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Differential reaction of crossing and non-crossing rat retinal axons on cell membrane preparations from the chiasm midline: an in vitro study

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SUMMARY

In the rat, a small subpopulation of retinal ganglion cell axons forms a persistent projection to the ipsilateral half of the brain. These fibres originate almost exclusively from the ventrotemporal margin of the retina. In contrast to all other retinal axons they seem to be deflected from the midline of the optic chiasm and thereby led into the ipsilateral optic tract.

In order to analyse the interactions between growing fibres and chiasm midline, we have developed the following in vitro model. Axons of the embryonic rat retina are grown on a carpet of tectal cell membranes used as a general growth-permissive substratum. At a certain distance from the explant (200-450 μm), the advancing fibres are confronted with two stripes of cell membranes prepared from the chiasm midline. Such chiasm membranes are shown to act as a barrier for the presumptive non-crossing axons, while they do not influence growth of fibres originating from any other regions of the retina, including the dorsotemporal part. The repulsion of non-crossing fibres by chiasm membranes is observed in vitro only when retinal explants from embryonic day (E) 17/18 and chiasm preparations from E14/15 are used. Fibres and tissue from different regions of the brain as well as from different developmental ages, and even from different species, can be combined in this assay system. In a first attempt to characterize the molecular basis of the repulsive effect of chiasm membranes on ventrotemporal fibres, similar assays were performed with membranes derived from other regions of the central nervous system midline, some of which are known to have repulsive properties against certain axon populations. Since these cell membranes did not act as a barrier for the ventrotemporal retinal axons, we suggest that the guidance cues at the chiasm are very specific.

Our results are consistent with the hypothesis that certain cells at the chiasm midline (very likely radial glial cells) express 'repulsive or inhibitory' molecules, which act in a specific way on ipsilaterally projecting axons.

Key words: rat, optic chiasm, ipsilateral projection, axonal growth

INTRODUCTION

One of the fascinating questions in neuroembryology concerns the mechanisms that govern the proper orientation of nerve fibres during development: what sort of signs are used by the outgrowing fibres? and do they react to mechanical structures and/or chemical cues or do they find their targets by trial and error? The chemoaffinity hypothesis put forward by Sperry in 1963 postulates that axons find their way and recognize their termination site by chemical positional cues present on fibre tracts and target cells and complementary markers on the growing axons. While some other mechanisms might still be involved, there is growing evidence that the pattern of neuronal connections is generated by a precise and coordinated interaction between the growing axons and their cellular surroundings (Harris, 1989; review: Guthrie, 1989). Fibres can react to cellular and extracellular cues using the highly mobile growth cones on their distal tips, which show specific morphological changes in response to different environments (Tosney and Landmesser, 1985; Caudy and Bentley, 1986; Bovolenta and Mason, 1987; Godement et al., 1990).

Some of these environmental cues have been characterized in the last two decades. Certain proteins of the extracellular matrix (ECM) such as fibronectin and laminin (Lander, 1989; Sanes, 1989; Reichardt and Tomaselli, 1991) and cell adhesion molecules (e.g. N-CAM; for review: Edelman, 1986; Jessell, 1988; Takeichi, 1990) expressed by neuroepithelial cells provide a more or less specific pathway for growing fibres. In addition, at certain points on this pathway, axons have to decide between different growth directions, or to decide on which target cells to settle. This implies the existence of more specific molecular signals than those just mentioned. These could be chemotropic, attractive factors or inhibitory or repulsive molecules.

Evidence for the expression of chemotropic factors by final and also intermediate targets have already been found (review: Dodd and Jessell, 1988). Moreover, in invertebrates, it has been demonstrated that the first axons that grow out, the so-called 'pioneer fibres', are instructed by certain guidepost cells (Bastiani and Goodman, 1986; Jacobs and Goodman, 1989) and, after the removal of these, axons grow without specific orientation (Bastiani and Goodman, 1986; Thomas et al., 1988). Axons growing out later frequently fasciculate with those pioneer axons (Raper et al., 1983a,b; Bastiani et al., 1986). An alternative way of guiding axons is the inhibition of growth (for review: Patterson, 1988; Keynes and Cook, 1990; Davies and Cook, 1991). Evidence for axon repulsion was found in the posterior tectum of chick, mouse, rat and fish (Walter et al., 1987a,b; Godement and Bonhoeffer, 1989; Vielmetter and Stürmer, 1989) and in some other regions of the central nervous system (CNS). Membrane fractions of these and other brain regions provoke a collapse of growth cones (Cox et al., 1990; Davies et al., 1990; Raper and Kapfhammer, 1990). The collapse will in turn either lead to a reversible or persisting paralysis or to a withdrawal of the axons and thereby to a course correction. Such course corrections are observed also in vivo, after experimentally induced deviations of the axons from their natural pathways (e.g. Fujisawa, 1981; Holt and Harris, 1983; Thanos and Bonhoeffer, 1986).

The present work has been undertaken to examine the mechanisms that are involved in the guidance of retinal axons within the chiasm, in particular in the decision of a selected population of axons to orientate ipsilaterally. At the chiasm, fibres of both eyes diverge and redistribute into pathways that lead to either side of the brain. The proportion of axons that form the ipsilateral projection depends on the binocular overlap of the visual field and is therefore species-specific. In the rat, the ipsilateral projection originates in the periphery of the ventrotemporal retina (Land et al., 1981; Jeffery and Perry, 1982; Bunt et al., 1983; Jeffery, 1984). In principle, several different mechanisms could account for the divergence of ipsilateral and contralateral fibres at the chiasm. First, animals that have only a contralateral projection as adults often form a transient ipsilateral projection during embryonic development (e.g. 2% in the chick; McLoon and Lund, 1982; Thanos and Bonhoeffer, 1984) which disappears completely during the cell death period. Therefore, retinal axons arriving at the optic chiasm could possibly be led more or less by accident into the ipsilateral or contralateral optic tract, and fibres that have taken the wrong way are later eliminated. Indeed, in the rat, the animal that we have used, one half of the ipsilateral projection appears to be only transient (Land et al., 1981; Jeffery and Perry, 1982; Bunt et al., 1983; Jeffery, 1984). Another possibility is that the spatial order of retinal fibres in the optic nerve or mechanical structures in the optic nerve and at the chiasm could work together in such a way that the axons are mechanically led into the proper optic tract (Silver, 1984; Horsburgh and Sefton, 1986; Navascués et al., 1987; Webster et al., 1988). The findings of Dräger (1985), Baker and Jeffery (1989) and Collelo and Guillery (1990) seriously question this last hypothesis (see also Chan and Guillery, 1990) by showing that contralaterally and ipsilaterally projecting axons grow together in mixed fascicles. Thus, it seems more likely that there is an active pathfinding of the retinal fibres within the chiasm. Time-lapse video microscopy independently performed by Godement et al. (1990) and Sretavan (1990b) has demonstrated that a region of about 200 μm around the chiasm midline is actively avoided by ipsilaterally projecting axons. Moreover, the growth cones of these fibres exhibit a specific morphology characteristic for growth cones searching for a proper environment (Godement et al., 1990).

The obvious importance of the midline region of the brain for the process of decussation is demonstrated in various positions along the neuraxis. Characteristic glial cells have been found at the midline between the two tecta and in the hindbrain, which may act as a barrier for certain axon populations (Mori et al., 1990; Snow et al., 1990). Mason et al. (1990) detected a similar radial glia population demarcating the boundary of the chiasm midline region, which may express a specific signal inducing non-crossing fibres to turn away. Since there are certain limitations to such studies in vivo, we have developed an in vitro assay system designed to reflect the situation of retinal axons approaching the chiasm midline. This assay system has allowed the combination of retinal explants and chiasm membrane preparations from different embryonic stages, and it was shown that, under certain conditions, presumptive non-crossing fibres, and only these, react to repellent guidance cues present on membrane preparations from the chiasm midline. As a control, rat retinal axons were also confronted with chiasm membranes from the chick, which does not have a persistent ipsilateral projection.

MATERIALS AND METHODS

All rats used for this study were from the Sprague Dawley strain and were raised in our laboratories. For the prenatal stages, time pregnancies were calculated by checking for the presence of sperms; plugged date was considered embryonic day 0 (E0). Pregnant females were deeply anesthetized with chloralhydrate (0.42 g/kg). By a Cesarian section, the lower part of the belly was opened to expose the uterine horns. The embryos were quickly retrieved from the uterus, transferred into Hank's buffered saline and decapitated. Eyes, tecta and chiasm regions were dissected from the embryos.

Preparation of membranes carpets

All steps were performed at 4°C. Adjoining tissue and pia were removed from the chiasms/chiasm regions and an area of 300 μm around the midline was dissected (Fig. 1). The chiasms were collected in medium and briefly centrifuged, the supernatant was removed and a urea solution (4 M urea, 4 mM spermidine ×3 HCl, 20 μl/ml aprotinin solved in phosphate-buffered saline (PBS); 25 μl/chiasm) was added. The mixture was vortexed at regular time intervals during 30 minutes. Then the nuclei were pelleted (30 minutes, 9000 ***g***, Heraeus centrifuge). The supernatant was pelleted again at 60 000 revs/minute in a TLA 100.1 rotor (Beckman, TL 100 centrifuge) for 30 minutes. The pellet containing the membrane vesicles was resuspended in PBS. The protein concentration was measured and adjusted to approximately 100 μg/ml.

Tectal membranes were prepared from the anterior half-tectum of 17 or 18 day old embryos. Only anterior tectum was used because this area has been found to be a good growth substratum

for fibres from all retinal regions, including the temporal ones (Walter et al., 1987a,b). The procedure for preparing membranes from the tectum and from other brain regions was essentially the same as described for the chiasm except that the addition of urea solution was adjusted to the amount of material. In some cases, a sucrose membrane fractionation was used to prepare the tectum membranes (Walter et al., 1987a) because the yield of this method is higher than by urea extraction. Membrane carpets were prepared as described in Walter et al. (1987a). The only difference was that we used two channels per silicon matrix instead of many. Through a glassfrit a vacuum can be applied to the channels (800-900 mPa). A nuclepore filter (pore size 0.1 μm; Nuclepore, Tübingen) is placed onto the matrix and covered by the membrane suspension (40 μl) used for the two stripes. The negative pressure drains the suspension above the channels, thereby forming stripes of membranes. The nuclepore filter was then transferred onto a nylon grid and charged with the membrane fraction (150 μl) of the second type. Thus, the surrounding of the stripes is coated with the other membrane vesicles. Green fluorescence-labelled beads were added to the membrane fraction comprising the two stripes (Covaspheres, Duke Scientific; 1:500 000).

Preparation of retinal explants

The eyes were dissected in medium (Dulbecco's modified Eagles medium/F12, Gibco). Sclera, pigment epithelium and lens were removed together with the blood vessels covering the ganglion cell layer of the retinae. The last step is sometimes difficult to perform but indispensable in order to spread the retinae evenly onto a nitrocellulose filter. The flat-mounted retinae were transferred to a Petri dish and covered by 2 ml of a DiI (1,1′-dioctadecyl-3,3,3′,3′,-tetramethylindo-carbocyanin Perchlorat, Nr. D-282; Molecular Probes) or Di10Asp (4-(4-didecylaminostyryl)-N-methyl-pyridinium iodide, Nr. D-291; Molecular Probes) suspension (5 mg DiI solved in 500 μl DMF (dimethylformamide), 1 mg Di10Asp solved in 500 μl DMSO (dimethylsulfoxide); 15 μl of the solution were suspended in 2 ml Hank's). After centrifugation (1500 revs/minute, 10 minutes; Sorvall GLC-2B General Laboratory Centrifuge) of the dye particles onto the retinae, unbound dye was removed by several rounds of washing in Hank's followed by draining on filter paper, until no dye could be seen on the paper. Strips (300 μm; see Fig. 1) cut out of the stained retinae were explanted onto the membrane carpets, 200-450 μm from and parallel to the two stripes, and held in place with two metal bars. In order to reduce the pressure on the retinal tissue, a spacer (Visking-dialysis tubing 20/32, Serva) was placed on either side of the tissue, between membrane carpet and nitrocellulose support (Fig. 2) (Godement and Bonhoeffer, 1989).

RESULTS

Assay system with chiasm membranes

Flat-mounted rat retinae of different developmental ages were cut into small strips, parallel to or at a certain angle to the dorsoventral axis, and explanted onto homogeneous carpets of membrane preparations from anterior tectum. At a distance of 200-450 μm from the explant, outgrowing axons were confronted with 2 small stripes of membranes from the chiasm midline (see Figs 1, 2), again prepared from different developmental stages. This assay set up was designed to resemble the in vivo situation of retinal axons approaching the chiasm midline. It was assumed that presumptive contralaterally projecting fibres should easily cross the chiasm membranes, as they would do in vivo, whereas presumptive ipsilaterally projecting fibres, those from the ventrotemporal retinal margin, should be deflected if there is a specific recognition of midline cues. Fig. 3 shows the result of such an experiment. When the explant was derived from the ventrotemporal retina, fibres did not cross the chiasm membranes. Cell membranes of the chiasm midregion obviously act as a barrier for non-crossing axons, resembling the in vivo situation where these axons do not cross the chiasm midline. Typically, fibres were seen to have stopped or turned. In contrast, fibres of the dorsotemporal and nasal retina crossed the chiasm midline membranes. A second example of retinal fibre behaviour upon contact with chiasm membranes is presented in Fig.

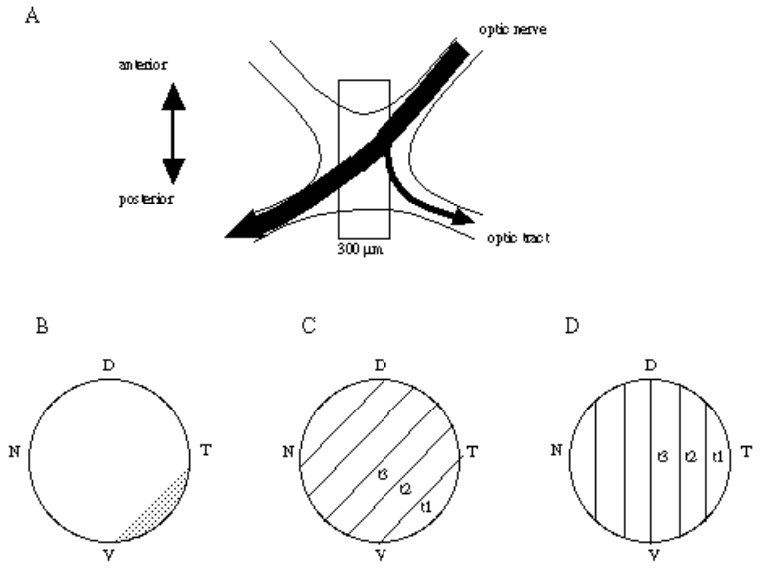

Fig. 1. Schematic drawing of the chiasm and of the rat retina, indicating the region from where the ipsilaterally projecting retinal axons originate, and the sections that were explanted in the test system. (A) Optic nerve entrances are shown at the anterior end of the chiasm, the optic tract exits at the posterior end. The midpart of the chiasm (around 300 μm) that was used for the membrane preparation is indicated by a rectangle. The black arrows symbolize the path of the ipsilateral and contralateral projection of one eye. (B) The stippled crescent within the ventrotemporal part of the retina depicts the region where only ipsilaterally projecting axons grow out at a certain age. This projection is also found in the adult animal. (C) In order to be able to explant only the peripheral ventrotemporal part of the retina, we cut the retinae into strips at an angle of roughly 45°to the dorsoventral axis. The strip that corresponds to the most peripheral section of the ventrotemporal retina was designated t1, the strips further to the retina center were continuously numbered (t2,t3). (D) Some retinae were cut in a dorsoventral direction. Thus the dorsal and ventral part of the temporal retina is located on one retinal strip. Depending on the area the strip originates from, more or less ipsilaterally projecting axons grow out of the explant.

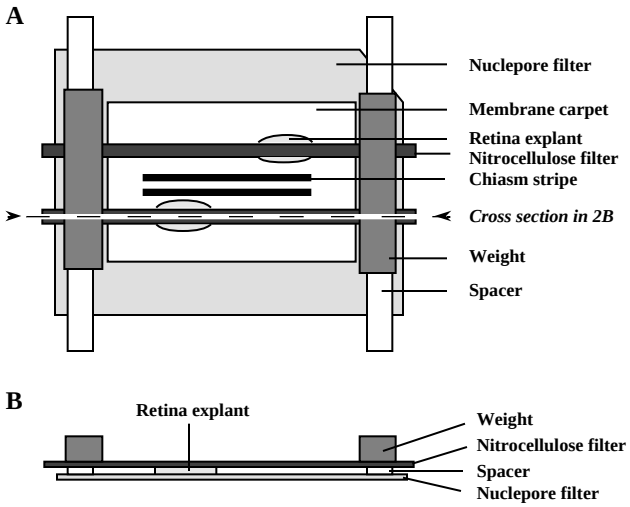

Fig. 2. Schematic drawing of the assay setup. (A) The retina explants are placed on a nuclepore filter, which is covered with a tectum membrane carpet, and are fixed with two metal bars. The position of the spacer, which serves to reduce the pressure on the retinal tissue (see Methods), is indicated. (B) Side view of the setup shown in A to demonstrate the exact location of the spacer.

4. It should be emphasized that the behaviour of the ventrotemporal fibres seen in this assay system is not due to a somewhat lower growth rate of this fibre population, as shown in Fig. 4 and by control experiments with 'inactive' chiasm membranes (see below) from older embryos. Also, in all experiments, the distance of ventrotemporal and dorsotemporal explants to the chiasma membranes stripes was equal.

The outcome of this assay system was dependent on the area within the retina from which the explant strips were cut. In general, more peripheral strips showed a more clearcut result than more central ones. For this reason, it was a prerequisite for all experiments to spread the retinae onto the nitrocellulose filter support with the periphery left completely intact. In order to localize more precisely the region of the retina where the fibres show the above-mentioned reaction to chiasm membranes, we have performed the following series of experiments. The retinae were cut into strips at an angle of roughly 45° to the dorsoventral axis. The strip that corresponded to the most peripheral part of the ventrotemporal region was designated t1, the strips further to the retina center were continuously numbered (t2, t3). In all experiments, explants from nasal retina were taken as controls. In cases where the nasal explants did not grow well or fibres did not reach the chiasm membranes, the temporal explants were discarded. From all 12 cases marked as t1, there was only one in which fibres crossed the chiasm membrane stripes. The majority of axons from the other explants stopped or turned. The ratio was 2 to 4

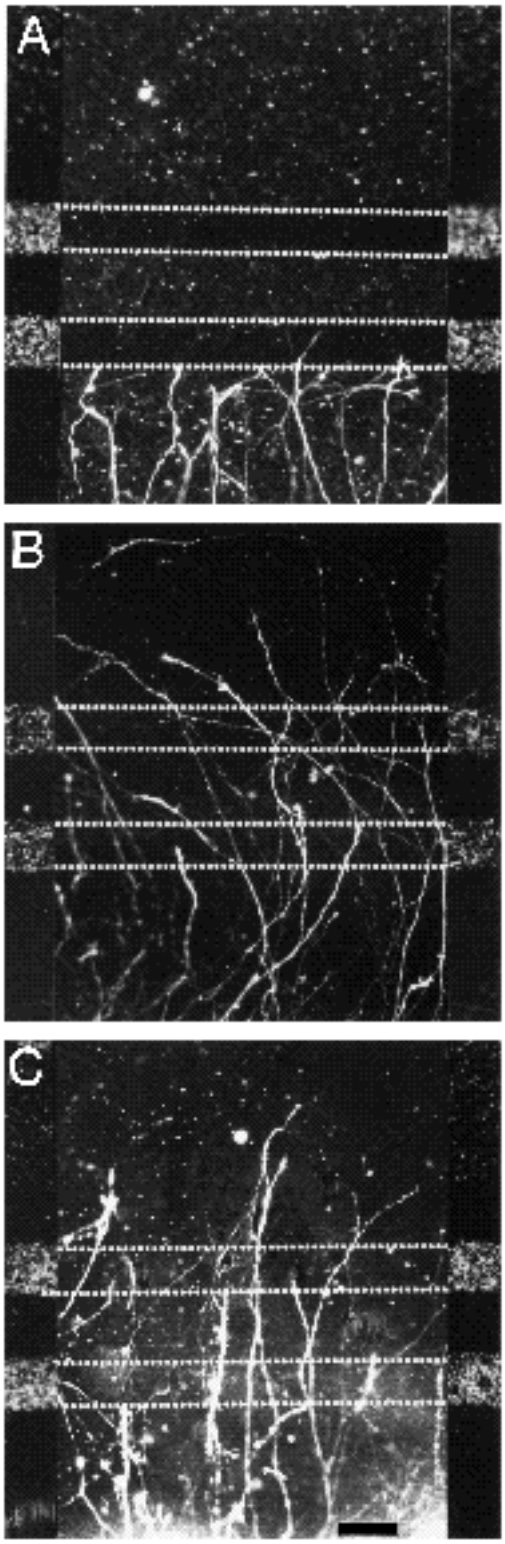

Fig. 3. Chiasm membranes act as a barrier for a distinct axon population. Chiasm membranes from E15 and retinae from E17 rat embryos were prepared for this experiment. The retinae were cut parallel to the dorsoventral axis and the resulting strips placed in an equal distance (about 300 μm) from the membrane stripes. Chiasm membrane stripes were labelled with green fluorescent beads indicated by the dotted lines. The retinae were labelled with DiI. The culture time was the same for temporal and nasal explants. (A) Axons of the ventrotemporal retina are shown to avoid the chiasm stripes. Only a few succeeded in growing over the chiasm membranes but have advanced very little compared to dorsotemporal fibres (see B). (B,C) Axons of the dorsotemporal and nasal regions of the retina which grow across the chiasm membranes. Scale bar: 100 μm.

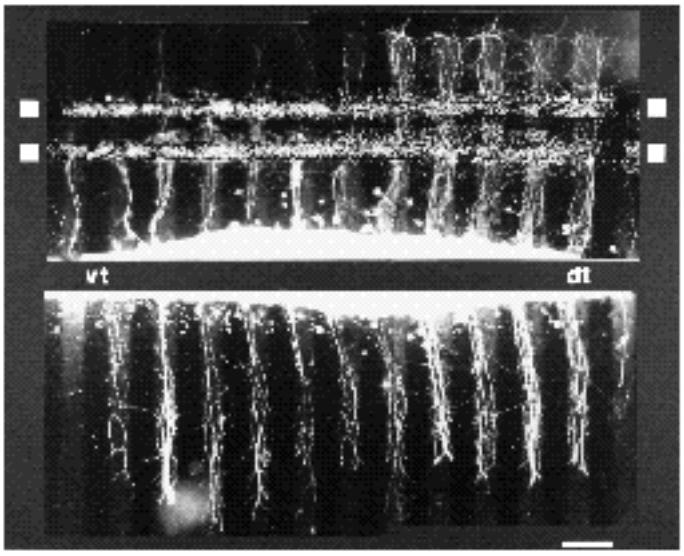

Fig. 4. Influence of chiasm membranes on different retinal axon populations. The explant strip was cut, in parallel to the dorsoventral axis, from an E18 retina and labelled by Di10Asp. The chiasm membranes from E15 embryos were prepared by a urea extraction and mixed with green fluorescent beads (marked by the squares on each side). Axons grow out from both sides of the retinal explant strip. The upper part of the picture shows the behaviour of axons confronted with chiasma stripes (300 μm away from the explant), the lower part shows the growth without any obstacle. In the upper part, the axons of the ventrotemporal (vt) retina are shown to avoid the chiasm stripes, in contrast to dorsotemporal fibres (dt) which grew across the chiasm membranes. The lower part of the picture shows that this behaviour of the ventrotemporal fibres is not due to a somewhat lower growth rate of this axon population, since without an obstacle ventrotemporal fibres show the same length as dorsotemporal ones. Scale bar: 100 μm.

for retinal explant strips of the t2 type. For the more central retinal strips (t3), no clearcut reaction to the chiasm membranes could be observed. From all explanted t3 strips, some fibres did stop, whereas most others crossed the chiasm membranes (see Table 1). These findings correlate well with the distribution of the ipsilaterally projecting ganglion cells within the retina which are concentrated in a half-moon-shaped region at the ventrotemporal margin (Fig. 1B). A schematic drawing of these results is presented in

Table 1. Growth behaviour of axons originating from different regions of the retina were confronted with chiasm membrane stripes

Retina region	Growth over chiasm stripes	No growth over chiasm stripes
t1	1	11
t2	2	4
t3	4	see text
n	12	0

Chiasm membranes of E14/15 and retinal explants of E17/18 embryos were used. For the designation of retinal regions refer to Fig. 1C. The number of explants that showed one or the other growth behaviour is listed.

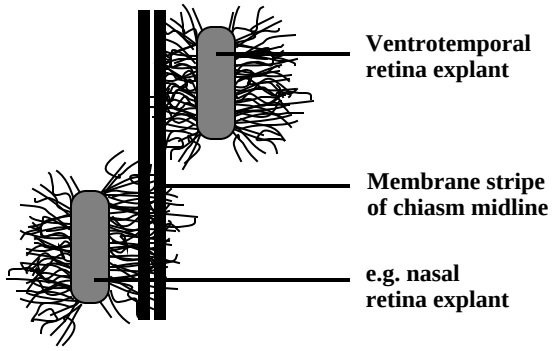

Fig. 5. Scheme of the results. This drawing summarizes the results of Figs 3 and 4. The two black bars in the middle represent chiasm membranes of E14/15 embryos. Growth of crossing and non-crossing fibres of E17/18 retinae is shown.

Fig. 5. The probability of fibres of the ventrotemporal retina not crossing chiasm membranes, compared to fibres of the rest retina, was significant with a 0.1% χ^2 test corrected after Yates (34 explants examined).

During the course of these experiments, it turned out that the age of the embryos used was a crucial point. Only a combination of retinal explants from E17 or E18 with chiasm membranes of 14 or 15 day old embryos yielded the expected result. Retinal axons of younger or older embryos behaved differently. Almost all temporal (n=5) axons of E14 and E15 retinae crossed like the nasal ones (n=5). The same is valid for retinae older than E18 (temporal: n=4; nasal: n=2). Chiasm membranes prepared from older embryos (E16-E20) seemed to have no effect on fibres of the ventrotemporal part of 17 of 18 day old retinae (Fig. 6). In most of these combinations, the membrane stripes were crossed by fibres originating from the ventrotemporal retinal margin. The χ^2 test corrected after Yates performed in this case did not show no a significant repulsion of retinal fibres from all different areas (P=0.1).

The following picture emerges from these experiments: axons of the ventrotemporal retina, which do not cross the midline of the brain in vivo, in vitro also avoid a membrane fraction prepared from the chiasm midline if both tissues have a specific embryonic age. These in vitro results correlate well with those of Godement and Mason (1990), Godement et al. (1990) and Sretavan (1990a,b), obtained in vivo.

Assay system with membranes from other brain regions

The principle of repulsion of growing axons is also observed in other brain regions along the neuraxis. Therefore, it seemed reasonable to compare the repulsive effect of chiasm membranes on ipsilaterally projecting retinal fibres with that of membranes derived from other brain regions. In particular, we used embryonic cortex, tectum midline and floorplate. The cortex is the termination region of fibres arising from the thalamus that arrive at the cortex before it is fully differentiated. In the rat, thalamic axons will grow into the cortex after a specific waiting period. Götz et al. (unpublished data) could show that in vitro cell

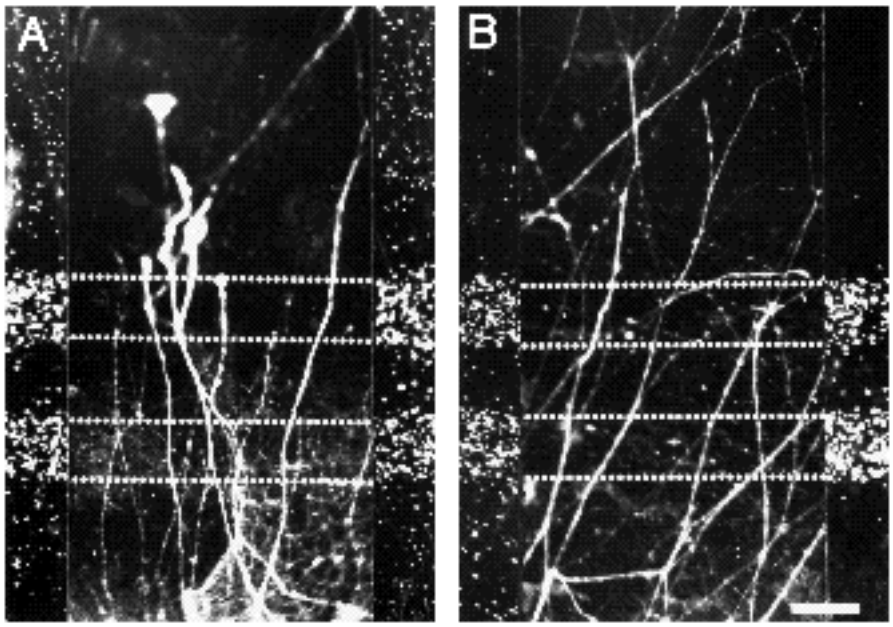

Fig. 6. Assay with chiasm membranes of E17 embryos. The membranes were prepared by a urea extraction and marked with green fluorescent beads. The retinae (E17) were labelled with DiI. (A) Axons of the nasal retina cross the chiasm membranes. (B) Ventrotemporal fibres exhibit the same behaviour as nasal ones when confronted with chiasm membranes of 17 day old embryos. Scale bar: 100 μm.

membranes from undifferentiated cortex are a non-permissive substratum for thalamic fibres, while being permissive for other fibre populations including those of the cortex itself. Membranes of embryonic (E15) and postnatal cortex did not display any repulsive effects on retinal axons. Almost all fibres (E15 and E17 retinae) crossed those stripes independently of the age of the cortex used or the retinal region from which the fibres originated.

In the midsagittal region of brainstem and spinal cord, a specific glial cell population is found (Mori et al. 1991; Snow et al. 1991). One important function attributed to this glia is the formation of interregional boundaries. Such barriers could contribute to a compartmentalisation into different brain regions by a selective repulsive influence on the corresponding axons. Snow et al. (1991) suggested that glial cells within the roofplate and the related region of the midbrain (e.g. the midline between the tecta) prevent axons from growing across the midline. Mori et al. (1991) have shown that the roofplate glia and the ventral radial glia in the spinal cord and brainstem share an epitope which, in principle, could have a similar inhibitory effect on certain axon populations. However, midsagittal brainstem membranes of 14 day old embryos did not influence the crossing of retinal axons in the assay system. With E17 retinae, in 5 out of 6 cases, all fibres grew over these stripes, while fibres from E14 retina crossed in all cases examined.

Studies on the development of the optic tectum in hamster have suggested that the glial cells localized between the two tectal hemispheres represent a barrier for retinal fibres to confine their termination within one tectum. Destruction of this midline results in crossing of retinal axons into the other tectum (Schneider, 1973; So, 1979; Poston et al., 1988). The glial cells at this midline show a strong similarity (e.g. radial morphology, keratan sulfate binding) with glial cells forming the roofplate of the spinal cord. From in vivo experiments it is known that keratan sulfate and other glycosaminoglycans (GAG) can be inhibitory for axon growth (Carbonetto et al., 1983; Tosney and Landmesser, 1985; Verna, 1985; Funderburgh, 1986; Perris and Johanssen, 1987; Snow et al, 1990, 1991; Perris et al, 1991). However, if membranes of the tectal midline cells (E15 to E18) were employed in the assay system, the axons of temporal retinal explants did not change their growth direction, like most from the nasal explants, where only fibres from one explant stopped growing. (Using the Fisher-Yates exact test, there was no significance found for the growth stop of fibres).

Control experiments

Two types of control experiments have been performed. First, chick retina, which does not form a persistent ipsilateral projection, was explanted next to chiasm membrane stripes of the rat. Out of 8 temporal and 5 nasal chick explants none behaved in a way similar to that described for the rat temporal retina. Second, we performed a stripe assay (Walter et al., 1987a,b) with membranes from anterior and posterior tectum. This assay had been developed to study the mechanisms underlying the development of the projection of chick retinal fibres onto their target. These experiments had revealed that there is a specific repulsive molecule for temporal fibres which is highly enriched in posterior tectum membranes. Fig. 7 shows rat retina explanted on a carpet of alternating stripes of anterior and posterior tectum membranes. Nasal axons, in this assay system (Fig. 7A), did not prefer membranes of a specific part of the tectum. As Fig. 7B shows, the temporal fibres grew only on anterior tectum membranes if they had the choice. In contrast to the assay system described above, no difference in the behaviour of ventrotemporal and dorsotemporal axons could be observed.

DISCUSSION

In the adult rat, a small subpopulation of retinal ganglion cell (RGC) axons forms a persistent projection to the ipsi-

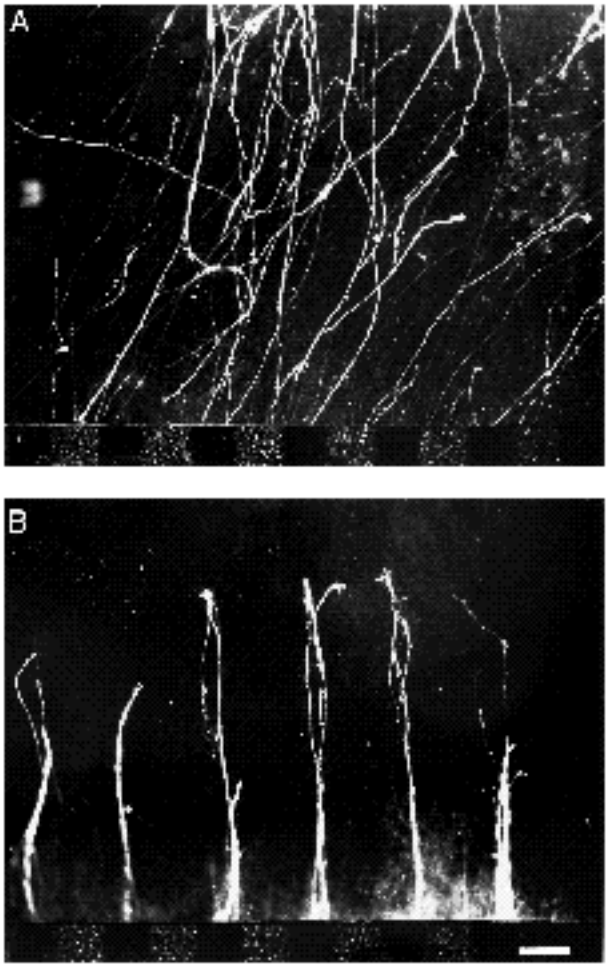

Fig. 7. Assay system with membranes of anterior and posterior tectum. Membranes of the anterior and posterior tectum were prepared according to Walter et al. (1987a) and sucked in alternating stripes onto a nuclepore filter. Perpendicular to these stripes, retina explants were placed, so that the axons could grow out in parallel to the membrane stripes. Membranes of the posterior tectum were labelled with green fluorescent beads, the retinae with DiI. (A) Fibres of nasal retina did not distinguish between membrane stripes of anterior or posterior tectum. (B) An explant of temporal retina the axons of which grew only on membrane stripes of anterior tectum. There was no difference found in the behaviour of axons originating from the ventrotemporal or dorsotemporal retina. Scale bar: 100 μm.

lateral half of the brain. These fibres originate almost exclusively from a ventrotemporal crescent of the retina. The guidance mechanisms that are involved in separation of this contingent of axons from the majority of contralaterally projecting neurites are not understood.

The experiments described here were based on the following assumptions.

(1) Ipsilaterally projecting fibres, rather than being guided by mechanical structures or specific fasciculation within the optic nerve, or being specifically attracted by the ipsilateral optic tract, should react to certain guidance cues to be found in the chiasm itself.

(2) The chiasm midline region (Mason et al., 1990) may express certain cell surface or ECM molecules which will be a non-permissive or repulsive substratum for non-crossing fibres.

(3) Ipsilaterally projecting fibres, in turn, should differ from all other retinal axons in at least one molecule, maybe a receptor molecule.

In order to study the molecular basis of this process of fibre segregation within the chiasm, it seemed reasonable to devise an in vitro test system that would enable us to manipulate and thereby finally characterize all the components involved. The test system was developed out of the stripe assay introduced by Walter et al. (1987a,b), which we had to modify for two reasons. First, the amount of material that can be obtained from the chiasm midline region is very limited. Therefore, we used a silicon matrix with only two stripes (90 μm) instead of many. The remaining surface of the filter was covered with tectum membranes as growth substratum. Second, the system was designed to resemble the in vivo situation of axons approaching the chiasm midline in that the retinal explant strips were placed in parallel to the chiasm stripes. Thus, the chiasm membranes should act like a barrier for non-crossing axons.

Behaviour of non-crossing axons in the in vitro assay

In accordance with the results of Godement and Mason (1990), Godement et al. (1990), Sretavan (1990a,b) and Guillaume et al. (1991), fibres of the ventrotemporal margin of the retina did indeed not grow over membrane stripes prepared of chiasm midline material (Figs 3-5). However, this effect turned out to be strictly dependent on the developmental age of both the retinal explants and the chiasm tissue. Only E14/15 chiasm membranes could influence the growth behaviour of non-crossing axons, membranes from older chiasms were not effective. This may be taken as evidence that the growth preference of ventrotemporal axons is due to specific repulsive properties of the chiasm membranes which, in principle, could also be a response to attractive properties of the tectal membranes. The possible existence of a specific attraction of ventrotemporal fibers by tectal membranes is further ruled out by experiments where the chiasm membranes were replaced by material from other brain regions (see below). None of the cultures evaluated so far showed a preferential growth on tectum membranes.

As for the age of retinal explants, a clear choice of ventrotemporal fibers was found only with E17/18 retinae. Our results correspond well with the course of development of the non-crossing projection. The embryonic age of E14 is the time point when the first central RGC send out their axons (Lund and Bunt, 1976; Horsburgh and Sefton,1986). Half a day later these axons reach the optic nerve. At that time RGC in the periphery are just differentiating, and the chiasm is still devoid of retinal axons. On E15 the first axons arrive at the chiasm. The majority of fibres reaches it at E15.5. At E16 the first axons are found in the lateral geniculate nucleus (LGN), at E16.5 on the Colliculus superior (Bunt et al., 1983). The region of those RGC that project ipsilaterally becomes discernible within the retina not before E16/17 (Bunt et al., 1983; Reese et al., 1991). In

albino rats, this projection is detectable only one day later in development than in pigmented rats (Bunt et al., 1983; Horsburgh and Sefton, 1986).

In our assay system, only fibres from retinae of embryonic day 17 or 18 exhibited the expected decision, axons from older or younger retinae showed hardly any reaction. This could be due to one of the following reasons: First, it is known that the majority of ipsilaterally projecting fibres in albino rats leaves the retina at E17/18, from younger retinae very few or no non-crossing axons should grow out. Axons emerging from the ventrotemporal retina after E18 are known to project mainly contralaterally (Bunt et al., 1983). In order to verify the correlation of our results with the histological-anatomical studies, a next step should be to repeat the experiments with pigmented rats. We would expect that in this case ventrotemporal axons of younger retinae than E17/18 should react to the chiasm membranes. It could even be possible that more axons would react because the number of non-crossing axons was shown to be higher in pigmented rats than in albino rats, while they originate from the same area in the retina (Lund and Bunt, 1976; Land et al., 1981; Bunt et al., 1983; Martin et al., 1983).

Second, on the two-dimensional substratum of our test system, the relatively few presumptive non-crossing axons of older retinae might well fasciculate with crossing axons (see above) of the ventrotemporal retina and thereby be led over the chiasm membrane stripes. It is known that growth cones look different on two- and three-dimensional substrata and it seems reasonable that, in vivo, growth cones of non-crossing fibers, even if growing in close contact with crossing fibers, would have a greater chance to sense repulsion factors in a three-dimensional system like glial curtains at the chiasm midline. It is also possible that crossing fibres release specific proteases that would help them penetrate the glial barrier at the midline. Such proteases should then be absent from ipsilaterally projecting growth cones. Proteases are indeed known to be released by growth cones that have to grow through a normally non-permissive substratum (Pittman, 1985, 1988; Monard, 1988; Fawcett and Housdon, 1990). Since in vitro there are no cells that could reproduce the hypothetical repulsive factor the way would be free for the ipsilaterally projecting fibres.

A similar point can be made about the age of the chiasm. If the ventrotemporal fibres were confronted with chiasm membranes of embryos that were older than E14/15, they easily crossed those membranes. One possible explanation could be that chiasm membrane preparations from such embryos will contain high amounts of axon membrane material since most axons have arrived at the chiasm by that time (Horsburgh and Sefton, 1986). Any repulsive or inhibitory effect could thus be obscured by an excess of growth-permissive axon membrane components.

Selection of a suitable membrane preparation technique

Since the chiasm midline is a small structure, the amount of membrane material that can be collected is limited. We therefore started the experiments with a membrane preparation technique that would give us the highest yield, a so-called sucrose gradient fractionation. These membranes had no or only a weak effect on non-crossing axons. The selection of an alternative membrane preparation was necessary and we chose treatment of the tissue with a urea solution (v. Boxberg, 1987). It was only then that the ipsilaterally projecting fibres showed a decision in our assay sytem. The overall substratum properties of both types of membranes are very much alike. So, for example, rate of outgrowth and fibre density are similar. Still, the two preparations might differ in protein composition. Concerning their effects on retinal fibres, Goverdhan (1989) has shown that membranes prepared by urea extraction exhibit a higher repulsive activity on temporal axons when employed in the stripe assay of Walter et al. (1987). In principle, there are two possible explanations for the observed effect of chiasm midline membranes on the presumptive non-crossing axons. Either these membranes could lack certain substratum molecules or growth-promoting factors and this renders them non-permissive for growth of non-crossing axons, or they could contain repulsive components that specifically repel non-crossing axons. The dependence of the results of our assay system on the method of membrane preparation points to the chiasm midline being a repulsive rather than a non-permissive substratum for non-crossing axons: It would be hard to explain why one but not the other preparation should allow growth of non-crossing fibres if it is not due to some repulsive molecule that is more enriched in the urea type of membranes. In principle, the urea-extracted membranes could also be depleted of molecules that are specifically permissive for non-crossing axon growth. However, such permissive molecules should not exist at all, since they would allow crossing of ipsilateral fibres in vivo.

Our results do not give any hints whether the hypothetical repulsive molecule is to be found on the glial cells within the chiasm or whether it is a component of the extracellular matrix which could be produced by the very same glial cells. The question what type of cells could be responsible for the repulsive effects on non-crossing fibres at the chiasm midline has been addressed by several groups and, so far, no clear answer has emerged. Mason et al. (1990, 1991) described a population of radial glia cells demarcating the boundary of the chiasm midline, which seem to have a repulsive influence on non-crossing fibres in culture (Guillaume et al., 1991). The same authors have also detected (Mason et al., 1991) a group of cells of the macrophage/monocyte lineage, located underneath the pia where later arriving axons grow and turn. They suggest that divergence of crossing and non-crossing retinal fibres involves contact interactions with different optic chiasm cells. Another type of cell described by Feng and Sretavan (1991) is distributed at the ventral surface of the diencephalon and seems to be of neuronal origin. Based on the ‘strategic’ position within the chiasm and the observation that pioneering retinal axons grow in association with their processes, these authors propose that these neurons could influence the navigation of retinal axons. McKanna (1991) found a population of primitive glial ependymal cells in the midline of the prospective chiasm which can be stained for p35 Annexin, a Ca^{2+} and phospholipid-binding protein. This protein is also expressed in a spatially and temporally restricted pattern at the midline of the floorplate, suggesting that it might

play a role in the formation of the spinal cord commissure. Immunoreactivity of p35 disappears early; thus this protein is not required for final stages of chiasm formation. McKanna proposes that these ependymal cells may be analogous to guidepost glia in invertebrates (Bastiani and Goodman, 1986; Jacobs and Goodman, 1989).

Demarcation of territories by repulsive/inhibitory molecules

There is increasing evidence that mechanical or chemical barriers confine axon growth within certain territories (review: Keynes and Cook, 1990; Davies and Cook, 1991). Mori et al. (1990) described a radial glia in the postnatal and adult brainstem demarcating the midline. Protrusions of these glial cells form a palisade, which separates the right from the left brainstem half. Between these protrusions some nuclei are located, such as the raphe nuclei. Snow et al. (1990) have studied the expression of specific markers on this glia. The glia of the roofplate and the mesencephalic midline (tectum) was found to express keratan sulfate unlike other glial cells in the neighbourhood. Their interpretation is that a specific molecule that binds keratan sulfate may prevent axons from crossing the midline. All reports agree in that axon crossing within these regions is only possible upon a certain loosening of the tissue. If such glia cell membranes of different regions of the brain midline were employed in our test system, it could have been expected that all retinal axons should be stopped. However, retinal axons did not react to any of these membrane preparations. This was especially unexpected in the case of the tectal midline membranes, since retinal fibres should be hindered in vivo from growing across this midline. It could be either that newly outgrowing retinal axons do not yet express the proper receptors for this tectal midline inhibitory molecule, or that, for our membrane preparation, we have just dissected too much growth-permissive tectal tissue around the midline, which has in fact a width of only some cell diameters. In any case, the nature of this tectal midline molecule should be different from that of the chiasm midline, because, to the latter, only the non-crossing retinal fibres should respond.

Since the results of our assay sytem suggest that the radial glia or the corresponding extracellular matrix express a repulsive molecule which should not be expressed by glia cells in the optic nerve and the chiasm side region, we performed a two-dimensional polyacrylamide-gelelectrophoresis (PAGE) with tissue of the chiasm midline and the chiasm side region. There are indeed certain proteins found exclusively on gels of the chiasm midline which are absent from the side region (results not shown).

In summary, we have shown that the in vitro model described here will be a useful means for a variety of studies on the process of fiber decussation at the chiasm midline. Biochemical treatment of the chiasm membranes employed in the stripe assay, such as heat inactivation (Walter et al, 1987b) or digestion with mild proteases, could reveal whether the repulsive factor from chiasm midline is a membrane protein. If the active compound is a proteoglycan, an incubation of the chiasm membranes with different glycosidases should have an effect on the outcome of the assay sytem (Keynes and Cook, 1991). A common feature of several regions within the CNS that are non-permissive for axon growth or cell migration is the expression of a peanut lectin (PNA)-binding epitope (Davies et al., 1990; Stahl et al., 1990). Since peanut lectin-binding proteins were found (not shown) on western blots from gels of optic chiasm material, a midline-specific PNA-binding molecule might be a good candidate for the repulsive factor.

Finally, in reversal of the experiments described here, it should be examined whether chiasm membranes of the rat have a repulsive effect on other axon populations than the retinal, and on retinal fibers from other species. All these studies taken together should help approach an elucidation of the chemical nature of the repulsive molecule from chiasm midline that guides fibres into the ipsilateral optic tract.

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