Development of the mammalian retinofugal pathways

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Thesis submitted for the degree of Doctor of Philosophy
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ABSTRACT

DEVELOPMENT OF THE MAMMALIAN RETINOFUGAL PATHWAYS

Sun On Chan, St. John's College, Oxford

Submitted for the degree of Doctor of Philosophy

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The retinofugal pathways in the rat and the ferret have been studied in order to understand how the decussation pattern at the chiasm and the retinotopic maps in the targets are developed.

By using DiI as a anterograde marker, it was found that fibres from one eye are arranged in a quadrant-specific order in the developing rat optic stalk. This order decays as the fibres approach the brain and is eventually lost at the chiasm. Thereafter, the fibres are arranged in another order which may predict the dorsoventral axis of their target termination. There is no obvious difference in the arrangement of fibres when the albino and normally pigmented optic pathways are compared, indicating that the misrouting of fibres at the chiasm of albino rats is not due to a change in gross fibre topography in the stalk.

By using DiI as a retrograde marker, it was found that the earliest uncrossed axons come exclusively from the central retina of rats, the axons from the temporal crescent arise later during prenatal development. The uncrossed fibres are found intermingling in the stalk with the crossed fibres at all ages studied, indicating that a non-chiasmatic cue must be involved in setting up the decussation pattern. It was also shown that only the temporal crescent but not the central ipsilateral component is abnormal in the albino rat during early stages of development, suggesting that the albino gene may act specifically on the cells in the retina.

By removing one eye prenatally in rats and ferrets, either pigmented or albino, the uncrossed pathway of the remaining eye is reduced at birth. This indicates that the axons require an interaction with fibres from the other eye to select an uncrossed path at the chiasm. The decussation line of the pigmented enucleates is not altered, indicating that the early enucleation does not produce an albino-like uncrossed pathway. Further, it has been shown in ferrets that the temporal crescent can be abolished after an early monocular enucleation, indicating that the binocular fibre interaction at the chiasm is crucial for the development of the temporal crescent.

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Table of Contents

CHAPTER 1	GENERAL INTRODUCTION	1
CHAPTER 2	FIBRE ARRANGEMENTS IN THE OPTIC PATHWAYS OF NORMAL AND ALBINO RAT EMBRYOS	
	Introduction	13
	Materials and Methods	16
	Results	19
	Discussion	26
	Figures	36
CHAPTER 3	PRENATAL DEVELOPMENT OF THE UNCROSSED RETINOFUGAL PATHWAYS IN NORMAL AND ALBINO RATS	
	Introduction	56
	Materials and Methods	60
	Results	64
	Discussion	74
	Figures	86
CHAPTER 4	THE EFFECTS OF PRENATAL MONOCULAR ENUCLEATION ON THE DEVELOPMENT OF THE UNCROSSED RETINOFUGAL PATHWAYS IN NORMAL AND ALBINO RATS	
	Introduction	110
	Materials and Methods	115
	Results	123
	Discussion	135
	Figures	145
CHAPTER 5	DEVELOPMENT OF THE UNCROSSED RETINOFUGAL PATHWAY IN PIGMENTED AND ALBINO FERRETS AFTER PRENATAL MONOCULAR ENUCLEATION	
	Introduction	161
	Materials and Methods	164
	Results	169
	Discussion	176
	Figures	183
CHAPTER 6	GENERAL DISCUSSION	197
	REFERENCES	205

CHAPTER 1 GENERAL INTRODUCTION

The visual system of mammals has been studied extensively in the past decades as a model to look for rules that govern the development of neural pathways. This system has been proven to be an excellent experimental model to work with in order to address the question of how some basic connection patterns in the nervous system are developed, such as the formation of topographic maps and the directional choice of growing axons at some crucial points along a neural pathway. In this thesis, I will concentrate on parts of the visual pathways of mammals connecting the eye and the subcortical visual nuclei, and discuss how some of the fundamental features of this retinofugal pathway, namely the decussation pattern and the retinotopic fibre arrangement, may be developed during prenatal life. Further, the development of the retinofugal projection in albino animals has also been examined in order to understand how this pathway may be altered by a mutation in one of the genes responsible for pigment production.

Retinotopic order in the primary visual pathway of mammals

It is well documented that in adult mammals the visual field is represented retinotopically in the primary visual nuclei, such as the dorsal lateral geniculate nucleus (Le Gros Clark and Penman, 1934; Polyak, 1957; Bishop et al., 1962; Montero et al., 1968; Guillery and Kaas, 1971; Sanderson, 1971; Lund et al., 1974; Reese and Jeffery, 1983; Holcombe and Guillery, 1984) and the superior colliculus

(Siminoff et al., 1966; Cynader and Berman, 1971; Lane et al., 1973 and 1974; Lund and Lund, 1976). The classical view for explaining how this retinotopicity is formed in visual targets of mammals is often based on the idea that retinal fibres are arranged topographically according to their retinal origins in the adult pathways (Polyak, 1957). This idea leads to the postulation that growing fibres in the developing optic pathway maintain the neigbourhood relationships that are also seen in the adult, and that this order determines or contributes to the final termination pattern of fibres in the target.

However, there is growing evidence that in adult mammals the retinotopic order is lost on the way from the eye to the chiasm (Horton et al., 1979; Naito, 1986 and 1989; Baker and Jeffery, 1989), indicating that the target maps are not likely to be formed by simply preserving the retinotopic fibre order in the pathway. These results raise serious problems concerning whether fibres in the developing optic stalk are really arranged in a retinotopic array. Accounts of developing rodent visual pathways have proposed that there is a retinotopic order of the fibres in the optic stalk (Silver, 1984; Horsburgh and Sefton, 1986). However, none of these studies provided clear evidence that the same population of fibres was being traced throughout the stalk since the fibres were not labelled by any marker. Further, there is significant evidence for interchange of fibre groups and fibres between individual fibre bundles (Silver, 1984; Williams and Rakic, 1985). Therefore, whether a retinotopic order exists in the developing optic stalk of mammals and whether this order is essential for the establishment of the target maps still remained to be defined.

The origin of the uncrossed retinofugal pathways in mammals

It has been well established for nonprimate adult mammals that the majority of retinal ganglion cells with axons projecting to ipsilateral targets arise from the temporal retina, but a small number of ganglion cells in the nasal retina also project ipsilaterally (rat: Perry and Cowey, 1979; Lund et al., 1980; Jeffery and Perry, 1982; Perry and Linden, 1982; mouse: Dräger and Olsen, 1980; Godement et al., 1987; hamster: Hsiao, 1984; Insausti et.al., 1984; ferret: Vitek et al., 1985; Morgan et al., 1987; cat: Stone, 1966; Cooper and Pettigrew, 1979a). In this thesis, the nasal border of the major population of ipsilaterally projecting ganglion cells of the temporal retina is defined as the decussation line, and this term will be used in this sense throughout the following descriptions. In rodents and carnivores, the majority of the contralaterally projecting cells are found in the region nasal to the line of decussation, but a number of cells temporal to this line also project contralaterally. In primates, the contralaterally projecting cells come almost exclusively from the nasal retina whereas the ipsilaterally projecting cells arise only in the temporal retina (monkey: Stone et al., 1973; Leventhal. et al., 1988; Fukuda et al., 1989). These two population of cells only overlap within a narrow strip of retina representing the vertical meridian of the visual field.

Development and modifiability of the uncrossed retinofugal pathway

Most studies on the mammalian uncrossed retinofugal pathway have been targeted to an understanding of the development and modifiability of this pathway during late

prenatal and postnatal development (for review see Lund, 1978). One of the most important findings is that there is an exuberant uncrossed retinal component in immature animals, in terms of the termination pattern and number of retinal ganglion cells. This has been demonstrated in rats (Land and Lund, 1979; Maxwell and Land, 1981; Bunt et al., 1983; Cowan et al., 1984; Jeffery, 1984; Chan et al., 1989), mice (Godement, 1984), hamsters (So et al., 1978 and 1984; Insausti et al., 1984), ferrets (Linden et al., 1981; Cucchiaro and Guillery, 1984; Morgan and Thompson, 1985; Henderson et al., 1988), cats (Shatz and Kliot, 1982; Shatz, 1983; Chalupa and Williams, 1984; Sretavan and Shatz, 1984 and 1986) and monkey (Chalupa and Lia, 1991). This immature pattern is later modified and refined during development by an extensive loss of retinal ganglion cells and their axons, so that a more precise and restricted uncrossed pathway is established in the adult (Land and Lund, 1979; Ng and Stone, 1982; Potts et al., 1982; Lam et al., 1982; Jeffery, 1984; Williams et al., 1986; Henderson et al., 1988; Chan et al., 1989; Chalupa and Lia, 1991).

Removal of one eye at this immature stage results in the formation of a larger than normal uncrossed pathway in the adult animal (Lund and Lund, 1973 and 1976; Lund et al., 1973; Hsiao, 1984; Jen et al., 1984; Jeffery, 1984; Chalupa and Williams, 1984; Chan et al., 1989), indicating that the normal cell death is modulated by a lack of a binocular interaction of fibres in the target of the experimental animals (Rakic, 1981; Rakic and Riley, 1983a and 1983b).

The characteristic adult pattern of the uncrossed projection, with the majority of the ipsilaterally projecting retinal ganglion cells confined to the temporal retina and

a sparse population distributed in areas outside, can be seen during prenatal development long before the onset of the major cell death period (Bunt et al., 1983; Godement et al., 1987b; Lia et al., 1987; Collelo and Guillery, 1990; Sretavan, 1990a; Chalupa and Lia, 1991). These observations indicate that when the temporal component of the uncrossed pathway first appears, its border is well defined. The set of cues that control the formation of the decussation pattern of the uncrossed component must act on the pathway very early in development and be independent of cell death.

Relationships of the birthdates of retinal ganglion cells and their decussation patterns

One hint to understanding how the decussation pattern of the uncrossed pathway is developed came from the studies of birthdates of different classes of retinal ganglion cells and the courses of their axons along the adult pathway (for review see Guillery, 1991).

In carnivores, retinal ganglion cells in the adult retina can be classified into three major classes - alpha, beta and gamma (or Y, X and W respectively), according to their soma sizes, dendritic morphology, physiological properties and terminal distribution of their axons (Boycott and Wässle, 1974; Fukuda and Stone, 1974; Bowling and Michael, 1980; Wässle et al., 1981a and 1981b; Fukuda et al., 1984; Vitek et al., 1985). The alpha cells have the largest soma size and axon diameter, gamma cells are the smallest in size and axon diameter, and the size and fibre

diameter of beta cells are in between (Fukuda et al., 1984).

It has been shown in the cat that the retinal ganglion cells are produced more or less sequentially according to their morphological classes during the neurogenesis of the retina (Walsh et al., 1983; Walsh and Polley, 1985). The beta cells are generated before the alpha cells, and the gamma cells are born throughout the period of ganglion cell genesis.

The different classes of ganglion cells have their characteristic decussation patterns. In the cat, by using retrograde degeneration technique, it has been demonstrated that the beta cells have a "primate-like" decussation pattern (Fukuda and Stone, 1974), with cells nasal to the line of decussation being crossed and cells temporal to the line of decussation being uncrossed. The alpha cells have both crossed and uncrossed axons. All uncrossed axons of this group arise from the temporal retina whereas the crossed axons come from the nasal retina and the parts of temporal retina close to the decussation line. The decussation pattern of gamma cells is very similar to that of the alpha cells, again with uncrossed axons arising from the temporal retina and crossed axons from the whole retina surface, but also including the most peripheral parts of the temporal retina.

If one relates the birthdates of different ganglion cell classes to their decussation pattern, one will readily see that the earliest born cells (beta cells) have the most distinct line of decussation, whereas the later generated cells (alpha and gamma classes) have a less clearly defined line of decussation and this line appears to be shifted gradually towards the temporal retinal margin as development proceeds. It

looks as if there is a signal that produces the distinct decussation pattern and this signal seems to decay and move the line of decussation to the temporal retinal margin, so that the later generated ganglion cells become more likely to take a crossed route than the earlier generated ones (Walsh et al., 1983).

The idea that the earlier the ganglion cells are born, the greater the chance is for their axons to take a uncrossed route is supported further by two lines of evidence. The first one comes from studying the birthdates of retinal ganglion cells in the mouse in relation to their decussation pattern (Dräger, 1985). It was found that the earliest generated retinal ganglion cells show a clear segregation according to their crossed or uncrossed pathway, with cells born in the nasal retina being all crossed and cells in the temporal crescent being all uncrossed. However, this clear segregation pattern is lost in later development, with more and more ganglion cells having crossed axons being born in the temporal crescent.

Other evidence comes from the study of the decussation pattern of axons in the optic tract. In cats, the partial segregation of fibre diameter classes in the optic tract can be interpreted as a chronologic order of retinal ganglion cell axons, with the oldest axons (mostly from the beta cells) occupying the deepest parts of the tract and the younger axons (from the alpha and gamma cells) being more superficially located (Walsh et al., 1983). When the deep parts of the tract are selectively labelled, it is found that the ganglion cells (mainly beta) that have their axons lying in this part of the tract show a clear decussation pattern, with the crossed axons coming from the contralateral nasal and the uncrossed axons coming from the ipsilateral temporal retina

(Reese et al., 1991). In contrast, injections into the superficial tract label almost exclusively crossed axons (mostly from the alpha and gamma cells) from the contralateral nasal and temporal retina.

The role of binocular fibre interactions in the development of the uncrossed retinofugal pathway

In addition to the birthdate of retinal ganglion cells, the chiasmatic choice of growing retinal axons is also influenced by the interaction with their environment at the chiasm. Studies of the trajectories of uncrossed retinal axons at the chiasm have revealed that as these axons reach the chiasm, they turn abruptly and head towards the ipsilateral tract, as if these axons hit a cue at the chiasm that deviates them before they can cross the midline (Godement et al., 1990; Godement and Mason, 1990; Sretavan, 1990a and 1990b). These observation led to the suggestion that there may be a local cue at the chiasm that instructs specifically the uncrossed axons to turn and the crossed axons to pass the midline of the chiasm.

This chiasmatic cue may be present on the glial elements or it may be carried by the fibres that pass the chiasm. Studies on the effects of prenatal monocular enucleation on the development of the uncrossed pathway, before the optic fibres terminate in their targets, have shown that the uncrossed component in the remaining eye of the adult animal is reduced, in terms of retinal ganglion cells for mice (Godement et al., 1987a) and the termination of the retinogeniculate projection for ferrets (Guillery, 1989). These results indicate that the development of the uncrossed

pathway is somehow dependent on the interaction with fibres from the opposite eye at the chiasm. However, the nature of this chiasmatic fibre interaction and the extent to which the uncrossed pathway depends on this binocular interaction at the chiasm is still unknown.

The uncrossed retinofugal pathway in albino mammals

It is well documented in a number of mammalian species that the uncrossed retinal component is abnormally small in animals that have a defect in the production of ocular melanin (rat: Lund, 1965; Guillery et al., 1973; Lund et al., 1974; Creel and Giolli, 1976; mouse: Guillery et al., 1971; LaVail et al., 1978; Dräger and Olsen, 1980; rabbit: Sanderson, 1975; cat: Guillery, 1969; Guillery and Kaas, 1971; Creel et al., 1982; ferret: Guillery, 1971; Morgan et al., 1987; mink: Sanderson et al., 1974; Guillery et al., 1979; tiger: Guillery and Kaas, 1973; monkey: Guillery et al., 1984). This reduction of the uncrossed component is probably caused by a misrouting of some of the normally uncrossed fibres at the chiasm, as demonstrated in the Siamese cat (Guillery and Kaas, 1971; Stone et al., 1978; Cooper and Pettigrew, 1979; Shatz and Kliot, 1982). This misrouting of axons affects most severely the ganglion cells close to the nasal border of the ipsilateral cell population in the temporal retina, it thus causes a temporal shift of the line of decussation in the Siamese retina (Stone et al., 1978; Guillery and Kaas, 1971; Cooper and Pettigrew, 1979).

The development of the uncrossed pathway in the albino animals has been studied by using anterograde tracing technique. In the animals examined so far, it is clear that

the pathway abnormality appears very early in development (rat: Bunt et al., 1983; Gayer et al., 1989; ferret: Cucchiaro and Guillery, 1984; cat: Shatz and Kliot, 1982; Kliot and Shatz, 1985). That is, the albino gene acts to affect the mechanisms that govern the normal development of the decussation pattern of the retinal pathway during the prenatal life of the animal.

It has been demonstrated that the pathway abnormality is closely linked to the severity of pigment production which is caused by mutations at different gene loci concerned with the melanin synthesis or packaging (Sanderson et al., 1974; LaVail et al., 1978; Balkema and Dräger, 1990). The chiasmatic pathway is also affected more severely in animals with a total absence of pigment production than in animals with a reduced retinal pigment production (Guillery and Kaas, 1971; Sanderson et al., 1974; LaVail et al., 1978; Creel et al., 1982). These results indicate that the ocular melanin is in some way related to the wrong chiasmatic choice made by the retinal axons in the albino mutants.

It has been suggested that the misrouting of fibres at the chiasm may be due to a change in fibre topography in the developing stalk as a result of absence of pigment in the albino stalk (Silver and Sapiro, 1981; Webster et al., 1988). However, there is growing evidence showing that fibre positions in the optic stalk of albino animals are not obviously different from those in the normally pigmented animals (Strongin and Guillery, 1981; Horsburgh and Sefton, 1986; Collelo and Jeffery, 1991), indicating that the albino mutation does not affect the fibre position in the optic stalk.

As mentioned in the previous section, an early monocular enucleation can produce a reduced uncrossed retinal pathway in a normally pigmented animal (Godement et al., 1987a; Guillery, 1989). It is surprising to know that the uncrossed pathway thus produced is remarkably similar to the abnormal albino uncrossed pathway, in terms of the numbers and the distribution of ipsilaterally projecting retinal ganglion cells for the mouse (Godement et al., 1987a) and of the termination of retinogeniculate axons for the ferret (Guillery, 1989). Further, the early enucleation appears to have no effect on the albino uncrossed pathway (Guillery, 1989). These results raise a very interesting idea, that in ferrets the albino pathway may be developed as a result of the absence or weakening of the binocular fibre interaction at the chiasm. However, it is still unclear whether the early enucleation in pigmented animals really produces an albino "phenocopy" of the pathway abnormality. It still remains to be defined whether the reduced uncrossed component in the pigmented ferret with an early enucleation has its line of decussation shifted temporally, comparable to the shift seen in the albino mutants.

Aims of the studies

This thesis consists of four individual studies, which are all aimed at understanding the development of the retinofugal pathway in mammals.

The first study is to look at the quadrant-specific fibre organization in the optic pathway of embryonic rats, and then ask if there is any difference in the fibre organization in the stalk between pigmented and albino animals in order to clarify the

question whether the pathway abnormality in the albino animals is caused by a change in fibre topography in the stalk (Silver and Sapiro, 1981; Webster et al., 1988).

The second study is to determine, by using a retrograde labelling technique, the prenatal development of the uncrossed retinal pathway in pigmented and albino rats. The question asked is whether the albino gene may act specifically on the routing of retinal ganglion cells from the temporal crescent or it may act on the ganglion cells throughout the retina as a whole.

The third chapter is dedicated to understanding the effects of a prenatal monocular enucleation on the development of the uncrossed pathway in the rat, either pigmented or albino, emphasizing the question of whether the absence of binocular fibre interaction at the chiasm of pigmented animals early in development really produces an albino phenocopy of pathway abnormality.

The last chapter is aimed at studying the effects of early monocular enucleation in ferrets, which have a much better developed uncrossed component and much better defined decussation line than do rodents. This study is to test directly the idea that the elimination of one eye in early development may produce an albino-like uncrossed component in the pigmented ferret (Guillery, 1989). Moreover, some preliminary results are presented to demonstrate the extent to which the uncrossed pathway in ferrets is dependent on the binocular interaction at the chiasm by making earlier monocular enucleation in ferret fetuses, which is technically extremely difficult to achieve in rats.

CHAPTER 2 FIBRE ARRANGEMNETS IN THE OPTIC PATHWAYS OF NORMAL AND ALBINO RAT EMBRYOS

Introduction

In this study, the fibre arrangements in the optic pathways of rat embryos have been investigated in order to understand first how the fibre order is related to the development of retinotopic maps in primary visual targets, and second, whether the fibre topography in the optic stalk can in some extent be related to the chiasmatic choice made by the growing optic axons.

It is known that the terminals of optic axons are arranged very precisely, according to their retinal origins, in the target fields so that retinotopic maps are formed. One of the approaches, that attempts to explain how the maps develop is based on the argument that fibres in the adult are arranged retinotopically and that during development they maintain their neighbourhood relationships along their paths to the targets (Horder and Martin, 1978; Bunt and Horder, 1983). This argument can explain the development of maps only if the retinotopic order is maintained rigidly along the pathway. However, there are a number of studies in teleosts and amphibians showing that although fibres in the nerve are arranged retinotopically, they are subject to rearrangements, either abruptly or gradually, at the nerve-tract junction. This reshuffling of fibres brings a change from one quadrant-specific fibre order in the nerve to another order in the tract, with this latter matching the target map (cichlid fish: Scholes, 1979; goldfish: Easter et al., 1981; Bunt, 1982; Springer and Mednick, 1986a and 1986b; *Rana*: Scalia and Fite, 1974; Reh et al., 1983; *Xenopus*: Fawcett,

1981; Fawcett and Gaze, 1982; Fawcett et al., 1984; Taylor, 1987). During this transition process, the near neighbourhood relationships of the retinal fibres in the nerve must be changed in some way to allow the establishment of a new order in the tract.

In mammals, the classical view on the fibre organization of optic pathways is that retinotopy is preserved along the whole primary visual pathways, i.e. fibres in the optic nerve, chiasm and tract are organised topographically as a representation of their origin in the retina (Polyak, 1957). However, there is growing evidence showing that axons from a small part of the adult retina, although they are apparently grouped tightly together when they enter the nerve, become scattered when they approach the chiasm (Horton et al., 1979; Naito, 1986 and 1989; Baker and Jeffery, 1989). These observations suggest that the retinal fibres are organised in a well defined quadrant-specific order at the distal part of the optic nerve but that in the adult this order is lost guadually as the fibres approach the brain. Beyond the chiasm, fibres are organised again within the tract but into different quadrant-specific maps as demonstrated in the adult cat (Torrealba et al., 1982). Based on these findings, one may envisage that a sorting process, similar to that found at the nerve-tract junctions of teleosts and amphibians, may occur also in mammals and that this is responsible for the change from one fibre order in the nerve to another order in the tract. However, the questions of whether this rearrangement of fibre order happens in mammals and where it takes place during development is far from clear.

The fibre order in the optic stalk of fetal mouse has been related to the chiasmatic choice made by the retinofugal fibres. It has been reported by Silver (1984) that

fibres are organised retinotopically in the rodent optic nerve and that this quadrant-specific order is maintained along the nerve upto the the optic chiasm. The ventral temporal fibres, which constitute the majority of the uncrossed component, are believed to occupy the caudal part of the nerve and chiasm and are thus thought to be guided to the ipsilateral optic tract along the most direct route by mechanical means. However, it has been shown in several recent studies that the growing optic axons can take an ipsilateral course at any point in the rostrocaudal dimension of the chiasm (Sretavan, 1990; Collelo and Guillery, 1990; Godement et al., 1990). These results indicate that factors other than the mechanical guidance must also be involved in directing the growing optic axons into the ipsilateral tract.

It is well known that the uncrossed retinofugal components in albino animals are abnormally small, probably due to misrouting of some of the uncrossed fibres at the chiasm (Lund, 1965; Guillery, 1969; Guillery and Kaas, 1971; Dräger and Olsen, 1980; Creel et al., 1982). The chiasmatic misrouting has been explained by the suggestion that fibre topography within the stalk in albino animals is distorted because a group of early fibres are shifted to an ectopic position in the dorsal part of the stalk in the absence of pigment (Silver and Sapiro, 1981; Webster et al., 1988). However, this idea is contradicted by a number of observations which report that there is no evidence that fibres occupy an abnormal position within the optic stalk of the albino rat (Horsburgh and Sefton, 1986), mouse, hamster or ferret (Strongin and Guillery, 1981; Colello, 1989). Though it has been shown that the albino abnormality does not affect the position of fibres in the stalk, the question of whether the albino mutation affects the quadrant-specific fibre organization in the stalk during early development

still remains unresolved.

In this study, we have used a lipophilic dye, DiI (Honig and Hume, 1986; Godement et al, 1987b), to label different retinal quadrants in rat embryos and look at the quadrant specific topographic organization of retinal fibres along the optic pathway. The first question to ask is whether a retinotopic representation exists in the fibres within the stalk, chiasm and tract, and to see if there is any change in fibre order in the pathway, as such commonly observed in teleost and amphibian visual systems. Further, it is of interest to determine whether the changes in the pathway can be related to the way in which the retina is mapped onto the primary visual targets. The second question to ask is whether there is a abnormality of the quadrant-specific fibre topography in the optic stalk of albino rat as suggested before (Silver and Sapiro, 1981) that may be correlated to the misguidance of fibres at chiasm in the albino mutants.

Materials and Methods

Lister Hooded rats and Sprague Dawley albino rats were used in this study. Time mated females were obtained from the animal house in this Department. The day on which a plug was found was designated as embryonic day 0 (E0).

Fixation

Two groups of embryos were collected, one at the age of E16.5 and the other at E18.5. The mothers were anaesthetized by an intraperitoneal injection of pentobarbital

(40 mg/kg body weight) and the embryos were taken out by Caesarian section. After washing in phosphate buffered saline, the embryos were fixed by immersing into 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.4. The heads were removed and fixed in the same fixative at 4°C. The tissues were usually stored for 3 days, but could be kept upto 2 months, before making the DiI injection.

DiI implant into the retina

A lipophilic carbocyanine dye, DiI (DiIC18(3): 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, from Molecular Probe Inc., Eugene, OR), was used to label the optic pathways.

Before the dye was introduced into the retina, the skin overlying the eye, togther with the cornea and lens were removed with a fine syringe needle. The interior of the eye was then dried with a cotton swab. For labelling the whole retina, a clump of DiI crystals was either deposited directly onto the optic disk or dissolved in a small amount of Triton X-100 intraocular (Godement et al., 1987b) under a dissecting microscope. Most embryos received a focal labelling of the retina. The retina was partitioned into four quadrants: ventral temporal, dorsal temporal, dorsal nasal and ventral nasal. A small, solid DiI crystal was inserted onto one retinal quadrant and the positions of labelled fibres coming out from this part of retina was later traced along the optic pathway.

After the dye was implanted, the embryos were transfered to 2% buffered formalin and stored in the dark at room temperature to allow the dye to diffuse. In this experiment, E16.5 tissues were normally stored for 6 weeks and E18.5 tissues for 12

weeks.

Preparation of sections

The heads of embryos were embedded in gelatin-albumen which is hardened with the addition of 10% glutaraldehyde (from 25% stock solution). Excess embedding material was removed, the tissue was oriented through the transparent embedding medium and one corner was cut to mark the orientation of the block.

In E18.5 embryos, transverse sections of the optic tract were collected first. The rostral part of the head was tilted ventrally and sectioned horizontally at a plane about 30° to the horizontal at a thickness of 200 um on a vibratome. Serial sections were collected until they were about 400-600 um above the floor of the diencephalon. After collecting the sections of the optic tract, the remaining block was re-embedded and sectioned parasaggitally on a vibratome in order to obtain serial 200 um transverse sections through the eyecup, optic stalk and chiasm. In E16.5 embryos, only the transverse sections from the eye to the chiasm were examined.

All the sections were collected in 0.1M phosphate buffer. They were transferred onto clean slides, mounted in an antiquenching agent (glycerol with 1% p-phenylenediamine, Johnson et al. 1982) and coverslipped.

The sections were viewed either with a Leitz fluorescent microscope or with a MRC 500 confocal imaging system (Biorad) using a rhodamine filter set. The images were recorded on black and white films, using Kodak TMAX 400 for fluorescent micrographs and Ilford FP4 for confocal micrographs.

Results

Checking of retinal label

The extent of the retinal label was checked in transverse sections of the eye-cups in all embryos. Only those embryos that showed a localized labelling in the retina were selected. The table below (Table 1) shows the number of successful cases in which only a small part of the retina had been labelled.

Table 1. Number of cases selected in which a focal label of DiI is successfully made in the retina

	Number of cases	
Age	Pigmented	Albino
E16.5	15	8
E18.5	15	18

The findings in E16.5 embryos are virtually the same as the results in E18.5 embryos, except that there are fewer fibres in the optic pathway in E16.5 rats. Therefore, most of the following descriptions will be based on the results obtained in E18.5 embryos.

Fig. 1 shows the retinal labels in eight E18.5 embryos, the four on the right column are normal and the other four on the left column are albino. The embryos in each group received a DiI implant in the ventral temporal, dorsal temporal, dorsal nasal and ventral nasal retina respectively. The confocal images show that the retinal label is largely limited to one retinal quadrant, except in the two embryos with the

ventral nasal label where some dye is found diffused across the neighouring ventral temporal retina (Fig. 1d and h). The whole retina in each embryo was checked by examining sections from the rim of the retina to the optic disk. In most cases the DiI granule was found sitting in the retinal layers and only labelled the tissues next to it without spreading to other retinal regions.

Fibre order in the distal optic stalk

In the retina, labelled fibres from each sector bundle together into radial fascicles. These fascicles then converge towards the optic disk and meet fibres from the other retinal sectors. They are arranged at first in a radial array, just like the spokes of a wheel (Fig. 2a). No dramatic exchange of fibres between fascicles is found at this region. Hence, the optic fibres maintain their relative neighbourhood relationships in accordance with their origins in the retina.

However, this organization is broken down quickly as the fibres enter the optic stalk. Here, within 200 um from the eye, the circular order found in the optic disk begins to split ventrally at the optic fissure and transform into a linear order in the stalk. The ventral side of the stalk within this region can be marked by the hyaloid artery which enter the back of the eye through the optic fissure (Fig. 2b). As a result of this split, the ventral temporal and ventral nasal fibres, which are neighbouring to each other at the disk, are now separated and occupy two distinct parts at the rostral and caudal sides of the stalk (Fig. 3). Between the ventral fibres are fibres from the dorsal retina. Although there is some overlap between fibres from adjacent retinal quadrants, it is still readily recognisable that fibres from ventral nasal, dorsal nasal,

dorsal temporal and ventral temporal retina are arranged in sequence rostrocaudally across the transverse section of the stalk. It should be pointed out that, as mentioned above, in the two cases of ventral nasal label some dye is found spread across to the adjacent ventral temporal retina. That is why in these animals a small group of filled fibres is found in the caudal part of the stalk (Fig. 3d and h).

In albino embryos, similar relationships are seen within the distal stalk. A retinotopic map is formed in which the circumferential organization of the retina is represented linearly across the rostrocaudal dimension of the stalk (Fig. 3). No distinguishable difference can been seen between the maps in normal and albino animals.

Fibre order in the proximal optic stalk

The distinct linear arrangement of the quadrantic representation of the retina in the distal stalk is not maintained very far into the stalk. In fact, at a point 600 um from the eye, about one third of the stalk's length, a change in the relative positions of the labelled fibres begins to appear (Fig. 4). The ventral fibres show a tendency to shift dorsally and eventually meet at the dorsal parts of the stalk. This is accompanied by the movement of the dorsal retinal fibres towards the ventral regions of the stalk. At this point, the dorsal temporal fibres mix almost entirely with the dorsal nasal fibres. This relationships is maintained along the optic pathway, as shown by the confocal micrographs in Fig. 5, which are taken from a region 1400 um from the eye, just before the optic stalk begins to connect to the ventral part of the diencephalon. At this point a more extensive intermixing of fibres between neighbouring quadrants is

observed. The overlap is most obvious between the dorsal temporal and dorsal nasal fibres. These fibres spread across the stalk rostrocaudally but appear to occupy apparently the ventral half of the stalk. Although the fibres from the two dorsal sectors overlap almost completely, the temporal and nasal fibres from the ventral retina remain almost entirely separate on the two sides of the stalk. Most of the ventral fibres are found in the dorsal tier of the stalk. It is worth noting that in this region, before the fibres have reached the diencephalon, a rough quadrant-specific retinotopic map is still recognizable.

In the materials examined so far, the albino embryos, when compared with normals, show no apparent difference in the fibre organization along the whole length of the stalk (Fig. 4 and 5). Minor difference can be seem between certain comparable micrographs, e.g. the ones in Fig. 4b and f which show that the dorsal temporal fibres in the albino stalk are more scattered than the fibres in the normal stalk. This discrepancy is possibly caused by a difference in the size of retinal label because in another albino embryos in which a smaller retina label was made at the dorsal temporal retina, the filled fibres are found in a more restricted area in the central and ventral parts of the stalk, just like the distribution of dorsal temporal fibres in normal embryos. Hence, a rough retinotopic order is recognizable in the albino stalk, but again there is a considerable overlap of fibres from adjacent retinal sectors, especially when the labelled fibres approach the brain. Based on these results, it can be concluded that the absence of pigment in the albino optic pathways is very unlikely to cause any change in the overall topographic arrangement of fibres in the optic stalk.

Changes in fibre orders in the prechiasmatic region and optic chiasm

It has been shown above that a rough retinotopic order is maintained along the stalk until it connects to the diencephalon. Several interesting questions then arise. The first one is whether this order is maintained at the optic chiasm and whether it can be related to the route that an optic axon takes at the chiasm. The second question is whether there is a change in order when the early axons reach the optic tract and how this may be related to the maps that are formed in the primary visual targets. The first question can be resolved by examining the organization of labelled fibres as they approach the midline of the brain.

At the prechiasmatic region, about 1600 um from the eye, the dorsal part of the optic stalk begins to join the floor of the diencephalon. Following this structural change, the fibre fascicles are now arranged in the shape of a cresent at the ventral margin of the stalk. The focal retinal labels reveal a change in the fibre arrangement in the this region. Fibres from the dorsal retinal sectors are distributed rostrocaudally across the ventral marginal crescent (Fig. 6), similar to their distribution in the proximal stalk. The nasal fibres still occupy the rostral half of the prechiasmatic region. However, it is obvious that the ventral temporal fibres start to shift from their caudal position towards the rostral region of the prechiasmatic stalk.

At the midline (Fig. 7), about 2200 um from the eye, although fibres from different retinal sectors seem to localize at certain region in the optic chiasm, it is evidence that they intermingle very extensively with one another. It appears that there is no recognizable map at the midline in the chiasm.

Similar results have been obtained in albino embryos (Fig. 6 and 7), which show

that at the prechiasmatic stalk fibres from each retinal quadrant, especially those from the ventral retina, begin to spread across the rostrocaudal extent of the optic chiasm. These observations suggest that optic fibres in albinos may follow a set of regional instructions similar to those guiding fibres across the chiasmatic region in normal animals.

Fibre order in the optic tract

The question of whether there is a fibre order in the optic tract and how this order, if it exists, is related to the target maps is answered by investigating the organization of labelled fibres from different retinal quadrants within the tract. A total of 33 E18.5 embryos has been examined, including 15 normal and 18 albino rats. In general, we have found that the fibres are arranged in a recognizable order in the tract and that order is not different in the normal and albino animals. Therefore, the results obtained from these two strains of animals are pooled and presented together.

In order to define the boundaries of the optic tract, in three rats at E18.5 the right eye was labelled by DiI and the tract position was identified in transverse sections of the diencephalon. The results are shown in Fig. 8. Fibres with fluorescent label are seen primarily in the optic tract contralateral to the injected eye. These fibres are grouped together as a bundle and remain closely packed through all the sections examined, from 400 um to 1400 um above the optic chiasm. Besides the fluorescent label, the optic tract is readily recognized by two special features. The first one is its location. The tract is at the lateral surface of the diencephalon just next to the rostral tip of the fissure that separates the diencephalon on the medial side from the

telecephalon on the lateral side (Fig. 8). The second special feature is its shape. In Fig. 8c-e, from 800 um to 1200 um above the chiasm, the optic tract has a wedge shape with an expanded rostral head and a slender and tapered caudal tail, very much like a comma. All of these features enable us to define the boundaries of the tract which are essential for the analysis of the results.

Four E18.5 embryos have been selected to illustrate the results. A small DiI implant was made in one retinal quadrant in each animals. Fig. 9 illustrates the spread of label across the retina in these animals, which is more or less confined within one retinal quadrant.

The position of labelled fibres in the optic tract contralateral to the injected eye of these four animals is shown in Fig. 10. This set of confocal micrographs were taken 800 um above the chiasm, where the tract begins to attain its comma shape. It is clear that the ventral temporal and ventral nasal fibres both occupy the rostral head of the optic tract. The most densely labelled regions are in the superficial part, which abuts the rostral tip of the fissure, and the lateral part of the rostral head. Very few of the labelled ventral retinal fibres are found in the caudal tail. However, there is no apparent segregation between these two populations of ventral retinal fibres. Fibres from the dorsal temporal and dorsal nasal retina occupy a totally different region in the tract. The dorsal fibres lie rostrally at the deep and medial parts of the head and gradually move to the superficial region at the caudal half of the tract. Only a few of them are occasionally seen in the medial and superficial regions in the rostral head. Again there is no evidence for any segregation in the position of these two groups of dorsal fibres.

There may be an argument that the apparent fibre order seen in the tract could be an artefact caused be the variation in the plane of sectioning. This is very unlikely because of the fact that consistent results have been obtained in more than 6 cases in each group of embryos. In addition to that, the special shape of the tract and its relation to the fissure are very useful landmarks to identify the different position of filled fibres in the tract. Further, the tract order at different levels in the brain has been examined. The arrangement of fibres found in the tract 200 um above the sections shown in Fig. 10 is similar to that shown for each of the four embryos (not shown). So it can be concluded that a specific fibre order is present in the optic tract of embryonic rats, in which the ventral retina is represented at the rostral and superficial parts of the tract whilst the dorsal retina is represented in the deep and caudal parts of the tract.

Discussion

Technical considerations

Since DiI acts by the incorporation into plasma membranes, it will label all cells indiscriminately around the site of injection. When the dye is applied to trace a long pathway, e.g. the retinofugal projection, transcellular labelling is reported to occur in those cells that appose closely to the the brightly filled axons (Godement et al. 1987b) and at the axon terminal (Fritzsch and Wilm, 1990). Similar observations have occasionally been found in this study. In some cases, especially the ones with prolonged storage after applying the dye, a few neuroepithelial cells which are in close

vicinity to some brightly labelled axons can be seen labelled. But it is easy to distinguish these palely labelled cells from the brightly labelled optic axons (Godement et al, 1987b; personal observation). In this study, the storage time after applying DiI to the retina has been adjusted so that optic axons can be filled to their maximal extent while transcellular diffusion is kept to a minimum.

Another point that is worth noting is the rate of diffusion of the dye. The rate of diffusion of DiI in fixed tissue is affected by several factors: a) the dye diffuses faster at higher temperatures. It should be noted that although the storage time can be shortened considerably when the tissue is stored at a higher temperature, eg. 40°C in an oven, the quality of labelling thus obtained is far less satisfactory than after storage at room temperature, and the incidence of trancellular spreading occurs more frequently in these preparations (Godement, personal communication). b) The dye seems to diffuse faster in lightly fixed tissues. It has been observed that when DiI is placed into the retina of an embryo fixed for example 3 days, satisfactory label is obtained in a much shorter time than after similar labelling in embryos fixed for months. The reason is not known, but may be due to the change in fluidity of the plasma membrane upon storage in fixative. That is the reason why the tissues have been stored at a low concentration of formalin in this study.

Fibres do not maintain their neighbourhood relationships in the rat optic stalk

In summary, it has been shown in this study that growing fibres from different retinal quadrants are arranged retinotopically when they invade the optic stalk, and that this order decays gradually as the fibres approach the chiasm. At the midline of the

brain, the quadrant-specific order appears to be completely lost. However, as the fibres grow into the tract, they begin to reorganize into another order in which there is a clear segregation of the dorsal from the ventral retinal fibres but not of the nasal and temporal fibres.

The early fibres in the rat optic stalk become scattered, as indicated by the increase in the degree of overlap between fibres from neighbouring retinal quadrants, when they approach the brain. This is reflected in the adult pathway in which the optic fibres arising from ganglion cells in a small part of the retina become dispersed in the nerve (Horton et al., 1971; Naito, 1986 and 1989; Baker and Jeffery, 1989). It has been shown that there is rich fibre interchanges from one fascicle to another in the optic nerve of fetal monkey (Williams and Rakic, 1985). The loss of retinotopic order in the developing stalk has also been reported in *Xenopus* embryos (Holt and Harris, 1983). It can be concluded that the neighbourhood relationships of the retinal fibres are not maintained throughout the optic nerve, and this argues strongly against the morphogenetic hypothesis proposed by Horder and Martin (1978). Factors other than contact guidance and fasciculation as suggested in their hypothesis must also be involved in directing growing axons to terminate in their specific target sites.

Fibre order is changed at the optic chiasm

The fibre order of the retinal projection is always changed where the nerve enters the brain. In adult fish, the change takes place when the nerve joins the tract (Scholes, 1979; Bunt, 1982; Rusoff, 1984). In adult frog, the change in fibre order is found at or near the optic chiasm (Reh et al, 1983; Fawcett et al., 1984; Taylor,

1987). It has been shown in this study that the arrangement of the quadrantic retinal representation in the stalk is also reorganized at the chiasm of fetal rats. A similar sort of rearrangement of growing fibres is also observed at the chiasm of *Xenopus* embryos (Holt and Harris, 1983). These results show that the optic fibres behave very differently when they reach the chiasm, as if they were instructed by a new set of cues, which are of course different from those in the optic stalk, and that these cues enable the fibres to organize into a new fibre order in the tract. The change in the behaviour of optic axons may be brought about by an alteration of the glial environment that the growing axons encounter at the chiasm. It has been reported that in cichlid fish astroglia in the nerve express a vimentin like intermediate filament but not glial fibrillary acidic protein, but interestingly only the glial fibrillary acidic protein is expressed by the astroglia in the optic tract (Maggs and Scholes, 1986). In other studies, a change in the age-related order of the growing fibres from the eye of fetal ferret has been related to a change in morphological type of glial elements in the intracranial part of the stalk (Walsh, 1986; Guillery and Walsh, 1987). It appears that the growing axons respond to the change in glial environment in the optic pathway at or close to the chiasm where the fibres are signaled to change their neighbour relationship, and this allows the fibres to form the age-related fibre order and hemiretinal order in the optic tract.

Fibre order in the rat optic tract

In the optic tract of fetal rats, there is a clear segregation of dorsal and ventral retinal fibres from one eye, but the nasotemporal segregation from this eye is not

obvious in our preparations. The dorsal fibres always occupy the deepest positions rostrocaudally but also run superficially at the caudal parts of the tract while the ventral fibres always lie superficially in the rostral regions of the tract. The reason why some of the dorsal retinal fibres are found in the deepest region of the tract is readily conceived when one understands that the deep to superficial dimension in the tract reads the sequence of axon arrivals in the tract, with the oldest axons lie furthest from the pial surface (Easter et al, 1981; Reh et al., 1983; Walsh and Guillery, 1985; Collelo, 1989). In rodents, it has been shown clearly that the first axons that enter the tract are those from the dorsal central retina (Godement et al., 1987b; Collelo and Guillery, 1990; Chan et al., 1990, see also chapter 3). So the dorsal retinal fibres being the first to reach the tract should occupy the deepest regions, and that is what happens in rat embryos. The dorsal axons that are found superficially at the caudal parts of the tract may represent the population of newly arrived axons from the dorsal retina.

Another point that is worth noting is that in the species examined so far, a clear segregation of fibres from dorsal and ventral retina from one eye is seen consistently in the adult tract while the segregation of fibres in the nasotemporal dimension is far less distinctive (fish: Easter et al., 1981; Bunt, 1982; Springer and Mednick, 1986b; frog: Reh et al., 1983, Fawcett et al., 1984). Interestingly similar fibre segregation pattern is reported in the cat optic tract which contains both crossed and uncrossed fibres from two eyes (Torrealba et al., 1982). This pattern of distribution of optic fibres in the tract is also found during development, as seem in the fetal rats of the present study and in *Xenopus* embryos described by Holt and Harris (1983 and 1984).

It appears that the dorsoventral axis of the retinal map may be defined in the optic tract even before the fibres innervate the central targets. The actual cues that underlie the sorting process of dorsal from ventral retinal fibres are unknown. However, what is known is that fibres do respond to local cues along the tract to guide them towards the target (Reh et al., 1983; Harris, 1989), but whether these positional cues are also responsible for setting up the dorsoventral axis of the retinal map in the tract is still a question.

The relation of fibre order to the development of target map

Further to the question of how the fibre order is related to the development of target maps in the rat, little can be concluded from the results in this study. In fish and frog it has been shown that the growing dorsal and ventral retinal fibres follow their positions in the tract, enter the ventral and dorsal tectal brachia respectively and terminate in their appropriate target regions (Holt and Harris, 1983; Easter and Stuermer, 1984; Holt, 1984). However, it has been shown recently that most growing optic axons in chick embryos and in fetal rats do not navigate directly to their appropriate tectal regions, instead some of them pass their appropriate sites and arborize in other regions of the tectum (Nakamura and O'Leary, 1989; Simon and O'Leary, 1989). Nonetheless, most developing axons show a preference for terminating in the appropriate target site, through direct axonal ingrowth or formation of collateral branches. The imprecise branches and terminal arbors are eliminated later so that a highly specific topographic termination is established in the mature tectum.

It appears that the development of topographic maps in the visual projections of rats is controlled by multiple mechanisms. The retinotopic order in the optic nerve seems to play a minor role at best since this order is lost at the chiasm. At the postchiasmatic tract optic axons are sorted and form a new fibre order in the tract. The tract order seems to lay down the dorsoventral axis of the future map. The formation of the nasotemporal axis is likely dependent on the specific interaction of retinal axons with their appropriate target cells. It has been shown in chick and mouse that the temporal retinal axons behave differently from the nasal retinal axons when they are grown on tectal membranes obtained from different regions of the tectum (Walter et al., 1987a and 1987b; Godement and Bonhoeffer, 1989). The temporal axons show a preference to grow on anterior tectal membranes while the nasal axons grow indiscriminately on both anterior and posterior tectal membranes. Moreover, this difference of outgrowth on tectal membranes is not observed between the dorsal and ventral retinal axons. These observations suggest that the development of the dorsoventral axis of the retinal map is determined by a cue that is different from the tectal cue required for the axons to establish the nasotemporal axis. The dorsoventral axis is likely to be set up in the pathway before the retinal axons innervate the target, and that is what suggested by the results in this study. All these interactions will establish a rough topographic map in the target. This map will then be refined and remodeled by other processes, such as activity dependent interactions of the optic axons (Meyer, 1984; Reh and Constantine-Paton, 1985; Shatz and Stryker, 1988) and elimination of collateral branches and inappropriately targeted axons (Sretavan and Shatz, 1984; O'Leary et al., 1986; Nakamura and O'Leary, 1989; Simon and O'Leary,

1990), before the highly precise retinotopic map is formed in the mature visual projection.

Relation of fibre order to the chiasmatic choice of growing axons

It has been suggested that in both mouse and rat the retinal axons reach the optic chiasm in a topographic order (Silver, 1984; Horsburgh and Sefton, 1986). The ventral temporal retinal axons, which constitute the majority of the uncrossed projecting component, arrive at the caudal side of the chiasm. In this part of the chiasm the fibres encounter a special glial environment and so deviate away from their original direction and grow into the ipsilateral tract. These kind of observations suggest that optic axons are directed into the ipsilateral tract simply by mechanical means. This argument can only be valid if the ipsilateral axons are totally separated from the contralateral axons in the pathway at the region of the optic chiasm, where the choice of route is made. The results of this study cannot provide direct evidence which argues against the above idea since the uncrossed component cannot be recognized by the anterograde labelling technique. However, it is noted that in fetal rat the ventral temporal fibres do not maintain a caudal position through the stalk, instead they begin to shift from their caudal position to the more rostral positions in the prechiasmatic stalk. Moreover, the caudal side of the optic chiasm contains not only the ventral temporal fibres but also fibres from all other quadrants of the retina. More direct evidence is provided in a study which shows that in no part of the stalk can one find a region that contains only the uncrossed fibres but not the crossed fibres

(Sretavan, 1990a; Collelo and Guillery, 1990; Godement et al., 1990; see also results in chapter 3). All these observations point to a conclusion that the growing ipsilateral axons cannot be guided into the tract simply by mechanical means. The retinal axons must carry some markers that enable the uncrossed fibres to separate from the crossed fibres when they need to make their choice at the same part of the chiasm.

The albino mutation has no effect on the fibre order in the developing optic pathway

The misguidance of optic axons at the chiasm of albino animals has been related to the supposed alteration of the gross topographic arrangement of fibres in the developing optic stalk in the absence of pigment production (Silver and Sapiro, 1981; Webster et al., 1988). So far there is little evidence to support this view. However, evidence arguing against it is accumulating. First, it has been shown that there is no obvious difference in the position of fibre fascicles in the developing optic stalk of normal and albino animals (Horsburgh and Sefton, 1986; Strongin and Guillery, 1981). These observations argue against the point that in the absence of pigment a group of fibres are shifted to an ectopic position in the albino stalk - it is on which the above argument is shaped. Second, it has been shown in this study that the quadrant-specific retinal order in the stalk of albino rat fetuses is not different from the one found in normal rat fetuses. It indicates clearly that the albino mutation does not affect the fibre topography in the developing optic stalk. Even if it did, it is still difficult to correlate the disorder of fibre arrangement to the chiasmatic choice made by the growing axons, since the fibre order is anyway scrambled at the optic chiasm.

Therefore, in understanding the abnormality caused by the albino mutation, one should look directly into the action of albino genes on the retinal ganglion cells and the behaviour of axons in relation to their environment at the albino chiasm where the abnormal choice is made.

Fig. 1 Confocal micrographs of the transverse sections of the eyes taken from eight E18.5 rat embryos, pigmented ones are on the left (a-d) and albino ones are on the right (e-h). The bright areas indicate the retinal regions that are labelled by the fluorescent dye DiI. From top to bottom on each column, the embryos have received a DiI label in the ventrotemporal, dorsotemporal, dorsonasal and ventronasal retina respectively. Note the fluorescent dye in the retinas shown in d and é spreads from the ventronasal retina to the adjacent ventrotemporal retina. The lines and shadows interrupting the images are artifact resulted during the collection of images to create a composite image of the whole retina under a low magnification. In all these pictures, dorsal is up and temporal is on the left. The positions of labelled fibres in the optic stalks of these eight animals are shown in the subsequent figures (Fig. 3 to 7), and they are arranged in the same order and orientation as shown in this figure. Scale bar is 250 μm.

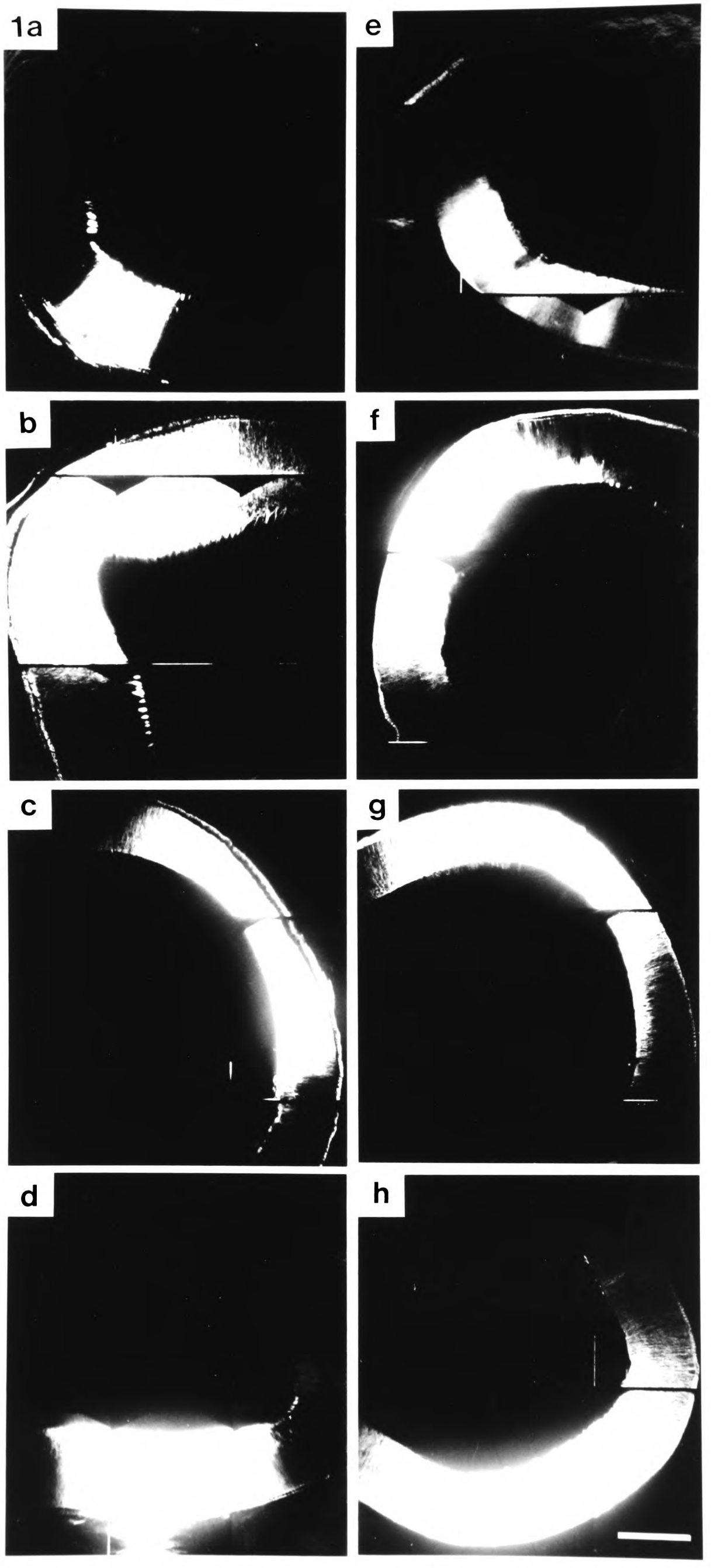
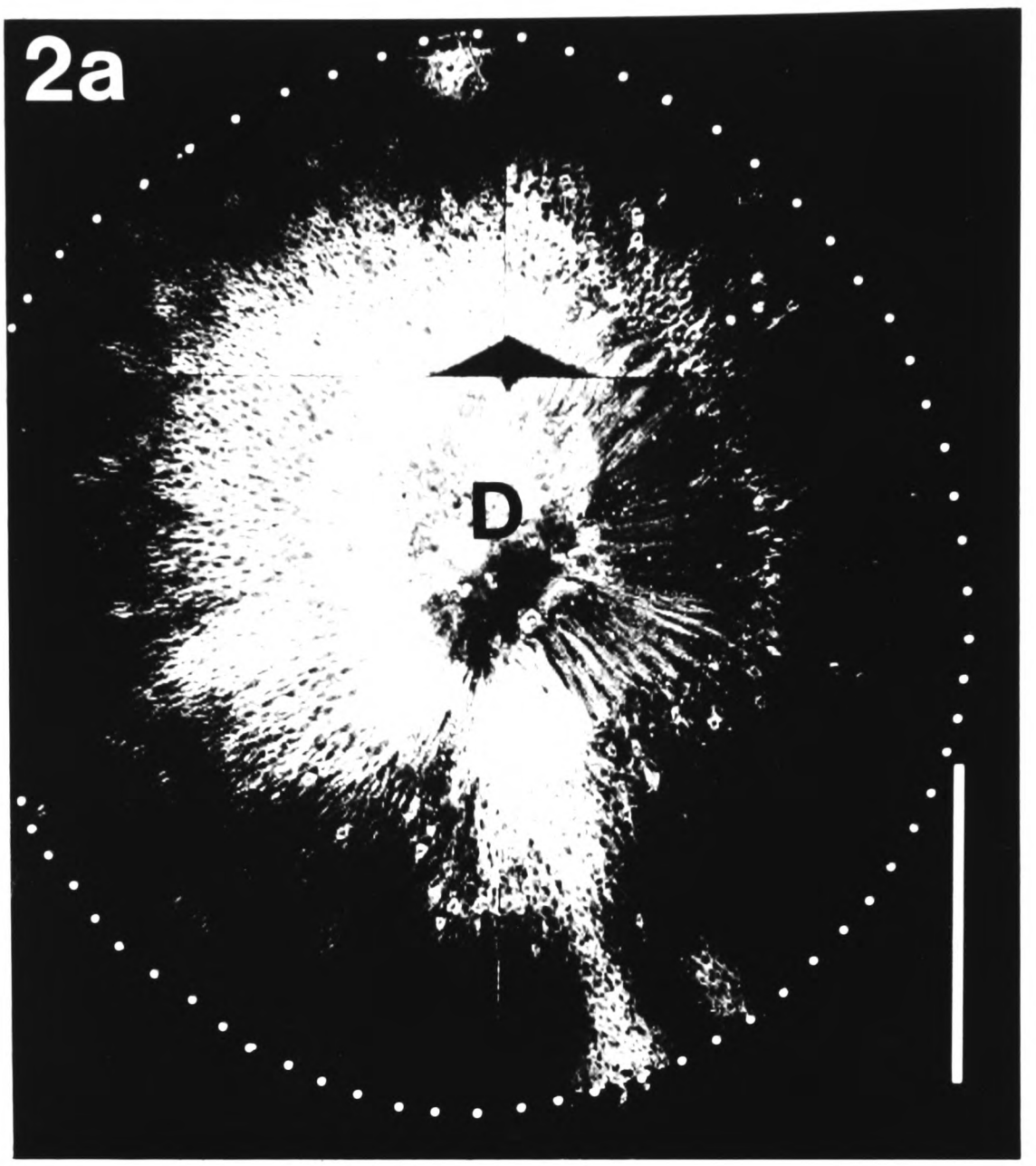

1a
e
b
f
c
g
d
h

Fig. 2 Confocal micrographs showing transverse sections of the retina and the optic stalk in a E16.5 pigmented rat embryo. This embryo received a very large DiI implant in the temporal retina and was stored for a prolonged period of time, therefore almost all except the nasal retinal regions were labelled. 'a' was taken from a retinal section including the optic disk (D) where the ganglion cell axons leave the eye and enter the optic stalk. Within 200 µm from the eye, the retinal fibres are grouped together in fascicles within the stalk (b). The ventral part of the stalk is marked by a hyaloid artery (ha). The scale bar in a is 250 µm, in b it is 50 µm.


2a
D

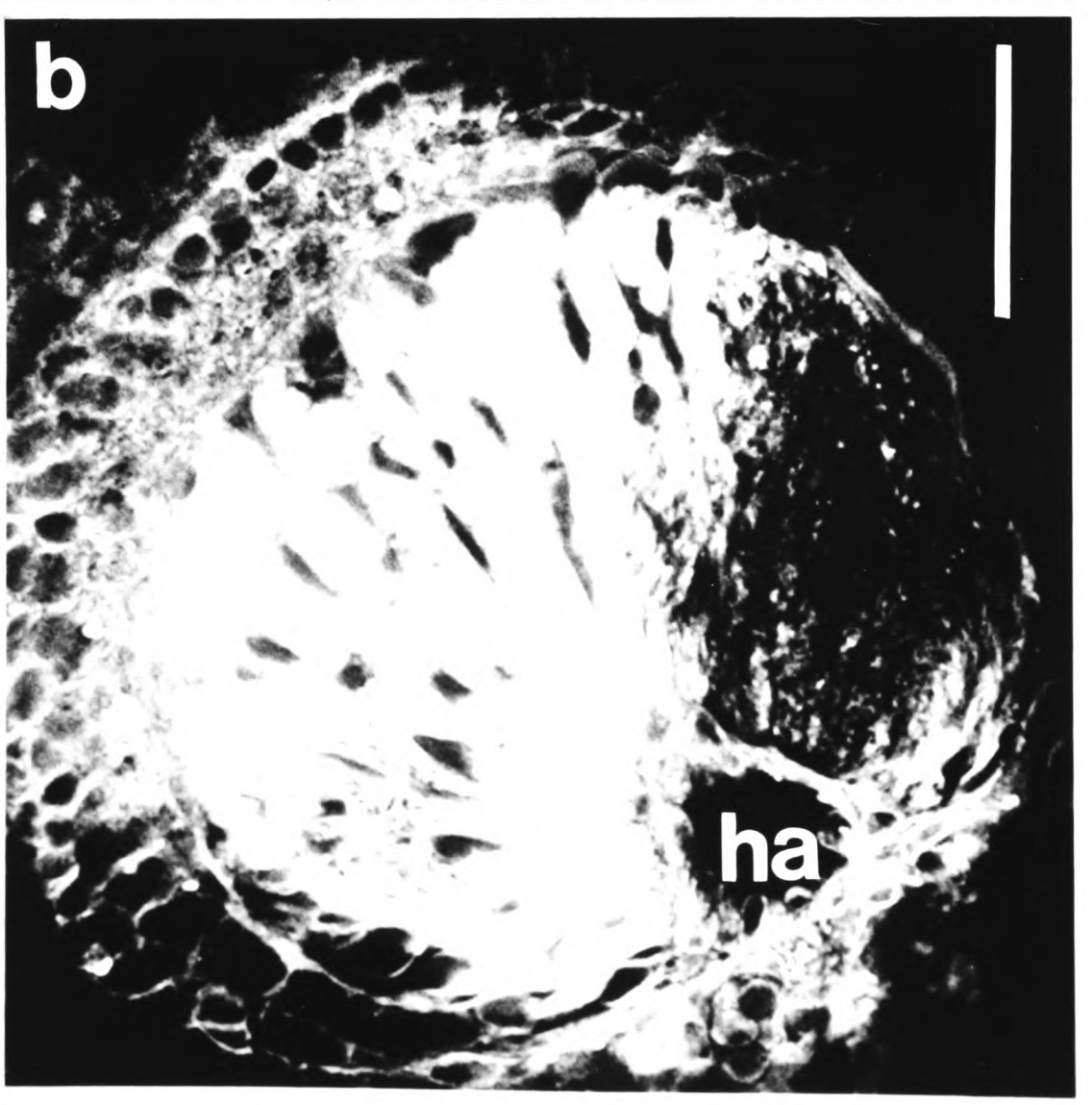

b
ha

Fig. 3 Confocal micrographs showing the positions of labelled fibres from different retinal quadrants in transverse sections of the optic stalk of E18.5 rat embryos, 200 µm from the eye. The white dots outline the boundary of the stalk. The order and orientation of these micrographs are the same as in Fig. 1. Note that the fibres in the temporal parts of the stalk shown in d and h are labelled by a spread of dye from the ventronasal to the ventrotemporal retina. The micrographs demonstrate that the optic fibres are arranged in a quadrant-specific order when they enter the stalk. Further, there is no obvious difference between the pigmented and albino embryos in terms of fibre order in the stalk. Scale bar is 50 µm.

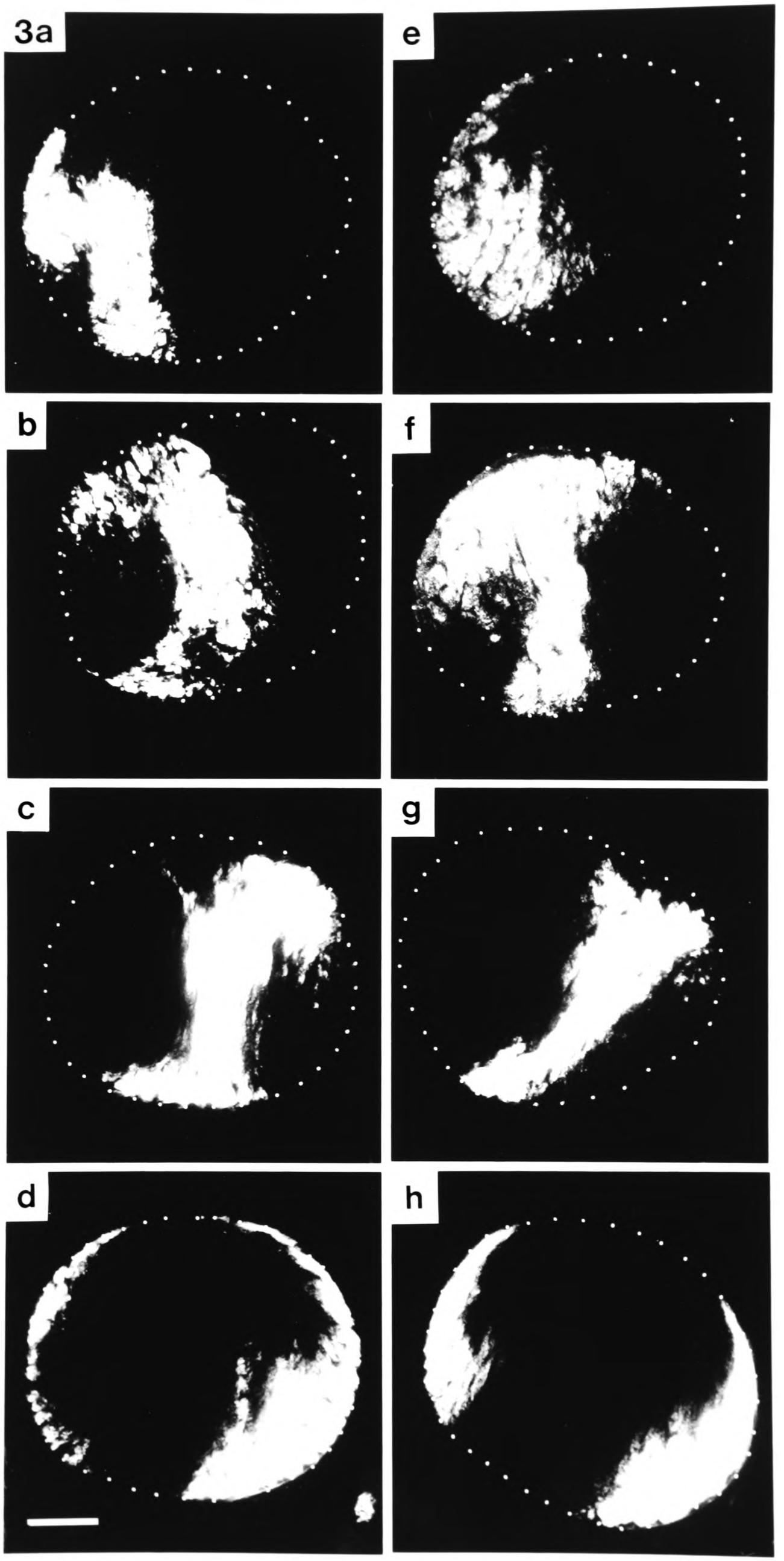

3a
e
b
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c
g
d
h

Fig. 4 Confocal micrographs showing the positions of labelled fibres from different retinal quadrants in the transverse sections of the rat optic stalk, 600 µm from the eye. Scale bar is 50 µm.

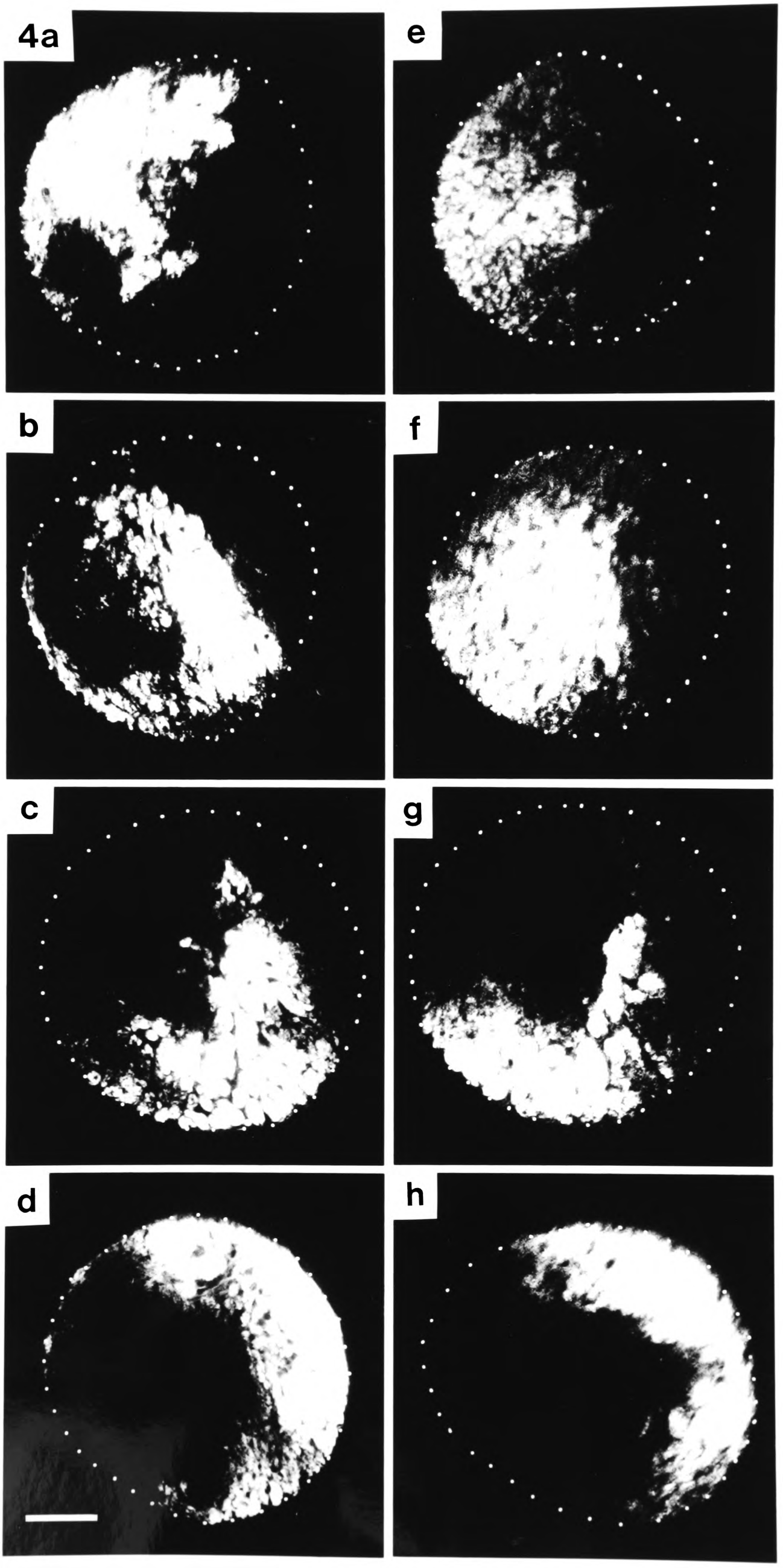

4a
e
b
f
c
g
d
h

Fig. 5 Confocal micrographs showing the positions of labelled fibres from the different retinal quadrants in the transverse sections of the rat optic stalk, 1400 µm from the eye. Note the drastic increase in the scattering of labelled fibres (compare with Fig. 4) in the stalk. Scale bar for a-d and h is 100 µm, for e-g is 50 µm.

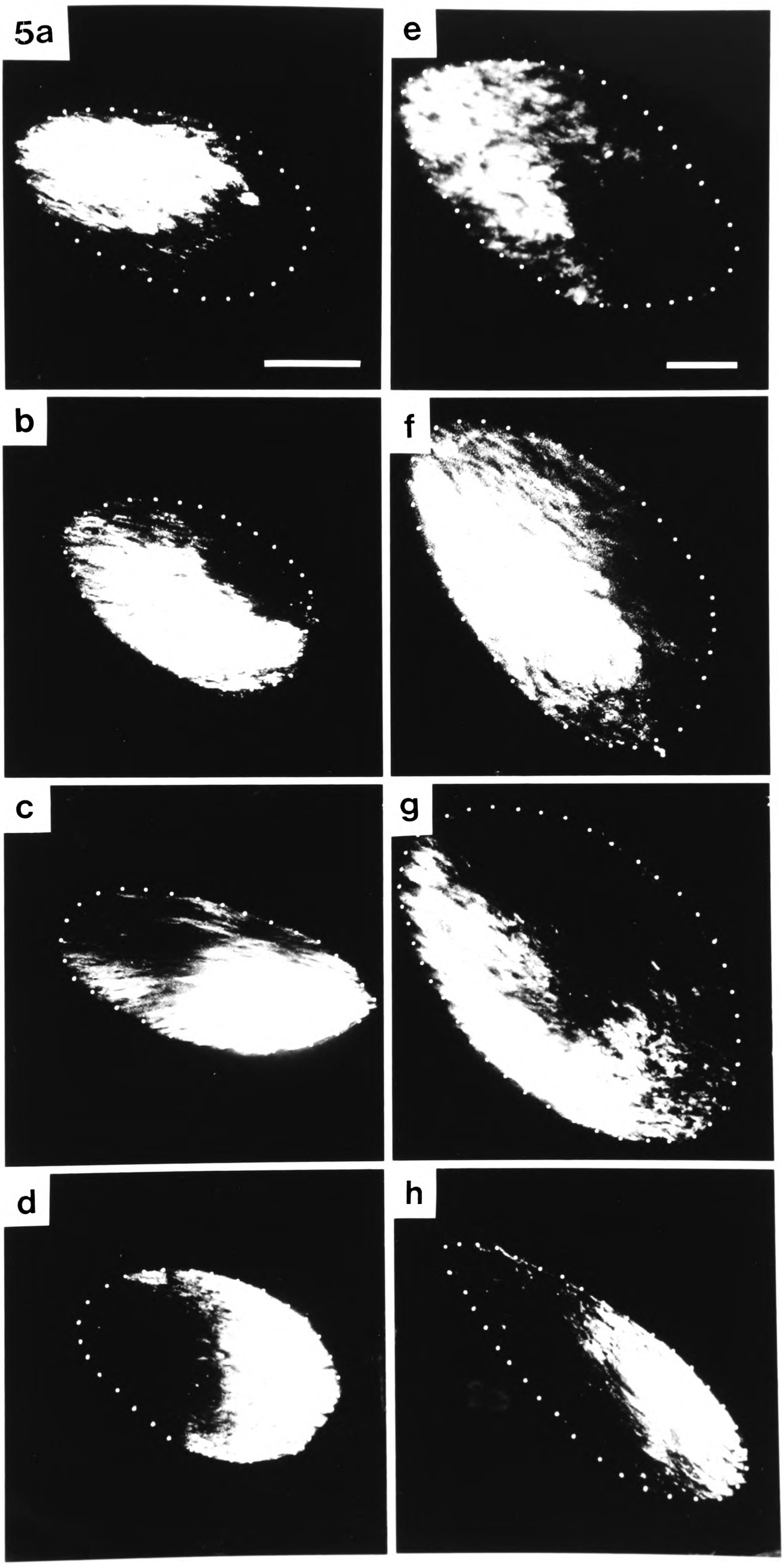

5a
e
b
f
c
g
d
h

Fig. 6 Confocal micrographs showing the positions of labelled fibres from different retinal quadrants in the transverse sections of the prechiasmatic stalk, 1600 µm from the eye. Note the increase in scattering of fibres (except the ventronasal fibres) and the extensive overlap between fibres from various retinal quadrants. Scale bar for e is 50 µm and for rest is 100 µm.

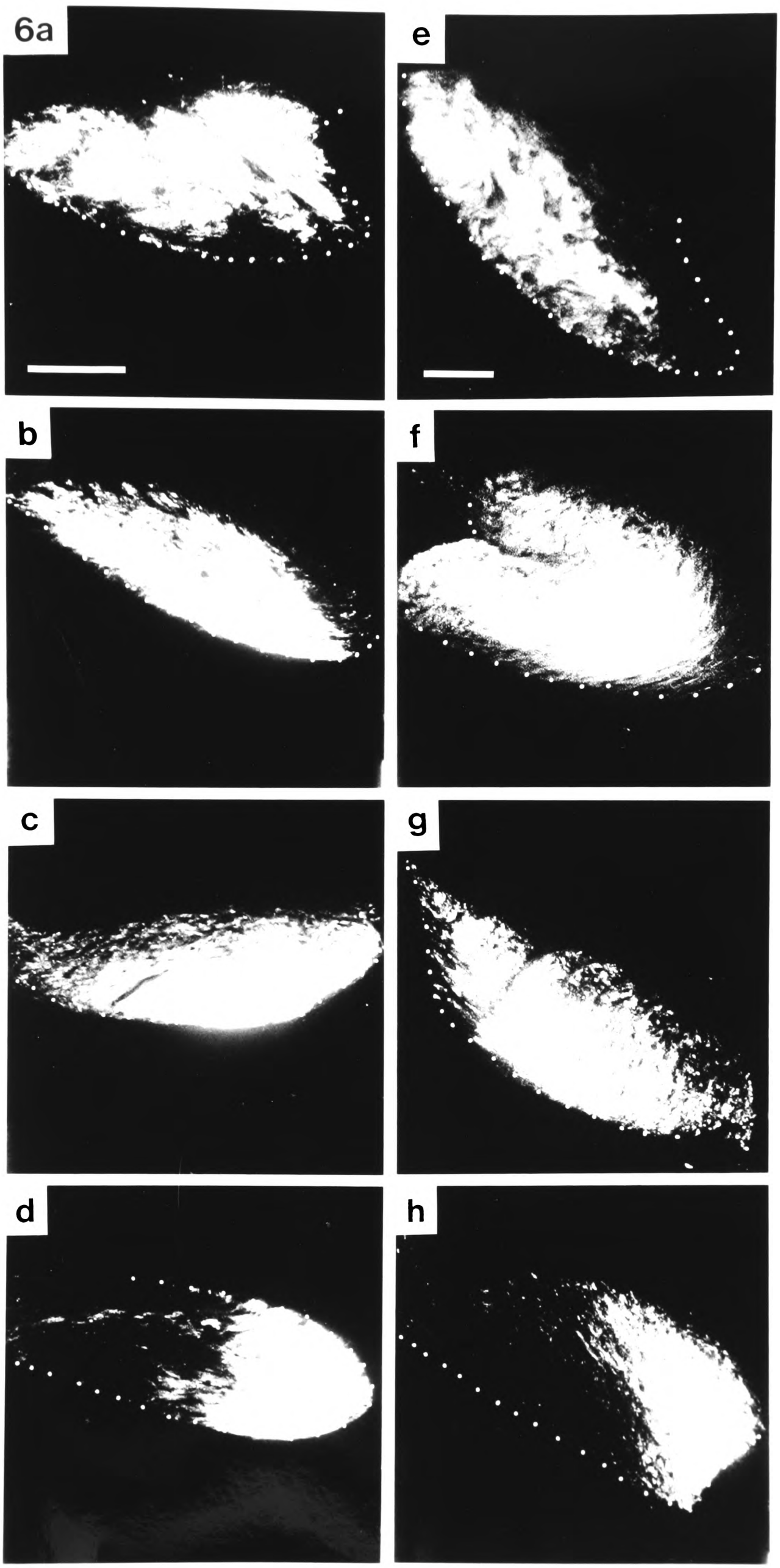

6a
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Fig. 7 Confocal micrographs showing the positions of labelled fibres from different retinal quadrants in the transverse sections of the optic chiasm, 2200 µm. from the eye. The fibres from various retinal sectors overlap extensively at the chiasm. There is virtaully no recognizable map at the chiasm. Scale bar is 100 µm.

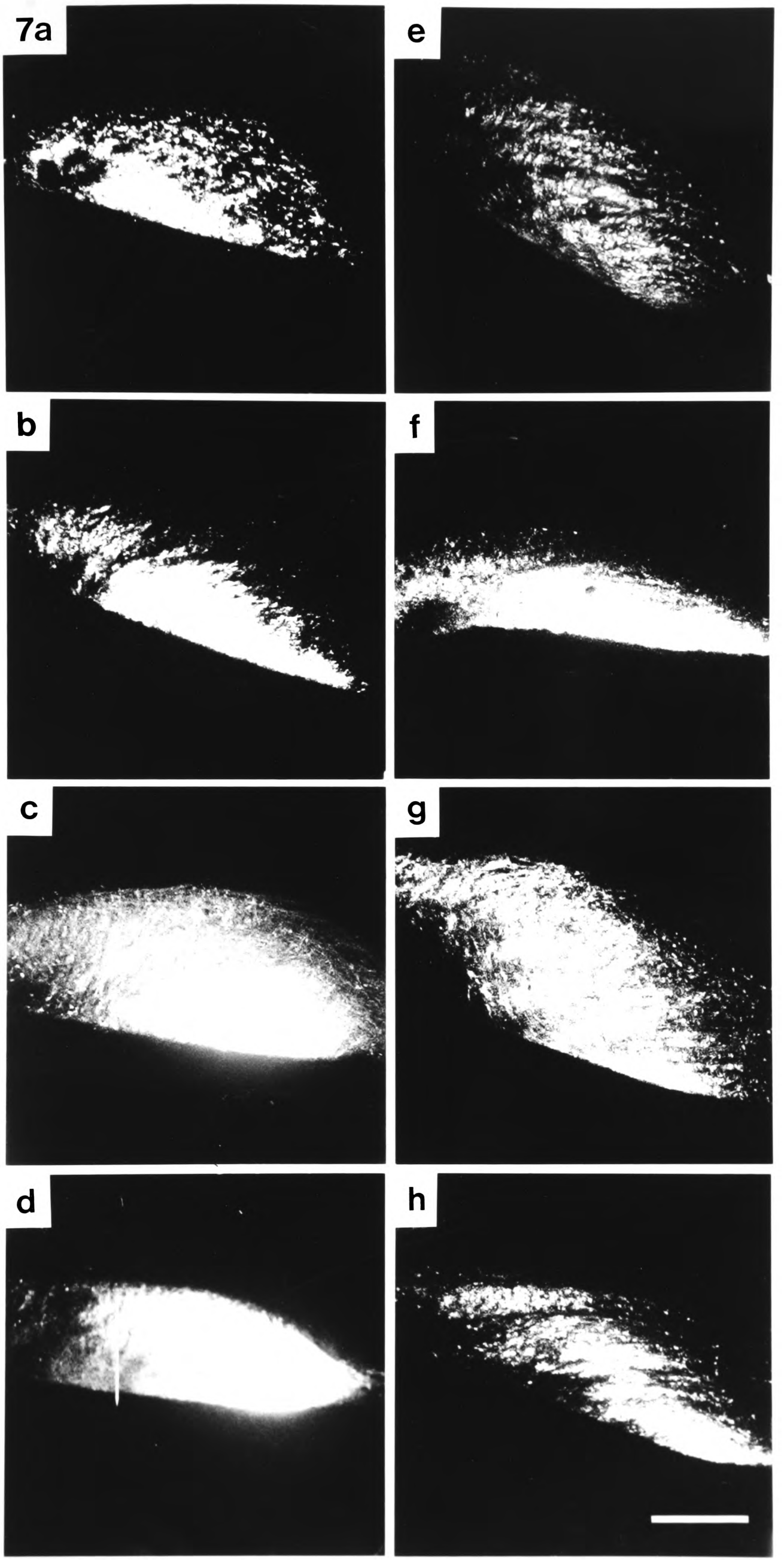

7a
e
b
f
c
g
d
h

Fig. 8 Confocal micrographs showing the labelled fibres in horizontal sections of the optic tract of an E18.5 rat embryo contralateral to the eye that had been labelled whole with DiI. Caudal is up and lateral to the right. The white dots indicate the pial surface of the diencephalon in a and b and the boundary of a fissure in the rest of the micrographs. This fissure separates the diencephalon on the medial side from the telencephalon on the lateral side. a was taken at 400 μm dorsal to the optic chiasm, and the subsequent micrographs (b-f) were taken progressively dorsal of the diencephalon at 200 μm interval. Note the optic tract in c, d and e has its characteristic inverted comma shape and lies medially and rostrally to the fissure. Scale bar is 100 μm.

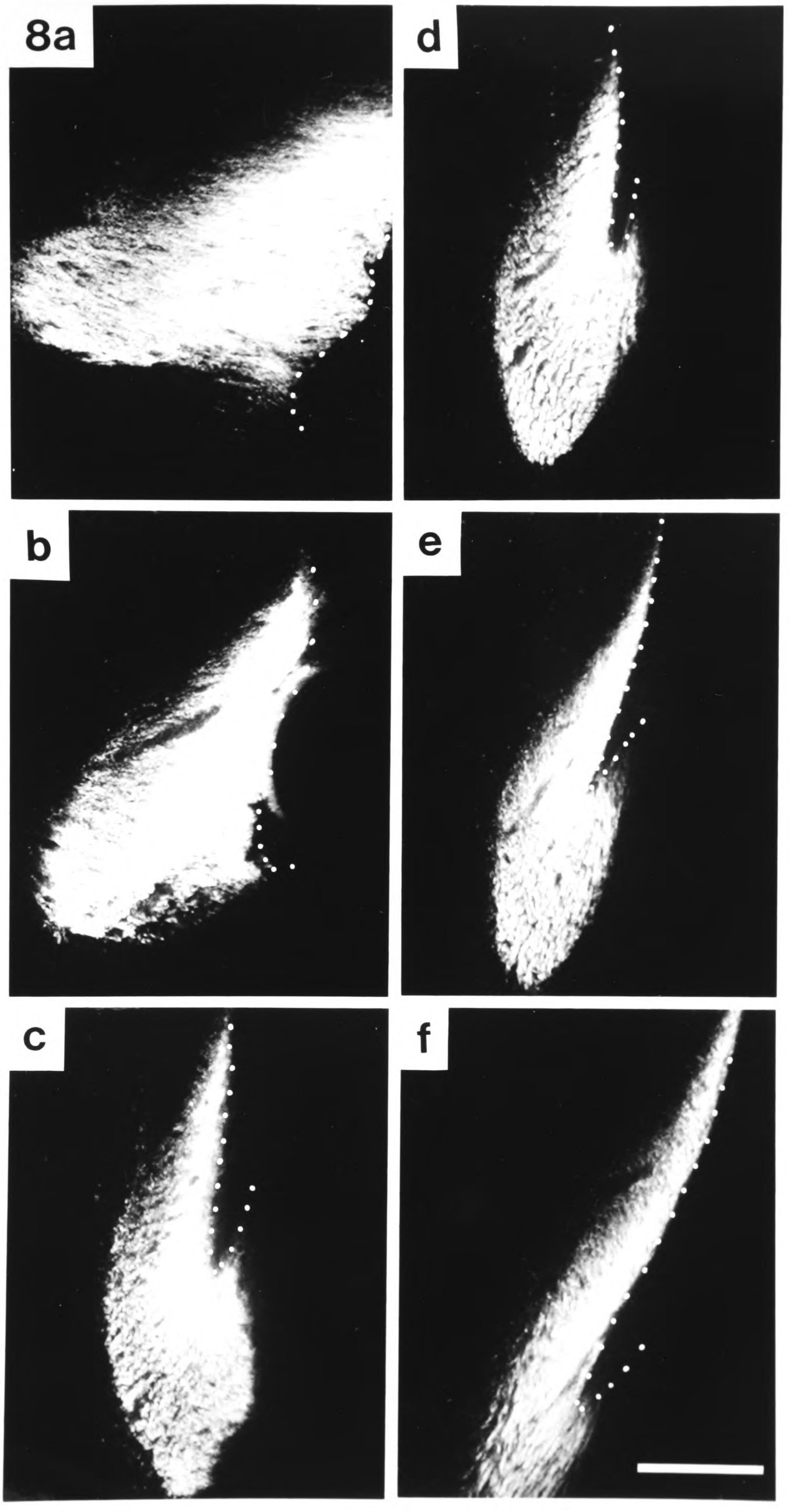

8a
d
b
e
c
f

Fig. 9 Confocal micrographs showing the sites of DiI label in transverse sections of the retinas in four rat embryos. Dorsal is up and temporal is on the left. From a-d, are shown the retinal label in the dorsotemporal, dorsonasal, ventrotemporal and ventronasal retina respectively. The positions of labelled fibres in the contralateral tract of these animals are shown in Fig. 10. Scale bar is 250 μm.

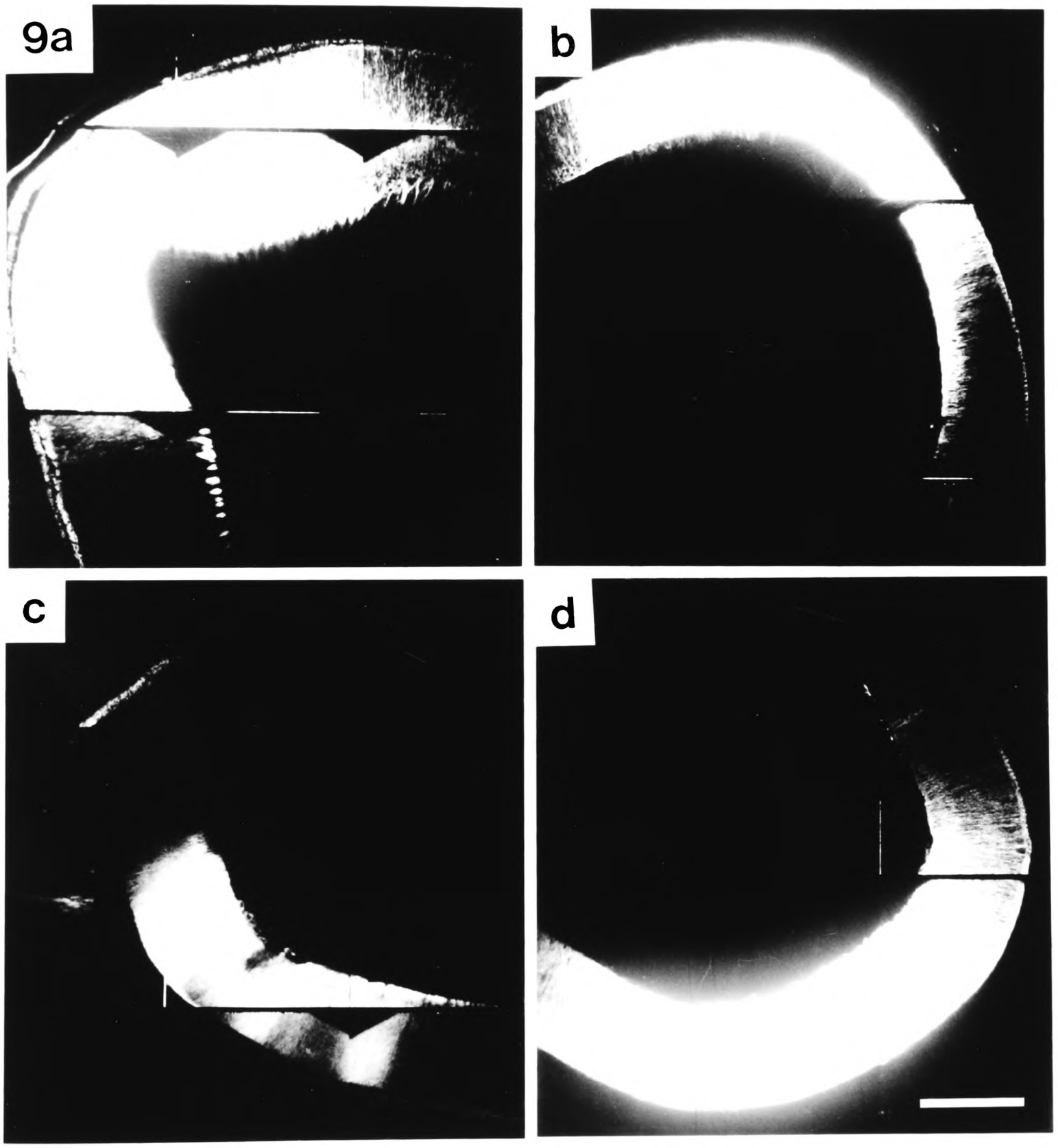
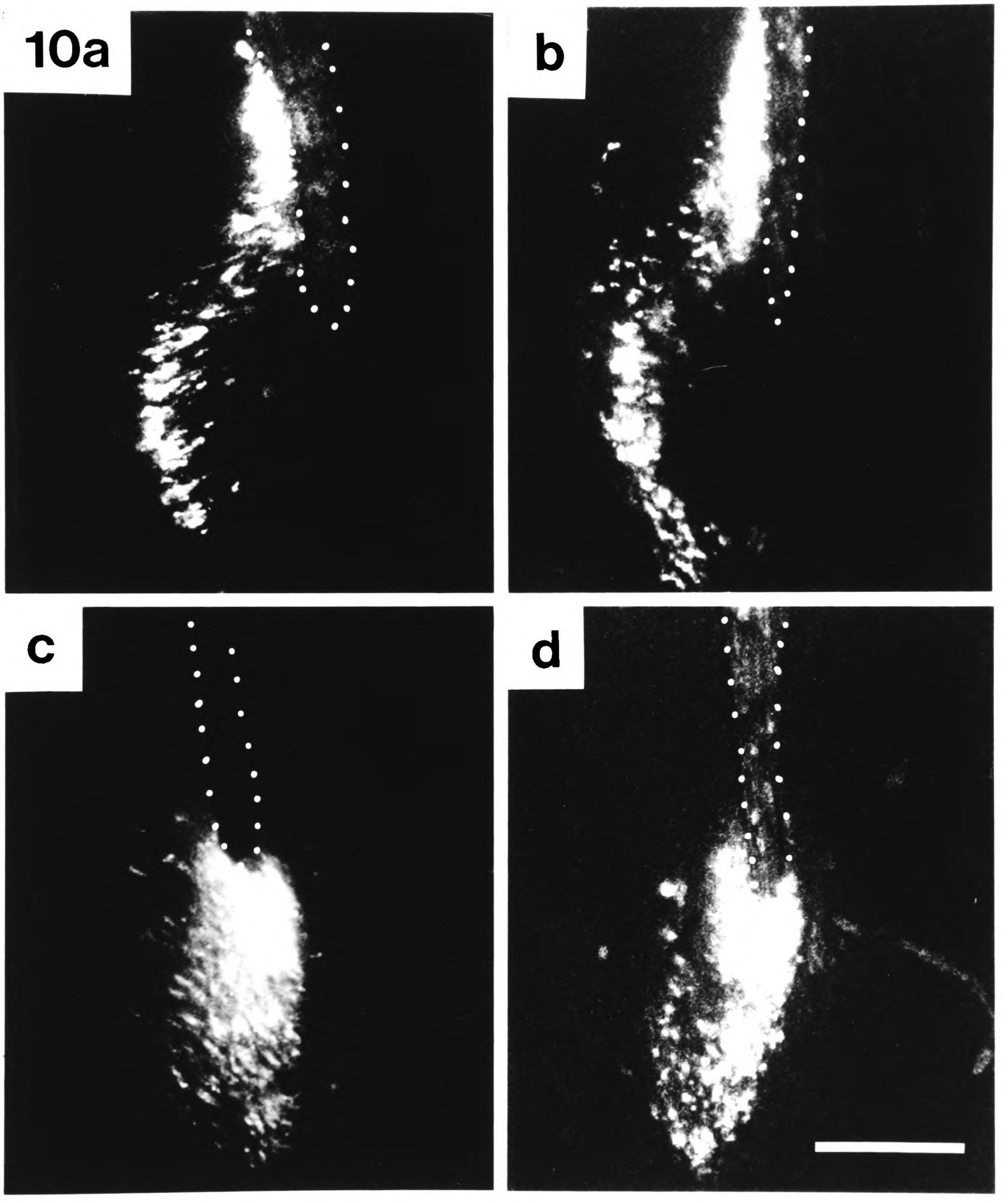

9a
b
c
d

Fig. 10 Confocal micrographs showing the positions of labelled fibres from different retinal quadrants in horizontal of the contralateral optic tract. Caudal is up and lateral is on the right. a-d demonstrate the labelled fibres from the dorsotemporal, dorsonasal, ventrotemporal and ventronasal retina respectively. It is clear that there is a segregation between the dorsal and ventral retinal fibres but none between the nasal and temporal fibres in the tract. Scale bar is 100 μm.


10a
b
c
d

CHAPTER 3 PRENATAL DEVELOPMENT OF THE UNCROSSED RETINOFUGAL PATHWAYS IN NORMAL AND ALBINO RATS

Introduction

In adult mammals, the optic axons from each eye segregate at the chiasm and innervate the primary visual targets on both sides of the brain. In primates, there is a clear segregation of the contralaterally and the ipsilaterally projecting components in the retina in which the retinal ganglion cells in the temporal retina have an uncrossed axon while the ganglion cells in the nasal retina have a crossed axon (Stone et al., 1973; Leventhal et al., 1988; Fukada et al., 1989). In rodents, however, the crossed axons come from cells in all parts of the retina, thus a decussation line can be defined only for the uncrossed component in the temporal crescent (Cowey and Perry, 1979; Dräger and Olsen, 1980; Hsiao, 1984).

A number of studies have attempted to address the question of when the decussation pattern of the uncrossed retinal projection in mammals is established. It has been reported that there is a stage when the cells with an uncrossed axon are confined to the temporal retina in animals at early embryonic ages (Bunt et al., 1983; Lia et al., 1987; Sretavan, 1990a; Chalupa and Lia, 1991). These results indicate clearly that the decussation line of the uncrossed retinal projection is established very early in development.

However, it has been shown in two independent studies that the precise decussation pattern is not observed at the very beginning of the development of the

retinofugal pathway. Indeed, it has been demonstrated in the mouse that the earliest axons entering the ipsilateral tract arise from cells exclusively in the central retina (Godement et al., 1987b; Collelo and Guillery, 1990), two days before the appearance of ipsilaterally projecting cells in the temporal crescent, where most of these cells are found in the adult retina. This intriguing result poses a series of questions. First, it is of interest to know whether this sequence of appearance of early ipsilaterally projecting cells, first in the central retina and then in the ventral temporal retina is also demonstratable in other species. Second, it would be worth knowing whether these early central ipsilateral cells play a role as guides or pioneers in the development of the uncrossed retinal projection. Third, whether the early central component and the late ventral temporal component of the uncrossed pathway require similar cues for finding their routes to the ipsilateral tract.

A simple view of how the uncrossed retinal pathway develops is that the uncrossed axons tend to occupy a caudal position in the developing optic stalk, and that this leads to the direct ingrowth of the axons into the ipsilateral tract by mechanical means (Silver, 1984). However, it has been shown in the adult rat, hamster and cat that although the uncrossed axons tend to lie in the caudal or lateral region when they first enter the optic nerve, they become scattered across the whole medio-lateral extent of the nerve as they approach the optic chiasm (Jeffery, 1989 and 1990; Baker and Jeffery, 1989). Similar observations are reported in the optic pathway of mouse embryos, which show consistently that the uncrossed axons from all positions in the optic stalk and chiasm are able to find their way into the ipsilateral tract (Collelo and Guillery, 1990; Godement et al., 1990; Sretavan, 1990a). These

results indicate that factors other than simple mechanical guidance must be invoked in directing the course of the uncrossed axons at the chiasm.

The development of the uncrossed retinal projection is also affected by a congenital genetic defect in pigment production. It has been well documented that the uncrossed component in albino animals is abnormally small (Lund, 1965; Guillery, 1969; Guillery and Kaas, 1971; Dräger and Olsen, 1980; Creel et al., 1982). It has been claimed that this reduction of the uncrossed component in albino animals is correlated with a change in gross topography of fibres in the optic stalk as a consequence of the absence of stalk pigment, and that this in turn causes the misguidance of fibre at the chiasm (Silver and Sapiro, 1981; Webster et al., 1988). This hypothesis is disproved by the fact that there is no obvious difference in the fibre order within the developing optic stalks of normal and albino rats (see chapter 2). However, whether the albino mutation affects selectively the axonal course and chiasmatic choice of a specific population of ipsilaterally projecting retinal ganglion cells, eg. those arising early in the central retina or the first ones that come from the temporal crescent, remains a question.

In the present study, the uncrossed retinal component in normal and albino rat embryos was examined by using DiI as a retrograde neuronal marker (Honig and Hume, 1986; Godement et al., 1987b). Three questions have been addressed. The first question is whether the uncrossed retinal pathway in normal rat embryos develops in a similar way as that seen in mouse (Godement et al., 1987b; Collelo and Guillery, 1990), with the earliest ipsilateral cells coming from the central retina and cells from the temporal crescent appearing later. Second, the development of the uncrossed

pathway in the albino rat embryos was studied in order to determine whether the albino mutation may have a selective effect on the uncrossed component in the temporal crescent or affects the early central component as well. Third, the position of the growing uncrossed axons along the optic stalk in the normal and albino embryos was examined to see whether the albino mutation affects selectively the route of uncrossed axons coming from the central retina and the temporal crescent when they navigate to the chiasm through the stalk.

Some of the preliminary results have been reported as an abstract (Chan et al., 1990). However, it should be noted that the results reported in this abstract are incorrect since they were collected from litters that were either all pigmented or all albino so that precise age matching of the albino and pigmented embryos was not possible. In the present study, experiments were done on carefully staged embryos and the differences between individual litter was kept mininum by making the comparison of data from normal and albino littermates within the same mother.

Materials and Methods

Normal and albino littermates

In order to minimize the possible error due to differences in developmental stages between individual litters, the study was undertaken in normal and albino rat embryos that came from the same mother. Lister hooded rats and Sprague Dawley albino rats were obtained from the rat colonies in the University farm. They were then crossbred in the department animal house. When the offspring that were heterozygous for the albino gene were sexually mature (about 2 months old), the heterozygous female rats were time mated with albino males and the heterozygous males were mated with albino females. The day on which a vaginal plug was found was taken as embryonic day 0 (E0). These resulted in litters of offspring with known ages that had both normally pigmented (heterozygous) and albino littermates in the same mother. The heterozygous embryos had ocular pigment by E14.5, the earliest stage examined, and their adult phenotypes, such as fur colour and ocular pigmentation, were very similar to the normal pigmented rats.

Staging of rat embryos and fixation

Embryos were collected daily from E14.5 to E18.5. The mothers were anaesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg body weight). The embryos were taken out by Caesarian section and the mothers were killed. The embryos were left enclosed within the visceral yolk sac and amniotic membrane when they were being transferred to a dish of phosphate buffered saline so that the distortion of the body shape and length of the embryos was minimized. In the dish, the

embryonic membranes were removed and all embryos in each litter were staged by measuring the crown-rump length using a dissecting microscope and a scale placed underneath the dish. After the measurement, the embryos were fixed whole in 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.4. The heads were blocked and fixed in the same fixative for 3 days to 2 months at 4°C and then DiI was applied to the optic tract.

DiI implant into the optic tract

A lipophilic carbocyanine dye, DiI (DiIC18(3): 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes Inc.; Honig and Hume, 1986; Godement et al., 1987b), was used in this study to label the uncrossed retinofugal pathways.

The heads were first blotted dry on filter paper and then embedded in a mixture of albumen and gelatin which was hardened with the addition of glutaraldehyde (10% by volume, from 25% stock solution). Excess embedding material was trimmed away and the tissue was oriented by cutting away one corner of the block.

In order to expose the optic tract, the head was sectioned horizontally on a vibratome, from the top of the head to a level about 400-600 um above the eyeball, that is about 800-1200 um above the optic chiasm, depending on the age of the embryos. A drop of Coomassie Blue was added to the cut surface so that the outline of the structures could be revealed. At this level, the transverse section of the optic tract was readily recognisable at the lateral edge of the diencephalon, medial to and abutting the rostral tip of the fissure that separates the telencephalon from the

diencephalon (see chapter 2). Several DiI granules were inserted just underneath the cut surface into the lateral edge of the diencephalon along the pial surface medial to the fissure to label the fibres in the whole optic tract.

After making the DiI implant, the tissues were stored in 2% buffered formalin in the dark at room temperature to allow the dye to diffuse along the axons to the cells in the retina. The storage time varied with the size of the tissues (Table 1).

Table 1. Storage time for diffusion of DiI from the optic tract to the retinal ganglion cells in the rat embryos

Embryonic Age	Storage time (weeks)
E14.5	6
E15.5	6
E16.5	6-7
E17.5	8-9
E18.5	11-12

Preparations of retinal whole-mounts

After the appropriate storage time, labelled retinal ganglion cells on both sides of the brain were examined. The tissue was transferred to buffered saline, the skin overlying the eyeball was removed and the optic stalk was severed. Before taking the

eye out, a cut was carefully made to mark the dorsal pole of the retina. The lens was then removed and the retina was peeled off the scleral coat with a pair of fine forceps. Two additional cuts were made and the retina was flat-mounted on a clean slide, with an antiquenching medium that contained glycerol and 1% p-phenylenediamine (Johnson et al., 1982), and coverslipped.

Fluorescent microscopy and cell count

The retinal whole-mounts on both sides of the brain were examined with a Leitz fluorescent microscope using a rhodamine filter set. The distribution of filled cells in the retina contralateral to the labelled tract was recorded on Kodak TMAX 400 films at a low magnification (4x10), while the retina on the side of the labelled tract was examined at a higher magnification (10x10), and pictures of the labelled ipsilaterally projecting cells were taken. These pictures were later reassembled to give a montage of that retina, and it was on these montages that the number of ipsilaterally projecting cells in each retina was counted.

Preparation of optic stalk sections for confocal microscopy

The position of labelled fibres in the optic stalks on both side of the brain were examined. After the eyeballs had been removed, the heads were first bisected at the midline and each half was re-embedded in the gelatin-albumen mixture. The block was then cut parasagittally on a vibratome at a thickness of 200 µm. Serial sections were collected in 0.1M phosphate buffer at pH 7.4. They were mounted on clean slides, with the antiquenching agent, and coverslipped.

The sections were viewed with a MRC 500 confocal imaging system (Biorad) using a rhodamine filter. The confocal images were recorded on Ilford FP4 black and white films.

Results

Preliminary results

Some of the preliminary data have been reported as an abstract (Chan et al., 1990), in which the development of the uncrossed retinal pathway of rats was studied in litters that were either all pigmented or all albino. Accurate age matching of the pigmented and albino embryos in these litters was not possible. Results of this preliminary study led to a conclusion that the albino mutation affects both the early central and the ventrotemporal components of the uncrossed pathway. This has now been proven to be wrong. The results described in the following sections were collected from precisely staged normal and albino littermates coming from the same mother, and it is clearly shown that only the ventrotemporal but not the early central component of the uncrossed pathway is affected by the albino mutation.

Variation of the crown-rump length in the littermates

The crown-rump (CR) length was measured in 209 embryos. In all litters examined, the normal embryos could be identified easily by the presence of pigment in their eyes whereas the ocular pigment is absent in albinos (Fig. 1) at all ages. Starting from E14.5, the earliest stage examined, the CR length of the embryos is

close to 11.5 mm (Fig. 2). The value increases steadily in older embryos and reaches about 26.5 mm at E19.5. There is clearly no difference in the CR length of normal and albino littermates at any age examined. This provides evidence to support the argument that comparisons in this study have been made between normal and albino embryos at comparable stages.

DiI labelled retinal ganglion cells

After sufficient storage time, DiI implanted in one optic tract diffuses retrogradely along the axons and fills up the retinal ganglion cells in both contralateral and ipsilateral retinas. The earliest age at which labelled cells have been found is E15.5. No labelled cell was seen in either retina in the E14.5 rat embryos (n=4). These results agree well with the observations of another study in which the optic axons have been shown to reach the midline of the chiasm and enter the tract on both sides of the brain at a time between E15.0 to E15.5 in the rat embryos (Horsburgh and Sefton, 1986).

The cells in the retina are brightly labelled, with intense red-orange fluorescence when viewed under ultraviolet light with a rhodamine filter (Fig. 3). Besides the cell bodies, the slender dendritic arbors and the intraretinal axonal profiles can be identified readily in the retinal whole-mounts. The label is distinct, appears to be limited to the filled ganglion cells and their processes, and shows no obvious sign of leaking out of any part of the cells in these retina wholemounts.

Evaluation of the tract labelling

The spread of DiI was checked in parasagittal sections of the embryo heads. In all embryos examined, except those at E15.5, the dye labels only the diencephalic tissue on the side of the injection and there is no obvious spread into the ventral diencephalon or across the midline. In E15.5 embryos the dye has diffused to the ventral margin of the diencephalon. This may be due to the small size of the brain and the prolonged storage time. However, there is no obvious sign that the dye has spread across the midline of the brain since the ventral diencephalon on the side opposite to the labelled tract is free of label.

Besides checking the spread of DiI in the brain, we have also used the label in the retina contralateral to the injected tract as a criterion to evaluate the tract label. In all cases selected, the cells in the contralateral retina had to be labelled brightly and evenly throughout the retina. Those cases that showed a patchy and pale labelling pattern in the contralateral retina were excluded for further analysis. For these reasons, only a number of cases have been selected and discussed in the following part the results and the numbers in each age group are listed in Table 2.

Table 2. Number of embryos at each age with a successful label of DiI into one optic tract

Age	Number of cases	
	Normal	Albino
E15.5	2	2
E16.65	7	7
E17.5	9	7
E18.5	4	4

The distribution of the ipsilaterally projecting retinal ganglion cells in E15.5 rat embryos

In both normal and albino embryos, the earliest ipsilaterally projecting retinal ganglion cells are first recognised at E15.5. Labelled cells are found exclusively in the central part of the retina. Most of them are in the dorsal half of the retina but some are in the ventral half as well around the optic disk (Fig. 4a and 4b). The filled cells are clustered together and the cell boundaries of individual ganglion cell are not recognizable in these preparations. There is no apparent difference in the extent of label in the retinas of normal and albino embryos. In the contralateral retinas of both strains of embryos, filled cells are also seen in the central retina (Fig. 4c and 4d). They occupy a similar extent of retinal area as that found in the ipsilataral retina. It is interesting to note that in these embryos, the label in the ipsilateral retina in most cases appears as extensive as the label in the contralateral retina, in one case the

ipsilateral component even seems to develop in advance of its contralateral component (compare Fig. 4a and 4c). Similar observations have been reported by using DiI as an antergrade marker to study the growing optic axons at the chiasm (Godement et al., 1987b), in which it has been observed that the uncrossed fibres from one eye entered the tract before any crossed fibre from the other eye crossed the midline of the chiasm.

As a summary, the present results confirm the findings in mouse that the earliest ipsilateral component comes exclusively from the central retina (Godement et al., 1987b; Collelo and Guillery, 1990). Moreover, these results also indicate that the uncrossed pathway develops at the same pace as, if not in advance of, the crossed pathway in rats during the very early development of the retinofugal pathway.

The changes in distribution and number of ipsilaterally projecting cells in E16.6 to E18.5 rat embryos

Cell counts were made on the embryos starting from E16.5 to E18.5. The cell number in E15.5 embryos were not assessed since no reliable count could be obtained in those perparations in which the boundaries of the closely packed labelled cells were not readily recognizable (see Fig. 4a and c). The number of cases in each age group is listed in Table 2. The data obtained in the normal and albino embryos within each age group were compared using an one-tailed non-parametric Mann-Whitney test.

In E16.5 embryos, both normal and albino, labelled ipsilateral cells are found in the central retina but unlike those at E15.5, they spread to the more peripheral parts of the retina. No labelled cell is seen at the far periphery (Fig. 5a and b). The filled

ipsilateral cells are scattered in all quadrants of the retina with a preference for the dorsal half of the central retina. They are labelled distinctly and the axons in most cells can be traced to the optic disk. No particular concentration of labelled cells can be found in these preparations in any part of the retina. The number of ipsilateral cells in normal embryos (mean= 84.1) is not different from the number in albinos (mean= 100.1) ($U=18$, $p>.05$) (Fig. 8), indicating that the albino mutation does not affect this early population of uncrossed axons in the central retina. The retina contralateral to the labelled tract shows an intense and even label of cells in the central retina of both strains of rat (Fig. 5c and d). These cells are found in about the central 2/3 to 3/4 of the retinal radius. As in the ipsilateral retina, no labelled contralateral cells can be found in the far periphery of the retina.

By E17.5, a concentration of filled ipsilaterally projecting cells is first seen in the ventrotemporal crescent in both normal and albino embryos (Fig. 6a and b). It is obvious that there are more temporal crescent cells in the normal embryos (mean= 203.9) than in the albinos (mean= 69.7) ($U=1$, $p<0.001$) (Fig. 8). However, this difference between the strains is not found in the number of ipsilateral cells in the central retina (mean in normals= 253.2; mean in albinos= 267.6) ($U=24.5$, $p>0.05$) (Fig. 8). The labelled cells in these embryos come from all parts of the retina, but the far periphery especially in the ventral retina are still free of any labelled cells. It can be concluded that the albino mutation selectively affects the axonal routes of the ipsilateral cells in the temporal crescent but not those in the central retina. The cells in the contralateral retina are intensely and evenly labelled (Fig. 6c and d). They are densely packed in all but the far periphery of the retina, and this sparsity is most

obvious in the ventral retina.

The distribution of labelled cells in the E18.5 embryos ipsilateral to the labelled tract is very similar to that seen in E17.5 embryos (Fig. 7a and b). Axons from cells in the central retina and temporal crescent are added to the ipsilateral tract continuously (Fig. 8). More labelled ipsilateral cells are found in the temporal crescent of the normal embryos (mean= 329.5) when compared with the number in albinos (mean= 150.8) ($U=0$, $p<0.001$), and no significant difference is seen in the number of cells in the central retina (mean in normals= 505.8; mean in albinos= 354.8) ($U=4$, $p>0.05$), although the mean in the normal embryos is slightly higher than that in the albinos. In the contralateral retina, in both strains of embryos, labelled cells can be found almost upto the peripheral margin of the retina, but an area of lower density of labelled cells is noticable in the ventrotemporal retina, and this may correspond to the area of retina that is densely populated by the ipsilaterally projecting cells (Fig. 7c and d).

The changes in total numbers of labelled cells in the ipsilateral retina at different ages are plotted in Fig. 9. In both normal and albino embryos, the total number of ipsilateral cells increases steadily from E16.5 to E18.5. At E16.5, the earliest age in which the cell number was counted, there is no significant difference in the number of cells in normal and albino embryos ($U=18$, $p>0.5$). However, it is obvious from the graph that the number of ipsilateral cells in the normal embryos increases at a faster rate than in the albino embryos within the period examined. In fact, the number of ipsilateral cells in the normal embryos is larger than the number in albino embryos in E17.5 ($U=14$, $p<0.5$), and more obvious differences are obtained at E18.5 ($U=0$,

$p<0.001$).

It is concluded from the present results that the albino mutation appears to affect only the routing of those ipsilaterally projecting cells in the temporal crescent, while it has no apparent effect on the cells in the central retina that have their axons enter the ipsilateral tract early in development.

The position of uncrossed axons in the normal and albino optic stalk

The confocal micrographs in Fig. 10 show the position of labelled uncrossed fibres in the optic stalk of E15.5 embryos. At this age, only a few retinal ganglion cells are labelled around the optic disk in the ipsilateral retina. The axons of these earliest central ipsilateral cells in both normal and albino embryos, when they first enter the optic stalk, are found throughout the rostrocaudal extent of the stalk (Fig. 10a and d). As the uncrossed fibres approach the brain, they remain distributed evenly in the stalk (Fig. 10b and e). At the prechiasmatic stalk (Fig. 10c and f), most of these earliest uncrossed fibres tend to accumulate at a position at the caudal part of the stalk before they enter the tract, but it is noted that there are still a few fibres in the rostral part of the stalk which also go into the ipsilateral tract.

In E16.5 embryos, the position of fibres was examined in the optic stalk on both sides of the brain. The uncrossed fibres in embryos at this age come exclusively from the central retina. When these fibres invade the stalk, in both normal and albino embryos, they occupy all parts of the stalk (Fig. 11a and b). In all cases examined the uncrossed fibres have no preference for any position in the distal optic stalk. As the fibres approach the brain, most of them tend to move to the caudal half of the

stalk and occupy the caudal end at the prechiasmatic region (Fig. 11c and d). However, a few uncrossed fibres can still be found in the rostral half of the stalk. The optic stalks contralateral to the labelled tract in both normal and albino embryos were also examined. In the distal optic stalk, the crossed fibres are found in all parts of the cross-section of the distal stalk except the far peripheral regions at both sides (Fig. 11e and f). These regions may represent the parts where the newly generated fibres, mostly from the ventral retina (see the fibre topography in the rat optic stalk in chapter 2), are in the stalk and that these fibres are unlabelled since they have not yet reached the site of DiI label in the tract. Labelled crossed fibres are observed in all parts of the prechiasmatic stalk (Fig. 11g and h). The crossed fibres are intermixed with the uncrossed fibres along the whole length of the stalk.

In E17.5 normal embryos, most of the labelled uncrossed fibres are seen in the caudal half of the post-optic stalk (Fig. 12a), though a number of them lie in the rostral half. This concentration of labelled fibres in the caudal part of the stalk occurs at a time that the temporal crescent first appears in the normal embryos. Such a group of densely packed fibres is not obvious in the albino ipsilateral stalk (Fig. 12b). This is probably due to the fact that there are fewer fibres at that age in the temporal crescent. The fibres are evenly distributed throughout the cross-sectional area of the albino stalk. However, in both strains of embryos, when the fibres reach the prechiasmatic stalk, they show a preference for a caudal position before they enter the tract although there are many axons in the rostral part of the stalk that also take an uncrossed route (Fig. 12c and d). The crossed fibres are found apparently in every fascicle in the stalk (Fig. 12e-h). Again these crossed fibres intermingle with the

uncrossed ones in all regions of the optic stalk.

In E18.5 embryos, a prominent temporal crescent is found in both normal and albino embryos. In the optic stalk of these animals, most uncrossed fibre gather preferentially in the caudal part of the postoptic stalk, but again a considerable number of them are located in the rostral half of the stalk (Fig. 13a and b). This distribution of fibres is maintained throughout the whole length of the stalk as shown in the confocal micrographs taken in the prechiasmatic region (Fig. 13c and d). Again, the crossed fibres lie in all fascicles within the stalk and they mixed completely with the crossed fibres at all part of the stalk (Fig. 13e-h).

It is concluded that the uncrossed axons, both the earliest component from the central retina and the late component including those from the temporal crescent, can take an ipsilateral route from any position in the stalk, although most of them are found to lie at a caudal position when they approach the brain. There is apparently no difference in the route that the uncrossed axons take in the albino embryos at any of the ages studied. The uncrossed component is always mixed with the crossed component in all fascicles in the stalk.

Discussion

The appearance of the temporal crescent is preceded by the central ipsilateral cells in the rat retina

The results of this study confirm the findings in mouse that the earliest retinal ganglion cells sending axons to the ipsilateral tract lie exclusively in the central retina (Godement et al., 1987b; Collelo and Guillery, 1990). Cells in the temporal crescent, which constitute the majority of projecting cells in the adult uncrossed component, send axons into the tract on same side of the brain several days after appearance of the central ipsilateral cells.

It has been argued in previous studies that the decussation line of the uncrossed retinal component is established very early in development (rat: Bunt et al., 1983; cat: Lia et al., 1987; mouse: Sretavan, 1990a; monkey: Chalupa and Lia, 1991). However, it is shown clearly that in mouse (Godement et al., 1987b; Collelo and Guillery, 1990) and rat (the present study) the decussation pattern of the uncrossed retinal projection is not established at the very beginning of the pathway development. The appearance of the central ipsilateral cells before those in the ventrotemporal retina has also been demonstrated in ferrets (Baker and Collelo, personal communication) which have a much more prominent uncrossed component than rodents.

It is not clear whether the early central ipsilateral component plays any role in the further development of the uncrossed retinal pathways. The results in this study show that the number of these cells continues to increase throughout the prenatal period examined. They may just represent cells in the central retina that have made an "error" in their chiasmatic choice during development or they may serve as

"pathfinders" that guide the later axons into the ipsilateral tract (Godement et al., 1990; Sretavan, 1990a). However, if they are pathfinders, it is puzzling to find that they continue to be produced well after a path has been formed. So far there is no evidence to support the claim that the early central ipsilateral cells may act as pathfinders for the development of the uncrossed pathway.

It is interesting to note that when one eye is removed at a very early developmental stage, i.e. at the time when only axons of the central cells from each eye have reached the optic chiasm, the adult uncrossed component of the remaining eye is reduced in the operated animals (Godement et al., 1987a; Guillery, 1989). These observations raise a very interesting suggestion that the growing uncrossed axons may need to interact with fibres from the opposite eye at the chiasm in order to make their path selection. That is, it appears that the early central crossed component from one eye is important for the development of the uncrossed pathway of the opposite eye.

Delay of ingrowth of fibres from the temporal crescent into the ipsilateral tract

Another interesting point is that in the study of the genesis of ganglion cells in the mouse retina Dräger (1985) has demonstrated that the earliest born ipsilaterally projecting cells (born on E10.5) are found in the adult exclusively in the temporal crescent. They are generated at the same time as the earliest contralateral cells in the central retina. Similar observations have been obtained in rats which also show that the earliest ipsilateral and contralateral cells are born at the same time on E13 and that they are distributed in two distinct regions in the adult retina, demarcated by the

central border of the temporal crescent, with ipsilateral cells lateral and contralateral cells medial to this border (Reese and Collelo, personal communication).

These results seem to be in conflict with those obtained in the mouse (Godement et al., 1987b; Collelo and Guillery, 1990) and rat (the present study) in which DiI is used as a retrograde marker to study the time of arrival of axons in the ipsilateral optic tract. One of the dicrepancies between the studies of birthdate of ganglion cells and the time of axon arrival in the tract is that the earliest cells sending axons to the ipsilateral tract are all in the central retina, whilst it is shown in the birthdating study that the earliest born ipsilateral cells are not in the central but the ventrotemporal retina. One possible explanation is that the earliest central ipsilateral cells which are labelled retrogradely by DiI are eliminated during the period of postnatal cell death (Land and Lund, 1979; Lam et al., 1982; Potts et al., 1982), so that they are not detected in the adult retina, from which the birthdate data are collected. In rat, more than 90% of ipsilateral cells present in the central retina of neonate are lost during the postnatal life (Jeffery, 1984; results in chapter 4).

Nonetheless, it is difficult to understand why the axons of the cells in the temporal crescent, in rats and mice, arrive at the ipsilateral tract two days later than the axons of the central cells, knowing that they are among the first born in the retina. One possible explanation is that these temporal axons arrive late in the tract because they are in the peripheral retina, they need more time to travel through the retina than those axons of the central cells. It has been estimated that axons from the temporal crescent traverse the retinal surface and reach the optic disk within one day (Collelo and Guillery, 1990). Given that the earliest cells in the rat retina are born on E13.0, those

in the central retina begin to send axons into the stalk on E14.5 and these axons reach the chiasm on E15.0 (Horsburgh and Sefton, 1986). Among these central retinal fibres, some grow into the ipsilateral tract and so are labelled by DiI on E15.5, as shown in the present study. If the axons from the first born ipsilateral cells in the temporal crescent do need to take a day of extra time to travel through the retina, they should be in the stalk on E15.5 and arrive at the chiasm on E16.0. It is shown in the next chapter that they really do so - by putting DiI into the far periphery of the temporal retina in a E16.0 rat, filled fibres can be traced to the optic chiasm. It is still not understood why these axons from the ventrotemporal retina are not labelled retrogradely by DiI in the E16.5 rat embryos. One possible explanation is that these axons, even though they have reached the chiasm, need to spend time in exploring the environment at the chiasm before growing into the ipsilateral tract. This idea is supported by the observation that growth cones of the uncrossed axons show a very complex morphology and a dynamic change in shape when they reach the chiasm, which indicates that they are exploring and may be seeking for a cue that help them to make a choice of route at the decision point (Godement and Mason, 1990; Godement et al., 1990).

Mechanisms that determine chiasmatic choice

It has been suggested that the chiasmatic choice made by the growing optic axons is achieved by simple mechanical means, that is, the growing axons are believed to be guided into the ipsilateral tract because they lie in a caudal (temporal) position in the stalk when they reach the chiasm (Silver, 1981; Horsburgh and Sefton, 1986).

However, there is growing evidence to argue against this hypothesis. It has been shown in earlier studies of mice (Collelo and Guillery, 1990; Godement et al., 1990; Sretavan, 1990) and in the present study on rats that during early development of the retinal pathway growing axons can take an uncrossed route from any position in the rostrocaudal dimension of the chiasm. There is clearly no privilege for axons that are in the caudal part of the stalk to select the uncrossed route.

A clue to understanding what makes the optic axons take an uncrossed pathway is provided from the studies of the axonal trajectories and behaviour of growth cones when the optic axons navigate through the chiasm. It has been shown that when the uncrossed axons grow to the chiasm, they turn abruptly at a right angle in the chiasm and grow into the ipsilateral tract whereas the crossed axons continue their paths towards the contralateral tract (Godement et al., 1990; Godement and Mason, 1990; Sretavan, 1990a and b). The distinct pathways that the crossed and uncrossed axons take at the chiasm have led to the interpretation that there is local cue within the chiasm that instructs the growing uncrossed axons to turn and grow into the ipsilateral tract, whilst the crossed axons simply ignore this cue and grow straight to the contralateral tract. This cue may be present on the membranes of the neuroepithelial cells at the midline of the chiasm (Godement et al., 1990; Mason et al., 1990) or may be a kind of axon-axonal interaction between the uncrossed fibre from one eye and the crossed fibres from the opposite eye (Godement et al., 1990; Sretavan, 1990a and b).

Nevertheless, whatever the chiasmatic cue is, it is still inadequate to explain why the uncrossed and crossed axons take a different route when they come across the

same cue at the same part of the chiasm, nor can this chiasmatic cue explain sufficiently why most uncrossed axons always arise from cells in a precise topographic region in the ventrotemporal retina in rodents.

The fact that uncrossed fibres are always mingled with the crossed fibres when reach the chiasm, as shown in the past studies in mice (Collelo and Guillery, 1990; Godement et al., 1990; Sretavan, 1990) and the present study in rats, points out very clearly that there must be some intrinsic difference in the crossed and uncrossed axons that enables them to respond differently when they recognize the same chiasmatic cue. And since the crossed and uncrossed fibres intermingle with each other along the whole length of the optic stalk (Collelo and Guillery, 1990; results of the present study), this intrinsic difference is very unlikely to be set up in the stalk. The most plausible site of this intrinsic difference is in the retina. The optic pathways of rodents may not be a good example to support this argument since the cells giving rise to the crossed and uncrossed components overlap almost completely in the retina, though there is a preference for the ipsilateral cells to be located in the temporal crescent (Cowey and Perry, 1979; Dräger and Olsen, 1980; Hsiao, 1984). A stronger evidence comes from studies of monkey optic pathway. In adult monkey, cells in the temporal retina always project to ipsilateral targets whilst cells in the nasal retina projecting contralaterally (Stone et al., 1973; Leventhal et al., 1988; Fukada et al., 1989). It has been shown that in the adult monkey optic nerve fibres from the far periphery of the temporal retina, which are purely uncrossed, intermingle with the fibres from the dorsal nasal retina, which are all crossed, along the whole length of the nerve (Naito, 1989). These results strongly support the idea that the intrinsic

difference between the crossed and uncrossed axons is in the retina. This intrinsic difference may be conceived as a retinal marker which is expressed specifically by the ipsilaterally projecting cells and that this marker instructs the growth cones of these cells to turn back and take an uncrossed route when they read the chiasmatic cue. The contralaterally projecting cells may not express this retinal marker at all or they may express another marker, and that enables their axons to ignore the chiasmatic cue and so grow straight to the contralateral tract.

Further, it is consistently found that during early development of rodents although a small population of cells in the central retina do make "errors" in projecting to the ipsilateral targets, the majority of the uncrossed population come from the ventrotemporal retina (Bunt et al., 1983; Collelo and Guillery, 1990; Sretavan, 1990a; results in the present study). These observations suggest that the intrinsic difference in response to the chiasmatic cue is likely to be set up in the ganglion cells according to their location in the retina. If we take the retinal marker into consideration, this marker should be expressed in the form of a gradient or a step function across the nasotemporal axis of the retina, being most preferentially expressed by ganglion cells in the temporal retina and the expression decreases drastically in those cells located outside the temporal crescent (see Guillery, 1990).

We know nothing about the nature of this retinal marker, nor do we have any direct evidence to show that there is a differential distribution of this marker across the nasotemporal retina. However, a protein which is specifically expressed on the temporal retinal axons has recently been demonstrated in chick embryos and it has been claimed that this protein is distributed in a step-gradient across the nasotemporal

axis of the retina (McLoon, 1990). It is of particular interest to see whether this protein is related to the development of target maps in the chick primary visual pathway, and it is definitely worth looking whether similar nasotemporal gradient also exists in the retina of mammals and if it is, whether it is in some way related to the chiasmatic segregation of the retinal axons.

The albino mutation only affects the routing of axons from the temporal crescent but not the earliest uncrossed axons from the central retina

It has been shown in this study that the albino albnormality appears at a very early developmental stage in the rat. The effect of the albino mutation on the development of the uncrossed component of the retinal projection is apparent on E17.5, that is at a time 4 days before the onset of massive cell and axonal loss in the primary optic pathway that starts on the day of birth in the rat (gestation period in rat is 22 days)(Lam et al., 1982; Potts et al., 1982; Sefton and Lam, 1984). The prenatal development of the rat optic pathway has also been investigated in rats using anterograde tracing technique, in which it has been shown that the uncrossed projection in the optic tract of albino rat appears to be smaller than that in the normal rat at all prenatal ages examined (Bunt et al., 1983). These results together with those in this study indicate strongly that the smaller uncrossed retinal component in the albino rat is very likely to be produced by a consequence of misrouting of growing axons at the chiasm, as proposed originally by Guillery (1969), rather than caused by preferential cell loss in the uncrossed component of the albino pathway, although we still cannot rule out some contribution due to a differential cell loss. Similar

conclusion has been obtained in the study of prenatal development of retinal pathway in normal and Siamese cats (Shatz and Kliot, 1982; Kliot and Shatz, 1985). It is reported in their studies that the uncrossed component in the Siamese cat is reduced when the fibres first appear in the ipsilateral tract on E30 and that this reduction in the uncrossed pathway is persistent throughout cat prenatal life. Moreover, the abnormality seen in the Siamese cat occurs long before the onset of major axonal loss in the optic nerve which starts from around E39-E44 in cats (Ng and Stone, 1982; Williams et al., 1986), indicating again that the cell loss can play no more than a secondary role in the formation of the abnormal uncrossed retinal pathway.

Another alternative to consider is that there may be a delay in the growth of uncrossed axons into the albino optic tract. This is very unlikely as an explanation of why the uncrossed component in albinos is abnormally small since the uncrossed pathway in the albino animals is persistently reduced throughout the prenatal development, and there is no catch-up in the growth of the ipsilateral projection at any period during development (Cucchiaro and Guillery, 1984; Kliot and Shatz, 1985; Gayer et al., 1989). The results in this study also argue against this idea. It is shown that the development of the uncrossed component in the albino rat embryos is in step with the normals, e.g. the first central ipsilateral cells can be recognized on E15.5 and a definable temporal crescent becomes apparent on E17.5 in both strains of embryos. Moreover, once the reduction in the uncrossed component appears in the albino rat, it is always smaller than the uncrossed component in normals at any stages investigated (see also chapter 4 for the number of ipsilateral cells in rat neonates).

It is of particular interest to note that the formation of the uncrossed pathway in

the rat is begun by a population of uncrossed axons from the cells in the central retina. An interesting question arises. That is since the axons of these early central ipsilateral cells appear first in the development of the pathway, they may guide the later axons from the temporal crescent and central retina to the ipsilateral tract at the chiasm, just as the subplate neurons are believed to pioneer the development of thalamocortical connections in cats (McConnell et al., 1989). If these early central ipsilateral axons did play a pioneer role, then it might have been argued that the albino mutation affects this group of central ipsilateral axons and thus causes the later temporal crescent component to select a wrong chiasmatic route. However, the results of this study show that in the rat only the axonal routing of cells from the temporal crescent is affected at the chiasm of albino embryos, and that the early central ipsilateral cells are not affected at any stage examined. Moreover, the axonal course of these early central ipsilateral cells in the albino stalk appears to be the same as the course of uncrossed fibres in the normal stalk. All these findings indicate that whatever the role that these early central ipsilateral cells may play in the development of the uncrossed retinal pathway, they are unlikely to be responsible for the misguidance of axons in the albino since they are not affected by the mutant gene.

It should be noted here that there is an effect of the albino mutation on cells in the central retina which is manifested during late prenatal development, i.e. between E18.5 to the day of birth. In albino embryos the number of ipsilaterally projecting cells in the central retina appears to be abnormally low at E18.5, and also the number of central ipsilateral cells in albino rats is significantly smaller than the number in normals on the day of birth (see chapter 4). These data suggest that there may be two

populations of central ipsilateral cells: one is generated very early in development and is not affected by the albino mutation, whereas the other is added to the uncrossed pathways at later prenatal stages, and the axonal routing of these cells can be changed by the mutation.

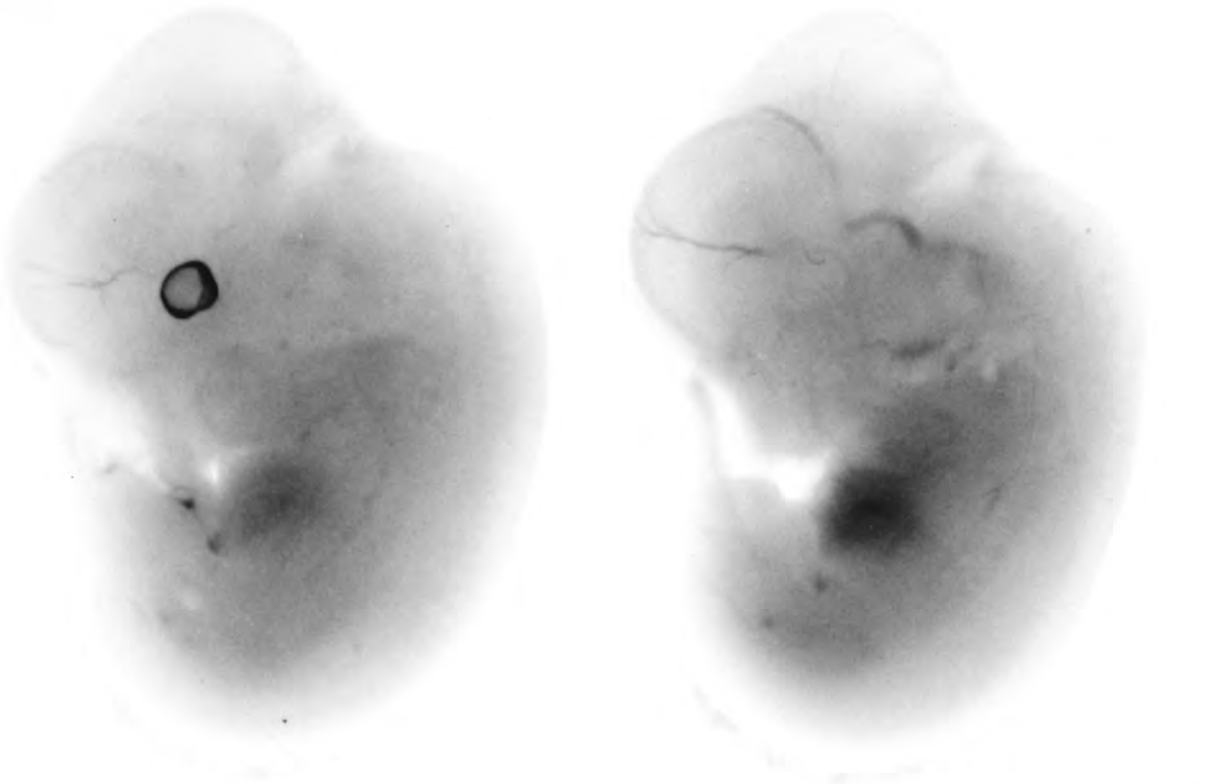
The actual mechanisms that generate the abnormally small uncrossed retinal component in albino animals are still not clear. What is known is that the abnormality is not due to a change in the fibre topography in the absence of pigment in the developing stalk (see chapter 2) as proposed in past studies (Silver and Sapiro, 1981; Webster et al., 1988). The uncrossed fibres in normal and albino embryos occupy similar position when they are navigating through the stalk at any age examined (Collelo and Guillery, 1990; Collelo and Jeffery, 1991; the present study). These results suggest that the fibres in albinos follow a similar set of rules that guide fibres from the eye to the chiasm. Therefore, the albino abnormality is definitely not triggered by any alteration in the local environment in the stalk. The only possible sites where the albino gene can act on in the pathway is either in the retina or at the optic chiasm, or both.

The results of the present study show that the albino mutation only causes a reduction of number of ipsilaterally projecting cells in the temporal crescent. A possible explanation for this is that the expression of the retinal marker in the ganglion cells, which is assumed to be responsible for instructing the growing axons to turn ipsilaterlally when they read the chiasmatic cue, is somehow altered in the albino retina. The expression of this marker may be weakened or it may be expressed in fewer cells in the temporal retina, especially those along the medial border of the

temporal crescent, and this in turn may produce a decrease in number of ipsilaterally projecting cells and a shift of the decussation line of the uncrossed component towards the temporal periphery as seen in the retina of albino rats at birth (see chapter 4). Another alternative is that the growing optic fibres need the interaction of fibres from the other eye at the chiasm for making their choice to grow into the ipsilateral tract (Godement et al., 1987a and 1990; Guillery, 1989). Weakening of this axo-axonal interaction may result in fewer axons growing into the ipsilateral tract, and that is what has been suggested for the optic chiasm in albino ferrets (Guillery, 1989). Therefore, either the retinal marker or the chiasmatic binocular interaction, or both, may be abnormal in the albino mutants and that may cause the misguidance of growing optic fibres at the chiasm. Studies have been done which are aimed at evaluating the role of these factors in governing the chiasmatic choice of growing optic axons and the results are discussed in the following chapters.

Fig. 1 Rat embryos at the age of E14.5 (a) and E18.5 (b). In each age group, the pigmented and albino embryos, as indicated by the presence or absence of ocular pigment, came from the same mother. There is no obvious difference in the external morphology between the pigmented and albino littermates. Scale bar is 2 mm.

1a



b

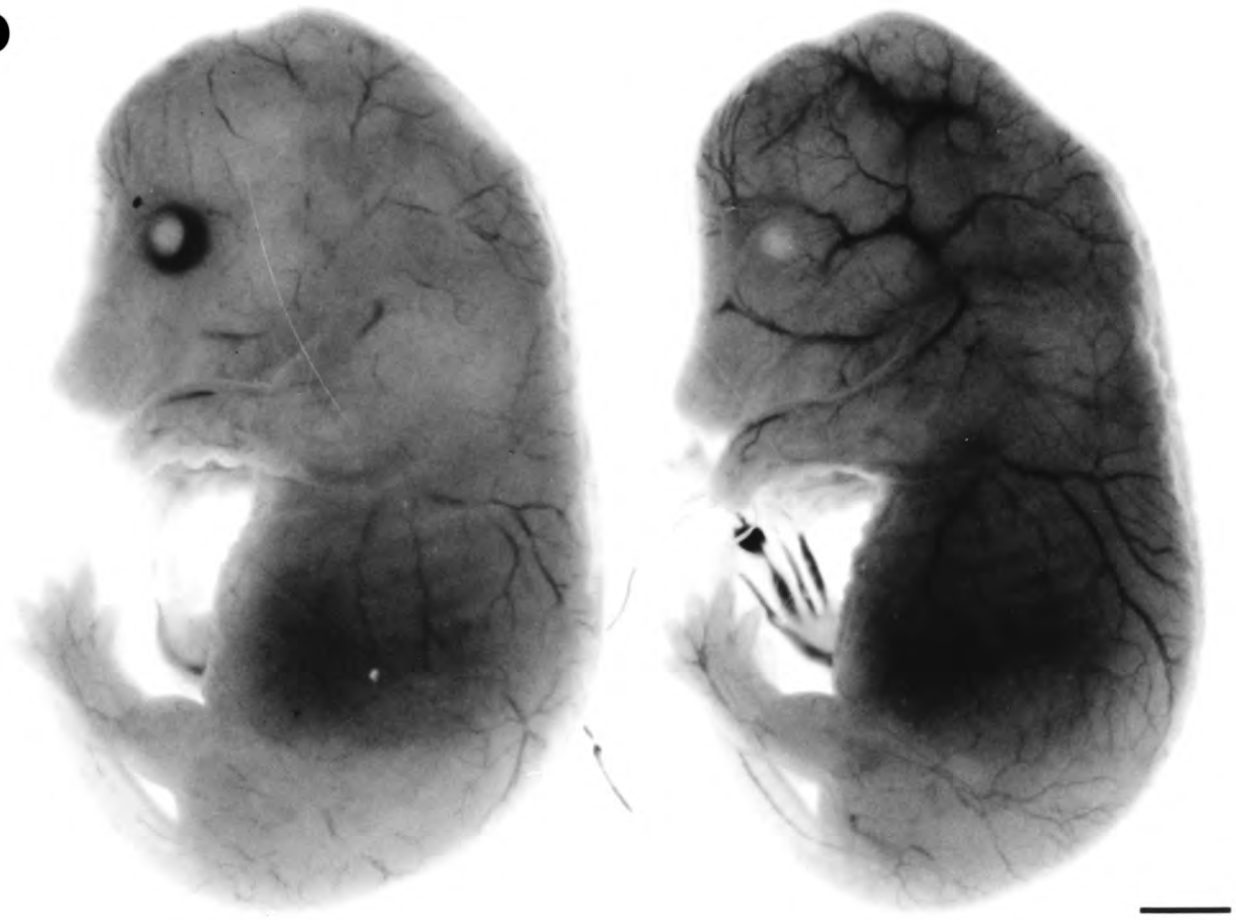


Fig. 2 Graph showing the change in crown-rump length in rat embryos at different prenatal stages. There is no obvious difference in terms of the crown-rump length between the pigmented and albino embryos at any of the ages studied.

Fig. 2

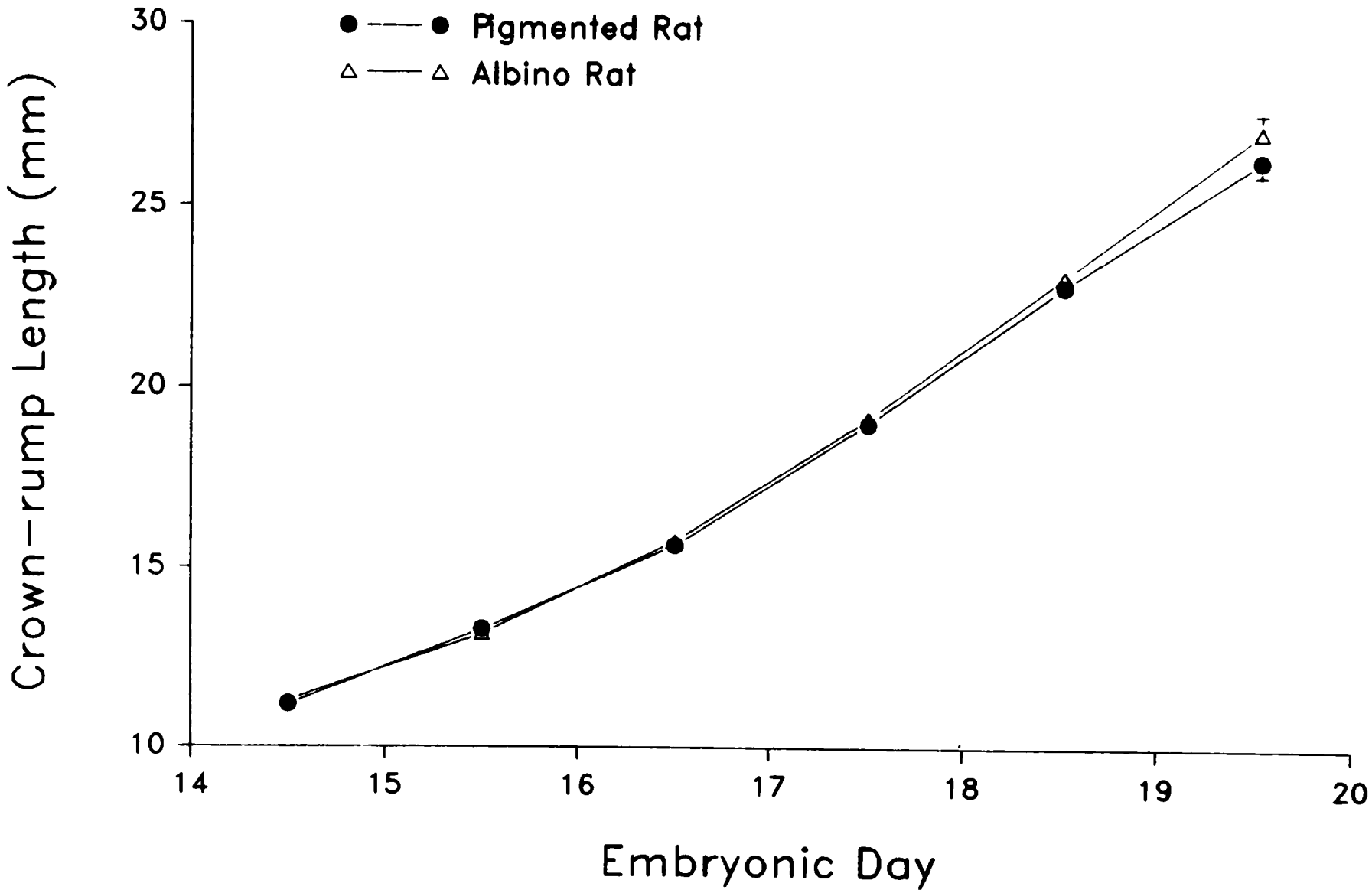
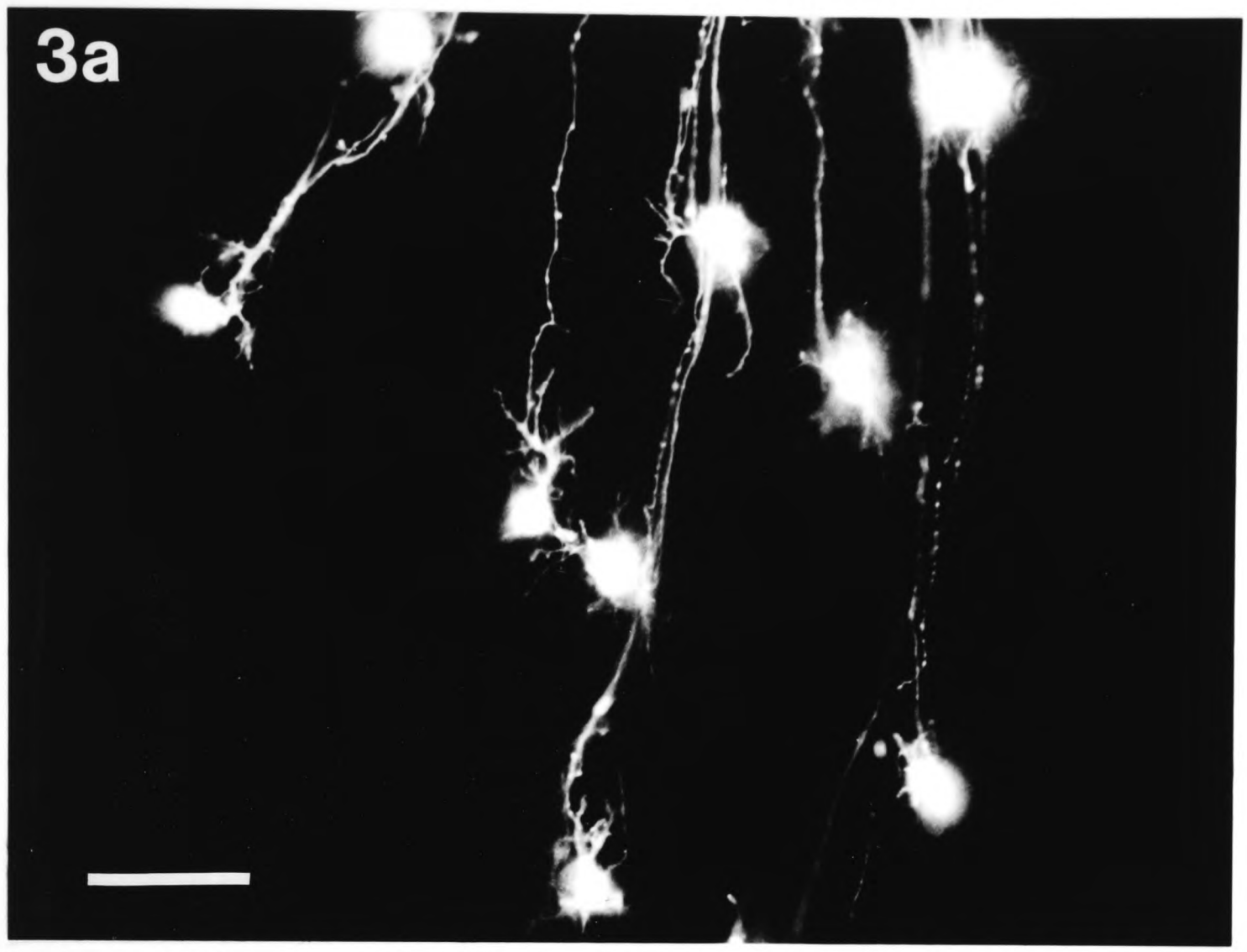

Pigmented Rat
Albino Rat
Crown–rump Length (mm)
30
25
20
15
10
14
15
16
17
18
19
20
Embryonic Day

Fig. 3 Fluorescent micrographs showing labelled ipsilaterally projecting retinal ganglion cells in the rat retina. a was taken from a E16.5 rat whereas b was taken from a E17.5 rat. Scale bar in a is 50 µm and in b is 20 µm.


3a

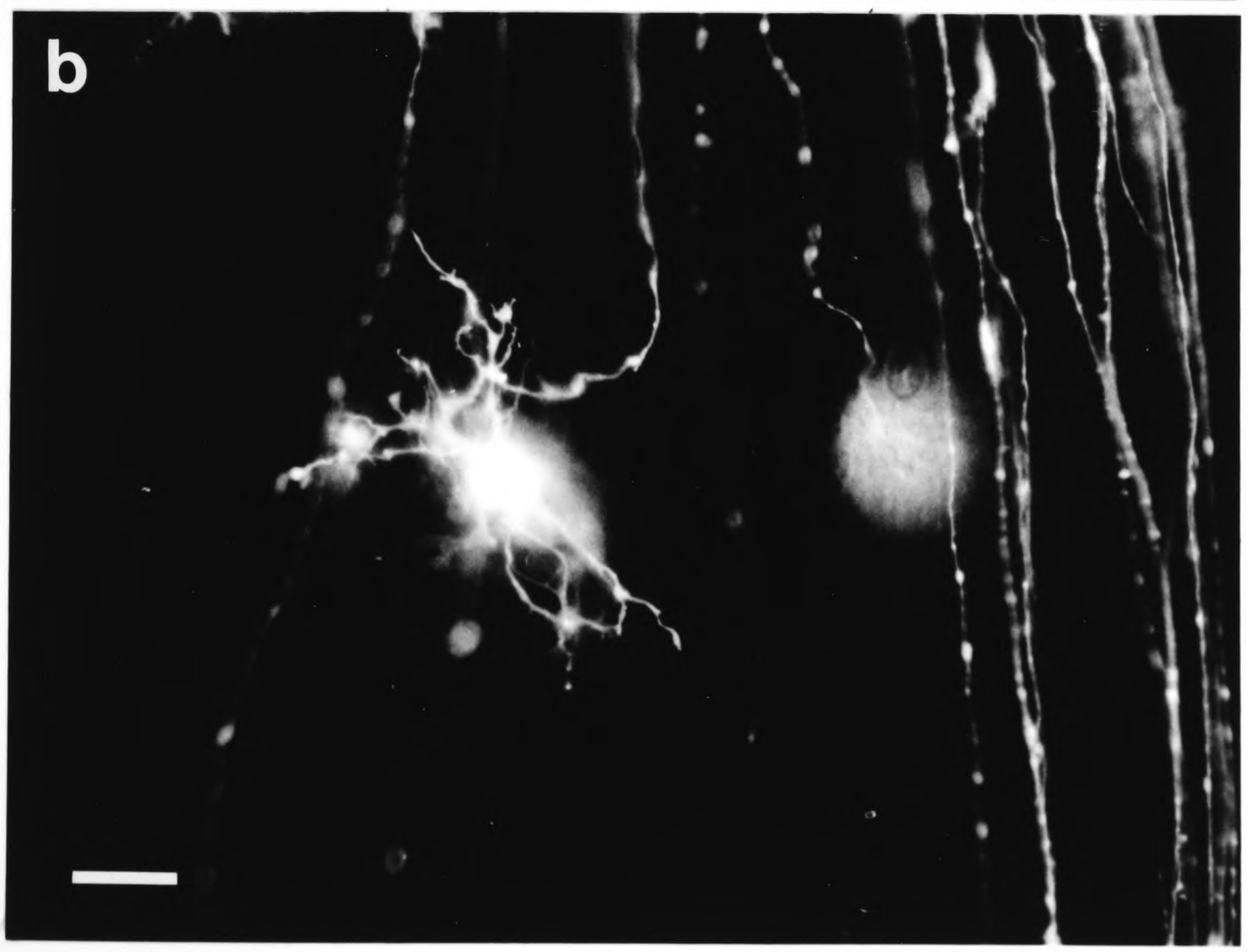
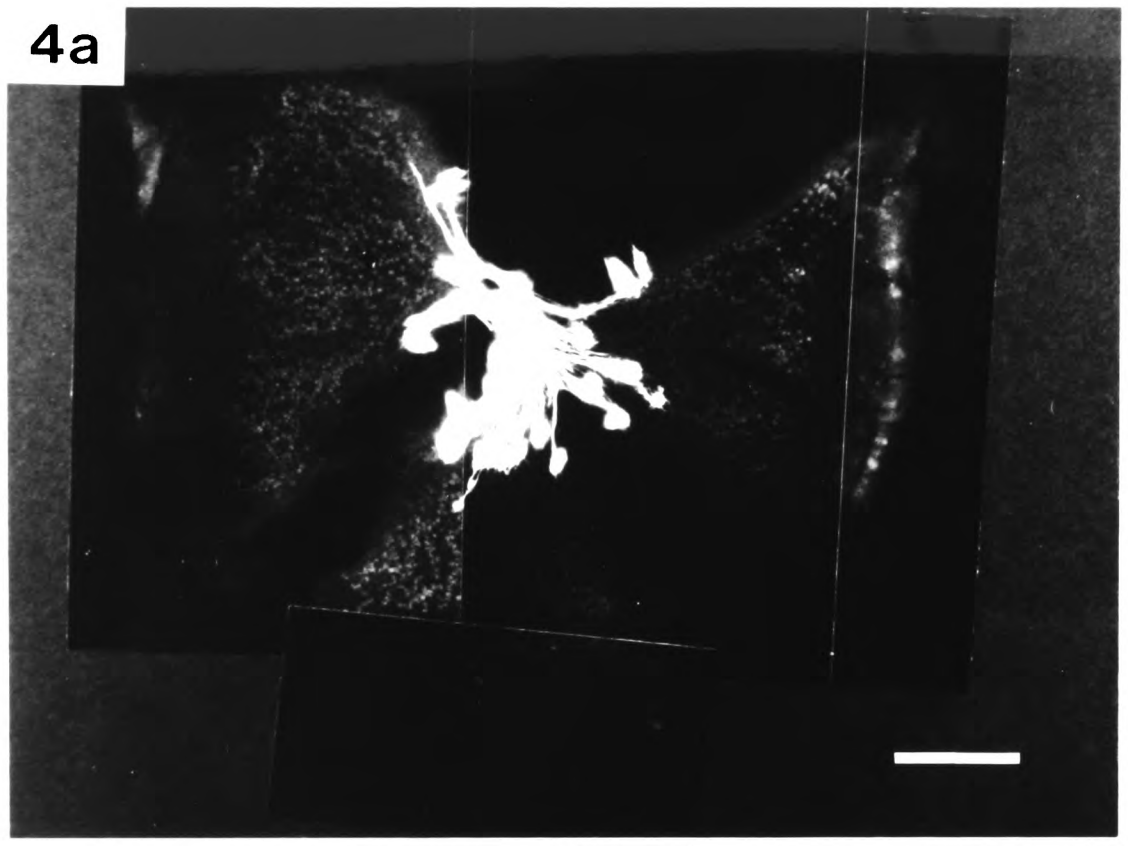
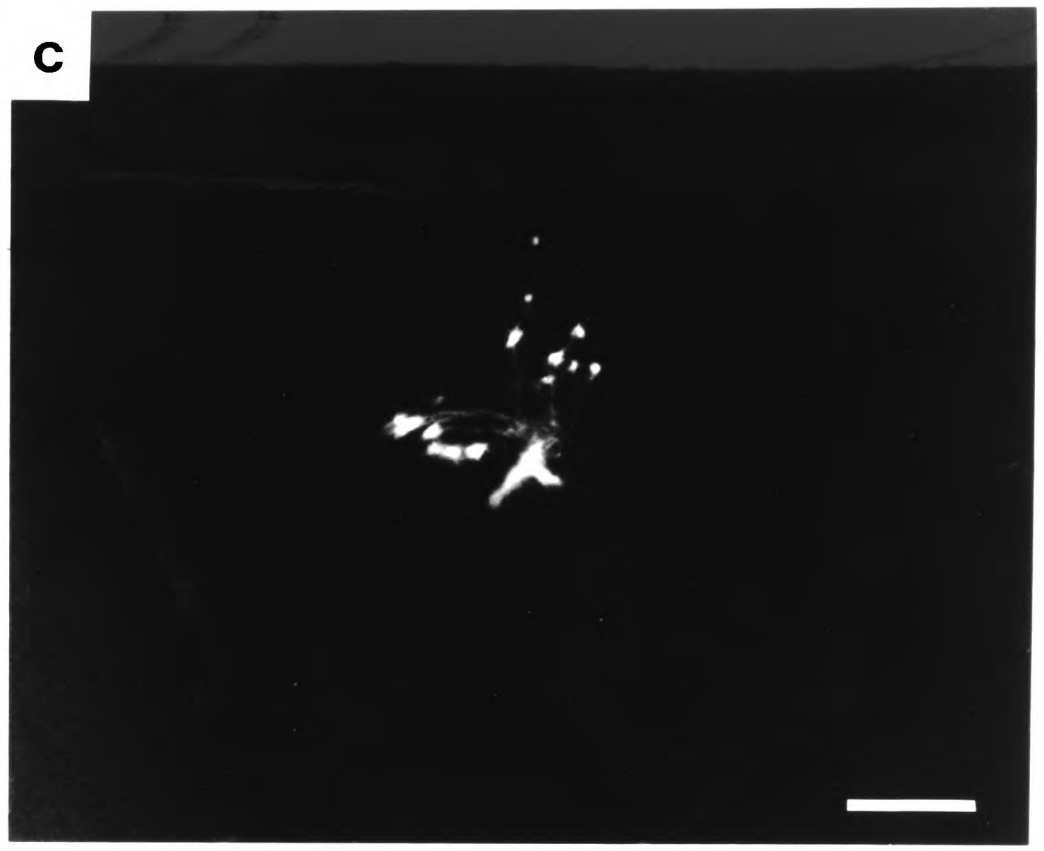
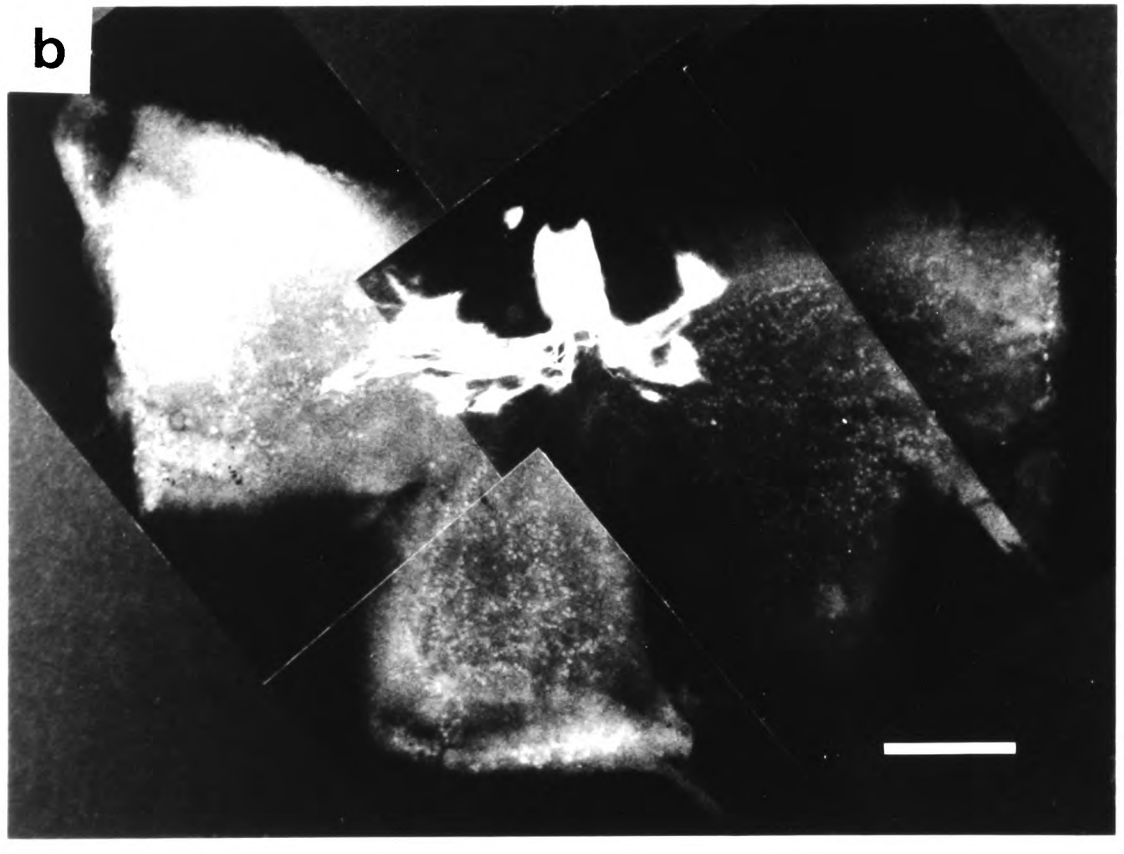

b

Fig. 4 Fluorescent micrographs showing the labelled retinal ganglion cells in the retinas of two E15.5 rat embryos ipsilateral (a and b) and contralateral (c and d) to the labelled optic tract. a and c are from a pigmented embryo and b and d are from an albino littermate. At this age, in the retina on either side of the labelled tract, only cells in the central retina around the optic disk send axons to the optic tract. For a and b, temporal is on the left; for c and d temporal is on the right; dorsal is up in these micrographs. Scale bar is 200 μm.


4a


c


b


d

Fig. 5 Fluorescent micrographs showing the labelled retinal ganglion cells in the retinas of two E16.5 rat embryos ipsilateral (a and b) and contralateral (c and d) to the labelled tract. a and c were taken from a pigmented embryo and b and d were taken from an albino littermate. In the retinas ipsilateral to the labelled tract, either pigmented or albino, some cells in more peripheral parts of the central retina are labelled, indicating that their axons have arrived at the tract. Temporal is on the left in a and b, and on the right in c and d; dorsal is up. Scale bar is 200 μm.

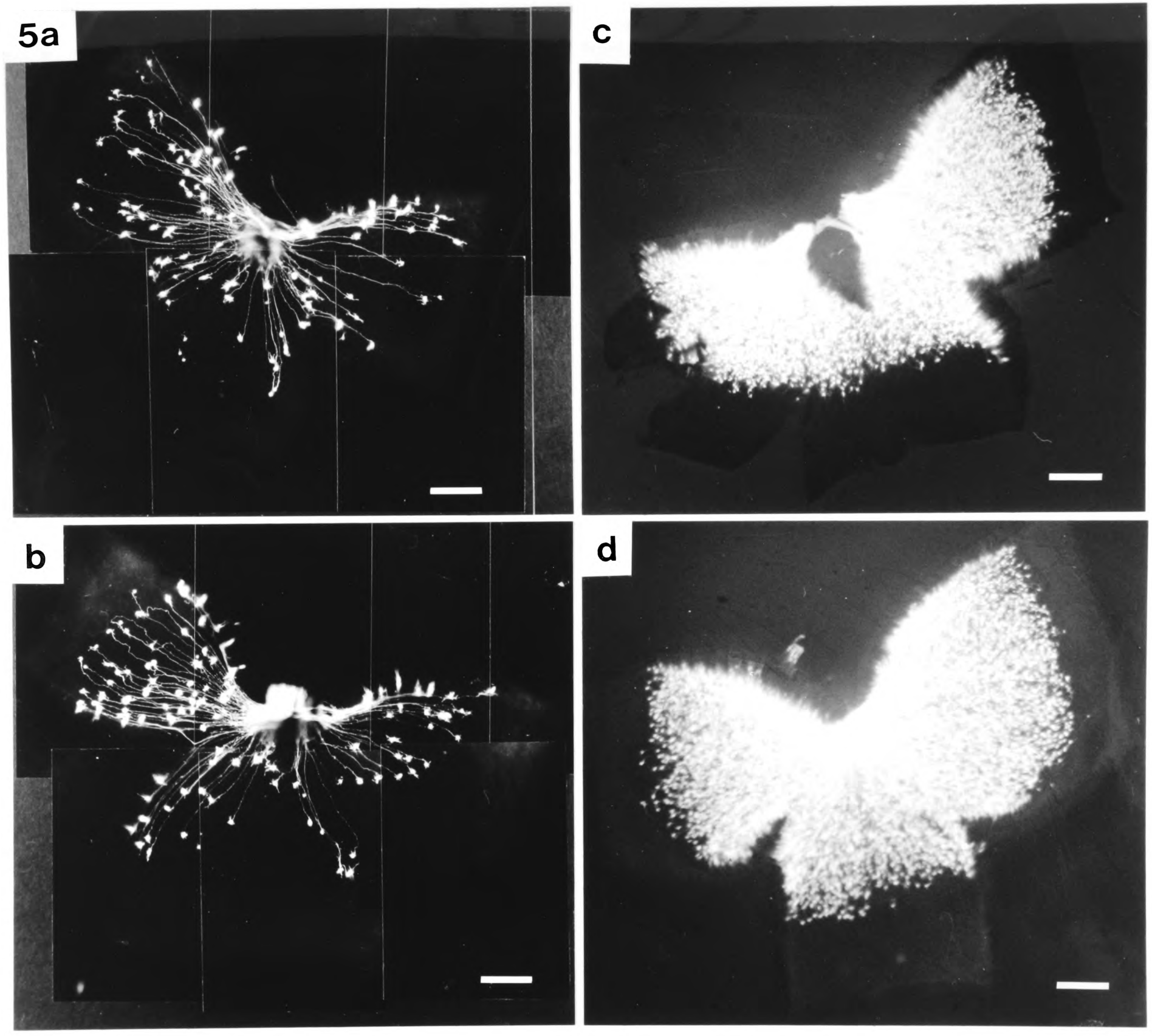
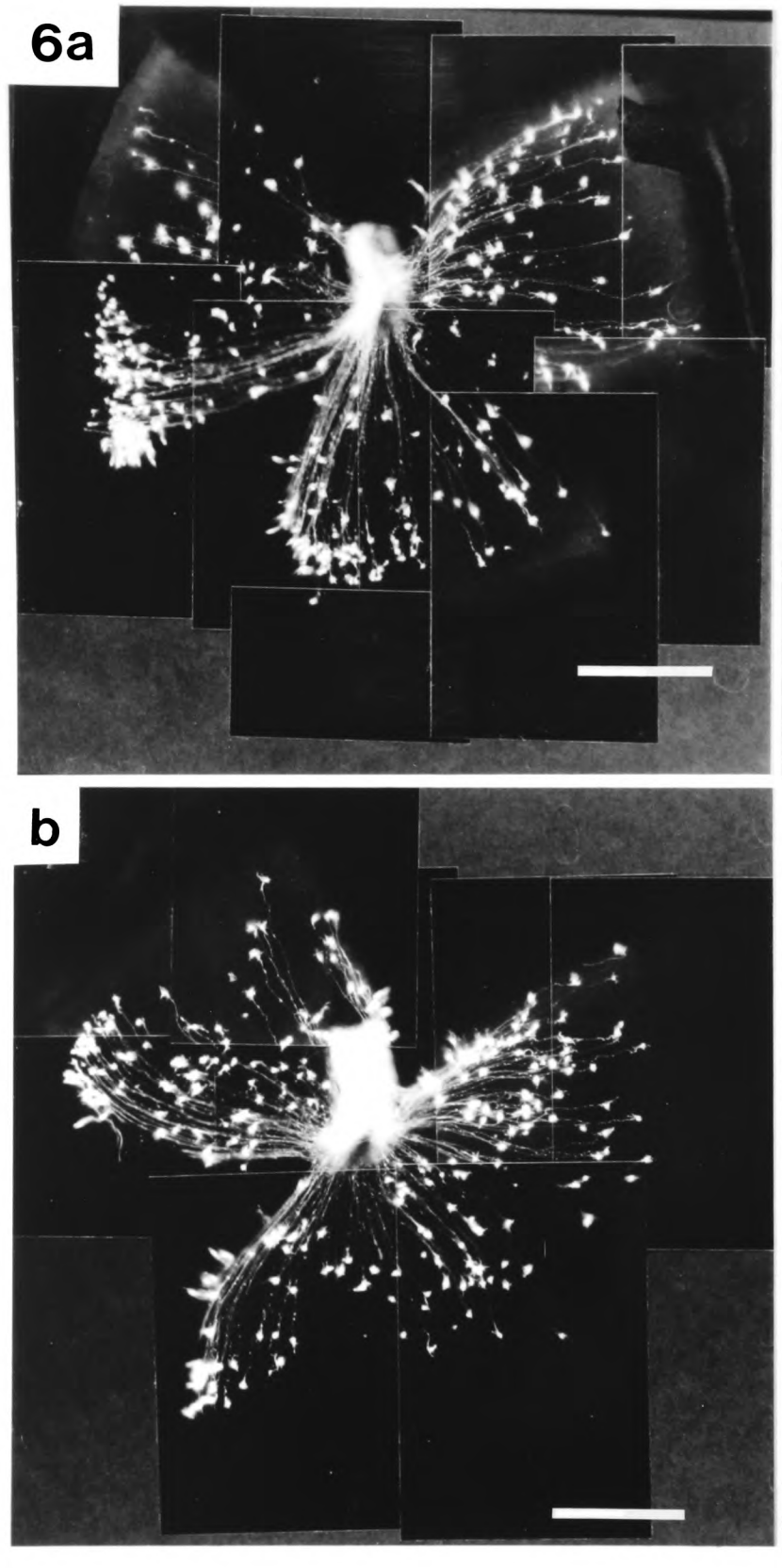
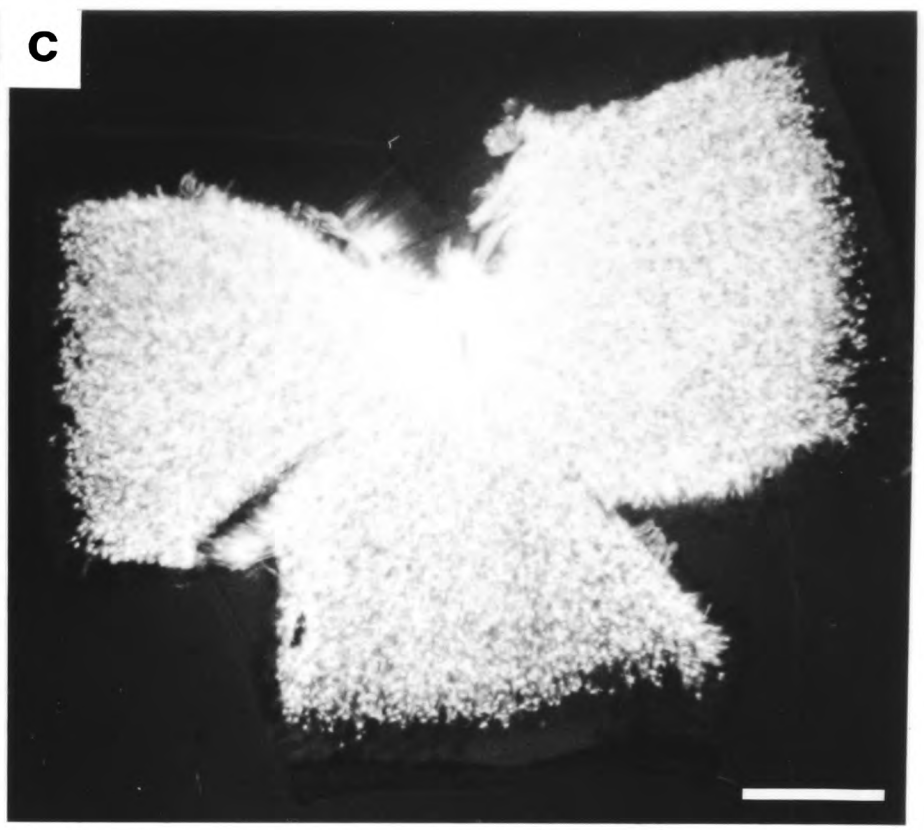

5a
c
b
d

Fig. 6 Fluorescent micrographs showing the labelled cells in the retinas of two E17.5 rat embryos ipsilateral (a and b) and contralateral (c and d) to the labelled tract. a and c are from a pigmented embryo and b and d are from an albino littermate. The concentration of ipsilaterally projecting retinal ganglion cells in the temporal crescent is first seen at this age. It is obvious that there are more cells in the temporal crescent of the pigmented retina projecting ipsilaterally than in the albino retina. Temporal is on the left in a and b, and on the right in c and d; dorsal is up. Scale bar is 400 μm.


6a


c

b

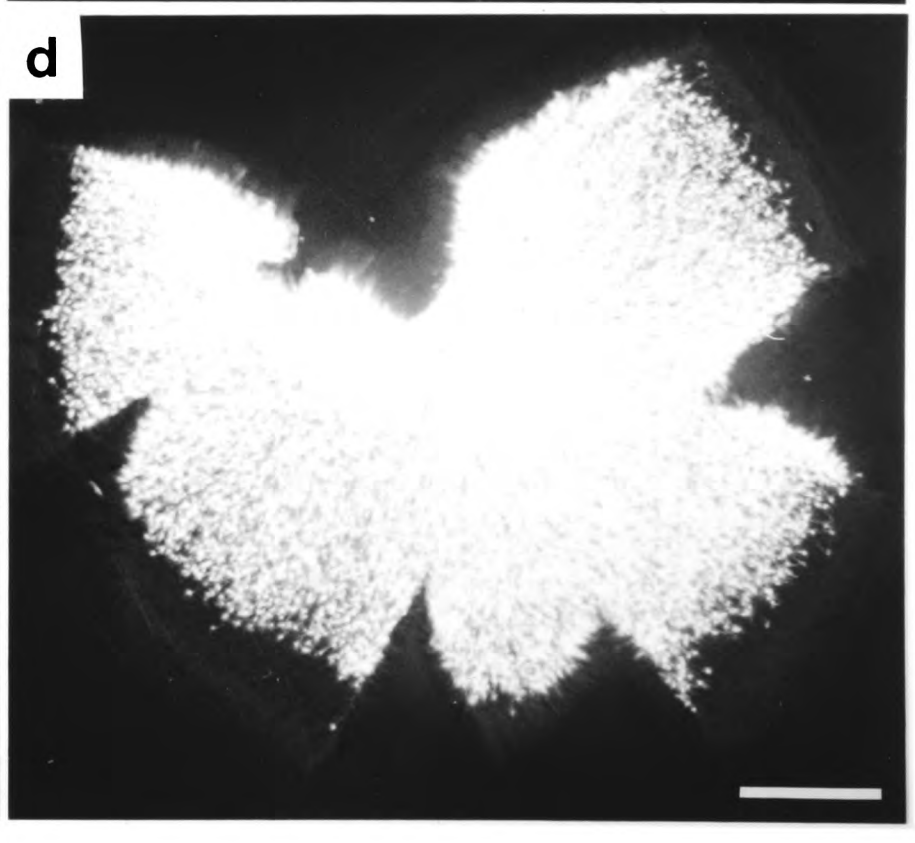
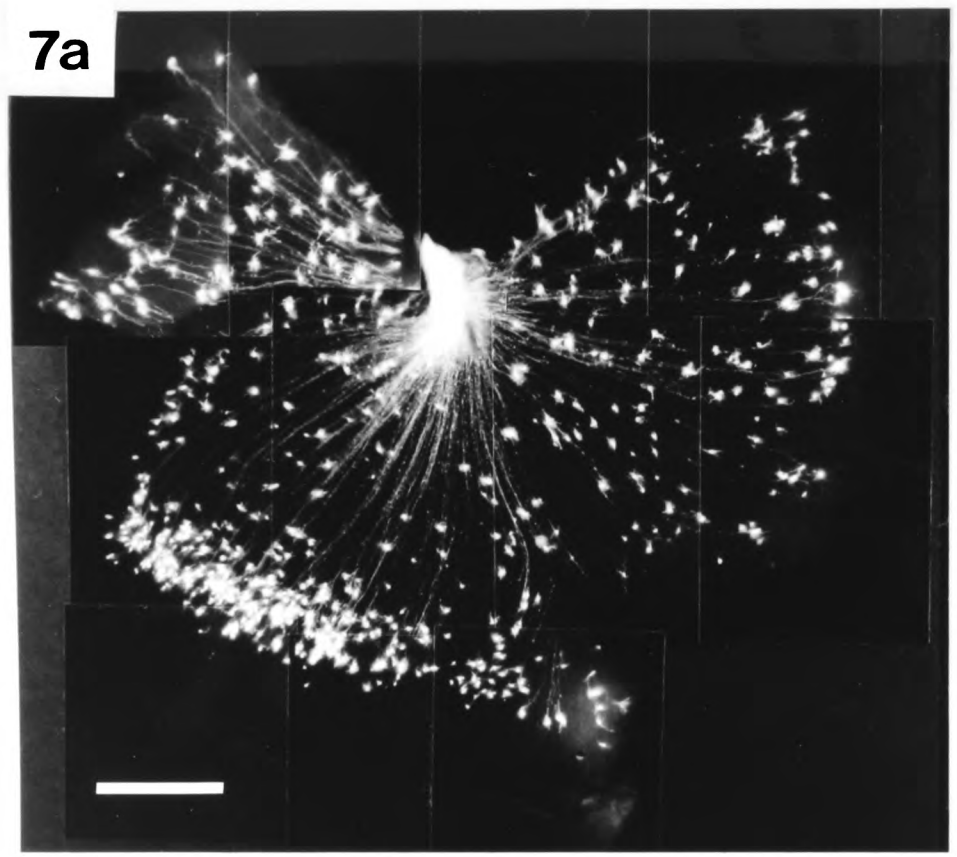
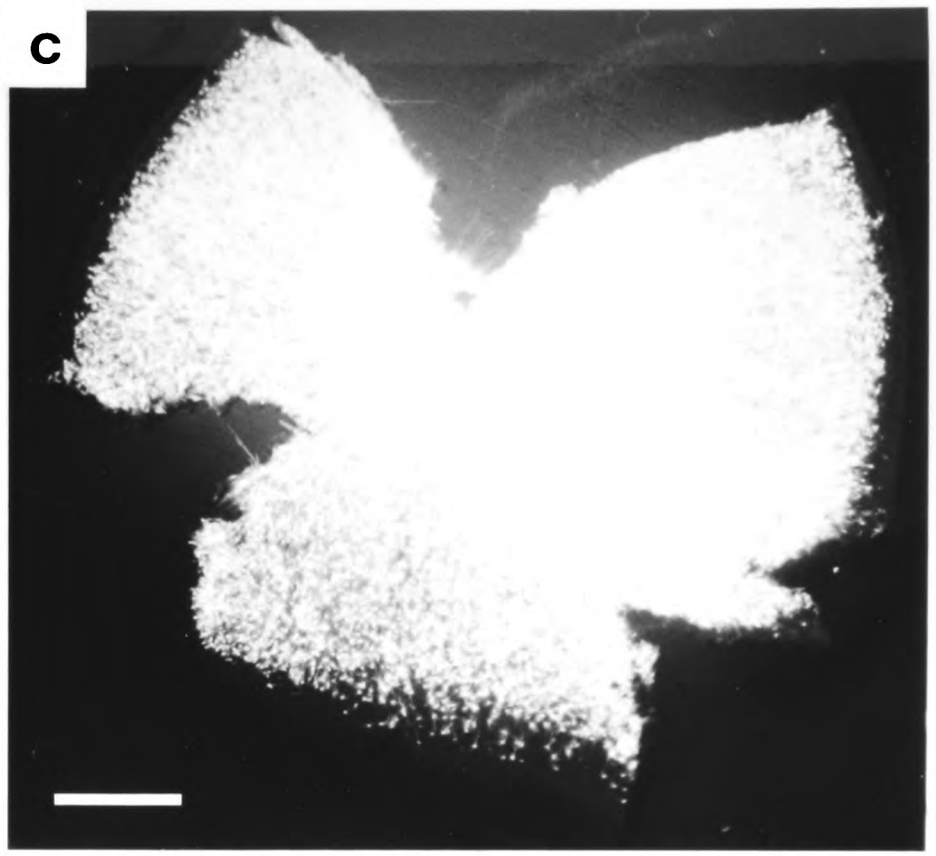
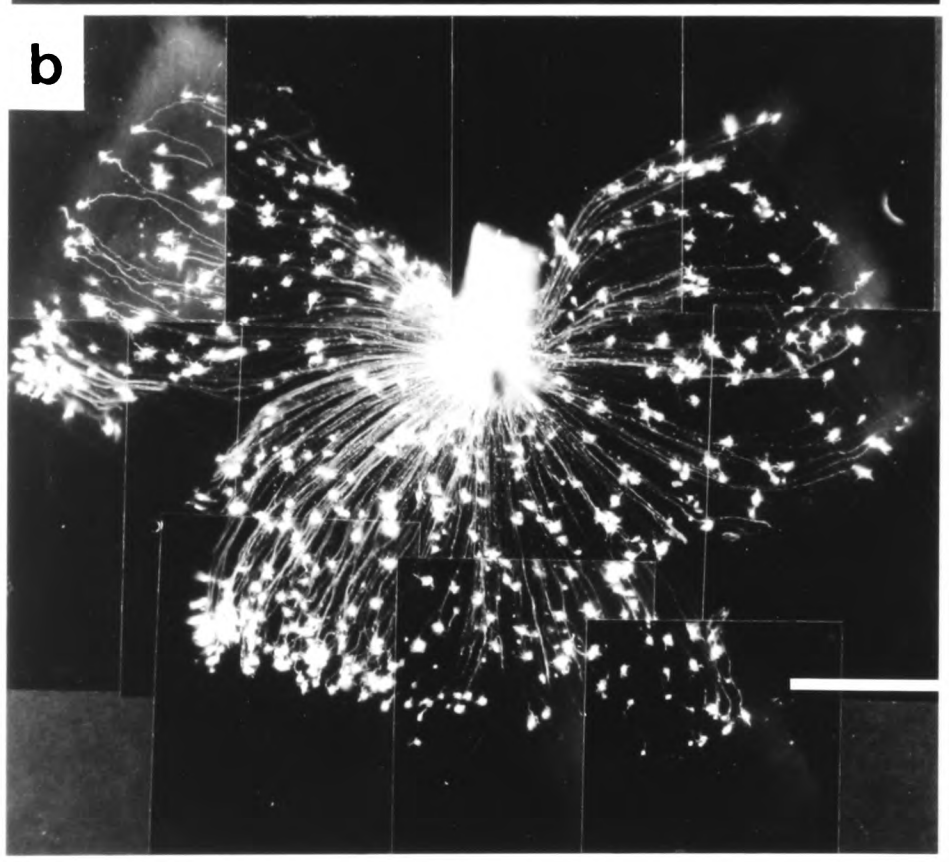

d

Fig. 7 Fluorescent micrographs showing the labelled ipsilaterally projecting ganglion cells in the retinas of two E18.5 rat embryos ipsilateral (a and b) and contralateral (c and d) to the labelled tract. a and c are from a pigmented embryo and b and d are from an albino littermate. At this age, it is obvious that more cells in the temporal crescent, in either pigmented or albino embryos, send axons to the ipsilateral tract. Temporal is on the left in a and b, and on the right in c and d; dorsal is up. Scale bar is 400 µm.


7a


c


b

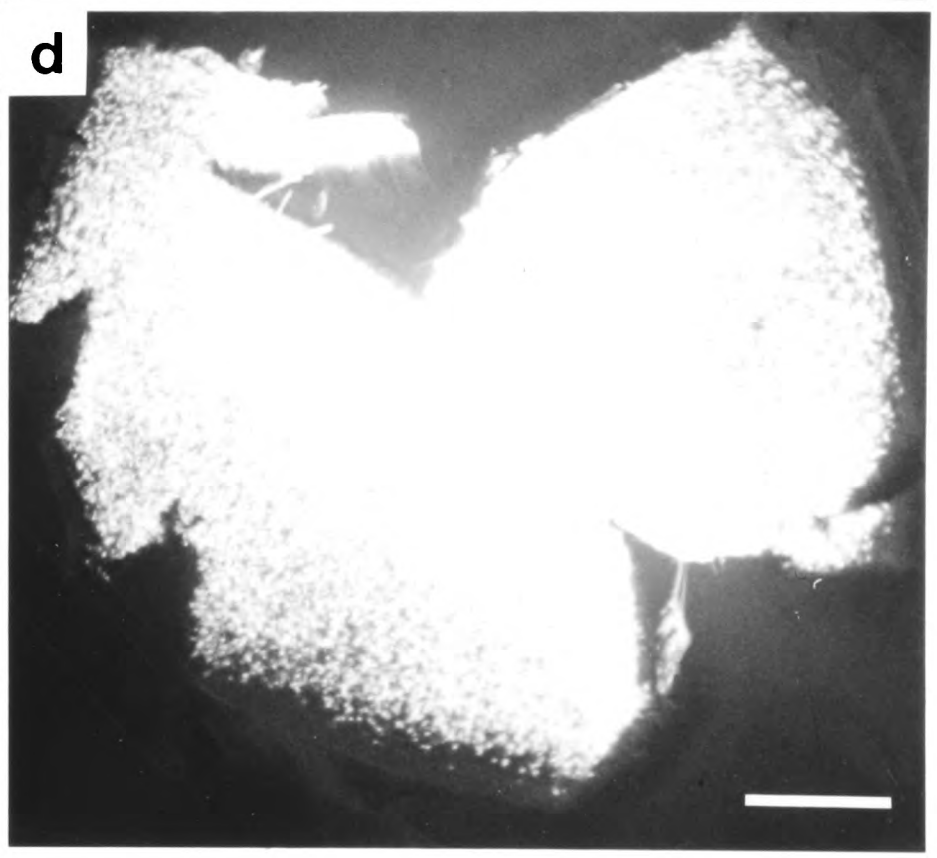

d

Fig. 8 Graph showing the number of labelled ipsilaterally projecting retinal ganglion cells in the central retina and the temporal crescent of rats at various prenatal stages. There is no significant difference in the number of ipsilateral cells in the central retina of pigmented and albino littermates at any ages studied, but the cell number in the temporal crescent is greater in the pigmented retina than in the albino retina.

Fig. 9 Graph showing the change in the total number of labelled ipsilaterally projecting retinal ganglion cells in the rat at different prenatal ages. Note a smaller than normal number of ipsilateral cells can be seen in the albino littermate at E18.5.

Fig. 8

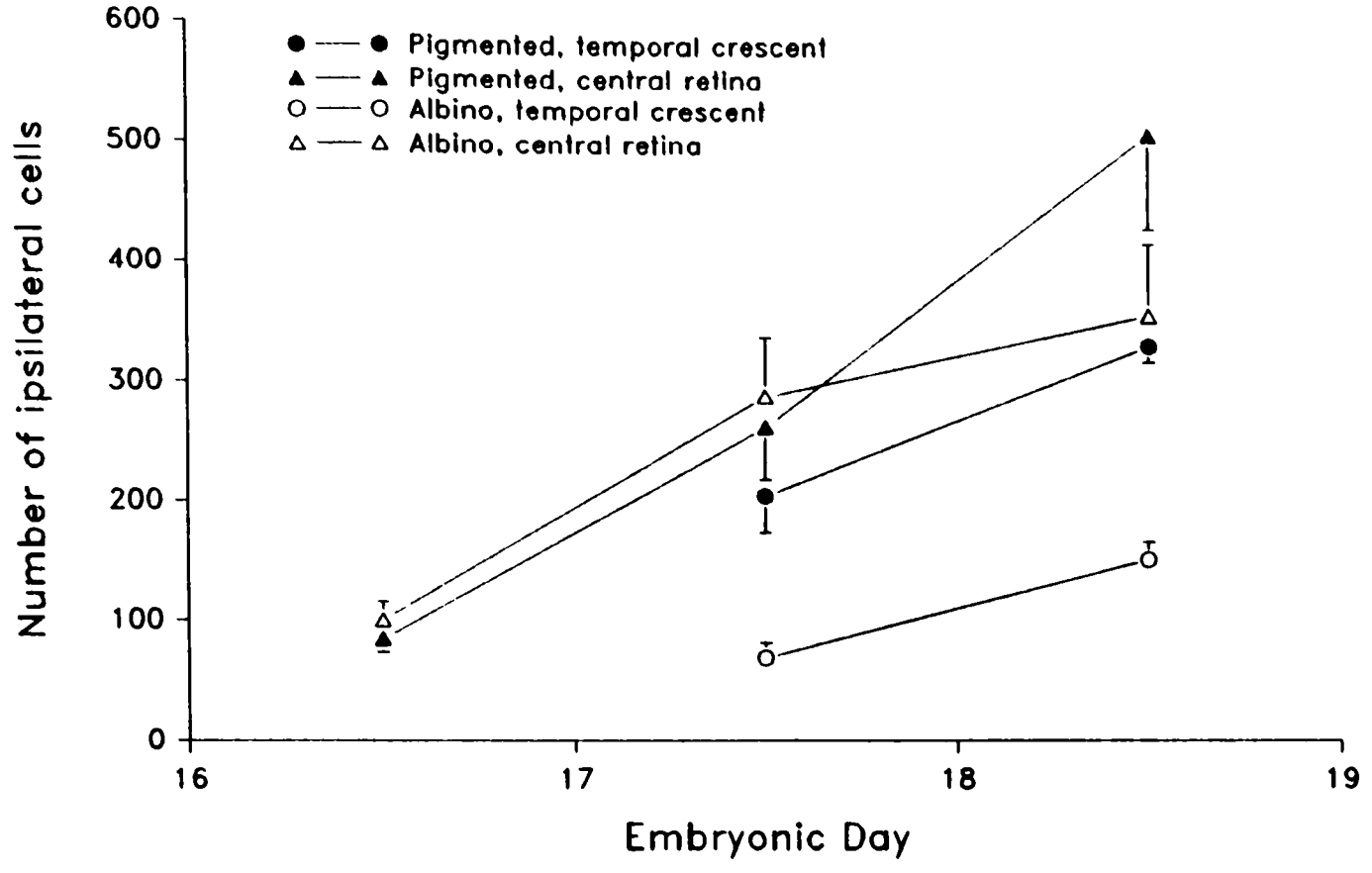

600
500
400
300
200
100
0
Number of ipsilateral cells
Pigmented, temporal crescent
Pigmented, central retina
Albino, temporal crescent
Albino, central retina
16
17
18
19
Embryonic Day

Fig. 9

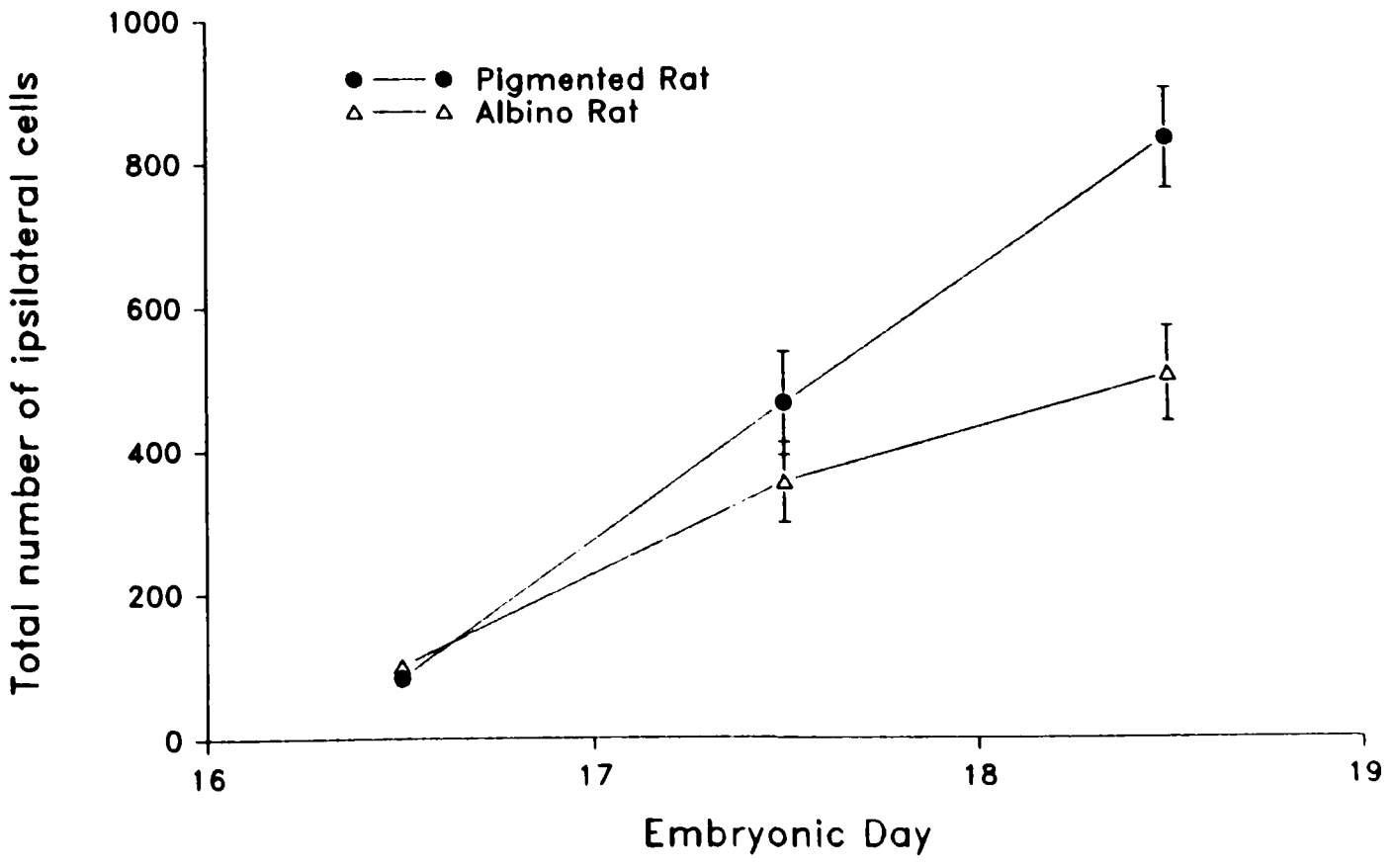

1000
800
600
400
200
0
Total number of ipsilateral cells
Pigmented Rat
Albino Rat
16
17
18
19
Embryonic Day

Fig. 10 Confocal micrographs showing the positions of labelled uncrossed fibres in transverse sections of the optic stalk of two E15.5 rat embryos. a-c were taken from a pigmented embryo whereas d-f were taken from an albino littermate. From top to bottom in each column, the figure shows fibres in the post-optic, middle and prechiasmatic stalk respectively. The white dots outline the boundary of the optic stalk. Dorsal is up and rostral is on the right. Scale bars are 25 μm, the bar in a is also for b, d and e; scale bar in c is also for f.

Although the uncrossed fibres are found throughout the rostro-caudal extent of the stalk, there is apparent variability in the pattern of uncrossed fibres in the pigmented and albino stalks, as shown in these two embryos.

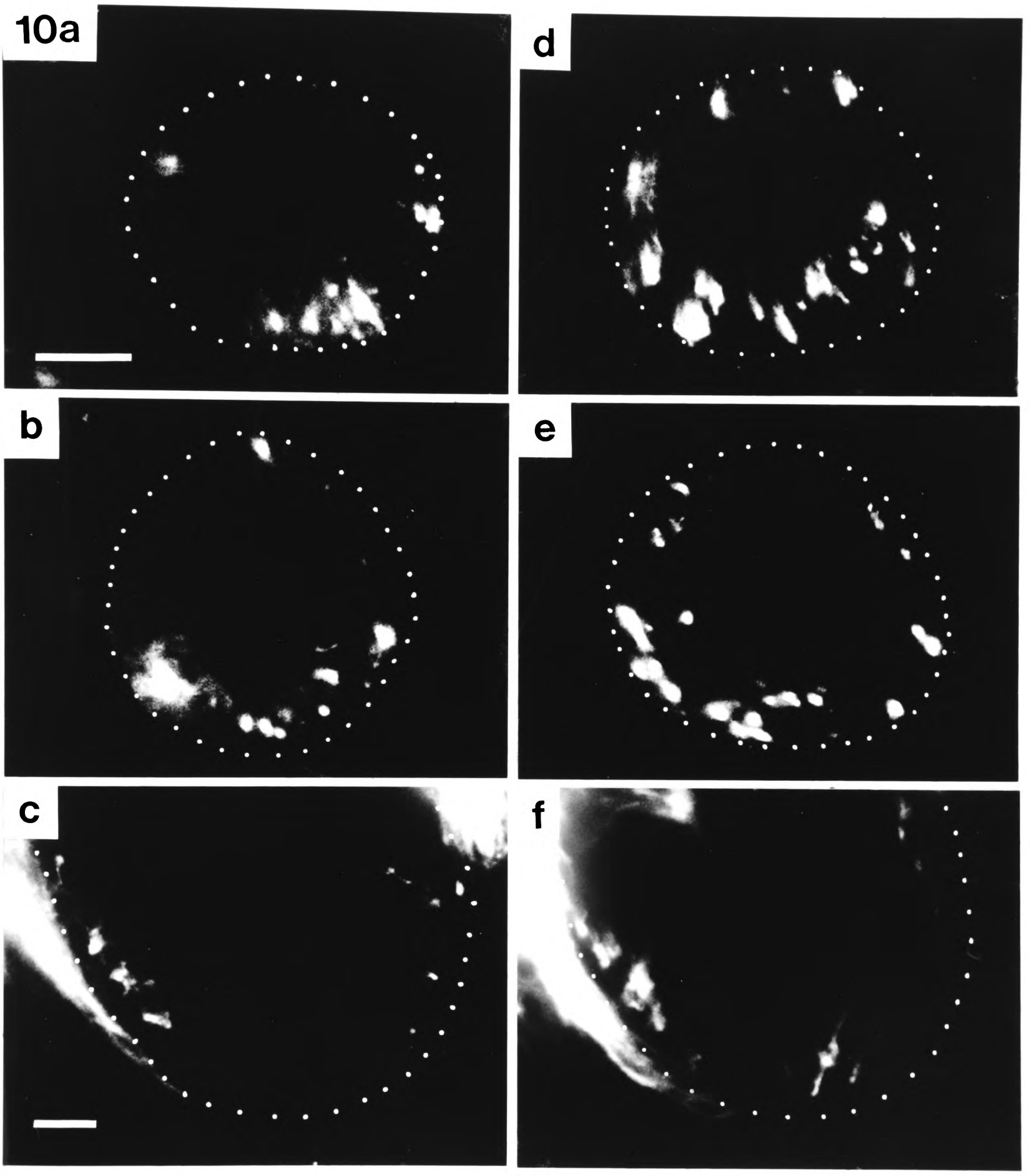

10a
d
b
e
c
f

Fig. 11 Confocal micrographs showing the labelled fibres in the optic stalk of E16.5 rat embryos ipsilateral (a-d) and contralateral (e-h) to the labelled tract. a and e were taken from the post-optic stalk of a pigmented embryo, and b and f were taken from a comparable location in the stalk of an albino embryo. c and g were taken from the prechiasmatic stalk of a pigmented embryos whereas micrographs taken at a comparable region in an albino embryo are shown in d and h. Dorsal is up and rostral is on the right. The arrangement and orientation of the micrographs in the subsequent figures (Fig. 12 and 13) are the same as in this figure. Note the intermixing of uncrossed fibres with the crossed fibres along the whole length of the optic stalk of either pigmented and albino embryo. Scale bars are 50 μm. The scale bar in a is also for b, e and f; the scale in c is also for d, g and h.

Although the uncrossed fibres are found throughout the stalk, there is apparent variability in the position of uncrossed fibres in the pigmented and albino stalks.

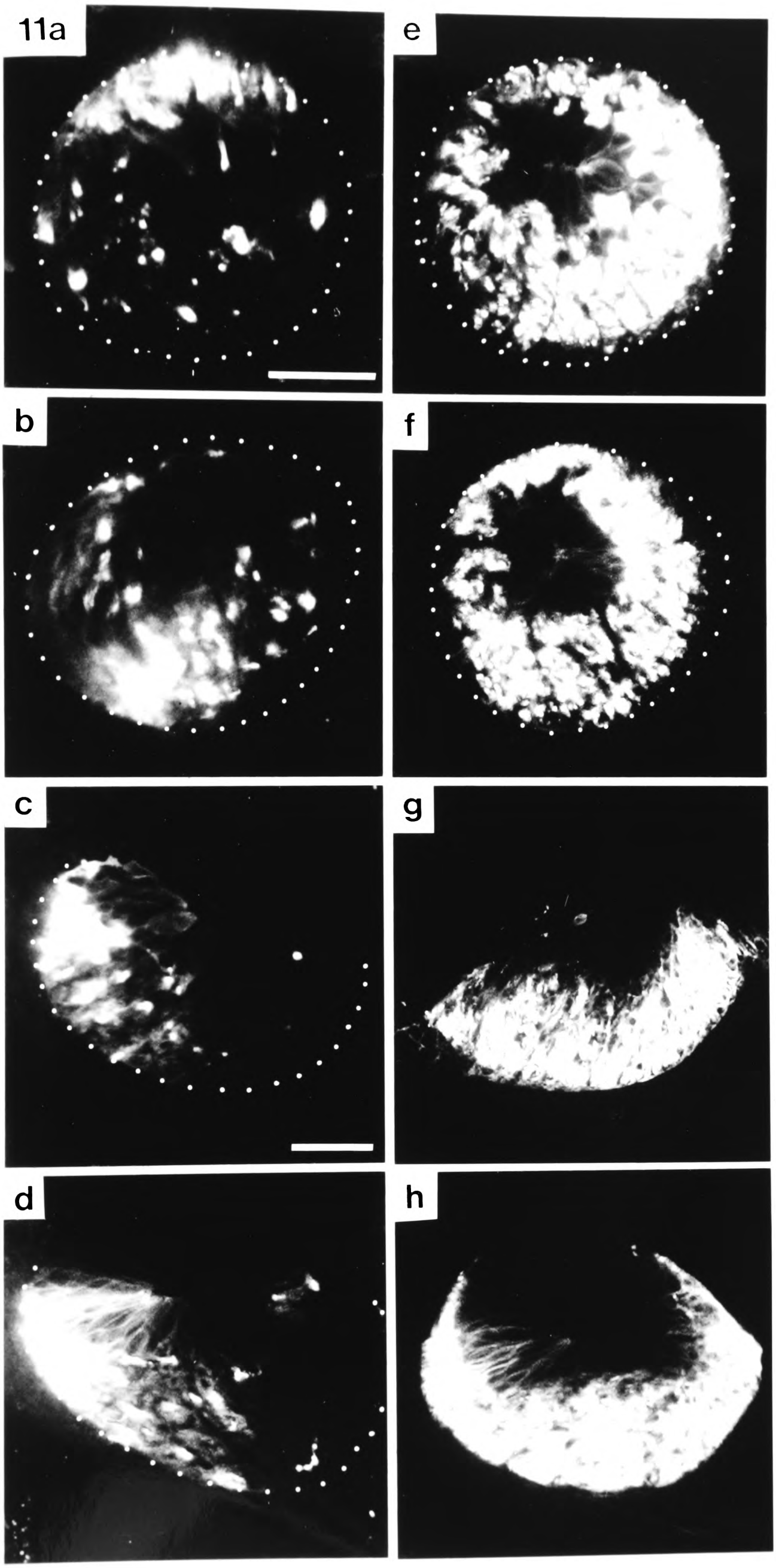

11a
e
b
f
c
g
d
h

Fig. 12 Confocal micrographs showing the labelled fibres in the optic stalk ipsilateral (a-d) and contralateral (e-h) to the labelled tract of two E17.5 rat embryos, one pigmented (a, c, e and g) and one albino (b, d, f and h). The arrangement and orientation of the micrographs are the same as in Fig. 11. Note the uncrossed fibres are intermingled with the crossed fibres along the whole length of the stalk. Scale bar for a-c and e-g is 50 μm; scale bar for d and h is 100 μm.

Although the uncrossed fibres are found throughout the stalk, there is apparent variability in the position of uncrossed fibres in the pigmented and albino stalks.

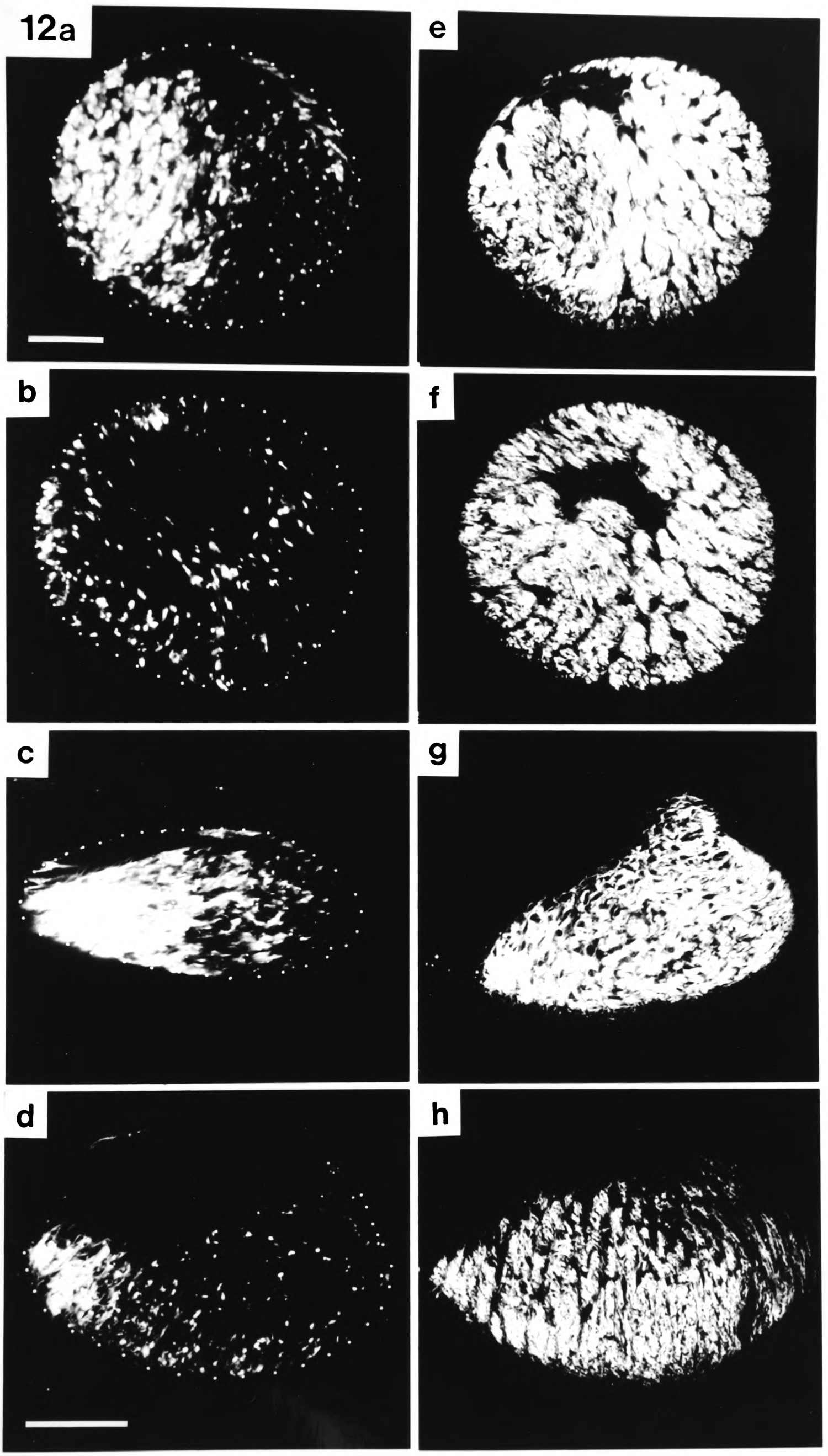
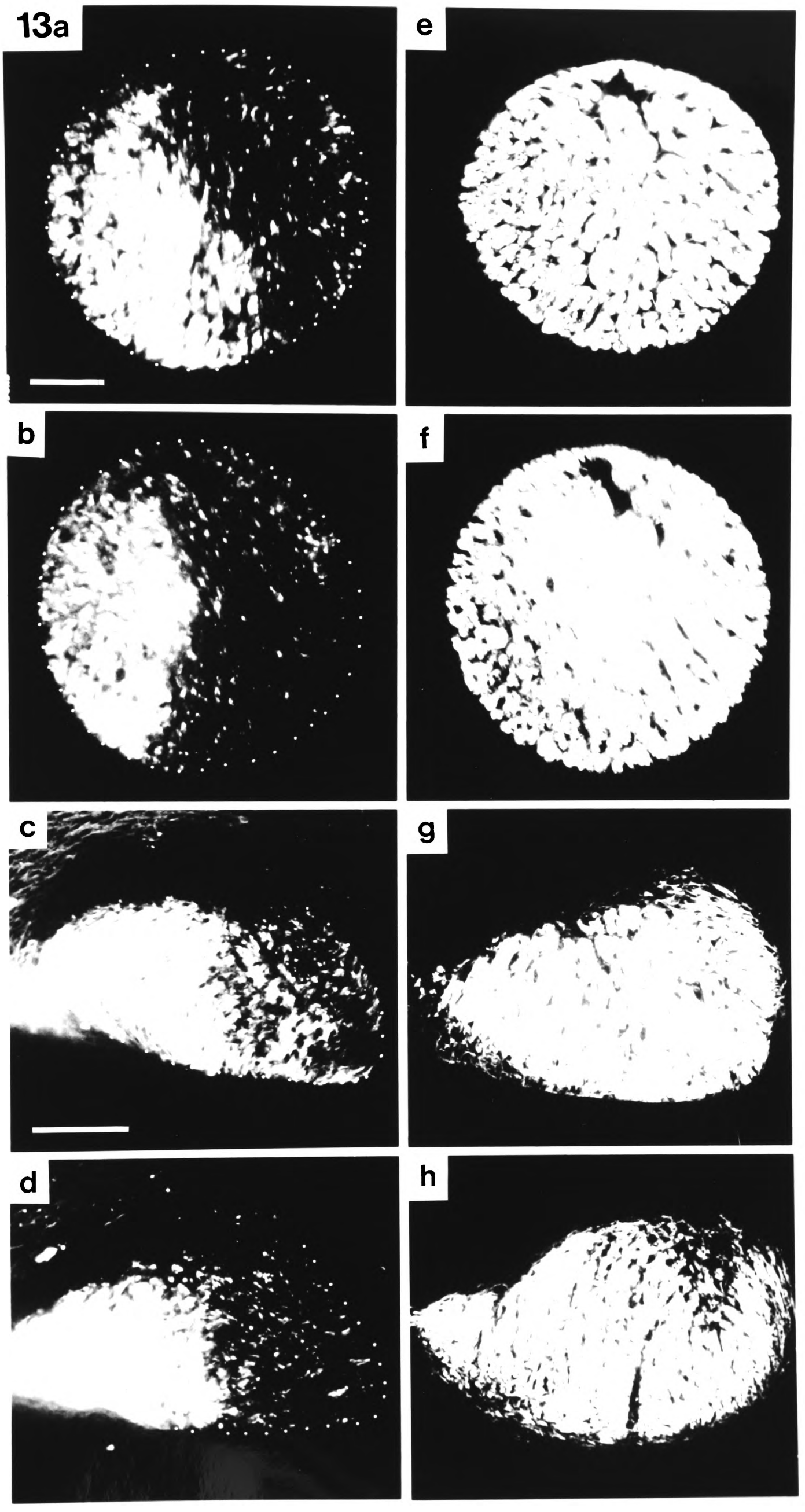

12a
e
b
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d
h

Fig. 13 Confocal micrographs showing the labelled fibres in the optic stalk ipsilateral (a-d) and contralateral (e-h) to the labelled tract of two E18.5 rat embryos, one pigmented (a, c, e and g) and one albino (b, d, f and h). The arrangement and orientation of the micrographs are the same as those in Fig. 11. Scale bar for a-b and e-f is 50 μm and for c-d and g-h is 100 μm.

Although the uncrossed fibres are found throughout the stalk, there is apparent variability in fibre position between the pigmented and albino stalks.


13a
e
b
f
c
g
d
h

CHAPTER 4 THE EFFECTS OF PRENATAL MONOCULAR ENUCLEATION ON THE DEVELOPMENT OF THE UNCROSSED RETINOFUGAL PATHWAYS IN NORMAL AND ALBINO RATS

Introduction

In adult rats, the ganglion cells that project to ipsilateral visual targets are found throughout the retina, however, most of them concentrate in the ventral temporal crescent. A decussation line can be drawn at the central border of the temporal crescent and that marks an abrupt change in the density of ipsilaterally projecting cells from a densely populated area in the temporal crescent to a sparsely populated area in the rest of the retina (Cowey and Perry, 1979; Jeffery and Perry, 1982; Linden and Perry, 1982; Perry and Linden, 1982). The contralaterally projecting cells, however, are distributed evenly in all parts of the retina except for a slight thinning in the ventrotemporal retina (Cowey and Perry, 1979).

Little is known about how this decussation pattern is established in the development of the rat retina. However, it is clear that the decussation line of the uncrossed component must be established early since a major concentration of the ipsilaterally projecting cells is found in the ventrotemporal retina of rats and other species during their prenatal life (rat: Bunt et al., 1983; Chan et al., 1990; see also chapter 3; mouse: Godement et al., 1987b; Collelo and Guillery, 1990; Sretavan, 1990a; cat: Lia et al., 1987; monkey: Chalupa and Lia, 1991).

How the uncrossed axons are able to find their way to the ipsilateral tract during the formation of the pathway is still a question. Studies of the axonal trajectories of growing optic fibres at the chiasm suggest that the growing uncrossed axons need to read a local cue at the chiasm that directs them to turn from their original course and head towards the ipsilateral tract (Sretavan, 1990a and 1990b; Godement et al., 1990; Godement and Mason, 1990). More interestingly, it has been reported that in the mouse if one eye is removed prenatally before the retinal fibres have reached the chiasm, most fibres from the ventral temporal retina of the remaining eye, where most of the ipsilateral axons arise, stall at the chiasm and fail to invade the ipsilateral tract (Godement et al., 1990). This observation suggests that the chiasmatic cue that guides growing optic fibres into the ipsilateral tract may be a binocular fibre interaction, that is either a direct interaction between fibres from the two eyes or else involves some intermediate interaction with the glial environment at the chiasm.

It has been observed that the uncrossed fibres are intermingled with the crossed fibres along the whole length of the developing stalk (Collelo and Guillery, 1990; see results in chapter 3). Based on this point, it is reasonable to argue that whatever the chiasmatic cue may be, there must be an intrinsic difference between the uncrossed and crossed axons. This intrinsic difference enables the uncrossed axons to select an ipsilateral course when they encounter the chiasmatic cue and the crossed axons to grow directly to the contralateral tract.

It have been argued that the possible site where this intrinsic difference of the crossed and uncrossed axons may be set up is in the retina (see discussion in chapter 3). Although the nature of this intrinsic difference is not known, but it may be

regarded as a difference in the expression of some marker in the retinal ganglion cells that project to different sides of the brain. One simple hypothesis is that the ipsilaterally projecting cells have a retinal marker and that this marker enables their axons to interpret the chiasmatic cue as an instruction to select an uncrossed path. The contralaterally projecting cells either may not express this marker or the expression of this marker is weakened in these cells so that their axons ignore the chiasmatic cue and grow directly to the contralateral tract. Further, this hypothetical marker is somehow expressed preferentially by cells in the temporal crescent, since most ipsilateral cells are found in this retinal region, and either only a few cells outside the temporal crescent carry this marker or else the ipsilaterally projecting ganglion cells in this part of the retina are regarded as "errors".

It seems reasonable to assume that the uncrossed retinal pathway in normal pigmented animals develops through the expression of a retinal marker on the ipsilateral cells, this marker enables the uncrossed fibres to interact with the crossed fibres from the other eye at the chiasm, and that allows the uncrossed fibres to take an ipsilateral route. This assumption can be used further to explain how the abnormal pathway in albino animals may be developed.

It is well documented that in albino animals the uncrossed retinofugal component is abnormally small, probably because some of the ipsilaterally projecting axons are misguided at the optic chiasm to the contralateral tract (Lund, 1965; Guillery, 1969; Guillery and Kaas, 1971; Dräger and Olsen, 1980; Creel et al., 1982). A reduced uncrossed pathway can also be produced in adult pigmented mice and ferrets by removing one eye prenatally at a time before or just after the first axons pass through

the chiasm (Godement et al., 1987a; Guillery, 1989). It has been shown further in the ferret that an early eye removal in pigmented embryos not only produces a reduced uncrossed projection to the adult dorsal lateral geniculate nucleus, but also creates a novel termination pattern which is remarkably similar to that seen in a mature albino uncrossed retinogeniculate pathway (Guillery, 1989). Removal of one eye in albino ferrets at a similar prenatal stage was reported to have no apparent effect on the development of the adult uncrossed retinogeniculate projection. Based on these observations, it was argued that the albino abnormality may be developed as a consequence of a weakening of the binocular fibre interaction at the chiasm, the result of which is mimicked by removing fibres from one eye in pigmented embryos, but cannot be affected by eye removal in albinos.

A question then arises. Although it is known that the early enucleation in pigmented embryos produces an uncrossed retinal component which is abnormally small in terms of retinal ganglion cells in mice (Godement et al., 1987a) and in terms of geniculate terminal fields in ferrets (Guillery, 1989), it is still not known for either species whether the decussation line of the reduced uncrossed component is shifted towards the temporal margin of the retina in pigmented animals with an early monocular enucleation. The temporal shift of the decussation line is commonly seen in the adult albino uncrossed projection (Guillery and Kaas, 1971; Stone et al., 1978; Cooper and Pettigrew, 1979b; Dräger and Olsen, 1980; Morgan et al., 1987). If there is really a shift of the decussation line in the uncrossed pathway of early pigmented enucleates, it will support the view that the albino abnormality is due to an alteration of binocular fibre interaction at the chiasm. If the early enucleation does not affect

the line of decussation, it will suggest that the effects of the enucleation and of the albino abnormality are not the same. This will then encourage us to look for a non-chiasmatic site of the action of the albino gene, although (of course) it will not prove or demonstrate that the albino gene acts at a non-chiasmatic site.

The present study was undertaken to answer the question, first, whether the early monocular enucleation in rats, as in mice, can produce a reduced uncrossed pathway, in terms of number of ipsilaterally projecting retinal ganglion cells in the neonatal retina of pigmented animals, and thus produce a pathway quantitatively like that seen in albino neonates. The second question is whether the early enucleation can cause a shift of the decussation line to the temporal periphery in the reduced uncrossed pathway of the pigmented enucleate, comparable to that seen in albinos. The third question is to see if the development of the uncrossed pathway in albino rats is independent of the chiasmatic binocular fibre interaction, as proposed on the basis of the ferret study (Guillery, 1989). In order to answer these questions, pigmented and albino rat embryos had one eye removed prenatally at a time when the first optic axons have passed the chiasm and not yet entered their targets (Bunt et al., 1983; Chan, unpublished data). The number and distribution of retinal ganglion cells in the uncrossed retinofugal component was then determined on the day of birth. Further, fibres from the far periphery of the ventrotemporal retina of rat embryos were labelled with DiI (Honig and Hume, 1986; Godement et al., 1987b) to see whether these fibres have reached the chiasm at the time that the enucleations were made. The number of ipsilaterally projecting cells in adult pigmented and albino rats was also examined to illustrate the influence of cell death on the uncrossed retinal pathway of early

enucleates which develops without any binocular fibre interaction in the primary visual targets.

Materials and Methods

Animals

Lister Hooded rats and Sprague Dawley albino rats were used in this study. Timed mated females were obtained from the Departmental Animal House. The day that the vaginal plug was found was taken as embryonic day 0 (E0).

Intrauterine surgery

One eye in a number of rat embryos, both pigmented and albino, was removed at the age of E16.5, the earliest stage for which survivals could be obtained. The mothers were first anaesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg body weight). The embryos were then exposed by making a small incision at the midline of the abdominal wall. At this age, the embryos could be seen as chains of swellings along the two unterine horns. The uterine wall was thin enough to enable one to locate the eye without much of a problem. Only two embryos were operated on in each litter, one on each side. The selected embryos, still in their uterine horn, were taken out of the abdomen, one at a time, and they were oriented in such a direction that one eye of the embryo faced the poorly vascularized region of the uterine wall, usually opposite to the placenta. A purse-string suture was then placed in the unterine wall just over the eye. Special precautions were taken to avoid

damaging any blood vessels, especially those of the embryonic membranes. The uterine wall within the suture and the eye underneath were removed by a hand-held microcauterizer. The wound was immediately closed by tightening the suture and the operated embryos were put back gently into the abdomen. After finishing the intrauterine enucleation, the abdominal wall and the skin were closed up and the mother were left to recover in a warm environment.

Optic tract injection in newborn rats

The normal and operated littermates were delivered naturally. Some litters of rats were left with their mothers until they were adult before they received the second operation. Other litters were operated on the day of birth and their mothers were killed by an overdose of pentobarbital. The newborn rats were first anaesthetized by ether. An incision was made to expose the skull on the right side of the head in normal animals, both pigmented and albino, and on the side of the remaining eye in animals with an early eye removal. Under a dissecting microscope, a window was made through the cartilaginous skull on the lateral side of the head rostral to the transverse venous sinus. The meningeal membranes were retracted with special care so as to prevent any damage to the nearby venous sinus. The cortex and hippocampus underneath the window were removed by aspiration and the lateral parts of the diencephalon were exposed. The surgical area was kept clear by surgical gelfoam and occasionally by using aspiration. Three to six small lesions were made in the lateral diencephalon with a syringe needle. The lesions were along a line perpendicular to the course of the optic tract and a small crystal of neuronal tracer was inserted into

each lesion site to label the lateral thalamus including the dorsal lateral geniculate nucleus and the optic tract on one side of the brain. The neuronal tracer was prepared by soaking a small piece of gelfoam in a solution of 2% wheat-germ agglutinin conjugated to horseradish peroxidase (WGA-HRP, Sigma) and 30% horseradish peroxidase (HRP, Sigma type VI) in 2% dimethylsulphoxide and air dried before the surgery (Chan et al, 1989). After making the tract injection, the lesion cavity was filled with gelfoam and the skin was sutured. The animals were kept in a warm and humid environment for recovery and were perfused on the following day.

Removal of one eye in newborn rats

Several litters of neonatal rats had one eye removed at birth. The rat pups were anaesthetized with ether. The eyelid was cut open and the eye was carefully dissected away. The orbit was then filled up with gelfoam and the eyelid was sealed by a surgical dressing (Nobecutane aerosol, Astra). After recovering in a warm environment, the animals were returned to their mother and reared until the second operation.

Optic tract injection in adult rats

Three groups of animals, both pigmented and albino, received the second operation as adults (at least 2 months old): a) normal controls; b) rats with one eye removed prentally; c) rats with one eye removed on the day of birth. In this operation, neuronal tracer (WGA-HRP and HRP) was introduced into the right optic tract in normal controls and on the same side as the intact eye in animals with the

monocular enucleation.

The animals were anaesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg body weight). The heads were then fixed in a stereotaxic headholder. Again the scalp on one side was retracted and a hole was made with a dental drill in the occipito-temporal region of the skull about 5 mm rostral to the lambdoid suture. The meningeal membranes were removed and the cerebral hemisphere and hippocampus overlying the lateral thalamus were removed by suction. The exposed optic tract on the lateral surface of the thalamus was transected completely with a syringe needle. Several small pieces of dried gelfoam with neuronal tracer (see above) were introduced into the transected site in order to label all the fibres in the optic tract. The lesion cavity was filled with clean gelfoam and the scalp was sutured. The animals were kept in a warm environment and allowed to recover. They were allowed to survive for two days before they were perfused.

Preparations of retinal wholemounts and brain sections

All the animals that received a tract injection were anaesthetized, in the neonates by ether and in the adult by an intraperitoneal injection of pentobarbital. They were perfused transcardially first with phosphate buffered saline, then with 1% paraformaldehyde in 0.1M phosphate buffer at pH 7.4. A puncture was made with a syringe needle at the dorsal pole of the limbus to mark the orientation of the eye. The eyes were dissected out from the orbit and transfered to the same fixative. The cornea and the lens were removed. An incision was made from the puncture mark upto the optic disk to mark the orientation of the retina. The retina was then separated from

the sclera and choroid, and the pigment epithelium was removed if possible. Two additional cuts were made to allow the retina to be flat-mounted onto a clean slide. The retina was further fixed in several drops of 2% glutaraldehyde and was kept flattened by the weight of a coverslip. It was kept in the fixative at 4°C for 1 hour and then transfered to phosphate buffer.

The animals were further perfused by 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1M phosphate buffer. The brains were then removed and fixed in the same fixative for about 5 hours before they were transfered to 30% sucrose in 0.1M phosphate buffer and stored overnight at 4°C. In the enucleated rats, the lesioned orbits were checked to see whether there was any eye remnant indicating an imcomplete removal. In all animals studied, the eye on the lesioned side had been completely removed. This was further evidenced by the absence of optic nerve on the lesioned side of the adult brain. All the brains were cut on a sledge microtome and frozen sections of a thickness of 25 μm were collected in 1:4 series in phosphate buffer.

The labels in the retinas and brain sections were revealed by reacting the tissues with the Hanker-Yates histochemical reaction (Hanker et al., 1977). The tissues were incubated in an aqueous solution of 1% cobalt chloride and 1% ammonium nickel sulphate in the proportion of 3:2 for 15 minutes. After several washes with distilled water and then with phosphate buffer, the tissues were reacted in the freshly prepared Hanker-Yates solution (0.05% p-phenylenediamine and 0.1% catechol in 100 ml 0.1M sodium cacodylate buffer at pH 5.2) with the addition of two drops of 30% hydrogen peroxide for 15 to 20 minutes. The reaction was stopped by several changes of

phosphate buffer. The retinas and brain sections were then mounted on gelatinized slides. The retina was coverslipped and a weight was put on top of it to keep it flat. This was then left in 10% formal alcohol overnight and then dehydated, cleared and mounted in DPX. The brain sections were dried in air and also mounted in DPX.

Cell counts and measurement of density

The number of labelled ipsilaterally projecting ganglion cells was counted in each retina. The retinas of neonatal animals were examined with a Nikon Optiphot microscope under an oil immersion lens at a magnification of 812.5x. The cell number was obtained from a grid of 150 x 150 μm^2 which was moved systematically across the whole surface of the retina, and the number in each grid was recorded on a corresponding area in a proportional plot of this retina. A similar analysis was done on the adult retinas, but in these cases the cell number was counted under a magnification of 312.5x using a grid of 300 x 300 μm^2 in area. The central border of the temporal crescent was estimated by simple observation on the retinal plots. The numbers of labelled cells in the temporal crescent and in the regions outside, or central retina, were calculated separately.

The change in density of labelled ipsilaterally projecting cells across the temporal half of the neonatal retina was examined. It was prepared by drawing a straight line on a retinal plot which extended from the optic disk to the temporal margin of the retina perpendicular to the central border of the temporal crescent. The cell density along this line was computed. Any error arising from the different extent of retinal shrinkage was corrected by expressing distances from the optic disk as a proportion

of the retinal diameter.

DiI labelling and photo-oxidation

The developing axons from the ventral temporal retina of three E16.0 pigmented rat embryos were labelled and their positions within the optic pathway were determined. The embryos were fixed whole with 4% paraformaldehyde. The heads were blocked and the skin overlying the eye was removed. The cornea and lens were dissected away. A small granule of DiI (DiIC18(3): 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate, Molecular Probes Inc.; Honig and Hume, 1986; Godement et al., 1987b) was inserted into the far periphery of the ventral temporal retina. The globe was then filled with gelfoam.

The tissues were stored in 2% buffered formalin for 5 weeks at room temperature. They were then embedded in a solution of gelatin and albumen which was hardened with the addition of 10% glutaraldehyde by volume (from 25% stock solution). Excess embedding medium was trimmed away. The blocks were cut horizontally on a vibratome and 200 µm serial sections were collected in 0.1 M phosphate buffer. The sections were then mounted on clean glass slides and the labels in the retina and the optic pathway were checked using a Leitz fluorescent microscope under ultraviolet illumination with a rhodamine filter set. After photographing, the sections that contained the optic chiasm and the injected region within the eye were transferred and incubated in 0.05% diaminobenzidine (Sigma) in Tris buffer at pH 7.8 for ten minutes. The sections were then exposed under the appropriate excitation wavelength in the presence of the incubating solution for about 1 hour. This process converts the

fluorescence in the labelled structures into a permanent dark brown reaction product. The photo-oxidized tissues were then mounted on gelatinized sildes, coverslipped with a weight on top and placed in formal alcohol. They were then dehydrated, cleared and mounted in DPX on the following day.

The course of labelled axons from the ventral temporal retina at the optic chiasm of one photo-oxidized section was reconstructed using a Leitz microscope with a drawing tube. The axons were traced under the magnification of 200x.

Statistical analysis

The date obtained in each series of experimental animals were analyzed using one-tailed non-parametric Mann-Whitney tests.

Results

Evaluation of the tract label

The spread of label in the diencephalon was checked in the brain sections of all experimental animals. Fig. 1a shows the brain of a pigmented rat neonate. In this animal, the neuronal tracer was largely restricted to the injected side of the diencephalon and the surrounding cortical tissues. Most parts of the thalamus including the lateral geniculate nuclei and the optic tract were labelled. Spread of label across the midline of the brain was often observed in neonatal brains, but as shown in Fig. 1a, the optic tract and the major primary visual targets were always clear of label. No retrogradely labelled neurons in the cortex overlying the intact side of the brain were seen in these preparations - another criterion indicating that the visual structures opposite to the injected side are free from label.

The spread of neuronal tracer in the adult brain was much more retricted. The micrograph shown in Fig. 1b was taken from an adult albino rat with one eye removed at birth, in which only the lateral parts of the thalamus around the injected site were seen to be labelled. These included the regions of the medial and lateral geniculate nuclei, the pretectal nuclear complex and the optic tract. Sometimes the label could be traced up to the superior colliculus. No midline spread has ever been seen in the adult preparations in this study.

Besides checking the injection sites in the brain, the labelling pattern in the retina was also used to assess whether the tract injection was complete. In control animals, both adult and neonate, the retina contralateral to the injected side of the brain had to be evenly labelled by the neuronal tracer. While in the ipsilateral retina of all

animals, the cells had to be distinctly labelled. Only those cases that satisfied above conditions were selected for the cell counts and for further analysis in this study.

Morphology of the labelled ipsilaterally projecting retinal ganglion cells

Fig. 2a shows the labelled ganglion cells in the retina of a neonatal pigmented control ipsilateral to the injected optic tract. It was taken from the temporal crescent, an area in the ventro-temporal retina which is densely populated by the ipsilaterally projecting cells. All the labelled cells have their cytoplasm filled with dark brown reaction product. Most of them are heavily labelled, with a distinct dark and homogenous cell body, while some have fewer reaction products in their cytoplasm and so appear very granular. Nevertheless, the boundaries of all labelled cells are readily identified. The cell bodies of these cells are mostly spindle-shaped, some of them have a round or oval shape and they appear to be relatively uniform in size. It is often seen that one to three processes extend out from the cell body. It is an important criterion for distinguishing the labelled retinal ganglion cells from those cells within the retina, such as cells in the retinal blood vessels, that also possess a similar reaction product.

The label of ipsilaterally projecting retinal ganglion cells in adult is shown in Fig. 2b. This micrograph was taken from the temporal crescent of a pigmented rat with one eye removed prenatally at E16.5. It can be seen that the cytoplasm of the labelled cells is filled distinctly with the dark reaction product and also the primary dendrites and sometimes the axons are filled up as well. Cells with different soma sizes are apparent in the adult rat retina (Perry, 1979).

The distribution and number of labelled ipsilaterally projecting cells in rat neonates

In all animals examined, both normal controls and early enucleates, labelled ipsilaterally projecting ganglion cells are found throughout the retina. Most of these ipsilateral cells concentrate in the temporal crescent (Fig. 3) but a number of them are distributed evenly in the retinal areas outside the temporal crescent, and these are refered to as in the "central retina" in the following description.

The number of labelled ipsilaterally projecting cells in each group of animals is listed in Table 1 and the same results were depicted in Fig. 4. It is found that the numbers of ipsilateral cells in pigmented controls are significantly larger than the numbers in albino controls, both in the temporal crescent ($U=0$, $p<0.001$) and in the central retina ($U=0$, $p<0.001$) (Fig. 4). The numbers of cells in albinos are reduced roughly 50% when compared to the pigmented animals. These results show clearly that the abnormally small uncrossed component in albino mutants can be seen obviously in the rat on the day of birth. The albino mutation affects ganglion cells in both temporal crescent and central retina.

The effect of early monocular enucleation on the development of the uncrossed pathway is different in pigmented and albino animals. In pigmented rats with one eye removed at E16.5, the number of ipsilateral cells in the temporal crescent is significantly reduced when compared with the pigmented controls ($U=0$, $p<0.001$) (Fig. 4). However, the ipsilateral component arising from the central retina shows no significant difference ($U=4$, $p>0.05$). An overall reduction in the total number of

Table 1. Number of labelled ipsilaterally projecting retinal ganglion cells in neonatal rats. TC: temporal crescent; CR: central retina; SEM: standard error of the means.

Animals		Number of ipsilateral cells TC	CR	Total
Pigmented controls	D241	7421	2276	9697
	D242	7512	2787	10299
	D243	8802	2818	12193
	Mean=	7911.7	2818.0	10729.7
	SEM=	445.9	322.2	752.0
E16.5 pigmented enucleates	D235b	5588	2964	8552
	D273a	4048	2233	6281
	D273b	3828	3557	7385
	Mean=	4488.0	2918.0	7406.0
	SEM=	553.7	382.9	655.7
Albino controls	D311	3748	1210	4958
	D312	4684	1433	6117
	D313	3214	1159	4373
	Mean=	3882.0	1267.3	5149.3
	SEM=	429.6	84.1	512.5
E16.5 albino enucleates	D303a	2570	2514	5084
	D303b	2704	2979	5683
	D305a	1743	1596	3339
	Mean=	2339.0	2363.0	4702.0
	SEM=	300.5	406.3	703.1

ipsilateral cells can be seen in the pigmented rats with early enucleation ($U=0$, $p<0.001$).

The changes in the uncrossed component of albino rats after early enucleation are more complicated. When compared with the albino controls, there is a significant decrease in number of cells in the temporal crescent of early enucleates ($U=0$, $p<0.001$), but the cell number in the central retina is increased ($U=0$, $p<0.001$). The reverse changes in the temporal crescent and central retina in the early enucleates result in an uncrossed component which has similar total number of ipsilateral cells as the albino controls ($U=4$, $p>0.05$), but it is clear that the uncrossed pathway in albinos is actually altered by the early monocular enucleation. Another point that is worth noting is that the number of cells in the central retina of albino enucleates is not significantly different from that in the pigmented controls ($U=3$, $p>0.05$) and pigmented enucleates ($U=3$, $p>0:05$).

The question of whether the early enucleation in pigmented rats can produce a reduced uncrossed component that is quantitatively similar to the albino uncrossed component can be answered by comparing the number of ipsilateral cells in the early pigmented enucleates and albino controls. It is clear that the number of ipsilateral cells in the temporal crescent of early pigmented enucleates is similar to that in albino controls ($U=2$, $p>0.05$) (Fig. 4). However, there are more ipsilateral cells in the central retina of pigmented enucleates than in albinos ($U=0$, $p<0.001$), and the total number of ipsilateral cells is obviously larger in pigmented enucleates than in albinos ($U=0$, $p<0.001$). Therefore, the early monocular enucleation at the age of E16.5 in pigmented rats does not produce an uncrossed pathway that is comparable to the

abnormal uncrossed component seen in albino rats, at least in terms of distribution of the ipsilaterally projecting cells.

The decussation line of the uncrossed retinal component in rat neonates

The changes in density of labelled ipsilaterally projecting ganglion cells across the temporal retina were measured in all rat neonates, including the controls and early enucleates of both pigmented and albino strains. The central border of the temporal crescent, which is defined as the decussation line of the uncrossed component in this study, is however difficult to define. As shown in Fig. 3, the increase in the density of labelled ipsilateral cells across the temporal crescent in the neonatal rat retina is gradual, especially in the pigmented animals, and that sometimes makes it difficult to mark the position of the decussation line. This can also be shown in the density plot of four animals in Fig. 5. In order to define the position of the decussation line more precisely, a baseline is drawn at the density of 1000 cells per mm^2 across the plots, a value slightly larger than the maximum density observed in the central retina, and the decussation line is thus defined as the point in a density curve at which the density of ipsilateral cells begins to increase to the value of 1000 cells/mm^2. Using this criterion, the position of the decussation line in all animals can be determined directly from the plots and the results are shown in Fig. 6.

The plots in Fig. 5 show that moving from the optic disk towards the temporal edge the cell density in all groups of animals is initially low, below 1000 cells/mm^2, and this low density is maintained with only slight variation for some distance into the temporal retina upto a point, which marks the decussation line, where the density of

ipsilateral cells begins to increase abruptly. As shown in Fig. 6, the decussation line of the pigmented controls is at an eccentricity of 62.5%, whereas in the albino controls the decussation line is shifted to an eccentricity of 79.5%, which is significantly larger than the value in pigmented animals ($U=0$, $p<0.001$). Moreover, it is also shown clearly in Fig. 6 that removing one eye prenatally at E16.5 does not change the position of decussation line in either the pigmented (mean= 62.9% eccentricity) or the albino (mean= 78.5% eccentricity) enucleates. The decussation line in the pigmented enucleates is at a more central position than that in albino controls ($U=0$, $p<0.001$).

One more point that is worth mentioning is that the initial phase of increase in cell density in the pigmented enucleates follows closely to that in the pigmented controls (Fig. 5). However, at about 82% eccentricity the density of cells in the pigmented enucleate begins to level off and drop in the far peripheral parts of the temporal crescent whilst the density of cells in the pigmented control continues to increase upto the temporal retinal margin. The peak density of cells in the pigmented controls (mean= 10533.3 cells/mm^2) is significantly higher than that in the pigmented enucleates (mean= 6830.0 cells/mm^2) ($U=0$, $p<0.001$). The density curve of the albino enucleate also follows closely to the curve of albino control (Fig. 5). The peak density in the albino enucleate (mean= 4726.3 cells/mm^2) is significantly lower than that in the albino control (mean= 7111.0 cells/mm^2) ($U=0$, $p<0.001$).

As a conclusion, the results suggest that first there is already a shift in the decussation line of the uncrossed component towards the temporal margin of the retina in albino rat neonates. Second, the early monocular enucleation done at E16.5 appears not to affect the decussation line in the retina of either pigmented or albino neonates.

Third, the removal of one eye prenatally does not produce an uncrossed pathway that has a decussation line comparable to the one in the albino.

The position of ventrotemporal fibres in the optic pathway of E16.0 rats

The fluorescent micrograph in Fig. 7a illustrates the filled optic fibres in the proximal stalk and the chiasm. It was taken from a pigmented rat embryo at E16.0 after a precise focal labelling with DiI at only the far periphery of the ventrotemporal retina, which later will give rise to the temporal crescent, without significant label of cells in the central retina. After photo-oxidizing this section, a camera-lucida drawing was made to show the course of filled ventrotemporal fibres at the chiasm (Fig. 7b). At this age most ventrotemporal fibres are found in the proximal stalk, but a few have just passed the midline and some of them have entered the ipsilateral tract. Most of these filled fibres, especially those in the ipsilateral tract and those past the midline, are led by a growth cone-like structure at their tips indicating that they have just arrived at or crossed the midline of the chiasm. It should be noted that as shown in Fig. 7b the ventrotemporal fibres are originally grouped into fascicles in the caudal part of the proximal stalk, but the fibres begin to fan out at the prechiasmatic region and occupy the whole rostrocaudal extent of the chiasm (see also results in chapter 2).

The number of labelled ipsilaterally projecting retinal ganglion cells in adult rats

The number of ipsilaterally projecting cells in adult control rats and rats with either one eye removed at birth or at E16.5 was deteremined in order to investigate the effects of postnatal cell death on the development of the uncrossed pathway. It

is demonstrated in Fig. 7 that most of the ipsilaterally projecting retinal ganglion, both in controls and monocularly enucleated adult rats, are found in the temporal crescent. The numbers of ipsilateral cells in each group of animals are listed in Table 2 and shown in the graph of Fig. 9.

It is evident that as in the neonates, the number of ipsilateral cells is reduced in adult albino controls when compared with the pigmented controls, both in the temporal crescent ($U=0$, $p<0.001$) and in the central retina ($U=0$, $p<0.01$). Removal of one eye at birth produces an enlarged adult uncrossed pathway, in both pigmented and albino rats, which has significantly higher than normal numbers of ipsilateral cells in both temporal crescent and central retina ($U=0$, $p<0.001$ for all comparable pairs in the temporal crescent and central retina within the same strain of rat). However, removing one eye at E16.5, when the fibres have just passed the optic chiasm but not yet entered their targets (Bunt et al., 1983; personal observation), does not produce a reduced uncrossed component in adults, which is in contrast to the observations made in the neonatal animals. It has been found that in E16.5 pigmented enucleates the number of ipsilateral cells in the temporal crescent is not significantly different from the number in the controls ($U=4$, $p>0.05$), whilst the number of ipsilateral cells in the central retina of early enucleates is significantly increased ($U=0$, $p<0.001$). As a result, the total number of ipsilateral cells in early enucleates is not significantly different from that in the pigmented controls ($U=2$, $p>0.05$). Similar comparisons can be made for the albino animals. In E16.5 albino enucleates, when compared with albino controls, the number of ipsilateral cells in the temporal crescent though is slightly larger but not significant ($U=1$, $p>0.05$), whereas the cell number in the

Table 2. The number of labelled ipsilaterally projecting retinal ganglion cells in adult rats. TC: temporal crescent; CR: central retina; SEM: standard error of the mean.

Animals		Number of ipsilateral cells TC	CR	Total
Pigmented controls	C476	2042	245	2287
	C483	2443	222	2665
	D71	2408	355	2763
	D352	2089	203	2292
		Mean= 2245.5	256.3	2501.8
		SEM= 104.6	34.0	124.2
Neonatal pigmented enucleates	C217	2744	825	3589
	C220	2775	1037	3812
	C222	3379	862	4241
		Mean= 3077.0	908.0	3874.0
		SEM= 246.6	65.4	196.5
E16.5 pigmented enucleates	C485	1847	597	2444
	D03a	2377	1077	3454
	D35a	2364	1205	3569
		Mean= 2196.0	959.7	3155.7
		SEM= 174.5	185.1	357.4
Albino controls	D65	929	186	1115
	D62	1028	184	1212
	D351	1191	166	1357
		Mean= 1049.3	178.7	1228.0
		SEM= 76.4	6.4	70.3
Neonatal albino enucleates	D09	1498	530	2028
	D10	1916	631	2547
	D197	1960	877	2837
	D198	2188	850	3038
		Mean= 1890.5	722.0	2612.5
		SEM= 143.8	84.4	219.4
E16.5 albino enucleates	D01	1382	526	1908
	D02	1143	442	1585
	D15	1502	440	1942
	D16	1749	707	2456
		Mean= 1444.0	528.8	1972.8
		SEM= 125.6	62.7	180.1

central retina is increased (U=0, $p<0.001$), resulting in the formation of a larger than normal uncrossed component (U=0, $p<0.001$).

The extent of cell loss in the uncrossed pathway of different groups of rats, both controls and enucleates, was calculated in terms of the numbers and percentages of ipsilateral cells lost during postnatal development. In Table 3, it is shown that in both pigmented and albino controls, about 70% of ipsilateral cells in the temporal crescent and 90% of ipsilateral cells in the central retina are lost postnatally. It is evident that the numbers and percentages of cells lost is reduced in animals with one eye removed at birth, and that this results in a production of relative enlargement of the uncrossed pathway during postnatal development. A similar reduction in cell loss is also observed in rats with one eye removed at E16.5, which acts to produce an enlarged (in albino) or close to normal (in pigmented) uncrossed pathway, in terms of number of ipsilateral cells. It should be noted that there is a greater loss in the number of cells in the central retina of E16.5 albino enucleates than in the albino controls. This is due to the presence of a greater population of ipsilateral cells in the central retina of the albino enucleate at birth (see Table 1), the percentage of cell loss in this group of cells in the early albino enucleate is still lower than that in the albino control.

The results in adult rats indicate that the uncrossed pathway in early enucleates can be further modified by cell death during postnatal development, which in turn adds further complexity to the results. The fact that postnatal cell death also occurs in the uncrossed component of early enucleates raises an interesting question, that is to what extent the cell death is dependent on the binocular fibre interaction in the targets, as cell death can be found in an optic pathway which has apparently no

interaction with fibres from the other eye in the targets throughout its development, as demonstrated in the uncrossed pathway of early enucleates in this study.

Table 3. The numbers and percentages of cells lost in the uncrossed pathway of rats during postnatal development.

Animals	Number and (percentage) of cell loss TC	CR	Total
Pigmented			
Controls	5666.2 (71.6)	2561.7 (90.9)	8227.9 (76.7)
Neonatal enucleates	4834.7 (61.1)	2010.0 (67.8)	6855.7 (63.9)
E16.5 enucleates	2292.0 (51.1)	1958.3 (67.1)	4250.3 (57.4)
Albino			
Controls	2832.7 (73.0)	1088.6 (85.9)	3921.3 (76.2)
Neonatal enucleates	1991.5 (51.3)	545.3 (43.0)	2536.8 (49.3)
E16.5 enucleates	895.0 (38.3)	1834.2 (77.6)	2729.2 (58.0)

Note: The number of cells lost is the difference in number of ipsilateral cells between neonates and adults, whilst the percentage of cell loss is calculated as the percentage of cells in neonates that disappears during the postnatal cell death period. The numbers in the adult controls and neonatal enucleates were compared with the number in control animals at birth, whereas the adult numbers of E16.5 enucleates were compared directly with the number obtained in the newborn E16.5 enucleates. TC: temporal crescent; CR: central retina.

Discussion

The results of the present study can be summarised as follows: first, it has confirmed for the newborn rat the previous findings for adult mouse and ferret that in pigmented animals the uncrossed retinal pathway of one eye can be reduced by removing the other eye during prenatal development (Godement et al., 1987a; Guillery, 1989). Second, it has demonstrated that the uncrossed pathway in albino rats can also be reduced by prenatal monocular enucleation. Third, although a smaller than normal uncrossed component can be produced in the pigmented and albino rats with early enucleation, the decussation line of the uncrossed pathway in these animals is not affected, each colour phase keeps its characteristic line of decussation. And fourth, it has been shown that in the absence of all binocular interactions within the target nuclei, the uncrossed pathway is still subject to the postnatal cell death.

The development of the uncrossed retinal pathway requires a binocular fibre interaction at the chiasm

It has been shown in this study that if one eye of a pigmented rat embryo is removed when the first axons have passed the chiasm but not yet entered the targets, a reduced uncrossed projection will be produced in the intact eye at birth. Comparable results have been obtained in mice (Godement et al., 1987a) and ferrets (Guillery, 1989) in which the adult uncrossed retinal pathways of the early enucleates were examined. All these results indicate a point that at least some developing optic fibres need to interact with fibres of the other eye, either directly or indirectly, at the chiasm in order to make their decision to select an uncrossed path.

The nature of this chiasmatic binocular interaction is unknown. It may be a direct interaction between fibres from the two eyes, or it may be mediated by the glial elements at the chiasm. Studies of the axonal trajectory in the mouse optic chiasm have shown that only the crossed fibres pass the midline of the chiasm and interact directly with the uncrossed fibres from the other eye (Godement et al., 1990; Sretavan, 1990a). There is no direct contact between the uncrossed fibres of the two eyes as the uncrossed fibres always turn back from their original course before they reach the midline. So if the chiasmatic binocular interaction is a direct fibre interaction, it must occur between the uncrossed fibres from one eye and the crossed fibres from the other.

It should be noted that in E16.5 rat embryos, only cells in central regions of the retina can be labelled on either the contralateral or ipsilateral side after an implant of DiI into one optic tract (chapter 3). That is, the axons that contribute to the crossed pathway in E16.5 rats are largely from the central retina. It then becomes very interesting to ask whether the crossed fibres from the central retina of one eye may play any role in guiding the uncrossed fibres of the other eye into the ipsilateral tract.

Alternatively, the binocular fibre interaction that guides growing axons into the ipsilateral tract may be mediated through the glial elements at the chiasm (Godement et al., 1990; Mason et al., 1990). There may be a certain signal on the glia at the chiasm that instructs the uncrossed axons to turn while it allows the crossed axons to continue their course to the contralateral tract. The expression of this signal on the glia must be dependent on the presence of fibres from one eye, either crossed or uncrossed.

The extent to which the development of the uncrossed pathway in rats is dependent on the chiasmatic binocular interaction

It has been shown in this study that not all cells in the uncrossed pathway are affected by a prenatal eye removal. Indeed, about 50% of ipsilateral cells in the temporal crescent and almost the whole complement of central ipsilateral cells remain in the early enucleate.

It may be argued that the monocular enucleation in this study was not made early enough to eliminate the whole uncrossed pathway. This argument gains support from the observation that in E16.0 pigmented embryos, half a day before the enucleation was done, some fibres from the far periphery of the temporal retina have already arrived at the prechiasmatic stalk and the chiasm. The cells that survive in the early enucleate can thus be interpreted as those that have sent axons to the chiasm and already made their choice for an uncrossed route at or before the time of enucleation. A similar argument can be applied to the ipsilateral cells in the central retina. As shown in the present study, these central ipsilateral cells are apparently not affected by the early enucleation. One possible explanation is that they may have sent their axons to the chiasm and made their chiasmatic choice at or before the enucleation was made.

The extent to which the development of the uncrossed retinal pathway depends on a chiasmatic binocular interaction can be addressed by looking at the uncrossed pathway in rats with earlier eye removal, but it is technically difficult to make the enucleation earlier than at E16.5 in rats. Nevertheless, earlier prenatal monocular enucleations have been successfully done in mice in which one eye of the embryo was removed at E12 or E13, when the first fibres are still growing towards or just reach

the presumptive chiasmatic region (Godement et al., 1987a). It has been shown in that study that the adult uncrossed pathways in the early enucleates are reduced, but that there is always a population of ipsilateral cells in the central retina and temporal crescent that project to the ipsilateral targets. It is then concluded in that study that in the mouse there may be two populations of ipsilateral cells. The first population of cells are autonomous in their pathway choice, i.e. they have the ability to select an uncrossed route without any interaction with fibres of the other eye, and so they are not affected by the early enucleation. These cells are found in both temporal crescent and central retina. The other population is dependent on the binocular fibre interaction at the chiasm for guiding them into the ipsilateral tract, and so these cells disappear after the enucleation. However, whether there are two populations of ipsilateral cells in the rat, as suggested for the mouse, remains as a question.

The fate of the ipsilateral cells that are lost from the temporal crescent of a newborn rat when one eye is removed prenatally is not clear. It has been reported in mice that if one eye is removed at E13.5, and the fibres from the far periphery of ventrotemporal retina are examined at E16.5, one will find that most fibres from the presumptive temporal crescent, which should have entered the ipsilateral tract at this age, will instead be seen stalled at the chiasm (Godement et al., 1990). However, the question remains whether these presumptive temporal crescent fibres will stay at the chiasm without proceeding any further in the optic pathway, retract their axons or even die, or whether they will continue their course into the contralateral tract in later development. However, from the results of an early enucleation study in the cat, it has been demonstrated that the density of ganglion cell-like neurons is normal across

the nasotemporal axis of a E59 retina after one eye is removed at E23 (Sretavan and Shatz, 1986). This suggests that cells in the temporal crescent do not die after the early enucleation. There is however not enough information available yet for us to choose among the other alternatives.

The decussation line of the uncrossed component is not affected by the prenatal enucleation in pigmented rats

It has been shown in the present study that the uncrossed pathway in the albino rat is already reduced, as in the adult (Collelo and Jeffery, 1991), in terms of retinal ganglion cells that can be labelled at birth. Further, it has been demonstrated that the decussation line of the albino uncrossed component is shifted towards the temporal retinal margin of the neonatal retina, similar to the shift demonstrated in the adult albinos of other species (Guillery and Kaas, 1971; Stone et al., 1978; Cooper and Pettigrew, 1979b; Dräger and Olsen, 1980; Morgan et al., 1987).

How the albino mutation causes a production of a reduced uncrossed retinal pathway during development is still not clear. The fact that removing one eye prenatally in pigmented ferrets produced a reduced uncrossed retinogeniculate termination which is very similar to that seen in albinos led to the suggestion that the albino abnormality may develop as a consequence of weakening of the binocular fibre interaction at the chiasm (Guillery, 1989). In the present study, it has been shown that early monocular enucleation in the pigmented rat produces a reduced uncrossed component at birth. However, although the number of ipsilateral cells is reduced, the early enucleation made at E16.5 does not produce an uncrossed component in the

pigmented rat which is comparable to the abnormally small uncrossed component in the albino. This can be shown by the fact that there is no obvious shift of the decussation line in the reduced uncrossed component of pigmented enucleate, comparable to that found in the retina of albino controls. Moreover, although the number of ipsilateral cells within the temporal crescent of early pigmented enucleates is similar to that in the albinos, more ipsilateral cells can be found consistently in the central retina of pigmented enucleates. These results lead to a conclusion that the effect of early monocular enucleation on the development of the uncrossed pathway in rats is different from that of the albino mutation, although both of them act to produce a reduced uncrossed retinal component.

How the reduced uncrossed pathway in albino rats develops

There remains a question of how the albino gene may act on the retinal pathway and leads to the misguidance of fibres at the chiasm. One possible explanation is that, as argued above, there may be a retinal marker on the ipsilateral cells and that it is through this marker that the ipsilateral fibres can interpret the chiasmatic cue as a signal to turn and grow to the ipsilateral tract (see also chapter 3). This retinal marker should be expressed preferentially in cells within the temporal crescent. In the albino, the expression of this marker is somehow altered so that fewer cells, especially those along the central border of the temporal crescent, carry this marker. The uncrossed component of the albino is thus reduced and the decussation line is shifted towards the temporal retinal margin. Based on this hypothesis, removal of the chiasmatic cue in either pigmented and albino animals, as some fibres are passing through the chiasm

while most are still on their way, should then produce a reduced uncrossed projection but have no change in the position of the decussation line in either strain, as the fibres are marked already to go ipsilaterally in their retinas. This is exactly what has been found in this study.

Another alternative that may explain the development of albino abnormality is that the albino gene may act at the optic chiasm rather than in the retina either by altering the glial environment along the midline of the chiasm or by weakening the fibre interaction between the two eyes, so that fewer fibres are guided into the ipsilateral tract. However, if the albino gene does act at the chiasm alone, it may be difficult to conceive how the decussation line can be shifted so precisely and consistently in the albino animals and yet be unaffected by the enucleation. Nevertheless, the possibility that this gene may have action at the chiasmatic structures, i.e. glia or fibres, cannot be eliminated.

There is another interesting point that is worth noting. As shown in this study, there is an obvious reduction in the number of ipsilateral cells in the temporal crescent of newborn albino rat after early enucleation. This result indicates that most if not all of the growing uncrossed axons from the temporal crescent in the albino rats, as in the normals, also depend on the interaction with the crossed fibres from the other eye to select an uncrossed route. However, it is puzzling to see that the ipsilateral component in the central retina of albino neonates is enlarged after early eye removal, and it appears to be similar quantitatively to the central component in the pigmented neonate. These results are difficult to understand. One possible explanation is that there may be a preferential loss of uncrossed axons from the central retina of albinos

during later prenatal stages which shapes the reduced central component seen in the neonates. Elimination of one eye prenatally may prevent the loss of these fibres. However, there is no evidence to support this argument.

Cell death occurs in the uncrossed pathway of rats without any interaction with fibres from the other eye in the targets

It has been shown in this study that the number of ipsilateral cells in the rat with prenatal enucleation can be further reduced during the postnatal cell death period. This massive loss of cells occurs in an uncrossed pathway that develops with very little if any binocular interaction within the targets, as it has been reported in rats that at E16.5, the time that the early enucleation is made, the optic fibres have just reached the surface of the primary visual targets (Bunt et al., 1983; Chan, unpublished result).

It is well documented that the uncrossed retinal projection in the newborn rat is more extensive than that found in the adult, both in terms of the terminal fields in the primary visual nuclei (Land and Lund, 1979; Maxwell and Land, 1981; Bunt et al., 1983) and the retinal ganglion cells (Land et al., 1981; Jeffery, 1984; Chan et al., 1989). This exuberant neonatal pattern is then modified and refined to a more precise and restricted adult pattern by an extensive cell death during the first week of postnatal development (Land and Lund, 1979; Maxwell and Land; 1981; Jeffery, 1984; Chan et al., 1989). Some of these transient ipsilateral cells and axons from one eye can however be preserved by eliminating the opposite eye at birth (Land and Lund, 1979; Jeffery and Perry, 1982; Jeffery, 1984; Linden and Serfaty, 1985; Chan and Jen, 1988; Chan et al., 1989). These results together with studies in other species led to

the idea that postnatal cell death is probably caused by a competitive interaction of fibres from the two eyes in the target nuclei, possibly for some trophic cues that are essential for the fibres to survive (Land and Lund, 1979; Rakic, 1981; Jeffery and Perry, 1982; Rakic and Riley, 1983a and 1983b; Jeffery, 1984; Leventhal et al., 1988). Removal of one eye at birth eliminates the crossed fibres innervating the contralateral targets. It thus allows more uncrossed fibres to acquire these cues and survive the cell death.

However, the results in this study have demonstrated clearly that the postnatal cell death occurs in an uncrossed pathway that has very little if any competitive interaction with the crossed component in the targets. This phenomenon of loss of retinal ganglion cells and their axons is also observed in the optic projection of E59 cat fetuses which have one eye removed at E23 (Sretavan and Shatz, 1986). These results indicate that most of the cells lost during the cell death period may not be lost as a consequence of binocular competition in the targets, indeed it may be caused by an intrinsic property of the pathway itself. It has been suggested that the interaction between the dendrites of ganglion cells may play a role in regulating the cell death, as more ipsilateral cells can be rescued by removing the neigbouring cells that project to the contralateral target, either by a unilateral tectal ablation or by a unilateral optic tract lesion (Linden and Perry, 1982; Perry and Linden, 1982; Linden and Serfaty, 1985; Chan and Jen, 1988; Chan et al., 1989). Alternatively, the axons of the uncrossed pathway may compete among themselves for a limited amount of trophic cue in the target that can specifically sustain the uncrossed axons. As a conclusion, the cell death of the uncrossed retinal pathway is not only caused by a competitive

interaction of fibres from the two eyes in the target, it also depends on the interaction of cells and fibres within the pathway itself. The two different types of interaction may well be operated simultaneously to shape the final pattern of the mature adult uncrossed retinal projection.

Fig. 1 Coronal sections of the brains taken from a normal newborn pigmented rat (a), and an adult albino rat with one eye removed at birth (b). The black areas indicate the spread of a neuronal tracer (WGA-HRP and HRP) in the diencephalon. Note that the lateral parts of the thalamus, including the optic tract, lateral geniculate nuclei and the pretectal nuclei (in the adult brain) were heavily labelled. As shown in these micrographs, the label is largely limited to one side of the brain. Dorsal is up and the left side of the brain is on the right. Scale bar is 1 mm.

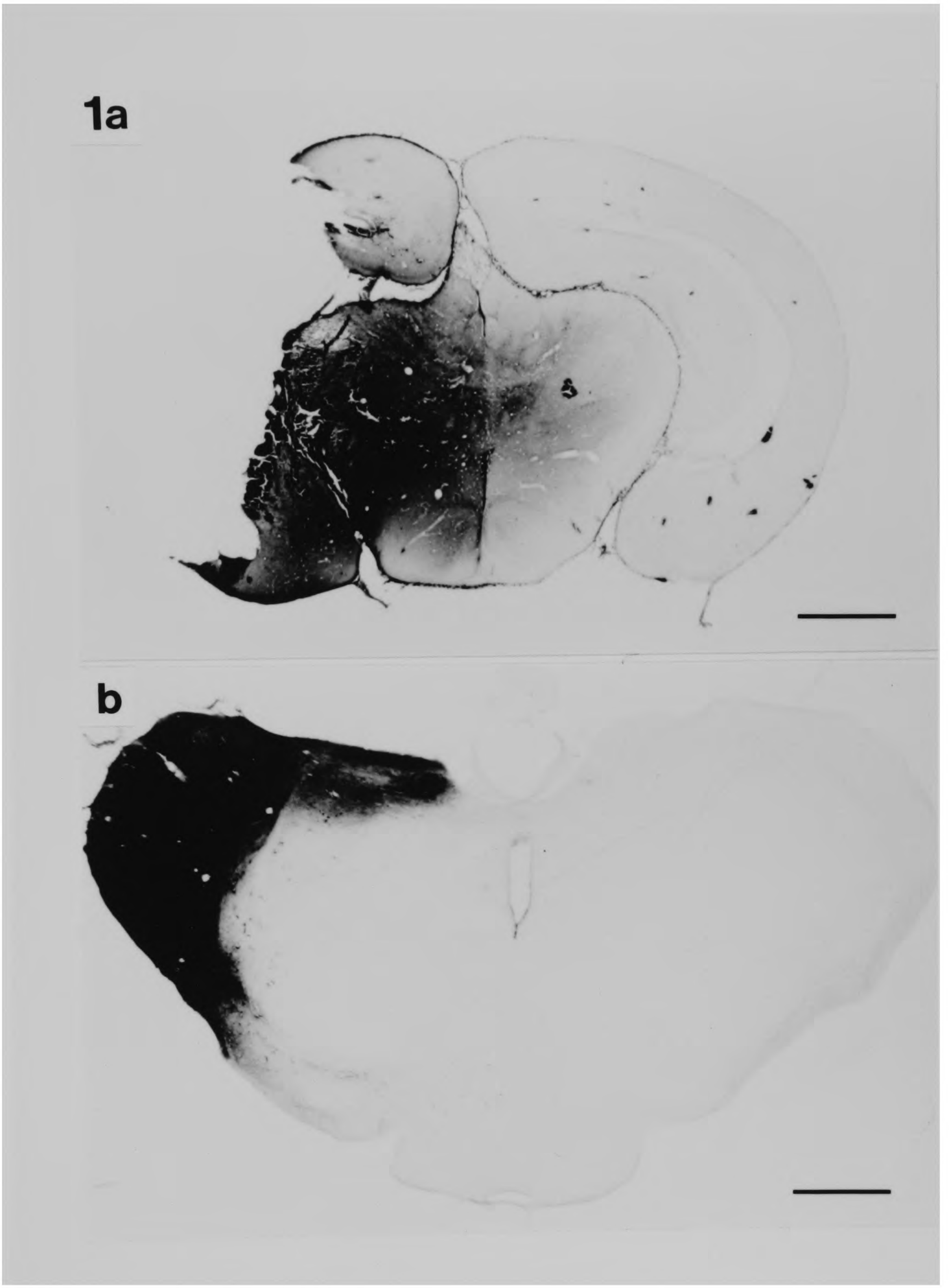
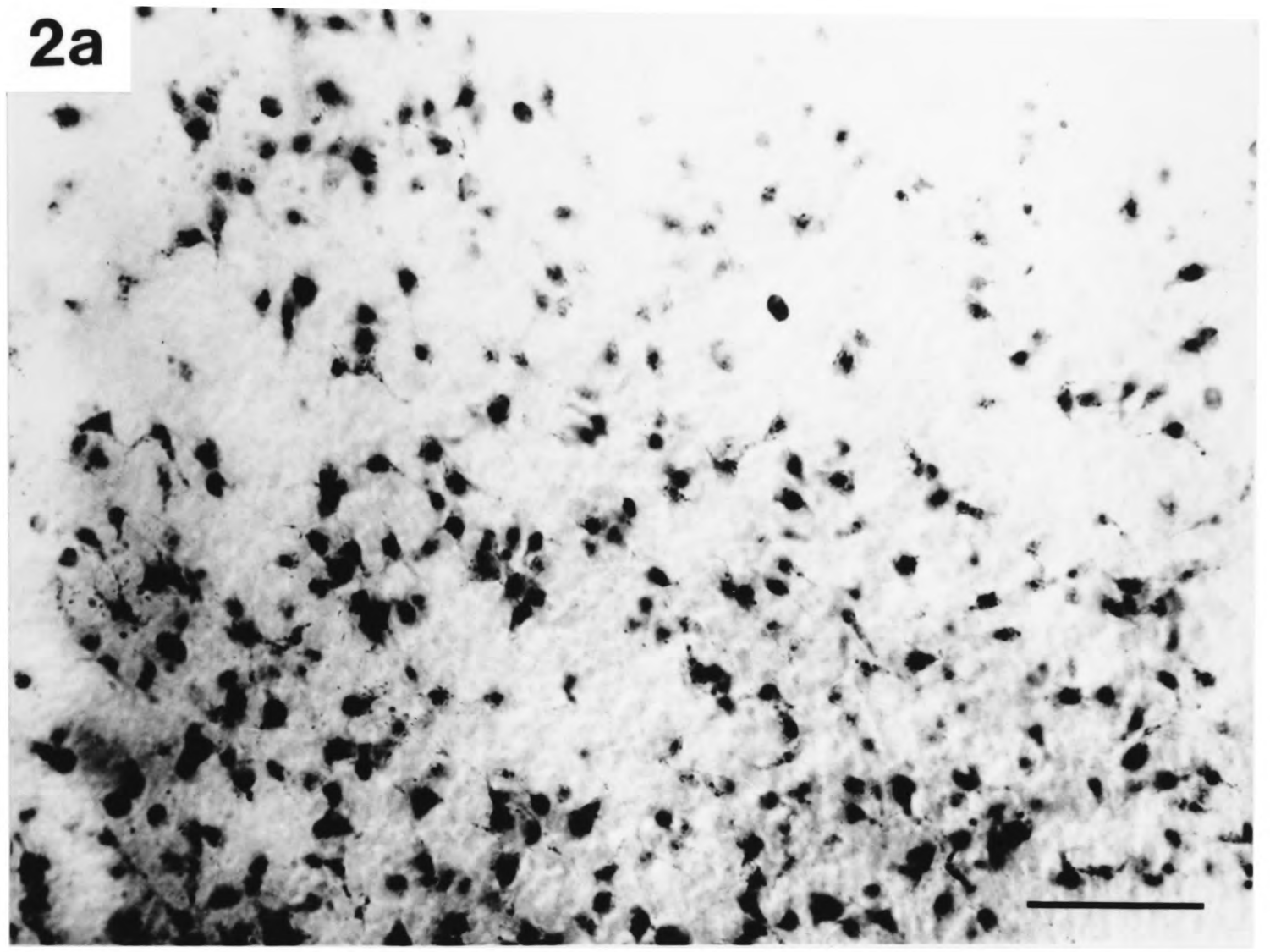

1a
b

Fig. 2 Light micrographs showing the labelled ipsilaterally projecting retinal ganglion cells in the temporal crescent of a normal newborn pigmented rat (a), and an adult pigmented rat with one eye removed at E16.5 (b) after a tract label. Optic disk is to the dorsal (not included in these micrographs). Scale bar is 50 µm.


2a

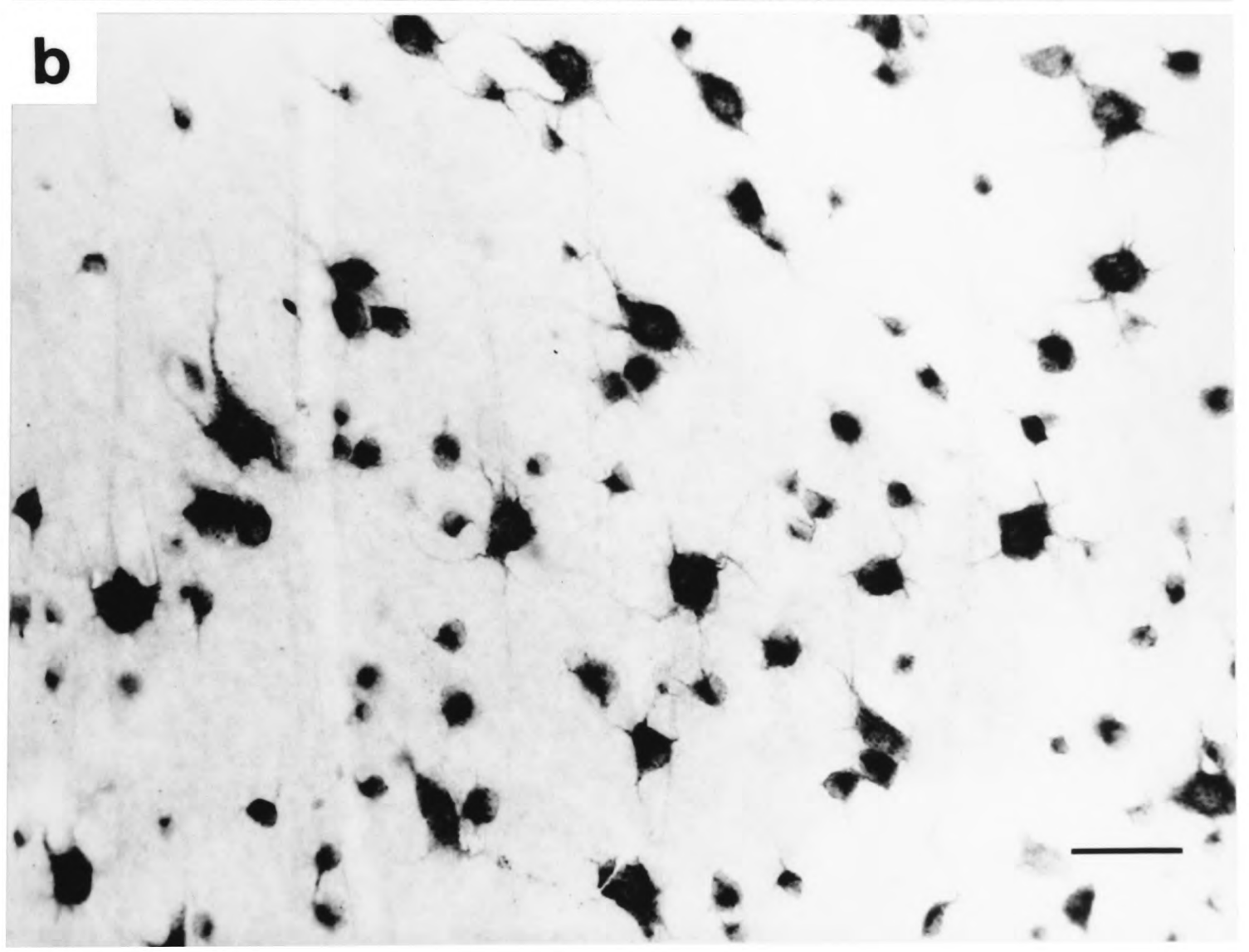

b

Fig. 3 Light micrographs taken from the ventrotemporal retina of four newborn rats showing the labelled ipsilaterally projecting ganglion cells in the temporal crescent. a is a pigmented control and b is a albino control; c is a pigmented neonate and d is a albino neonate, both of them have one eye removed at E16.5. The temporal retinal margins in these micrographs were aligned on the left. The optic disk is on the right (not included in these micrographs). Scale bar is 100 μm.

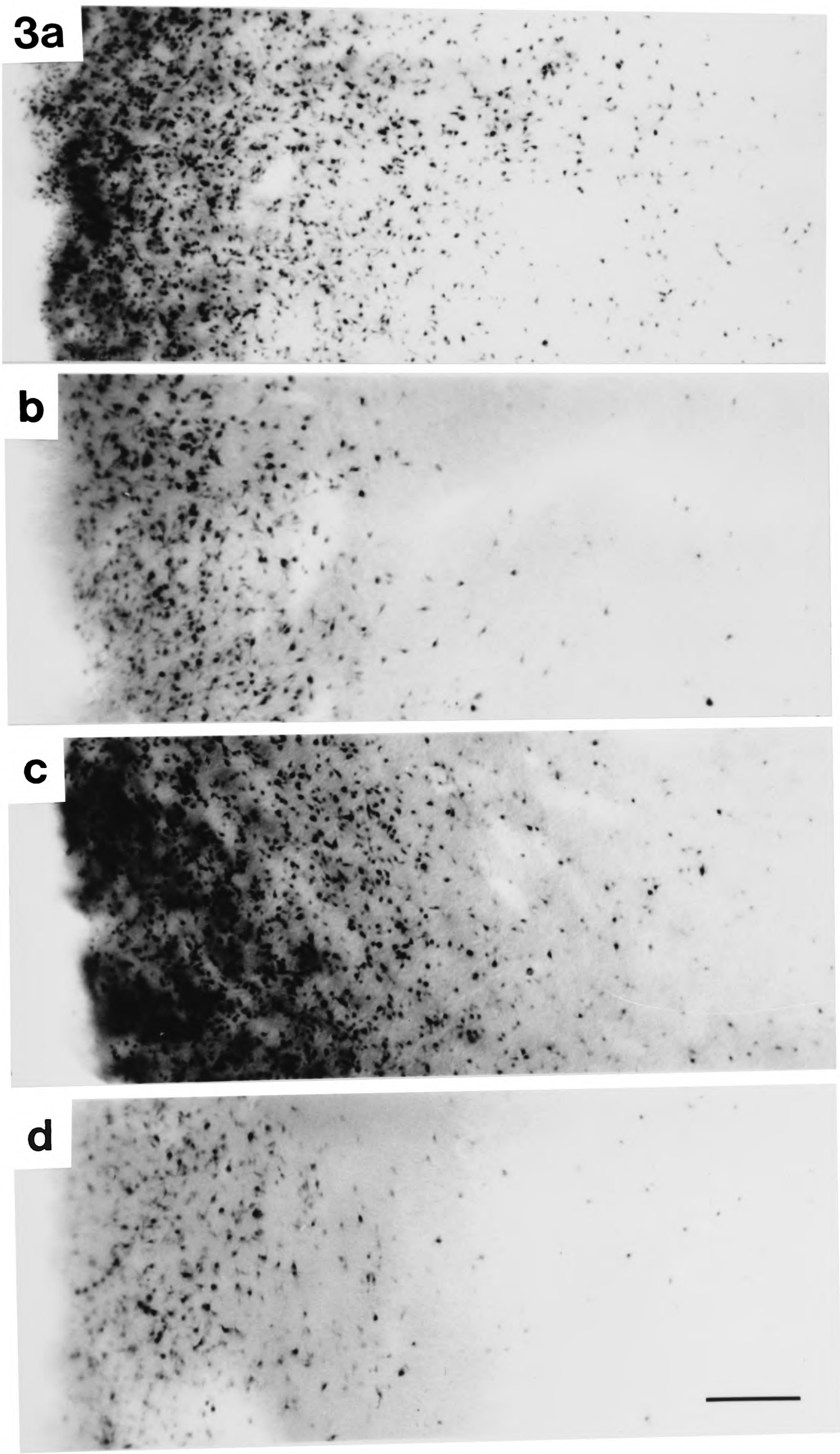

3a
b
c
d

Fig. 4 Graph showing the number of labelled ipsilaterally projecting retinal ganglion cells in the newborn rats, either controls or E16.5 enucleates, of the pigmented and albino strain.

Fig. 4

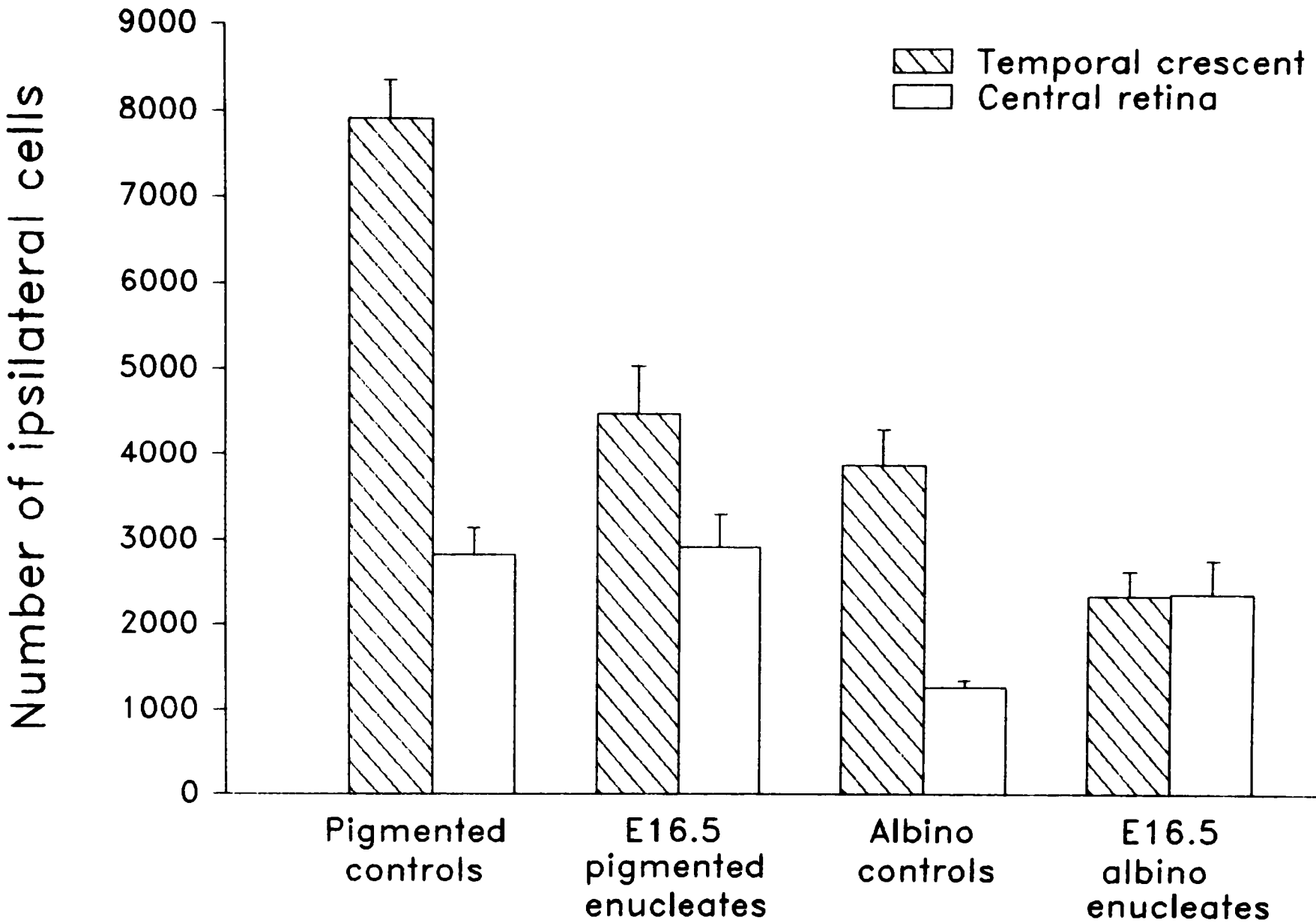

Number of ipsilateral cells
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Temporal crescent
Central retina
Pigmented controls
E16.5 pigmented enucleates
Albino controls
E16.5 albino enucleates

Fig. 5 Graph showing the changes in density of labelled ipsilaterally projecting ganglion cells from the optic disk (OD) to the retinal edge across the temporal retina of four newborn rats, one control and one E16.5 enucleate from either the pigmented or albino strain. A horizontal line was drawn at the density of 1000 cells/mm^2 in order to mark the decussation line of the uncrossed retinal component.

Fig. 6 Graph showing the positions of the decussation line in the retinas of control neonates and neonates with one eye removed at E16.5, in pigmented and albino rats. OD: optic disk.

Fig. 5

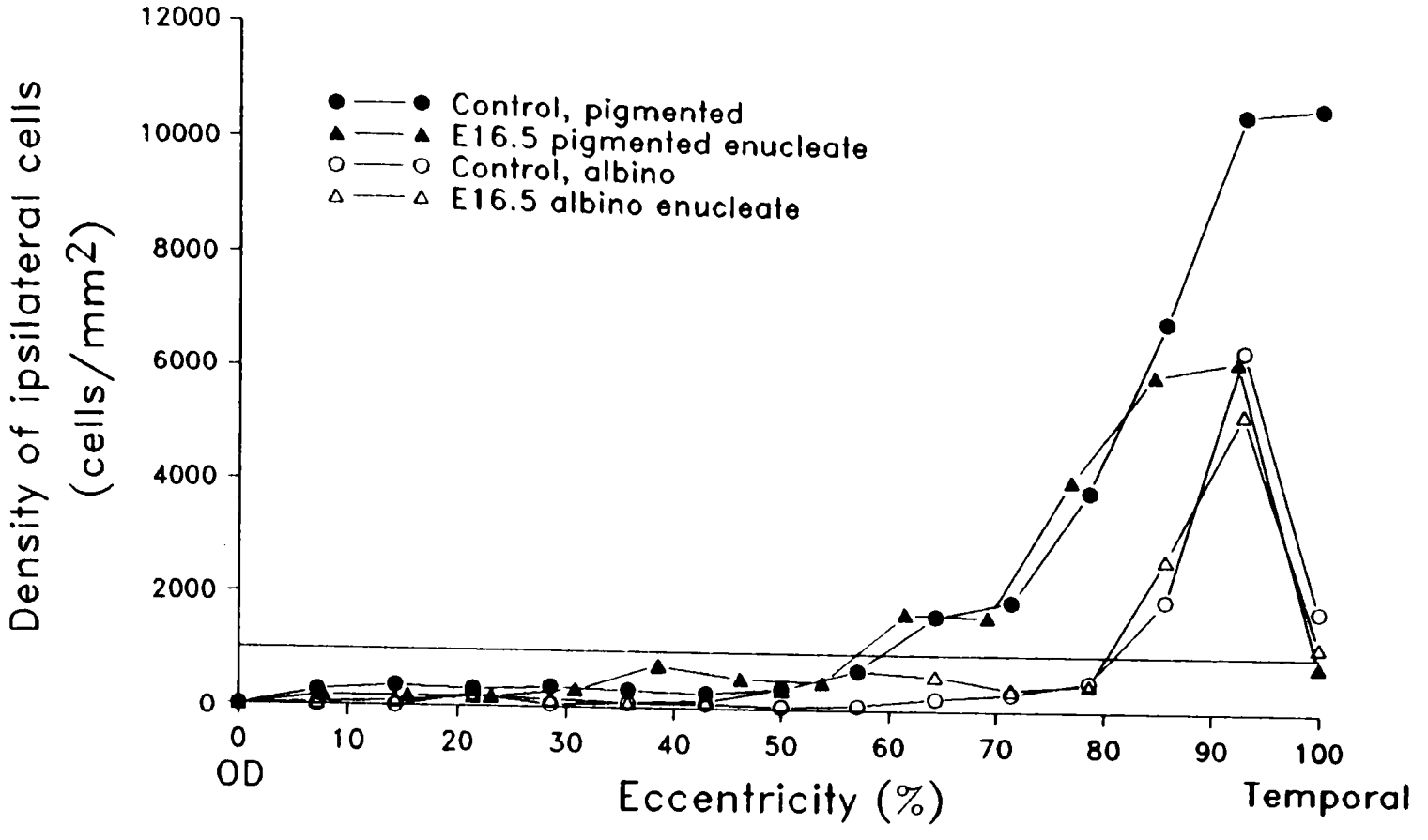

Density of ipsilateral cells (cells/mm2)
12000
10000
8000
6000
4000
2000
0
Control, pigmented
E16.5 pigmented enucleate
Control, albino
E16.5 albino enucleate
0
OD
10
20
30
40
50
60
70
80
90
100
Temporal
Eccentricity (%)

Fig. 6

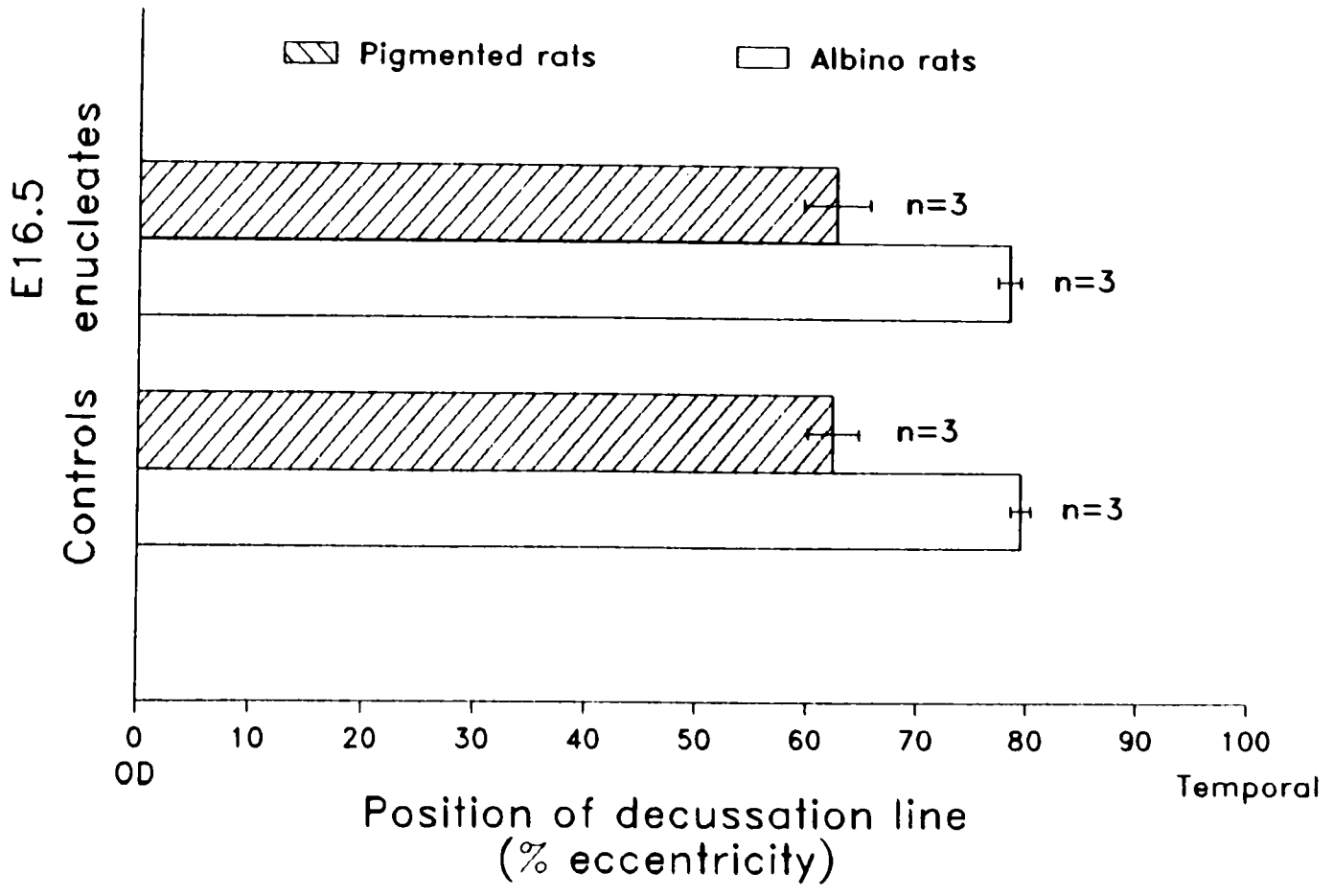
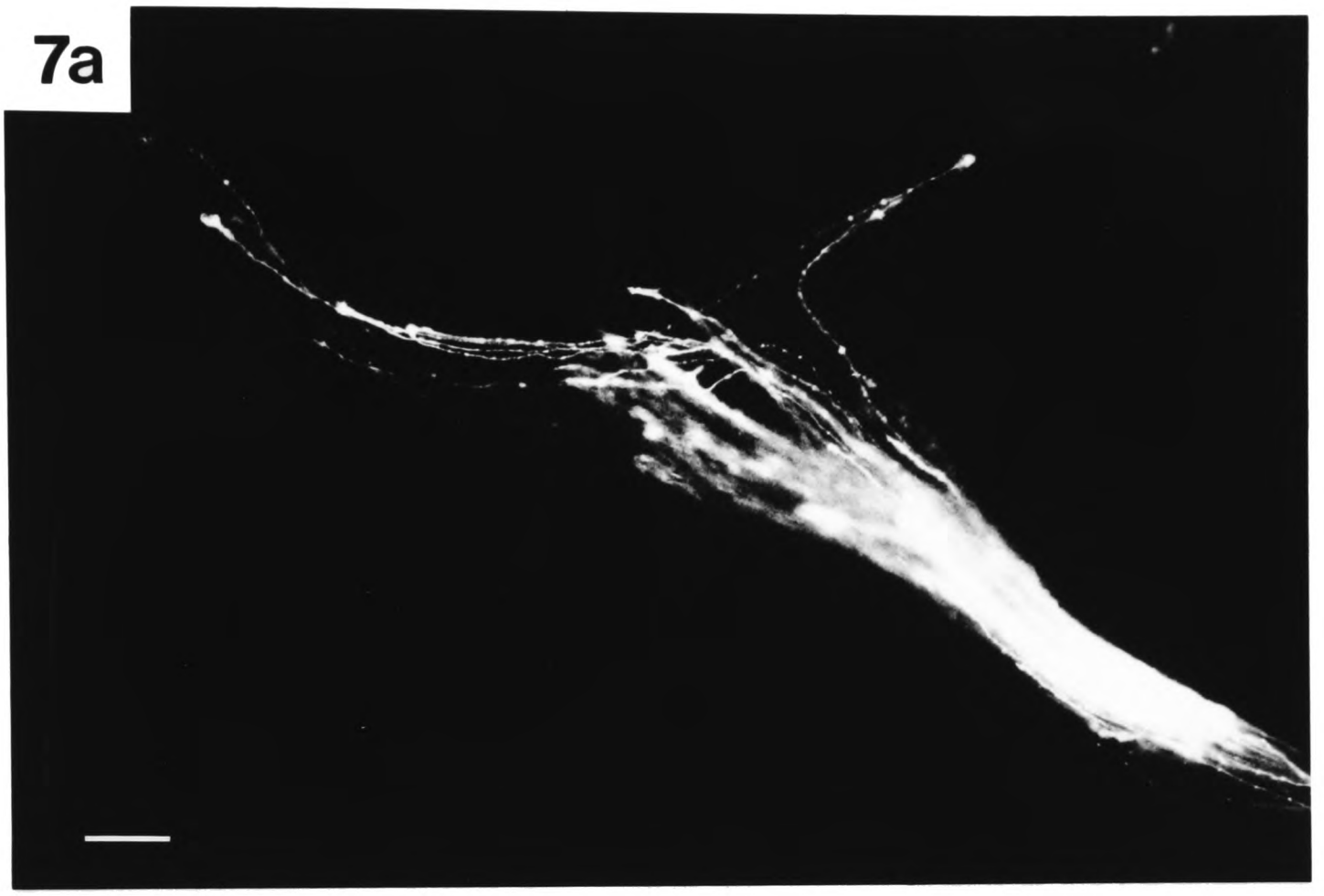

Pigmented rats
Albino rats
E16.5 enucleates
Controls
n=3
n=3
n=3
n=3
0
OD
10
20
30
40
50
60
70
80
90
100
Temporal
Position of decussation line
(% eccentricity)

Fig. 7 a illustrates the axons labelled in a horizontal section of the head, which includes the optic stalk and the chiasm, of an E16.0 pigmented rat embryo by putting DiI into the far perphery of the ventrotemporal retina. This section was photoconverted and the labelled axons were drawn in b using a drawing tube. Caudal is up and lateral is on the right. ML marks the midline of the brain. It is evident that at this developmental age, some axons from the ventrotemporal retina have just arrived at the chiasm and two have entered the ipsilateral tract as shown in these pictures. Scale bar is 100 μm.

7a



b

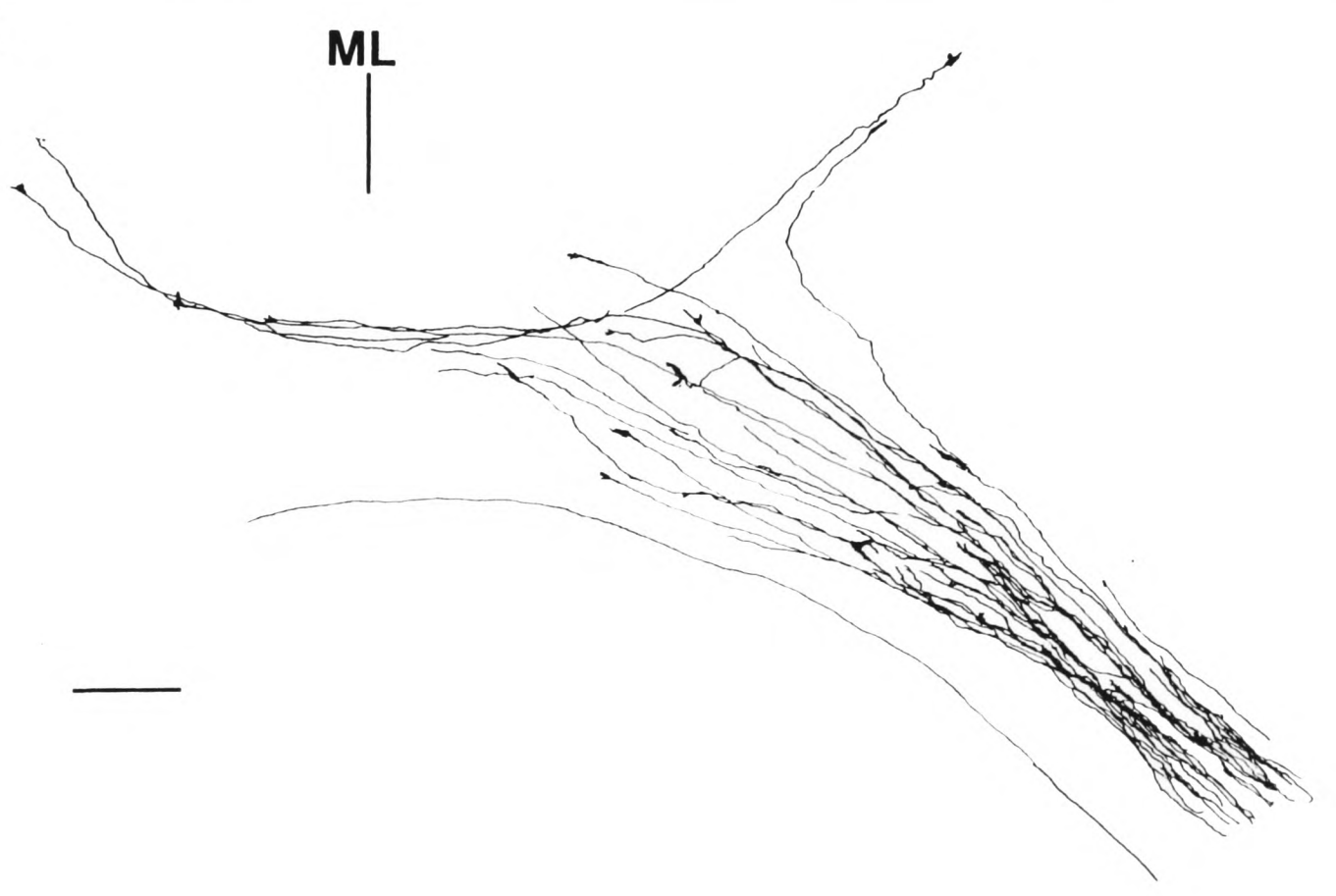


Fig. 8 Light micrographs showing the labelled ipsilaterally projecting retinal ganglion cells in the temporal crescent of adult rat retinas. a was taken from a pigmented control whereas b was taken from an albino control; c and d illustrate the temporal crescent in a pigmented and an albino rat respectively, both of which had received a monocular enucleation at birth; e and f were taken from a pigmented and an albino rat respectively, which had one eye removed at E16.5. The temporal edges of the retinas were aligned on the left. Optic disk (not shown) is on the right. Scale bar is 200 μm.

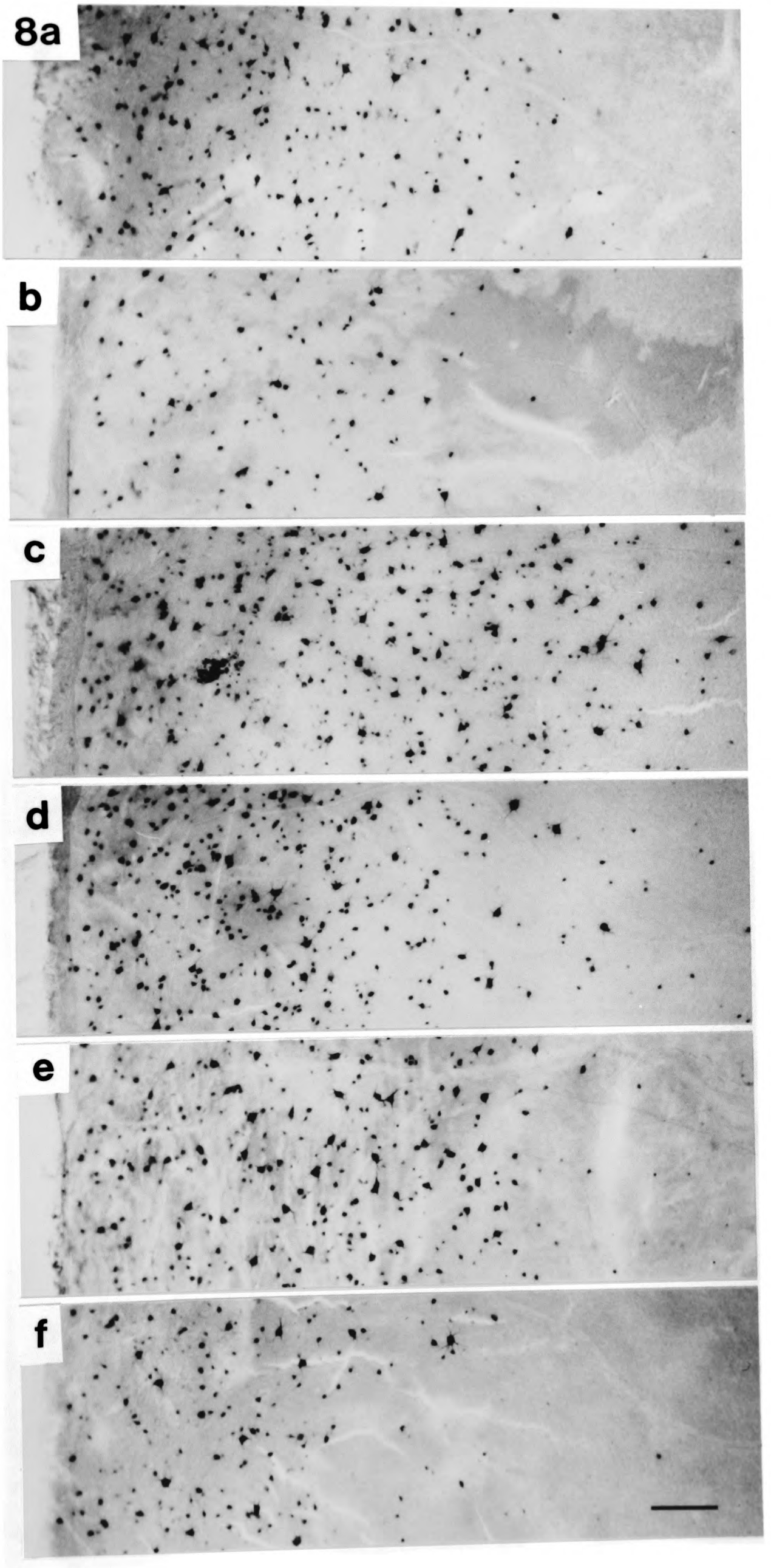

8a
b
c
d
e
f

Fig. 9 Diagrams showing the number of labelled ipsilaterally projecting retinal ganglion cells in the retinas of adult pigmented (a) and albino (b) rats.

Fig. 9a

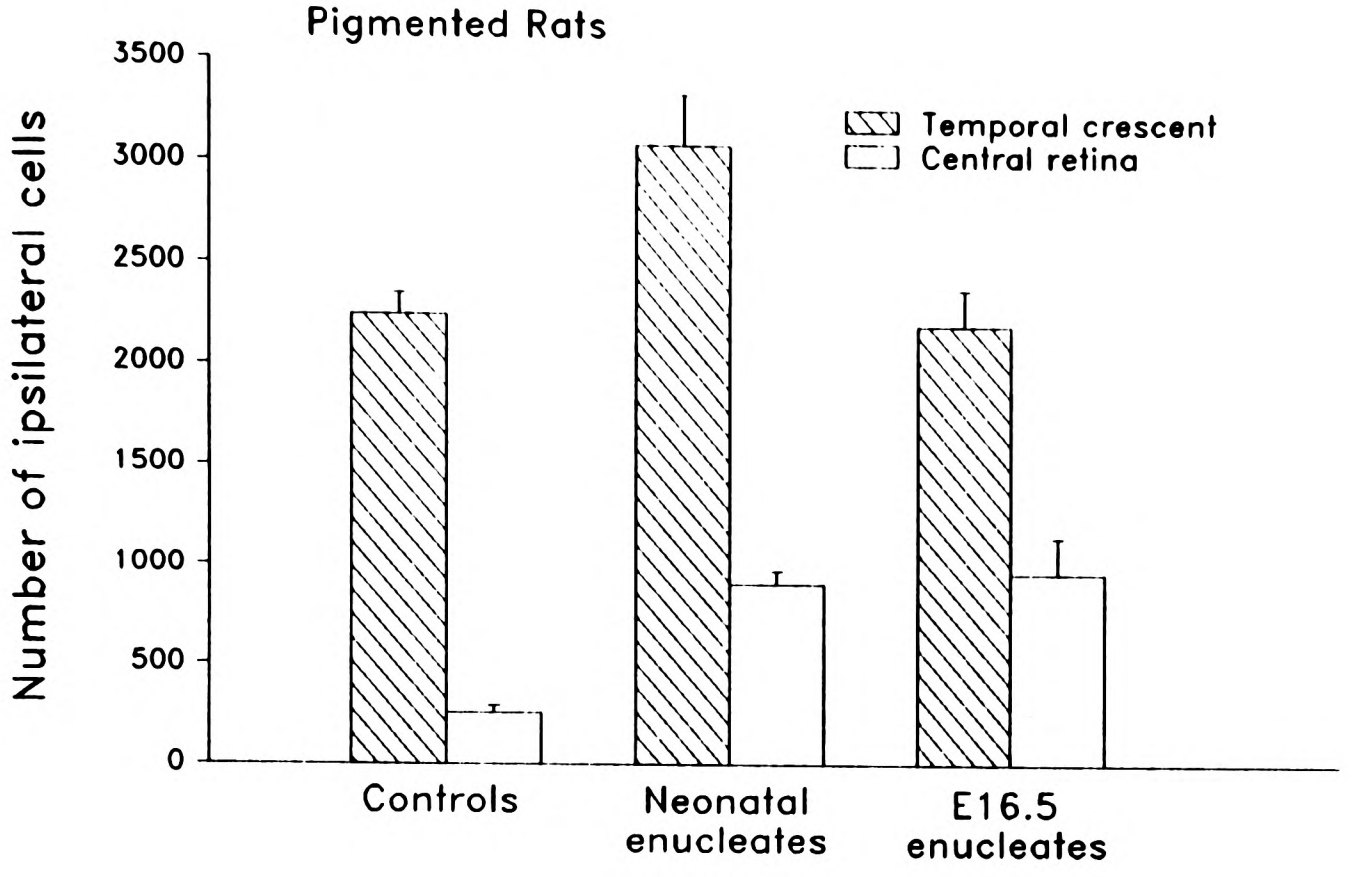
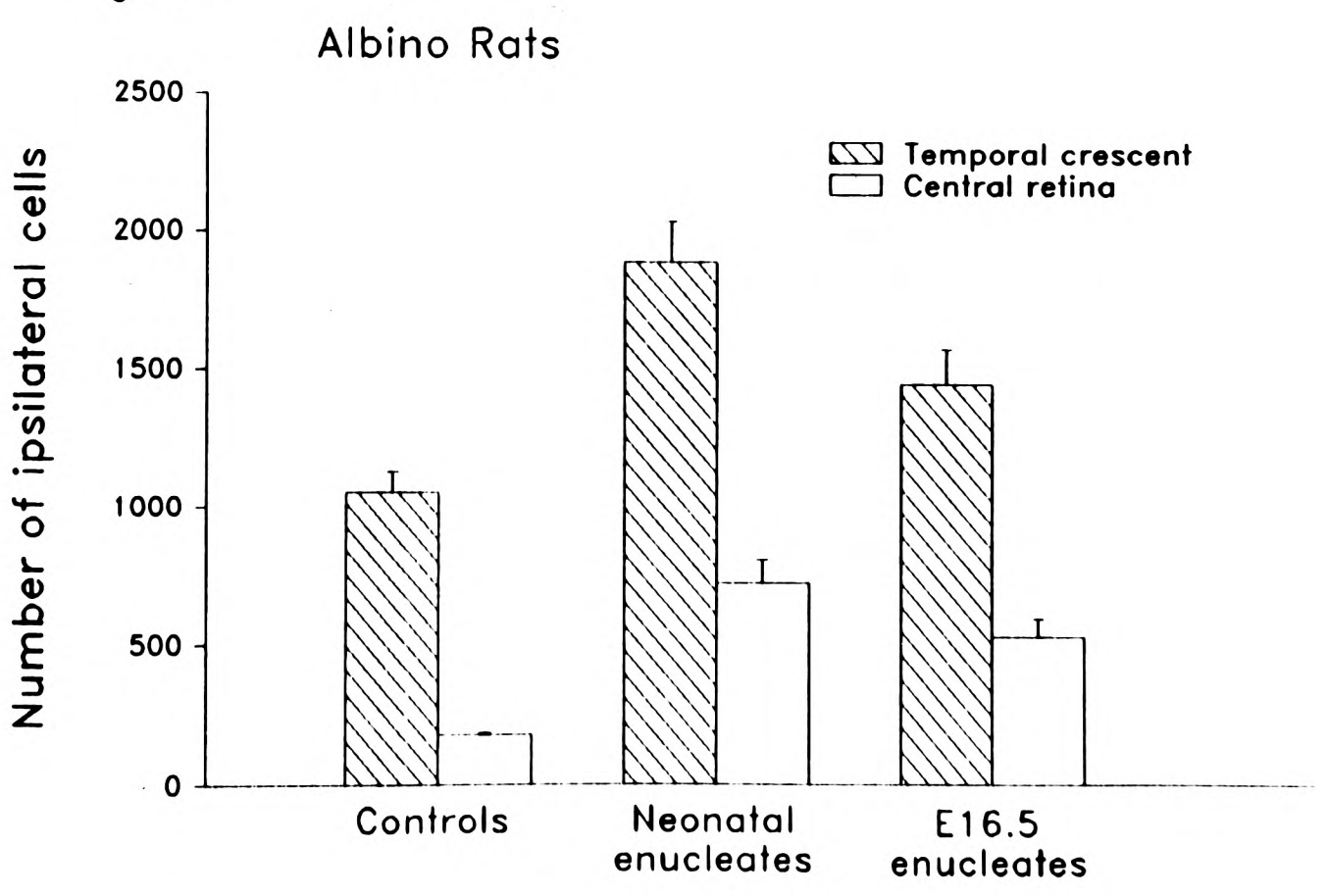

Pigmented Rats
Number of ipsilateral cells
3500
3000
2500
2000
1500
1000
500
0
Temporal crescent
Central retina
Controls
Neonatal enucleates
E16.5 enucleates

Fig. 9b


Albino Rats
Number of ipsilateral cells
2500
2000
1500
1000
500
0
Temporal crescent
Central retina
Controls
Neonatal enucleates
E16.5 enucleates

CHAPTER 5 THE DEVELOPMENT OF THE UNCROSSED RETINOFUGAL PATHWAY IN PIGMENTED AND ALBINO FERRETS AFTER PRENATAL MONOCULAR ENUCLEATION

Introduction

It is still unclear how the specific segregation pattern of the crossed and uncrossed retinal pathway is established at the chiasm during development. It has been argued in earlier chapters that there may be a cue at the chiasm and that this cue is interpreted by the uncrossed axons as a turning signal whereas the same cue is ignored or interpreted by the crossed fibres as a signal to continue their course to the contralateral tract (Godement et al., 1990; Sretavan, 1990; see chapter 3 and 4). This intrinsic difference between the crossed and uncrossed axons in response to the chiasmatic cue is likely to be set up in the retina (see chapter 3). It may exist in the form of a marker on the cells destined to be ipsilateral and this marker may make the axons of these cells behave differently from the axons of those destined to cross at the chiasm.

One approach to understanding the nature of the chiasmatic cue is to alter the environment at the chiasm and observe the change in the developing optic pathway. It is evident that when one eye is removed prenatally before or shortly after the first axons pass the chiasm, a reduced uncrossed component is produced in the remaining eye (mouse: Godement et al., 1987a; ferret: Guillery, 1989; rat: Chan, see chapter 4). These results indicate clearly that the binocular fibre interaction at the chiasm plays a crucial role in guiding fibres into the ipsilateral tract. However, it has been claimed

that in mice the uncrossed pathway can never be abolished even if the lesion is made early before any fibres reach the chiasm (Godement et al., 1987a). These observations led to the idea that in the mouse there may be a population of ipsilateral cells, both from the temporal crescent and the central retina, that do not require the binocular fibre interaction at the chiasm, i.e. they are autonomous, for making their chiasmatic choice. A similar argument has been presented on the basis of early enucleation studies in cats. It has been shown by using an anterograde tracing technique that in cats with one eye removed at E23 the uncrossed component of the remaining eye can still develop even in the absence of any interaction of fibres from the other eye at the chiasm (Sretavan and Shatz, 1986). However, whether this population of uncrossed axons that develop without the chiasmatic fibre interaction in the cat arise from the whole retina, as in the mouse (Godement et al., 1987a), or whether they come only from a specific part of the retina remains unresolved.

Little is known about the retinal marker that is assumed to mark the ipsilateral cells and that enables these cells to behave differently from the contralateral cells when their axons arrive at the chiasm. The fact that most ipsilateral cells are found in the temporal retina, even at early fetal stages (Bunt et al., 1983; Godement et al., 1987b; Chalupa and Lia, 1991; see chapter 3) indicates that this marker is preferentially expressed by cells in the temporal retina.

Another issue that has been addressed in the previous chapter is how the albino abnormality might affect the development of the uncrossed retinal pathway. In albino animals, the number of ganglion cells contributing to the uncrossed pathway is abnormally small (Dräger and Olsen, 1982; Morgan et al., 1987), and the decussation

line is shifted towards the temporal retinal margin (Stone et al., 1978; Cooper and Pettigrew, 1979b; Morgan et al., 1987). One view of how this abnormal uncrossed pathway develops comes from the early enucleation study of ferrets (Guillery, 1989). It was shown that a reduced uncrossed retinogeniculate pathway is formed in an adult pigmented ferret which has one eye removed prenatally, and that this reduced uncrossed projection is strikingly similar to the termination pattern in an albino uncrossed pathway. Moreover, removal of one eye early in development was reported to have no apparent effect on the albino uncrossed projection. These results raised the idea that the albino abnormality may develop as a consequence of a weakening of the binocular fibre interaction at the chiasm, and that this result can be mimicked by an early monocular enucleation in pigmented animals. However, there is indirect evidence to argue against this idea. It has been demonstrated in rats that although early monocular enucleation can produce a smaller than normal uncrossed projection in pigmented rats at birth, the decussation line of the reduced uncrossed pathway is not shifted towards the temporal margin of the retina, comparable to the shift seen in the albino controls. Further, the albino uncrossed component in rats is also reduced after an early eye removal.

In this chapter a series of studies was undertaken in parallel to those described in chapter 4, in which the effects of prenatal enucleation on the development of the uncrossed pathway in ferrets were examined. The first question to be addressed was the extent to which the uncrossed pathway in ferrets depends on the binocular fibre interaction at the chiasm. That is whether there is a population of ipsilateral cells from both the temporal and central retina which are autonomous in their pathway

choice. That is, the axons of these cells do not require an interaction with fibres from the other eye at the chiasm to select an ipsilateral route, as has been shown for the mouse (Godement et al., 1987a). The second issue was to find out whether the decussation line is unaffected by the early enucleation in pigmented ferret, as in rats. The decussation line of the uncrossed component in the ferret retina is much well defined than in the rat (Vitek et al., 1985; Morgan et al., 1987). This can provide direct and perhaps better evidence to test the hypothesis that early monocular enucleation can produce an albino phenocopy of uncrossed retinogeniculate projection in ferrets (Guillery, 1989). In order to answer these questions, monocular enucleations were done in ferret embryos at E27 and E28, both pigmented and albino, before the growing optic fibres have reached the subcortical visual targets (Cucchiaro and Guillery, 1984). The number and distribution of ipsilateral cells were determined at birth before the major cell death period commences (Morgan and Thompson, 1985; Henderson et al., 1988).

Materials and Methods

Normally pigmented and albino ferrets (*Mustela putorius furo*) were used for this study. The animals were bred in the Animal house of the Department. Timed mated pregnancies were obtained by putting a female with a male ferret for 24 hours. The date that the female was separated from the male was counted as embryonic day 0 (E0). Pregnancy was checked at about E23 when the embryos in the uterus became big enough to be palpated through the abdomen. The gestation period in our colony

is approximately 41 days.

Intrauterine surgery

The mother was first anaesthetized with a combination of xylazine (1 mg/kg body weight; Rompun, from Bayer) and ketamine hydrochloride (10 mg/kg body weight; Vetelar, from Parke-Davis Veterinary) injected intramuscularly. This was supplemented with one third of the normal dose of pentobarbital (normal dose is 40 mg/kg body weight) injected intraperitoneally. The xylazine-ketamine mixture and the pentobarbital were given at intervals as necessary during the surgery to keep the animal in a deeply anaesthetized condition.

A midline abdominal incision was made and one conceptus was exposed. A purse-string suture was then placed in the exposed uterine wall in a region over the embryo's head and with relatively little vascularization. Immediately outside this suture, a small wire loop was then stitched onto the uterine wall to provide a support to manipulate the uterus during the surgery. Several drops of local anaesthetic and a vascular contrictor (xylocaine, 2% with adrenaline 1:200,000, from Astra Pharmaceuticals Ltd.) were dropped onto the uterine surface. The uterus was then opened by an incision within the suture and the embryo's head was exposed briefly. The postcranial part of the embryo was prevented from coming out by tightening the suture. One eye of the embryo was then removed whole by a pair of forceps. After the enucleation, the suture was loosened and the embryo's head was returned to the uterus gently. The uterus was closed by tightening the suture again and the wire loop was removed. Usually three to four embryos were operated in a litter. After finishing

the fetal operation, the abdominal wall and the skin of the mother were closed and the animal was allowed to recover in a warm environment. Sterile precautions were taken throughout the operating procedure. A dose of antibiotic (0.5 ml duplocillin LA, from Mycofarm) was given intramuscularly to the mother to prevent postoperative infection.

Neonatal surgery

On E41, at the end of the gestation period, the mother was anaesthetized by an intramuscular injection of xylazine-ketamine mixture (see above for dosage). The normal and experimental animals were delivered by Caesarian section. The mother was then killed by an overdose of pentobarbital.

A neuronal tracer was used to label one optic tract of the neonatal ferrets. The normal (control) and operated animals, both pigmented and albino, were first anaesthetized by ether. The scalp was retracted on the right side of control embryos and on the side of the remaining eye in the enucleated animals. A window was made through the cartilagenous skull at a lateral position anterior to the transverse venous sinus. The meningeal membranes were retracted and the cortex and hippocampus underneath the window were removed by aspiration. The lateral parts of the diencephalon and the surrounding areas were kept clear by surgical gelfoam and occasionally by using aspiration. Five to six small lesions were made in the lateral diencephalon perpendicular to the course of the optic tract. A small granule of neuronal tracer, made by soaking a small piece of gelfoam in a solution of 2% wheat-germ agglutinin conjugated to horseradish peroxidase (WGA-HRP, Sigma) and 30% horseradish peroxidase (HRP, Sigma type VI) in 2% diemethylsulphoxide and air-

dried, was inerted into each lesioned site to label the whole optic tract and the primary retinorecipient nuclei in the thalamus on one side of the brain (Chan et al., 1989). A piece of gelfoam was then used to fill the lesioned cavity and the scalp was closed. The animals were allowed to recover in a warm and humid environment until they were perfused on the following day.

Perfusion and preparation of tissues

The procedures of perfusion and tissue preparation were similar to those described in chapter 4. All animals were allowed to survive for at least 18 hours after the diencephalon had been labelled. The animals were first deeply anaesthetized by ether and then perfused transcardially by phosphate buffered saline followed by 1% paraformaldehyde in 0.1M phosphate buffer at pH 7.4. A puncture was made to mark the dorsal pole of the eye. The eyes were then carefully dissected away and put in 1% paraformaldehyde in 0.1M phosphate buffer. The animal was further perfused with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1M phosphate buffer at pH 7.4. The brain was removed, further fixed in the aldehyde mixture for 5 hours, and transferred to 30% sucrose in 0.1M phosphate buffer overnight at 4°C.

The retina was dissected out and flat-mounted on a slide. It was further fixed in 2.5% glutaraldehyde for 1 hour at 4°C. After the fixation, the retina was reacted with Hanker-Yates histochemical reaction (Hanker et al., 1977; for details see chapter 4). It was then mounted on a gelatinized slide.

All the brains were cut frozen on a sledge microtome and 50μm transverse sections were collected as a 1:4 series in phosphate buffer. The sections were then

reacted with the Hanker-Yates reaction and mounted on gelatinized slides.

Cell counts and measurement of density

The number of labelled ipsilaterally projecting cells in each retina were counted using a Nikon Optiphot microscope under an oil immersion lens at a magnification of 812.5x. The number of labelled cells within a grid of 150x150 μm^2 was counted. The grid was moved systematically across the whole retina and the number in each grid was recorded on a corresponding area in a proportional plot of the retina. The central border of the temporal crescent, which is defined as the decussation line of the uncrossed component in this study, was estimated by simple observation on the retinal plots. The numbers of ipsilateral cells in the temporal crescent and in the areas outside, which are referred to the central retina in this study, are calculated separately.

The distribution of the labelled ipsilateral cells in some animals was represented by dot diagrams. These diagrams were prepared by pooling the cell counts in four adjacent grids in the retinal plot together so that each dot in the diagram represents the cell number in an area of 300x300 μm^2.

The change in density of the labelled ipsilateral cells from the optic disk to the temporal margin of the retina was measured. The measures were obtained by extending a line on the retinal plots from the centre of the optic disk to the temporal edge of the retina perpendicular to the central border of the temporal crescent. The densities of cells along this line were then calculated. The retinal eccentricity was expressed as a proportion of the distance from the centre of the optic disk to the retinal diameter.

Results

The spread of label in the brain

The spread of label was checked in the brains of all newborn ferrets. Fig. 1a shows a brain section taken from a pigmented control (D292). In this animal, the label was limited to the right side of the diencephalon, with very little label spreading across the midline. The optic tract and the major retinorecipient nuclei, including the lateral geniculate nuclei, the pretectum and the superior colliculus were heavily labelled. However, in most cases, the label was seen also spreading across the midline of the brain. The extent to which the diencephalon opposite to the side of labelled tract was labelled varied in individual animals. An extreme case is shown in Fig. 1b, which showed the label in the brain of a newborn albino ferret which had one eye removed at E28 (D228a). In this animal, the diencephalon on the side of the labelled tract and the centromedial part of the diencephalon on the opposite side were heavily labelled. However, it was evident that the lateral and ventral parts of the diencephalon opposite to the labelled tract, which include the optic tract and the lateral geniculate nuclei, were clear of label. Moreover, in no cases could we see any retrogradely labelled neurons in the visual cortex contralateral to the injected tract, indicating that the subcortical visual nuclei on that side of the brain were devoid of any label.

Morphology of the labelled ipsilaterally projecting retinal ganglion cells

Fig. 2 shows the labelled retinal ganglion cells in the dorsal region of the temporal crescent ipsilateral to the labelled tract. The labelled ipsilateral cells are mostly round, with a relatively uniform soma size. The cytoplasm of most labelled cells is filled

with dark brown reaction products. In some of these cells one or two processes are seen extending from the soma. The cytoplasm of most labelled cells is filled with dark brown reaction products. However, there are some lightly labelled cells, with a pale and granular cytoplasm. These cells were distinguished carefully from the surrounding non-labelled cells under an oil immersion lens.

The number and distribution of labelled ipsilaterally projecting retinal ganglion cells

The number of labelled ipsilaterally projecting retinal ganglion cells in the newborn ferrets, both controls and early enucleates, is listed in Table 1. The distributions of ipsilateral cells in the retinas of these animals are presented in the dot diagrams in Fig. 3 and 4.

In the retinas of control animals, both pigmented and albino, most ipsilateral cells are found in the temporal crescent (Fig. 3a and b; Fig. 5a and b). A number of ipsilaterally projecting cells can be found throughout the central retina, and it appears that most of these cells are located in the ventral half of the retina. The number of ipsilateral cells in pigmented and albino ferrets were compared by Mann-Whitney non-parametric tests. It has been found that the number of ipsilateral cells in the temporal crescent of pigmented controls is significantly higher than that in albino controls ($U=0$, $p<0.001$), but the number in the central retina does not show significant difference between these two strains of animals ($U=0$, $p<0.001$). As a whole, there are obviously more ipsilateral cells in the retina of the pigmented controls than in the albinos ($U=0$, $p<0.001$). It can be concluded that the uncrossed pathway in the albino ferret is

already reduced in terms of retinal ganglion cells on the day of birth.

Only two successful cases of E28 enucleation were obtained in the pigmented ferrets in this study, because of the low survival rate after surgery. In these animals, labelled ipsilateral cells can be found throughout the retina, but most of them concentrate in the temporal crescent (Fig. 3c and 5c). It is evident that the uncrossed pathway in these E28 pigmented enucleates is reduced. The number of ipsilateral cells in these animals is lower than the number in pigmented controls, with about half of the normal number of ipsilateral cells in either the temporal crescent and the central retina (Table 1).

The results in albino enucleates with an enucleation on E28 are more complicated. All E28 albino enucleates have an uncrossed pathway with axons arising from cells in the central retina, however only two out of four enucleates show a concentration of ipsilateral cells in the temporal crescent (Fig. 3d and 5d). The number of ipsilateral cells in these two animals is obviously lower than that in the albino controls (Table 1). In the other two albino enucleates, there are a number of ipsilateral cells in the central retina, but there is no obvious concentration of ipsilateral cells in the peripheral part of the ventrotemporal retina (Fig. 4a and b; Fig. 5e). The ipsilateral cells in these two animals are located throughout the retina, with a bias towards the ventral and central regions (Fig. 4a and b). The number of ipsilateral cells in these two animals, as in the other two albino enucleates, is again lower than that in the albino controls (Table 1). These results indicate that the uncrossed pathway in the albino ferret can be further reduced by an early monocular enucleation. Further, in some cases the concentration of ipsilateral cells in the temporal crescent can even be abolished,

Table 1. The number of ipsilaterally projecting retinal ganglion cells in newborn ferrets. TC: temporal crescent; CR: central retina; SEM: standard error of the mean.

Animal		Number of ipsilateral cells TC	CR	Total
Pigmented controls	C479	7848	3062	10910
	D292	11240	2249	13489
	D293	13158	2722	15880
	D294	11085	4340	15425
	Mean=	10832.8	3093.3	13926.0
	SEM=	1101.0	447.8	1131.1
E28 pigmented enucleates	C410	4641	1625	6266
	D256b	5761	1642	7403
	Mean=	5021.0	1633.5	6834.5
	SEM=	560.0	8.5	568.5
E27 pigmented enucleate	C480	-	-	478
Albino controls	C273	1731	2345	4076
	D252	2378	1203	3581
	D253	2673	2249	4922
	D255	3035	2467	5502
	Mean=	2454.3	2066.0	4520.3
	SEM=	276.0	291.1	428.6
E28 albino enucleates	D228c	1074	1480	2554
	D228b	394	942	1336
	C441	-	-	3308
	D228a	-	-	2855
	Mean=	734.0	1211.0	2513.3
	SEM=	340.0	269.0	421.9
E27 albino enucleate	D275a	-	-	1549

Note: The means in the TC and CR of E28 albino enucleates were calculated from the two cases which had a recognizable temporal crescent, while the mean of the total in these animals was the average of all four cases.

creating an uncrossed retinal projection without any decussation line, a pattern that has so far never been described either as a result of an enucleation or as an affect of the albino gene.

This novel distribution pattern of ipsilateral cells can also be seen in the retinas of two ferrets, one pigmented and one albino, which had one eye removed earlier at E27. Again, the labelled ipsilateral cells in these animals are found throughout the retina, and there is no sign of any concentration of ipsilateral cells in the ventrotemporal retina. In the pigmented enucleate, there is no obvious concentration of ipsilateral cells in any parts of the central retina (Fig. 4c and 6a), and the cell number is highly reduced when compared with pigmented controls (Table 1). In the albino enucleate, the ipsilateral cells are found in all parts of the retina but with a slight bias towards the central parts of the ventral hemiretina (Fig. 4d and 6b). The number of ipsilateral cells in this animal is again smaller than the albino controls (Table 1).

Change in the decussation line of the uncrossed pathway after early enucleation

Four newborn ferrets, control and E28 enucleate in either pigmented and albino animals, were selected to illustrate the changes in density of ipsilateral cells across the temporal retina (Fig. 7). As for the early enucleation studies in rats (chapter 4), the decussation line of the uncrossed pathway in the ferret retina is defined as a point on the density plot at which the density of ipsilateral cells first reaches 1000 cells/mm^2, a value slightly higher than the maximum density in the central retina. Based on this criterion, the positions of decussation line in the newborn ferrets were determined and

listed in Table 2.

Table 2. The positions of decussation line of the uncrossed pathway in the control and early enucleated newborn ferrets. SEM: standard error of the mean.

Animal		Position of the decussation line (% eccentricity)
Pigmented controls	D292	59.0
	D293	62.4
	D294	58.4
		Mean=59.9
		SEM= 1.2
E28 pigmented enucleates	C410	64.0
	D256b	61.7
		Mean=62.9
		SEM= 1.2
Albino controls	C273	84.5
	D252	75.3
	D253	80.7
	D255	79.2
		Mean=79.9
		SEM= 1.9
E28 albino enucleates	D228b	85.6
	D228c	79.6
		Mean=82.6
		SEM= 3.0

It is shown in Fig. 7 that the density of ipsilateral cells in the central retina of all animals is initially low, with only very slight variation. In pigmented animals, either the control or the early enucleate, at about 60% eccentricity the cell density reaches the arbitrary line that marks the point of the

decussation line of these animals as defined in this study (Table 2). These results indicate that the decussation line in the pigmented ferret is not affected by the early enucleation. Further, it should be noted that the density curve of the early pigmented enucleate follows very closely to the curve of the pigmented control during the initial phase of density surge (Fig. 7). The cell densities in the three pigmented controls examined in this study all peak within the range of 75-80% eccentricity (not shown). This high density of ipsilateral cells is maintained in these animals almost up to the edge of the temporal retina (see also Fig. 3a and 5a). The density plot in the early enucleate, however, peaks at a lower density than in controls and begins to drop rapidly at 72% eccentricity (Fig. 7). This is also found in another early enucleate, which shows a drop of cell density at 74% eccentricity. This drop of density in the middle of the temporal crescent in the E28 pigmented enucleates results in an area along the peripheral margin of the temporal crescent which is very sparsely populated with the labelled ipsilateral cells (see also Fig. 3c and 5c).

The surge in density of ipsilateral cells in the albino controls begins at about 80% eccentricity (Fig. 7), indicating that the decussation line of the albino ferret is already shifted by about 20% eccentricity towards the temporal edge of the retina (compare Fig. 5a and 5c). There is no obvious change in the position of the decussation line in the early albino enucleates (Table 2; Fig. 5d). Moreover, the peak density of cells in the albino enucleates (maximum is 2667 cells/mm^2) is much lower than that in the controls (maximum is 5022 cells/mm^2) (Fig. 7), but unlike that in the pigmented enucleates, there is no obvious reduction in cell density in the peripheral parts of the temporal crescent (Fig. 5d and 7).

Discussion

The results of the present study have shown that the uncrossed retinal pathway in pigmented ferrets can be reduced by an early monocular enucleation made at E28. Although the number of ipsilateral cells is reduced, the decussation line of the uncrossed pathway in the pigmented animal is however not affected by the early eye removal. Further, it has been demonstrated that the albino uncrossed pathway can also be reduced by removing one eye at E28, and again the decussation line in the albino is not changed after an early monocular enucleation. Finally, it has been found in some E28 albino enucleates and in two E27 enucleates, one pigmented and one albino, that an early monocular enucleation can reduce the uncrossed pathway to such an extent that no recognizable temporal crescent is established in the retinas of these animals. This results in the formation of an uncrossed pathway which has only axons from the cells sparsely populated throughout the whole retinal surface and without a decussation line.

The development of the uncrossed pathway in pigmented and albino ferrets depends on the binocular fibre interaction at the chiasm

It has been shown in this study that removal of one eye in a pigmented ferret at E28, when the first fibres have just crossed the chiasm and not yet reached the targets (Cucchiaro and Guillery, 1984), a reduced uncrossed pathway is produced at birth. These results confirm the findings in the earlier study (Guillery, 1989) that early enucleation in pigmented ferrets produces a reduced uncrossed retinogeniculate projection, but they disprove the claim that the albino uncrossed projection is not

affected by an early eye removal. Indeed, the albino uncrossed pathway can be further reduced by removing one eye at E28. These results agree well with the findings in rats, which also show a reduction in the number of ipsilateral cells after a monocular enucleation at E16.5 (chapter 4). Therefore, it can be concluded that in ferrets at least some axons in the developing uncrossed pathway, either in the pigmented or albino animal, require an interaction with fibres of the other eye in order to make their chiasmatic choice.

Early monocular enucleation in pigmented ferrets does not produce an albino-like uncrossed pathway

It was reported that an albino phenocopy of the uncrossed retinogeniculate projection was produced in the pigmented ferrets with one eye removal at E28 (Guillery, 1989). In the present study, it has been demonstrated that although the number of ipsilateral cells is reduced in the E28 pigmented enucleates, the line of decussation is not changed in these animals. These results agree well with findings in rats that the decussation line in the pigmented retina is unaffected by removing one eye at E16.5 (chapter 4), indicating clearly that the early monocular enucleation does not produce an uncrossed pathway which is comparable to that in the albino animals.

It should be noted that although the early monocular enucleations in pigmented rats and ferrets do not produce an albino-like uncrossed retinal pathway, these results do not prove that the albino gene does not act by altering the fibre interaction at the optic chiasm, nor can they prove that the albino gene acts on the retinal ganglion cells. However, the facts that the decussation line in the albino animals is consistently

shifted peripherally and also the decussation line in both pigmented and albino uncrossed pathway is not affected by the early eye removal suggest strongly that the albino gene indeed affects the cells in the retina.

Elimination of the binocular interaction at the chiasm early enough can abolish the temporal crescent in newborn ferrets

The early enucleation studies in the rat have shown that if one eye is removed at E16.5, when the first axons from the peripheral parts of the ventrotemporal retina have reached the chiasm, the uncrossed component in the temporal crescent at birth will be reduced but it is still prominent (see chapter 4). These results suggest that the early enucleation affects those fibres which are still growing towards the chiasm, whereas it has apparently no effect on those which have arrived at the chiasm and have already made their chiasmatic choice.

This idea is supported by the present results showing that a reduced uncrossed component can be obtained in the temporal crescent of either pigmented or albino ferrets which are enucleated at E28. In ferrets, the first axons that enter the ipsilateral tract (E26 - the earliest age studied) come exclusively from the cells in the central retina, axons from the temporal crescent enter the optic tract at a later embryonic age (Baker and Collelo, personal communication). The uncrossed pathway of ferrets at E28 arises mostly from the central retina, but there is a first appearance of retrogradely labelled ipsilateral cells in the temporal crescent, indicating that the uncrossed component from the temporal crescent has reached the chiasm and just begun to innervate the optic tract (Baker and Collelo, personal communication).

Therefore, enucleations made at E28 should produce a reduced uncrossed component in the temporal crescent, as has been demonstrated in the present study. Moreover, it has been noted that the density of ipsilateral cells drops rapidly in the peripheral margin of the temporal crescent in pigmented ferrets after enucleation at E28. These observations suggest that the early eye removal affects particularly the routing of those fibres arising from cells in the far periphery of the temporal crescent. These fibres very likely come from cells that are born later than the more centrally located cells in the temporal crescent during the retinal ganglion cell genesis, as suggested indirectly from the birthdating studies in the cat (Walsh et al., 1983; Walsh and Polley, 1985). Axons of these later born cells may well be on their way to the chiasm and so are affected by the absence of the chiasmatic fibre interaction.

It can be argued that if the monocular enucleation is made early enough, either in rat or ferret, before any fibres from the temporal crescent have arrived at the chiasm, then the temporal crescent may be abolished. It has been shown in this study that the temporal crescent can really be abolished by making an enucleation in some E28 albino ferrets or in E27 ferrets of either strain. Although the number of successful cases is small, simply because it is extremely difficult to obtain survivals after intrauterine and neonatal operations, it shows clearly that almost all cells in the temporal crescent which would normally project to the ipsilateral targets are lost after the early enucleation.

It is unclear why there is a concentration of ipsilateral cells in the temporal crescent of some albino enucleates while in the others there is no recognizable temporal crescent. However, these results may be interpreted by taking into account

the possible variation in the developmental stage of these embryos. It is possible that in some E28 albino embryos, some of the temporal crescent cells have sent their axons to the chiasm and that these axons are committed to take an uncrossed route, and so they are not affected by the enucleation; in the other cases, most axons from the temporal crescent are still approaching the chiasm and they are lost after missing the chiasmatic binocular interaction. The results obtained in this study do not allow us to verify this point - whether the difference obtained in the E28 albino enucleates actually reflects the variation in staging of embryos. Although the number of successful enucleates is low, the results obtained in E28 enucleates together with those obtained in the E27 enucleates suggest strongly that the development of the uncrossed component in the temporal crescent of ferret depends totally on the binocular fibre interaction at the chiasm. In order to strengthen this point, experiments are currently undertaken to gather enough successful cases of early enucleates.

It may be argued further that if the enucleation is done earlier in ferret, before any fibres have reached the chiasm (eg. at E23 in ferrets), the whole uncrossed component may be abolished. It is not known whether the uncrossed pathway in ferrets can be totally abolished by an enucleation at E23, but it has been reported in cats that the uncrossed retinal pathway can still develop in animals with one eye removed at E23, before any fibres reach the chiasm (Sretavan and Shatz, 1986), indicating that the uncrossed pathway can never be completely abolished. It is likely that there is a population of cells in the cat, may be also in ferret, that can navigate to the ipsilateral tract without any interaction with fibres from the other eye. If this population of cells does exist in the ferret, most of them, if not all, must be from the central retina, since

it has been shown that almost all cells in the temporal crescent require the binocular interaction at the chiasm to select their uncrossed paths. However, the cells in the central retina which are assumed to be autonomous in their pathway choice should constitute only a portion of the total population of ipsilateral cells in the central retina, as it has been demonstrated in the present study that the number of cells in the central retina of early enucleates can be reduced by an early eye removal.

Comparison of the early enucleation studies in rodents and ferrets

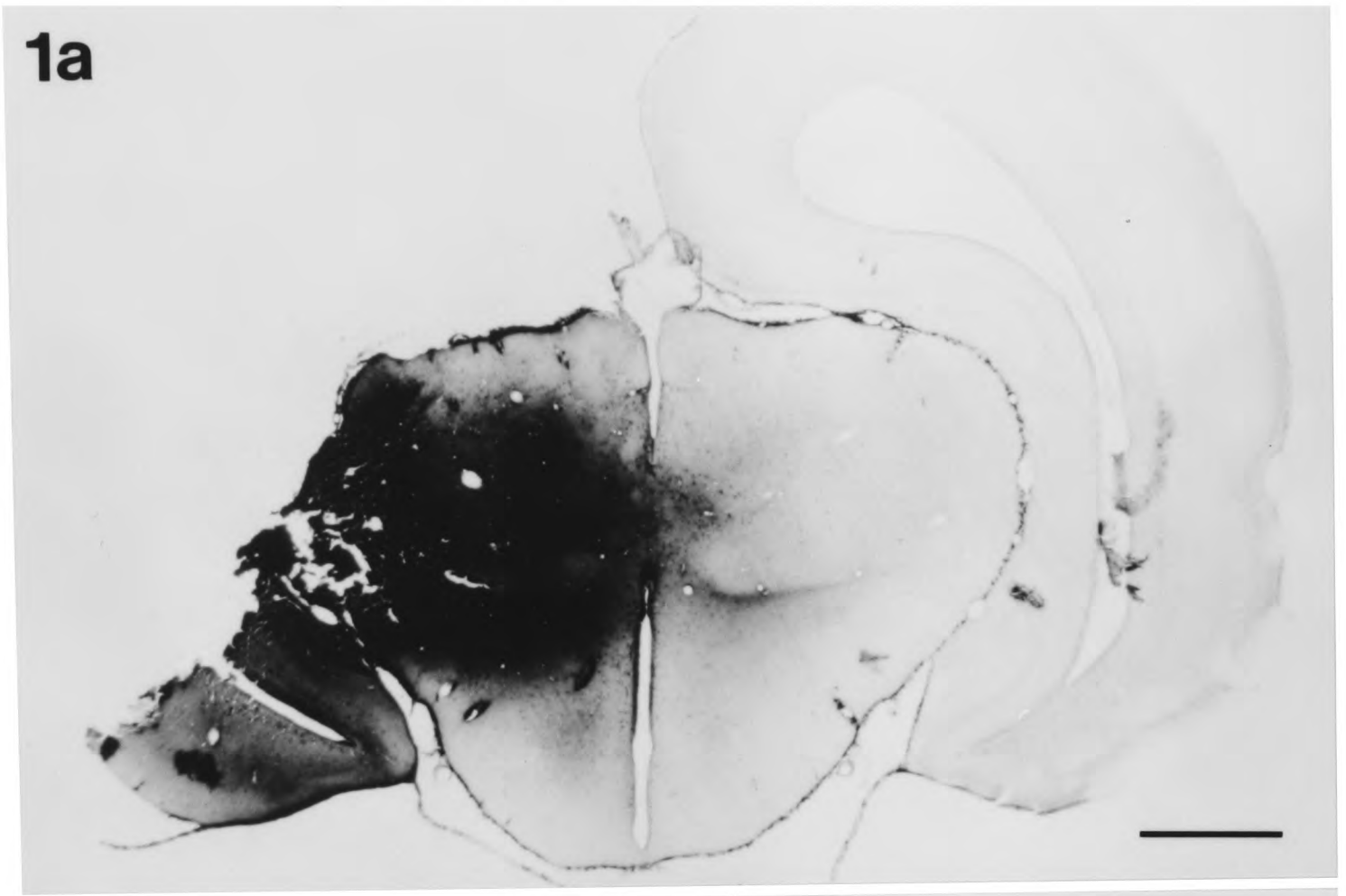
It has been shown in chapter 4 that in rats when one eye is removed at E16.5, the uncrossed component in the temporal crescent is reduced in number. It may be argued that if the monocular enucleation is made early enough in the rat, the whole uncrossed component in the temporal crescent may disappear, as what has been found in ferrets.

This idea seems to be discouraged by the results of early enucleation studies in mice (Godement et al., 1987a), in which one eye was removed in mice at E12 or E13, before or at the time that the first axons arrive at the chiasm. It was shown that even after such an early enucleation, a number of ipsilateral cells in the central retina and a well defined uncrossed component in the temporal crescent can still survive in the adult retina. These observations suggest that at least some of these ipsilateral cells in the temporal crescent and the central retina of mouse are autonomous in their pathfinding, i.e. they do not require an interaction with fibres from the opposite eye in order to select an ipsilateral route.

The factor that sets up the difference between the ferret and the mouse in terms of the retinal distribution of ipsilateral cells that are autonomous in selecting their

paths is unknown. It is possible that there may be fundamental differences in specifying the cells that contribute to the uncrossed pathway in mouse and ferret during development, such as the birthdates of cells that have crossed or uncrossed axons. However, it is clear that these animals need the retinal marker to specify the cells destined to be ipsilateral, together with the binocular fibre interaction at the chiasm, to a different extent, as cues to establish the uncrossed retinofugal pathway during development.

Fig. 1 Transverse sections of the brain taken from a normal newborn pigmented ferret (a) and a newborn albino ferret with one eye removed at E28 (b). These animals had one side of the diencephalon labelled by a neuronal tracer (WGA-HRP and HRP). It is demonstated that one side of the brain is heavily labelled, which includes the optic tract and the lateral geniculate nuclei. The label in a is limited to the implanted side, but the label in b spreads across the midline of brain. However, it is evident that the lateral parts of the thalamus and the overlying visual cortex opposite to the implanted side are free from any labelling, indicating that major retinal recipient nuclei were not labelled. Dorsal is up, left side of the brain is on the right. Scale bar is 1 mm.


1a

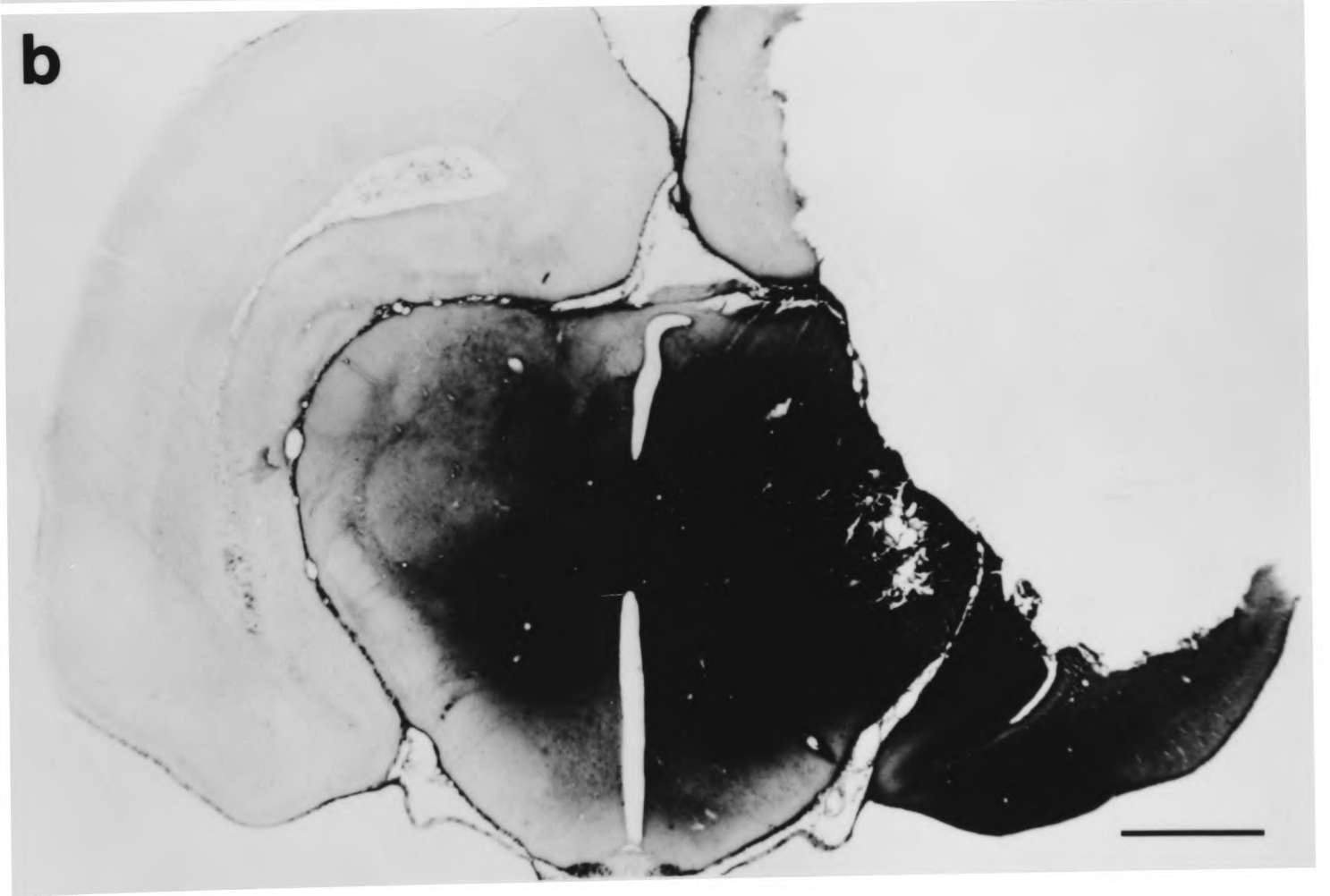

b

Fig. 2 Light micrograph showing the labelled ipsilaterally projecting retinal ganglion cells in the temporal crescent of the retina of a normal newborn pigmented ferret. Scale bar is 50 μm.

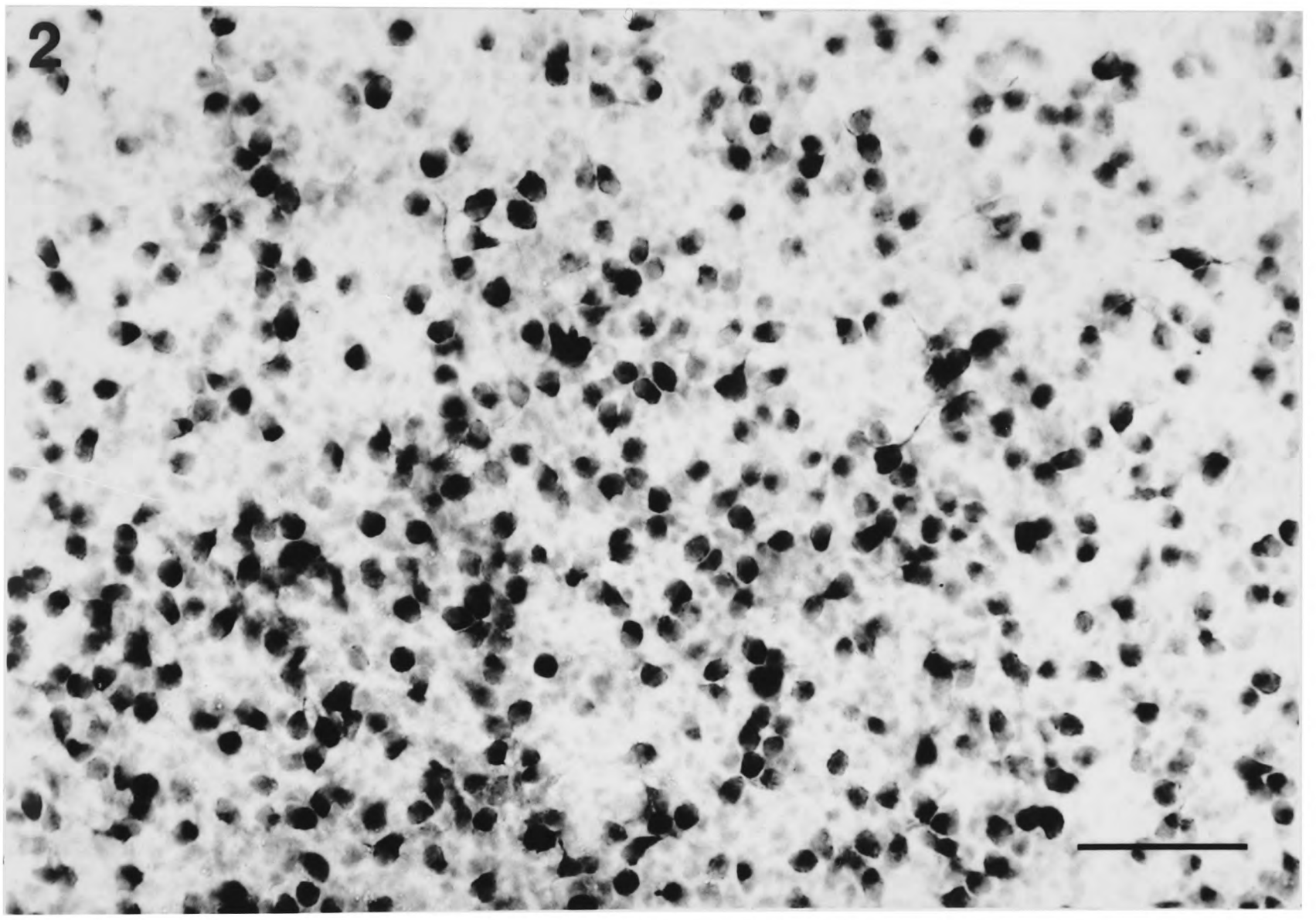
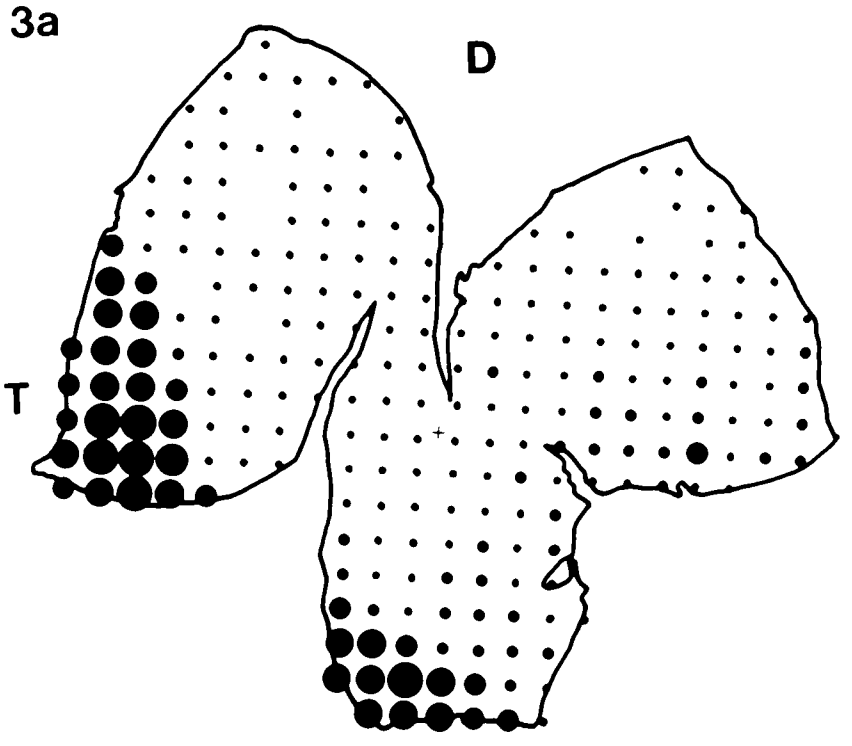
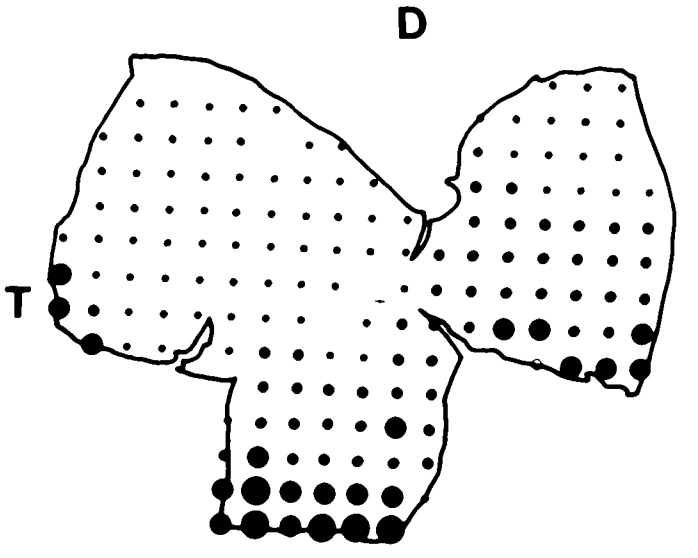

2

Fig. 3 Dot diagrams illustrating the distribution of ipsilaterally projecting retinal ganglion cells in the retinas of newborn ferrets. a is a pigmented control; b is a albino control; c is from a pigmented animal with one eye removed at E28; and d is from an E28 albino enucleate. D is dorsal and T is temporal; the optic disk is indicated by a small cross. The dots indicate the number of cells in an area of 300x300 μm^2. Scale bar is 500 μm.

3a

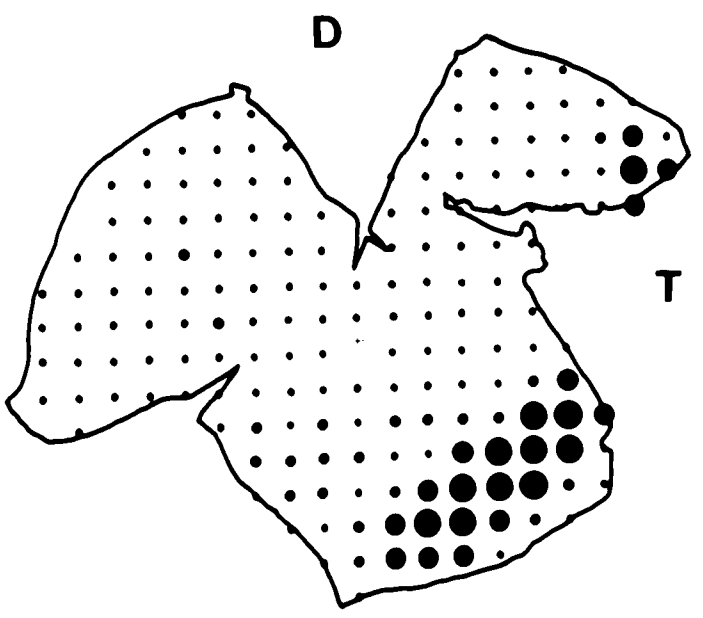


b



1-20
21-50
51-200
201-500
Above 500

c



d

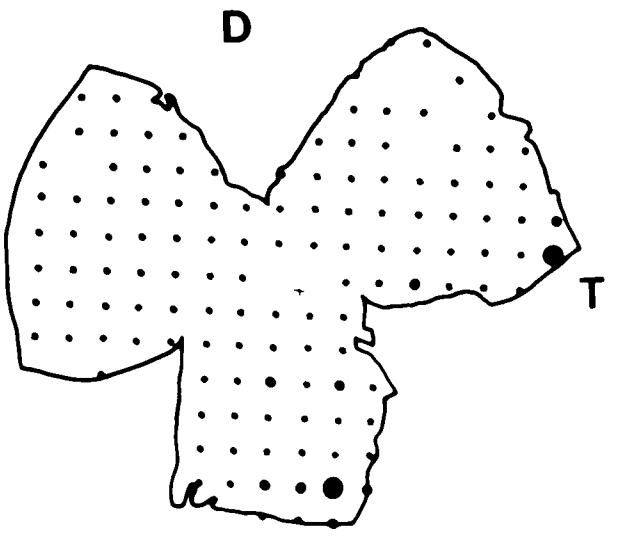
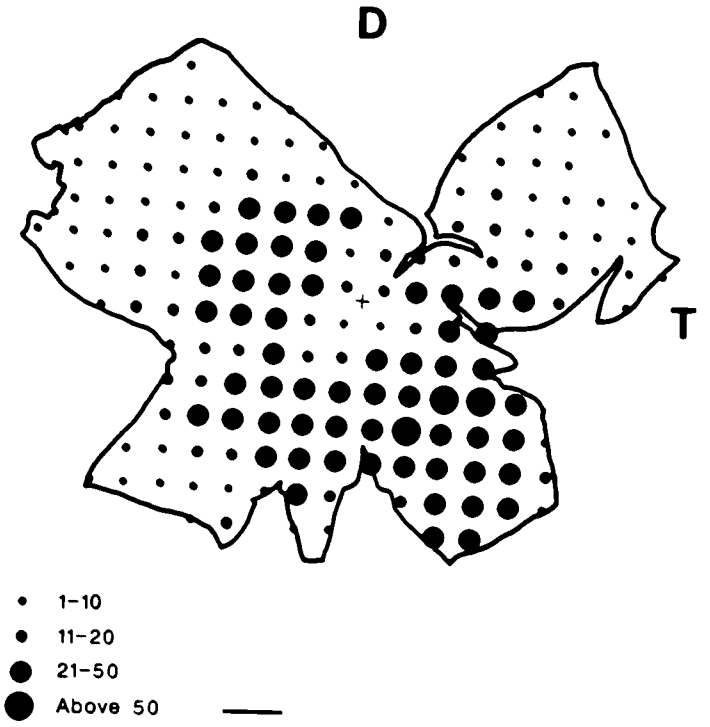
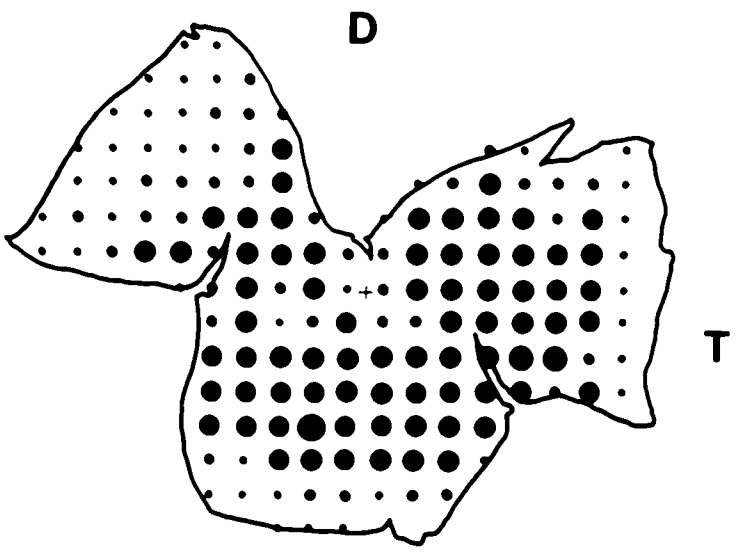


Fig. 4 Dot diagrams showing the distribution of ipsilaterally projecting retinal ganglion cells in the retinas of newborn ferrets. a and b are from two E28 albino enucleates; c is from a pigmented ferret with one eye removed at E27; d is from an albino ferret with one eye removed at E27. In these four animals, there is no recognizable temporal crescent. Note the dot scale is much smaller than the one in Fig. 3. D is dorsal and T is temporal. The dots indicate the number of cells in an area of 300x300 μm^2. Scale bar is 500 μm.

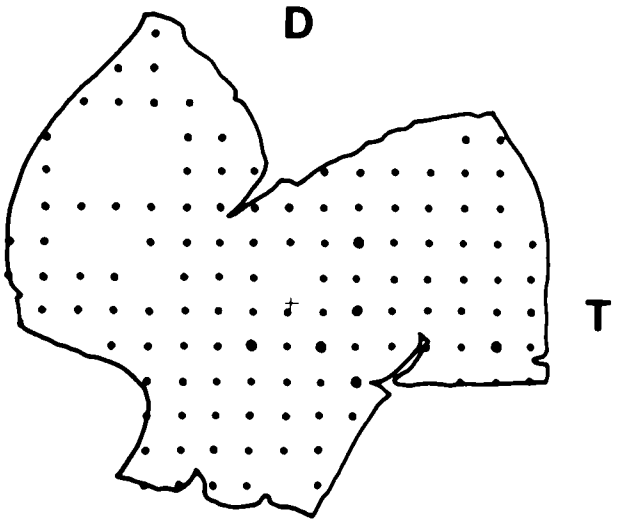
4a



b



c



d

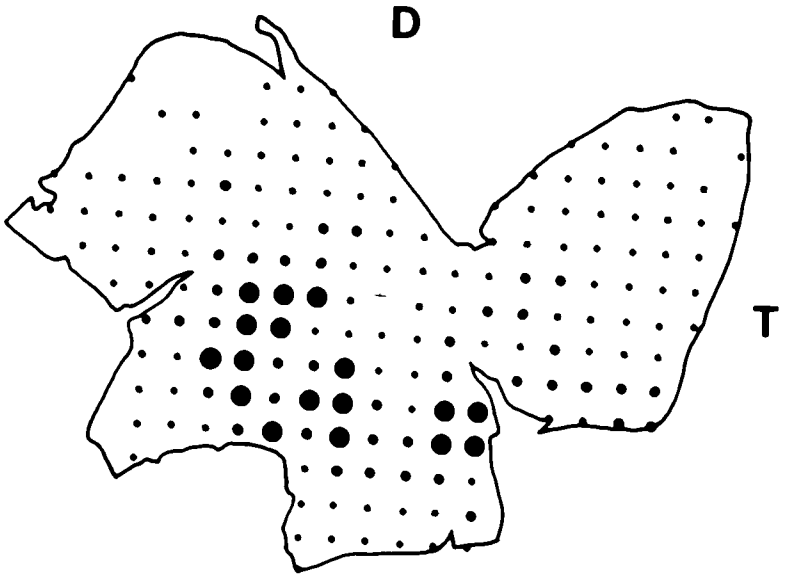


Fig. 5 Light micrographs showing the labelled ipsilaterally projecting retinal ganglion cells in the ventrotemporal retina of newborn ferrets. The temporal retinal edges were aligned on the left. a was taken from a pigmented control and b was from an albino control; c and d illustrate the concentration of labelled ipsilateral cells in the temporal crescent of a pigmented and an albino ferret respectively, each had one eye removed at E28; e shows the ventrotemporal retina from an albino ferret with one eye removed at E28, in which no obvious concentration of labelled cells can be recognized. Scale bar is 200 μm.

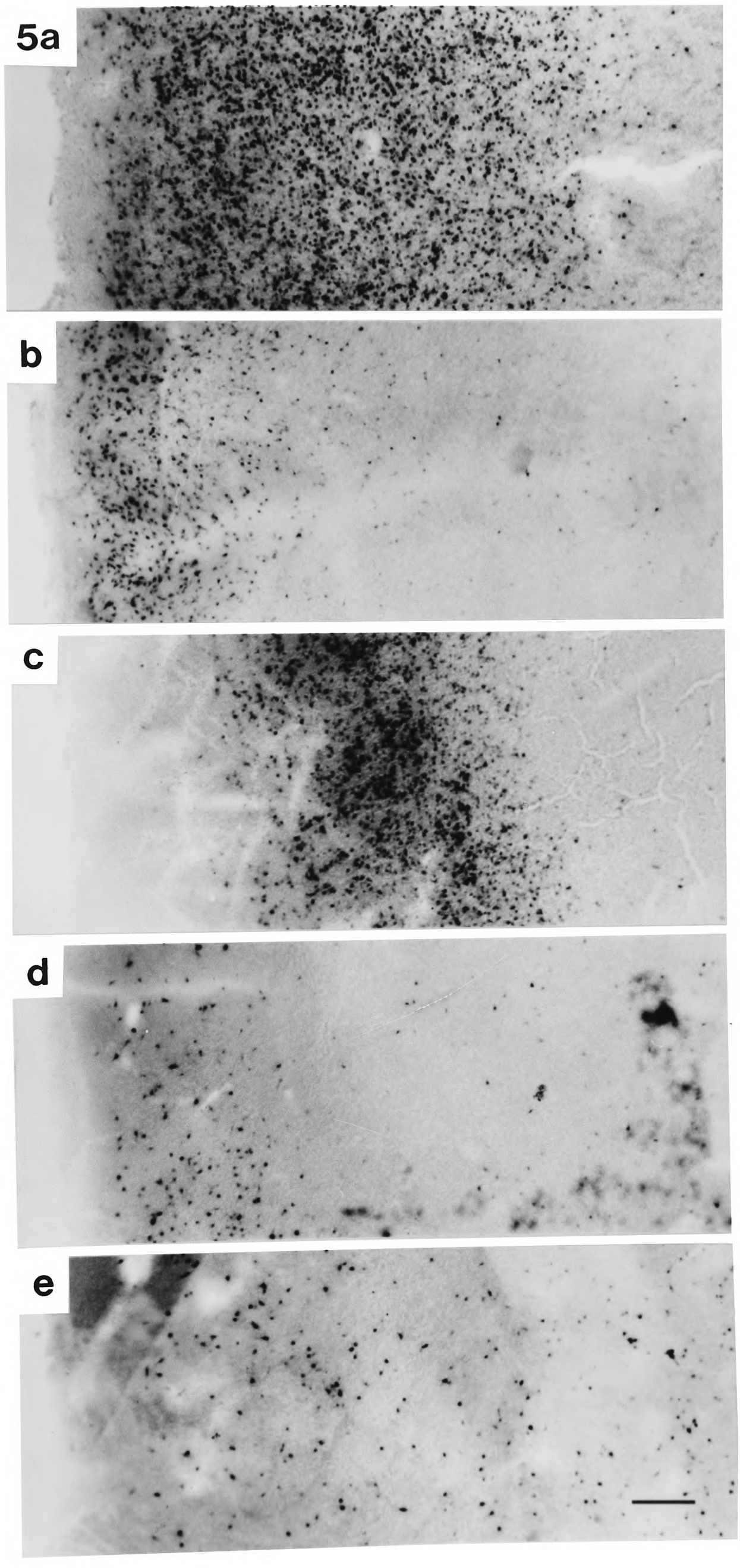

5a
b
c
d
e

Fig. 6 Light micrographs showing the labelled ipsilaterally projecting retinal ganglion cells in the ventrotemporal retina of a pigmented (a) and an albino (b) newborn ferret which had received a monocular enucleation at E27. In these animals, there are very few labelled cells and no recognizable temporal crescent can be seen in the retina. Two of the labelled ipsilateral cells, which were identified under an oil immersion lens, are indicated by arrows. The temporal retinal margins are aligned on the left. Scale bar is 200 μm.


6a

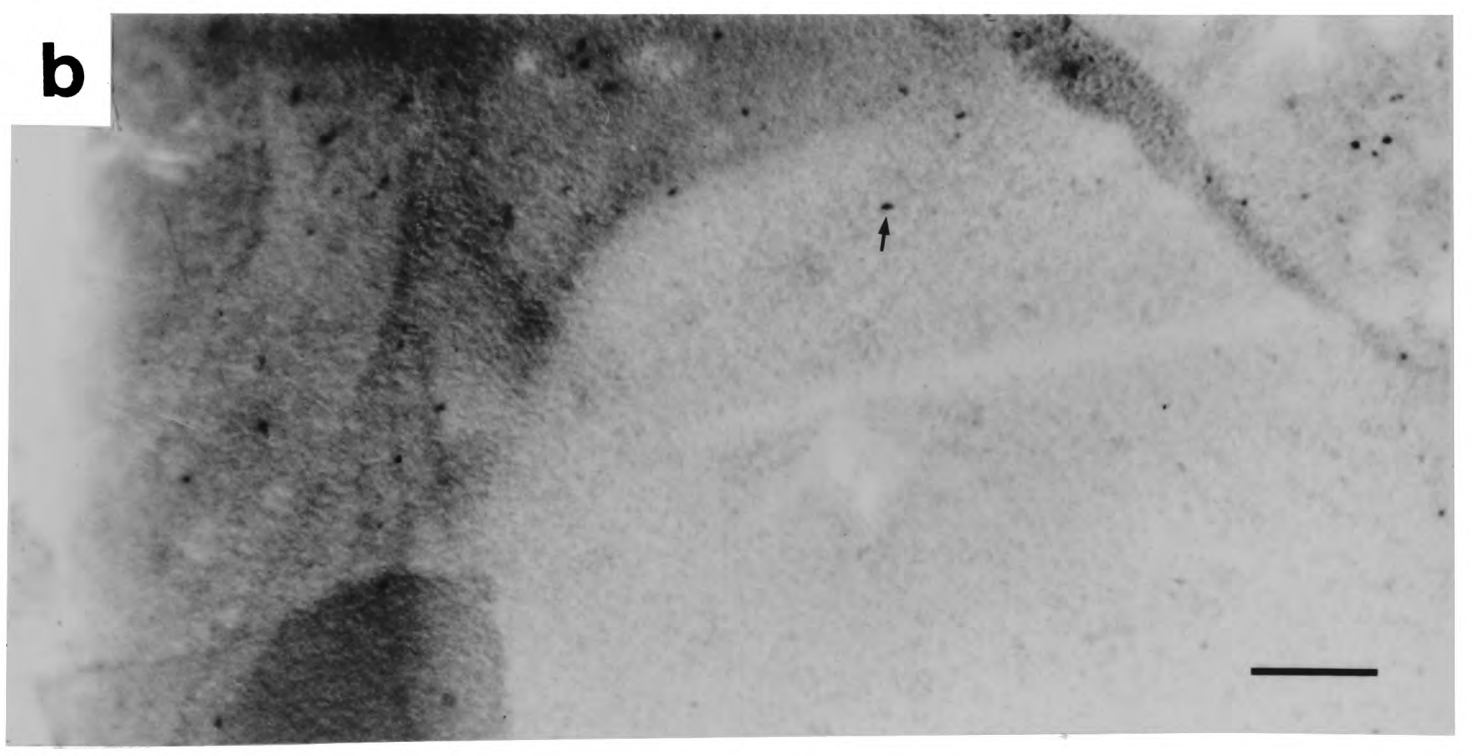
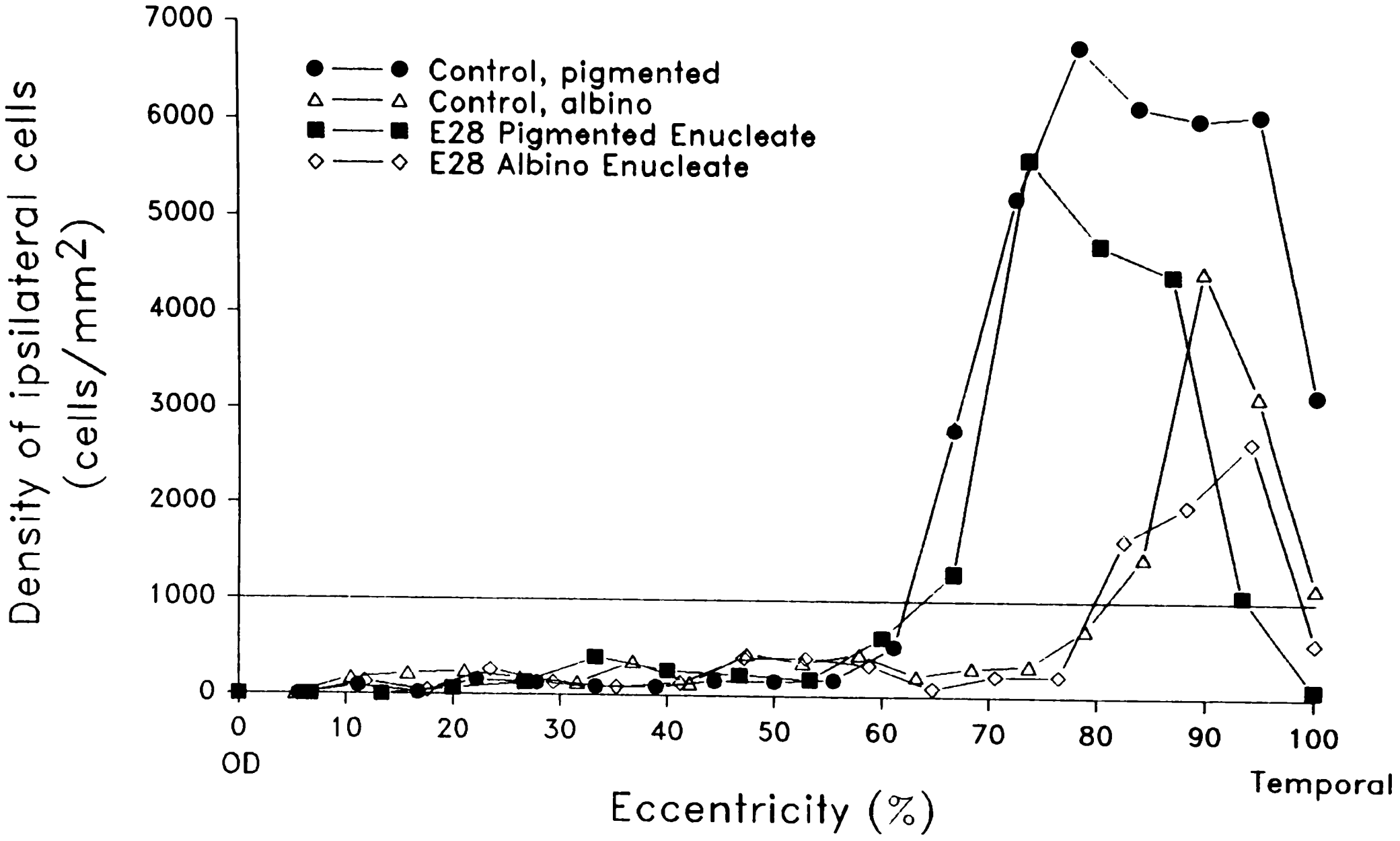

b

Fig. 7 Graph showing the changes in density of labelled ipsilaterally projecting ganglion cells in four newborn ferrets from the optic disk (OD) to the retinal margin across the temporal retina. A horizontal line was drawn across the density of 1000 cells/mm^2 in order to define the decussation line of the uncrossed component in these animals.

Fig. 7


Control, pigmented
Control, albino
E28 Pigmented Enucleate
E28 Albino Enucleate
Density of ipsilateral cells
(cells/mm2)
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
100
OD
Temporal
Eccentricity (%)

CHAPTER 6 GENERAL DISCUSSION

This thesis has been dedicated to understanding how optic axons in mammals navigate from the eye to their targets. Three main issues have been addressed: The first one is whether there is a quadrant-specific fibre order in the retinal pathway, which may be related to the formation of retinal maps in the primary visual targets. The second issue is to look at how the uncrossed retinofugal pathway is formed during prenatal development, and what the possible factors are that determine and guide growing axons to the ipsilateral tract. And the third, which is closely related to the second, is how the albino gene affects the uncrossed retinal pathway. The understanding of how the abnormal uncrossed pathway is established in the albino may provide useful information to indicate how the normal pathway is developed.

Retinotopic order in the rat optic pathway

One view of how the retinotopic maps are formed in the primary visual targets of mammals is based on the assumption that an ordered and rigid retinotopic arrangement of retinal fibres is maintained along the adult optic pathway (Polyak, 1957; see also Horder and Martin, 1978). This idea is argued against by the growing evidence that fibres from one eye do not maintain their neigbourhood relation in the adult optic nerve (Horton et al., 1979; Naito, 1986 and 1989; Baker and Jeffery, 1989; Jeffery, 1990), and these observations suggest that a similar loss of fibre order in the nerve may also occur during development.

The loss of quadrant-specific fibre order in the developing optic stalk has been

clearly shown in the embryonic rat (chapter 2), signaling that the cues that govern the formation of fibre order at the postoptic stalk are gradually weakened in the more central segment the stalk. This loss of fibre order in the embryonic stalk indicates that the formation of retinotopic maps in the targets cannot simply rely on a maintenance of neigbourhood relation along the optic pathway.

In the chiasmatic regions, there is an active sorting process of retinal axons in which a new fibre order is established in the tract according first to the chronologic order of fibre arrival at the tract (Walsh et al., 1983; Walsh and Guillery, 1985) and second, to the location of their cell bodies in the dorso-ventral axis of the retina, as a clear segregation of dorsal and ventral fibres can be seen in the tract (see chapter 2). This loss (or partial loss) and recovery of retinotopy in the retinofugal pathway was also reported in teleosts and amphibians (Scholes, 1979; Bunt, 1982; Springer and Mednick, 1986a and 1986b; Reh et al., 1983; Fawcett, 1981; Fawcett et al., 1984), indicating that the loss and regaining of fibre order is a common process during the formation of the optic pathway in these species. The reason why there is always a change in fibre order within the optic pathway is unknown, but as suggested in the past studies (Scholes, 1979; Springer and Mednick, 1986b), this change in fibre order may represent a process in which the retinal axons are rearranged in such a way that enables them to match their terminal positions in the target structures.

How these changes in fibre order from the stalk to the tract are achieved during development is far from being understood. It has been suggested that there is a change in the local cue at the chiasm, possibly through the alteration of the glial environment (Walsh, 1986; Guillery and Walsh, 1987), that may trigger a reshuffling

of fibres into a chronotopic order in the chiasmatic region. Moreover, since the fibres from the dorsal and ventral retina are mingled completely at the chiasm but are segregated in the tract, it appears that the dorsal fibres are marked differently from the ventral fibres so that they have different responses to the cues that set up the tract order. It leads to an interesting idea that the development of fibre order in the optic tract depends on a dorso-ventral difference in retinal axons.

Development of the uncrossed retinofugal pathway - an interaction between the retinal marker and binocular fibre interaction at the chiasm

The lack of topography of retinal axons in the chiasmatic region indicates clearly that the uncrossed fibres cannot be guided to the ipsilateral tract simply by mechanical means. It has been demonstrated that the uncrossed axons are mingled extensively with the crossed axons along the whole length of the stalk (mouse: Collelo and Guillery, 1990; rat: see chapter 3; ferret: Baker and Colello, personal communication), indicating that there must be an intrinsic difference that marks the uncrossed axons from the crossed axons, so that they can respond differently when they read the local cue at the chiasm (Godement et al., 1990; Sretavan, 1990a). It can be concluded here that the chiasmatic choice of growing axons cannot be achieved simply by a chiasmatic cue. There must also be a non-chiasmatic property interacting with the chiasmatic read out to determine the development of the uncrossed pathway.

As argued earlier (see chapter 3), the intrinsic difference between the crossed and uncrossed axons is very likely to be set up in the retina. It has been assumed that cells that are destined to take an uncrossed route might express a marker that makes

these cells different from the cells that go contralaterally. The nature of this marker is of course unknown. Nevertheless, this retinal marker must be expressed early in development as a well defined concentration of ipsilateral cells within the temporal crescent is found during the prenatal life of the animals (mouse: Godement et al., 1987b; Colello and Guillery, 1990; rat: Chan, see chapter 3; ferret: Baker and Colello, unpublished data; monkey: Chalupa and Lia, 1991). And it must be expressed preferentially by cells in the temporal crescent so that a gradient of distribution of this marker is established across the nasotemporal retina.

Cells that possess this retinal marker have the ability to read the chiasmatic cue as a signal to turn at the chiasm and enter the ipsilateral tract. One of the possible cues that guides fibres to enter the ipsilateral tract is the interaction with fibres from the other eye at the chiasm. Absence of this binocular interaction at the chiasm, as in the adult mouse (Godement et al., 1987a) and ferret (Guillery, 1989) that have one eye removed prenatally, produces a reduced uncrossed retinal pathway. It has been confirmed in the present studies that in rats and ferrets after an early monocular enucleation, the uncrossed component of the remaining eye is reduced in the newborn animals (chapter 4 and 5). Further, it has been shown here that the development of the uncrossed pathway, in either pigmented or albino animals, requires the binocular fibre interaction at the chiasm.

It is of great interest to know how the cells that possess the retinal marker are distributed in a gradient across the nasotemporal retina. The answer is of course unknown. However, the fact that an early monocular enucleation can either reduce the number of ipsilaterally projecting cells in the temporal crescent (in rats and ferrets)

or abolish this concentration of ipsilateral cells in the temporal retina as a whole (in ferrets), but can never move the line of decussation, suggests strongly that the setting of the distribution of retinal marker is independent of the binocular fibre interaction at the chiasm.

The formation of the gradient of distribution of cells that have the potential to go ipsilaterally is best explained by a factor in the retina which induces the ganglion cells to express the retinal marker. This factor may exist across the nasotemporal retina in the form of a gradient or step-function. How this gradient is set up and what its possible nature would be is far from clear. However, the fact that different ganglion cell classes have distinct decussation patterns, and that the earlier the cells are born the higher chance their axons have to take an uncrossed route at the chiasm, indicates that the distribution of this factor is shifted gradually towards the temporal retinal margin as development proceeds.

Is there a pioneer pathway for the uncrossed axons?

It has been found in the rat that the adult pattern of the uncrossed projection, in which most axons arise from the temporal crescent and only a few from the central retina, does not appear at the beginning of the pathway development (chapter 3). Indeed, the earliest ipsilateral cells lie in the central retina. The appearance of central ipsilateral cells before the ipsilateral cells in the temporal crescent is also seen in the mouse (Godement et al., 1987b; Colello and Guillery, 1990) and the ferret (Baker and Colello, personal communication). So far it is unclear what possible role the axons of these early central ipsilateral cells may have, but it has been suggested recently that

these early uncrossed axons from the central retina may act as "pioneer" or "pathfinder" axons, for the development of the late arriving axons in the uncrossed pathway of the mouse (Godement et al., 1990; Sretavan, 1990a).

There is no evidence to support the idea that the early uncrossed axons from the central retina may pioneer the late uncrossed axons. This idea could perhaps be tested by eliminating selectively the early central ipsilateral cells and their axons and observing the changes that occur in the uncrossed pathway of this eye, but this is not simple and has not yet been done.

It has been shown in the present (chapter 4 and 5) and the past studies (Godement et al., 1987a; Guillery, 1989) that eliminating the fibres from one eye can reduce the uncrossed component in the surviving eye. Further, it has been demonstrated in ferrets that the early eye removal can even abolish the concentration of ipsilaterally projecting cells in the temporal crescent. All these results indicate clearly that the uncrossed axons from one eye, indeed, depend very much on the interaction with fibres from the opposite eye.

This binocular fibre interaction may be a direct axo-axonal interaction or it may be through the glial elements in the chiasm. If it is a direct interaction between fibres from the two eyes, then it would be very interesting to know whether fibres from one eye are guided or "pioneered" by the crossed fibres from the central retina of the other eye in order to innervate the ipsilateral tract, as these crossed fibres are the only ones that crossed the midline early to interact with the uncrossed fibres of the opposite eye (Godement et al., 1990; Sretavan et al., 1990a and 1990b).

Development of the albino uncrossed pathway

It has been shown that there is apparently no difference in the quadrant-specific fibre order along the whole length of the optic stalk in albino and pigmented rat embryos (chapter 2). These results rule out the view that the misguidance of of optic fibres in the albino chiasm is due to a gross alteration in fibre topography within the stalk, which is claimed to be produced by an absence of pigment in the albino stalk (Silver and Sapiro, 1981; Webster et al., 1988). The albino gene is unlikely to affect fibres within the stalk, but indeed very likely to act either on the fibres at the chiasm or on the cells in the retina, or both.

It has been shown in a recent study that if one eye is removed prenatally in a pigmented ferret, a reduced uncrossed retinogeniculate projection is produced which is very similar to the abnormal uncrossed termination pattern in the albinos (Guillery, 1989). Further, the prenatal monocular enucleation has been shown to have no apparent effect on the uncrossed retinogeniculate projection in the albino ferret (Guillery, 1989). These results led to the suggestion that the albino abnormality may be developed as a consequence of the absence or weakening of the binocular fibre interaction at the chiasm. The idea that early monocular enucleation in pigmented animals can produce an albino-like uncrossed pathway and the related idea that an early monocular enucleation does not affect the albino pathway have now been proven wrong. It has been shown in both rats and ferrets that a reduced uncrossed retinal component, in terms of retinal ganglion cells, can be produced after an early enucleation, but the decussation line in the pigmented enucleate is not shifted, comparable to the shift seen in the albino retina (chapter 4 and 5). However, these

results do not rule out the possibility that the albino gene may act on weakening the binocular fibre interaction at the albino chiasm, but they suggest that the albino gene is more likely to act at another locus in the optic pathway, possibly in the retina.

By looking at the early prenatal development of the uncrossed pathway in the rat, it is evident that the albino mutation affects only the route of axons from the temporal crescent but not the axons from the central retina (chapter 3). These results lead to the suggestion that the albino gene may act on the retinal ganglion cells by altering the expression of a retinal marker that marks the cells that go ipsilaterally, and this effect is especially strong on the cells near the decussation line, causing a shift of the decussation line towards the temporal retinal margin in albino animals as shown in the newborn albino rat and ferret (chapter 4 and 5). However, how the expression of the retinal marker is altered in the albino retina is far from clear, but it appears to be closely linked to the underproduction of the retinal pigment (Sanderson et al., 1974; LaVail et al., 1978; Creel et al., 1982). The issue of how hypopigmentation affects the directional choice of retinal axons at the chiasm is definitely worth looking into in the future studies though it would not be easy.

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