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GAP-43 mediates retinal axon interaction with lateral diencephalon cells during optic tract formation

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SUMMARY

GAP-43 is an abundant intracellular growth cone protein that can serve as a PKC substrate and regulate calmodulin availability. In mice with targeted disruption of the GAP-43 gene, retinal ganglion cell (RGC) axons fail to progress normally from the optic chiasm into the optic tracts. The underlying cause is unknown but, in principle, can result from either the disruption of guidance mechanisms that mediate axon exit from the midline chiasm region or defects in growth cone signaling required for entry into the lateral diencephalic wall to form the optic tracts. Results here show that, compared to wild-type RGC axons, GAP-43-deficient axons exhibit reduced growth in the presence of lateral diencephalon cell membranes. Reduced growth is not observed when GAP-43-deficient axons are cultured with optic chiasm, cortical, or dorsal midbrain cells. Lateral diencephalon cell conditioned medium inhibits growth of both wild-type and GAP-43-deficient axons to a similar extent and does not affect GAP-43-deficient axons more so. Removal or transplant replacement of the lateral diencephalon optic tract entry zone in GAP-43-deficient embryo preparations results in robust RGC axon exit from the chiasm. Together these data show that RGC axon exit from the midline region does not require GAP-43 function. Instead, GAP-43 appears to mediate RGC axon interaction with guidance cues in the lateral diencephalic wall, suggesting possible involvement of PKC and calmodulin signaling during optic tract formation.

Key words: GAP-43, Retinal ganglion cell, Growth cone, Axon guidance, Optic tract, Optic chiasm, Mouse

INTRODUCTION

GAP-43 is an intracellular protein that is highly expressed in developing and regenerating axon growth cones and in synaptic terminals of adult brain regions that exhibit synaptic plasticity (Skene, 1990; Benowitz and Routtenberg, 1997). Although biochemical studies have demonstrated the potential for GAP-43 to be targeted to membranes through lipid modifications (Skene and Virag, 1989), to serve as a PKC substrate, and to bind or release calmodulin depending on phosphorylation state (Alexander et al., 1987, 1988; Chapman, 1991), the exact role of this protein during axon growth and synaptic function remains to be determined.

Mice deficient in GAP-43 have grossly normal-appearing brain structures indicating that GAP-43 is not essential for simple axon elongation (Strittmatter et al., 1995; Kruger et al., 1998; Meiri et al., 1998; Zhu and Julien, 1999). The finding that, in the visual system of these animals, RGC axons fail to progress normally from the optic chiasm into the optic tracts (Strittmatter et al., 1995; Kruger et al., 1998) have focused attention on the involvement of GAP-43 in specific axon guidance tasks. The failure of a particular segment of the embryonic retinal pathway to form in the absence of normal GAP-43 function suggests that insight into the mechanisms of GAP-43 action during development can be gained by identifying the specific RGC axon pathfinding task requiring GAP-43 function.

During normal development of the vertebrate visual system, RGC axons from both eyes cross the ventral midline of the diencephalon in the region of the future hypothalamus forming an X-shaped pathway intersection called the optic chiasm. Upon exiting from the midline hypothalamic region, RGC axons then find their way into the adjacent lateral diencephalic wall to form the optic tracts, which represent the last segment of the pathway prior to reaching CNS targets. In GAP-43-deficient animals, RGC axons arrive at the chiasm region on time and proceed to cross the midline (Kruger et al., 1998). At approximately 400 μm lateral to the midline, roughly at the junction between the chiasm and the beginning of the optic tract, RGC axons turn away from entering the lateral diencephalon and grow in semicircular trajectories to abnormally recross the midline within the chiasm region (Sretavan and Kruger, 1998).

The guidance mechanisms that normally govern embryonic retinal ganglion cell (RGC) axon exit from the optic chiasm to form the optic tracts are poorly understood. RGC axon guidance during formation of the optic chiasm itself involves

cues provided by both intrinsic neuronal (Sretavan and Reichardt, 1994; Sretavan et al., 1995; Wang et al., 1995) and glial (Wang et al., 1995) populations. As RGC axons leave the chiasm to form the optic tract, they are gathered into a tight bundle of axons suggesting increased fasciculation as RGC axons traverse the lateral diencephalic wall. Axon guidance in the optic tract has best been studied in amphibia where RGC axon growth within the optic tract in dorsal diencephalon close to the optic tectum appears to involve axon interactions with FGF (McFarlane et al., 1995), which is thought to be bound by heparan sulfates (Walz et al., 1997). Treatment of exposed brain preparations with tyrosine kinase inhibitors have also been shown to alter axon growth within the optic tract (Worley and Holt, 1996). The appearance of growth cones differs between the chiasm and the optic tract. RGC growth cones exhibit complex morphologies within the optic chiasm but change into relatively simple spear-like endings within the optic tract (Bovolenta and Mason, 1987; Mason and Wang, 1997). Although this change in growth cone morphology may reflect differences in the glial composition of the optic chiasm compared to the lateral diencephalon optic tract region (Reese et al., 1994), the pathfinding mechanisms operating at the optic chiasm-optic tract transition have not been established.

How the absence of normal GAP-43 function results in failed optic tract development is unclear. Although GAP-43 appears to act cell-autonomously within RGC axons (Kruger et al., 1998), it is not known whether the lack of GAP-43 function interferes with the ability of RGC axons to exit from the midline chiasm region or with their ability to enter the lateral wall of the diencephalon to initiate optic tract development. For example, GAP-43 may mediate changes in RGC growth cone responsiveness to chiasm guidance cues necessary for these axons to leave the midline region, similar to those described for commissural axons at the ventral midline CNS of invertebrates (Kidd et al., 1998) and the mammalian spinal cord (Shirasaki et al., 1998). Alternatively, GAP-43 function might be required for RGC axons to enter the lateral diencephalic wall by enabling these axons to respond to growth-promoting cues in this territory or by dampening the effects of inhibitory cues that have been reported in the lateral diencephalon (Tuttle et al., 1998).

As a step towards better understanding GAP-43 function in axon guidance, we have attempted to distinguish between these two general types of mechanisms by examining how GAP-43-deficient axons interact with lateral diencephalon cells and whether GAP-43-deficient RGC axons exit the optic chiasm region following modifications of the adjacent lateral diencephalon environment. The results from both in vitro retinal explant assays and tissue transplantation experiments show that GAP-43-deficient RGC axons have no difficulty leaving the chiasm region. The failure of GAP-43-deficient RGC axons to form the optic tract appears to be due to the fact that GAP-43 is normally required for either RGC axon response to a growth-promoting guidance cue or to overcome an inhibitory cue in the lateral diencephalon.

MATERIALS AND METHODS

Animals

GAP-43-deficient mice containing a targeted deletion of exon II of the GAP-43 gene (Strittmatter et al., 1995) were the generous gift of Dr Mark Fishman (Harvard University). Heterozygous adult females were bred with heterozygous adult males and the morning of plug detection counted as embryonic day 0 (E0). (Homozygous animals do not survive after birth to adulthood.) Pregnant female mice were anesthetized with intraperitoneal injections of 4 mg sodium pentobarbital. Embryos were harvested by Cesarean section and the adult female euthanized by an overdose of sodium pentobarbital followed by thoracotomy. Embryo genotype was identified by a PCR-based procedure using tail tissue DNA digested in proteinase K (Kruger et al., 1998). All experiments reported here including in vitro outgrowth assays and donor-host tissue transplantations were set up prior to knowledge of tissue genotype.

Optic chiasm neurite outgrowth preparations

The ability of retinal axons to exit from the chiasm region was examined in modified tissue preparations based on retina-chiasm preparations described in previous studies (Sretavan and Reichardt, 1993; Godement et al., 1994; Sretavan et al., 1995). Briefly, these preparations contain the retinas together with the optic nerves and the part of the ventral hypothalamus/diencephalon containing future regions of the optic chiasm and the optic tracts. In these preparations, the CNS tissue within which retinal axons extend have been exposed and RGC axons continue to grow superficially near the pial surface within their native cellular environment. These retina-chiasm preparations have been used in time-lapse videomicroscopy studies to examine RGC growth cone behavior at the chiasm (Sretavan and Reichardt, 1993; Godement et al., 1994) and for experimental ablation of embryonic neurons residing at the future site of the optic chiasm (Sretavan et al., 1995).

Following isolation of retina-chiasm preparations, DiI (1:50 dilution in F12 culture medium of a DiI saturated stock solution of 9:1 Etoh and DMSO) was pressure injected intraocularly using glass micropipettes and a picospritzer (general valve) to label RGC axons. After injection, diencephalon tissue on the side opposite to the injected eye was removed 400 μm lateral to the midline. This is less than the distance of 450 μm lateral to the midline where the GAP-43-deficient axon guidance defect is seen in E14 embryos. This adjustment was necessary since the overall size of the diencephalon is smaller at E12.5 compared to E14. The amount of correction was determined by comparing the distance between fixed landmarks such as the midline and the optic nerve attachment site at these two ages. Distances were determined using a microscope eyepiece containing a calibration scale.

Injected preparations were transferred into coverglass bottom tissue culture dishes containing either collagen gel or laminin coating as a substratum. Collagen (1 mg/ml of bovine collagen; Vitrogen) was diluted with F12 culture medium, N2 supplement, penicillin/streptomycin (pen/strept), the pH adjusted to 7 using sterile NaOH, and kept at 4°C until use. Injected preparations were transferred into coverslip dishes containing collagen gel and placed in a CO_2 incubator at 37°C for 1 hour to allow the collagen to gel. F12/N2/pen/strept was then added and the preparations allowed to grow further for 24-48 hours. For growth on laminin, tissue preparations were transferred into coverglass dishes, which have been precoated with poly-L-lysine for one hour, washed with 0.1 M phosphate buffered saline (PBS) and incubated with laminin (Gibco/BRL) at a concentration of 20 μg/ml in 0.1 M PBS overnight at 4°C. Laminin-treated dishes were rinsed three times with 0.1 M PBS before use.

Following growth for 24-48 hours at 37°C, tissue preparations in collagen gel or in laminin-coated dishes were fixed using 4% paraformaldehyde in 0.1 M PBS pH 7.4. The amount of RGC axon growth from the chiasm region onto the laminin substratum or in the collagen gel was visualized using rhodamine optics on either a Nikon SA epifluorescence microscope or on an inverted Olympus fluorescence microscope employing Deltavision image acquisition and image processing software (API, Issaquah, WA).

Outgrowth assays with cell membrane fragments

Retinal explants from wild-type, heterozygous or homozygous GAP-43-deficient retinas were co-cultured with a variety of CNS cell membrane fragments from wild-type C57/bl6 embryos. Pairs of retinas were harvested from individual E13 embryos and maintained separately from retinas of other embryos. To maximize the potential of individual retinal explants to elaborate neurites radially from all sides, the peripheral retina was dissected away and the optic disc region was removed (punched out) from each retina using a glass micropipette whose tip size was slightly larger than the optic disc diameter. The retina was then further divided into 8-12 pieces for use as explants.

Cell membrane fragments were isolated from a variety of CNS regions in E13 wild-type C57/bl6 embryos. The ventral halves of the diencephalon were isolated, a cut was made approximately 400 μm lateral on both sides of the midline, resulting in isolation of ventral midline optic chiasm region tissue and lateral diencephalon tissue. Cortex tissue was isolated from the dorsal lateral aspects of the cortical hemispheres. Midbrain tissue was isolated from the dorsal anterior midbrain region. Collected tissues were homogenized in Ca^{2+}/Mg^{2+}-free PBS with 0.32 M sucrose, 1 mM EDTA, pepstatin (0.1 mg/100 ml), phenylmethylsulfonyl fluoride (0.2 mg/ml), N-ethylmaleimide (2.5 mg/ml), and leupeptin (0.1 mg/ml). The homogenate was then centrifuged at 4°C for 10 minutes at 100 ***g*** to remove large cell fragments. The supernatant was then re-centrifuged at 4°C for 10 minutes at 10,000 ***g*** to pellet membrane fragments. The protein content of membrane fragment preparations was determined prior to use.

Co-culture experiments were performed in either 35 mm glass coverslip dishes or 8-well borosilicate coverglass chamberslides (NUNC Lab-Tek 136439) precoated with poly-L-lysine and laminin (described above). Membrane fragments were added to individual wells and allowed to attached to the bottom. In experiments involving a mixture of cell membranes, the amount of cortical or lateral diencephalon membrane required to achieve each dosage combination (Fig. 5) was determined by the protein content. Retinal explants were then placed into wells or dishes with all retinal explants in a given dish or well coming from the same embryo. Retinal explants were grown for 14-18 hours, 24-28 hours or 40-48 hours in F12 N2 Pen/Strep at 37°C in 5% CO_2.

Conditioned medium

Lateral diencephalon cell conditioned medium was obtained from dissociated diencephalon cells cultured in F12/N2/Pen/Strep medium. The ventral half of the diencephalon minus the ventral midline region (see cell membrane fragments isolation described above) was isolated from E13 C57/bl6 embryos and subject to papain digestion for approximately 1 hour at 37°C. (The digestion buffer contained 40 units of papain, 0.3 mg L-cysteine, 100 μg DNase, 3 μl EDTA, 3 μl NaOH in 1.5 ml Hanks BSS.) Digestion was stopped by the removal of the papain-containing buffer and the addition of a solution of ovamucoid 30 mg, DNase 0.3 mg, fetal bovine serum 0.3 ml, in 2.7 ml of F12 medium with N2 supplement. The tissue was then gently triturated using a fire-polished glass pipette 7-10 times. The stop solution was replaced by culture medium, the cells subjected to washes by two rounds of centrifugation (600 revs/minute, 5 minutes), the supernatant aspirated and fresh F12/N2/pen/Strept medium added. Approximately 0.5 to 1.0×10^6 cells were used to condition 0.5-1.0 ml of medium for 45-48 hours. The conditioned medium was harvested and centrifuged at 4°C for 1 minute at 13,000 ***g*** prior to use.

Phalloidin staining

Retinal explants and neurites were visualized by Texas-red Phalloidin staining. The fixative was removed and Texas-red Phalloidin staining solution (0.2% L-A-Lysophosphotidylcholine, palmitoyl (Sigma L-5254); 5 U Texas red Phalloidin (Molecular Probes T-7471); 4% formaldehyde in 0.1 M PBS) was added at 4°C for 20 minutes. The explants were then washed five times with 0.1 M PBS and the tissue stored in the dark until analysis. Phalloidin-stained explants and neurites were visualized using rhodamine optics on an inverted microscope. Retinal explants, which showed asymmetric outgrowth and did not have neurites extending from all sides of the explant, were excluded for analysis. Roughly 17% of explants were of this category. For quantitation of total neurite length, all phalloidin-stained neurites from a given explant were traced and scanned into Photoshop 5.0. The number of black pixels in each scanned image (ie. each explant) was determined using the histogram function and the total neurite length derived by dividing this number by the number of pixels representing a known length.

Host preparations for transplantation

Host tissue preparations for transplantation consisted of standard E12.5 retina-chiasm preparations (described above) from which a 200×200 μm^2 region of the lateral diencephalon 400 μm lateral to the midline was removed using sharpened tungsten needles. The removed tissue represented the approximate site where retinal axons first enter the lateral diencephalon to form the optic tract (Optic tract entry zone). Distances from the midline were determined using an ocular micrometer on a dissecting microscope.

Harvesting and DiO labeling of donor tissues

The anterior region of the dorsal midbrain or the region of the lateral diencephalon containing the future optic tract in E12.5 embryos were used as donor tissues for transplantation. For transplants of lateral diencephalon tissue, the donor tissue was obtained from a different donor embryo because of the technical difficulties associated with removing, labeling and then re-transplanting the same tissue back into the original preparation. The anterior midbrain was chosen for transplantation because its curvature and thickness approximates that of the lateral diencephalon region that was removed and is therefore technically suitable for implantation. Secondly, at E12.5-13, RGC axons have yet to innervate the midbrain therefore ruling out any possibility that RGC axons from host preparations use remnants of RGC axons in the transplant as a growth substratum. Lastly, the anterior portion of the midbrain/superior colliculus is a region that in vivo is permissive for the growth of RGC axons from all regions of the retina. The anterior region of the midbrain was identified using landmarks as described in Schambra et al. (1992).

For transplantations, the ventricular surface of the lateral diencephalon and the midbrain tissues was marked with animal carbon using a glass micropipette in order that the pial surface of the transplant can be identified and aligned with the pial surface of the host tissue. Tissue to be used as transplants were labeled by placement in F12 media supplemented with N2 containing a 1:10 dilution of a fresh saturated DiO solution (in 9:1 Etoh: DMSO) for 10 minutes at 37°C. Labeled tissues were then washed in 37°C F12/N2 media three times prior to transplantation.

Transplantation and analysis

Labeled donor tissues were positioned onto host retina-chiasm preparations and nudged into place using forceps. Care was taken to insert the donor tissue so that its pial surface matched that of the host tissue. Following transplantation, preparations were maintained in F12/N2/pen/strept media in 5% CO_2 at 37°C for 56-78 hours. Transplantations were performed using all embryos of a given litter without prior knowledge of tissue genotype. Genotyping was carried out the following day and preparations consisting of specific combinations of wild-type or GAP-43-deficient hosts which received either lateral diencephalon or midbrain transplants, were identified and formed three control groups and one experimental group for analysis. After culturing, preparations were fixed using 4% PFA in 0.1 M PBS. Retinas were removed from the donor preparations and crystals of DiI inserted into the optic disc to label RGC axons. After incubation of DiI-labeled tissues for 3-4 days at 37°C, fluorescently

labeled RGC axons were traced, digitized and quantified as described in Kruger et al. (1998). Host preparations in which the transplanted tissue moved during culture (n=9), in which DiI injections failed to label an adequate number of RGC axons (n=5) or in which RGC axons failed to grow during the in vitro culture period (n=11) were excluded from analysis.

RESULTS

GAP-43-deficient RGC axons can exit the chiasm

First, we examined whether the simple removal of lateral diencephalon tissue immediately adjacent to the midline region will permit GAP-43-deficient RGC axons to leave the optic chiasm area or whether these RGC axons will remain trapped. In normal development, RGC axons enter the ventral hypothalamus around E12, begin to cross the midline by E13 and have formed the optic tracts along the lateral wall of the diencephalon by E14 (Mason and Sretavan, 1997). In GAP-43-deficient embryos, RGC axons arrive at the ventral hypothalamus and cross the midline on schedule but, at E14, grow only 450 μm lateral to the midline and then fail to progress further to form the optic tracts (Kruger et al., 1998) (see also Fig. 1A). E12.5 embryos were collected from GAP-43 heterozygous females bred with heterozygous males. Retina-chiasm tissue preparations were isolated which contained only one retina with its optic nerve together with the optic chiasm region extending 400 μm past the midline but not including lateral diencephalic tissue on the contralateral side (Fig. 1B). These preparations therefore included the midline optic chiasm region up to the site where GAP-43-deficient RGC axons fail to progress into the lateral diencephalon. DiI was injected into the retinal vitreous space to label RGC axons and the labeled preparations placed in collagen matrix or on laminin-coated coverslips for 24-48 hours. At the beginning of the culture period (E12.5), RGC axons in both wild-type and mutant embryos have begun to arrive at the ventral hypothalamus but have yet to reach the cut site (Kruger et al., 1998; see also Fig. 6C,D below).

Wild-type, heterozygous and homozygous GAP-43-deficient RGC axons in retina-chiasm preparations grew past the midline and freely exited from the chiasm region (dotted line shows border between optic chiasm tissue and the laminin or collagen substratum). The pattern of growth on laminin-coated coverslips consisted of small axon fascicles or individual axons growing in fairly straight trajectories radiating from the chiasm region (Fig. 1C,D). The pattern in collagen gels was similar (Fig. 1E,F). In both conditions, no apparent difference in RGC axon lengths was observed among the three genotypes. This observation that wild-type and GAP-43-deficient RGC axons after exiting from the chiasm region grew equally well on laminin or collagen in vitro is reminiscent to the report that wild-type and GAP-43-deficient DRG neurites in vitro are similar in their growth characteristics on laminin (Strittmatter et al., 1995). The finding that GAP-43-deficient RGC axons freely exited the midline region if the adjacent lateral diencephalon tissue was removed suggested that changes in RGC growth cone responsiveness to midline cues that may be required for exit from the chiasm can occur independently of GAP-43 function.

Reduced growth of GAP-43-deficient axons cultured with diencephalon cell membranes

The interactions of wild-type and GAP-43-deficient retinal axons with lateral diencephalon cells were analyzed in vitro to determine whether RGC axon behavior might be altered in the absence of proper GAP-43 function. Wild-type retinal explants grown on laminin-coated coverslip dishes in the presence of membrane fragments isolated from lateral diencephalon cells elaborated a radial pattern of neurite outgrowth after 24 hours. (Fig. 2A-D). Quantitation of Texas-Red Phalloidin-stained neurites showed a mean total neurite length of 34.44±10.36 mm (s.d.); n=11 (Fig. 3B). Retinal explants obtained from

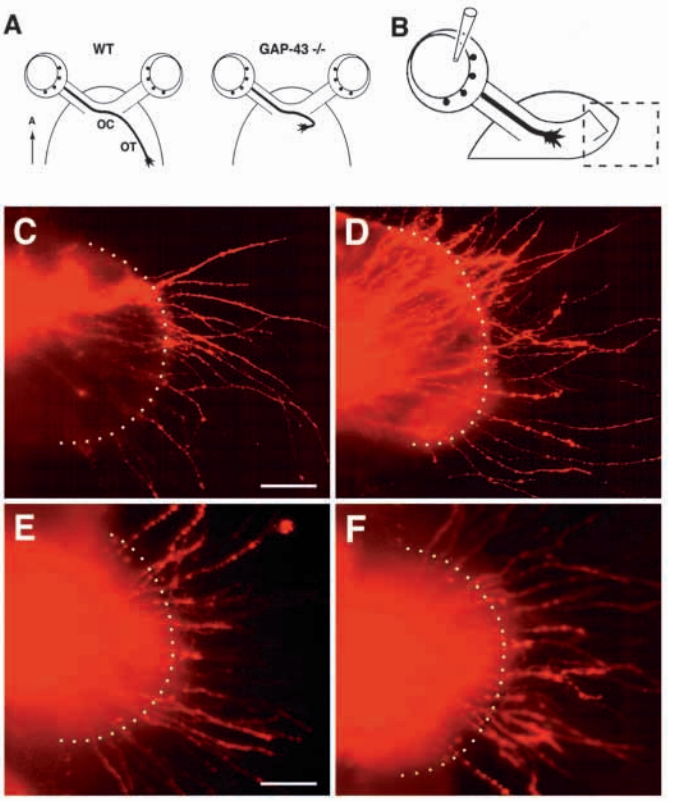

Fig. 1. Summary of RGC axon pathfinding defect in GAP-43-deficient embryos in vivo and RGC axon outgrowth from modified retina-chiasm preparations in vitro. (A) Diagrams depicting the normal RGC axon pathway at E14 in wild-type (left) and GAP-43-deficient (right) embryos. Normally by E14, RGC axons have grown through the midline chiasm region (OC) and have extended into the lateral wall of the diencephalon forming the optic tract (OT). In GAP-43-deficient embryos, RGC axons grow through the midline but are able to progress from the midline region into the lateral wall of the diencephalon, and an optic tract is not evident. (B) Schematic diagram showing the modified in vitro retina-chiasm tissue preparation. The area of the rectangle is shown in C-F. (C,D) RGC axon outgrowth from the optic chiasm regions of wild-type (C), and homozygous GAP-43-deficient (D) embryos following removal of lateral diencephalon tissue and grown on laminin substratum. The dotted white lines represent the cut edge of the tissue preparations. DiI-labeled axons can be seen growing out of the midline chiasm region. (E,F) RGC axon outgrowth from the optic chiasm regions of wild-type (E), and homozygous GAP-43-deficient (F) embryos following removal of lateral diencephalon tissue and grown in collagen. DiI-labeled RGC axons are less well defined when visualized through the collagen. Scale bars (C,D) 100 μm; (E,F) 100 μm.

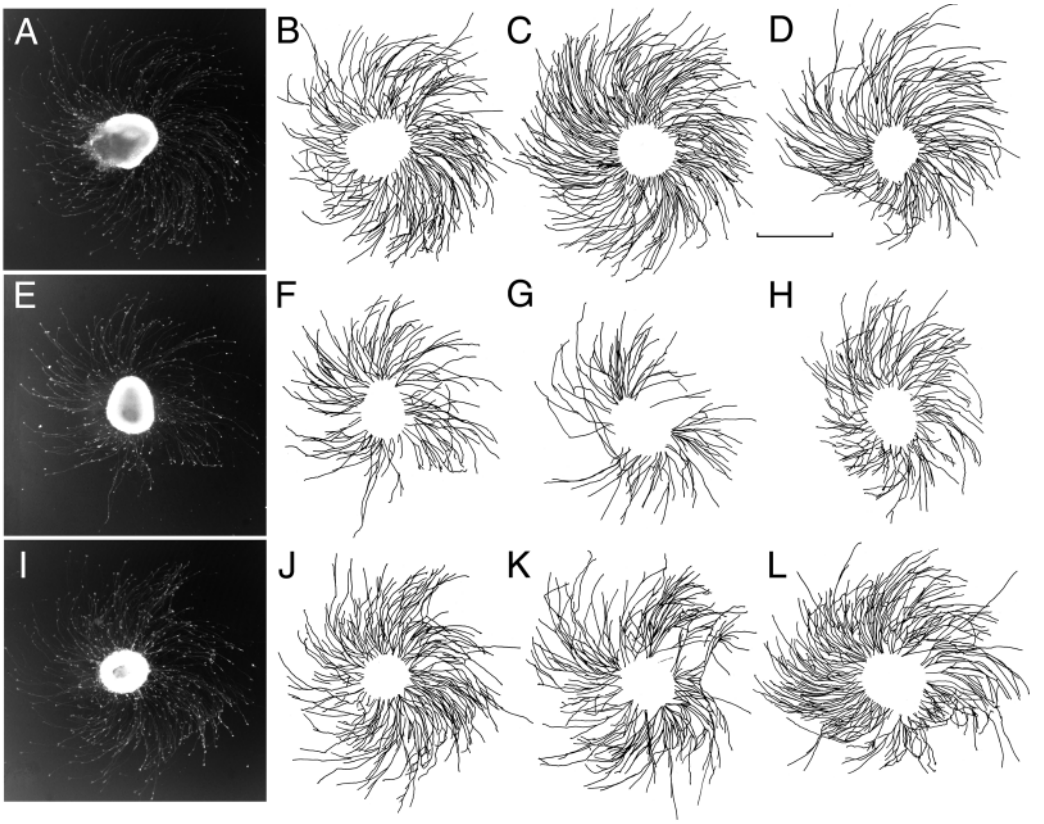

Fig. 2. Neurite outgrowth from retinal explants of wild-type and GAP-43-deficient E13 embryos. (A-D) Wild-type retinal explants and neurites after 24-28 hours. of growth in the presence of lateral diencephalon membrane fragments. (A) Texas-Red Phalloidin-stained explant. (B-D) Tracings of neurite outgrowth from other wild-type explants. (B is a tracing of the explant in A.) (E-H) GAP-43-deficient retinal explant and neurites after 24-28 hours of growth in the presence of lateral diencephalon membrane fragments. (E) Texas-Red Phalloidin-stained explant. (F-H) Tracings of neurite outgrowth from other wild-type explants. (F is a tracing of the explant in E.) (I-L) GAP-43-deficient retinal explant and neurites after 24-28 hours. of growth on laminin alone. (I) Texas-Red Phalloidin-stained explant. (J-L) Tracings of neurite outgrowth from additional wild-type retinal explants. (J is a tracing of the explant in I.) Scale bar, 500 μm.

heterozygous embryos showed a similar amount of outgrowth (37.36±10.34 mm; n=8; Fig. 3B). In contrast, explants obtained from GAP-43-deficient embryos (Fig. 2E-H) showed a significantly reduced amount of neurite outgrowth (18.36±5.55 mm; n=10; P<0.001; 2-sample t-test with unequal variance). This reduced neurite outgrowth was not due to a reduced ability of GAP-43 retinal axons to grow on laminin since GAP-43-deficient retinal axons cultured in the absence of lateral diencephalon membrane fragments (Fig.2I-L) exhibited a larger amount of neurite outgrowth (31.58±10.47 mm (s.d.); n=8). This amount of outgrowth was not statistically different to wild-type or heterozygous retinal explants grown in the presence of lateral diencephalon cell membranes (P=0.732, P=0.782, respectively).

The reduced growth of GAP-43-deficient axons in the presence of lateral diencephalon cell membranes was not due to a delay in initiation of neurite outgrowth. At 14-18 hours following culture, a similar amount of neurite outgrowth was present from wild-type, heterozygous and GAP-43-deficient explants (Fig. 3A). GAP-43-deficient neurites also did not appear to catch up in growth. At 40-48 hours, GAP-43-deficient neurites still showed a significantly reduced amount of growth (P<0.001) compared neurites from heterozygous and wild-type retinal explants (Fig. 3C).

Outgrowth in lateral diencephalon conditioned medium

To investigate the nature of this growth inhibitory activity on GAP-43-deficient axons, conditioned medium derived from lateral diencephalon cells was tested on wild-type and GAP-43-deficient retinal explants. In the presence of conditioned medium, compared to cell membrane fragments, neurite

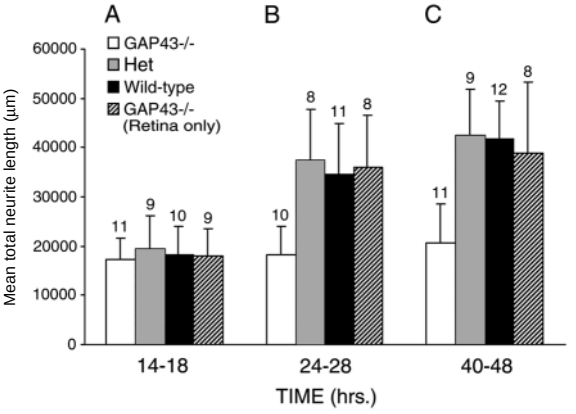

Fig. 3. Graph of mean total neurite length from retinal explants in the presence of lateral diencephalon membrane fragments. Measurements were made after growth for 14-18 hours, 24-28 hours, and 40-48 hours. The number of explants measured is indicated on the top of each error bar representing the standard deviation. GAP-43$^{-/-}$ (retina only) represents GAP-43-deficient retinal explants grown on laminin without lateral diencephalon cell membrane fragments. At 14-18 hours, the total amount of neurite outgrowth from GAP-43-deficient retinal explants in the presence of lateral diencephalon cell membrane fragments was not significantly different than for the other three conditions (pairwise comparison versus wild-type P=0.69; versus heterozygotes P=0.30; versus GAP-43$^{-/-}$ retina alone P=0.61; two-sample t-test with unequal variance). At both 24-28 hours and 40-48 hours, the total amount of neurite outgrowth from GAP-43-deficient retinal explants in the presence of lateral diencephalon cell membrane fragments was significantly less than for the other three conditions (P<0.001 for all pairwise comparisons, two-sample t-test with unequal variance).

outgrowth was reduced for both wild-type (Fig. 4A-C) and GAP-43-deficient explants (Fig. 4D-F). This finding likely reflected the inhibitory effects of a previously described diffusible factor secreted from the ventral half of the diencephalon (Tuttle et al.,1998). The conditioned medium used in the present study however did not differentially affect wild-type and GAP-43-deficient explants. At each of three time points from 14 to 48 hours, the amount of neurite outgrowth from wild-type and GAP-43-deficient explants was similar (Fig. 4G). These results of neurite outgrowth in the presence of conditioned medium or membrane fragments are consistent with a requirement for GAP-43 function in retinal growth cones for an appropriate response to a membrane associated cue on lateral diencephalon cells.

Cell type specificity

The outgrowth of wild-type and GAP-43-deficient neurites was also tested on membrane fragments derived from three other cell types. No significant differences in total neurite outgrowth were observed at 24-28 hours between wild-type and GAP-43-deficient explants grown in the presence of membrane fragments from E13 ventral midline diencephalon (chiasm) (P=0.344), cortex (P=0.478) or dorsal midbrain cells (P=0.387) (Fig. 4H). Thus GAP-43 does not appear to be involved in axon growth responses to CNS tissues in general. Rather, it seems specifically involved in RGC axon interactions with lateral diencephalon cells.

Response to increasing amounts of lateral diencephalon membranes

The results so far do not readily distinguish between the possibilities that GAP-43 is required for retinal axons to respond appropriately to a lateral diencephalon cue that promotes retinal axon growth or a cue that is inhibitory. The findings suggests the latter possibility since the retinal axons are cultured on a favorable substratum, laminin. Therefore, the added cell membranes are less likely to be an additional source of growth-promoting activity but could be a source of growth inhibitory cues. To investigate further, GAP-43-deficient retinal axon growth was examined in experiments in which both cortical and lateral diencephalon membranes were added together in varying amounts but in which the total amount of cell membrane fragments added was held constant (Fig. 5). Results from these dose-response experiments showed that, as the percentage of lateral diencephalon cell membranes was increased from 25% to 75% of the total membranes present (Fig. 5, dosage combinations 1-3), the mean total amount of GAP-43-deficient neurite growth decreased significantly from

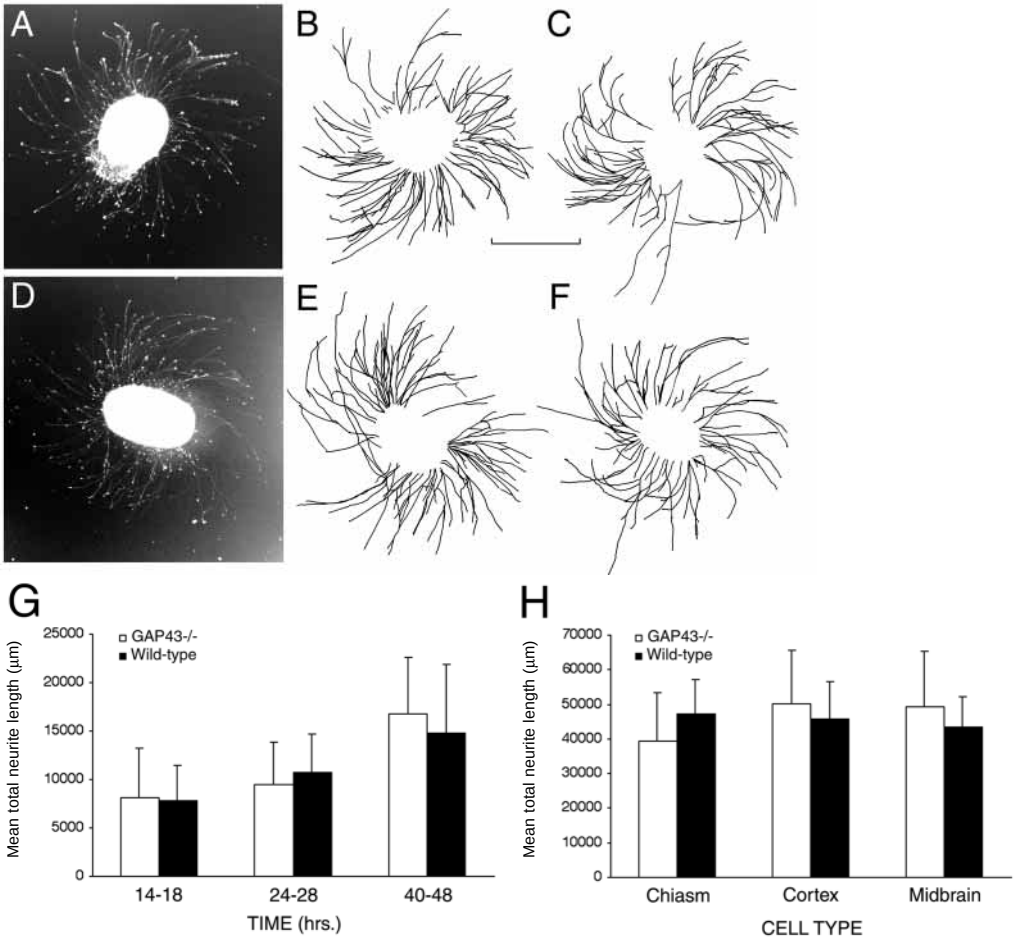

Fig. 4. Wild-type and GAP-43-deficient retinal neurite outgrowth in lateral diencephalon condition medium or with membranes of other CNS cell types. (A-C) Wild-type retinal explants after 24-28 hours of growth in lateral diencephalon conditioned medium. (A) Texas-Red Phalloidin-stained explant. (B,C) Tracings of neurite outgrowth from additional wild-type retinal explants. (D-F) GAP-43-deficient retinal explants after 24-28 hours of growth in lateral diencephalon conditioned medium. (D) Texas-Red Phalloidin-stained explant. (E,F) Tracings of neurite outgrowth from additional GAP-43-deficient retinal explants. (G) Mean total neurite length from wild-type and GAP-43-deficient retinal explants after 14-18 hours, 24-28 hours and 40-48 hours of growth in lateral diencephalon cell conditioned medium. No significant differences were observed between wild-type and GAP-43-deficient explants at 14-18 hours, P=0.893; at 24-28 hours, P=0.527; at 40-48 hours, P=0.547 (two-sample t-test with unequal variance). Error bars show standard deviation. (H) Mean total neurite length from wild-type and GAP-43-deficient retinal explants after 24-28 hours in the presence of membrane fragments from E13 diencephalon ventral-midline (optic chiasm region), cortex or dorsal midbrain. No significant differences were observed between wild-type and GAP-43-deficient explants in membrane fragments from ventral midline diencephalon (chiasm) (P=0.344), cortex (P=0.478), or dorsal midbrain cells (P=0.387). Error bars, standard deviation. Scale bar (A-F) 500 μm.

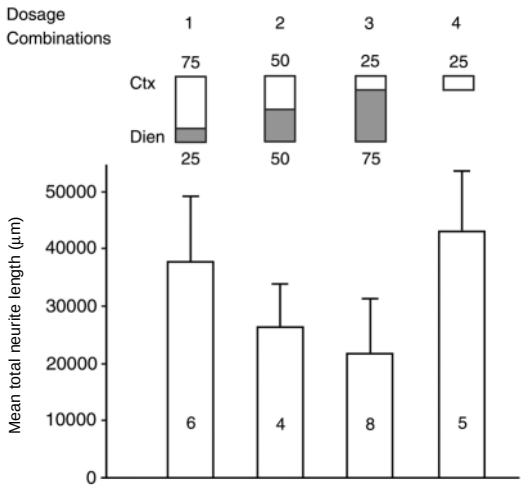

Fig. 5. Mean total retinal neurite outgrowth in response to increasing amounts of lateral diencephalon membranes. In experimental conditions represented by dosage combinations 1-3, the total amount of membrane fragments was held constant; however the amount of cortical and lateral diencephalon membranes in each was varied. The numbers above each dosage schematic represent the percentage of cortical membranes, while the numbers below represent the percentage of lateral diencephalon membranes. Dosage combination 4 represent experiments in which only cortical membrane fragments were added in an amount equivalent to 25% of the total membrane fragments present in dosage combinations 1 to 3. The results showed that the addition of increasing amounts of lateral diencephalon membrane fragments resulted in a decreased amount of mean total retinal neurite outgrowth. The addition of cortical membranes alone in an amount equivalent to 25% of the amount of total membrane fragments used in dosage combination 3 resulted in a significantly higher amount of mean total retinal neurite outgrowth. See text for details.

38.21±13.88 mm; n=6, to 22.34±10.14 mm; n=8; P<0.05. As a control experiment (dosage combination 4), cortical membrane fragments alone were added at an amount equivalent to 25% of the total amount of membranes used in the previous dosage combinations 1-3. In this situation, the mean total neurite outgrowth was 41.59±11.54 mm; n=5 (Fig. 5, dosage combination 4), and was significantly greater than that of dosage combination 3 (P<0.01). Since in each of dosage combinations 1-3, an amount of cortical membranes sufficient for a larger amount of neurite outgrowth was present, the reduced neurite outgrowth observed with increasing lateral diencephalon membranes is not likely due to insufficient cortical membrane fragments promoting neurite outgrowth. In the same vein, the results are difficult to explain on the basis of an inability of GAP-43-deficient RGC axons to recognize a hypothetical growth-promoting effect of lateral diencephalon membranes. Although not definitive, these experiments are most consistent with the interpretation that lateral diencephalon membranes have an inhibitory activity and that GAP-43-deficient RGC axons respond abnormally to this inhibitory cue.

Transplantation of the lateral diencephalon optic tract entry area

The results from in vitro experiments using retinal tissue explants indicate that GAP-43 function might be specifically required for entry into the lateral diencephalon. However, given the possibility that RGC axons growing through the optic chiasm region in vivo may modify their guidance properties, it may be argued that the use of retinal tissue explants in vitro to investigate postchiasmatic or optic tract axon guidance may not be entirely satisfactory. To obtain independent evidence that GAP-43 function is required for appropriate response to lateral diencephalon cues, we reasoned that GAP-43-deficient axons may be able to leave the midline region if the adjacent lateral diencephalon was removed and substituted by tissue further along the visual pathway. To test this, transplantation studies were performed in E12.5 retina-chiasm preparations which contained both retinas and the entire ventral hypothalamus (Sretavan and Reichardt, 1993; Sretavan et al., 1995). A 200×200 μm^2 region of the lateral diencephalon (starting 400 μm lateral to the midline) containing the optic tract entry area (Fig. 6A) was microsurgically replaced with a transplant consisting of a 200×200 μm^2 piece of tissue from the anterior region of the dorsal midbrain (approximately the anterior part of the future superior colliculus) from E12.5-E13.5 embryos (Fig. 6B). (Tissue from wild-type embryos can be used as transplants since GAP-43 functions cell autonomously within RGC axons (Kruger et al., 1998).) Following transplantation, GAP-43-deficient RGC axons exiting the optic chiasm region no longer encountered the lateral diencephalon but faced a different non-midline portion of the visual pathway. RGC axons in these preparations were labeled by intraocular injections of DiI and the host preparations containing tissue transplants were placed ventral side up in cell culture medium at 37°C for 56-78 hours. At the beginning of the culture period (E12.5), RGC axons have yet to enter the lateral diencephalon (Fig. 6C,D).

RGC axons grow into transplanted optic tract entry region

A set of control experiments consisted of wild-type embryos obtained from a GAP-43 heterozygous female bred with a heterozygous male to demonstrate that the tissue transplantation procedure did not interfere with the ability of wild-type RGC axons to grow from the midline region into the lateral diencephalon. The 200×200 μm^2 area starting at 400 μm lateral to the midline was removed from host embryos containing the portions of the lateral diencephalon where the future optic tract will be formed. In its place, a DiO-labeled 200×200 μm^2 piece of the tissue from a wild-type donor embryo starting from 500 μm lateral to the midline was inserted. (The use of lateral diencephalon tissue 500 μm lateral to the midline for transplantation eliminated the possibility that part of the midline region was inadvertently included in the transplant. This procedure was used in all experiments involving transplants of lateral diencephalon tissue, including that in GAP-43-deficient mice.) In these control experiments (n=4), DiI-labeled RGC axons from host preparations grew from the midline region into the donor lateral diencephalon tissue (Fig. 6E,F). Some RGC axons that entered the donor tissue exited from the other end to grow back into the host preparation. The presence of RGC axon growth indicated that the donor diencephalon tissue integrated well into the host and RGC axons were able to grow through the donor-host boundary.

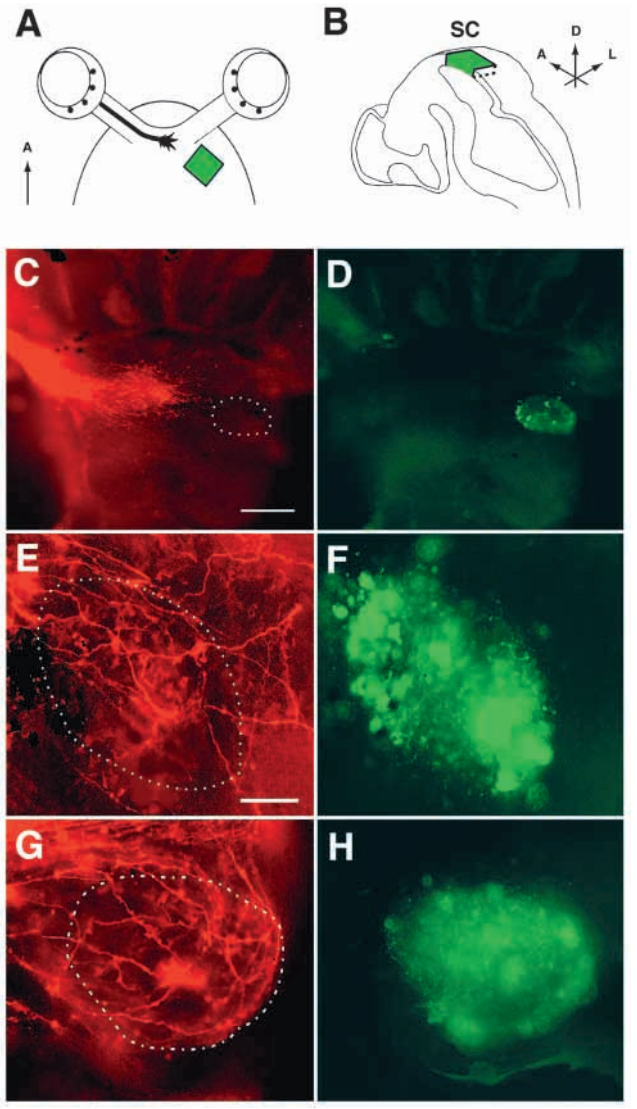

Fig. 6. RGC axon growth following transplantation replacement of the lateral diencephalon optic tract entry region with developing anterior midbrain tissue. (A) Schematic diagram showing in horizontal view the retina-chiasm preparations from E12.5 embryos used in transplantation experiments. The green square represents the location in the lateral diencephalon where an approximately 200×200 μm^2 piece of tissue containing the entrance into the optic tract is replaced by DiO-labeled lateral diencephalon/optic tract tissue in control experiments or by developing midbrain tissue in transplantation experiments. A, anterior. (B) Schematic of the developing brain as viewed from the cut sagittal surface. The region depicted in green and by the dotted line is the approximate area of the developing midbrain where a 200×200 μm^2 piece of tissue was taken, labeled with DiO, and transplanted into host retina-chiasm preparations shown in A. (C) Host RGC axons in a retina-chiasm preparation labeled with DiI have reached the midline but none have yet entered the lateral diencephalon wall to form the optic tract at the time of transplantation (E12.5). (D) The same retina-chiasm preparation using fluorescein optics to visualize the DiO-labeled anterior midbrain tissue transplant replacing lateral diencephalon tissue. (E,F) High-magnification views of the pattern of wild-type RGC axon ingrowth into a lateral diencephalon transplant obtained from a donor embryo. The dotted line in E represents the outline of the transplanted optic tract tissue. (F) The DiO-labeled transplanted lateral diencephalon tissue. Labeled wild-type RGC axons grow into the transplant and some exit the transplant to continue into the lateral wall of the host diencephalon. (G,H) The pattern of wild-type RGC axon growth into an anterior midbrain transplant obtained from a donor embryo. (G) The dotted line represents the outline of the transplanted midbrain tissue. (H) The DiO-labeled transplanted tissue. Wild-type RGC axons grow from the midline chiasm region into the transplanted midbrain tissue similar to their growth into transplanted lateral diencephalon tissue. RGC axons only occasionally exit from the midbrain tissue into the lateral diencephalic wall of the host preparation. Scale bars (C,D) 200 μm; (E-H) 50 μm.

RGC axons enter transplanted midbrain tissue

A second set of control experiments involved wild-type embryos in which donor tissue obtained from the anterior region of the midbrain was substituted for host lateral diencephalon tissue (*n*=4). The results showed that RGC axons grew from the midline region into the donor midbrain tissue (Fig. 6G,H) similar to host RGC axon growth into donor lateral diencephalon tissue described above. Thus wild-type RGC axons exiting the midline region apparently do not distinguish between lateral diencephalon tissue and midbrain tissue in that both can serve as cellular substrata for growth of wild-type RGC axons with GAP-43 function. We found that RGC axons that entered the anterior midbrain transplant tended to remain within this tissue, although some axons do extend into the host. This likely reflects a property of midbrain tissue that normally develops into a target for RGC axons.

GAP-43-deficient axons fail to enter transplanted optic tract entry area

In the third set of control experiments, the effects of the transplantation procedure on RGC axon growth out of the midline region in GAP-43-deficient embryos were tested by removing host lateral diencephalon tissue and then transplanting lateral diencephalon tissue obtained from a donor GAP-43-deficient animal (*n*=4). Results showed that, in this situation, GAP-43-deficient RGC axons, like wild-type axons, grew within the retina-chiasm preparations across the midline through the chiasm region. However, unlike wild-type RGC axons, which grew into the transplanted lateral diencephalon tissue, GAP-43-deficient RGC axons did not enter the lateral diencephalon transplant (Fig. 7A-C). This absence of GAP-43-deficient RGC axon growth resembled the lack of GAP-43-deficient RGC axon growth into the lateral diencephalon in vivo to form the optic tracts (Kruger et al., 1998). The presence of a guidance defect at the transition between the midline chiasm region and the lateral diencephalon demonstrated that the transplantation procedure by itself did not induce GAP-43-deficient RGC axons to invade the lateral diencephalon.

GAP-43-deficient axons enter transplanted midbrain tissue

The replacement of the lateral diencephalon region in GAP-43-deficient hosts with anterior midbrain tissue from wild-type or GAP-43-deficient donors produced quite a different pattern of RGC axon growth. In this case, GAP-43-deficient RGC axons did not stop at the transition between the chiasm region and the transplanted midbrain tissue and grew into all regions of the midbrain tissue transplant (Fig. 7D-L). The GAP-43-

deficient RGC axon growth pattern in the transplant was similar to that of wild-type RGC axons growing into a midbrain tissue transplant (compare to Fig. 6G,H). Thus when not faced with lateral diencephalon tissues, GAP-43-deficient RGC axons freely exited the chiasm region to grow into the transplanted anterior midbrain tissue.

Quantitation of RGC axon growth

The difference in GAP-43-deficient RGC axon growth into lateral diencephalon versus midbrain tissue transplants was quantified by measuring the amount of DiI-labeled RGC axons within the transplant itself and the amount found in an equivalent-sized area of the host midline chiasm region just outside the transplant. (This latter measurement represented RGC axons that were in a position to grow into the transplant.) The amount of axon growth in the transplant was then expressed as a ratio to the growth measured in the midline chiasm region to obtain a growth index (Table 1). A small growth index of close to zero reflects an inability of RGC axons to enter the transplant, while a larger index closer to or greater than one indicates robust RGC axon growth into the transplant. (An index greater than one was obtained in situations where RGC axons instead of growing straight through the transplant, meandered within the transplant itself.)

The amount of DiI-labeled RGC axons in the 200×200 μm^2 area of the midline region was not significantly different between host preparations which received midbrain transplants (8.8±1.9 mm (s.d.), n=5) and host preparations which received lateral diencephalon transplants (11.5±4.2 mm, n=4; P=0.24; see also Table 1). This indicated that, in both situations, roughly equal amounts of RGC axon were in a position to enter the transplant. On the contrary, the amount of DiI-labeled RGC axons within the transplanted tissue itself was significantly greater in host preparations that received midbrain transplants (8.6±2.7 mm (s.d.), n=5) compared to host preparations that received lateral diencephalon transplants (0.62±0.54 mm, n=5; P<0.001; Table 1). No significant difference was observed in the sizes (surface area) of lateral diencephalon transplants (19.6±2.3×10^3 μm^2 (s.d.), n=4) compared to midbrain transplants (21.6±5.4×10^3 μm^2 (s.d.), n=5; P=0.52).

DISCUSSION

In this study, we compared wild-type and GAP-43-deficient RGC axons in their ability to exit the ventral midline optic chiasm and in their interactions with lateral diencephalon tissue representing approximately the future optic tract region. GAP-43-deficient RGC axons were no different to wild-type axons in their ability to exit the chiasm following either simple removal of the lateral diencephalon or transplant replacement of lateral diencephalon tissue with tissue from other parts of the retinal pathway. However, compared to wild-type RGC axons, GAP-43-deficient RGC axons were less capable of growing in the presence of membrane fragments from lateral

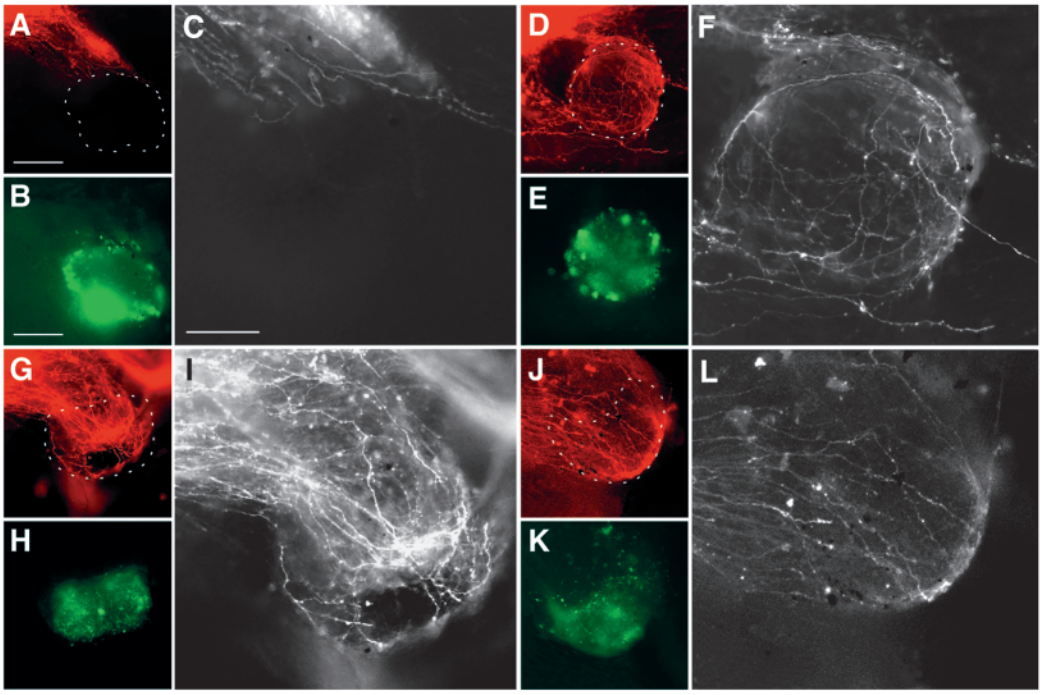

Fig. 7. GAP-43-deficient RGC axon growth into lateral diencephalon and midbrain transplants. (A-C) The pattern of RGC axon growth (higher magnification in C) in a GAP-43-deficient host preparation that received a DiO-labeled lateral diencephalon transplant (B) originating from a GAP-43-deficient donor and grown for 78 hours. The dotted line in A marks the location of the transplanted diencephalon tissue. GAP-43-deficient RGC axons approach the lateral diencephalon tissue but do not enter, mimicking the in vivo failure of optic tract formation. (D-F) The pattern of RGC axon growth (D, higher magnification in F) in a GAP-43-deficient preparation in which the lateral diencephalon (entrance of the optic tact) was substituted by a DiO-labeled midbrain transplant originating from a wild-type donor (E) and grown for 69 hours. The dotted line in D marks the location of the transplanted midbrain tissue. DiI-labeled GAP-43-deficient RGC axons grow extensively into the midbrain transplant. (G-I) The pattern of RGC axon growth (G, higher magnification in I) in a second GAP-43-deficient preparation in which the lateral diencephalon region (entrance of the optic tract) was substituted by a DiO-labeled midbrain transplant originating from a GAP-43 wild-type donor (H) and grown for 72 hours. The dotted line in G marks the location of the transplanted midbrain tissue. As in the preparation shown in D-F, DiI-labeled GAP-43-deficient RGC axons grow extensively into the midbrain transplant. (J-L) A third preparation in which the lateral diencephalon region (entrance of the optic tract) was substituted by a DiO-labeled midbrain transplant originating from a GAP-43-deficient donor (K) and grown for 73 hours. The dotted line in J marks the location of the transplanted midbrain tissue. GAP-43-deficient RGC axons (J, higher magnification in L) grow into the transplanted midbrain tissue. Scale bars, 100 μm; except for C,F,I,L, 50 μm.

Table 1. Quantitation of the amount of RGC axon growth in the midline chiasm region compared to RGC axon growth within the transplanted tissue

(A) GAP-43-deficient hosts with lateral diencephalon transplants

A: Optic chiasm midline region (mm)	B: Total amount of GAP-43-deficient RGC axon growth — Lateral diencephalon transplant (mm)‡	Growth index B/A
6.8	0	0
13.6	0.5	0.04
16.1	0.7	0.04
9.3	1.3	0.14
mean 11.5±4.2 (s.d.)*	0.62±0.54 (s.d.)§	0.05±0.06 (s.d.)

(B) GAP-43-deficient hosts with midbrain transplants

A: Optic chiasm midline region (mm)	B: Total amount Of GAP-43-deficient RGC axon growth — Midbrain transplant (mm)‡	Growth index B/A
11.3	6.0	0.53
10.2	8.9	0.87
7.5	6.7	0.89
6.9	8.4	1.22
8.1	12.9	1.59
mean 8.8±1.9 (s.d.)*	8.6±2.7 (s.d.)§	1.02±0.4 (s.d.)

*The amounts of GAP-43-deficient RGC axon growth in the chiasm region outside of the transplant in hosts that received either lateral diencephalon (11.5±4.2 mm (s.d.)) or midbrain (8.8±1.9 mm (s.d.)) transplants were not significantly different to each other (P=0.24).

‡The average size (surface area) of the lateral diencephalon transplants ($19.6\pm2.3\times10^3$ μm^2 (s.d.), n=4) was not significantly different to the average size of the midbrain tissue transplants ($21.6\pm5.4\times10^3$ μm^2, n=5; P=0.52).

§The average amount of RGC axon growth within lateral diencephalon transplants (0.62±0.54 mm (s.d.)) was significantly less than the average amount of RGC axon growth in anterior midbrain tissue transplants (8.6±2.7 mm; P<0.001).

diencephalon cells. This appeared to be due to a cell-associated activity since conditioned medium was not effective in producing this difference. This behavior was also not mimicked by membranes obtained from the midline chiasm region, cortex or dorsal midbrain. Together, these results show that GAP-43 function is apparently not required for RGC axons to leave the midline chiasm region. Instead, GAP-43 function mediates RGC axon interaction with lateral diencephalon cells and thus is likely involved in RGC axon growth within the lateral diencephalon and formation of the optic tract. This result is informative since identification of the retinal pathfinding event requiring GAP-43 is a necessary step towards understanding how this intracellular growth cone protein acts during RGC axon guidance. Furthermore, although the mechanisms underlying optic tract formation are poorly understood, the present study indicates that lateral diencephalon guidance cues mediating optic tract development very likely trigger signal transduction cascades involving GAP-43.

One line of evidence that GAP-43 is not used by RGC axons in order to leave the midline optic chiasm region is that GAP-43-deficient RGC axons are capable of exiting the midline region when the adjacent lateral diencephalon tissue is replaced by transplantation of dorsal midbrain tissue. We consider it unlikely that the effects observed with midbrain tissue transplants reflect that fact that they behave like midline chiasm tissue and the transplant merely increases the size of the 'chiasm region'. The transplanted midbrain tissue is not a midline commissural region for RGC axons and axonal growth characteristics are known to be quite different within the chiasm and the midbrain. For instance, time-lapse analysis of living RGC axons show that RGC axons entering the superior colliculus (optic tectum in birds & amphibia) branch extensively (Harris et al., 1987; O'Rourke and Fraser, 1990;), but this mode of axon growth has not been observed at the chiasm region (Sretavan and Reichardt, 1993; Godement et al., 1994). A second possibility is that GAP-43 function is normally required for RGC axons to modify their responses to midline cues and exit from the chiasm region, but that the presence of midbrain tissue somehow overrides this requirement. Although we cannot directly rule out this possibility, it should be noted that GAP-43-deficient axons are capable of exiting the chiasm region in the absence of midbrain tissue placed next to the chiasm region (Fig. 1).

Pathway segments with distinct axon guidance mechanisms

The finding that GAP-43-deficient retinal axons in vivo do not progress normally from the chiasm region into the lateral diencephalon (Strittmatter et al., 1995; Kruger et al., 1998) is complemented by the present results showing that these two brain regions differ in their effects on GAP-43-deficient retinal axon behavior. Together this data indicate that the optic chiasm and the optic tract represent segments of the visual pathway exhibiting distinct RGC axon guidance mechanisms. This idea has been suggested by previous anatomical data comparing retinal axon behavior at these two sites. For example, RGC axons organize into a more compact bundle as they leave the chiasm to grow into the diencephalon. In addition, RGC growth cones change from their more complex morphologies in the chiasm to exhibit simple spear-like endings as they grow within the optic tract (Bovolenta and Mason, 1987, Mason and Wang, 1997). Differences in glial composition have also been described between the chiasm region and more lateral diencephalon regions (Reese et al., 1994) raising the possibility that axon-glial interactions underlie RGC axon guidance at these two sites. A fundamental reason why RGC axon guidance might differ significantly between the chiasm and the tract comes from a study of homeobox gene expression, which shows that the initial portion of the optic tracts is formed along the embryonic longitudinal axis; parallel to the expression domain of the homeobox gene *Nkx2.2* (Marcus et al., 1999). Thus the chiasm/optic tract junction may represent a site where embryonic RGC axons exit a commissure (the optic chiasm) to enter a longitudinal pathway.

Transition from commissures into longitudinal tracts

Axon guidance at the transition from commissural pathways into longitudinal tracts have been investigated during development of both the invertebrate CNS and the mammalian spinal cord. In the invertebrate CNS nerve cord, commissural axons originating from one side cross the midline to extend along longitudinal pathways on the opposite side. The ability of commissural axons to exit the midline region is thought to involve the upregulation of Robo protein on commissural axons, which mediates axon response to the midline inhibitory

protein Slit, thus preventing commissural axons from re-entering the midline (Kidd et al., 1999; Brose et al., 1999). Mutations in robo function result in commissural axons with multiple midline crossings and a failure of longitudinal pathways to form properly (Seeger et al., 1993; Kidd et al., 1998; Zallen et al., 1998). In vertebrates, commissural axons that have grown through the ventral midline floorplate lose their ability to respond to chemoattractants within the floorplate itself (Shirasaki et al., 1998), a feature that has been proposed to facilitate commissural axon exit from the midline. While RGC axon exit from the midline chiasm region may well involve modifications of pathfinding mechanisms necessary for guidance to the midline, GAP-43 does not appear to be involved in this process. Instead it operates as part of a guidance mechanism needed for entry into the next segment of the retinal pathway. Thus separate guidance mechanisms may exist to mediate RGC axon exit from the chiasm region and entry into longitudinal pathways.

Pathfinding cues in the lateral diencephalon

Why might GAP-43 function be specifically required for RGC axon growth into the lateral diencephalon? A recent study demonstrated that cerebellar granule cell axons from GAP-43-deficient mice, compared to wild-type axons, grow less well on 3T3 cells in response to soluble NCAM; raising the possibility that GAP-43 mediates NCAM-dependent axon growth (Meiri et al., 1998). This is of note since NCAM has been described in the developing diencephalon and correlated with the approximate region of the optic tract (Silver and Rutishauser, 1984; D. W. S., unpublished). NCAM is also found in other parts of the RGC axon pathway and antibody perturbation studies have implicated this adhesion molecule in RGC axon guidance within the chick retina (Thanos et al., 1984, Brittis and Silver, 1995) and in the frog optic tectum (Fraser et al., 1984). GAP-43-deficient mice, however, do not show gross abnormalities in RGC axon pathfinding within the retina (Kruger et al., 1998); although mild defects have not been ruled out. Similarly, NCAM-deficient mice have not been reported to have a GAP-43-like visual system phenotype (Tomasiewicz et al., 1993; Cremer et al., 1994; Stork et al., 1997). Nevertheless, the reduced growth of GAP-43-deficient axons in response to NCAM suggests it is worthwhile examining the possible involvement of members of the immunoglobulin superfamily of guidance molecules in optic tract RGC axon pathfinding.

Results from the present study, though not definitive, are consistent with the requirement of GAP-43 function for appropriate RGC axon response to a lateral diencephalon inhibitory cue. A previous study has shown that disruption of GAP-43 function by antisense oligonucleotides causes DRG neurites in vitro to be more sensitive than normal to the growth inhibitory effects of myelin proteins (Aigner and Caroni, 1995). An interpretation of these results is that GAP-43 may normally function to dampen responses to inhibitory cues. Since the lateral diencephalon/optic tract region has been reported to be inhibitory for RGC axon growth in vitro (Tuttle et al., 1998), the fact that RGC axons normally are capable of growing through this region may reflect the inhibition dampening effects of GAP-43 in RGC growth cones. Of note, this inhibitory activity is thought to be secreted by lateral diencephalon cells. Our results are consistent with the presence of a diffusible inhibitory activity from the lateral diencephalon since RGC neurites formed in the presence of lateral diencephalon cell conditioned medium are reduced in length (see Fig. 4G) compared to neurites growing in the presence of lateral diencephalon membranes. However, no differences were noted between wild-type and GAP-43-deficient axons in their response to the lateral diencephalon conditioned medium used in the present study, suggesting that GAP-43 function may be required for interaction, not with a diffusible component, but with a cell membrane cue. However further examination is needed since a diffusible factor may be bound to the cell surface matrix and be present on membrane fragments.

The presence of one or more inhibitory cues in the lateral diencephalon raises the question of their function. One possibility is that inhibitory cues are involved in local developmental events such as cell migration within the lateral diencephalon and that GAP-43, through a inhibition dampening effect, simply allows RGC axons to successfully traverse this region. A second possibility suggested by Tuttle et al. (1998) is that this lateral diencephalon inhibitory activity prevents RGC axons from entering the ventral diencephalon tissue and making inappropriate synaptic connections. A third possibility is that growth through inhibitory regions promote RGC axon fasciculation which could be important for the topographic sorting of RGC axons according to functional classes that is known to occur within the optic tract (Reese, 1987; Reese and Cowey, 1988).

GAP-43 signaling function

Biochemically, GAP-43 has been shown in vitro to act as a highly abundant calmodulin binding protein which upon phosphorylation by PKC, releases calmodulin and thus regulates calmodulin availability and signaling (Skene, 1990; Benowitz and Routtenberg, 1997). The involvement of GAP-43 in the progression of RGC axons from the chiasm into the lateral diencephalon raises the possibility that PKC and calmodulin-dependent signaling is involved in RGC growth cone guidance in optic tract development. Of note, alteration of calmodulin function in *Drosophila* has been reported to affect commissural axon progression from the midline region into longitudinal pathways (VanBerkum and Goodman, 1995). Thus, in mice, further insight into optic tract axon guidance may come about through experimental inhibition of PKC activity, studies of GAP-43 phosphorylation states and alterations in GAP-43 calmodulin binding in RGC axons.

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REFERENCES

Aigner, L. and Caroni, P. (1995). Absence of Persistent Spreading, Branching, and Adhesion in GAP-43-depleted Growth Cones. *J. Cell Biol.* **128**, 647-660.

Alexander, K. A., Cimler, B. M., Meier, K. E. and Storm, D. R. (1987). Regulation of calmodulin binding to P-57: A neurospecific calmodulin binding protein. *J. Biol. Chem.* **262**, 6108-6113.

Alexander, K. A., Wakim, B. T., Doyle, G. S., Walsh, K. A. and Storm, D. R. (1988). Identification and characterization of the calmodulin-binding

domain of neuromodulin, a neurospecific calmodulin-binding protein. *J. Biol. Chem.* **263**, 7544-7549.

Benowitz, L. I. and Routtenberg, A. (1997). GAP-43: An intrinsic determinant of neuronal development and plasticity. *Trends in Neurosci.* **20**, 84-91.

Bovolenta, P. and Mason, C. (1987). Growth cone morphology varies with position in the developing mouse visual pathway from retina to first targets. *J. Neurosci.* **7**, 1447-1460.

Brittis, P. A. and Silver, J. (1995). Multiple factors govern intraretinal axon guidance: a time-lapse study. *Mol. Cell Neurosci.* **6**, 413-432.

Brose, K., Bland, K. S., Wang, K.-H., Arnott, D., Henzel, W., Goodman, C. S. and Tessier-Lavigne, M. (1999). Evolutionary conservation of the repulsive guidance function of Slit protein and their interactions with robo receptors. *Cell* **96**, 795-806.

Chapman, E. R., Au, D., Alexander, K. A., Nicolson, T. A. and Storm, D. R. (1991). Characterization of the calmodulin binding domain of neuromodulin. *J. Biol. Chem* **266**, 207-213.

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S., Bathels, D., Rajewsky, K. and Wille, W. (1994). Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature* **367**, 455-459.

Fraser, S. E., Murray, B. A., Chuong, C.-M. and Edelman, G. (1984). Alteration of the retinotectal map in Xenopus by antibodies to neural cell adhesion molecules. *Proc. Natl. Acad. Sci.* **81**, 4222-4226.

Godement, P., Wang, L. C. and Mason, C. A. (1994). Retinal axon divergence in the optic chiasm: dynamics of growth cone behavior at the midline. *J. Neurosci.* **14**, 7024-7039.

Harris, W. A., Holt, C. E. and Bonhoeffer, F. (1987). Retinal axons with and without their somata growing to and arborizing in the tectum of Xenopus embryos: A time-lapse video study of single fibers in vivo. *Development* **101**, 123-133.

Kidd, T., Bland, K. and Goodman, C. S. (1999). Slit is the midline repellent for the Robo receptor in Drosophila. *Cell* **96**, 785-794.

Kidd, T., Brose, K., Mitchell, K. J., Fetter, R. D., Tessier-Lavigne, M. and Goodman, C. S. (1998). Roundabout controls axon crossing of the CNS midline and defines a novel family of evolutionarily conserved guidance receptors. *Cell* **92**, 205-215.

Kruger, K., Tam, A. S., Lu, C. and Sretavan, D. W. (1998). Retinal ganglion cell axon progression from the optic chiasm to initiate optic tract development requires cell autonomous function of GAP-43. *J. Neurosci.* **18**, 5692-5705.

Marcus, R., Shimamura, K., Sretavan, D. W., Lai, E., Rubenstein, J. and Mason, C. A. (1999). Domains of regulatory gene expression and the developing optic chiasm: Correspondence with retinal axon paths and candidate signaling cells. *J. Comp. Neurol.* **403**, 346-358.

Mason, C. A. and Sretavan, D. W. (1997). Glia, neurons, and axon pathfinding during optic chiasm development. *Curr. Opin. Neurobiol.* **7**, 647-653.

Mason, C. A. and Wang, L. C. (1997). Growth cone form is behavior-specific and, consequently, position-specific along the retinal axon pathway. *J. Neurosci.* **17**, 1086-1100.

McFarlane, S., McNeill, L. and Holt, C. E. (1995). FGF Signaling and Target Recognition in the Developing Xenopus Visual System. *Neuron* **15**, 1017-1028.

Meiri, K. F., Saffell, J. L., Walsh, F. S. and Doherty, P. (1998). Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. *J. Neurosci.* **18**, 10429-10437.

O'Rourke, N. and Fraser, S. (1990). Dynamic changes in optic fiber terminal arbors lead to retinotopic map formation: an in vivo confocal microscope study. *Neuron* **5**, 159-171.

Reese, B. E. (1987) The distribution of axons according to diameter in the optic nerve and optic tract of the rat. *Neuroscience* **22**, 1015-1024.

Reese, B. E. and Cowey, A. (1988) Segregation of functionally distinct axons in the monkey's optic tract. *Nature* **331**, 350-351.

Reese, B. E., Maynard, T. M. and Hocking, D. R. (1994). Glial Domains and axonal reordering in the chiasmatic region of the developing ferret. *J. Comp. Neurol.* **349**, 303-324.

Schambia, U., Lauder, J. and Silver, J. (ed.) (1992). *Atlas of the Prenatal Mouse Brain.* San Diego, CA: Academic Press.

Seeger, M., Tear, G., Ferres-Marco, D. and Goodman, C. (1993). Mutations affecting growth cone guidance in Drosophila: Genes necessary for guidance toward or away from the midline. *Neuron* **10**, 409-426.

Shirasaki, R., Katsumata, R. and Murakami, F. (1998). Change in chemoattractant responsiveness of developing axons at an intermediate target. *Science* **279**, 105-107.

Silver, J. and Rutishauser, U. (1984). Guidance of optic axons in vivo by a preformed adhesive pathway on neuroepithelial endfeet. *Dev. Biol.* **106**, 485-499.

Skene, J. H. P. (1990). GAP-43 as a 'calmodulin sponge' and some implications for calcium signaling in axon terminals. *Neurosci. Res.* **13**, S112-S125.

Skene, J. H. P. and Virag, I. (1989). Posttranslational Membrane Attachment and Dynamic Fatty Acylation of a Neuronal Growth Cone Protein, GAP-43. *J. Cell Biol* **108**, 613-624..

Sretavan, D. W. and Kruger, K. (1998). Randomized retinal ganglion cell axon routing at the optic chiasm of GAP-43-deficient mice: Association with midline recrossing and lack of normal ipsilateral axon turning. *J. Neurosci.* **18**, 10502-10513.

Sretavan, D. W., Pure, E., Siegel, M. W. and Reichardt, L. F. (1995). Disruption of retinal axon ingrowth by ablation of embryonic mouse optic chiasm neurons. *Science* **269**, 98-101.

Sretavan, D. W. and Reichardt, L. F. (1993). Time-Lapse video analysis of retinal ganglion cell axon pathfinding at the mammalian optic chiasm: growth cone guidance using intrinsic chiasm cues. *Neuron* **10**, 761-777.

Stork, O., Welzl, H., Cremer, H. and Schachner, M. (1997). Increased intermale aggression and neuroendocrine response in mice deficient for the neural cell adhesion molecule (NCAM). *Eur. J. Neurosci.* **9**, 1117-1125.

Strittmatter, S. M., Fankhauser, C., Huang, P. L., Mashimo, H. and Fishman, M. C. (1995). Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. *Cell* **80**, 445-452.

Thanos, S., Bonhoeffer, F. and Rutishauser, U. (1984). Fiber-fiber interaction and tectal cues influence the development of the chicken retinotectal projection. *Proc. Natl. Acad. Sci. USA* **81**, 1906-1910.

Tomasiewicz, H., Ono, K., Yee, D., Thompson, C., Goridis, C., Rutishauser, U. and Magnuson, T. (1993). Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system. *Neuron* **11**, 1163-1174.

Tuttle, R., Braisted, J. E., Richards, L. J. and O'Leary, D. D. M. (1998). Retinal axon guidance by region-specific cues in diencephalon. *Development* **125**, 791-801.

VanBerkum, M. F. A. and Goodman, C. S. (1995). Targeted disruption of Ca2+-calmodulin signaling in Drosophila growth cones leads to stalls in axon extension and errors in axon guidance. *Neuron* **14**, 43-56.

Walz, A., McFarlane, S., Brickman, Y. G., Nurcombe, V., Bartlett, P. F. and Holt, C. E. (1997). Essential role of heparan sulfates in axon navigation and targeting in the developing visual system. *Development* **124**, 2421-2430.

Wang, L.-C., Dani, J., Godement, P., Marcus, R. and Mason, C. A. (1995). Crossed and Uncrossed Retinal Axons Respond Differently to Cells of the Optic Chiasm Midline In Vitro. *Neuron* **15**, 1349-1364.

Worley, T. and Holt, C. (1996). Inhibition of protein tyrosine kinases impairs axon extension in the embryonic optic tract. *J. Neurosci.* **16**, 2294-2306.

Zallen, J. A., Yi, B. A. and Bargmann, C. I. (1998). The conserved immunoglobulin superfamily member sax-3/robo directs multiple aspects of axon guidance in C. elegans. *Cell* **92**, 217-227.

Zhu, Q. and Julien, J.-P. (1999). A key role for GAP-43 in the retinotectal topographic organization. *Exp. Neurol.* **155**, 228-242.