The receptor tyrosine kinase Off-track is required for layer-specific neuronal connectivity in *Drosophila*

Patrick Cafferty, Li Yu and Yong Rao*

McGill Centre for Research in Neuroscience, and Department of Neurology and Neurosurgery, McGill University Health Centre, 1650 Cedar Avenue, Montreal, Quebec H3G 1A4, Canada
*Author for correspondence (e-mail: yong.rao@mcgill.ca)

Accepted 17 August 2004

Development 131, 5287-5295
Published by The Company of Biologists 2004
doi:10.1242/dev.01406

Summary

The nervous system in many species consists of multiple neuronal cell layers, each forming specific connections with neurons in other layers or other regions of the brain. How layer-specific connectivity is established during development remains largely unknown. In the *Drosophila* adult visual system, photoreceptor (R cell) axons innervate one of two optic ganglia layers; R1-R6 axons connect to the lamina layer, while R7 and R8 axons project through the lamina into the deeper medulla layer. Here, we show that the receptor tyrosine kinase Off-track (Otk) is specifically required for lamina-specific targeting of R1-R6 axons. Otk is highly expressed on R1-R6 growth cones. In the absence of *otk*, many R1-R6 axons connect abnormally to medulla instead of innervating lamina. We propose that Otk is a receptor or a component of a receptor complex that recognizes a target-derived signal for R1-R6 axons to innervate the lamina layer.

Key words: Off-track, Neuronal target selection, Layer-specific connectivity, *Drosophila* visual system

Introduction

The vertebrate central nervous system has a multi-layered architecture in which different neuronal cell layers receive innervation from axons that project from distinct neuronal populations. To establish such layer-specific neuronal connections, it is thought that each neuronal cell layer must express specific membrane-bound surface labels, which are then recognized by specific receptors expressed on the growth cone of incoming axons (Bolz and Castellani, 1997). Due to the complexity of the vertebrate nervous system, however, it is only recently that the molecular mechanism underlying the formation of layer-specific connectivity has begun to be elucidated. For instance, it has been shown that the expression of the cell adhesion molecule N-Cadherin by subtypes of laminae in the chick visual system is necessary for layer-specific targeting of distinct subtypes of retinal axons in the optic tectum (Inoue and Sanes, 1997). Recent studies also implicate a role for Ephs and their ligands ephrins in regulating the formation of layer-specific thalamocortical connections in mice (Mann et al., 2002).

The formation of photoreceptor-to-optic-lobe connections in the *Drosophila* adult visual system is an excellent and simple model to study the molecular mechanisms that control the establishment of layer-specific neuronal connectivity during development (Clandinin and Zipursky, 2002; Tayler and Garrity, 2003). The *Drosophila* adult visual system is comprised of the compound eye and the optic lobe. The compound eye consists of ~800 ommatidia or single eye units, each containing eight different photoreceptor cells (R cells). R cells project axons into one of two optic ganglion layers in the brain. R1-R6 cells connect to the superficial layer of the optic lobe, the lamina, and are responsible for the absorption of light in the green range. While R7 and R8 cells connect to the deeper medulla layer, and are responsible for the absorption of light in the ultraviolet and blue range. The formation of layer-specific R-cell connection pattern begins at the third-instar larval stage. Precursor cells in third-instar larval eye-imaginal discs begin to differentiate into R cells. Within each ommatidium, the R8 precursor cell differentiates first and projects its axon through the optic stalk and the developing lamina into the medulla. Axons from the later differentiated R1-R7 cells within the same ommatidium form a single bundle with the pioneer R8 axon until they encounter a layer of glial cells (i.e. marginal glia) within the lamina layer. There they have to make a binary choice: either stop or keep going into the medulla. The R1-R6 growth cones terminate within the lamina in response to an unknown stop signal from lamina glial cells (Poeck et al., 2001), their intermediate target at larval stage. By contrast, R7 growth cones extend further to join R8 growth cones in the medulla. During pupation, R1-R6 growth cones undergo further stereotyped rearrangements and subsequently form synaptic connections with lamina neurons (Clandinin and Zipursky, 2000; Meinertzhagen and Hanson, 1993).

Recent studies have identified several cell surface proteins that are required for R-cell connectivity. Specifically, N-Cadherin, the receptor tyrosine phosphatase Lar and the Cadherin-related protein Flamingo have each been shown to be required for the establishment of local synaptic connections between R1-R6 axons and lamina cartridge neurons (Clandinin et al., 2001; Lee et al., 2001; Lee et al., 2003). An additional role for N-Cadherin, Lar and the receptor tyrosine phosphatase PTP69D in R7 axons and Flamingo in R8 axons for forming

local connections with target cells within the medulla has also been reveled (Clandinin et al., 2001; Lee et al., 2001; Lee et al., 2003; Maurel-Zaffran et al., 2001; Newsome et al., 2000a; Senti et al., 2003). However, loss of N-Cadherin or Flamingo does not affect the initial choice between lamina versus medulla target selection. In their absence R1-R6 still connect to the lamina, while R7 and R8 still choose the medulla for establishing synaptic connections. While loss of *Ptp69D* or *Lar* does affect the initial projections of R1-R6 axons (Clandinin et al., 2001; Garrity et al., 1999), the completed pattern of lamina-versus-medulla target selection in adult *Ptp69D* or *Lar* mutants remains largely unchanged (Clandinin et al., 2001; Newsome et al., 2000a). These data argue against a direct role for either PTP69D or Lar in specifying lamina-specific targeting of R1-R6 axons. In addition to the above cell surface receptors, two *Drosophila* receptor tyrosine kinases, the insulin receptor and Eph receptor, are also required for regulating different aspects of R-cell axon guidance (Dearborn et al., 2002; Song et al., 2003). However, neither has been shown to play a role in regulating layer-specific R-cell connectivity. Thus, it remains unclear how R-cell axons detect layer-specific targeting signals to make the binary decision for choosing either lamina or medulla to establish synaptic connections.

In a search for genes that are required for R-cell projections in the developing visual system, we have identified the receptor tyrosine kinase Otk as a key determinant in specifying the binary lamina versus medulla target selection. While Otk was originally isolated based on its homology with the *trk* family of neurotrophin receptors in vertebrates (Pulido et al., 1992), more recent studies suggest strongly that Otk is not a homolog of the vertebrate Trk A receptor (Kroiher et al., 2001). It has been shown that in vitro Otk mediates cell–cell adhesion in a Ca^{2+}-independent homophilic manner (Pulido et al., 1992), while in vivo it functions downstream of Semaphorin-1a (Sema-1a) to regulate motor axon guidance at the embryonic stage (Winberg et al., 2001). In this study, we show that Otk is predominantly localized to R1-R6 growth cones in the fly visual system and is specifically required for lamina-specific targeting of R1-R6 axons. We propose that Otk recognizes a lamina-derived signal for R1-R6 targeting.

Materials and methods

Genetics

$otk^{EP(2)2017}$ was obtained from the Berkeley *Drosophila* Genome Project. $sema^{P1}$ was provided by Alex Kolodkin. otk^{3}, UAS-*otk* and UAS-*fasII* were provided by Cory Goodman. Large clones of otk^{3} or $sema^{P1}$ were generated in an otherwise heterozygous or wild-type eye by eye-specific mitotic recombination using the eyFLP/FRT system (Newsome et al., 2000a). Using this method, ~80-90% of ommatidia in a mosaic eye were otk^{3} or $sema^{P1}$ mutant cells in all individuals examined. Since mitotic recombination is under control of the eye-specific eyFLP, mutant clones were generated in the eye but not in the target region, thus allowing us to determine if *otk* is required in the eye for regulating R-cell connectivity. Rescue experiments were performed by crossing a GMR-GAL4 and a UAS-*otk* transgene into the $otk^{EP(2)2017}/otk^{3}$ transheterozygous individual, which allows the eye-specific expression of the *otk* transgene in $otk^{EP(2)2017}/otk^{3}$ mutants. The potential effect of the *otk* mutation on R7 projections was examined by crossing the adult R7 marker PANR7-GAL4::UAS-Synaptobrevin-GFP into otk^{3} mosaics as described (Lee et al., 2001). To express Otk in R7 axons, UAS-*otk* flies were crossed with PM181-GAL4, UAS-*lacZ* flies.

Histology and immunohistochemistry

Adult retinae were dissected, fixed and embedded in plastic for tangential sectioning as described (Tomlinson and Ready, 1987). Cryostat sections of adult mosaic heads were stained with mAb 24B10 or anti-β-galactosidase antibody as described (Garrity et al., 1996). Eye–brain complexes from third-instar larvae were dissected and stained with antibodies as described (Ruan et al., 1999). Antibodies to Chaoptin (24B10) (1:100 dilution, DSHB), Prospero (1:200 dilution, DSHB), Boss (1:2000 dilution), Repo (1:10 dilution, DSHB), Otk (1:100 dilution) (Pulido et al., 1992), GFP (1:1000 dilution, Molecular Probes) and β-galactosidase (1:100 dilution) were used as primary antibodies. For HRP/DAB visualization, HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were used at 1:200 dilution. For fluorescent staining, Texas-red- or FITC-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Jackson Immunochemicals) were used at 1:200 dilution. Epifluorescent images were captured using a high-resolution fluorescence imaging system (Canberra Packard) and analyzed by 2D Deconvolution using MetaMorph imaging software (Universal Imaging, Brandywine, PA).

The percentage of mistargeted R2-R5 axons or axon bundles in the medulla in *otk* and *sema* mutants was estimated by following the method described previously (Garrity et al., 1999) with only minor modification. Since mistargeted R2-R5 axons were observed in individuals that were much younger than that reported previously (Garrity et al., 1999), the mean number of ommatidial rows were subtracted by four instead of nine ommatidial rows. R-cell axons projected from these subtracted younger ommatidial rows presumably had not reached the brain.

Results

otk is required for R-cell growth-cone targeting

To identify genes that are required for layer-specific targeting of R-cell axons, we examined R-cell projection pattern in available mutants, including novel P-element insertions from the Berkeley Drosophila Genome Project as well as mutations that disrupt known genes that are expressed specifically in the nervous system. Among them, we found that mutations in the *otk* gene caused a specific R-cell projection phenotype. Since the null mutation otk^{3} in which the putative translational start codon and part of the signal peptide is deleted causes embryonic lethality (Winberg et al., 2001), we performed genetic mosaic analysis to examine axonal projections from otk^{3} mutant R-cell clones. *otk* homozygous mutant tissues were generated in an otherwise heterozygous or wild-type eye-imaginal disc by eye-specific mitotic recombination using the eyFLP/FRT system (Newsome et al., 2000a). By examining mutant clones in adult mosaic eyes, we estimated that ~80-90% of ommatidia in each mosaic eye examined were *otk* mutant clones, which was consistent with the absence of most ant-Otk immunoreactivity in the lamina in all otk^{3} mosaic third-instar eye–brain complexes examined (see below).

R-cell projection pattern in *otk* mosaic larvae was examined using monoclonal antibody 24B10, which visualizes all R-cell axons in the developing optic lobe (Van Vactor et al., 1988). In wild type (Fig. 1A), R1-R6 growth cones terminated within the lamina and then expanded significantly in size, which were seen as a continuous layer of 24B10 immunoreactivity within the lamina. Whereas expanded R7 and R8 growth cones form a highly organized pattern within the medulla. In otk^{3} mosaic individuals (n=25 hemispheres, Fig. 1B), small gaps were frequently observed in R1-R6 terminal field. The terminal field within the medulla was also disorganized as thicker bundles

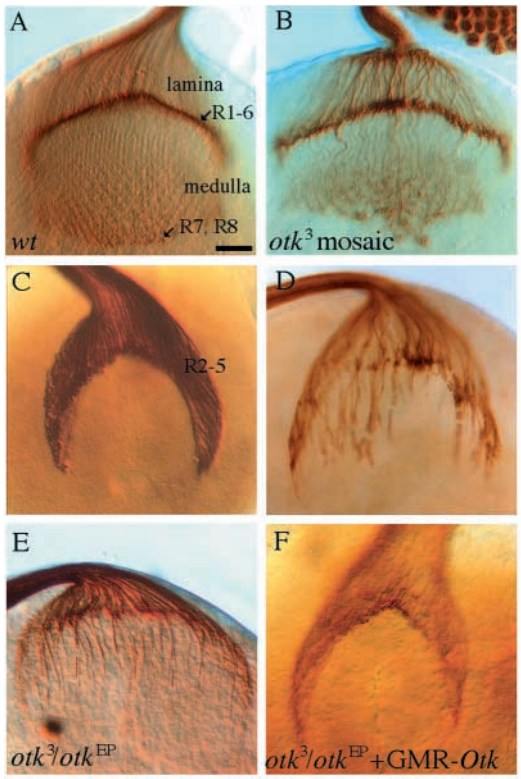

Fig. 1. The effect of *otk* mutations on R-cell projection pattern at larval stage. All R-cell axons in third-instar larvae (A,B) were stained with MAb 24B10. R2-R5 axons in third-instar larvae (C-F) were labeled with the larval R2-R5 marker *ro-τ-lacZ*. In wild type (A), after exiting the optic stalk (os), R7 and R8 growth cones passed through the lamina into the medulla, whereas R1-R6 growth cones stop within the lamina, which could be identified as a continuous line of MAb 24B10 immunoreactivity. In B, an otk^3 mosaic individual in which ~80-90% eye tissues were homozygous otk^3 mutant ommatidia, displayed defects in R-cell projections. The lamina plexus was uneven with the presence of small gaps. Abnormal thicker bundles were observed within the medulla. In wild type (C), *ro-τ-lacZ* labeled R2-R5 axons terminated within the lamina. In an otk^3 mosaic individual (D), many labeled R2-R5 axons projected aberrantly into the medulla. Similar mistargeting phenotype was also observed in $otk^3/otk^{EP(2)2017}$ transheterozygous larvae (E). In an $otk^3/otk^{EP(2)2017}$ transheterozygous larvae expressing an UAS-*otk* transgene in R cells under control of the GMR-GAL4 driver (Mismer and Rubin, 1987) (F), most labeled R2-R5 axons now terminated within the lamina. Scale bar: 20 μm.

were frequently observed within the medulla. Unlike some known mutations (e.g. *dock* and *pak*) that affect R-cell guidance (Garrity et al., 1996; Hing et al., 1999), loss of *otk* did not cause an obvious defect in the overall organization of R-cell axons within the developing optic lobe. The formation of topographic map also appeared normal.

To determine if the above defect is caused by mistargeting of R1-R6 axons, we used the larval R2-R5 marker *ro-τ-lacZ* to assess the initial targeting of a subset of R1-R6 axons at third-instar larval stage. In wild type (Fig. 1C), the vast majority of R2-R5 axons stop within the lamina, and only a few labeled axons (average three mistargeted axons or axon bundles per hemisphere, n=19 hemispheres) projected into the medulla. In otk^3 mosaic individuals (Fig. 1D), however, more than 32% (average 33 axons or axon bundles per hemisphere, n=16 hemispheres) of ommatidia projected one or more R2-R5 axons or axon bundles aberrantly into the medulla. Similar mistargeting phenotype (average 18 mistargeted R2-R5 axons or axon bundles per hemisphere, n=19 hemispheres) was also observed in $otk^3/otk^{EP(2)2017}$ transheterozygous larvae (Fig. 1E). However, the phenotype was less severe than that in otk^3 mosaic larvae, which was probably due to the hypomorphic nature of the $otk^{EP(2)2017}$ allele. To further determine if the above phenotype was indeed due to the lesion in the *otk* gene locus, we performed transgene rescue experiments. We found that eye-specific expression of an *otk* transgene completely rescued the R1-R6 mistargeting phenotype in *otk* mutants (Fig. 1F). The average number of mistargeted R2-R5 axons or axon bundles was reduced to three in $otk^3/otk^{EP(2)2017}$ transheterozygous larvae expressing the *otk* transgene, which is similar to that in wild type. This result, taken together with that from eye-specific genetic mosaic analysis, indicates that Otk is required in the eye for lamina-specific targeting of R1-R6 growth cones.

Otk is expressed in the developing *Drosophila* adult visual system

Previous studies demonstrated that Otk is specifically expressed in the nervous system at the embryonic stage (Pulido et al., 1992; Winberg et al., 2001). To determine if Otk is also expressed in the developing adult visual system at larval stage, we stained third-instar larval eye–brain complexes with an affinity purified anti-Otk antibody (Pulido et al., 1992). In wild type (Fig. 2B,C), anti-Otk staining was detected on R-cell axons in the developing optic lobe. In the lamina, the staining overlapped largely with 24B10 immunoreactivity, which reflects the expression pattern of Chaoptin, a cell surface adhesion molecule expressed exclusively on all R cells and their axons (Van Vactor et al., 1988). The strongest staining was observed in the lamina plexus, comprised primarily of R1-R6 growth cones. Although anti-Otk immunoreactivity was also detected in the developing medulla, we could not tell if Otk is present on R7 and R8 growth cones due to the uniform staining pattern in the medulla neuropil, which consists of both R-cell and non-R-cell axons (Fig. 2B,C). The specificity of anti-Otk staining was supported by the fact that the staining within the lamina was largely absent in otk^3 mosaic larvae (Fig. 2E,F). We conclude that Otk is expressed in developing R cells and is localized predominantly to R1-R6 growth cones.

R-cell differentiation and fate determination occur normally in *otk* mutants

The R1-R6 mistargeting phenotype may reflect a direct role for Otk in regulating R-cell growth-cone targeting. Alternatively, the defect might be caused by abnormal R-cell differentiation or cell fate determination; for instance, the transformation of a R1-R6 cell into a R7 or R8 fate. To distinguish among those possibilities, we examined R-cell development by using R-cell-specific developmental markers. Differentiating R7 and R8 cells in the developing eye disc were identified with anti-Prospero and anti-Boss antibodies, respectively. As in wild type (Fig. 3A,C), only one R7 (100%, n=2052 ommatidia in 13 eye discs, Fig. 3B) and one R8 (100%, n=3862 ommatidia in 16 eye discs, Fig. 3D) were observed in each ommatidium

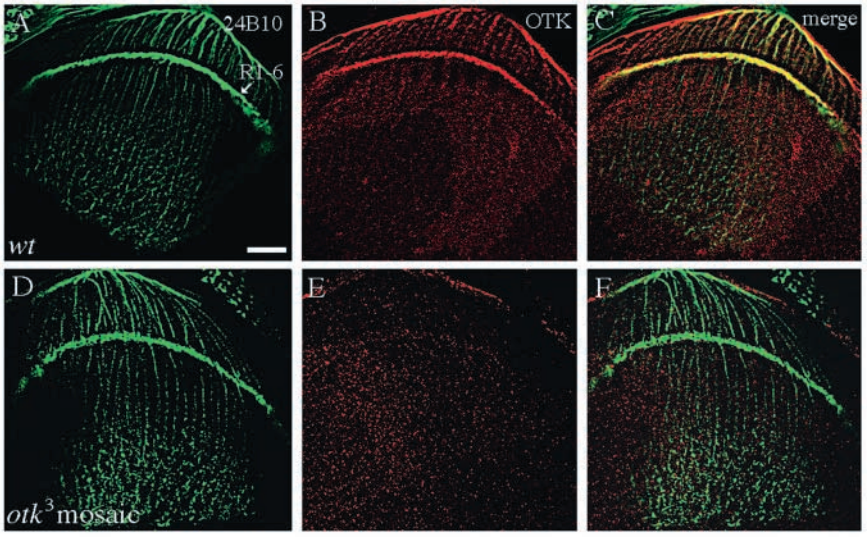

Fig. 2. Otk is highly enriched in R1-R6 growth cones. Third-instar eye–brain complexes were double-stained with MAb 24B10 (green) and anti-Otk antibody (red). In a wild type optic lobe (A-C), anti-Otk immunoreactivity was detected in both lamina and medulla. The strongest staining was detected in the lamina plexus, consisting mainly of R1-R6 growth cones at this developmental stage. In an *otk*^{3} eye-specific mosaic individual in which most R-cell axons were *otk*^{3} mutant axons (D-F), anti-Otk immunoreactivity was largely absent in the lamina plexus. Scale bar: 20 μm.

in all *otk*^{3} mosaic eye discs examined. Consistently, examination of *otk* adult mosaic eyes did not reveal any defect in either the number or the organization of R cells in all *otk*^{3} mutant ommatidia examined (*n*=978 ommatidia in 10 eyes) (compare Fig. 3F to 3E). Thus, *otk* is not required for R-cell differentiation and cell fate determination.

otk is not required for the differentiation and migration of lamina glial cells

Previous studies demonstrate a dynamic interaction between R-cell axons and lamina glial cells, the intermediate target of R1-R6 axons at larval stage (Poeck et al., 2001; Suh et al., 2002). On one hand, lamina glial cells produce an unknown stop signal to induce the initial termination of R1-R6 growth cones within the lamina (Poeck et al., 2001). On the other hand, R-cell axons also produce an unknown signal to induce the migration of lamina glial cells into the R1-R6 target region (Suh et al., 2002). To determine if the expression of Otk in R-cell axons is necessary for lamina glial cell differentiation and/or migration, we examined the development of lamina glial cells in *otk* mutants. Glial cells were visualized using a monoclonal antibody that recognizes the glia-specific nuclear protein Repo. In wild type (Fig. 4A), R1-R6 axons stop in the lamina and expand their growth cones in between two layers of lamina glial cells (i.e. epithelial and marginal glia). Although lamina glial cells in *otk* mutants appeared less organized than that in wild type (compare Fig. 4B with 4A), the number of lamina glial cells surrounding the lamina plexus in *otk* mutants was similar to that in wild type (*n*=12 hemispheres), indicating that the migration of lamina glial cells occurred normally in *otk* mutants.

The function of Otk in R-cell growth cones appears to be independent of Sema-1a signaling

Previous studies showed that Otk interacts with Plexin A (Cytochrome P450-9b2 – FlyBase) in mediating a Sema-1a-induced repulsive response during motor axon guidance at embryonic stage (Winberg et al., 2001), raising the possibility that the role of Otk in R1-R6 growth cones is also dependent on Sema-1a signaling. If so, one would predict that loss of

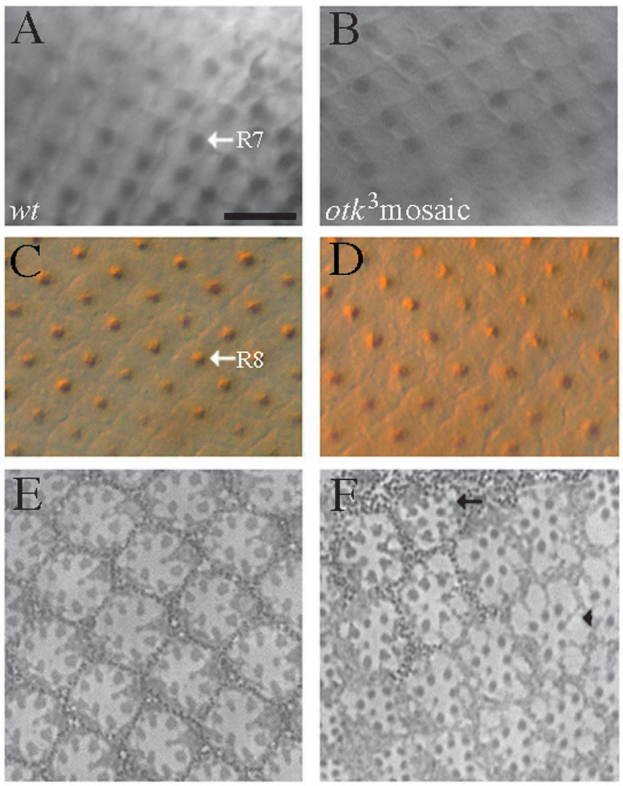

Fig. 3. *otk* is not required for R-cell differentiation and cell fate determination. Third-instar eye-imaginal discs were stained with anti-Prospero (A,B) or anti-Boss antibody (C,D). In wild type, each ommatidium contains only a single R7 (R7 nucleus indicated by arrow in A) and a single R8 cell (cell apical surface indicated by arrow in C). In an *otk*^{3} mosaic eye-imaginal disc, only one R7 (B) and one R8 (D) were present in each ommatidium. Tangential sections of wild-type (E) and *otk*^{3} adult mosaic eyes (F) did not reveal any defect in either the number or the organization of R cells within each ommatidium. The arrow in F indicates a wild-type ommatidium surrounded by dense pigment granules. The arrowhead in F indicates an *otk*^{3} mutant ommatidium that can be recognized by the absence of pigment granules. Scale bar: 10 μm.

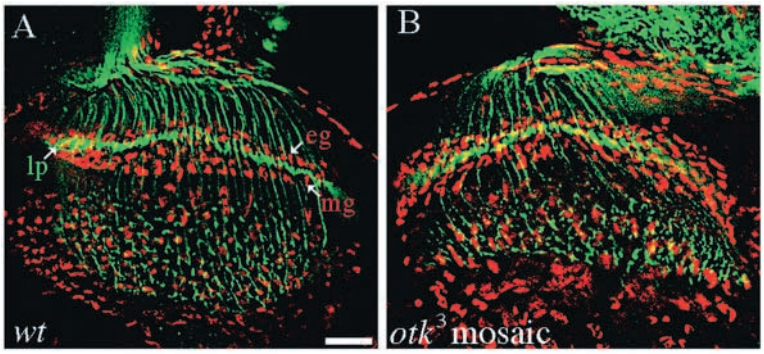

Fig. 4. Lamina glial cell migration occurs normally in *otk*^{3} mutants. Third-instar eye–brain complexes were double-stained with anti-Repo (red) and anti-β-galactosidase antibody (green). Anti-β-galactosidase antibody was used to visualize all R-cell axons in both wild type and *otk*^{3} mosaic individuals that carry a *glass-lacZ* transgene, in which the expression of *lacZ* is under control of the eye-specific *glass* promoter (Mismer and Rubin, 1987). Anti-Repo recognizes the nuclear protein Repo expressed in all types of glial cells. In wild type (A), all R1-R6 growth cones (green) stop in between two layers of glial cells (red), epithelial (eg) and marginal glia (mg), forming the lamina plexus (lp). In an *otk*^{3} mosaic individual (B), lamina glial cells were still present at the R1-R6 termination site. Scale bar: 20 μm.

Plexin A or Sema-1a should cause a similar R1-R6 targeting phenotype. Unfortunately, we were unable to assess the role of *plexin A* during R1-R6 growth-cone targeting, as the available *plexin A* mutation causes early lethality. And the *plexin A* gene is located on the fourth chromosome and thus not amenable to FLP/FRT-mediated mosaic analysis. However, we were able to examine R-cell projections in both *sema-la* homozygous null mutants (i.e. *sema*^{P1}) and *sema-la* eye-specific mosaic animals in which large clones of *sema*^{P1} mutant eye tissues were generated similarly using the eyFLP/FRT system as described above. Labeling of R-cell axons with MAb 24B10 staining revealed an R-cell projection phenotype in both *sema*^{P1} homozygous mutant and mosaic larvae. The R1-R6 terminal field in the lamina was severely disrupted; clumps and loop-like structures were frequently observed in *sema* mutants (Fig. 5C). In comparison, *otk* mutations caused only relatively mild defects in the organization of R-cell axons within the lamina (Fig. 5B).

To specifically assess the potential effect of *sema-1a* mutations on R1-R6 targeting, we used the *ro-τ-lacZ* marker to label R2-R5 axons in *sema*^{P1} homozygous mutant larvae. Surprisingly, although the organization of R-cell axons within the lamina was severely disrupted in *sema*^{P1} mutants (Fig. 5C), lamina-specific targeting of R2-R5 axons occurred in a largely normal fashion (Fig. 5F). In *sema*^{P1} homozygous mutants, the average number of mistargeted axons or axon bundles in each hemisphere is seven (*n*=15 hemispheres), a few more than that in wild type (i.e. three, Fig. 5D), but much fewer than that in *otk*^{3} mosaic animals (i.e. 33, Fig. 5E). Those observations argue against the possibility that Otk is regulated by Sema-1a for targeting R1-R6 axons to the lamina.

Loss of *otk* severely disrupts the completed pattern of R-cell connectivity in adult flies

To determine the effect of the *otk* mutation on the completed pattern of R-cell-to-brain connectivity in adults, we examined R-cell axonal projections in *otk* mosaic heads. Again, large clones of *otk*^{3} mutant tissues were generated in the compound eye by eye-specific mitotic recombination. The completed R-cell projection pattern in adults was examined by staining frozen sections of *otk* mosaic heads with MAb 24B10. Although R-cell axons appeared to project into correct topographic locations, an increase in the number of axon terminals within the medulla was observed in all sections examined (*n*=16 hemispheres) (compare Fig. 6B,D with 6A,C), suggesting that many mistargeted R1-R6 axons remained within the medulla.

To confirm this, we specifically labeled R1-R6 axons using an adult R1-R6 marker *Rh1-lacZ*. To accurately count the total number of axons that project abnormally into the medulla, we performed whole-mount staining of the brain instead of staining frozen sections. In wild type (Fig. 6E), all *Rh1-lacZ*-

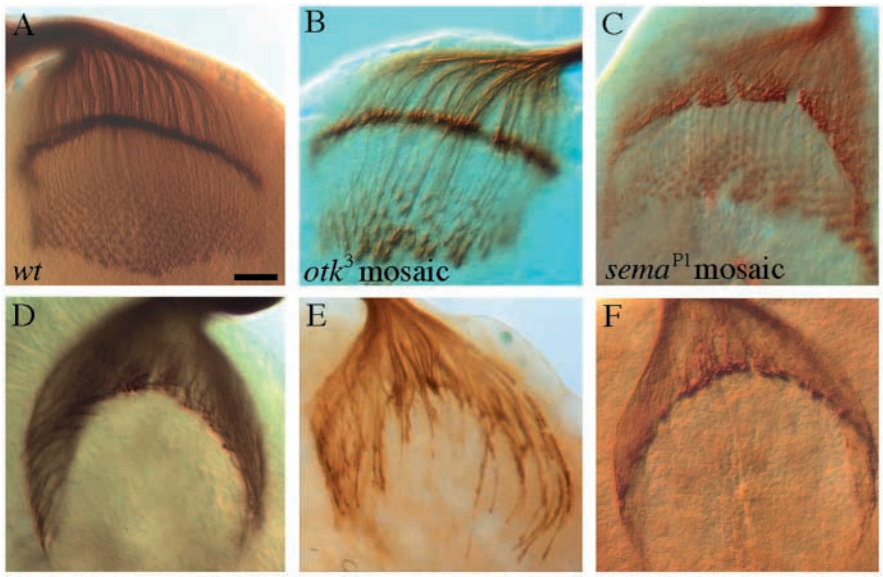

Fig. 5. R1-R6 targeting appears largely normal in *sema* mutants. Third-instar eye-brain complexes of wild type (A,D), *otk*^{3} eye-specific mosaic (B,E), *sema-1a*^{P1} eye-specific mosaic (C) and homozygous mutants (F) were stained with MAb 24B10 (A-C) or with anti-β-galactosidase antibody (D-F). Individuals in D-F carried the *ro-τ-lacZ* marker, which labels R2-R5 axons at larval stage. Although *sema-1a*^{P1} caused a defect in the organization of R-cell axons within the lamina (C) that was more severe than that caused by the *otk*^{3} mutation (B), it did not significantly affect R1-R6 targeting (compare F with E). Scale bar: 20 μm.

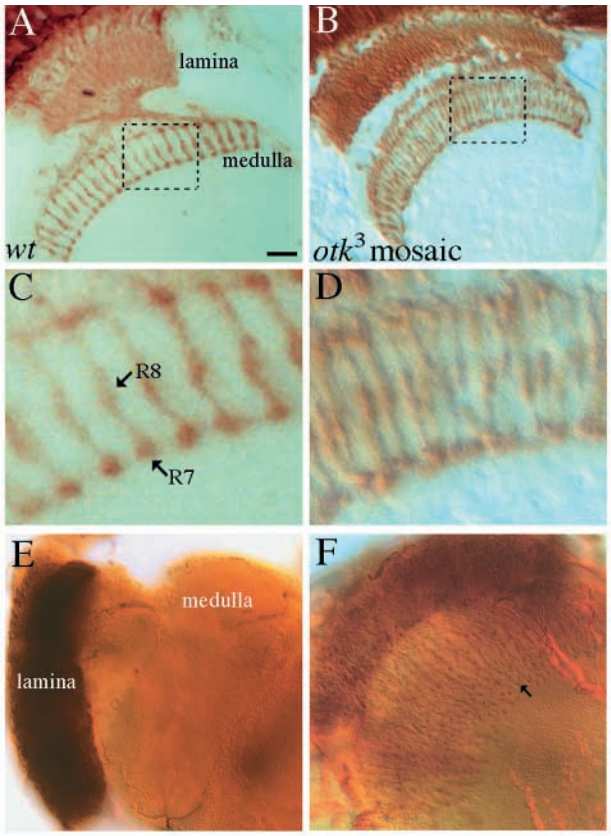

Fig. 6. The completed pattern of R1-R6 connectivity was severely disrupted in adult *otk* mutants. Cryostat sections of wild-type (A,C) and otk^3 eye-specific mosaic heads (B,D) were stained with MAb 24B10 to visualize all R-cell axons. In wild type (A), R7 and R8 axons projected into different layers within the medulla. Individual R7 and R8 axon terminals could be readily identified. In an otk^3 mosaic head (B), the medulla appeared to be innervated by an increased number of R-cell axons. C and D are higher magnification views of the boxed region in A and B, respectively. Whole-mount wild-type (E) and otk^3 mosaic brains (F) carrying an adult R1-R6 specific marker, Rh1-*LacZ*, were stained with anti-β-galactosidase antibody. In wild type (E), *LacZ* staining was exclusively observed in the lamina, as all R1-R6 axons terminate within this layer. In an otk^3 mosaic brain (F), a large number of R1-R6 axons (arrow) connected abnormally to the medulla. Scale bar: 20 μm in A,B,E,F; 5 μm in C,D.

labeled axons connected to the lamina. In all 11 wild-type hemispheres examined, no labeled axons projected into the medulla. In *otk* mosaic heads (Fig. 6F), however, a large number of R1-R6 axons were present in the medulla in *otk* mosaic animals (16 out of 17 hemispheres). Among 16 *otk* mosaic hemispheres that displayed the mistargeting phenotype, 13 hemispheres were mounted properly such that the total number of mistargeted R1-R6 axons or axon bundles could be accurately counted. The average number of mistargeted R1-R6 axons or axon bundles per hemisphere was 336 (ranging from 119 to 363 in different hemispheres). Mistargeted axons were distributed evenly within the medulla. By dividing the average number of mistargeted R1-R6 axons or axon bundles (i.e. 336) with 800 (the approximately total number of ommatidial fascicles within an adult eye), we estimate that approximately 42% of ommatidia projected one or more R1-R6 axons aberrantly into the medulla. This is in marked contrast to that in *Ptp69D* adult mutants, in which only a few mistargeted R1-R6 axon bundles (<5%) were observed within the medulla (Newsome et al., 2000a).

otk is not required for R7 axon targeting

To determine if loss of *otk* also affects the targeting of other R cells, we used the adult R7 marker PanR7-GAL4::UAS-Synaptobrevin-GFP to specifically assess the projections of R7 axons in *otk* adult mosaic heads in which the vast majority of R cells are *otk* mutant cells. In wild type (Fig. 7A), R7 axons projected into a region (i.e. M6 layer) in the medulla that is deeper than the R8 terminal field (i.e. M3 layer). In all sections examined (10 hemispheres), we found that labeled R7 axons still projected into the correct locations within the medulla (Fig. 7B). Thus, unlike loss of *Ptp69D* or *Lar* (Clandinin et al., 2001; Maurel-Zaffran et al., 2001; Newsome et al., 2000a), mutations in *otk* do not affect R7 targeting.

The expression of Otk in R7 axons was not sufficient for targeting R7 axons to lamina

In our previous studies (Ruan et al., 2002), we showed that the expression of the Ste20-like ser/thr kinase Misshapen (Msn) or the cytoskeletal regulator Bifocal (Bif) in R7 cells under control of a larval R7-specific driver PM181-GAL4 caused some R7 growth cones to target into the lamina. To determine if the expression of Otk alone is sufficient for specifying lamina-specific targeting of R-cell axons, we examined the

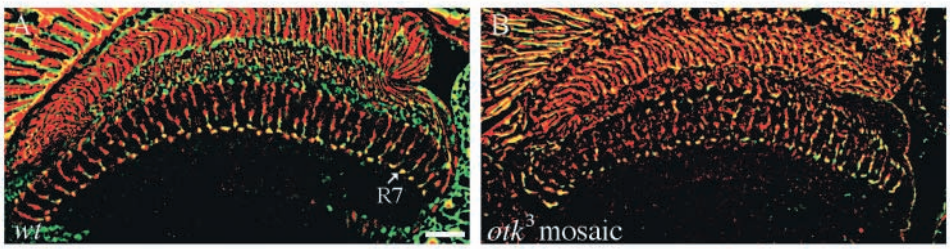

Fig. 7. *otk* is not required for R7 targeting. Cryostat sections of wild-type (A) and otk^3 eye-specific mosaic heads (B) were double-stained with MAb 24B10 (red) and anti-GFP antibody (green). Both wild-type and otk^3 mosaic individuals carried the adult R7 marker PANR7-GAL4::UAS-Synaptobrevin-GFP, in which the expression of UAS-Synaptobrevin-GFP was controlled by the R7-specific driver PANR7-GAL4 (Lee et al., 2001). In wild type (A), all labeled R7 axons innervated a region that is deeper than the R8 targeting layer within the medulla. Although R7 axons in an otk^3 eye-specific mosaic head (B) appeared less organized than that in wild type (A), they still projected into the correct target region. Scale bar: 20 μm.

effect of expressing Otk in R7 axons using the PM181-GAL4 driver. In wild type (Fig. 8A), all labeled R7 axons projected through the lamina and terminated within the medulla. In all larvae expressing Otk in R7 cells (*n*=11 hemispheres), R7 axons still extended normally into the medulla (Fig. 8B).

Discussion

In this study, we show that the receptor tyrosine kinase Otk is specifically required for lamina-specific targeting of R1-R6 growth cones. R1-R6 targeting errors in *otk* mutants were first observed at third-instar larval stage when R cells begin to project axons into the developing optic lobe. Many R1-R6 growth cones passed through the lamina and extended into the medulla instead. This initial R1-R6 targeting error was not corrected at later developmental stages, as many R1-R6 axons remained within the medulla in adult *otk* mutants. While *otk* is necessary for lamina-specific R1-R6 targeting, it is not required in R7 axons for establishing connections with local target cells within the medulla. The presence of Otk on R1-R6 growth cones and the specific *otk* loss-of-function phenotype support a key role for Otk in R1-R6 growth cones to specify their lamina-specific targeting decision.

The role of Otk in R1-R6 growth cones appears to be different from that of PTP69D, the only other cell surface receptor that has also been shown to be required for the initial termination of R1-R6 axons within the lamina (Garrity et al., 1999; Newsome et al., 2000a). In *Ptp69D* mutants, although ~25% of ommatidia projected one or more R1-R6 axons into the medulla at larval stage (Garrity et al., 1999), only a few axon bundles (32 mistargeted R1-R6 axons or axon bundles in a total of 34 hemispheres examined) remained within the medulla at adult stage (Newsome et al., 2000a). In addition, mutations in *Ptp69D* also disrupted R7 targeting (Newsome et al., 2000a). Many R7 axons did not project into their normal M6 layer, but instead stayed with the pioneer R8 axon at the superficial M3 layer within the medulla. These observations led to the suggestion that PTP69D plays a permissive role in R1-R6 targeting: that is, PTP69D may mediate defasciculation between R1-R6 and the pioneer R8 axon in the lamina and between R7 and R8 axons in the medulla, thus allowing them to respond to a targeting signal. While we cannot entirely exclude this possibility for the action of Otk, it appears unlikely that R1-R6 targeting error in *otk* mutants is simply caused by defects in R-cell defasciculation. Unlike that in *Ptp69D* mutants (Newsome et al., 2000a), severe R1-R6 targeting errors (one or more mistargeted R1-R6 axons in ~42% of total ommatidial axon bundles) were also observed in *otk* adult mutants, whereas R7 target selection remained normal. Moreover, although mutations in the *trio* or *pak* gene caused a severe hyper-fasciculation phenotype, they did not affect the completed pattern of R1-R6 connectivity (Hing et al., 1999; Newsome et al., 2000b). Thus, we favor the model in which Otk is actively involved in detecting a targeting signal for R1-R6 axons to select the lamina layer.

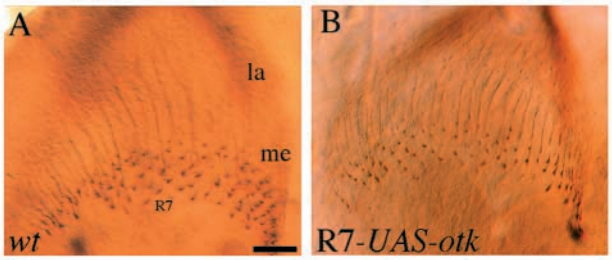

Fig. 8. The Expression of Otk in R7 did not retarget R7 growth cones to the lamina. Otk was expressed in R7 (B) under control of the PM181-GAL4 driver. Third-instar eye–brain complexes were stained with anti-β-galactosidase. Individuals in A and B carried the PM181-GAL4 driver and an UAS-*lacZ* transgene. Individuals in B also carried an UAS-*otk* transgene. In an individual expressing Otk in R7 axons (B), like that in wild type (A), all labeled R7 axons projected correctly through the lamina (la) into the medulla (me). Scale bar: 20 μm.

While in *otk* mutants a large number of R1-R6 axons connected abnormally to medulla, many R1-R6 axons still select the lamina for establishing synaptic connections. One probable explanation is that the absence of Otk may be partially compensated by another receptor that also plays a role in specifying R1-R6 targeting. Partial redundancy is not uncommon for genes that regulate axon guidance. For instance, it has been shown that four neural-specific receptor tyrosine phosphastes (i.e. PTP10D, LAR, PTP69D and PTP99A) are partially redundant with each other in regulating axon guidance in the fly embryo (Sun et al., 2001). In mammals, recent studies demonstrate that the floor-plate-derived morphogen sonic hedgehog cooperates with netrin to guide commissural axons toward the ventral midline in the developing spinal cord (Charron et al., 2003).

Previous studies show that mutations in the *brakeless (bks)* (*scribbler* (*sbb*) – FlyBase) gene caused a more severe R1-R6 targeting phenotype (Rao et al., 2000; Senti et al., 2000). Most, if not all, R1-R6 axons in *bks* mutants projected aberrantly into the medulla. The *bks* gene encodes a nuclear protein expressed in all R cells (Rao et al., 2000; Senti et al., 2000). Later studies by Banerjee and colleagues further indicate that Bks functions in R-cell growth-cone targeting by repressing the expression of another nuclear protein, Runt, in R2 and R5 cells (Kaminker et al., 2002). These studies thus raise the interesting possibility that Bks and Runt are components of a gene expression regulatory pathway, which controls the expression of specific cell surface receptors on R1-R6 growth cones for detecting a stop signal from the target region. To examine if the expression of Otk in R1-R6 cells is dependent on Bks, we examined the level of the Otk protein in *bks* mutants. However, no alteration in the expression level of Otk was detected (data not shown), arguing against Otk as a downstream target of the Bks pathway.

Although *otk* is necessary for lamina-specific targeting of R1-R6 axons, its expression in R7 axons was not sufficient to target R7 axons to the lamina. There are several possible explanations for this result. Otk may need to collaborate with another cell surface protein that is present on R1-6 but not R7 growth cones to mediate the lamina-specific targeting decision, and thus act as a component of a receptor complex. This situation may be similar to that of the Nogo (Rtn4 – Mouse Genome Informatics) receptor complex, which is involved in inhibiting neurite outgrowth in mammals (Wang et al., 2002). Upon ligand binding, the Nogo receptor initiates an inhibitory response only in the presence of p75 (Ngfr – Mouse Genome Informatics), another cell surface receptor. Alternatively, the signaling components that function downstream of Otk in R1-6 growth cones may not be present in R7 growth cones. Or the

presence of some inhibitory mechanisms within R7 growth cones prevents them from responding to an Otk-mediated lamina-targeting signal. The possibility that Otk plays a permissive but not instructive role in R1-R6 growth-cone targeting cannot be excluded either.

Previous studies demonstrated that Otk forms a receptor complex with Plexin A, which functions downstream of Sema-1a during motor axon guidance in the fly embryo (Winberg et al., 2001). In the fly adult visual system, however, the *sema-1a* phenotype appears quite different from that of *otk*, as the R1-R6 targeting pattern remained largely normal in *sema-1a* mutants (see Fig. 5). The simplest interpretation of this data is that *otk* functions in a different pathway in R1-R6 growth cones for specifying lamina-specific targeting decision. An alternative explanation is that Sema-1a may function redundantly with other proteins (for instance, other members of the Semaphorin protein family), to regulate the function of Otk during R1-R6 targeting. Our present data do not allow us to distinguish among these possibilities.

Otk belongs to the evolutionarily conserved CCK-4 family of 'dead' receptor tyrosine kinases (Kroiher et al., 2001). Members of this family carry alterations in several evolutionarily conserved residues within the kinase domain that have been shown to be essential for the activity of most (if not all) active tyrosine kinases. Indeed, several of them have been shown to be inactive kinases by biochemical analysis (Miller and Steele, 2000). How does a defective receptor tyrosine kinase such as Otk transduce targeting signals for specifying layer-specific R-cell connectivity? One possibility is that Otk associates with an unknown active tyrosine kinase, which induces tyrosine phosphorylation on Otk upon ligand binding. One precedent for this is the dead kinase ErbB3, a member of the vertebrate EGFR family. Although the kinase activity of ErbB3 is greatly impaired, it can transduce mitogenic signals by forming a heterodimer receptor complex with another EGFR family member (e.g. ErbB2) carrying an active kinase domain (Alimandi et al., 1995; Kim et al., 1998; Sliwkowski et al., 1994). ErbB2 then induces tyrosine phosphorylation in the cytoplasmic domain of ErbB3, which serves as docking sites for downstream signaling proteins. Interestingly, it has been shown that Otk is phosphorylated on tyrosine residues in both fly and mammalian cultured cells (Pulido et al., 1992; Winberg et al., 2001). It is highly possible that in response to a targeting signal these phosphorylation sites recruit downstream signaling proteins, which then transduce the signal into the termination of R1-R6 growth cones within the lamina. In this context, it is notable that the intracellular signaling protein Dreadlocks (Dock), a SH2/SH3 adapter protein, also plays a role in lamina-specific targeting of R1-R6 axons (Garrity et al., 1996). Dock contains a single SH2 domain that can bind to specific phosphorylated tyrosine residues on activated proteins. Our previous studies suggest that a Dock-mediated signal activates the Ste20-like kinase Msn, which in turn phosphorylates the cytoskeletal regulator Bif, leading to the termination of R1-R6 growth cones in the lamina (Ruan et al., 2002; Ruan et al., 1999). We have performed experiments to investigate the potential interaction between Otk and Dock during R1-R6 targeting. However, we did not observe any genetic interaction between them (data not shown). Moreover, quantification of the R1-R6 targeting phenotype in adults shows that the phenotype in *dock* mutants was less severe than that in *otk* mutants (data not shown). While these data appear inconsistent with the notion that Otk and Dock function in the same pathway, it does not exclude the possibility that Dock cooperates with another SH2-containing protein to transduce the signal from the activation of Otk to downstream effectors for lamina-specific targeting of R1-R6 axons. Further studies will be necessary to critically address this matter.

In summary, our present study demonstrates an essential role for Otk in specifying R-cell connectivity. We propose that Otk is involved in recognizing a layer-specific signal for R1-R6 axons to select the lamina for synaptic connections. Further biochemical, molecular and genetic dissection of the Otk pathway will help to understand the action of Otk in R-cell growth cones and shed light on the general mechanisms controlling the establishment of layer-specific neuronal connectivity in the nervous system.

We thank: Don van Meyel for critical reading of the manuscript; the BDGP and Bloomington Stock Center for fly stocks; DSHB at University of Iowa for MAb 24B10, anti-Prospero and anti-Repo antibodies; M. Barbacid for anti-Otk antibody; C. Lee for PM181-GAL4 and PANR7-GAL4 lines; D. Montell and C. Goodman for *otk* transgene and mutant lines; A. Kolodkin for *sema-1a*^{P1} mutant line; and S. L. Zipursky for anti-Boss antibody. This work was supported by an operating grant (MOP-14688) awarded to Y.R. from the Canadian Institutes of Health Research.

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