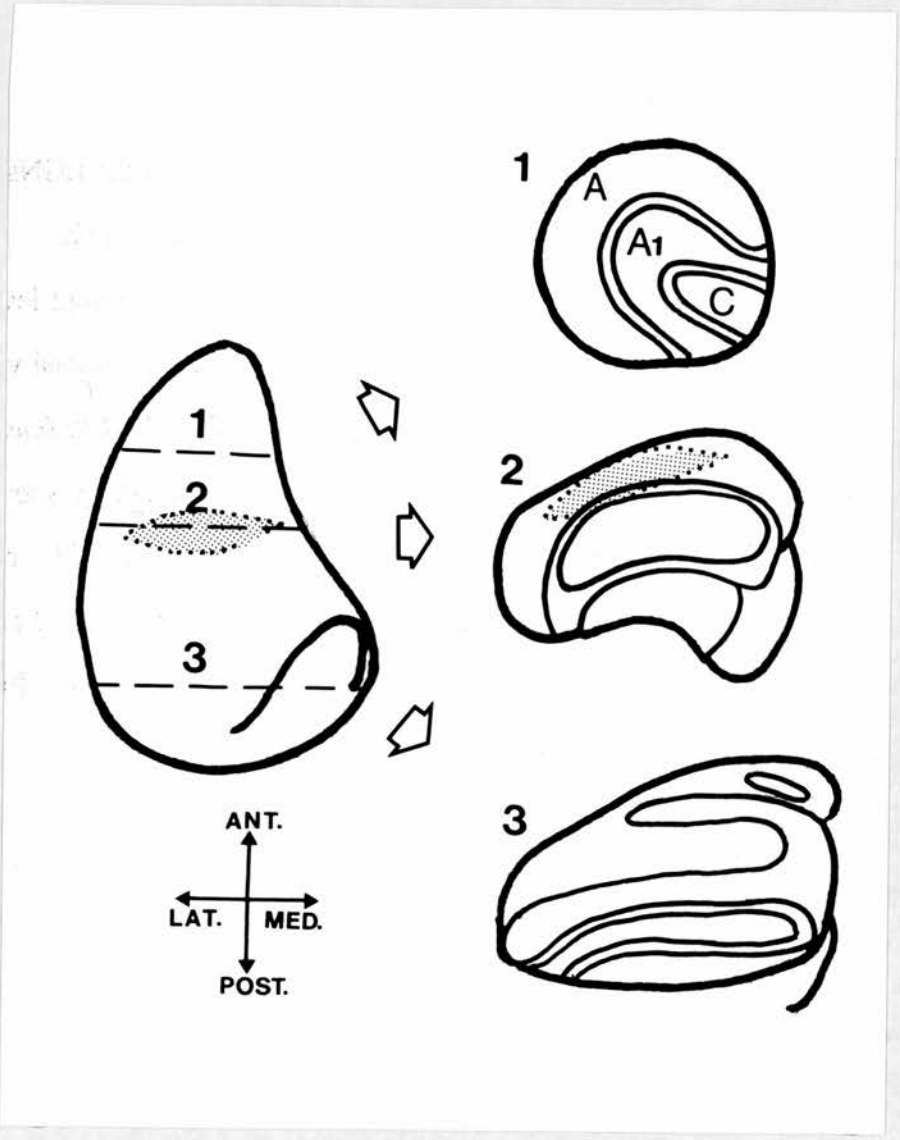
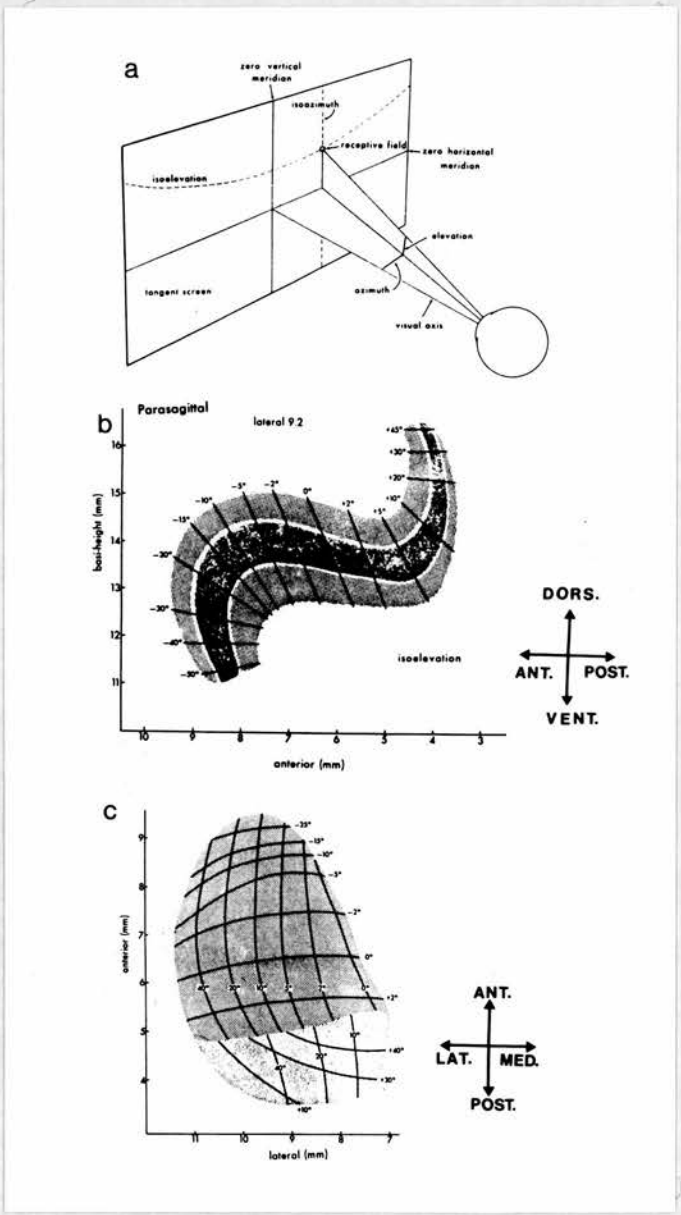

1
2
3
ANT.
LAT.
MED.
POST.
1
A
A1
C
2
3

Figure 4. (*a*) Diagram of the tangent screen showing how the visual direction of a receptive field is expressed in terms of the two angles *azimuth* and *elevation.* Note that on the tangent screen *iso-azimuth lines* are straight and *iso-elevation* lines become hyperbolae. Definitions of terms used in this diagram are given here: *fixation plane*, the plane which includes the visual axis and the zero horizontal meridian (HM); *azimuth*, the angle between the line of sight through the projection of the centre of the receptive field on the fixation plane on the one hand and the visual axis on the other (positive to the right of the visual axis, negative to the left); *elevation*, the angle between the line of sight through the centre of the receptive field and the fixation plane (positive upwards from the fixation plane, negative downwords). (*b*) Pattern of isoelevations on a parasagittal section through the middle of the LGN. (*c*) Plan view of the LGN showing the pattern of isoelevations and isoazimuths for cells that lie close to the dorsal surface of the nucleus. The posterior part of the LGN is shaded differently from the rest of the nucleus, since the dorsal surface of the posterior part is 1.5-2 mm above the dorsal surface of the main body of the nucleus. (From Sanderson, 1971)


a
zero vertical meridian
isoazimuth
receptive field
zero horizontal meridian
isoelevation
elevation
tangent screen
azimuth
visual axis
b
Parasagittal
lateral 9.2
basi-height (mm)
anterior (mm)
isoelevation
DORS.
ANT.
POST.
VENT.
c
anterior (mm)
lateral (mm)
ANT.
LAT.
MED.
POST.

measured the thickness of the different layers (6, 5, 4, 2/3 and 1) and the overall cortical thickness. I also counted cells and calculated cell densities in the SP, layers 6, 4 and upper and lower part of layers 2/3. Cell counts were made using a 10x eye piece and 40x objective with a grid in the eye piece. Neurones were counted in different layers in a surface of 0.024 μm^2 (25 squares). The cell numbers were divided by the volumes of the cortex containing these cells to calculate cell densities. Values for the cortical thickness in the lesioned and the control non-lesioned sides were compared using a Mann-Whitney test. Other data were compared using Student's *t*-test or, in the cases where the standard deviations were not equal, the alternative Welch's test.

4.4 RESULTS

4.4.1 Characteristics of ibotenic acid induced lesions

The amount of IBO given and the extent of the lesion

IBO acts similarly to the neurotoxin kainic acid to cause local neural degeneration and glial proliferation but spares axon terminals and axons of passage, whose cells bodies lie at a distance from the injection site (Coyle et al., 1978; Schwarcz et al., 1979; Kohler and Schwarcz, 1983; Woodword et al., 1989). However IBO has the advantages of being less toxic to the animals than kainic acid and produces more discrete lesions (Kohler and Schwarcz, 1983). In all animals, the LGN on the control side appeared normal (Fig. 1b). On the lesioned side, the extent of the LGN degeneration varied from no signs of a lesion to cases where lesions extended beyond the LGN's boundaries. I measured the overall rostrocaudal extent of the lesion (n=20), which correlated well with the amount of IBO given ($r=0.936$, $p<0.0001$) (Fig. 2).

Small amounts of IBO (volume: 1.2-1.8 μl) caused either small LGN lesions (2 animals) or no signs of a lesion (8 animals).

Volumes of IBO solution from 3 to 4.3 μl produce lesions with an average rostrocaudal extent of 4.33 ± 0.26 mm (n=12). This dose was enough to cause severe (Fig. 1c) or total degeneration (Fig. 1d) of the LGN (if the injection was in the centre of the LGN) as the average rostrocaudal size of the LGN in the contralateral non-lesioned side was slightly smaller (3.99 ± 0.18 mm; n=19) than the average extent of the lesion. This amount of IBO caused low postoperative mortality (only 2 of 16 injected animals died after the operation).

A larger volume of IBO solution (4.9-5 μl) was more toxic to the animals and in 3 of 4 injected animals death occurred soon after the operation.

The reconstruction of the LGN lesion following IBO injections

The reconstructions of the LGN lesions (n=25) revealed that the nucleus was: i) lesioned completely (n=2) (Fig. 1d), ii) lesioned severely with only a small part of the nucleus remaining (n=8) (Fig. 1c), iii) lesioned to about half its normal size (n=3) and iv) very little (n=3). In the remaining 9 lesioned animals, the LGN appeared normal; the rostrocaudal size of the LGN was smaller (3.57± 0.19 mm, n=9) than the size of the LGN in the contralateral non-lesioned side (3.99 ± 0.18 mm; n=19) but this was not statistically significant (Student's *t*-test, P>0.15).

I estimated the anatomical positions and retinotopographic representations in the remaining parts of the LGNs, using projection maps of the visual field by Sanderson (1971) (Fig. 4). The visual hemifield projects to each LGN and is divided by the horizontal meridian (HM) into the upper and lower part (Fig. 4a; see also in Chapter 6, Fig. 1c,e perimeter charts showing the visual hemifield). The upper visual hemifield, above the HM, projects to the caudal half of the LGN, and the lower visual hemifield field, below the HM, projects to the rostral half of the LGN (Fig. 4b,c). The medial edge of the LGN represents the region of the visual hemifield near

the vertical meridian (VM); the lateral edge of the LGN represents more lateral regions of the visual hemifield (Fig. 4c). In the LGN the representation of the lateral part of the visual field is compressed if compared to the representation of the visual field near the VM (Fig. 4c).

In 2 cases I observed a complete (Fig. 1d) disappearance of neurones from the entire LGN, which was replaced by extensive gliosis.

In animals with a small part of the LGN remaining (n=8), the position of each residua was located along the AP axis. As the residua often contained only a small number of cells, it was rarely possible to say whether they belonged to the medial or the lateral part of the LGN. In three cases the LGNs residua were estimated to be from the middle part of the LGN along the AP axis, which represents the region of the visual hemifield near the HM. In four animals the remaining LGNs were from the rostral LGNs that represents the region of the visual hemifield from about 5^{o} below the HM (Fig. 3). In one animal the residua was from the rostral and lateral half of the LGN, which represents a small region of the lower visual hemifield about 20^{o} lateral to the VM.

In three animals measurements of the rostrocaudal length of the remaining LGN revealed that about half of the LGN was lesioned. All lesions seemed to be in the caudal half of the LGN which normally represents the region of the visual hemifield above the HM.

Three animals had small lesions, one restricted to the rostrolateral third of the LGN, a second in the rostromedial part of the LGN and a third in the most caudal part of the LGN. The rostrolateral lesion probably affected only a very small representation of the visual hemifield from about 10-20^{o} below the HM and from 20^{o} lateral to the VM. The rostromedial lesion affected the representation of the lower visual hemifield near the VM. The caudal lesion affected the representation of the most peripheral part of the upper visual hemifield.

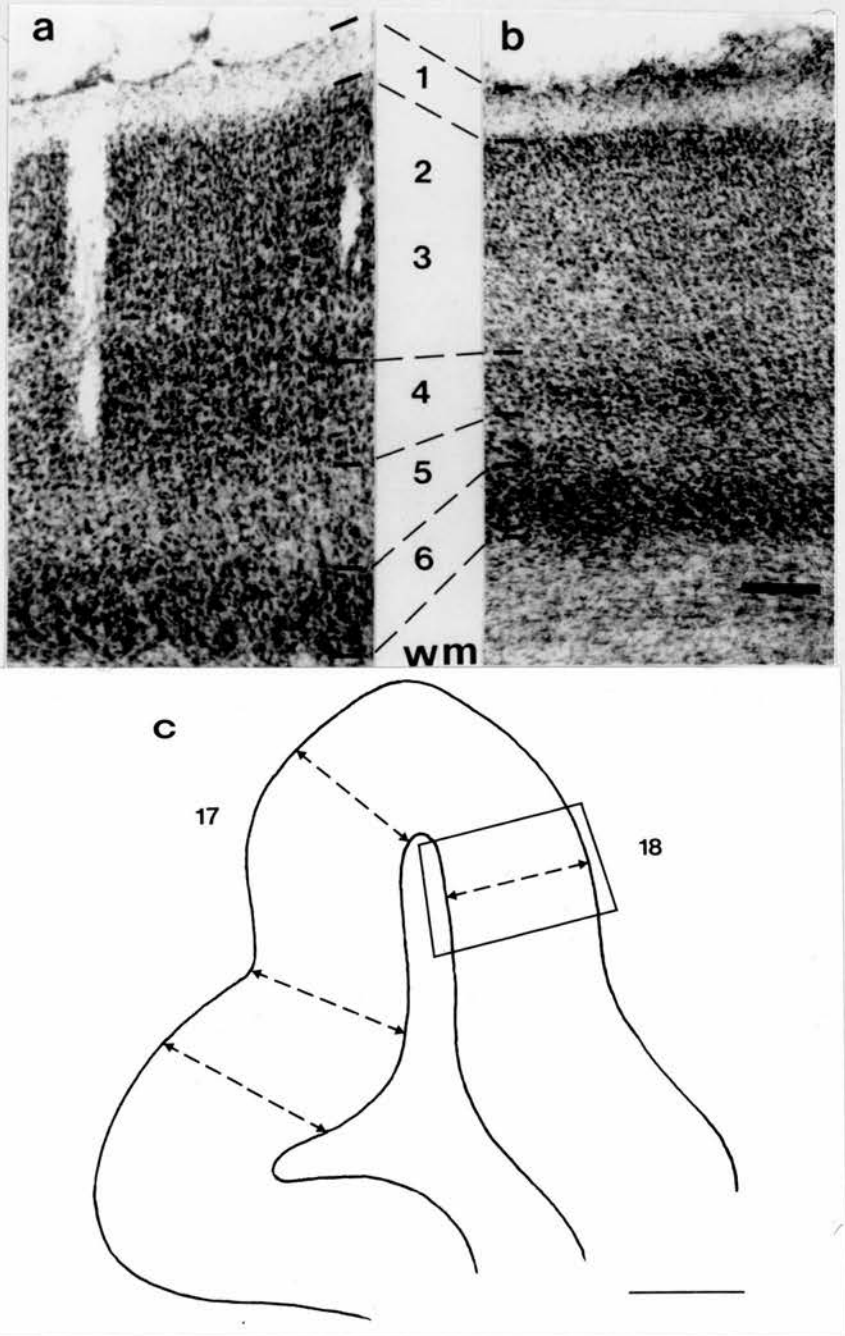
4.4.2 Changes in visual areas 17 and 18 after the LGN lesion

Overall the cortex in the lesioned hemispheres appeared thinner and the curvatures were not well pronounced (Fig. 5). Quantitative analysis of the thickness of the cortex in area 17 lateral to the suprasplenial sulcus and just before the apex of the lateral gyrus as well as in the middle part of area 18 confirmed that indeed the cortex was narrower on the lesioned side, by about 8.5 % (range, 6-10%). The cortex in the medial part of area 17 (medial to the suprasplenial sulcus) was about 16 % thicker after the lesion. I shall first describe thickness of the cortex in area 18 and the lateral part of area 17 and then in the medial part of area 17.

The cortex of area 18 was significantly thinner ($P<0.0002$) in the lesioned (1.26 ± 0.02 mm) than in the non-lesioned (1.41 ± 0.02 mm) hemispheres. Similarly the cortex in the lateral part of area 17 was significantly thinner ($P<0.03$) after the lesion (1.23 ± 0.03 mm) compared to the cortex on the non-lesioned sides (1.36 ± 0.04 mm). The cortex of area 17 above the suprasplenial sulcus seemed thinner in the lesioned (1.23 ± 0.03 mm) than the non-lesioned hemispheres (1.32 ± 0.03 mm) but this was not statistically significant. In all three examined zones layer 4 was significantly thinner ($P<0.002$) after the lesion; in area 18 its thickness was about 0.13 ± 0.01 mm after the lesion and 0.17 ± 0.01 mm in the control sides, in the lateral part of area 17 it was about 0.13 ± 0.01 mm and near the sulcus it was about 0.11 ± 0.01 mm. In the control, the thickness was 0.19 ± 0.05 mm in the lateral part of area 17 and 0.19 ± 0.01 mm near the sulcus. The overall decrease of this layer was about 49% (range 30-79 %). Other cortical layers 2/3, 5 and 6 had a tendency to be narrow in all three regions; this was significant only in area 18 in layers 2/3 ($P<0.0004$) and in layer 5 ($P<0.03$). In all examined regions layer 1 seemed wider but this was not statistically significant.

The medial cortex in area 17 was significantly thicker ($P<0.0001$) in the

Figure 5. (*a-b*) Bright-field photomicrographs of cresyl violet-stained coronal sections of the visual cortex in a 1-month-old kitten that had a complete unilateral lesion of the LGN on the day of birth. Cortical layers in a non-lesioned hemisphere are shown in *a* and in a lesioned hemisphere are shown in *b*. Both photographs were taken in regions of area 18 indicated in *c* by the *rectangle*. Layers are indicated by *broken lines*; wm, white matter. (c) Camera lucida drawing of a coronal section through the visual cortex in the same kitten; sampling sites are shown with *broken lines,* one in the cortex of area 18 and three in the cortex of area 17 (one medial to the suprasplenial sulcus, a second in the same sulcus and a third just before the apex of the lateral gyrus). Scale bar in *b* refers also to *a*, 0.2 mm; scale bar in *c*, 1 mm.


a
b
1
2
3
4
5
6
wm
c
17
18

lesioned (1.42 $\pm$ 0.03 mm) than in the control sides, at a similar rostrocaudal location (1.22 $\pm$ 0.02 mm). In this region there was a significant increase in the thickness of layer 1 ($P<0.003$), layers 2/3 ($P<0.004$) and layer 6 ($P<0.001$). Layer 4 seemed thinner on the lesioned side (1.13 $\pm$ 0.01 mm), but this was not statistically significant.

My impression on cell density was that it was higher in layer 4 in area 18 in lesioned than in non-lesioned hemispheres (Fig. 5). Quantitative analyses confirmed that the cell density in layer 4 was significantly higher (*t-test*; $P<0.048$) in the lesioned (11 198 $\pm$ 274 cell mm $^{-3}$) than in the contralateral side (9 479 $\pm$ 640 cell mm $^{-3}$). I also quantified the cell densities in layers 6, 4, lower part of layer 2/3 and upper part of layer 2 and the SP in areas 17 and area 18. All values seemed higher in lesioned hemispheres than in the control non-lesioned hemispheres but the increases were not statistically significant (except the value for cell density in layer 4 in area 18).

4.5 DISCUSSION

In these experiments I determined the amounts of IBO needed to cause complete or large ablation of the LGN in newborn kitten. Secondly, I reconstructed the position of the lesion in the LGN along the AP and the ML axes and estimated the retinotopographic projections to the remaining LGN. The reconstruction of the LGN lesion was relevant to determine denervated regions in the visual cortex (discussed in Chapter 5). Thirdly, after complete or large LGN lesions I found an overall decrease in cortical thickness; the thinning of the cortex was significant in the lateral part of area 17 and in area 18 but not in the medial part of area 17. The decrease of cortical thickness was mainly due to the reduced thickness of layer 4. Thinning of other layers in these region was evident (except for layer 1) but this was significant only in layers 2/3 and 5 in area 18. Cell density was higher in all layers after the lesion, but this was significant only in layer 4 in area 18.

Similarly to present findings, neonatal electrolytic thalamic ablation in hamsters was reported to induce a significant thinning of the cortex and profound changes in layer 4 such as loss of stellate cells in layer 4 and an overall decrease in cell number per column unit (Windrem and Finlay, 1991). Differences in cortical cell numbers after thalamic lesions in two studies may be consequences of the differences in the maturation of the cortex and the positions of LGN axons at the time of birth in the cat and hamster. For example in hamster at the time of birth LGN axons are in residence under the cortical plate (Naegele et al., 1988); in the cat at the time of birth axons have reached their target, cells in layer 4 (Ghosh and Shatz, 1992). Windrem and Finlay (1991) suggested that in the hamster the presence of LGN axons in the vicinity of the cortex is important for the proliferation and differentiation of cells in the superficial layers as these cells are undergoing their last cell division. However, in the cat visual cortex, the division of cells destined for superficial layers and the arrival of LGN axons occur before birth, and we would not expect the same effect. Thus the decrease in cortical thickness and increase in cell density seem to be the consequences of the removal of the large number of afferent axons. However the interesting question is how the removal of these afferents affects the connectivity in the cortex (i.e. corticocortical). The importance of LGN afferents on corticocortical cells is the subject of Chapter 5.

CHAPTER 5

LGN LESION AND CORTICOCORTICAL CONNECTIONS

5.1 ABSTRACT

In this study I examined corticocortical connections in 1-month-old kittens with unilateral LGN neonatal lesions. Corticocortical connections were labelled with fluorescent dyes in fixed brains or in vivo. After either partial or complete LGN destruction, the clustering in deafferented visual area 18 was more crude and overall projections were decreased. Corticocortical connections showed a wider rostrocaudal distribution. The likely explanation of these changes is that corticocortical connections which are starting to form at birth, in order to stabilise and increase at particular points need the activity from the LGN; if the LGN is silent the activity in the cortex has different pattern and level which may cause different distribution of corticocortical cells.

5.2 INTRODUCTION

From a number of experiments performed on kittens in the past, it is clear that early visual experience is important for the refinement of ipsilateral corticocortical and callosal connections in the visual cortex. Various means of visual deprivation were used: dark rearing, eyelid suturing, surgical strabismus and enucleations (Lund et al., 1978; Innocenti and Frost, 1979; Lund and Mitchell, 1979; Innocenti and Frost, 1980; Innocenti et al., 1985; Price and Blakemore, 1985; Price, et al., 1994). Overall, it seems that continual binocular visual deprivation (BD) from birth leads to an abnormally high loss of callosal (Innocenti et al., 1985) and ipsilateral corticocortical projections (Price and Blakemore, 1985). On the other hand, monocular visual deprivation (MD) and strabismus lead to the maintenance of the projections that would normally be eliminated; for example strabismus (Lund et al., 1978; Innocenti and Frost, 1979), MD or mononuclear enucleation (Innocenti and Frost, 1979) cause a more widespread distribution of callosal neurones than is the case in normal kittens. In ipsilateral corticocortical projections, MD prevents the normal decrease in cell density in clusters (Price, et al., 1994). These experiments

suggest that the effects of visual experience on the developmental refinement of corticocortical connections must be the consequence of alterations in either the level and/or the pattern of neural activity. The role of activity has been demonstrated in the segregation of geniculocortical axons in layer 4, which was blocked with tetrodotoxin (TTX) (Stryker and Harris, 1986). Experiments done in the past which examined the effect of binocular eyelid sutures on the formation of corticocortical connections did not entirely deprive the retina of visual stimuli (Loop and Sherman 1979; Spear et al., 1978). Even without visual stimuli, the developing visual system is not silent; for example, in the neonatal ferret there are spontaneously generated waves of activity in the retinal ganglion cells and furthermore the activity of neighbouring cells is more correlated than those of distant cells (Wong et al., 1993). Could it be that spontaneous electrical activity from the LGN shapes the formation of corticocortical connections? In the present experiment I address this question. I examined the effects of the neonatal LGN lesion on the formation of corticocortical connections. These connections were visualised with tract-tracers in fixed brain and in vivo in one month old kittens.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Successful injections were carried out on 17 kittens aged one month; 2 of them were normal kittens and 15 had a unilateral neonatal lesion of the LGN. All animals were from an isolated laboratory colony. In 2 normal kittens, aged P29, DiI and DiA were applied to cortical area 17 in fixed tissue. In 10 lesioned kittens, DiI (alone or with DiA) were applied to area 17 in the same way as in normal kittens, both in the lesioned and contralateral non-lesioned hemispheres. Injections were made in 2 kittens with total LGN lesions (P1/29; day of the lesion/day of injection); 4 kittens with small LGN residua (P1/29, P2/30, P3/28, P3/30); 3 kittens with lesioned caudal halves of the LGN (P3/28; P3/30, 2 kittens); and one kitten with a

rostromedial LGN lesion (P2/28). The other five lesioned kittens received two in vivo injections, one of DiI and one of DY, in the cortex of area 18 on the lesioned side. Injections were made in four kittens with small LGN residua (P2/28, 2 kittens; P4/29, P5/29) and one kitten with a small rostrolateral LGN lesion (P1/29).

5.3.2 Application of DiA and DiI in fixed brains

Kittens were perfused transcardially and injected following the procedure already described (see Material and Methods in Chapter 2). Small crystals, one of DiI and the other of DiA, were placed side-by-side in area 17 in the medial bank of the lateral gyrus (above the suprasplenial sulcus) where area 17 represents the region of the visual field about 5° lateral to the vertical meridian (VM) (Fig. 1a,b,c,d). All pairs of injections (except in one lesioned hemisphere) were placed in the rostral half of area 17, at anteroposterior (AP) levels between -2 to 0, where this area represents the region of visual field 2-5° below the vertical meridian (HM). One pair of injection was made in the caudal half of area 17, about 7 mm posterior to anteroposterior level zero (AP0), a region that represents the visual field about 4-5° above the HM. The position of injection sites in area 17 were confirmed by examining the distribution of labelled cells in the LGNs in two non-lesioned animals. Fig. 2. illustrates LGN label after injection in area 17 at about AP level -2 and its position in the retinotopographic map on the LGN. The LGN labelling was achieved after diffusion of dyes in fixed tissue for period over 2 years.

Labelling with carbocyanine dyes DiI and DiA in fixed brain in 1-month-old kitten

The majority of injected brains were stored at room temperature for 3 to 4.5 months. There were three exceptions; two non-lesioned hemispheres were stored for two years to reveal LGN labelling and one lesioned hemisphere was stored for 2 weeks.

Figure 1. (*a,b*) Photomicrographs of a dorsolateral view (in *a*) and a medial view (in *b*) of the cat brain showing the typical locations of injection sites in areas 17 and 18. The *open circle* represents the position of DY tracer; the *black circles* represent the positions of carbocyanine (DiI and DiA) dyes. (*c,e*) Perimeter charts showing the extent of the visual hemifield (*stippled region*) in areas 17 (in *c*) and 18 (in *e*). The *open circles* represent the vertical meridian (VM); the *black squares* represent the horizontal meridian (HM); the *black triangles* represent the most peripheral part of the visual field; the *stars* represent injection sites (note that in area 17, DiI and DiA injection sites are marked by a single *star* symbol). (*d,f*) Drawings showing the visual hemifield (*stippled region*) represented in areas 17 (in *d*, a medial view) and 18 (in *f*, a dorsolateral view) on the surface of the cat brain; positions of typical injection sites are marked in each area (*white stars*). Lines of iso-azimuth are represented as *thin solid lines* and lines of iso-elevation are represented as *thin broken lines*. An approximate position of AP0 is indicated by an *arrow* in *d;* the *black star* in *f* represents the area centralis. Other abbreviations: ANT, anterior; POST., posterior; LAT., lateral; MED., medial; DORS., dorsal; VENT., ventral. Scale bar in *b* refers also to *a*, 10 mm. (*c-d* are simplified from Figures of Tusa et al., 1978, 1979)

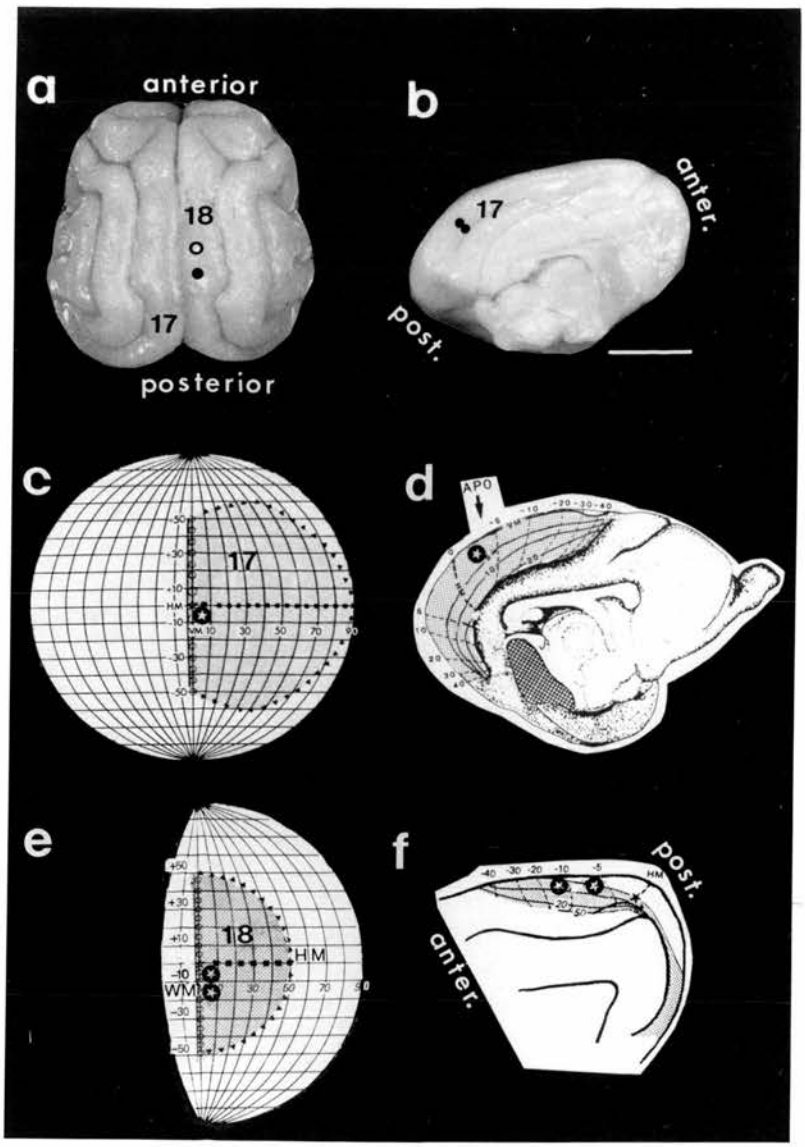
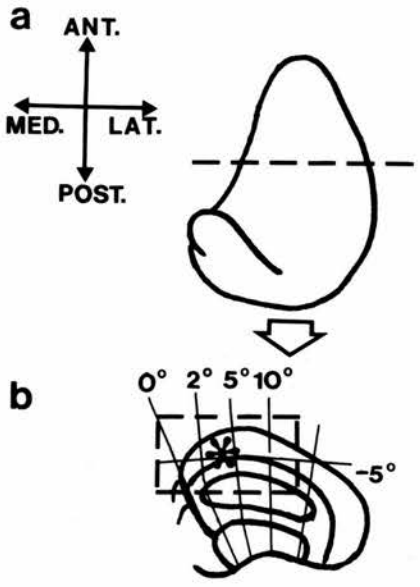
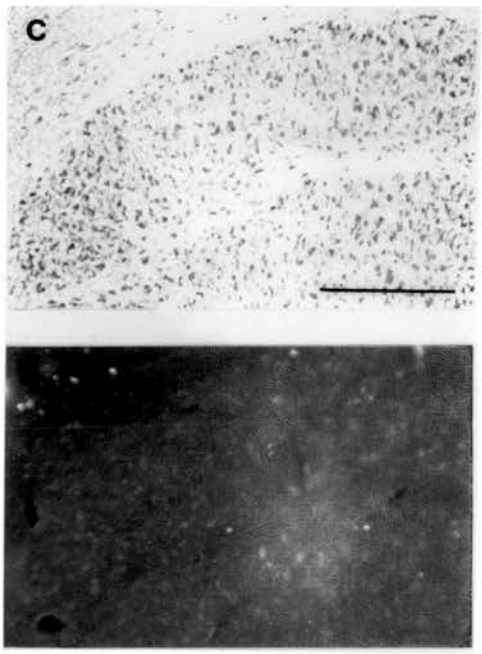

a
anterior
18
17
posterior
b
17
anter.
post.
c
17
HM
WM
d
APO
e
18
HM
WM
f
post.
anter.

Figure 2. (*a)* Drawing of the LGN (a dorsal view); the coronal section of the nucleus (shown in *b*) has been taken at the level indicated by the *broken line*. (*b*) Drawing shows the position of cells (*asterisk*) in the LGN retrogradely labelled after the injection of DiI in area 17. The region oulined by the *box* (*broken line*) is shown in photomicrographs in *c* and *d*. Lines of iso-elevation and a line of iso-azimuth are represented as *thin solid lines*; note the position of label in a region that represents the visual field at about -5^{o} below the horizontal meridian and about 5^{o} lateral to the vertical meridian. (*c*) A bright-field photomicrograph of a cresyl violet-stained coronal sections of the LGN in a 1-month-old kitten; the photomicrograph illustrate laminae A and A_1 of the LGN. (*d*). A fluorescence photomicrograph of the adjacent section coronal to section shown in *c* demonstrates DiI retrogradely labelled cells resulting from injection in area 17 in fixed tissue after diffusion for period over 2 years. Abbreviations: ANT, anterior; POST., posterior; LAT., lateral; MED., medial; Scale bar in *c* refers also to *d*, 0.5 mm


a
ANT.
MED.
LAT.
POST.
b
0°
2°
5°
10°
-5°


c

When the carbocyanine dyes were allowed to diffuse for 2 weeks, only labelled axons were seen in area 18 (Fig. 3). After about 3 months it was possible to see small branches and a few faintly labelled cell bodies in area 18 (Fig. 4). After 4 months, the cell bodies of reciprocal corticocortical cells in area 18 were clearly labelled (Fig. 5). The difference in the diffusion times needed to label axons projecting to area 18 (anterograde label) and cells projecting from area 18 (retrograde label) allowed me to some extent to distinguish two populations of projections. Fibres and arborization visualised in area 18 after short diffusion times (less than 3 months) were likely to be anterogradely labelled; after longer diffusion times, additional label was more likely to belong to reciprocal cortical cells in area 18. Labelling in area 19 occurred after diffusion times of about 3 months. This was mainly of fibres and only a few retrogradely labelled cells.

The longer diffusion time needed to start to visualize cells in areas 19 than in area 18 could be caused by a difference in the distance; thus the longer time was needed for diffusion of dyes over the larger distance from area 17 to area 19 than from area 17 to area 18. The second reason could be that the diffusion of lipophilic dyes is much slower after the onset of myelination "probably as the myelin acts as a sink for lipophilic dyes" according to the description by Balice-Gordon et al. (1993). Balice-Gordon et al. (1993) reported that after the beginning of myelination in motor axons, DiI diffuses anterogradely at approximately 0.2 mm per day (in unmyelinated embryonic axons about 0.6 mm per day), whereas DiA diffuses at approximately 0.4 mm per day (in unmyelinated embryonic axons about 1 mm per day). In the kitten's cortex, myelination starts around P21-23 (Cragg, 1972). The maturation of the cortex follows a lateromedial gradient; thus, area 19 matures before area 18 and area 18 matures before area 17; we would expect that around P30, myelination would be most advanced in area 19, followed by area 18 and least advanced in area 17.

Figure 3. A montage of fluorescence photomicrographs of a coronal section through visual area 18 after injection of DiI in area 17 in a 1-month-old kitten that had a severe unilateral lesion of the LGN on the day of birth. Cortical laminae are marked by *short lines* in area 18. Note only labelled fibres after diffusion time of 2 weeks. Scale bar, 0.25 mm.

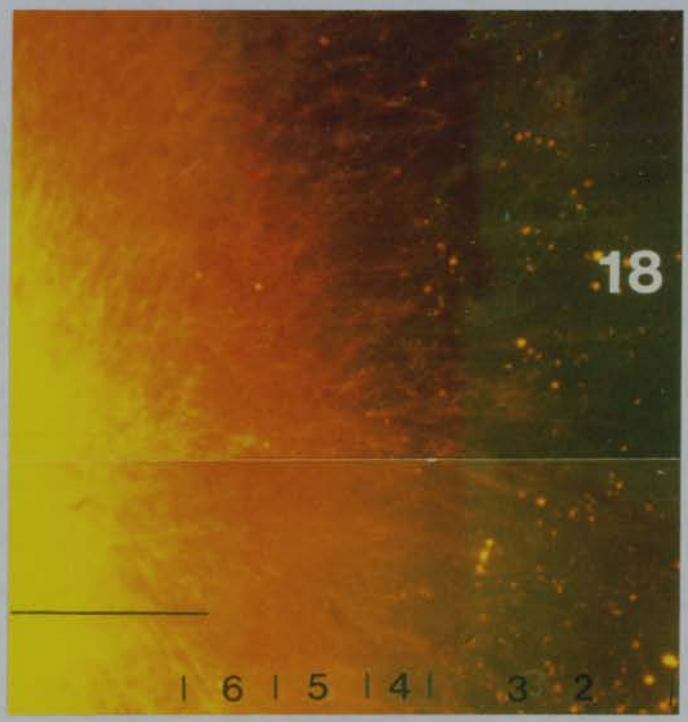

18
| 6 | 5 | 4 | 3 2

Figure 4. A montage of fluorescence photomicrographs of a coronal section through the lateral part of visual area 17 and through visual area 18 after injection of DiI in area 17 in a 1-month-old kitten that had a complete unilateral lesion of the LGN on the day of birth. Cortical laminae are marked by *short lines* in areas 18. The position of the area 17/18 border is indicated by *an arrow.* Labelling is of mainly fibres after a diffusion time of 3.5 months. Scale bar, 0.5 mm.

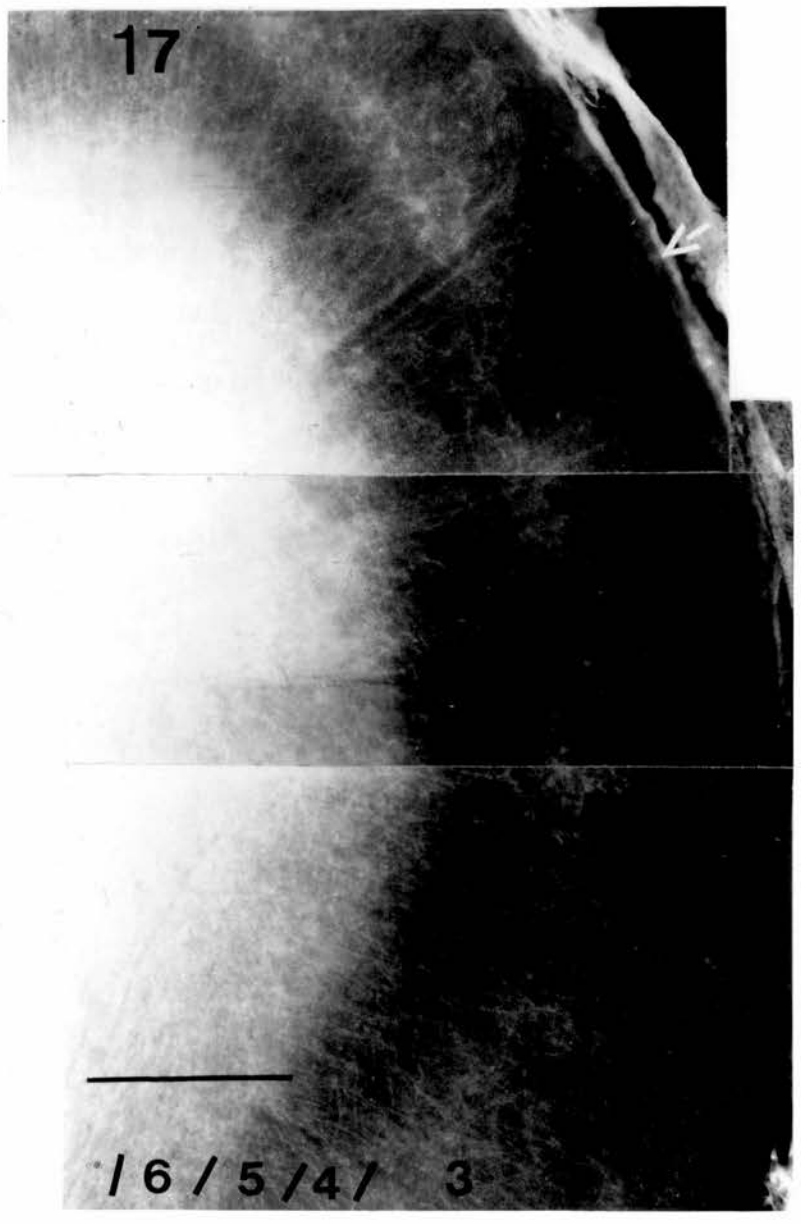
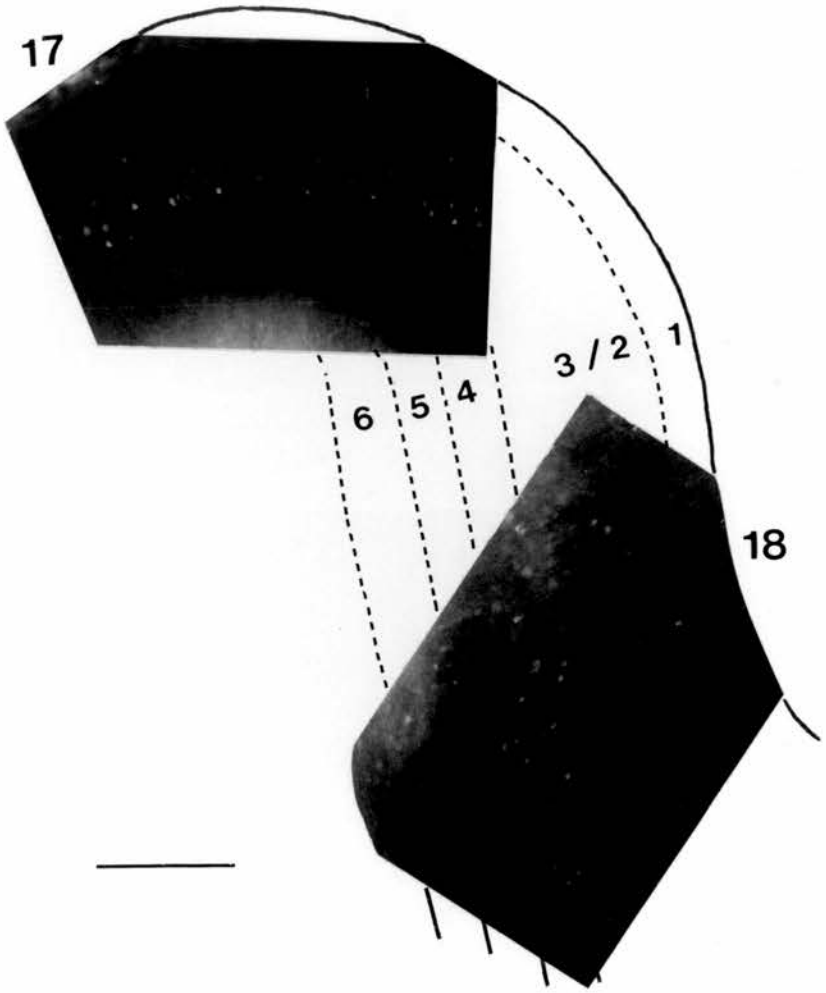

17
/6/5/4/ 3

Figure 5. A camera lucida drawing of a coronal section through the lateral part of visual area 17 and through visual area 18 after injection of DiI in area 17 in a 1-month-old kitten that had a severe unilateral lesion of the LGN on the day of birth. Photomicrographs show labelling of cell bodies in areas 17 and 18 after a diffusion time of 4.5 months. Scale bar, 0.5 mm.


17
1
3 / 2
4
5
6
18

5.3.3 Surgical procedures and application of DY and DiI in vivo

The lesioning of the LGN was described in Chapter 4. Injections in area 18, one of DY and the other of DiA, were mainly placed in the middle of the crown of the lateral gyrus where this area represents the region of the visual hemifield between 5-10° from the VM (Fig. 1a,e,f). In one animal injections were placed medial in area 18 where this area represent the visual hemifield less than 5° from the VM. DY was placed at AP levels between 5-7, and DiI was placed near AP0 or 2-3 mm further rostral (Fig. 1a,e,f). The surgical procedures and application of tracers were the same as described previously (see Chapter 2).

5.3.4 Histological preparation

Fixed brains were blocked and placed in a solution of 20% sucrose in phosphate buffer to equilibrate for 1-2 day. Brains with DiI and DY in vivo and two hemisphere with DiI and DiA labelling in fixed tissue were cut on a freezing microtome at a thickness of 50 μm. Others were cut on a vibroslice at a thickness of 200 μm, as after longer diffusion times (as more lipophilic dye was getting in the membrane) dyes would more easily leak out from the cell membrane during cutting at low temperature.

5.3.5 Analysis

Cortical reconstruction

In all lesioned animals, the denervated regions in areas 17 and 18 were located on the visual maps using the retinotopographic reconstruction of the remaining LGNs and the lesion (see Chapter 4). The position of denervated cortical region and each pair of injections were marked on the standardized drawings similar to that shown in Fig. 1d and f. Overall the visual map in the cortex has a similar rostrocaudal orientation to that in the LGN (see Fig. 1), the rostral half of the LGN projecting to the rostral half of the visual cortex (representing the lower visual field),

whereas the caudal half of the LGN projects to the caudal half of the visual cortex (representing the upper visual field) (Garey and Powell, 1968; Sanderson, 1971; Tusa et al., 1978, 1979). The VM is represented along the medial side of the LGN and projects to the area 17/18 border, and the lateral border of area 19 (Garey and Powell, 1968; Sanderson, 1971; Tusa et al., 1978, 1979).

Two types of injections were analysed: one where the location of the cortical injection sites matched the location of the LGN lesion and the second where the location of the cortical injection sites matched the location of the remaining LGN. The second type of injections was used to examine whether the lesions would have any effect on the non-retinotopographic region of the cortex.

The quantification of the peak density of labelled corticocortical cells in superficial layers was done following the procedure described in Chapter 3. The rostrocaudal convergence was calculated following the procedure described in Chapter 2.

5.4 *RESULTS*

5.4.1 Cortical reconstruction

Cortical reconstruction and injections in fixed brain

In four animals with small amounts of LGN left, the size and the position of the remaining LGN cells suggested that the most of the visual cortex was denervated except a small region. In three of these animals the remaining parts of the LGNs were from its rostral third, and represented the visual hemifield from about 5^0 below the HM. The expected remaining projections would be in the region of area 17 rostral to AP0; all injections in area 17 were about 1-2 mm posterior to AP0, thus slightly caudal to the expected innervated region (Fig 1c,d). In one animal, the remaining LGN was from its middle region in the AP direction and represented visual hemifield near the HM; in this animal injections were placed further caudal to

the denervated part, about 7 mm posterior to AP0 (in the region that represents upper visual hemifield).

In three animals where the caudal half of the LGN was lesioned (expected to denervate the caudal half of the cortex, from about 3 mm posterior to AP0 onwards) injections were placed near AP0 or 2 mm further caudal, thus in the region that was still receiving LGN axons. Similarly, injections were placed in non-denervated cortex in one animal with a small rostral and medial LGN lesion.

Cortical reconstruction and injections in vivo

In three animals injected in vivo in area 18, the residua were composed of a small group of cells. In 2 of these animals I estimated that their AP location was the rostral third of the LGN (represents the region in the visual hemifield about 2-5^{o} below the HM); this suggested that the most of the visual cortex was denervated except a small region around AP0. Injections were placed in the middle of area 18: DiI was about 2 mm anterior to AP0, and DY about 5 mm anterior to AP0 (Fig. 1a,e,f). In the third animal the residua was from the middle of the LGN (represents the region of the visual hemifield near the HM). Both injections were placed in the middle of area 18, DiI near AP0 (5^{o} below the HM) and DY about 5 mm anterior to AP0. In all three animals I expected that both DiI and DY injections were in the denervated part of the cortex. However, in all of them there were a few DiI labelled cells in the LGN; these cells could be those with non-retinotopographic projection to the cortex, or another possibility was that they were cells dispositioned further caudally after the ablation of the caudal part of the LGN. In any case, labelling in the LGN suggested that regions injected with DiI were not totally denervated, whereas regions injected with DY were.

In the fourth animal a small residua was from the rostral and lateral half of the LGN. This suggested that most of the visual cortex was denervated except a small region of the lower visual hemifield about 20^{o} lateral to the VM. Injections

were placed in the rostromedial part of area 18 (DiI about 3 mm anterior to AP0, and DY about 7 mm anterior to AP): these were expected to be in the denervated cortex. In the remaining LGN there were DiI and DY labelled cells. However, labelled cells in the LGN were expected to be in the middle or medial edge of the LGN, but these cells were located at the lateral edge of the residua; this suggested the presence of geniculocortical axons to injected cortex from non-retinotopographic part of the LGN.

The fifth animal had a small LGN lesion located in the rostral and lateral third of the LGN. The lesion probably affected only a small representation of the visual field from about 10-20^{o} below the HM and from 20^{o} lateral to the VM. The LGN label confirmed that injections were made in the part of area 18 that contains LGN axons; DiI and DY labelled cells were located at the lateral edge the LGN, near the glial scar.

5.4.2 Labelling with DiI and DiA in fixed brains

A) Distribution of corticocortical projections in area 18 in one month old kittens

In all non-lesioned contralateral hemispheres and non-deafferented cortex, fibres were distributed in patches in the cortex of area 18, as seen in the normal one month old kitten (see Fig. 5 in Chapter 2). Fibres were clustered just before entering the cortex; sometimes individual fibres were seen crossing this region to arborize in deep layer 5 and 6. Between patches, the density was lower due to the presence of fewer fibres and less arborization. From deep layers, individual fibres were seen crossing layer 4 (many running in parallel) towards the upper part of layers 2/3. Diffusion times of 3 months allowed visualisation of dense arborization in layers 2/3. After 4 months there were clearly labelled cells in area 18 in superficial layers 2, 3 and upper 4, with fewer in the lower part of layer 5 and upper 6.

B) Distribution of corticocortical projections in area 18 in one month old kittens after the neonatal LGN ablation

After injections in the hemispheres with complete LGN destruction or injections whose positions in area 17 matched the location of the LGN lesion, labelling in area 18 was different from normal in the mediolateral direction. Label covered most of the mediolateral extent of area 18 (Fig. 3). The distribution of labelling in the cortex was fairly uniform in the deep layers of area 18 (layers 5 and 6) and in the underlying white matter as well as in the superficial cortical layers (there was only a slight hint of patchiness). In the rostrocaudal direction, labelling in area 18 was at the retinotopographically corresponding level to the injected regions in area 17 but extended over a larger territory in area 18 than in the contralateral non-lesioned hemispheres. Measurements of rostrocaudal convergence indeed revealed that convergence was significantly larger (*t*-test; $p<0.0007$) in the denervated visual cortex (6.2 ± 0.2 mm, n=3) than in the non-lesioned hemispheres (4.7 ± 0.2, n=8). I also measured the convergence after injections where positions did not match the location of the LGN lesion. Although laminar and clustered labelling in the rostral cortex after severe lesion of the caudal half of the LGN was similar to that in the non-lesioned hemispheres, the convergence was significantly larger (*t*-test; $p<0.0007$) in the non-denervated rostral cortex (7 ± 0.4 mm, n=3) than in the non-lesioned contralateral hemispheres. In contrast, after a small rostromedial lesion, the convergence was 3.6 mm, thus similar to the non-lesioned hemisphere.

In deep layers 5 and 6, and in the underlying white matter, labelling was dense. The majority of labelled fibres were in deep layers. In the experiment with a short diffusion time, where no cells were retrogradely labelled in area 18, fibres were seen to run with scattered orientation and cross each other (Fig. 3) as in younger normal animals aged P6 to P10 (see Fig. 4 in Chapter 2). In brains where dyes diffused for 3.5 months, corticocortical fibres were seeing crossing layer 4, as in non-lesioned hemispheres (Fig. 4). What seemed to be the main difference was that

the arborizations were seen only in the lower part of layer 3, in contrast to non-lesioned hemispheres where they extended throughout the whole width of layers 2 and 3 (see Fig. 5 in Chapter 2). My impression was that after the LGN lesion there was decrease of labelled corticocortical cells in area 18 (Fig. 5) The quantification revealed that the peak density of corticocortical labelled cells in superficial layers was about four times lower after the LGN ablation (295 cells mm^{-3}) compared to the control (1,330 $\pm$ 156 cells mm^{-3}). Thus label with DiI and DiA in fixed tissue indicated that thalamic ablation caused a decrease of corticocortical projections from area 18 to area 17 in deafferenated cortex. The second main consequence of the lesion was that the corticocortical cells and the arborization were distributed in an almost uniform fashion, rather than in distinct clusters.

5.4.3 Labelling with DY and DiI in vivo

In experiments where the LGN was severely lesioned (only a very small number of the LGN cells were left) labelling in area 17 was very sparse. DiI labelled cells were scattered in deep and superficial layers across the entire mediolateral extent of area 17. There were a few small clusters of corticocortical cells in the superficial layers, composed of a very few cells; the peak cell density of corticocortical cells was between 184-692 cells mm^{-3}. In two of four injected animals I did not find labelled DY cells. In the other two cases, DY labelled cells were distributed in crude clusters, the peak density of corticocortical cells ranged between 4709-4755 cells mm^{-3}.

In an experiment where a small rostrolateral part of the LGN was lesioned, both DiI and DY cells were distributed in distinct clusters, in the medial part of area 17. The peak cell density of DiI labelled cells in the superficial layers was 1754 cells mm^{-3} and of DY was 4155 cells mm^{-3}.

Similarly, as observed in area 18 after labelling in fixed tissue, the overall distribution of labelling in area 17 after severe LGN lesions seemed much wider. The

rostrocaudal convergence ranged between 6.45-9.35 mm. However, after a small lesion the convergence was 3.5 mm, i.e. much lower than after severe lesions.

5.5 DISCUSSION

In the present study the LGN was lesioned in the newborn kitten and the effects of the lesion on the formation of the corticocortical connections were examined at P30. My main conclusions are:

1. Thalamic ablation caused an overall decrease in corticocortical connections between areas 17 and 18. In vivo injections suggested that after the lesion a very small number of cells projected from area 17 to area 18. Although the lesioned hemispheres had a tendency to bleeding which could prevent DY labelling, this was less likely as a decrease in corticocortical labelling was obtained after application of dyes in area 17 in fixed tissue.

2. Furthermore, lipophilic tracers in fixed tissue allowed me to examine the morphology of corticortical axons that project from area 17 to area 18; after the lesion, the distribution of fibres appear less mature than in the normal kitten of a similar age, as the peak of fibre density was in the deep layers and their distribution was in crude rather than the distinct cluster as in the animals of similar age.

3. The interesting finding was that after the thalamic lesion, the rostrocaudal convergence was significantly larger.

The larger convergence found in the present study is in agreement with findings for callosal connections in hamster after the neonatal thalamic lesion (Miller et al, 1991). These authors reported callosal connections were distributed much wider in the contralateral visual cortex in the lesioned animals compared to the control. The explanation of these changes occur after the LGN lesion could be caused by the lack of matching signals from the LGN to corticocortical cells in the retinotopographic regions that are going to become interconnected during the first few postnatal weeks. It could be that corticocortical cells in order to make reciprocal

connections need to be repeatedly activated by matching signals from the retinotopographically appropriate part of the LGN. According to Hebb's rule, when presynaptic afferents and postsynaptic cells are active in temporal contiguity the efficacy of excitatory transmission increases. Activity of corticocortical cells in temporal contiguity may be the key for increase in a number of corticocortical connections at appropriate retinotopographic regions. The silence of the LGN after the lesion may cause lack of signals after birth that normally enables the formation of cortical connections in localised region of the visual cortex. When there is a lack of specific and localised retinal input from the LGN, input will still come from other thalamic nuclei and other subcortical regions. However, these signals are spread over the larger cortical regions than those from the LGN; the result is that some of early exuberant projections are retained and much wider distribution of corticocortical projections.

At present, the activity dependent modifications and stabilisation of selective pathways in the developing nervous system is most appealing mechanism to explain these changes and it is also found to be important in development of other pathways. For example, the influence of activity on the development of afferents from the LGN to the cortex has been shown in experiments where blocking cortical activity prevented the normal reorganisation in this pathway (see review by Stryker, 1991). Blocking activity in the visual cortex and examining corticocortical connections has not yet been done and is certainly an experiment to be done in the future.

CHAPTER 6

PROSPECTS FOR IN VITRO STUDIES OF CORTICOCORTICAL CONNECTIONS IN THE CAT'S VISUAL CORTEX

6.1 ABSTRACT

To date, the development of corticocortical connections in the cat has been studied using only neuroanatomical and electrophysiological methods. Relatively little is known about the underlying molecular mechanisms that initiate and enhance the growth of these connections. One way to begin to answer these questions is to use in vitro methods; this involves culturing slices of the cerebral cortex next to explants from other cortical regions that are connected to them in vivo. However, the cat's cortex had not yet been cultured and the first aim of this study was to assess whether this was possible. In this series of experiments I found that slices from the cat's occipital cortex can produce neurite outgrowth in culture. I was also able to quantify this outgrowth. When I cultured cortical explants under different conditions, i.e. in conditioned medium or in presence of different tissues, I observed changes in the neurite outgrowth. The main effect found under different conditions was that the outgrowth from cortical explants was suppressed from the pial side but not from the white matter side.

6.2 INTRODUCTION

Over the last few years, there has been a gradual increase of interest in using in vitro methods to study the development of connections in the central nervous system. Particularly interesting examples are studies of the growth of geniculocortical axons into the visual cortex (Yamamoto, 1989, 1992; Molnar and Blakemore 1991; Bolz et al, 1992; Rennie at al., 1994; Lotto and Price, 1994). These studies provide anatomical and physiological evidence that the development of the geniculocortical pathway can be mimicked in vitro. For example, developing LGN axons can grow, recognize their target and terminate in cortical layer 4 in a tissue co-culture system.

One of the advantages of using in vitro methods compared to in vivo methods is that it reduces the numbers of experimental animals required. Secondly,

isolating and examining the growth of tissue of interest in a controlled environment allows us to address questions on the molecular mechanisms underlying their development; for example, work in our laboratory suggests that in the mouse the development of thalamocortical and corticothalamic axons is influenced by factors produced by their target cells in vitro (Rennie et al., 1994; Lotto, 1994). Third, in co-culture systems it is possible to use tissues derived from different species to examine whether their development is under the influence of inter-species conserved molecules. The first inter-species co-culture experiments were performed in our laboratory (Lotto and Price, 1994). For example, when the murine LGN was co-cultured with the cat's occipital cortex, it seemed that diffusible factors were released by the cat's cortex and stimulated the growth of axons from the LGN. Furthermore, murine geniculate axons grew freely on the cat's cortex.

During the third year of my PhD I also carried out a small series of in vitro experiments. The purpose of these experiments was to see whether corticocortical connections in the cat can be studied in vitro. Numerous neuroanatomical studies on cortical connections (callosal and ipsilateral corticocortical) have been done in the past in the cat, but still relatively little is known about the underlying molecular mechanisms that initiate and enhance the growth of these connections. The tissue co-culture system provides a potentially interesting opportunity to address these questions for cortical connections e.g. whether the development of corticocortical connection is enhanced by growth promoting factors. The cat's cortex had not yet been cultured and the first aim of this study was to asses whether this was possible. I first cultured slices from the cat's occipital cortex in isolation. The majority of slices produced outgrowth and I measured the length and density of neurite outgrowth. Later experiments in our laboratory suggested the possibility of an inter-species conservation of diffusible molecules affecting the mouse LGN, i.e. cat's cortex promoted growth from the embryonic murine LGN (Lotto and Price, 1994). I wanted to examine whether the opposite is the case, i.e. whether the murine LGN can

promote growth of the cat's cortex (the mouse LGN is capable of stimulating outgrowth from the murine cortex in vitro; Lotto, 1994). I co-cultured slices of the cat's occipital cortex with the murine embryonic LGN (at a distance from cortex or touching cortex) or in serum-free medium that had been preconditioned with murine embryonic LGN explants. A defined serum-free medium was used for all these experiments. Serum is known to contain many trophic factors and thus may mask any growth promoting interactions between co-cultured tissues.

6.3 MATERIALS AND METHODS

I made organotypic cultures (n=44) of slices of cat's occipital cortex either alone (n=16), or with embryonic murine LGN explants at a distance (n=6) or touching (n=6), or in serum-free medium that had been preconditioned with murine embryonic LGN explants (n=16). All cortical slices were from newborn kittens (P1). Murine LGN explants were taken on embryonic day 16 (E16), since this is the age when LGN axons reach the cortex in vivo (Catalano et al., 1991; De Carlos and O'Leary, 1992). Older LGN explants do not survive in culture, perhaps due to extensive denervation (Lotto, 1994).

6.3.1 Animals and Surgery

Cortical slices were collected from P1 kittens, that had been anaesthetized following the procedure described in chapter 2. Large bilateral craniotomies were made above the lateral gyrus and two blocks of tissue (one from each side, about 5 wide and 10 mm long) of the occipital cortex were quickly removed. The tissue was sliced coronally at 350 μm using a McIlwain tissue chopper.

Murine LGN explants were taken on E16. BALB/c mice from an isolated laboratory colony were mated overnight. On the following day (deemed to be embryonic day 1; E1) female with a vaginal plug were separated. On E16, the mother was deeply anaesthetized with urethane (0.3 ml of a 25 % solution in normal

saline, i.p.), the fetuses were removed by Caesarean section and decapitated. Using a Wild dissecting microscope, explants containing the LGN (about 0.5 x 0.5x 0.5 mm) were dissected from the dorsolateral thalamus. Prior tract-tracing experiments with DiI placed in the eye of fixed foetuses confirmed the LGN location in this region (Rennie et al., 1994).

6.3.2 Culture method

Culture in serum-free medium: Cortical slices and thalamic explants were collected as rapidly as possible and placed in a chemically defined serum-free culture medium (Romijn et al., 1984) in an incubator at 37°C and 5% CO_2 for no more than 40 min. The collected cortical slices were plated on a collagen coated filter (Costar U.K., Transwell-col chambers with 3 μm pores) suspended in a chemically defined serum-free medium. The filters and medium had been preincubated at 37°C and 5% CO_2 for 2 h. All cultures were set up with four cortical slices plated at a distance (about 3 mm); thus, fibres growing from the cortical explants never touched (Fig. 1a). Thalamic explants were placed at a small distance (about 0.5 mm) from the cortex (n=6) or touching the cortex (n=6) (Fig. 1b); in both cases, LGN explants were placed on the pial side of each cortical slice. Two millilitres of medium were placed in the lower chamber of each culture-well and 200-250 μl were placed in the upper chamber, ensuring that the tissues were just covered. The culture dishes were then returned to the incubator at 37°C and 5% CO_2, where they remained for three days. At the end of this period, the medium was replaced with a similar volume of 4% paraformaldehyde in 0.01M phosphate buffer. In a few cases, after qualitative analysis, small crystals of DiI (about 300 μm in diameter) were placed in the fixed cortical slices (in the upper layer, below the pia) and left for 1 week to label the growing axons.

Culture in conditioned serum-free medium: Four murine LGN explants were cultured alone in serum-free medium on collagen-coated filters. After 3 days, the

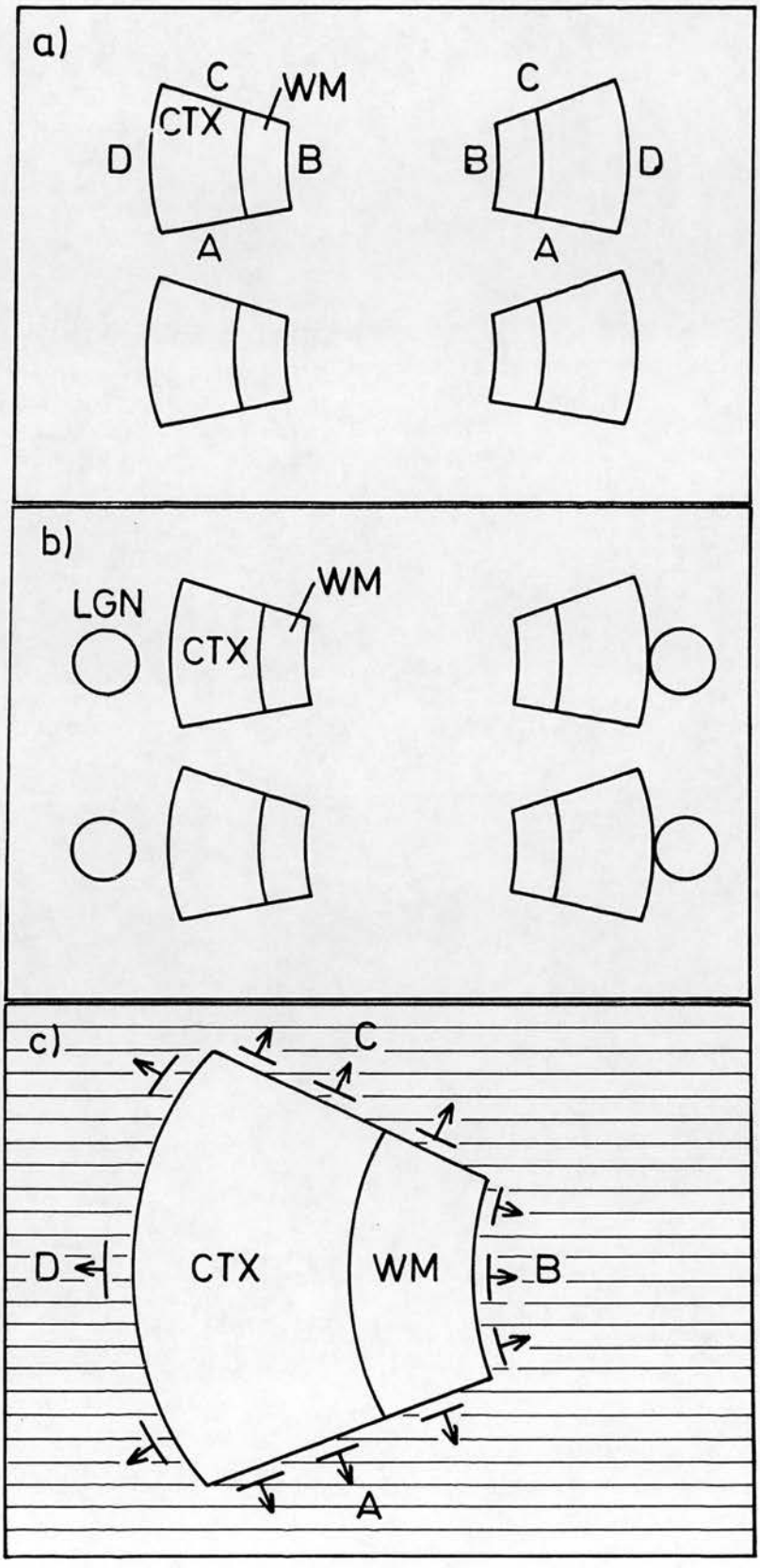
conditioned medium was decanted. Cortical slices were dissected and placed on collagen-coated filters (four per well; Fig. 1a) following the procedure described before. Once cortical slices were in place, the culture medium was replaced with conditioned medium and incubated at 37°C and 5% CO_2 for three days and then fixed in 4% paraformaldehyde in the same buffer.

6.3.3 Analysis

In the Transwell collagen filters, most of the collagen fibres have parallel equally spaced narrow grooves (35 μm wide; Figs. 1a; 2; 3a). This structure is created when the microporous membrane is treated with collagen, and the grooves act as a guide for the majority of fibres. However, some of the neurites emerging from the explant did not follow the grooves.

Quantifications of neurite outgrowth from the cortical slices were made through the microscope, under phase-contrast (x20 objective). In most cases, cortical slices produced outgrowth from all four sides: the white matter side, the pial side and the two sides where the cortex was cut radially. The outgrowth was quantified on each side of the cortical slices (Fig. 1a; sides positioned towards adjacent cortical slices: A - where the cortex was cut radially and B - where the white matter side was cut; sides positioned away from adjacent cortical slices: C - where cortex was cut radially and D - the pial surface). For each explant, the density and the length of neurite outgrowth were measured in 12 windows (three equally spaced 500 μm wide windows on each side of the explant; Fig. 1c). For density, the numbers of neurites were counted at the edge of the explant in each of these windows. When fascicles were encountered, the numbers of neurites within each fascicle were estimated based on the width of each fascicle as compared to the width of individual neurites. However, these fascicles were rare. The length of the neurites emerging closest to the midpoint of each window were measured.

Figure 1. Schematic diagrams illustrating four slices of cat's occipital cortex cultured in each well alone or in conditioned medium (*a*) and with the murine thalamic explants (*b*); the method of quantifying outgrowth is shown in (*c*). The grey/white matter border is indicated in each cortical slice. (*a*) In each cortical explant, four sides were analysed; two sides positioned towards adjacent cortical slices are indicated as *A* (where the cortex was cut radially) and *B* (where the white matter side was cut); two remaining sides positioned away from adjacent cortical slices are indicated as *C* (where cortex was cut radially) and *D* (the pial surface). (*b*) LGN explants were cultured at small distances from the cortical pial surface (on the *left*) or next to the pial side of cortical slices (on the *right*). (*c*) In each cortical explant there were twelve 500 μm windows used to asses density of outgrowth (three on each side of the explant; exceptions were cases in which the LGN was placed next to the pial surface and only two windows were examined on that side). There are indicated at the edge of the explant (*short lines*); the positions at which the neurite lengths were measured are indicated by *arrows*. The parallel horizontal lines indicate the orientation of the collagen grooves on the filter (seen in Fig. 2 and 3*a*).


a)
C
WM
CTX
D
B
A
C
B
D
A
b)
LGN
WM
CTX
c)
C
D
CTX
WM
B
A

Data from all windows were combined to calculate the average densities and lengths of neurite outgrowth in cortical slices within each type of experiments (i.e. cortex cultured alone, cortex cultured in conditioned medium or with thalamic explants). An overall measure of the amount of outgrowth produced (referred to as total outgrowth) was calculated by multiplying the average neurite length by their average density on each side of explant (density x length); the calculations were made for each type of experiments.

In all cortical slices, the outgrowth from the white matter sides (towards adjacent cortical slices) was more profuse than that from the pial surfaces (Fig. 2). In order to assess the effects of the LGN on cortical outgrowth towards adjacent cortical slices, I calculated the ratio of outgrowth between the white matter and the pial side. The ratio was calculated for the neurite densities, lengths and total outgrowths and compared between different types of experiments.

Statistical analysis were carried out using a Mann-Whitney test on unpaired data.

6.4 *RESULTS*

6.4.1 Outgrowth from cortical explants

The results were from 44 cultures. An example of a slice of cortex cultured in serum-free medium without addition of thalamic explants is shown in Fig. 2a. The outgrowth from each slice of the cortex was both towards and away from the other cortical explants. The pial and the white matter sides were always placed perpendicular to the grooves (Figs. 1c and 2). The majority of neurites from these two sides had a tendency to follow the grooves on the collagen filter; this guidance phenomenon by grooves has been described before in an other system, i.e. the outgrowth from thalamic explants (Rennie, et al. 1994). In all cortical explants, on the white matter sides and sides cut perpendicularly to the cortex, there were fibres that did not follow grooves (Figs. 2 and 3).

Figure 2. Phase-contrast photomicrographs of outgrowth from P1 slices from cat's occipital cortex cultured (*a*) alone and (*b*) in conditioned medium. The collagen grooves on the filter can be seen. Scale bar in *b* refers also to *a*, 0.25 mm; *c*, 0.1 mm; *d*, 0.25 mm.

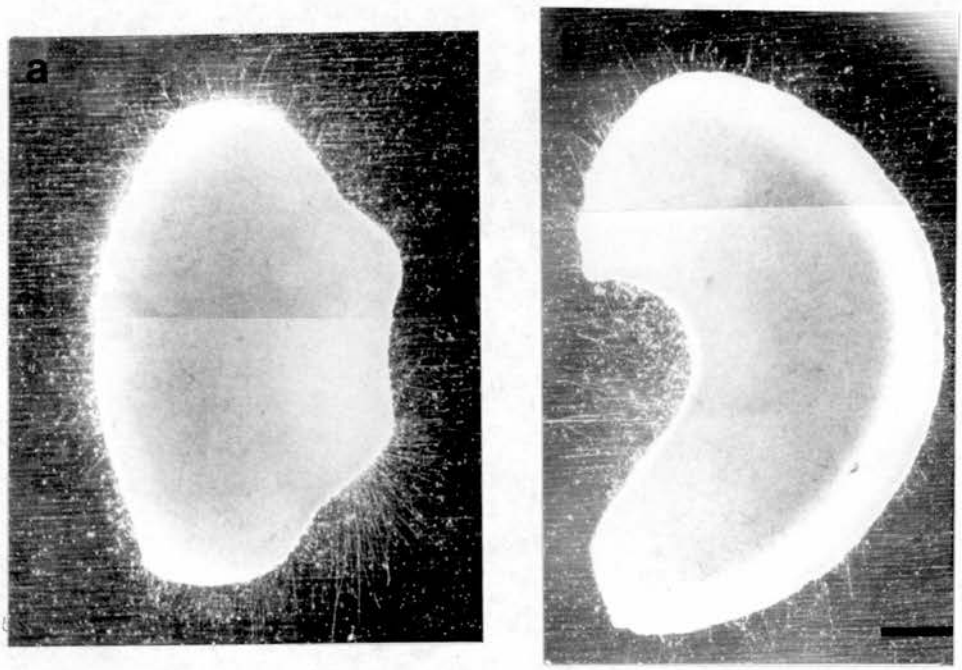
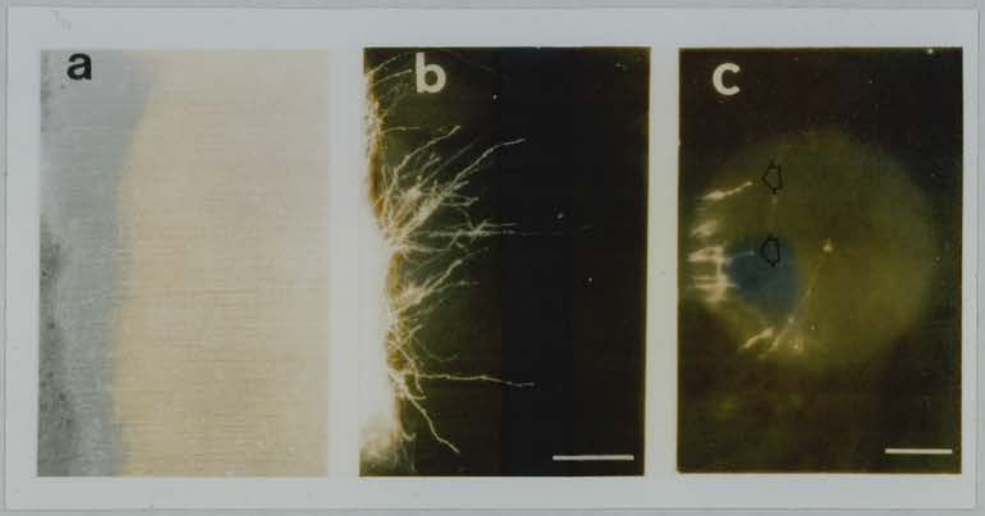
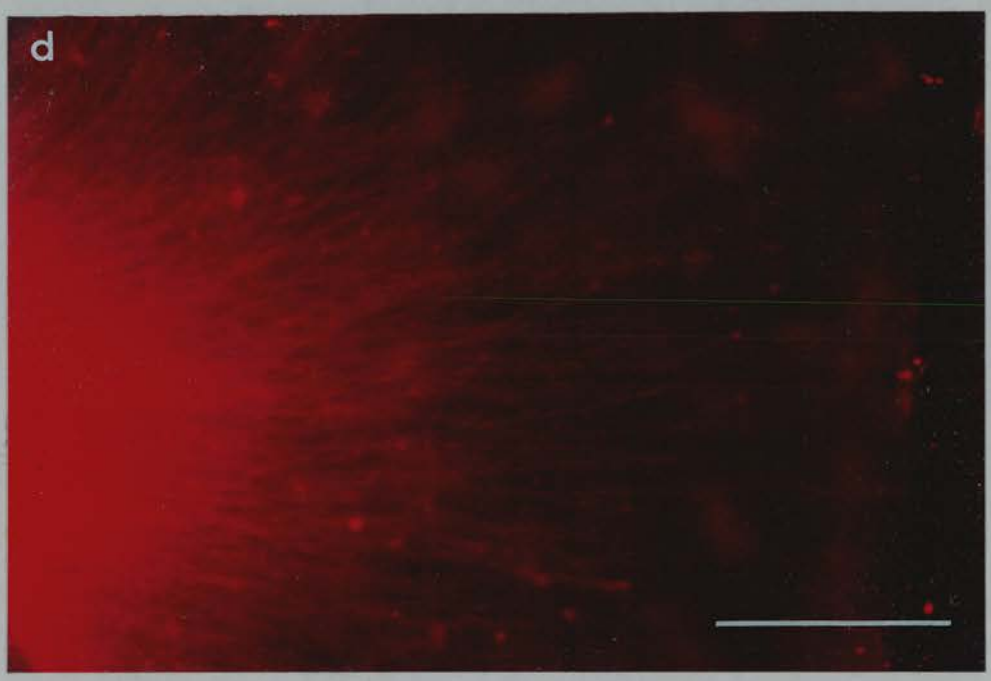

a

Figure 3. (*a*) Phase-contrast photomicrograph of outgrowth from P1 slices from cat's occipital cortex cultured alone. (*b, c, d*) Fluorescence photomicrographs of neurites labelled with DiI from a crystal placed in the upper cortical layers in fixed explant. (*b*) The same region of outgrowth as in *a*; only some neurites are labelled with DiI. (*c*) The growing neurites terminate with growth cones (*arrows*). (*d*) Fibres labelled in the cortical slice with DiI. Scale bars, *a* and *b*, 0.25 mm; *c*, 0.1 mm; *d*, 0.25 mm.


a
b
c


d

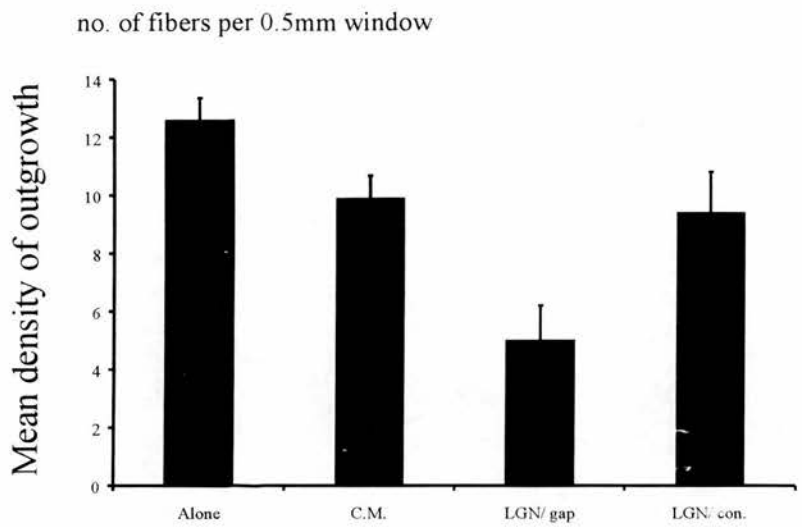
The overall appearances of the outgrowth from the cortex cultured with murine thalamic explants, or in a conditioned medium (Fig. 2b), were very similar to the appearances of the outgrowth from the cortex cultured alone. However, quantitative data revealed that the density of neurites emerging from the cortex (measured on all four sides; A, B, C and D see Fig. 1c), was significantly higher when the cortex was cultured alone than when cultured in conditioned medium ($P<0.02$), or co-cultured at a distance from the murine LGN ($P<0.0001$), or in contact with the murine LGN ($P<0.01$) (Fig. 4a). The mean length of the outgrowth did not differ significantly when the cortex was cultured alone or in conditioned medium or in contact with the murine LGN (Fig. 4b). However, a highly significant decrease in the mean length of neurites was found when the cortex was cultured at a small distance from the LGN ($P<0.0001$). Thus, when the LGN was placed at a distance from the cortex, there was significantly shorter and less dense outgrowth from the cortex compared to when the cortex was cultured alone. Conditioned medium and direct contact with the LGN cause the outgrowth to be less dense but similar in length to that from the cortex cultured alone.

For each explant, total outgrowth (density x length) was calculated to give an overall measure of the amount of outgrowth produced (Fig. 4c). The total outgrowth from the cortex was decreased significantly in the presence of the murine LGN at a short distance from the cortex ($P<0.0001$) compared to the total outgrowth from the cortex cultured alone. The lower amount of growth was caused both by a decrease in density and length of neurites. Conditioned medium and direct contact with the LGN did not have significant effect on the total amount of outgrowth from the cortex.

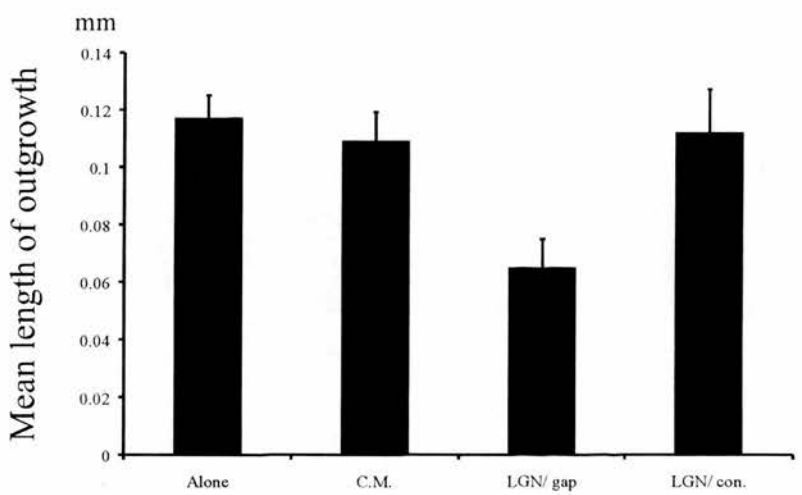
After quantifying growth from all sides in each explant, I addressed the question of whether there were significant differences between the outgrowth towards other cortical slices and away from them after the cortex was cultured alone or with murine explants. I calculated the ratio between the growth on the white matter side (towards other cortical slices) and the pial side (away from other cortical slices).

Figure 4. Histograms showing (*a*) the mean densities of outgrowth, (*b*) the mean lengths of outgrowth and (*c*) the mean of the total outgrowth (density x length) from cortical slices cultured alone, in conditioned medium (C.M.), or co-cultured with the murine E16 thalamic explants at as small distance from cortical pial side (LGN/ gap) or thalamic explants next to the cortical pial side (LGN/ con.).

a


no. of fibers per 0.5mm window
Mean density of outgrowth
14
12
10
8
6
4
2
0
Alone
C.M.
LGN/ gap
LGN/ con.

b


mm
Mean length of outgrowth
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Alone
C.M.
LGN/ gap
LGN/ con.

c

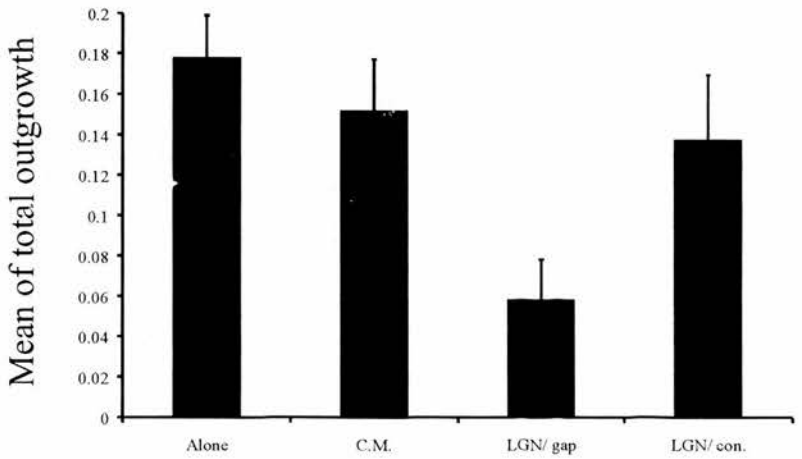
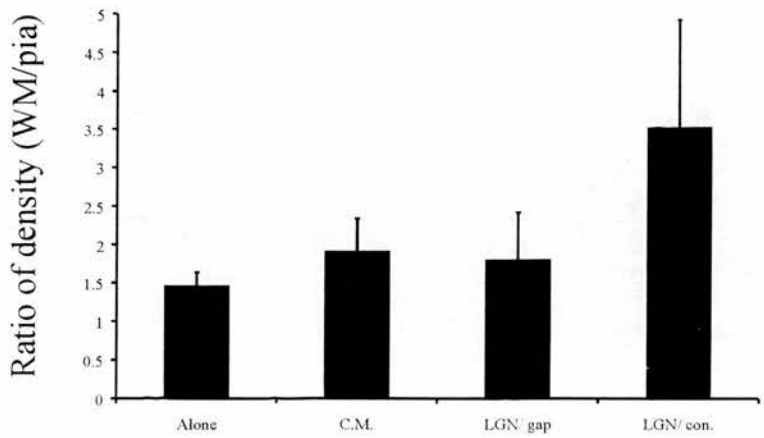
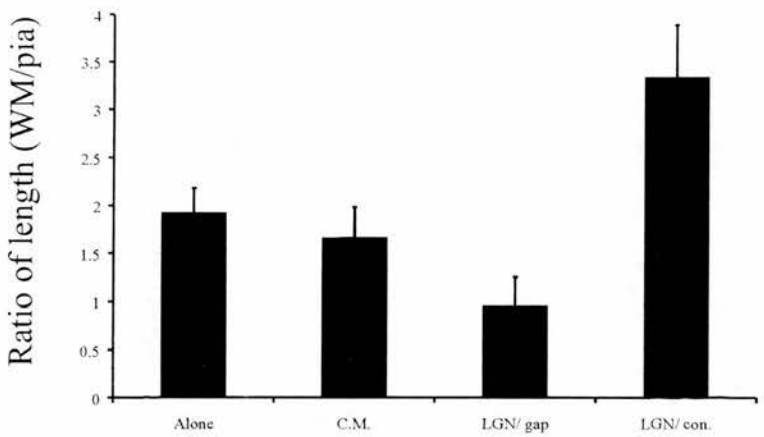

Mean of total outgrowth
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Alone
C.M.
LGN/ gap
LGN/ con.

Figure 5. Histograms showing the ratio between the outgrowth on the white matter sides and the pial sides (*a*) for the mean densities of outgrowth, (*b*) for the mean lengths of outgrowth and (*c*) for the mean of the total outgrowth (density x length) from cortical slices cultured alone, in conditioned medium (C.M.), or co-cultured with the murine E16 thalamic explants at a small distance from cortical pial side (LGN/ gap) or thalamic explants next to the cortical pial side (LGN/ con.).

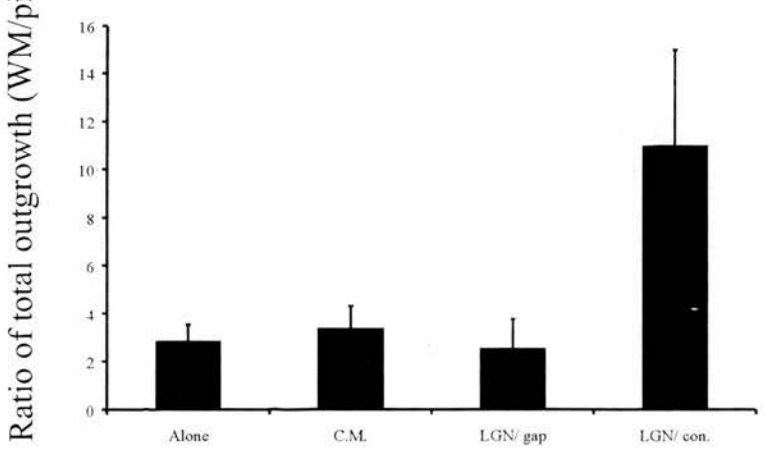
a


Ratio of density (WM/pia)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Alone
C.M.
LGN/ gap
LGN/ con.

b


Ratio of length (WM/pia)
4
3.5
3
2.5
2
1.5
1
0.5
0
Alone
C.M.
LGN/ gap
LGN/ con.

c


Ratio of total outgrowth (WM/pia)
16
14
12
10
8
6
4
2
0
Alone
C.M.
LGN/ gap
LGN/ con.

The ratio of the neurite density was not significantly increased when the cortex was cultured in the conditioned medium or with the murine LGN (Fig. 5a). The significantly higher ratio of the outgrowth from the white matter, was found both for the neurites lengths ($P<0.03$) (Fig. 5b) and total outgrowths ($P<0.01$) (Fig. 5b) in cases when the LGN was in contact with the cortex, but not in others.

4.2 DiI label

Crystals of DiI were placed in the upper part of cortical slices (below pia). DiI label revealed fibres emerging from the region where the crystal was placed (Fig. 3d). The majority of fibres were heading in a similar direction towards the white matter, almost in a fibre track. With DiI it was possible to see fibres growing from the cortical slices (Fig. 3b). The outgrowth described here seemed likely to be axonal; however, it is not possible to exclude the some was from glial cells as previous studies have shown that under phase-contrast glial cell outgrowth can appear similar (Torran-Allerand, 1990). I believe that this was less likely to be the case, as when DiI was placed on an explant, growth cones were revealed at the ends of growing fibres (Fig. 3c).

In all experiments crystals were placed in the superficial cortical layers; labelled fibres that emerge from the explant were mainly those which did not follow grooves (Fig. 3a,b) which suggests that other, non-labelled fibres that follow the grooves, were from deep layers.

6.5 DISCUSSION

This small series of in vitro experiments allowed me to become familiar with culture techniques. These experiments also revealed that fibres can grow from slices of the cat's occipital cortex in culture. Neurites growing from the cortex can grow following or without following the grooves. Thus some fibres growing from the cortex do not need guidance of the grooves. The presence of the murine LGN

significantly decreased the average density of outgrowth from cortical slices. The average lengths of neurites and the total outgrowth (density x length) were significantly decreased only when the LGNs were placed at a small distance from the cortex. However, the ratios of the density and total outgrowth from the white matter seemed to be increased in the presence of the murine LGN but this was significant only when the LGN is in a contact with the cortex but not in the conditioned medium or when the LGN is placed at distance.

It has been shown that murine thalamic explants grow freely to layer 4 in the cat's visual cortex (Lotto and Price, 1994); in the reciprocal situation, thalamic explants seem to suppress the overall growth from the cat's occipital cortex. There are many possible explanations; one of them is that toxins are released from the murine explants which affect the overall outgrowth from the cortex, but not from the white matter where fibres are likely to originate from the deep cortical layers that normally project to the LGN in vivo. The evidence for that is that DiI placed in the superficial layers labelled fewer fibres in the white matter, of which most did not follow the grooves. However, this explanation requires further evidence by retrograde labelling of cells that send these fibres.

In the mouse the growth promoting effect of the embryonic LGN on the embryonic cortex seems to be on the lateral but not on the medial (visual) part of the cortex (Lotto, 1994). Further experiments in the mouse are needed to assess whether there is a promoting effect of the LGN on the postnatal cortex. It would be interesting to see whether there is a difference in the effect of the LGN on the cortical superficial layers (that contain mainly corticocortical projections) or on deep layers (that normally project to the subcortical targets).

AFTERTHOUGHTS

The importance of the subplate zone and marginal zone (layer 1) in the development of corticocortical connections

In 1973 Molliver et al., reported that the first synapses in the human fetal brain are present above and the below the cortical plate, in the marginal and the subplate zones (the description and the name of the subplate zone were referred for the first time in 1974 by Kostovic and Molliver). These observations initiated further studies on the role of the subplate during development. The subplate cells were also found to be present in large numbers during fetal and early postnatal life in cat (Luskin and Shatz, 1985b). The subplate cells mature well before cortical neurones, but their numbers decline after birth. It has been hypothesized that the subplate cells serve as temporary target for ingrowing thalamic axons (reviewed by Shatz, 1991). From the time when I began this project, new direct evidence suggested that the subplate zone is involved in development of geniculocortical connections and in particular, in the formation of ocular dominance columns in the cat (Ghosh et al., 1992a). It would be useful to know whether the subplate zone is important in the formation of corticocortical connections It is possible that the subplate neurones are involved as temporary targets for corticocortical axons, as seems to be the case in the connections between the LGN and the visual cortex. This hypothesis can be tested by prenatal lesion of the subplate zone.

However, during the present study I concentrated on the role of layer 1, which, similarly to the subplate zone, matures very early. As corticocortical connections mainly originate in superficial cortical layers, layer 1 seems in a better position to influence their development. It is known that layer 2 neurones receive a great proportion of their synaptic input in layer 1 (see Vogt, 1990). From the beginning of my study it was clear that in young kittens corticocortical projections are dense in layer 1. This observation was reported by other authors studying corticocortical connections in the kittens of similar age (reviewed by Payne et al.,

1987). Kato (1986, 1990) reported the role of layer 1 in plasticity. Thus it seemed important to examine further the possible role of layer 1 in the formation of corticocortical connection. Therefore, I lesioned layer 1 in newborn kittens and indeed found that the formation of corticocortical connections was disrupted (Chapter 3). Explaining the mechanisms by which lesions of layer 1 produced these changes is complicated by the fact that at present the function of layer 1 is not known in the adult brain and with present knowledge it is difficult to synthesis a model of the role played by this layer in development and plasticity of the brain. The interesting point is that layer 1 contains fibres that transmit retinal input from C laminae of the LGN and those that transmit non-retinal input from the ILN thalamic nuclei, the noradrenergic and serotoninergic brainstem centres and cholinergic cell groups of the basal forebrain (see Chapter 1). It has been known for some time that mononuclear deprivation (MD) in paralysed and/or anaesthetized kittens is not a sufficient to produce functional changes of the visual cortex as it normally does in awake animals during the critical period (reviewed by Singer, 1985). From other experiments it seems that non-retinal gating signals are those that are necessary to facilitate the changes in the cortex after the (MD); for example pairing monocular light stimulation with electrical activation of the mesencephalic reticular formation or non-specific thalamic nuclei induces functional changes in the visual cortex (Singer and Rauschecker, 1982). Thus it seems that the retinal signal influences development only when the animal is awake and pays attention to that particular signal. It even seems that there is a unit in the ILN thalamic nuclei which discharge vigorously at the end of eye movements (Schlag and Schlag-Rey, 1983). Fibres that transmit non-retinal input in the cortex are located mainly in layer 1 which may suggest that this layer is important part of the cortex where non-retinal signals can be transmitted to apical dendrites of cortical cells. It would be interesting to test the hypothesis that non-retinal signals play role in the development of the corticocortical connections as

they seem to be involved in the plasticity of the visual cortex. These experiments would require lesioning of the ILN thalamic nuclei or the locus coeruleus.

In conclusion, the understanding of the mechanisms that control the development of corticocortical connections would require better understanding of the role of the subplate zone and the marginal zone (or layer 1) in these processes.

BIBLIOGRAPHY:

Adrien, J. and Roffwarg, H. P. (1974) The development of unit activity in the lateral geniculate nucleus of the kitten. *Exp. Neurol.*, **43**:261-274.

Allman, J. M. and Kaas, J. H. (1971) A representation of the visual field in the caudal third of the middle temporal gyrus of the owl monkey (Aotous trivirgatus), *Brain Res.*, **31**:85-105.

Allman, J. M. and Kaas, J. H. (1974) The organization of the second visual area (VII) in the owl monkey: A second order transformation of the visual hemifield. *Brain Res.*, **76**:247-265.

Anker, R. L. and Cragg, B. G. (1974) Development of extrinsic connections of the cat visual cortex. *J. Comp. Neurol.*, **154**, 29-42.

Balice-Gordon, R. J., Chua, C. K. Nelson, C. C and Lichtman, J. W. (1993) Gradual loss of synaptic cartels precedes axons withdrawal at neuromuscular junction. *Neuron*, **11**:801-815.

Barlow, H. B., Hill, R. M. and Levick, W. R. (1964) Retinal ganglion cells responding selectively to direction and speed of image motion in the rabbit. *J. Physiol.*, **173**:377-407.

Bear, M. F. and Singer, W. (1986) Modulation of visual cortical plasticity by acetylcholine and noradrenaline. *Nature*, **320**:172-176.

Blakemore, C., and Molnar, Z. (1990) Factors involved in the establishment of specific interconnections between thalamus and cerebral cortex. Cold Spring Harbor Symp. Quant. Biol., **55**, 491-504.

Blakemore, C. and Price, D .J. (1987) The organization and postnatal development of area 18 of the cat's visual cortex. *J. Physiol. (Lond.)* **384**, 263- 292.

Bloom, F. E. (1979) Chemical integrative processes in the central nervous system. In The Neuroscience: Fourth Study Program, (Schmitt, F. O. and Worden, F. G. eds.), MIT Press, Cambridge, pp:51-58.

Bolz, J., Novak, N., Gotz, M. and Bonhoeffer, T. (1990) Formation of target-specific neuronal projections in organotypic slice cultures from rat visual cortex. *Nature*, **346**:359-62.

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

Brodmann, K. (1909) Vergleichende Lokalisationslehre der Grosshirnrinde in ihren Prinzipendargestellt auf Grund des Zellenbaues. Leipzig: J. A. Barth.

Bullier, J., Kennedy, H. and Salinger, W. (1984) Branching and laminar origin of projections between visual cortical areas in the cat. *J. Comp. Neurol.*, **228**: 29-341.

Callaway, E. M. and Katz, L. C. (1990) Emergence and refinement of clustered horizontal connections in cat striate cortex. *J. Neurosci.*, **10**, 1134-1153.

Catalano, S. M., Robertson, R. T. and Killackey, H. P. (1991) Early ingrowth of thalamocortical afferents to the neocortex of the prenatal rat. *Proc. natn. Acad. Sci. U.S.A.*, **88**:2999-3003.

Caviness, V. S. Jr. and Sidman, R. L. (1973) Time of origin of corresponding cell classes in the cerebral cortex of normal and reeler mutant mice: An autoradiographic analysis. *J. Comp. Neurol.*, **148**:141-152.

Caviness, V. S. Jr. and Yorke, C. H. Jr. (1976) Interhemispheric neocortical connections of the corpus callosum in the reeler mutant mouse: a study based on anterograde and retrograde methods. *J. Comp. Neurol.*, **170**:449-460.

Clarke, S. and Innocenti, G. M. (1986) Organization of immature intrahemispheric connections. *J. Comp. Neurol.*, **251**, 1-22.

Coogan, T. A. and Burkhalter, A. (1988) Sequential development of connections between striate and extrastriate visual cortical areas in the rat. *J. Comp. Neurol.*, **278**:242-252.

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

Cragg, B. G. (1972) The development of synapses in cat visual cortex. *Invest. Ophtal.*, **11**:377-385.

Cunningham, E. T. and LeVay, S. (1986) Laminar and synaptic organization of the projection from the thalamic nucleus centralis to primary visual cortex in the cat. *J Comp. Neurol.*, **244**:65-77.

Daw, N. W. and Fox, K. (1991) Function of NMDA receptors in the developing visual cortex. In Development of the visual system, Vol. 3 (Man-Kit Lam, D. and Shatz., C. J., eds.), MIT Press, London, pp:243-252.

De Carlos, J. A. and O'Leary, D. D. M. (1992) Growth and targeting of subplate axons and establishment of major cortical pathways. *J. Neurosci.*, **12**:1194-1211.

Dehay, C., Bullier J. and Kennedy H. (1984) Transient projections from the fronto-parietal and temporal cortex to areas 17, 18 and 19 in the kitten, *Exp. Brain Res.*, **57**, 208-212.

Felten, D. L., Hallman, H. and Jonsson, G. (1982) Evidence for a neurotrophic role of noradrenaline neurons in the postnatal development of rat cerebral cortex. *J. Neurocytol.*, **11**: 119-135.

Ferrer, J. M. R., Price, D. J. and Blakemore, C. (1988) The organization of corticocortical projections from area 17 to area 18 of the cat's visual cortex. *Proc. Roy. Soc. Lond. Ser. B*, **223**, 77-98.

Ferrer, J. M. R., Kato, N., Price, D. J. and Blakemore, C. (1992) The organization of association projections from area 17 to areas 18 and 19 and suprasylvian areas in the cat's visual cortex. *J. Comp. Neurol.*, **316**, 261-278.

Ferster, D. (1981) A comparison of binocular depth mechanisms in areas 17 and 18 of the cat visual cortex. *J. Physiol.*, **311**:623-55.

Foote, S. L. and Morrison, J. H. (1987) Extrathalamic modulation of cortical function. *Ann. Rev. Neurosci.*, **10**: 67-95.

Friauf, E., McConnell, S. K. and Shatz, C. (1990) Functional synaptic circuits in the subplate during fetal and early postnatal development of cat visual cortex. *J. Neurosci.*, **10**, 32601- 2613.

Garey, L. J. and Powell, T. P. S. (1968) The projection of the lateral geniculate nucleus upon the cortex in the cat. *Proc. Roy. Soc. Lond. Ser. B*, **169**, 107-126.

Garey, L. J. (1971) A light and electron microscopic study of the visual cortex of the cat and monkey. *Proc. Roy. Soc. Lond. (Biol.)*, **179**, 21-40.

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

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

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

Gilbert, C. D. and Kelly, J. P. (1975) The projections of cells in different layers of the cat's visual cortex. *J. Comp. Neurol.*, **163**, 81-106.

Greuel, J. L., Luhmann, H. J. and Kasamatsu, T. (1988) Pharmacological induction of use-dependent receptive field modifications in the visual cortex. *Science*, **242**:74-77.

Guillery, R. W. (1970) The laminar distribution of retinal fibres in the dorsal lateral geniculate nucleus of the cat: A new interpretation. *J. Comp. Neurol.*, **138**:339-368.

Hartline, H. K. (1940) The receptive fields of optic nerve fibers. *Am. J. Physiol*, **130**:690-699.

Henderson, Z. (1982) An anatomical investigation of projection from lateral geniculate nucleus to visual cortical areas 17 in newborn kitten. *Exp. Brain Res.*, **46**: 117-185.

Heffner, C. D., Lumsden, A. G. S. and O'Leary, D. D. M. (1990) Target control of collateral extension and directional axon growth in the mammalian brain. *Science*, **247**, 217-220.

Henry, G. H., Saline, P. A. and Bullier, J. (1991) Projections from areas 18 and 19 to cat striate cortex: divergence and laminar specificity. *Eur. J. Neurosci.*, **3,** 186-200.

Horton, J. C. and Hubel, D. H. (1981) A regular patchy distribution of cytochrome-oxidase staining in the primary visual cortex of the macaque monkey. *Nature*, **292**:763-764.

Hubel, D. H. and Wiesel, T. N. (1959) Receptive fields of single neurones in the cat's striate cortex. *J. Physiol.*, **148**:574-591.

Hubel, D. H. and Wiesel, T. N. (1962) Receptive fields, binocular interaction and functional architecture in the cat's visual cortex. *J. Physiol.*, **160**:106- 154.

Hubel, D. H. and Wiesel, T .N. (1963) Receptive fields of cells in striate cortex of very young, visually inexperienced kittens. *J. Neurophysiol.*, **26**:994-1002.

Hubel, D. H. and Wiesel, T. N. (1965) Receptive fields and functional architecture in two non-striate visual areas (18 and 19) of the cat. *J. Neurophysiol.*, **28**:229-289.

Hubel, D. H. and Wiesel, T. N. (1977) Ferrier Lecture. Functional architecture of macaque monkey visual cortex. *Proc. R. Soc. Lond. B.*, **198**:1-59.

Innocenti, G. M. (1980) The primary visual pathway through the corpus callosum: Morphological and functional aspects in the cat. *Arch. Ital. Biol.*, **118**: 124-188.

Innocenti, G. M. (1981) Growth and reshaping of axons in the establishment of visual callosal connections. *Science*, **212**, 824-827.

Innocenti, G. M. and Clarke, S. (1984) The organization of immature callosal connections. *J. Comp. Neurol.*, **230**, 287-309.

Innocenti, G. M., Fiore, L. and Caminiti, R. (1977) Exuberant connections into the corpus callosum from the visual cortex of newborn cats. *Neurosci. Lett.*, **4**:237-242.

Innocenti, G. M. and Frost, D. O. (1979) Effects of visual experience on the maturation of the efferent system to the corpus callosum. *Nature*, **280**:231-34.

Innocenti, G. M. and Frost, D. O. (1980) The postnatal development of visual callosal connections in the absence of visual experience or of the eyes. *Exp. Brain Res.*, **39**:365-375.

Innocenti, G. M., Frost, D. O. and Illes, J. (1985) Maturation of visual callosal connections in visually deprived kittens: a challenging critical period. *J. Neurosci.*, **5**:255-267.

Jensen, K. F. and Killackey, H. P. (1984) Subcortical projections from ectopic neocortical neurons. *Proc. Natl. Acad. Sci. USA*, **81**:964-968.

Kalil, R. (1978) Development of the dorsal lateral geniculate nucleus in the cat. *J. Comp. Neurol.*, **182**:265-292.

Kasamatsu, T. (1987) Norepinephrine hypothesis for visual cortical plasticity: Thesis, antithesis and recent development. *Curr. Top. Dev. Biol,*. **21**: 367-389.

Kasper, E. m., Lübke, J., Larkman, A. U. and Blakemore, C. (1994) Pyramidal neurons in layer 5 of the rat visual cortex. III. Differential maturation of axon targeting, dendritic morphology, and electrophysiological properties. *J. Comp. Neurol.*, **339**:495-518.

Kato, N. (1986) Increase in the density of geniculocortical afferents to layer 1 after reverse suture in kittens. *Brain Research,* **386**, 393-396.

Kato, N. (1990) Effects of visual deprivation on the postnatal development of geniculocortical projections in kittens. *Neuroscience,* **37**, 101-114.

Kato, N. Kawaguschi, S. and Miyata, H. (1984) Geniculocortical projections to layer 1 of area 17 in kittens: orthograde and retrograde HRP studies. *J. Comp. Neurol.*, **225**, 441-447.

Kaufman, E. F. S. and Rosenquist, A. C. (1985a) Efferent projections of the thalamic intralaminar nuclei in the cat. *Brain Res.*, **335**:257-279.

Kaufman, E. F. S. and Rosenquist, A. C. (1985b) Afferent connections of the thalamic intralaminar nuclei in the cat. *Brain Res.*, **335**:281-296.

Katz, L. C. (1991) Specificity in the development of vertical connections in cat striate cortex. *Eur. J. Neurosci.*, **3**, 1-9.

Koester, S. E. and O'Leary, D. D. M. (1992) Functional classes of cortical projection neurons develop dendritic distinctions by class-specific sculpting of an early common pattern. *J. Neurosci.*, **12**, 1382-1393.

Kohler, C. and Schwarcz, R. (1983) Comparison of ibotenate and kainate neurotoxicity in rat brain: a histological study. *Neurosci.*, **8**: 819-835.

Kostovic, I. and Molliver, M. E. (1974) A new interpretation of the laminar development of cerebral cortex: Synaptogenesis in different layers of neopallium in the human fetus. *Anat. Rec.*, **178**, 395.

Kuffler, S. W. (1953) Discharge patterns and functional organization of the mammalian retina. *J. Neurophysiol.*, **16**:37-68.

LeVay, S. and Gilbert, C. D. (1976) Laminar pattern of geniculocortical projections in the cat. *Brain Res.*, **113**, 1-19.

Levitt, P and Moore, R. Y. (1971) Development of the noradrenergic innervation of neocortex, *Brain Res.*, **162**, 243-259.

Livingstone, M. S. and Hubel, D. H. (1982) Thalamic inputs to cytochrome-oxidase reach regions in monkey visual cortex. *Proc. Natl. Acad. Sci. USA*, **79**:6098-6101.

Livingstone, M. S. and Hubel, D. H. (1983) Specificity of corticocortical connections in monkey visual cortex. *Nature*, **304**:531-534.

Livingstone, M. S. and Hubel, D. H. (1984) Anatomy and physiology of a color system in the primate visual cortex. *J. Neurosci.*, **4**:309-356.

Loop, M. S. and Sherman, S. M. (1977) Visual discrimination during eyelid closure in the cat. *Brain Res.*, **128**:329-339.

Lorente de Nó, R (1938) The cerebral cortex: Architecture, intracortical connections, motor projections. In: Physiology of the Nervous System, (J. F. Fulton, ed.), Oxford Univ. Press, London, pp. 291-339.

Lotto, R. B. (1994) A search for factors controlling the formation and maintenance of connections between the thalamus and cortex in vitro. PhD thesis, University of Edinburgh.

Lotto, R. B. and Price, D. J. (1994) Evidence that molecules influencing growth and termination in the developing geniculocortical pathway are conserved between divergent mammalian species. *Dev. Brain Res.* In Press.

Lund, R D., Mitchell, D. E. and Henry, G. H. (1978) Squint-induced modification of callosal connections in cats. *Brain Res.*, **144**:169-72.

Lund, R. D. and Mitchell, D. E. (1979) The effects of dark-rearing on visual callosal connections of cats. *Brain Res.*, **167**:172-175.

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

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

Marin-Padilla, M. (1978) Dual origin of the mammalian neocortex and evolution of the cortical plate. *Anat. Embryol.*, **152**: 109-126.

Marin-Padilla, M. (1984) Neurones of layer 1: a developmental analysis. In: Cerebral Cortex, Vol. 1. (Peters, A. and Jones, E. G., eds.), Plenum Press, New York, pp. 447-477.

Marin-Padilla, M. (1992) Ontogenesis of the pyramidal cell of the mammalian neocortex and developmental cytoarchitectonics: a unifying theory. *J. Comp. Neurol.*, **321**:223-240.

Marshall, W. H., Woolsey, C. N. and Bard, P. (1941) Observation on cortical somatic sensory mechanism of cat and monkey. *J. Neurophysiol.*, **4**:1-24.

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

Meyer, G. and Gonzalez-Hernandez, T. (1993) Developmental changes in layer 1 of the human neocortex during prenatal life: a DiI-tracing and AChE and NADPH-d histochemistry study. *J. Comp. Neurol.* **338**:317-336.

Miller, B., Windrem, M. S. and Finlay, B. L. (1991) Thalamic ablations and neocortical development: alterations in thalamic and callosal connectivity. *Cereb. Cortex*, **1**, 241-261.

Molliver, M. E., Kostovic, I. and Van der Loss, H (1973) The development of synapses in cerebral cortex of the human fetus. *Brain Res.*, **50**:403-407

Molnar, Z. and Blakemore, C. (1990) Relationship of corticofugal and corticopetal projections in the prenatal establishment of projections from thalamic nuclei to specific cortical areas in the rat. *J. Physiol.*, **430**, 104P.

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

Mountcastle, V. B. (1957) Modality and topographic properties of single neurons of cat's somatic sensory cortex. *J. Neurophysiol.*, **20**:408-434.

O'Leary, D. D. M. and Koester, S. E. (1993) Development of projection neuron types, axon pathways and patterned connections of the mammalian cortex. *Neuron*, **10**:991-1006.

Payne, B. Pearson, H. and Cornwell, P. (1988). Development of visual and auditory cortical connections in the Cat. In: Cerebral Cortex, Vol. 7. (Peters, A. and Jones, E. G., eds.), Plenum Press, New York, pp. 309-389.

Palmer, L. A., Rosenquist, A. C. and Tusa, R. J. (1978) The retinotopic organization of the lateral suprasylvian areas in the cat. *J. Comp. Neurol.*, **177**:237-256.

Peinado, A and Katz, L. C. (1990) Development of cortical spiny stellate cells: retraction of transient apical dendrites. *Soc. Neurosci. Abstr.*, **16**:1127.

Price, D. J. (1985) Patterns of cytochrome oxidase activity in areas 17, 18 and 19 of the visual cortex of cats and kittens. *Exp. Brain Res.*, **58**:125-133.

Price, D. J. and Blakemore, C. (1985a) The postnatal development of the association projection from the visual area 17 to area 18 in the cat. *J. Neurosci.*, **5**:2443-2452.

Price, D. J. and Blakemore, C. (1985b) Regressive evens in the postnatal development of association projections in the visual cortex. *Nature*, **316**:721-724.

Price, D. J. and Ferrer, J. M. R. (1993) The incidence of bifurcation among corticocortical connections from area 17 in the developing visual cortex of the cat. *Eur. J. Neurosci.*, **5**:223-231.

Price, D. J., Ferrer, J. M. R., Blakemore, C. and Kato, N. (1994a) Functional organization of cortcocortical projections from area 17 to area 18 in the cat's visual cortex. *J. Neurosci.*,
14:2732-2746.

Price, D. J., Ferrer, J. M. R., Blakemore, C. and Kato, N. (1994b) Postnatal development and plasticity of cortcocortical projections from area 17 to area 18 in the cat's visual cortex. *J. Neurosci.*, **14**:2747-2762

Price, D. J. and Zumbroich, T. J. (1989) Postnatal development of cortcocortical efferents from area 17 in the cat's visual cortex. *J. Neurosci.*, **9**:600-613.

Raczkowski, D. and Rosenquist, A. C. (1983) Connections of the multiple visual areas with the pulvinar-lateral posterior complex and adjacent thalamic nucli in the cat. *J. Neurosci.*, **3**:1912-1942.

Rakic, P.(1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. *J. Comp. Neurol.*, **145**:61-84.

Rakic, P. (1974) Neurons in rhesus monkey visual cortex: Systematic relation between time of origin and eventual disposition. *Science*, **183**:425-427.

Rakic, P. (1978) Neuronal migration and contact guidance in the primate telencephalon. *Postgrad. Med. J.*, **54**:25-37.
Ramón y Cajal, S. (1911) Histologie du Système Nerveux de l'homme et des Vertébrés. Vol. 1. A Maloine, Paris.

Reinoso-Suarez, F. (1961) Topographischer hirnatlas der katze. (Kornmuller A. E., ed.) Darmstadt.

Rennie, S., Lotto, R. B. and Price, D. J. (1994) Growth-promoting interactions between the murine neocortex and thalamus in organotypic co-cultures. *Neuroscience*, **61**:547-564.

Romijn, H. J., de Jong, B. M. and Ruijter, J. M. (1988) A procedure for culturing rat neocortex explant in a serum-free nutrient medium. *J. Neurosci. Meth.*, **23**:75-83.

Rose, G. H. and Goodfellow, E. F.(1973) A stereotaxic atlas of the kitten brain. Los Angeles: University of California.

Rosenquist, A. C. (1985) Connections of visual cortical areas in the cat. In Peters, A. and Jones, E. G.(eds), Cerebral Cortex, Vol. 3. Plenum Press, New York, pp:81-117.

Salin, P., Bullier, J. and Kennedy, H. (1989) Convergence and divergence in the afferent projections to cat area 17. *J. Comp. Neurol.*, **283**:486-512.

Sanderson, K. J. (1971) The projection of the visual field to the lateral geniculate nucleus and medial interlaminar nuclei in the cat. *J. Comp. Neurol.*, **143**:101-118.

Sauer, F. C. (1935) Mitosis in the neural tube. *J. Comp. Neurol.*, **62**:377-405.

Schlag, J and Schlag-Rey, M. (1983) Thalamic units firing upon refixation may be responsible for plasticity in visual cortex. *Expl. Brain Res.*, **51**:146-148.

Shatz, C. J. (1977) Anatomy of interhemispheric connections in the visual system of Boston Siamese and ordinary cats. *J. Comp. Neurol.*, **173**: 497-518.

Shatz, C. J., Ghosh, A., McConnell, S. K., Allendoerfer, K. L., Friauf, E. and Antonini, A. (1991) Subplate neurons and the development of neocortical

connections. In: Development of the visual system, Vol. 3 (Man-Kit Lam, D. and Shatz., C. J., eds.), MIT Press, London, pp:175-196.

Shatz, C. J., Lindström, S. and Wiesel, T. N. (1977) The distribution of afferents representing the right and left eyes in the cat's visual cortex. *Brain. Res.*, **131**:103-116.

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

Schwarcz, R., Hokfelt, K., Fuxe, K. Jonsson, G. Goldstein, M. and Terenius, L. (1979) Ibotenic acid-induced neuronal degeneration: a morphological and neurochemical study. *Exp. Brain Res.*, **37**:199-216.

Sherrington, C. S. (1933) The brain and its mechanism. Cambridge University Press, London.

Sherk, H. (1986) Location and connections of visual cortical areas in the cat's suprasylvian sulcus. *J. Comp. Neurol.*, **247**:1-31.

Singer, W. and Rauschecker, J. P. (1982) Central core control of developmental plasticity in the kitten visual system: II. Electrical activation of mesencephalic and diencephalic projections. *Exp. Brain Res.*, **47**:223-233.

Singer, W. (1985) Central control of developmental plasticity in the mammalian visual cortex. Vision Res., 25:389-396.

Spear, P. D., Tong, L. and Langsetmo, A. (1978) Striate cortex neurons of binocularly deprived kittens respond to stimuii through closed eyelids. *Brain Res.*, **155**:141-146.

Stryker, M. P. (1991) Activity-dependent reorganization of afferents in the developing mammalian visual system. In: Development of the visual system, Vol. 3 (Man-Kit Lam, D. and Shatz., C. J., eds.), MIT Press, London, pp:267-287.

Stryker, M. P. and Harris, W. A. (1986) Binocular impulse blockade prevents the formation of ocular dominance columns in cat visual cortex. *J. Neurosci.*, **6**:2117-2133.

Symonds, L. L., Rosenquist, A. C., Edwards, S. B. and Palmer, L. A. (1981) Projections of the pulvinar-lateral posterior complex to visual cortical areas in the cat. *Neuroscience*, **6**: 1995-2020.

Symonds, L. L. and Rosenquist, A. C. (1984a) Corticocortical connections among visual areas in the cat. *J. Comp. Neurol.*, **229**:1-38.

Symonds, L. L. and Rosenquist, A.C. (1984b) Laminar origins of visual corticocortical connections in the cat. *J. Comp. Neurol.*, **229**:39-47.
Talbot, S. A. (1940) Arrangement of the visual field on cat's cortex. *Am. J. Physiol.*, **129**:477-478.

Talbot, S. A. (1940) Arrangement of the visual field on cat's cortex. *Am. J. Physiol.*, **129**:477-478.

Torran-Allerand, C. D. (1990) Neurite-like outgrowth from CNS explants may not always be of neuronal origin. *Brain Res.*, **513**:353-357

Tsumoto, T., Suda, K. and Sato, H. (1983) Postnatal development of corticotectal neurons in the kitten striate cortex: a quantitative study with the horseradish peroxidase technique. *J. Comp. Neurol.*, **185**, 657-679.

Tusa, R. J., Palmer, L. A. and Rosenquist, A. C. (1978) The retinotopic organization of area 17 (striate cortex) in the cat. *J. Comp. Neurol.*, **177**: 213-236.

Tusa, R. J., Rosenquist, A. C. and Palmer, L. A. (1979) Retinotopic organization of areas 18 and 19 in the cat. *J. Comp. Neurol.*, **185**: 657-679.

Tusa, R. J. and Palmer, L. A. (1980) Retinotopic organization of areas 20 and 21 in the cat. *J. Comp. Neurol.*, **193**:147-164.

Tessier-Lavigne, M. and Placzek, M. (1991) Target attraction: are developing axons guided by chemotropism? *Trends Neurosci.*, 14: 303-310.

Vogt, B. A. (1991) The role of layer 1 in cortical function. In A. Peters and E. G. Jones (eds): Cerebral cortex, Vol. 9, Normal and Altered States of Function. *New York: Plenum Press*, pp 49-90.

Yamamoto, N., Kurotani, T. and Toyama, K. (1989) Neuronal connections between the lateral geniculate nucleus and the visual cortex in vitro. *Science*, **245**:192-194.

Yamamoto, N., Yamada, K., Kurotani, T. and Toyama, K. (1992) Laminar specificity of extrinsic cortical connections studied in co-culture preparations. *Neuron*, **9**:217-228.

Yurkewicz, L., Valentino, K. L., Floeter, M. K., Fleshman, J. W. Jr. and Jones, E. G. (1984) Effects of cytotoxic deletions of somatic sensory cortex in fetal rats. *Somatosen. Mot. Res.*, **1**:303-327.

Windrem, M. S. and Finlay, B. L. (1991) Thalamic ablations and neocortical development: alterations of cortical cytoarchitecture and cell number. *Cereb. Cortex*, **1**, 230 -240.

Woodword, W. R., Meshul, C. K. and Coull, B. M. (1989) Ultrastructural and functional evidence for the survival of corticogeniculate neurons in kainic acid-lesioned lateral geniculate nucleus. *Brain Res.*, **494**:42-54.

Zeki, S. (1992) The visual image in mind and brain. *Sci. Amer.*, **267**: 69-76.