

Retinal axon divergence in the optic chiasm: midline cells are unaffected by the albino mutation

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SUMMARY

The visual pathway in albino animals is abnormal in that there is a smaller number of ipsilaterally projecting retinal ganglion cells. There are two possible sites of gene action that could result in such a defect. The first site is the retina where the amount of pigmentation in the retinal pigment epithelium is correlated with the degree of ipsilateral innervation (LaVail et al. (1978) *J. Comp. Neurol.* 182, 399-422). The second site is the optic chiasm, the site of retinal axon divergence. We investigated these two possibilities through a combination of *in vivo* and *in vitro* techniques. Our results demonstrate that the growth patterns of retinal axons and the cellular composition of the optic chiasm in albino mice are similar to those of normally pigmented mice, consistent with the albino mutation exerting its effects in the retina, and not on the cells from the chiasmatic midline. We directly tested whether the albino

mutation affects the chiasm by studying 'chimeric' cultures of retinal explants and chiasm cells isolated from pigmented and albino mice. Crossed and uncrossed axons from pigmented or albino retinal explants display the same amount of differential growth when grown on either pigmented or albino chiasm cells, demonstrating that the albino mutation does not disrupt the signals for retinal axon divergence associated with the albino optic chiasm. Furthermore, *in vitro*, a greater proportion of albino retinal ganglion cells from ventrotemporal retina, origin of uncrossed axons, behave like crossed cells, suggesting that the albino mutation acts by respecifying the numbers of retinal ganglion cells that cross the chiasmatic midline.

Key words: optic chiasm, albino, retinal axon divergence

INTRODUCTION

Retinal axon divergence is a powerful model for studies of axon guidance, because both growth and inhibition of axon extension occurs across the neuraxis in the optic chiasm. Albino mammals offer a useful neurological mutation for studying axon guidance and formation of the visual pathways. The albino mutation leads to an abnormally small uncrossed retinofugal pathway (Guillery et al., 1995), such that the number of retinal ganglion cells projecting ipsilaterally is reduced by roughly one half (mouse: Rice et al., 1995; ferret: Cucchiari, 1991; rat: Chan et al., 1993; hamster: Thompson et al., 1995). Understanding the site of gene action in the albino would lead to insights about normal mechanisms of axon guidance.

The gene affected in albino mutants encodes tyrosinase, the key enzyme in melanin synthesis (Levine, 1993). The amount of tyrosinase activity and the size of the uncrossed retinofugal pathway are closely correlated (LaVail et al., 1978; Guillery et al., 1987), suggesting that chiasmatic pathway choice is causally linked to tyrosinase activity. Furthermore, insertion of a functional tyrosinase transgene into albino mice is sufficient to correct the abnormal visual phenotype, demonstrating that the tyrosinase gene is involved in determining the laterality of retinal ganglion cell projection (Jeffery et al., 1994). The

mechanism by which tyrosinase activity affects the size of the uncrossed projection, however, remains unknown.

There are two possible sites of gene action that could lead to an imbalance in the numbers of cells that cross or do not cross the midline. The first site is the retina where both the amount of tyrosinase activity and pigmentation of the retinal pigment epithelium are thought to direct the degree of ipsilateral innervation (Sanderson et al., 1974; LaVail et al., 1978). Moreover, recent analyses of retinal axon trajectory in normally pigmented and albino rats and ferrets during normal development and following early monocular enucleation also point to the retina as the site of action of the albino mutation. In albinos, the retinal line of decussation separating crossed and uncrossed retinal ganglion cells is shifted peripherally. Prenatal enucleations reduce the uncrossed pathway in both normally pigmented and albino animals without altering the line of decussation, indicating that the developmental mechanisms affected by the early enucleations are distinct from those producing the albino abnormality (Chan et al., 1993; Chan and Guillery, 1993).

The second possible site of action of the albino mutation is the optic chiasm, the site of retinal axon divergence. Novel cellular configurations have been uncovered in the chiasmatic midline (McKanna, 1992; Silver et al., 1993; Reese et al.,

1994; Sretavan et al., 1994; Marcus et al., 1995; Marcus and Mason, 1995). In vitro studies revealed that membranes (Wizenmann et al., 1993) or cells (Wang et al., 1995a) isolated from the chiasmatic midline can induce differential growth from crossed and uncrossed retinal ganglion cells, suggesting that the chiasm itself may be affected in the albino, a possibility that has not been examined directly.

Our studies of dye-labeled axons in the intact embryonic mouse brain demonstrated that retinal axon divergence occurs in a midline zone occupied by early differentiated neurons and glia (Godement et al., 1990, 1994; Marcus et al., 1995; Marcus and Mason, 1995). In a culture setting, we confronted retinal explants containing crossed and uncrossed axons with cells dissociated from the chiasmatic midline (Wang et al., 1995a). Compared to axon growth from other retinal regions, the lengths and numbers of axons from ventrotemporal retina, the origin of uncrossed axons, were reduced. Furthermore, uncrossed, but not crossed, axons avoided clusters of chiasm-derived neurons and glia, supporting the hypothesis that cues associated with cells in the optic chiasm are involved in chiasmatic pathway choice.

In this study, we examined whether the albino mutation affects retinal axon guidance at the retinal or chiasmatic level. First, we studied the patterns of retinal axon growth and the cellular composition of the optic chiasm during both the early and late periods of optic pathway development. Second, using our culture paradigm, we directly tested whether the albino mutation affects the chiasm by studying 'chimeric' cultures of retinal explants and chiasm cells isolated from pigmented and albino mice. Cocultures of normal and mutant tissue have been used to examine the site of action of another neurological mutation, the *weaver* mouse, where defects in neuron-glia interactions result in the failure of granule cell migration in the cerebellum (Hatten et al., 1986; Gao et al., 1992). Our results reveal that retinal axon trajectories and resident chiasm cells in the albino are indistinguishable from those in normally pigmented animals. In addition, pigmented and albino retinal ganglion cells are similarly affected by chiasm cells from either albino or pigmented animals, demonstrating that the chiasm is unaffected in the albino. Because a greater proportion of retinal ganglion cells from ventrotemporal retina in vitro behave like crossed cells, our results suggest a model in which the albino mutation acts by respecifying the numbers of retinal ganglion cells that cross the chiasmatic midline.

MATERIALS AND METHODS

All experiments were carried out with normally pigmented C57BL/6J or albino C57BL/6J-*c^{2J}/c^{2J}* mice. Mice were originally obtained from Jackson Laboratory and subsequently kept in a timed-pregnancy breeding colony in this department. The time of conception was considered midnight before the day on which a plug was found, and noon the following day embryonic day 0.5 (E0.5). Embryonic age was confirmed by comparing the crown-to-rump length with measurements given in Theiler (1972).

Pregnant mothers carrying embryos between the ages of E12.5 and E16.5 were anesthetized with a mixture of ketamine and xylazine, and the embryos removed one at a time by Cesarean section.

DiI labeling of retinal axons in fixed tissue

Embryos used for dye labeling were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, and crystals of DiI (1,1'-dioctadecyl-

3,3,3',3'-tetramethylindocarbocyanine perchlorate) applied to the retinas of E12.5-E16.5 embryos as described previously (Marcus et al., 1995; Marcus and Mason, 1995). Dye was applied to the optic nerve head of embryos between E12.5 and E13.5 to ensure labeling of the maximum number of axons. Small crystals of dye were applied to the ventrotemporal retina in embryos older than E14.5 to label a small number of ipsilaterally directed axons. Tissue was stored in the dark at room temperature in buffer containing sodium azide for 5-10 days to allow transport of the dye.

Dye-labeled optic axons were visualized in both whole mounts (E12.5-E13.5) and 75 µm thick vibratome sections (E14.5-E16.5). Whole-mount preparations were prepared by dissecting free the optic stalks and the region of the ventral diencephalon including the presumptive optic chiasm. Dye-labeled axons in whole mounts and sections were viewed with fluorescent illumination on a Zeiss Axioplan microscope.

DiI was photoconverted to a permanent brown reaction product as described previously (Marcus et al., 1995). Following photoconversion, labeled axons were drawn with a camera lucida. Four to five embryos at each age were examined.

Immunocytochemistry: antibodies

Previously we identified a cellular specialization centered around the chiasmatic midline (Marcus et al., 1995; Marcus and Mason, 1995). Tissues from pigmented and albino mice were prepared and immunostained as described previously (Marcus et al., 1995; Marcus and Mason, 1995). Monoclonal antibody (mAb) RC2 (originally a gift of Dr J.-P. Misson and V. Caviness, Massachusetts General Hospital) specifically stains immature astrocytes in the mouse central nervous system (Misson et al., 1988) and a midline palisade of radial glia in the optic chiasm. mAb 480-1.1 (Developmental Studies Hybridoma Bank; Solter and Knowles, 1978) recognizes stage-specific embryonic antigen-1 (SSEA-1), which is expressed in both the immune and nervous systems (Dodd and Jessell, 1985), and in a V-shaped wedge of cells intersecting the radial glial palisade.

Culture methods

Retinal explants

Retinal explants from E14 embryos were collected in ice-cold DMEM/F12 medium with 15 mM Hepes buffer (Gibco). A small orienting cut was made before dissection, at the ventral point of the diamond-shaped opening of the eye cup formed by the pigmented epithelium in the pigmented mice. In albino mice, this position was approximated. The eye was dissected free and the pigmented epithelium, lens and vitreous together with the blood vessels were removed. A 250 µm wide strip of retina was cut, incorporating the periphery of both ventrotemporal (VT, source of uncrossed fibers) and dorso-temporal (DT, a source of crossed fibers) retina (Fig. 1).

Dissociation of chiasm cells

When possible, dissociated chiasm cells were obtained from the same embryos used for the retinal explants. Brains were dissected free from the skull and the region of the developing optic chiasm isolated by cutting a piece of tissue, about 200 µm to either side of the midline, that extended caudally about 600-700 µm from the juncture of the optic nerves with the brain. Chiasm tissue chunks were dissociated into a single cell suspension in cold DMEM/F12 medium containing trypsin and collagenase as described (Wang et al., 1995a).

Retinal explants and dissociated chiasm cells were cocultured in small-hole (3.5 mm diameter) glass coverslip microwells sequentially pretreated with polylysine (100 µg/ml, Sigma cat. no. P-1024) overnight at 4°C and laminin (20 µg/ml, Sigma) for 30 minutes at 35.5°C. Retinal explants were plated ganglion cell side down, in serum-free DMEM/F12 medium [DMEM/F12 medium supplemented with 1% BSA, 5 mg/l insulin, 5 mg/l transferrin, 5 µg/l sodium selenite (Sigma medium supplement, Cat. No. I-1884) and 20 units/ml penicillin/streptomycin] containing 0.4% methylcellulose (Sigma),

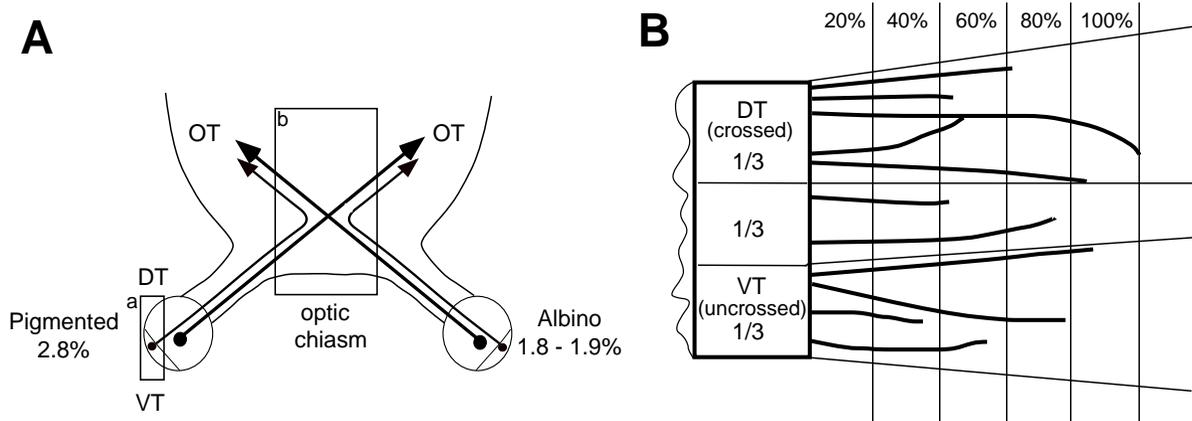


Fig. 1. Diagrams depicting the paths of crossed and uncrossed retinal axons from the retina to the optic tract (OT) (A) and the culture paradigm (B). (A) The numbers indicate the percent of retinal ganglion cells that project ipsilaterally in pigmented and albino animals (from Rice et al., 1995). The boxed regions depict (a) the region of the retina from which retinal explants were made and (b) the area of the brain used for dissociated chiasm cells. (B) Retinal explants containing a source of crossed (DT, dorsotemporal) and uncrossed (VT, ventrotemporal) retinal axons were cultured either on polylysine/laminin alone or in combination with cells dissociated from the chiasmatic midline (see Materials and Methods for details).

the latter added to ensure adhesion of the retina to the substratum. After allowing the retinal explants to attach to the substratum for 2-3 hours, the medium was removed from the explants and chiasm cells were added at a density of 15,000 cells/mm² in a total volume of 15 μ l of medium. Following adhesion of the cells to the substratum, fresh DMEM/F12 serum-free medium was added to the cultures which were incubated at 35.5°C for 22-24 hours.

Cultures were fixed for 30 minutes in 4% paraformaldehyde in phosphate buffer and drawn with a camera lucida attached to an Olympus CK2 inverted microscope fitted with phase optics. In order to reduce investigator bias, the identity (i.e. right or left eye) of the retinal explant was not revealed until after the cultures were drawn, thereby blinding the DT or VT origin of the retinal neurites. Selected cultures were coverslipped with Gelmount (Biomedica Corp.) and photographed on a Zeiss Axioplan microscope.

Retinal explants and dissociated chiasm cells from albino and pigmented mice were cultured in all four possible combinations, i.e., pigmented retina/pigmented chiasm cells, pigmented retina/albino chiasm cells, albino retina/pigmented chiasm cells, albino retina/albino chiasm cells. A total of 75 explants from 21 experiments was analyzed. An additional 29 retinal explants were grown on polylysine/laminin alone.

Analysis

Only cultures containing retinal explants exhibiting good growth along the entire cut edge were included in the analysis. Halfway along the long axis of the explant was considered the dividing point between VT and DT retina. Each explant was divided into thirds, and the neurites in the outer thirds analyzed, because the middle third was thought to contain a mixture of the two populations (Fig. 1).

Two different methods were used to quantify the degree of axon outgrowth. First, in the end thirds of the explant, the distance to which the longest neurite extended, as well as the distance from the explant edge to the point where the 10 longest neurites extended was measured, providing two different numerical values for the degree of neurite outgrowth (Table 1). The distance to where the 10 longest neurites extended was included because it gave a value for a population of neurites from each sector of retina, rather than simply reflecting the longest neurites. Data from at least 5 experiments from each category were analyzed by comparing the lengths of VT and DT retina within each explant using the paired Student's *t*-test (Cricket Graph, Cricket Software).

Table 1

Condition	n	Average length		DT - VT	% difference DT and VT		P value
		DT	VT		DT	VT	
(A) Average lengths and differences between DT and VT based on the lengths of the 10 longest neurites							
PRet/Laminin	18	1100±146	956±242	144	14	0.04	
ARet/Laminin	10	1033±123	947±221	86	8	0.29	
PRet/PChiasm	20	856±282	614±224	242	28	0.005	
PRet/AChiasm	23	711±188	515±202	196	28	0.002	
ARet/AChiasm	17	703±192	511±151	192	27	0.003	
ARet/PChiasm	15	961±230	739±208	222	23	0.01	
(B) Average lengths and differences between DT and VT based on the lengths of the longest neurites							
PRet/Laminin	18	1278±223	1151±273	127	10	0.13	
ARet/Laminin	10	1236±124	1150±200	86	8	0.25	
PRet/PChiasm	20	1112±294	832±252	280	25	0.003	
PRet/AChiasm	23	902±227	699±247	203	23	0.005	
ARet/AChiasm	17	889±213	723±199	166	19	0.03	
ARet/PChiasm	15	1178±245	993±214	185	16	0.04	

Second, a graphical representation of the pattern of outgrowth was obtained (Fig. 1B). First, the distance from the explant edge to the longest neurite in either of the end thirds was measured. Using this length as 100%, this distance was divided into five equal intervals. The number of VT and DT neurites in each interval was counted, and the average number of neurites in each 20% interval was calculated and presented graphically (Fig. 8).

RESULTS

The ipsilateral retinofugal pathway in albinos appears normal at all ages of development

Previous studies identified two distinct components of the developing uncrossed retinofugal pathway in normally pigmented animals (Godement et al., 1987; Colello and Guillery, 1990; Baker and Colello, 1994). The first is an early, transient component arising from dorso-central retina. The

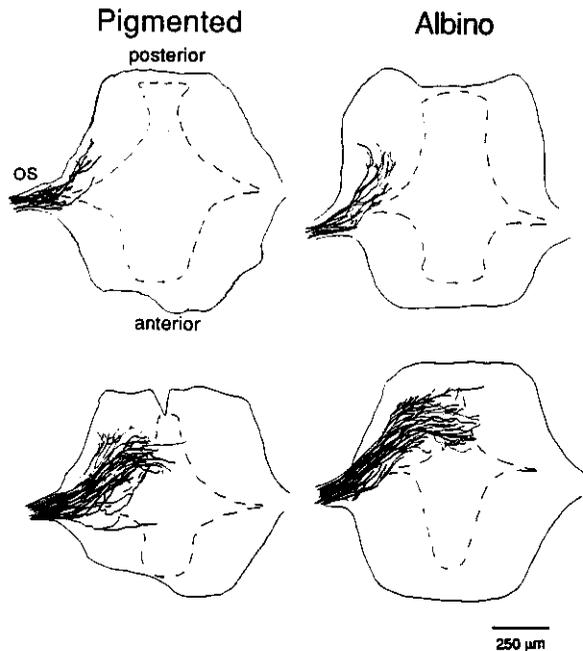


Fig. 2. The early pattern of optic axon growth in the ventral diencephalon of albino animals is similar to that of pigmented animals. Camera-lucida drawings of whole mount preparations of the ventral diencephalon of E12.75 embryos containing DiI-labeled, photoconverted retinal axons. Whole mounts are viewed from the ventral surface, and the dashed lines indicate the location of the third ventricle. The top two panels are of embryos at a slightly earlier developmental stage than those in the bottom two panels. In the top panels, optic axons leave the optic stalk (os) and initially course posteriorly in the lateral region of the ventral diencephalon. In the bottom two panels, crossed axons have turned medially in the posterior portion of the presumptive chiasm (see Results and Marcus and Mason, 1995, for details).

second component forms later in development, arises from ventrotemporal retina and contributes to the adult, permanent ipsilateral projection. Like their normally pigmented littermates, albino animals have two distinct components (Chan et al., 1993). The first, early component is normal in the albino, whereas the second component is significantly reduced, suggesting that the albino mutation affects the late VT component of the uncrossed pathway selectively.

In addition to the different retinal origins of the early and late components, the pathways taken by the early and late uncrossed retinal fibers through the developing optic chiasm differ in a number of respects (Marcus et al., 1995; Marcus and Mason, 1995). Therefore, we investigated the effects of the albino mutation on retinal axon trajectory by comparing the projection patterns of the developing ipsilateral pathway in albino and pigmented animals at different ages.

The pathways taken by DiI-labeled axons in albino animals were indistinguishable from those in their pigmented counterparts (Figs 2, 3). The first retinal axons entered the rostroventral base of the diencephalon between E12.5 and E12.75. Both crossed and uncrossed axons initially coursed posteriorly in the lateral portion of the ventral diencephalon. Crossed axons turned toward the midline, whereas ipsilaterally projecting axons grew directly into the ipsilateral optic tract (Fig. 2). This pattern was markedly different from the later pattern of growth

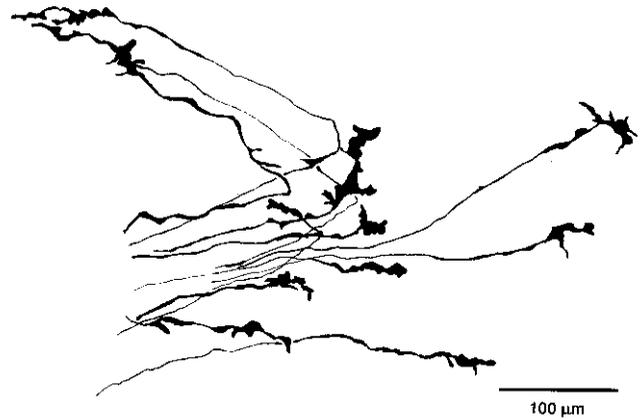


Fig. 3. The trajectories of crossed and uncrossed axons in the optic chiasm of E15.5 albino mice are similar to those of their pigmented counterparts. Composite figure made from Camera-lucida drawings of DiI-labeled, photoconverted axons in the region of the optic chiasm, where crossed and uncrossed axons diverge. Crystals of DiI were placed near the ventrotemporal retinal border. For comparison with trajectories of crossed and uncrossed axons in pigmented mice, see Fig. 3 in Godement et al. (1990).

in both albino and pigmented animals at E15-16 (Fig. 3). In albino and pigmented animals between E15 and E16, crossed and uncrossed axons initially grew toward the midline before diverging in a region 150-200 µm to either side of the midline, rather than growing in the lateral portion of the brain. Within this midline zone, growth cones on uncrossed axons formed highly complex shapes prior to turning back toward the ipsilateral optic tract (Fig. 3 and figure 3 in Godement et al., 1990). Thus, although the albino VT component is decreased in size, its pathways and growth cone forms appear normal. Overall, the early and late pathways of retinal axons in the developing chiasm appear unaffected by the albino mutation.

The cellular organization of the optic chiasm appears normal in albino animals

We identified a specialized cellular arrangement that occupies the zone within which crossed and uncrossed axons diverge (Marcus et al., 1995; Marcus and Mason, 1995). This cellular specialization consists of a palisade of radial glia and a subpopulation of early differentiating neurons that express SSEA-1 and CD44. Although the same antigens are expressed during both the early and late components of retinal axon growth in the chiasm, the configurations of these cells and the degree of interaction between the optic axons and the resident chiasm cells differ during these two periods. To investigate whether altered interactions between optic axons and resident chiasm cells might contribute to the reduced ipsilateral pathway, we used monoclonal antibodies RC2 and 480-1.1 to examine the cellular architecture of the developing chiasm at different ages in the albino mouse.

As seen in normally pigmented animals, in the albino, a palisade of RC2-positive radial glia straddled the midline during early and late components of retinal axon growth in the chiasm (Fig. 4A,B). Likewise, SSEA-1-positive cells in the albino chiasm were arrayed in the same age-specific conformations seen in normally pigmented animals. Between E12.5 and E13.5, SSEA-1-positive cells formed a 'V'-shaped wedge of cells, in which the tip of the 'V' was located most anteriorly

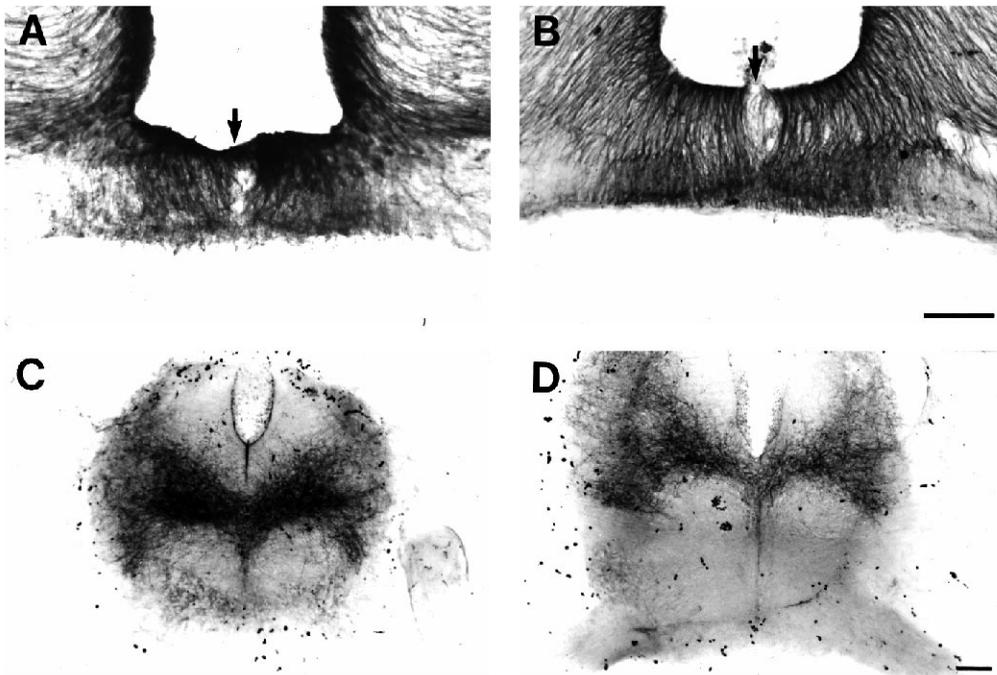


Fig. 4. The cellular composition of the optic chiasm is similar in E15.5 pigmented and albino mice. (A,B) Frontal sections from a pigmented (A) and albino (B) mouse labeled with the RC2 antibody. A palisade of RC2-positive radial glia extends 150–200 μm to either side of the midline (indicated by the arrows). (C,D) Horizontal sections from a pigmented (C) and albino (D) mouse labeled with an antibody against SSEA-1. SSEA-1-labeled cells form a V-shaped pattern in which the anterior tip of the V extends into the midline of the optic chiasm. The section in C is slightly more ventral than the section in D. Scale bars, 100 μm .

orly and the flanking regions extended back along the developing optic tracts (Marcus and Mason, 1995 and data not shown). Between E15 and E17, SSEA-1-positive cells formed a broad band caudal to the chiasm itself, from which a raphe extended rostrally (Marcus et al., 1995 and Fig. 4C,D). These results, together with the axonal projection patterns described above, indicate that the cellular environment of the optic chiasm is normal in albino animals and is consistent with the hypothesis that the albino mutation exerts its effects in the retina.

Albino chiasm cells elicit differential growth from crossed and uncrossed axons from normally pigmented retinas

Retinal explants from normally pigmented and albino animals grew effusively on polylysine/laminin (Fig. 5A,B). After 24 hours in culture, numerous unfasciculated neurites extended from the cut edge of the explant. Consistent with our earlier findings (Wang et al., 1995a), there were slight intrinsic differences in ventral and dorsal retinal axon growth on polylysine/laminin. When the distance from the explant edge to the point where the 10 longest neurites extended was quantified, neurite lengths from VT retina on polylysine/laminin were shorter than those from DT retina by 14% and 8% for pigmented and albino retinas, respectively ($n=18$, $P<0.05$ - pigmented; $n=10$, $P>0.05$ - albino; Fig. 6A, Table 1).

When retinal explants and cells from the chiasmatic midline of pigmented animals were cocultured together, overall retinal lengths of DT and VT retina were reduced relative to growth on polylysine/laminin alone (Figs 5C, 6B, Table 1). More striking, however, was the degree to which the lengths of VT retinal neurites were reduced when cocultured with dissociated chiasm cells. In cocultures of pigmented retinal explants with pigmented chiasm cells, outgrowth from VT retina was reduced by 28% when compared to growth from DT retina ($n=20$, $P=0.005$; 25% and $P=0.003$ for the longest neurites - Fig. 6B; Table 1). This differential reduction in the

growth of VT neurites grown on dissociated chiasm cells reflects a specific growth-reducing effect of chiasm cells on uncrossed axons, rather than simply reflecting intrinsic differences in the ability of dorsal and ventral axons to extend on a given substratum, since the reduction in neurite growth on chiasm cells from VT retina is greater than that for neurites from any other region of the retina (Wang et al., 1995a).

To determine if cells from the albino chiasmatic midline also support the differential growth of crossed and uncrossed axons, and therefore whether or not the albino mutation affects chiasm signals involved in chiasmatic pathway choice, we cocultured strips of retina from normally pigmented animals with cells dissociated from albino chiasm (Figs 5D, 7). Pigmented neurites from VT retina were significantly reduced compared to those from DT retina when grown on albino chiasm cells (28%, $n=23$ for the 10 longest neurites, $P=0.002$; 23% and $P=0.006$ for the longest neurites - Fig. 6B; Table 1). This is the same degree of reduction obtained for growth of pigmented retinas on pigmented chiasm cells. Moreover, the percent reduction in length of VT versus DT neurites, and the overall average difference in length and pattern of growth, were similar whether pigmented retinas were grown in the presence of chiasm cells from pigmented or albino animals (Figs 5C,D, 8; Table 1). These results indicate that chiasm cells from both albino and pigmented animals can elicit differential growth of crossed and uncrossed axons. Therefore, the albino mutation does not affect the ability of chiasmatic midline cells to signal retinal axon divergence.

Chiasm cells elicit a weaker differential response from albino retinal neurites

The data presented above are consistent with the albino mutation exerting its effects at the retinal, rather than the chiasmatic, level. To investigate this possibility further, we cocultured retinal explants from albino animals with dissociated chiasm cells from albino or pigmented animals. In cocultures of albino retinal explants with dissociated chiasm cells, the

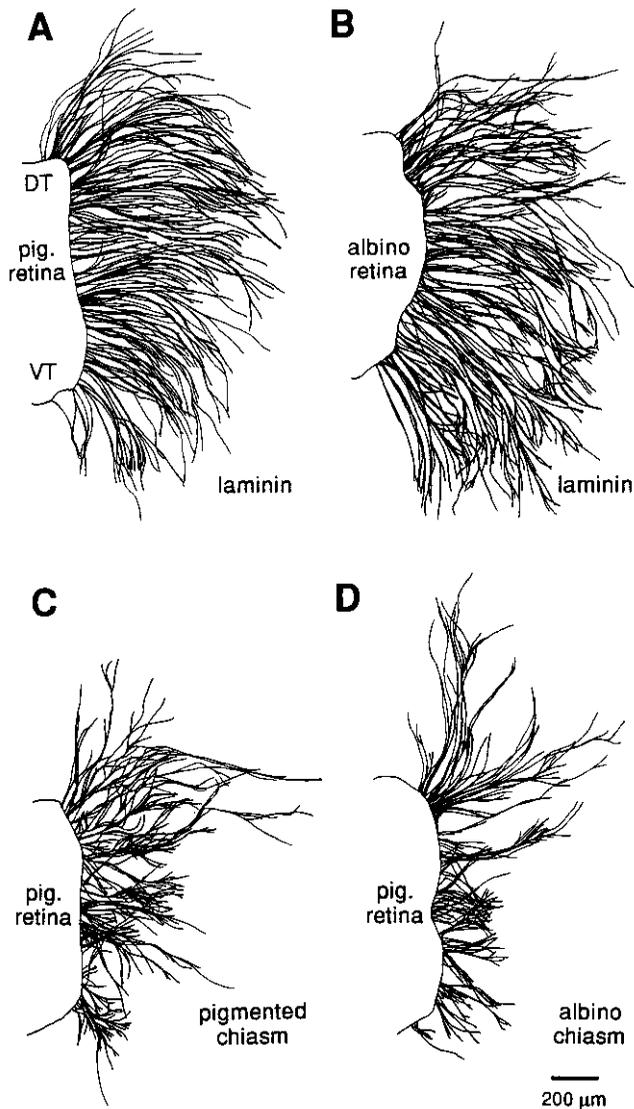


Fig. 5. Camera-lucida drawings of retinal explants grown on polylysine/laminin or on dissociated chiasm cells. All four retinal explants are oriented as shown in A, and the source of the retinal explants and the dissociated chiasm cells are as indicated. Numerous unfasciculated axons extend from both pigmented (A) and albino (B) retinal explants on polylysine/laminin. When pigmented retinal explants are cocultured with either pigmented (C) or albino (D) chiasm cells, ventrotemporal (VT) retinal neurites are shorter than those from dorsotemporal (DT) retina.

overall retinal lengths of both DT and VT neurites were reduced when compared to their growth on polylysine/laminin (Figs 6B, 8). Furthermore, albino retinal neurites responded similarly whether grown on pigmented or albino chiasm cells, as expected if the chiasm cells are unaffected by the albino mutation. The average length of VT neurites from albino retinas was reduced relative to the average length of DT neurites (by 16-27%, Table 1), as was found for VT and DT neurites from pigmented retinas. However, the degree to which the lengths of VT albino retinal neurites were reduced when cultured with either pigmented or albino chiasm cells, was consistently less than that seen with pigmented retinas, no matter what method was used to assess the differential response (by

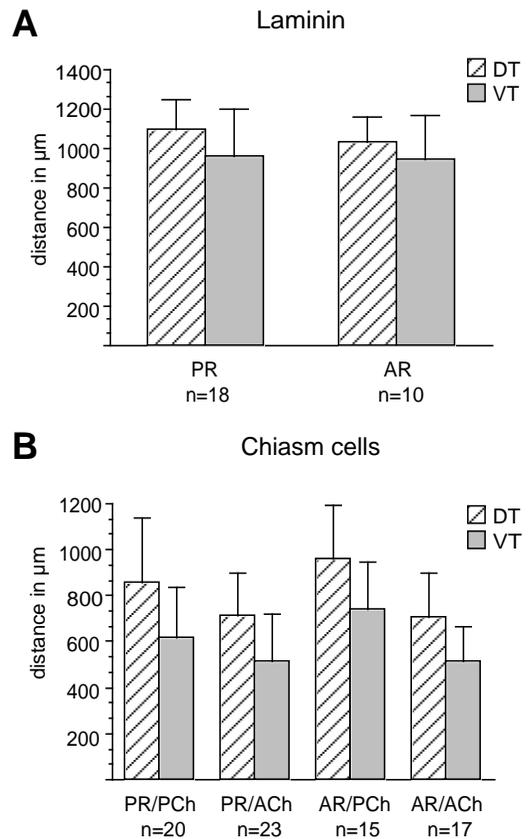


Fig. 6. Comparison of retinal neurite lengths when extending on laminin (A) and on dissociated chiasm cells (B). Neurite length was calculated by measuring the distance from the explant edge to the point where the 10 longest neurites extended, at the end thirds of an explant containing dorsotemporal (DT) and ventrotemporal (VT) retinal regions (see Fig. 1B). Table 1 presents the raw numbers from which the graphs were generated. Note the different scales on the Y axis for the two graphs. Abbreviations. PR, pigmented retina; AR, albino retina; PCh, pigmented chiasm cells; ACh, albino chiasm cells.

1%-9% when compared to the pigmented levels, Table 1; Fig. 8). Moreover, as described above, while the lengths of VT albino retinal neurites grown on polylysine/laminin alone were reduced compared to those from DT retina, this reduction was less than that seen between VT and DT neurites from pigmented retinas grown on polylysine/laminin alone (Table 1). Thus, VT and DT neurites from albino retinal explants respond differentially to chiasm-derived signals and to the laminin substratum, but to a lesser degree than pigmented retinal neurites. Taken together, our results suggest a model in which the albino mutation exerts its effects on the visual pathway by altering the numbers of retinal ganglion cells that are specified to respond to cues for crossing or remaining ipsilateral to the chiasmatic midline (see Discussion).

DISCUSSION

In theory, the albino mutation could affect the proportion of ipsilaterally projecting retinal axons at the retinal or the chias-

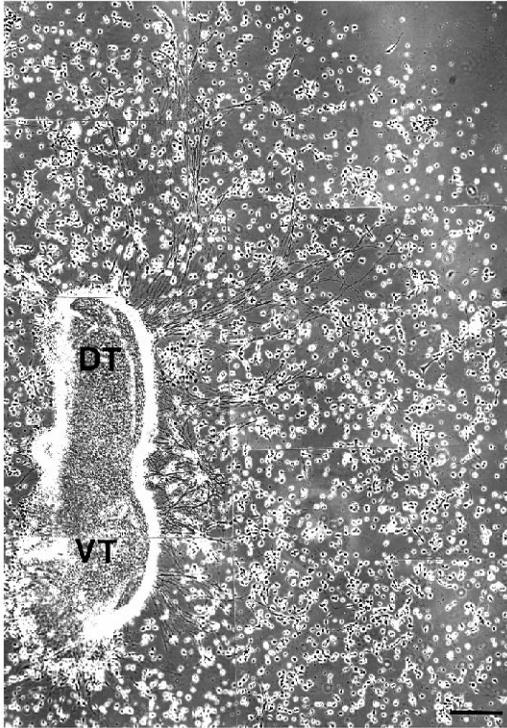


Fig. 7. Coculture of a pigmented retinal explant with albino chiasm cells. Photomontage of the culture drawn in Fig. 5D taken with phase optics, depicting the density of dissociated chiasm cells used in the cocultures. Neurites from ventrotemporal (VT) retina are shorter than those from dorsotemporal (DT) retina. Scale bar, 200 μ m.

matic level. Our results indicate that the growth patterns of retinal axons and the cellular composition of the optic chiasm in albino mice are similar to those in normally pigmented mice. This is consistent with the albino mutation exerting its effects in the retina, and not on chiasmatic midline cells. Moreover, comparison of the early and late components of the developing uncrossed retinofugal pathway demonstrates that the albino mutation does not selectively perturb the early or late growth pattern, consistent with earlier results using albino rats (Chan et al., 1993). Interestingly, despite the normal paths taken by uncrossed axons during early and late visual pathway development, the albino mutation specifically acts to decrease the number of ipsilaterally directed fibers contributing to the late VT component of the uncrossed pathway (Chan et al., 1993).

Pigmentation in optic stalk cells located behind the retina has also been implicated in directing optic axons across the midline (Silver and Sapiro, 1981). In embryonic Siamese cats, abnormal pigmentation and unusual optic stalk morphogenesis were correlated with the misrouting of uncrossed axons prior to their arrival at the chiasm (Webster et al., 1988). Studies in rat, however, revealed no correlation between the size of the adult uncrossed retinofugal pathway and the existence of optic stalk melanin during development (Colello and Jeffery, 1991). Thus, optic stalk pigmentation does not appear to direct the crossed or uncrossed fate of retinal axons.

Our results, as well as those of others, suggest that the albino mutation acts in the retina (LaVail et al., 1978; Guillery et al., 1987; Chan et al., 1993; Chan and Guillery, 1993). Localization of the site of action of the albino mutation to the retina is

further suggested by our *in vitro* data indicating that the chiasm, the other possible site of action, is unaffected in the albino. We directly tested whether the albino mutation affects the ability of albino chiasm cells to signal divergence by comparing the growth of retinal explants from pigmented animals on dissociated chiasm cells from either pigmented or albino mice. When cocultured with chiasm cells from either pigmented or albino animals, the lengths of uncrossed axons from pigmented VT retina were reduced by a similar degree relative to those of crossed axons from pigmented DT retina. This result indicates that the albino mutation does not disrupt the chiasm-derived signals detectable in this assay. Likewise, crossed and uncrossed neurites from albino retinas displayed similar amounts of differential growth when grown on dissociated chiasm cells from albino or pigmented animals. This is the expected result if albino and pigmented chiasm cells are equivalent in their signalling abilities.

The decreased growth of uncrossed versus crossed neurites in our cocultures could imply that compared to crossed neurites, uncrossed neurites have a reduced rate of axon elongation. Three different mechanisms could result in a reduced growth rate. First, uncrossed axons could extend at a slower rate than crossed axons. Second, uncrossed axons could display an increased frequency of stops and starts relative to crossed axons. Third, uncrossed axons could pause for longer durations than crossed axons. Time-lapse video microscopy of retinal axon growth in the intact optic chiasm revealed that uncrossed fibers pause for longer durations than crossed fibers (Godement et al., 1994) suggesting that the third mechanism may be operating in our cultures. Whether or not a retardation in growth either *in vitro*, or at the midline *in vivo*, is prerequisite to or merely associated with the phenomenon of turning into the ipsilateral tract is unknown.

Chiasm cells, however, may present general growth dampening cues to all axons. First, as seen here, the lengths of crossed and uncrossed axons grown on chiasm cells are reduced relative to growth on polylysine/laminin alone. Second, cocultures of retinal and chiasm explants in collagen reveal that the chiasm secretes a signal that dampens the growth of all retinal axons (Wang et al., 1995b). In addition, relative to growth in the optic nerve and tract, all axons have a slow growth rate in the chiasm (C. A. Mason and L.-C. Wang, unpublished data). The presence of general growth-dampening cues in a decision region may be a first step in preparing axons to respond to other cues (see Wang et al., 1995a for discussion).

A decreased length of uncrossed neurites might also be expected if a gradient of maturation existed in the retina such that ventrotemporal retina, the source of uncrossed fibers, was the last to differentiate and extend neurites. Two points argue against this possibility. First, birthdating studies reveal that crossed and uncrossed retinal ganglion cells are generated at the same time (Dräger, 1985). Second, crossed and uncrossed axons grow at the same rate in the path from the optic nerve to the chiasm (C. A. Mason and L.-C. Wang, unpublished observations). Furthermore, while we cannot rule out direct effects of the dissociated chiasm cells on the cell bodies of the retinal ganglion cells, analysis of the interactions between individual growth cones with clusters of dissociated chiasm cells suggests that chiasm cells have effects on the growth cones themselves (Wang et al., 1995a). Growth cones of uncrossed axons avoid clusters of neurons and glia, whereas crossed

axons grow over them, thus mimicking the *in vivo* response of uncrossed and crossed axons to chiasmatic midline cells.

Thus, we have identified a chiasm-derived signal that differentially affects the growth of crossed and uncrossed axons which is unaltered by the albino mutation. However, it is possible that the trypsin and collagenase used to dissociate cells from the optic chiasm cleaves a protein involved in generating the reduced ipsilateral projection in the albino, and which does not fully repopulate the cell surface within our 24 hour culture period (Zajac and Crowell, 1965). If such a protein is disrupted, this implies that there is more than one chiasmatic signal affecting retinal axon growth, because in our *in vitro* assay an effect is seen. Nevertheless, the reduction in the differential response of DT and VT neurites from albino versus pigmented retinal explants when cultured on polylysine alone or on dissociated chiasm cells indicates that the albino mutation does have effects on the retina itself.

Consistent with the albino mutation exerting its effects at the retinal level, the average reduction in length of VT neurites from albino retinas grown on either albino or pigmented chiasm cells was less than that seen with pigmented retinal explants (Table 1). Taken together, our results suggest a model by which the albino mutation results in a reduced ipsilateral pathway. If we consider retinal specification to signify those properties expressed by retinal ganglion cells that direct them to assume either a crossed or uncrossed phenotype, then our results are consistent with a model in which fewer retinal ganglion cells are specified to cross the neuraxis in albino animals.

The decreased amount of differential growth between DT and VT retina in albino versus pigmented retinal explants grown on either type of chiasm cell agrees with this model. Although the difference was not large, a difference was detected by all three methods used to evaluate axon growth in our culture system, and may

reflect the small numbers of uncrossed fibers normally present in pigmented animals (2.8% versus 1.8% in albinos; Rice et al., 1995). Furthermore, in normally pigmented animals, dorsal and ventral axons display an intrinsic difference in their ability to extend on polylysine/laminin alone (Wang et al., 1995a). The differential growth of DT and VT axons from albino retinas on polylysine/laminin was also reduced compared to those from pigmented retinas, further supporting the hypothesis that more axons from VT retina behave like crossed axons in the albino mutant.

If the albino mutation acts to alter the numbers of crossed

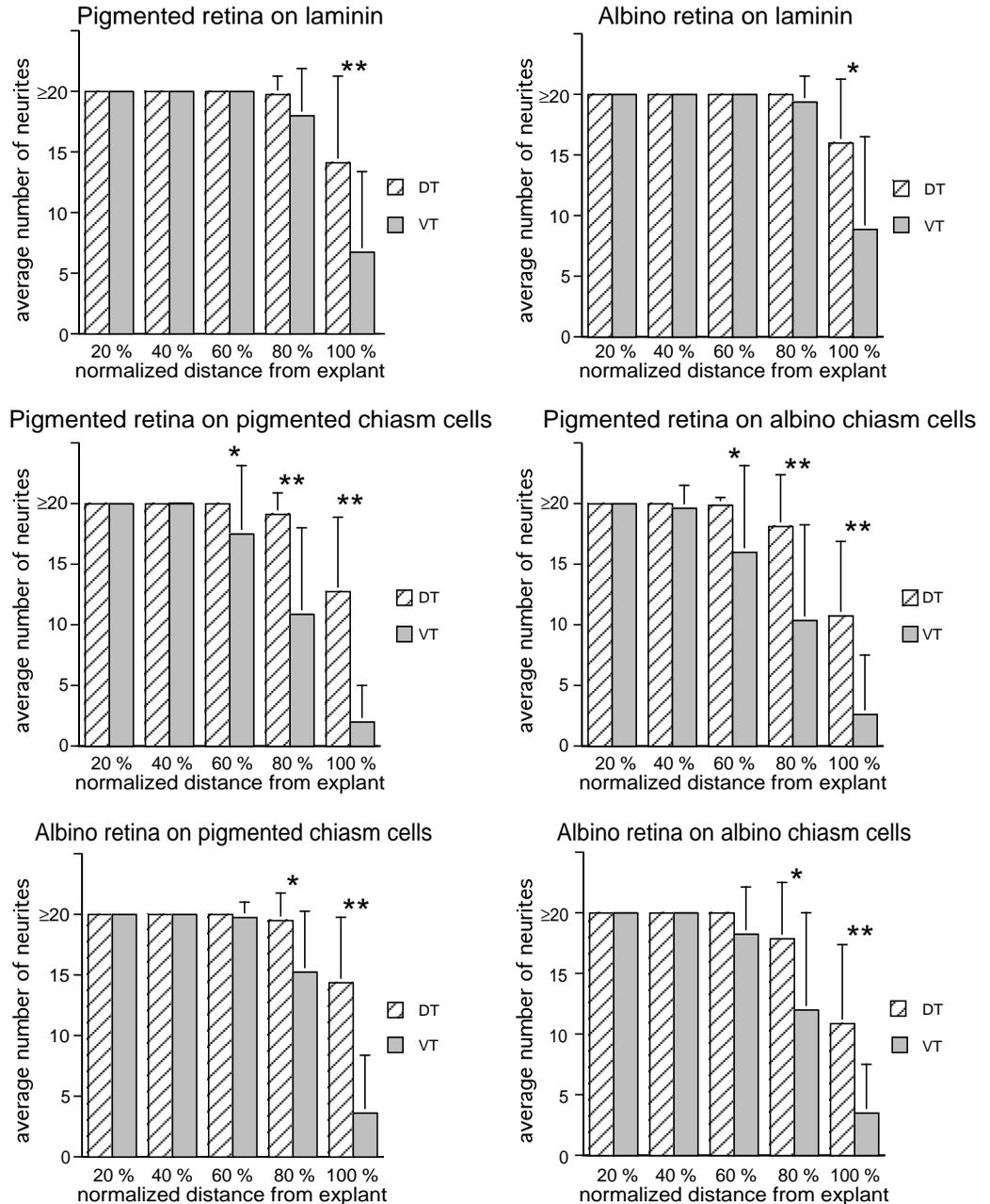


Fig. 8. Graphical representation of the pattern of outgrowth of pigmented and albino retinal explants when grown on polylysine/laminin, pigmented chiasm cells, or albino chiasm cells. The data is from the same experiments shown in Table 1. Error bars represent standard deviations, and the number of DT and VT neurites in each interval was compared using the paired Student's *t*-test. **P* < 0.05, ***P* < 0.001. See Fig. 1 and Materials and Methods for further details.

and uncrossed retinal ganglion cells, the remaining ipsilaterally directed axons arising from albino ventrotemporal retina should behave like those found in normally pigmented animals. Three observations from the current study support this prediction. First, the axonal trajectories of both the early and late albino ipsilateral retinofugal pathway were similar to those of normally pigmented animals. Second, the average maximum lengths of DT and VT neurites from albino or pigmented retinas were similar when grown either on polylysine/laminin or on chiasm cells. Third, cells isolated from the chiasmatic midline elicit differential growth from DT and VT albino retinas, indicating that uncrossed neurites from albino retinal explants can respond to divergence signals associated with chiasmatic midline cells.

The mechanism by which retinal ganglion cells are specified to cross or not cross the midline is still unknown. Recently different genes and molecules with specific dorsal-ventral or nasal-temporal distributions have been found (Hatini et al., 1994, Kaprielian and Patterson, 1994, Savitt et al., 1995), but the contributions of these genes and molecules to retinal specification are unknown. Transcription factors, such as BF-1/BF-2 and SOHo-1 (Deitcher et al., 1994, Hatini et al., 1994) are candidates for producing a crossed or uncrossed phenotype. For example, such molecules might specify retinal ganglion cell identity by promoting expression of receptors for permissive factors that allow axons to cross, or receptors for inhibitory factors that prevent axons from crossing, the midline (see also Godement et al., 1994; Wang et al., 1995a).

A change in the relative numbers of crossed and uncrossed retinal ganglion cells in the albino might also result from alterations of a retinal gradient leading to a shift in the border between the crossed and uncrossed retinal populations (Chan et al., 1993). The border between crossed and uncrossed retinal ganglion cells in the retina is relatively well defined, and is shifted peripherally in albino animals (Shatz and Klotz, 1982; Bunt et al., 1983; Morgan et al., 1986; Cucchiari, 1991; Chan and Guillery, 1993). Moreover, there may be a correlation between the degree of pigmentation and the peripheral location of this border. The border between crossed and uncrossed axons may represent a threshold with cells on one side of the threshold able to cross, and cells on the other side unable to cross, the chiasmatic midline (Chan et al., 1993). A peripheral shift in such a threshold in the albino would thus lead to a rerouting of normally ipsilateral fibers across the midline.

The relative numbers of crossed and uncrossed axons in the albino, and thus the retinal line of decussation, might be altered by disruption of the normal spatiotemporal sequence of retinal ganglion cell production (Jeffery et al., 1994). In pigmented animals, retinal ganglion cells are produced in a central-to-peripheral gradient (Greiner, 1980; Robinson, 1987). As retinal development proceeds, more and more of the later-born cells are committed to a crossed course (Dräger, 1985; Reese and Colello, 1992; Baker and Reese, 1993). Studies in albino animals have revealed that the spatiotemporal patterns of cell production and layer differentiation in the retina are affected by the degree of pigmentation (Webster and Rowe, 1991; Jeffery and Kinsella, 1992).

In conclusion, we have shown that one site of action of the albino mutation is in the retina. This result furthers our understanding of how the albino mutation works to affect the proportions of retinal ganglion cells that project to either side of

the brain. Our results are most consistent with a model in which the albino mutation leads to an alteration in the numbers of cells specified to cross, or not cross, the midline. Furthermore, this study establishes the usefulness of our in vitro system for testing candidate cells and molecules with potential roles in retinal axon divergence. With the site of action of the albino mutation established, the stage is set to explore the mechanism(s) by which defects in the tyrosinase gene lead to a reduction in the ipsilateral retinofugal pathway in albino animals.

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