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The Netrin receptor Frazzled is required in the target for establishment of retinal projections in the *Drosophila* visual system

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SUMMARY

Retinal axons in *Drosophila* make precise topographic connections with their target cells in the optic lobe. Here we investigate the role of the Netrins and their receptor Frazzled in the establishment of retinal projections. We find that the Netrins, although expressed in the target, are not required for retinal projections. Surprisingly, Frazzled, found on both retinal fibers and target cells, is required in the target for attracting retinal fibers, while playing at best a redundant role in the retinal fibers themselves; this finding demonstrates that target attraction is necessary for topographic map formation. Finally, we show that Frazzled is not required for the differentiation of cells in the target. Our data suggest that Frazzled does not function as a Netrin receptor in attracting retinal fibers to the target; nor does it seem to act as a homotypic cell adhesion molecule. We favor the possibility that Frazzled in the target interacts with a component on the surface of retinal fibers, possibly another Netrin receptor.

Key words: *Drosophila*, Visual system development, Retinal projection, Topographic map formation, Netrin, *frazzled*

INTRODUCTION

The *Drosophila* visual system consists of the compound eye and three optic ganglia, namely the lamina, medulla and lobula complex. The compound eye is composed of 800 units called ommatidia, each containing 8 photoreceptors (R1-R8), whose axons directly innervate the first two optic ganglia, the lamina and the medulla, in a strictly retinotopic fashion. The development of the *Drosophila* visual system is regulated both temporally and spatially. During the third instar larval stage, photoreceptors are generated in the eye imaginal disc in a posterior-to-anterior progression. The newly generated photoreceptors in turn send their axons through the optic stalk into the optic lobe. Axons from the same ommatidium project together to their appropriate retinotopic map site in the lamina, where the R1-R6 axons terminate, while the R7 and R8 axons penetrate deeper and terminate in the medulla. Incoming retinal axons project to the anterior edge of the developing lamina, where they trigger the maturation of lamina precursors. Thus a new row of cells is added anteriorly to the differentiated portion of the lamina with each row of ingrowing retinal axons, establishing appropriate topographic connections along the anteroposterior (a-p) axis. As the retinal axons grow towards the anterior edge of the developing lamina, they also spread and innervate along the dorsoventral (d-v) axis according to the position of their cell bodies in the eye disc (Fig. 1A-C; Kunes and Steller, 1993; Meinertzhagen and Hanson, 1993).

Various models have been proposed to account for retinotopic map formation, including morphogenetic assembly and chemoaffinity (Cowan and Hunt, 1985). The morphogenetic assembly model holds that, due to a defined spatiotemporal order of outgrowth and passive fasciculation with neighbors, ommatidial fibers maintain their topographic relationship while growing toward and innervating the target (Cowan and Hunt, 1985). The chemoaffinity model proposes that position-dependent chemical labels on retinal axons and target cells mediate retinotopic target recognition (Sperry, 1963; Cowan and Hunt, 1985). Based on ablation experiments in lower arthropods, earlier studies suggested that the morphogenetic process itself is sufficient for establishing retinotopic projections (Anderson, 1978; Macagno, 1978). However, more recent genetic ablation experiments in *Drosophila* strongly argue for the existence of positional cues, at least along the d-v axis, which guide photoreceptor axons to their spatially appropriate targets independently of retinal neighbors (Kunes et al., 1993).

In an effort to identify molecules required for establishing retinal axon projections, we tested the Netrins and their receptor Frazzled as candidates. The Netrins were initially discovered as long-range chemoattractants that are secreted by midline cells and attract commissural growth cones toward the midline (Hedgecock et al., 1990; Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1994; Harris et al., 1996; Mitchell et al., 1996). In vertebrates, Netrin-1 is also expressed in the visual system, including the optic disc, where it is required for the normal entry of retinal ganglion axons into the optic stalk

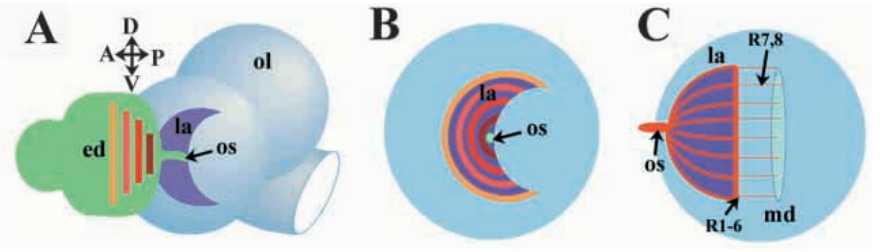

Fig. 1. Development of the adult visual system during the third instar larval stage. (A) Schematic whole-mount view of the developing visual system. Photoreceptors are generated progressively from the posterior to the anterior (brown to yellow lines) of the eye imaginal disc (ed), which is attached to the optic lobe through the optic stalk (os). Photoreceptors are organized in ommatidia, clusters of 8 cells. Photoreceptor axons from each ommatidium fasciculate and travel together through the optic stalk to innervate the optic lobe (ol). (B) Schematic lateral view of the optic lobe. Newly generated photoreceptors project their axons to the anterior edge of the developing lamina (la), the first optic ganglion. The innervation in the lamina progresses from posterior to anterior as more rows of retinal axons enter. (C) Schematic coronal section of the optic lobe. Photoreceptor axons spread out along the D-V axis after they exit the optic stalk. R1-6 photoreceptor axons stop in the lamina, R7 and R8 axons project to the developing medulla (md).

(Deiner et al., 1997). In *Drosophila*, the two identified Netrins (Net A/Net B; Harris et al, 1996; Mitchell et al., 1996) are also expressed in a subset of embryonic muscles, where both act as short-range attractive cues for target recognition of certain motor axons, while Net B, but not Net A, appears to act as a repulsive cue for other motor axons (Winberg et al., 1998). A single Netrin receptor, called Frazzled (Fra), has been identified in *Drosophila* (Kolodziej et al., 1996). Fra is a homolog of human Deleted in Colorectal Cancer (DCC; Fearon et al., 1990) and worm UNC40 (Chan et al., 1996). Previous studies have identified DCC as a Netrin receptor that mediates the attraction response of commissural fibers in the vertebrate spinal cord (Keino-Masu et al., 1996; Fazeli et al., 1997). Similarly, in *Drosophila*, Fra is required for Netrin-mediated attraction but not repulsion, which suggests the existence of additional Netrin receptors (Winberg et al., 1998).

MATERIALS AND METHODS

Mosaic analysis

For the *fra* mosaic analysis, clones of homozygous mutant cells were generated by FLP-mediated mitotic recombination (Xu and Rubin, 1993). fra^4 was recombined onto the *FRT40A* chromosome by mitotic recombination; the presence of the fra^4 allele was tested by complementation with a second *fra* allele (fra^3) by examining the phenotypes of transheterozygous embryos (Kolodziej et al., 1996). Clones of fra^4 mutant cells were marked in the eye disc or the optic lobe by the loss of an *hsp70-CD2* transgene (Jiang and Struhl, 1995). *y,w, hsFLP122; hsp70-CD2, FRT40A/*fra^4*, FRT40A* flies were heat shocked at 34°C for 30 minutes at 24-36 hours of development to induce mitotic recombination. *CD2* marker gene expression was induced by heat shocking third instar larvae at 37°C for 60 minutes. The larvae were allowed to recover for 60 minutes after which the eye-brain complexes were dissected and processed for immunohistochemistry. For ectopic Netrin expression, clones of *Net A*- or *Net B*-expressing cells were generated by FLP-out events (Tear et al., 1996). *Actin>CD2>GAL4; MKRS hsFLP1/+* males were mated to *UAS Net A/TM6B* or *UAS Net B* females. The progeny was heat shocked at 37°C for 30 minutes at 24-36 hours of development to induce FLP-out events. *CD2* marker gene expression was detected as described above.

RNA in situ hybridization and immunohistochemistry

Whole-mount RNA in situ hybridization was carried out essentially as described by Tear et al. (1996). Antisense digoxigenin (DIG)-labelled RNA probes for *net A* and *net B*, and an antisense Fluorescein (FITC)-labelled probe for *fra* were prepared as described (Harris et al., 1996; Mitchell et al., 1996). Double in situ hybridizations were carried out essentially as in the single-labeling experiments except both *net* and *fra* probes were hybridized to tissue at the same time. A mixture of anti-DIG goat IgG and anti-FITC mouse IgG (Boehringer) was used to recognize the probes. Following three rinses with PBS plus 0.1% Tween-20, tissues were incubated with a mixture of Cy3-conjugated anti-goat Fab fragment and Cy2-conjugated anti-mouse Fab fragment (Jackson). Immunohistochemistry of eye disc-brain complexes was carried out as described (Kunes et al., 1993). The eye discs and the optic lobes were examined with Zeiss LSM510 and Bio-Rad 1000 confocal microscopes. Photoreceptor axons were immunostained with antibodies against either HRP (FITC-conjugated, ICN Biomedicals) or Chaoptin (a gift from S. L. Zipursky). The lamina cells were immunostained with antibodies against Dachshund (a gift from G. M. Rubin) or Repo (a gift from G. Technau). Antibodies against Fra were a gift from P. A. Kolodziej. Antibodies against CD2 were obtained from Serotec.

RESULTS

In order to determine whether the Netrins and their receptor Fra are involved in the establishment of retinal projections, we first examined whether *net A* and *net B* are present in the optic lobe during the third instar larval stage. Since the existing antibodies do not permit reliable immunohistochemical detection of Netrins in the optic lobe, we examined the Netrin expression patterns by RNA in situ hybridization. We find that *net A* and *net B* are expressed in identical patterns: both transcripts are expressed in lamina precursors, which in wild type form an arc-shaped ribbon of cells (Fig. 2A). Thus, the Netrins are expressed in a pattern that would allow them to act as signals for incoming fibers.

Fra protein, in contrast, is strongly expressed in photoreceptor axons, suggesting that retinal fibers have the ability to sense Netrin in the target (Fig. 2D). Interestingly, Fra is also expressed in the target structure, the lamina (Fig. 2B). *fra* transcripts are found in an arc-shaped band of cells similar to *net* transcripts, but double RNA in situ hybridizations reveal that *fra* and *net* transcripts do not colocalize to the same cells. Instead, *fra* transcripts are expressed in more mature lamina precursor cells located posteriorly adjacent to the *net*-expressing lamina precursor cells (Fig. 2C). While the transcript is only expressed very transiently, Fra protein expression persists and is thus present throughout the differentiated lamina and in all lamina cells (Fig. 2E,F).

To examine the role of Netrins in the developing adult visual system, we analyzed the retinal projections in animals carrying a small synthetic deficiency (T9-B118; Winberg et al., 1998) which jointly removes the two Netrins. We find that retinal

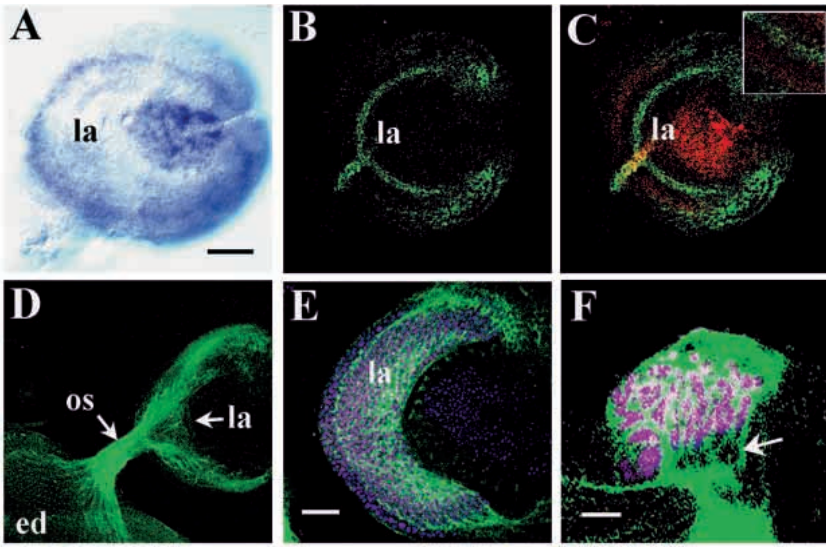

Fig. 2. Expression patterns of *net* and *fra* in the developing visual system. (A-E) Lateral views of the optic lobe. (A) *net A* transcript (purple) is present in the lamina precursor cells. (B) *fra* transcript (green) is also detected in the lamina precursor cells. (C) Double RNA in situ hybridization reveals that *net A* and *fra* transcripts are present in different populations of lamina precursor cells. Cells expressing *fra* transcript (green) are located posteriorly adjacent to the *net A*-expressing cells (red; see insert) and thus represent a more mature population of lamina precursor cells. (D) Fra protein (green) is present in photoreceptors and their axons. (E) Fra protein is also present on the membranes of all lamina neurons. Lamina cells are marked by Dac expression (purple). (F) A horizontal optical section shows that Fra is not only expressed in lamina neurons but also in lamina glia (arrow). ed, eye disk; la, lamina; os, optic stalk. Bar, 20 μm (A-E); 10 μm (F).

projections are normal in these animals (data not shown), indicating that the expression of *net A* and *net B* is not required in lamina precursor cells.

To study the effects of loss of *fra*, we utilized the FRT/FLP system (Xu and Rubin, 1993) to create mosaic *fra* null clones in an otherwise heterozygous animal. Despite strong expression of Fra in retinal fibers, *fra* mutant clones in the eye show no discernible projection defects (data not shown). Since *fra* is also expressed in the developing lamina, it could, however, be required in the target for proper retinotopic innervation. We therefore examined whether *fra* mutant clones in the developing lamina were innervated abnormally (Fig. 3). Intriguingly, we find that wild-type retinal fibers are incapable of innervating lamina target regions that lack *fra* function. Retinal fibers consistently avoid *fra* mutant patches and reroute to fra^+ areas. In most cases, this leads to a collapse of retinal fibers onto one another along the a-p axis (Fig. 3C,D,G,H). In some cases, retinal fibers reroute along the d-v axis, leading to a deformation of the normal crescent-shaped fan (Fig. 3E,F). Incoming retinal fibers respect clonal boundaries when innervating a *fra* mosaic target, suggesting that *fra* function is required cell-autonomously in lamina target cells.

These findings indicate that *fra* function is required in lamina target cells for their innervation by retinal fibers. Since retinal fibers strictly avoid areas lacking *fra*, we conclude that Fra is necessary for making lamina precursor cells attractive to incoming retinal fibers. Furthermore, the fact that retinal fibers reroute along both the a-p axis and the d-v axis suggests that target-mediated attraction is required for proper positioning along both axes.

Given these results, what might be the exact role of Fra in the target? One possibility is that Fra is required for differentiation

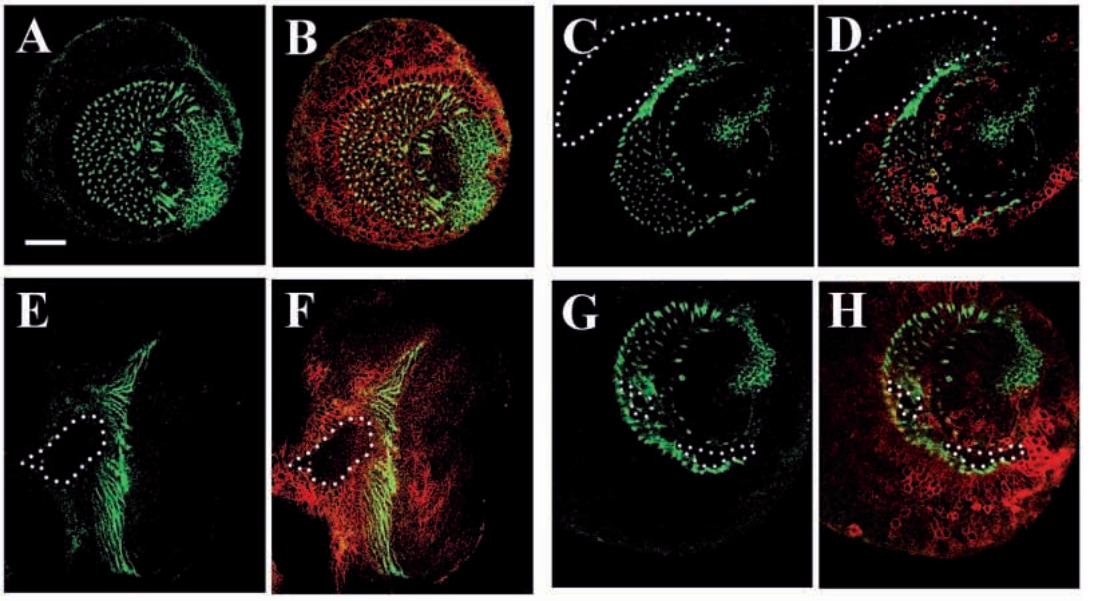

Fig. 3. Lack of *fra* function in the lamina causes retinal axons to avoid the mutant region. Clones of fra^4 cells were created in the optic lobe by the FRT/FLP technique. fra^+ cells are marked by the expression of a *CD2* transgene. Lateral views of optic lobes showing either retinal axon staining alone (anti-HRP, green) (A,C,E,G), or retinal axon staining (green) and marker gene expression (anti-CD2, red) together (B,D,F,H). fra^4 clones, which lack CD2 expression, are outlined in white dots. (A,B) Wild type; (C-H) *fra* mutant clones. Retinal axons avoid the fra^4 mutant regions (C/D, E/F, G/H). Depending on the position of the clone, this either results in a collapse of fibers along the a-p axis (C/D and G/H) or a wider spreading of the axons along the d-v axis (E/F) compared to wild type. Bar, 30 μm.

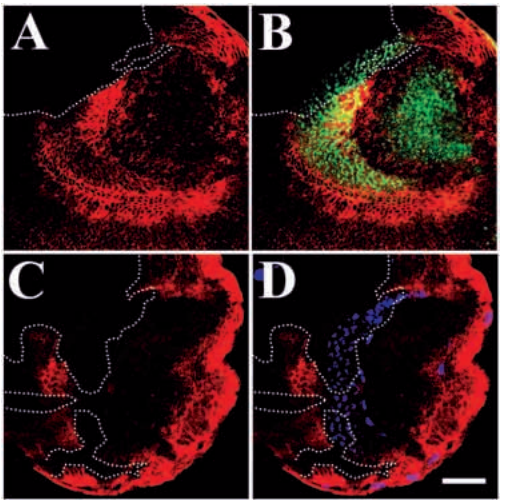

Fig. 4. Lack of *fra* function does not affect neuronal or glial differentiation of lamina target cells. Clones of *fra*$^{-}$ cells were created by the FRT/FLP technique. *fra*$^{+}$ cells are marked by the expression of a *CD2* transgene. Lateral views of optic lobes showing either mosaic marker alone (anti-CD2, red; A,C), together with neuronal marker (anti-Dac, green; B), or together with glial marker (anti-Repo, blue; D). *fra*$^{-}$ clones, which lack CD2 expression, are outlined in white dots. *fra*$^{-}$ cells near the border of the clone adjacent to the innervated portion of the lamina express the early differentiation marker Dac (B). Bar, 30 μm.

of lamina precursors into neurons and glial cells. Although previous studies have shown that retinal innervation triggers the differentiation of lamina precursors (Huang and Kunes, 1996), it is possible that the differentiation of lamina precursors is in turn required for innervation by retinal fibers or stabilization of axon-target interactions. Thus, Fra's contribution might be indirect. However, we find that *fra* mutant cells are capable of differentiating into lamina neurons: *fra* mutant cells along clonal borders adjacent to innervated *fra*$^{+}$ lamina areas express the early lamina neuron marker Dachshund (Dac; Fig. 4A,B; Mardon et al., 1994; Huang and Kunes, 1996), indicating that *fra* mutant cells are able to respond, over a distance of several cell diameters, to the differentiation signals delivered by incoming retinal fibers. Furthermore, *fra* mutant cells are also capable of differentiating into lamina glia as judged by their expression of the glial differentiation marker Repo (Halter et al., 1995; Perez and Steller, 1996). Interestingly, even within very large *fra* mutant clones the number and distribution of glial cells appears to be relatively normal (Fig. 4C,D), suggesting that glial differentiation in the lamina is much less dependent on retinal input than neuronal differentiation. These findings eliminate the possibility that Fra has a role in the differentiation of target cells and, instead, suggest a more direct role of Fra in attracting retinal fibers.

We also sought to further examine Fra's role in retinal axons. Although *fra* mutant clones in the eye disc have no discernible projection defects, Fra function might be redundant with another Netrin receptor on retinal fibers. Therefore, in an attempt to interfere with the function of Netrin receptors on the surface of retinal axons, we expressed high levels of *net A* and *net B* in the retinal fibers using the UAS/GAL4 system (Brand and Perrimon, 1993). To effect eye-specific expression, we used *GMR-GAL4*, which results in high levels of expression in all cells posterior of the morphogenetic furrow in the developing eye (Ellis et al., 1993; Hay et al., 1994). Under these experimental conditions, retinal axons were either unable to reach the target at all and stalled in the optic stalk (*n*=8/80 animals) or failed to spread and innervate the target region properly (*n*=72/80 animals) (Fig. 5). In most cases, the innervation pattern was affected along both axes. Along the d-v axis, retinal fibers fail to spread to their normal extent and thus form narrow fans (Fig. 5C,D). Along the a-p axis, younger, anterior retinal fibers are found to innervate older, more posterior portions of the lamina (Fig. 5F). The same phenomena were observed with expression of either Netrin.

These results suggest that Netrin receptors expressed on retinal neurons can be saturated to a varying degree by misexpression of Netrins in the same neurons. The fact that misexpression of Netrins in retinal neurons causes phenotypic defects, while the loss of *fra* function in retinal neurons does

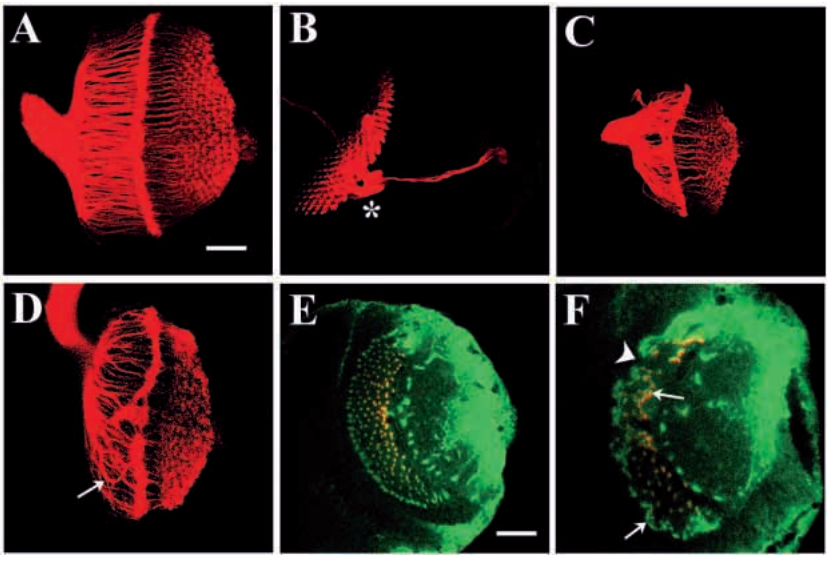

Fig. 5. Overexpression of Netrins in the retinal axons disrupts proper retinotopic innervation along a-p and d-v axes. Using the GAL4/UAS system, high levels of Netrins were expressed in the photoreceptors. Flies containing *GMR-GAL4* and 2 copies of *UAS-net A* transgenes were examined for retinal projection phenotypes. (A,E) Wild type; (B-D,F) *GMR-GAL4*/2x*UAS-net A*. (A-D) Coronal views of the optic lobe showing retinal axons (red) traveling through the optic stalk and spreading along the d-v axis. Retinal axons are labeled with antibodies against Chaoptin (red). (E,F) Lateral sections through the optic lobe showing retinal axons stained with antibodies against HRP, which labels all axons, and Chaoptin, which labels only older (more posterior) axons. When Netrins are expressed at high levels in the photoreceptor axons, the retinal axons stall in the optic stalk (B,*), spread abnormally (C) or criss-cross each other (D, arrow) along the d-v axis. Photoreceptor axons also collapse onto each other along the a-p axis (F, arrows) compared to wild type (E). As seen in F, the axons also spread abnormally along the d-v axis, which is evident from the irregular spacing of the axons (arrowhead). Axons seen projecting into the optic lobe in (B) belong to the larval optic nerve. Bar, 20 μm (A,C,D); 40 μm (B); 30 μm (E,F).

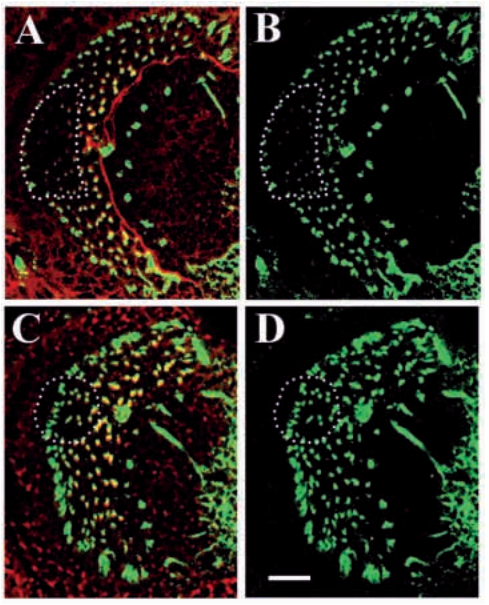

Fig. 6. Ectopic Netrin expression in the lamina causes irregularities in the retinal projection pattern. Lateral optic sections of the lamina with clones ectopically expressing *Net B* in two examples (A,B and C,D). Retinal fibers and FLP-out clones are visualized by staining with antibodies against HRP (green) and by lack of CD2 staining (red), respectively. Bar, 20 μm.

not, also supports the notion that Netrin receptors other than Fra are functioning in retinal fibers. Moreover, the phenotypes resulting from interference with the function of Netrin receptors on retinal fibers are similar to those resulting from the removal of Fra from the target cells; both are characterized by a failure of retinal axons to innervate the proper target cells, resulting in abnormal a-p and d-v projections. This finding suggests that the Netrin receptors in the retinal fibers and Fra in the target take part in the same process.

Finally, we sought to examine the effects on retinal projections of ectopic expression of Netrins in the target. To this end, we independently expressed both Netrins in patches of cells using a *Act>Flpout>GAL4* cassette (see Materials and Methods). Ectopic expression of either Netrin in the anterior, undifferentiated portion of the eye disc or the lamina anlage had no effect on retinal fibers (data not shown). However, Netrin-expressing clones in the differentiating portion of the lamina show mild hypoinnervation and irregular spacing of retinal fibers (Fig. 6). A likely interpretation of this result is that the increase of Netrin concentration in the differentiating lamina partially saturates Fra on the target cells, thus interfering with the attractive interaction between Fra and the retinal fibers.

DISCUSSION

Our results show that despite the strict spatiotemporal regulation of retinal axon outgrowth and target development in *Drosophila*, morphogenetic assembly is insufficient to establish the retinotopic map (cf. Kunes et al., 1993). In contrast, we demonstrate that, in order for innervation to occur, the lamina target has to be made attractive to retinal fibers, a finding that in principle supports the chemoaffinity hypothesis. Our experiments show that it is the Netrin receptor Fra that performs this function. We also find that Fra is at best redundant in retinal fibers and that the Netrins, while expressed in the lamina target, are not required for proper innervation of the lamina. Taken together, these results demonstrate that the familiar paradigm of axonal projection being established through chemoattraction between Netrins expressed as ligands in the target and Fra as the receptor expressed on the growing axons does not apply in this context.

What is the role of Fra in the target cells? Our experiments show that Fra is not required for neuronal or glial differentiation of lamina precursor cells. Non-innervated lamina precursor cells lacking *fra* can express the early neuronal differentiation marker Dac or the glial differentiation marker Repo, as long as the cells are within range of the diffusible differentiation signals emanating from ingrowing retinal fibers. Interestingly, for neuronal differentiation, this range appears to be restricted to a few cell diameters, while for glial differentiation, this range must be much larger, since even very large clones of *fra* appear to have a normal complement of glial cells. In fact, glial differentiation may be largely independent of retinal innervation, as has been suggested by a previous study which showed that even in uninnervated animals some glial cells are present in the lamina anlage (Perez and Steller, 1996). Together, these findings demonstrate that the presence of differentiated neuronal and glial cells in the target is not sufficient for the attraction of retinal fibers. Moreover, they exclude the possibility that Fra is merely indirectly involved in retinal fiber attraction by mediating target cell differentiation and point instead to a more direct role of Fra in the target for attracting retinal fibers.

What is the molecular function of Fra in the target cells? The fact that removal of both Netrins does not affect the retinal projection, makes it unlikely that Fra functions as a Netrin receptor in the lamina target. Further, the fact that removal of Fra from the retinal fibers does not affect their projection, makes it unlikely that Fra functions as a homotypic cell adhesion molecule, directly effecting the attractive interaction between retinal fibers and their target cells. Given these findings, we favor a third possibility: Fra in the target cells may interact in a heterotypic fashion with a component on the surface of retinal fibers. It is possible that this component is another Netrin receptor. This idea is supported by the finding that Netrin misexpression in retinal fibers results in projection defects which phenotypically mimic the removal of Fra from the target, suggesting the presence in retinal fibers of another Netrin receptor in addition to Fra. The existence of additional Netrin receptors in the fly is expected. Apart from an UNC-5 type receptor, which has been found in both worms and vertebrates (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; McIntire et al., 1992; Hamelin et al., 1993; Leonardo et al., 1997), a second DCC/UNC-40 homolog may also exist in the fly, based on genetic evidence that UNC40 function is partially redundant in the worm: molecular null alleles of *unc40* display a less severe phenotype than some truncation alleles, suggesting that the truncated proteins interfere with a second pathway (Chan et al., 1996). Of course, alternative models are possible.

Whatever the identity of the interacting partner, the presence of Fra on target cells is a prerequisite for any innervation by retinal fibers. Fibers whose designated target area lacks *fra* avoid the area by rerouting into fra^+ regions. It is interesting that, in avoiding *fra* mutant regions, retinal fibers do not scramble randomly to reach fra^+ areas, but rather reroute in an orderly fashion. When foregoing their a-p position, retinal fibers

appear to reroute as a cohort and, when misprojecting along the d-v axis, they maintain their relative order. This finding argues that the process of retinotopic map formation relies on two functionally separable mechanisms, one mediating attraction to the target, the other providing positional information. In vertebrates, positional information in the retinotectal system appears to be largely provided by graded repulsive interactions between retinal fibers and target cells mediated by Ephrins and their receptors (for review see Frisen and Barbacid, 1997; Flanagan and Vanderhaeghen, 1998). Such a repulsive mechanism for defining positional values requires an underlying attraction of innervating fibers to the target. Thus, it will be interesting to learn whether DCC receptors, similar to their role in the *Drosophila* visual system, serve to attract retinal fibers to their target in the vertebrate visual system as well.

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