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Postembryonic sensory axon guidance in *Drosophila*

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Abstract. The peripheral sensory system of the *Drosophila* adult has been used for the genetic analysis of axon guidance because of its accessibility for experimental manipulation and mutant screens. Wing, leg, antenna, or eye sensory axons are able to pathfind normally under different perturbations, indicating that sensory axon guidance is a highly canalized process. Similarly to other model systems, sensory growth cones seem to use multiple, simultaneous cues for guidance. In addition, sensory axons from peripheral structures seem to be capable of using alternative substrates for pathfinding. Developmental regulation could account for the high stability of axon guidance under experimental and natural perturbation conditions. Despite this flexibility, functional characterization of genes involved in sensory axon guidance is being carried out in situations where there appears to be less system redundancy.

Key words. Sensory axon; axon guidance; canalization; developmental regulation; *Drosophila*.

Early studies on the problem of axon guidance in *Drosophila* were primarily focused on the sensory system of the adult. During the last decade, however, there has been a shift toward the use of the embryo as a model for the genetic analysis of axon guidance in the fly. More than a decade ago, adult sensory structures and their larval progenitor organs, the imaginal discs, were easier to manipulate and mark (for example cobalt or horseradish peroxidase (HRP) backfilling). The advent of molecular marking techniques and tools (mainly monoclonal antibodies, enhancer trap P-lacZ insertions and the GAL4 system) encouraged an increased use of the embryo as a model system for central nervous system (CNS) as well as for peripheral nervous system (PNS) development. The main advantage of the embryo is the possibility to study phenotypes produced by embryonic lethal mutations (which would require the generation of mitotic recombination clones for study in the adult). Nevertheless, the adult sensory system continues to be uniquely suited for the study of certain neural development problems (as, for example, the construction of topographical sensory maps within the CNS). Moreover, the recent availability of green fluorescent protein [1] as a marker for the direct visualization of nerves in the living adult, larvae, or pupae is likely to produce a renewed interest in the use of the adult PNS as a model. Screens for mutants affecting adult axon projections will be an exciting area of research in the near future. Here, I present an overall picture of several adult sensory systems used as models for the analysis of axon guidance.

A common result in many genetic axon guidance studies has been a lack of major phenotypes, suggesting that axon guidance is a highly canalized process (very stable under perturbation [2]). Indeed, the current understanding of axon guidance relies on the idea that axons are guided by cues which act simultaneously, in a concerted fashion, and can be of different types: contact or diffusible, and repulsive or attractive [3]. Many of these cues seem to have partially redundant functions. Studies on adult sensory axon guidance in *Drosophila* depict the same scenario and suggest that developmental regulation is likely to play an important role in ensuring a high efficiency during growth cone guidance. The lack of overt axon guidance phenotypes in many mutants could be due to the existence of these regulatory capacities. Ideally, in the future, it will be important to use model systems that help maximize the chances of revealing phenotypes by minimizing the possibility of regulation and functional redundancy.

Mechano- and chemoreceptors of thoracic and antennal appendices

Most adult epidermal structures and their associated sensory organs derive from the imaginal discs in the larva. During the larval stages, the epithelium of imaginal discs proliferates from a few tens of cells to generate the tens of thousands that form the discs before differentiation. Imaginal discs and their associated sensory organs differentiate during metamorphosis, although the compound eye in the eye-antenna imaginal disc starts to do so during the third larval instar.

The epidermis of the *Drosophila* imago bears a large number of sensory receptors located at stereotyped positions. Mechanoreceptor organs include bristles, sensilla, and chordotonal organs. Chemoreceptors resemble bristles or sensilla, and can be found in the wing margin and legs (taste) as well as the antenna (olfactory). In the wing, there is also a complement of neurons which do not bear any specialized external cuticular structure and could represent type II sensory neurons (E cells) [4, 5]. The first neurons start to differentiate in the wing, leg, and antennal imaginal discs early after puparium formation (APF) [4, 6–8].

Sensory axons of the wing grow inside the prospective wing veins along the epithelium surrounded by extracellular matrix (ECM) [9]. Wing sensory axons project in a proximal direction toward the base of the wing. Axons from distal neurons sequentially contact and extend along with the axons of more proximal neurons in a converging pattern. Nevertheless, it has been shown that distal wing axons do not require more proximal axons [10, 11], nor the presence of the physical channels of veins for projection [12]. Moreover, the simultaneous absence of both physical channels (prospective veins) and putative guidepost cells (other neurons) does not cause abnormal pathfinding of sensory axons in the wing. Furthermore, although glial cells are present in the wing disc before sensory axons start extension [13], they migrate by following sensory axons [14]. Finally, wing sensory axons can project normally in cultured isolated disc fragments [12]. Thus, axon guidance in the pupal wing does not seem to strictly depend on guidepost cells, physical channels, or long-range diffusible attractive cues. Cues on the epithelium or in the ECM of the wing are likely candidates to support the guidance of these axons (at least under perturbed conditions). Sensory neurons of diverse peripheral origin (retina, antenna, or leg), but not from the CNS, seem able to recognize and project following these cues, since patches of these tissues transplanted to aneural wing discs (from the mutant *scute*$^{10.1}$) can project axons in the wing that follow normal wing trajectories [15]. The cues that operate during axon guidance in the pupal wing do not seem to be restricted to the normal axon pathways but are distributed over most of the wing surface [16]. Interestingly, there seems to be a region located around the base of future vein 2 that does not support (or repels) axon extension [17].

Wing sensory axons enter the CNS through the dorsal anterior mesothoracic nerve (fig. 1). The presence of a persistent larval sensory axon associated with a closely

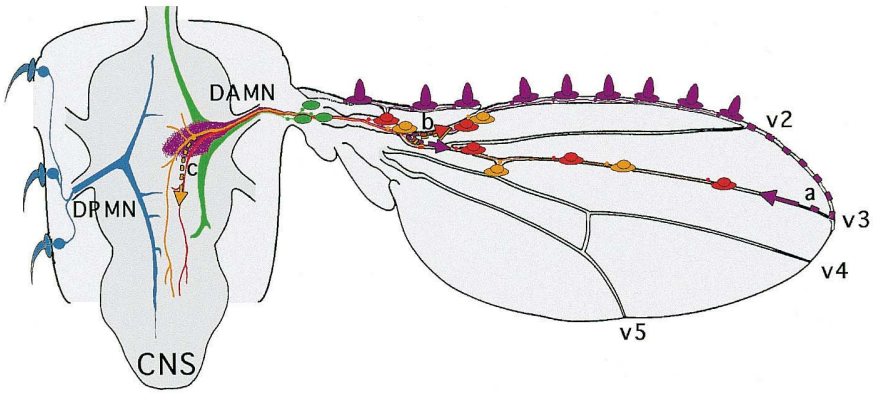

Figure 1. Sensilla and bristle axon projections from the wing and notum. The scheme depicts a horizontal section through the wing and thorax of an adult fly (anterior is up). Three classes of phenotypes are represented by small letters: (a) axons from the wing margin bristles project distally in *fasIII;fasI*, and *nac* mutants; (b) axons from wing margin bristles and 3rd-vein sensilla form a loop at the junction of vein 3 and marginal vein when *nrt* is ectopically expressed by the wing sensilla and the 3rd vein epidermis; (c) early developing phasic sensilla axons project along the pathway of late differentiating tonic sensilla in *fasIII* and *nac* mutants. See text for further details. Red: late differentiation tonic sensilla campaniformia; orange: early differentiation phasic sensilla campaniformia; blue: notum bristles; purple: wing margin bristles; green: wing base sensilla; DAMN: dorsal anterior mesothoracic nerve; DPMN: dorsal posterior mesothoracic nerve; v2–v5: vein 2 to vein 5.

located thoracic muscle might constitute a bridge for sensory axons from the wing to the CNS [18]. This persistent larval axon may correspond with the transient population of axons described by Withlock and Palka [19] as putative pioneers from the base of the wing to the CNS [18].

Several cell adhesion molecules (CAM) have been shown to influence axon extension in the wing. Fasciclin (Fas)I and III are expressed by sensory neurons in the margin of the wing and FasI is also expressed by sensory neurons of vein 3. Another fasciclin, FasII is expressed by wing margin neurons and by the epithelium [20]. *fasIII;fasI* double mutants display some pathfinding errors in the wing margin where axons can sometimes project distally along the margin and invade vein 4 (see fig. 1). Occasional misroutings of the same type can also be found in mutants of *fasII* [20]. Neurotactin (Nrt) is a CAM expressed by neurons in the wing margin [21]. Although loss-of-function mutations in *nrt* do not show any obvious abnormality in axon extension, ectopic expression of Nrt in vein 3 (neurons and epithelium) causes axons in vein 3 to turn back at the base of vein 3 and connect with the margin axons, creating a loop that prevents either nerve to project further proximally (fig. 1) [21]. Another mutation that causes abnormal pathfinding phenotypes in the wing is *neurally altered carbohydrate* (*nac*). The *nac* mutation disrupts a process of carbohydrate addition to cell surface proteins [22] and possibly alters the function of many cell surface molecules. The *nac* mutation causes phenotypes similar to that of the *fasIII;fasI* double mutant but with higher expressivity [20]. The ECM at the inner surface of the wing epithelium bears laminin during the stages of sensory axon extension [9]. Partial loss-of-function mutations for one of the laminin chains, laminin A, show occasional alterations in wing axon projection [23].

In the prospective notum a few hours APF, the first macrochaete axons extend along the basement membrane and converge onto a thoracic larval nerve which takes them to the CNS where they enter through the dorsal posterior mesothoracic root (fig. 1). Microchaete axons extend later and converge in one of four groups where axons are funneled along the prospective tendons that are differentiating from epidermal cells. From these epithelial projections, microchaete axons can reach the surface of developing muscles and contact the ingrowing motor axons [24, 25] plus the macrochaete axons already present. The separation of microchaete axons in four fasciculation groups correlates with their functional organization, since each group ellicits specific cleaning reflexes when stimulated [26].

Within the pupal CNS, different sensory neurons deploy specific projection patterns (fig. 1). Phasic sensilla of the wing differentiate early and project in a medial tract within the CNS, while the latter differentiating tonic sensilla campaniformia project in a lateral pathway [7]. Bristles also project in specific patterns [27]. Interestingly, macrochaete axons originated in an ectopic location (metathorax) and entering the CNS through the haltere nerve show projections that overlap those of the normal thoracic macrochaetes [27]. Neither axon-to-axon interactions between the ingrowing sensory fibers, nor electrical activity seem to be strictly required for the establishment of CNS projections [28]. By the time peripheral axons are projecting within the CNS, membrane-bound macromolecules are being expressed in stereotyped patterns [19, 20]. Indeed, mutations in either *fasIII* or *nac* produce abnormal wing axon projection phenotypes within the CNS. In both cases, axons from the early phasic sensilla are misrouted toward the more lateral tract corresponding to the late differentiating tonic sensilla [20]. Mutations in either *fasI*, *fasII*, or *fasIII* also cause more subtle phenotypes of increased wing sensory axon branching within the CNS. *disconnected* (*disco*) mutants show alterations in thoracic proprioceptive sensory axon projections, which may be a secondary consequence of the alteration of CNS Disco-expressing cells if these have a pioneering function [29].

Appropriate arrest of sensory axon extension at specific target cells within the CNS may depend on a mechanism involving the titration between activities in the extending sensory axon and its target cells. Giant axons extending in a normal pupal CNS (created by a mitotic recombination clone of the mutation *gigas*) project to and far beyond their normal targets, suggesting that these mutant axons fail to receive sufficient stop signals from the wild-type targets [30].

Leg imaginal discs bear a complement of sensory neurons during the whole larval life [6, 31]. These neurons correspond to the receptors of the Keilin organs of the epidermis of the embryo and larva. The neuronal cell bodies are located in the leg discs but connect with the Keilin organs through long dendritic processes. The axons from these larval sensory neurons project basally in the leg disc, forming two pathways that connect with a larval nerve which takes them to the CNS. Adult sensory organs of the legs appear early APF and extend axons which follow the two pre-existing larval pathways [6]. However, the pioneering function of the larval sensory nerves within the disc is not essential. *Antennapedia* or ss^a mutant flies show a transformation of antenna into leg lacking the complement of larval sensory neurons of the normal legs. Despite this, axonogenesis from the adult sensory neurons can proceed along the developing ectopic leg epithelium [31, 32]. This result suggests that adult leg sensory axons can read cues in the epithelium (or basement membrane) of the leg disc which can be used as an alternative to the larval sensory

nerves. However, axon projection from the leg disc to the CNS probably requires the use of a larval nerve as a bridge [31]. In antenna to leg transformations caused by the mutant ss^a, leg bristle nerves project following the larval antennal nerve indicating that leg sensory axons can read antennal nerve cues [32]. Persistent proprioceptive larval neurons have been identified which project in the larval segmental nerve and could guide the axons of adult leg proprioceptors to the CNS. These same larval axons seem also to prefigure some of the adult proprioceptive projection patterns within the CNS [18]. CNS proprioceptive projection patterns are affected in *disco* mutants, suggesting that CNS cells which express the Disco protein may have a role in sensory axon guidance [29].

Different sensory neurons of different modalities from the leg project to different layers within the leg neuropile of the thoracic neuromeres. While proprioceptive (hair plates and sensilla campaniformia) mechanoreceptors do not form a topographic map within the CNS leg neuropil, tactile mechanoreceptors (bristles) project somatotopically in a different layer [33, 34]. For tactile sensory neurons, adjacent positions around the leg correlate with adjacent axon projections around the leg neuropil of the CNS thoracic neuromeres [33]. Thus, although sensory axons leave the leg along one of two nerves, they show a more graded ability to make choices for projection within the CNS. The choice of axon fascicle used to leave the leg is not determinative of the site of projection, since sensory axons can change from one fascicle to another at the entrance of the CNS [33]. Topographic projection within the leg neuropil does not seem to strictly depend on axon to axon interactions, since removal of many sensory organs does not affect the projection of the remaining ones. Thus target domain recognition seems to be an autonomous feature of each single axon [33].

Mutations in the gene coding for a dynein light chain (*cut up*) or for a protein component of the dynactin complex (*Glued*), show strong axon pathfinding and branching phenotypes within the leg neuropils of the CNS. Remarkably, in both cases, abnormal pathfinding and branching axons are still able to recognize and terminate at their correct target domains [34, 35].

Antennae bear olfactory chemosensilla which project axons to the antennal lobe in the brain. The first neurons that differentiate in the antenna arise around 3 h APF from the prospective aristal region located at the center of the antennal anlage [32]. Axons from these neurons extend along the antennal disc epithelium and reach the antennal larval nerve around 6–9 h APF. Axons from the prospective second antennal segment seem to be the next to join this larval nerve [36]. The larval antennal nerve, which carries the axons from the larval olfactory organ, is then used as a bridge toward the remodeling antennal lobe in the pupal brain [36]. Again, a putative pioneering function of the aristal axons is not essential for other antennal axons to project correctly, since the pattern of projections from the proximal antennal regions is normal in cases where distal antenna is transformed into leg (in the mutant ss^a) [32].

Photoreceptors of the compound eye

The compound eye of *Drosophila* is the superposition of two different sensory fields: a regular array of ommatidia (roughly 800), which harbors photoreceptor neurons and other cell types, and another array of interommatidial bristles (roughly 600) with a mechanosensory function [37].

Each ommatidium harbors eight photoreceptors (R1–R8). Photoreceptors start appearing after a sequence of stereotyped cell-cell interactions during the patterning of the eye disc in the third larval instar [38, 39]. Six of the photoreceptors (R1–R6) project axons to the first optic ganglion, the lamina, in the optic lobe. Photoreceptors R7 and R8 project through the lamina to the second optic ganglion in the optic lobe, the medulla, where they terminate in different layers [40].

Extension of R axons begins during the third larval instar. Before any R cell differentiates, the eye-antenna imaginal discs are already connected with the developing optic lobes by a tube-shaped epithelial structure called the optic stalk (OS). The OS is already present early after embryogenesis [41] and during most larval life only carries the axons of the larval visual nerve (Bowlig's nerve). Although Bowlig's nerve grows during embryogenesis along an embryonic OS, the relationship of this with the larval OS is not clear [42]. During the early third larval instar, several transient axons project in the OS along with Bowlig's nerve. The origin of these axons seems to be in the eye disc [40].

Around the middle of the third larval instar, photoreceptor axon extension begins behind the morphogenetic furrow (a place of cell-cell interactions and cell rearrangements) which moves from posterior (close to the OS) to anterior (towards the position of the antennal anlage). Thus, the first axons to extend are the closest to the OS. Within each differentiating ommatidium, R8 is the first neuron that differentiates and extends an axon. The other R axons of the same ommatidium fasciculate and follow R8. Progressively, following the morphogenetic furrow displacement, more anteriorly located R axons begin to extend posteriorly funneling towards the OS. R axons project across the differentiating retinal field straight towards the OS which is their exit from the eye imaginal disc (fig. 2). Axons from single isolated ommatidia (in the mutant *Ellipse*) are able to leave the

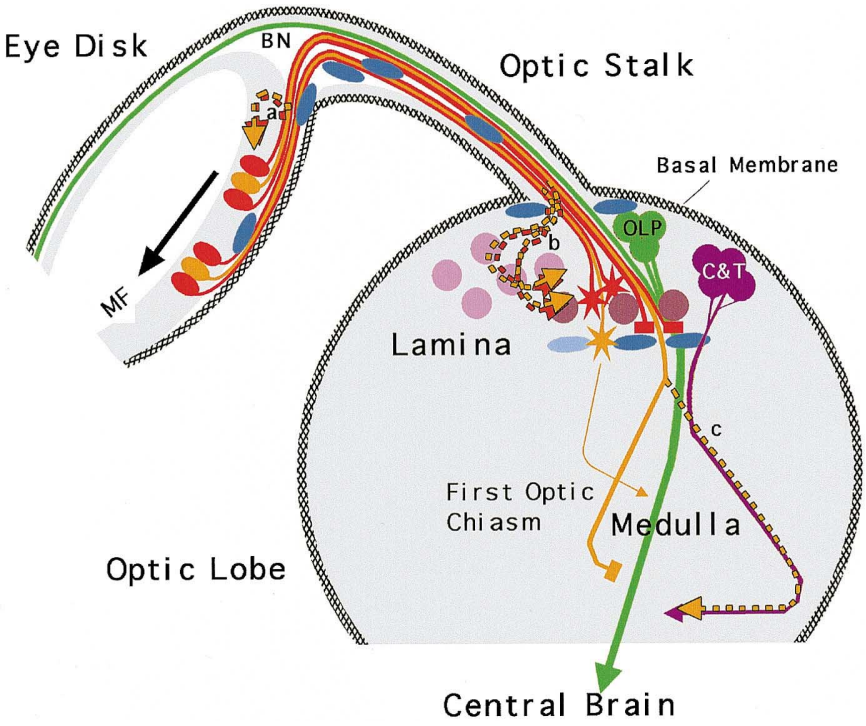

Figure 2. Photoreceptor axon projections in the compound eye and optic lobe. The scheme shows a horizontal section through the eye imaginal disc and optic lobe from a third-instar larva (anterior is left, right side is up). Projections of three photoreceptors from two different ommatidia are drawn. Three classes of phenotypes are represented by small letters: (a) photoreceptor axons tangled at the retina, typical of *lamA* and *eddy* mutants; (b) abnormal projections within the lamina in *dock* mutants; (c) R7 and R8 axons project along the C&T fibers in *irreC* mutants. See text for further details. Red: R1–R6; orange: R7 or R8; green: Bowlig's nerve (BN) and optic lobe pioneers (OLP); blue: glia (clearer in differentiating cells); purple: lamina neurons (clearer in differentiating cells) and C&T cells; MF: morphogenetic furrow.

imaginal eye disc in the absence of other neighbor ommatidial fascicles, although they extend in the disc with a more meandering trajectory. Therefore, younger axon fascicles do not absolutely require older R axon fascicles for guidance toward the OS [43].

R axon extension toward the OS is abnormal in *laminin A* (*lamA*) mutants. Laminin A is found in the ECM covering the basal surface of all imaginal discs. In partial loss-of-function *lamA* mutations, R axons can lose the correct navigation orientation within the retina and form large masses of tangled axon fascicles or even project outside the eye into epidermal regions (figs 2, 3). Therefore, lamA function is required, directly or indirectly, for R axons to find their way to the OS [23]. Another molecule required for R axons to reach the OS is encoded by the gene *eddy* which has a non-autonomous function and, therefore, could represent an ECM or other extracellular component [44].

lamA mutants also display errors in the distribution of retinal glia. Retinal basal glial (RBG) cells originate in the OS and migrate to the retina using the ingrowing R axons as a guiding substratum [45]. In addition, there are subretinal glia, which could be generated in the eye disc and/or OS and then migrate to the surface of the optic lobes [37, 46]. Subretinal glia and RBG cells seem to have a common origin [47] but it is not known whether subretinal glia depend on the R axons to migrate as RBG do. In *lamA* mutants, glial cells are seen in close association with R axons in clumps and whirls within the eye disc. These glial cells may have used the abnormal projections of R axons to attain their abnormal arrangement or vice versa. Ectopic ommatidial clusters, induced in clones for the mutant *patched*, which are isolated from the normal differentiating retina are devoid of glia [48]. R axons from these clones fasciculate with each other, although they fail to reach and fasciculate with the normal R axons of the endogenous retina. Therefore, the initial projection and fasciculation of R axons can take place in the absence of glia. Glial cells enter the clone territory as soon as it contacts the normal retina, suggesting that glia migrate toward R cells. The contact between the clone and the retinal

field also allows the R axons from the *patched* clone to reinitiate extension in a normal direction [48]. The correlation between R axon re-extension and invasion of the clone by glial cells may suggest that glia signal growing R axons to project in the normal direction. Alternatively, an ECM (or another short-range) cue which guides R axons within the retina may appear only after the process of retinal differentiation behind the morphogenetic furrow. Isolated patches of retinal cells induced at a distance from the normal differentiating retina would not have all the continuity in the deployment of this information for R axons to project outside the clone territory.

The connection provided by the OS is probably indispensable for R axons to reach the differentiating optic lobe [49]. Differentiating retinas devoid of an OS, as in the unconnected phenotype of *disco*, *so* or *ee* mutants [49–51], contain R axons that initially extend normally but eventually clump in subretinal positions. In gain-of-function *dpp* clones [52], or *dachshund* ectopic expression [53], or loss-of-function *patched*$^-$ clones [48] (see above), induced ectopic retinas which are not continuous with the normal retinal field and OS show R axons that remain at subretinal positions. In some cases, R axons from these ectopic retinal patches seem to be able to follow cues in the epithelium of the antennal imaginal disc [53]. Interestingly, eye-antenna imaginal discs cultured in vitro and cut at the OS show R axon extension out of the OS [54]. While the experimental conditions did not allow the authors to distinguish between regenerating axons (already present in the OS) from newly extending axons, staining the whole population of axons in the retina showed a correct funneling of all axon fascicles through the OS [54]. Thus, as in the case of wing sensory axons, all previous results are consistent with the idea that projection of R axons towards the CNS does not strictly require the presence of long-range diffusible attractive signals. In addition, electrical activity is not required for normal R axon pathfinding from the retina to the optic lobe targets [55].

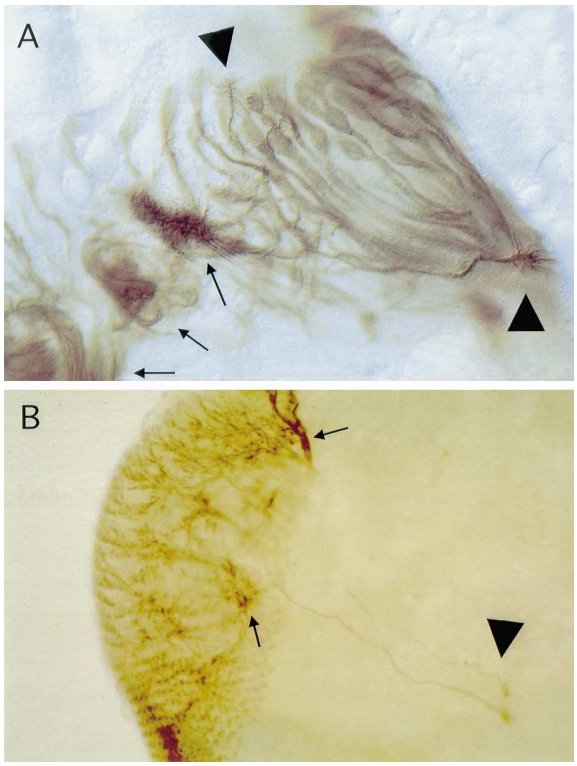

Figure 3. Photoreceptor axon guidance phenotypes in *lamA* mutations. Two examples of abnormal pathfinding by R axons in *lamA* mutants corresponding to a pupa before head eversion (anterior is up and lateral is right) (*A*) and to a pupa after head eversion (dorsal is up and lateral is left) (*B*). Arrows point to axon clumps at subretinal positions, arrowheads point to axons projecting to ectopic positions towards and under the epidermis. (*A*) mAb 22C10. (*B*) mAb 24B10.

Within the OS, ommatidial axon fascicles do not seem to require Bowlig's nerve or other neighbor R axon fascicles to extend and reach the optic lobe anlagen [43]. R axons may continue using ECM cues or glial cells, or the physical conduction provided by the channel, to reach the differentiating optic lobe. *repo* mutations impair glial differentiation in the visual system [56] and display axonal phenotypes within the OS [57]. However, the phenotype consists of fasciculation, rather than pathfinding, defects. Similar fasciculation defects within the OS, resulting in a broader projection and gaps between the axon fascicles, are also observed in *lamA* [23] and *doc* [58] (see below) mutants. A detailed analysis of the doc phenotype at the electron microscope level reveals loose packing of R axon fascicles by glia [58]. Therefore, it is possible that the lamA and repo phenotypes also reflect defects in fascicle packing.

Once R axons enter the developing optic lobe, they induce the terminal differentiation of target cells in the prospective lamina. Ingrowing R axons contact and induce lamina progenitor cells to progress to the S phase of the cell cycle giving rise to the lamina monopolar neurons [59, 60]. R axons also induce the terminal differentiation of lamina-glia (L-glia) [46], as well as the migration of some glial cell types to specific positions within the developing lamina [47]. Some of these inductive actions are produced through the release of Hedgehog by the R axons. However, it is likely that R axons produce additional signals for the induction of lamina cell differentiation [61, 62].

It is not known what cellular cues guide R axons in the developing lamina. Interaction of ingrowing R axon fascicles with older (more posterior) ones may con-

tribute to the positioning of the newly arriving R axons (more anterior ones) into more anterior positions within the lamina [40]. Different types of glial cells, like the subretinal glia, could play a role in R axon guidance in the optic lobe, since they are definitively positioned in the optic lobe before the arrival of R axons [47]. L-glia require R axon innervation for full differentiation. However, in the absence of retinal input, a small fraction of immature L-glia are correctly positioned prefiguring the position of early projections, thus anticipating the arrival of the first R axons [47]. Perez and Steller [47] have proposed a model in which some immature glia migrate and help guide the first ingrowing R axons, which, in turn, induce their terminal differentiation and trigger the migration of more glia. This new wave of glia will guide the new wave of R axons and so on. In this sequential inductive model, the number of glial cells and R axons within each lamina cartridge could be precisely controlled [47]. In addition to glia, larval neurons (optic lobe pioneers) could also play some role in guidance of R axons inside the optic lobe [63].

How R axons find their way inside the developing optic lobe depends to a large extent on a guidance mechanism involving the Dreadlocks (Dock) protein [58]. R axon pathfinding defects can be seen in *dock* mutants as soon as these axons enter the developing optic lobe. *dock* mutations do not seem to affect axon extension but only steering. R axons can lose their normal fasciculation and retinotopic arrangement, displaying frequent crossings between one another (see fig. 2). Gaps between axons can also be seen in the lamina, as well as R1–R6 fibers projecting beyond the lamina and towards ectopic positions in the medulla. Dock function is autonomously required in R axons where the protein localizes in the growth cone. The *dock* gene codes for an adapter protein with domains of src homology 2 and 3 (SH2 and SH3) and is homologous to human Nck [58]. Different SH domains of the protein have been shown to be differentially required by different neuronal types [64]. The Dock protein is likely to function as an integrator of tyrosine kinase signaling towards the control of the growth cone cytoskeleton [58, 64]. Although the penetrance of the phenotypes in the different *dock* mutant combinations is complete their expressivity is variable, indicating that many R axons are still able to roughly find their way inside the developing optic lobe. Moreover, although *dock* mitotic recombination clones in the retina produce strong phenotypes in the medullar projections of R7 and R8, like gaps, crossings, hyperinnervation and ectopic projections beyond the medulla, it is still possible to recognize a grossly normal retinotopy. Thus, it will be of great interest to know the molecular nature of the different *dock* alleles to confirm whether they correspond to real nulls or to leaky hypomorphs. This point will reveal if it is necessary to invoke other Dock-independent axon guidance mechanisms within the optic lobe to explain the lack of a total collapse of projections in *dock* mutants. A protein tyrosine phosphatase, dPTP61F, as well as two other phosphoproteins, which seem to interact with Dock, have been identified using the yeast two-hybrid system [65]. Other genes which are autonomously required in R1–R6 for targeting in the lamina have been identified by the mutations *limbo* and *divagary* [44].

In the lamina anlage, R1–R6 axons stop extension at their targets while R7 and R8 axons continue projecting to the medulla where they eventually stop at their respective targets. Each ommatidial fascicle is able to target to its corresponding retinotopic D/V lamina termination site independently of neighbor fascicles [43]. The same is true for the D/V retinotopic targeting of R7 and R8 in the medulla where single R7 termination sites are able to support innervation from extra R7 neurons from the same ommatidium. This is so, even when neighbor termination sites are not innervated [66]. Despite the autonomy of R axon targeting within the D/V axis, targeting in the A/P axis may involve fascicle-to-fascicle interactions [40]. A gene whose function is required in the lamina for R1–R6 targeting is *nonstop* [44].

Navigation of R7 and R8 from lamina to medulla requires the function of the CAM irreC [67, 68]. The gene *irreC* codes for a transmembrane immunoglobulin with similarity to chicken DM-GRASP/SCI/BEN. The protein is expressed by the differentiating retina and by subsets of axons in the lamina, medulla, and lobula within the optic lobes. Between the lamina and medulla, IrreC is expressed by the most recently extended axons, including R7 and R8, and from lamina monopolar neurons, being downregulated in older axons. It is not expressed by C&T neurons (fig. 2). Disruption of this IrreC pattern of expression, either by lack of expression of the protein or by its ubiquitous expression, leads to gross alterations in the projections within the first optic chiasm between the lamina and medulla. In both mutant conditions, newly projecting axons seem unable to distinguish themselves from older projected fibers and generate a grossly disorganized chiasm. Interestingly, although these axons are FasII positive and they normally do not fasciculate with C&T axons which are also FasII positive, in the mutant conditions, they follow C&T axons along their pathway (see fig. 2). This behavior suggests that IrreC helps maintain newly projecting axons as fasciculating units and independent of other FasII positive axons in the neighborhood [69]. Therefore, the lack of IrreC uncovers an alternative cellular interaction that guides axons toward the same targets but by a different route. In *irreC* mutants, giant chiasm glial cells collocalize with the abnormal projections.

Since these glial cells do not seem to express the IrreC protein, the defect suggests that giant chiasm glia follow axonal cues even though they are born before axons grow in the chiasms [70].

R axons remain arrested at their targets until 10 h APF. Then, the arrested R axons begin the exploration of their possible synaptic partners [40]. At this stage, nitric oxide (NO) signaling from R target cells is required to prevent R axons reinitiating extension and projecting beyond their target sites [71]. Cells in the lamina and medulla express a NO synthase (NOS) around 10 h APF. In the medulla, it has been shown that NOS is present in cells that collocalize with R7 and R8 axon terminals. Moreover, R growth cones express an NO-sensitive guanylate cyclase (GC) also around this time. Inhibition of NOS or GC activity leads to R axon projection beyond the medulla. Occurrence of these phenotypes can be prevented by providing a cGMP analog along with the inhibitor. Thus, NO released by target cells in the optic lobe seems to signal R growth cones causing a rise in cGMP through the activity of a specific GC [71]. An additional role for cGMP in regulation of growth cone steering has been recently propossed based on in vitro experiments [72].

Although R axon extension can occur in the absence of target cells, survival of retinal neurons depends on them. In the absence of these cells, R axons can form projections but eventually degenerate, indicating the existence of trophic input from their target cells [49, 73, 74].

Photo- and mechanoreceptors of the ocellar sensory system

Ocelli in the dorsal head of the adult develop from the eye-antenna imaginal discs. Left and right ocelli differentiate from the corresponding left and right eye-antenna imaginal discs, whereas the medial ocellus derives equally from both imaginal discs. The adult dorsal head of *Drosophila* also bears mechanosensory bristles arranged in a stereotyped fashion around the ocelli. Ocelli and bristles project axons with divergent trajectories towards different targets in the brain. In the adult, ocelli project away from the epidermal surface toward the ocellar ganglion beneath the surface of the head. In contrast, bristle axons initially project following the contour of the head epidermis (fig. 4). The basis of this different pathway selection by ocellar and bristle axons is laid down during early pupal life [23].

After puparium formation, a transient population of ocellar pioneer (OP) neurons differentiate and extend axons in four fascicles along the prospective internal side of the dorsal head. At these stages of pupal development (prepupa and cryptocephalic pupa), the head capsule still has an inside-out configuration and is invaginated inside the thorax (see fig. 4). The brain is located just posterior to the head capsule. OP axons in each fascicle (roughly 50) associate tightly with each other and travel in the ECM straight along and close to the underlaying epithelial surface but without contacting it. At these stages, no glial cells seem to be associated with the OP fascicles. Axons from the bristles also start extending at these stages. They follow the contour of the epithelium with a meandering trajectory. During head eversion (12 h APF), the head capsule pops out of the thorax and the brain moves inside it. Thus, the topological configuration of the three structures changes dramatically to attain the adult arrangement. The head is now anterior to the thorax and bears the brain inside. The OP fascicles, which are not attached to the epidermis, now become perpendicular to the internal head surface and to the dorsal brain. Bristle axons, in contrast, continue attached to the inside of the epidermal surface and follow its contour (fig. 4). Thus, the choice of OP axons to project in the ECM versus the choice of bristle axons to follow the epidermis is key to attain the adult configuration after head eversion.

OP axons require laminin A in the ECM to project normally. Partial loss-of-function mutations in the *lamA* gene (study of the null condition is complicated by the non-autonomous nature of laminin function [75]) cause dramatic phenotypes in OP axon pathfinding. OP axons attach to the epithelium in *lamA* mutants and either stall close to the OP cell bodies, or fasciculate and follow the bristle axons for some distance (eventually stalling as well). The association of OP axons with the epithelium in *lamA* mutants is better visualized after head eversion, when the abnormally projecting OP axons can be found following the contour of the head towards the antenna (see fig. 4).

In the wild-type, OP axons choose to project detached from the underlaying epithelium although they have access to both substrata, ECM and epithelium. In *lamA* mutants, OP axons project attached to the epithelial cell surfaces, although the ECM is obviously accessible to their growth cones. This suggests that LamA somehow inhibits OP axons from adhering to the epithelial surface. Therefore, either LamA (or another molecule that requires LamA to be functional) binds to the same OP receptor(s) as those used to adhere to the epithelial cells or LamA signals the OP axons to grow independently of the epithelium. The choice of OP axons for ECM instead of epithelium is an absolute requirement to attain a normal projection. The stalling of OP axons when attaching to the epidermis in *lamA* mutants suggests either that LamA is required for OP axon growth or that the epidermis has an inhibitory influence over OP axon extension (see below). Bristle axons seem to extend normally in *lamA* partial loss-of-function muta-

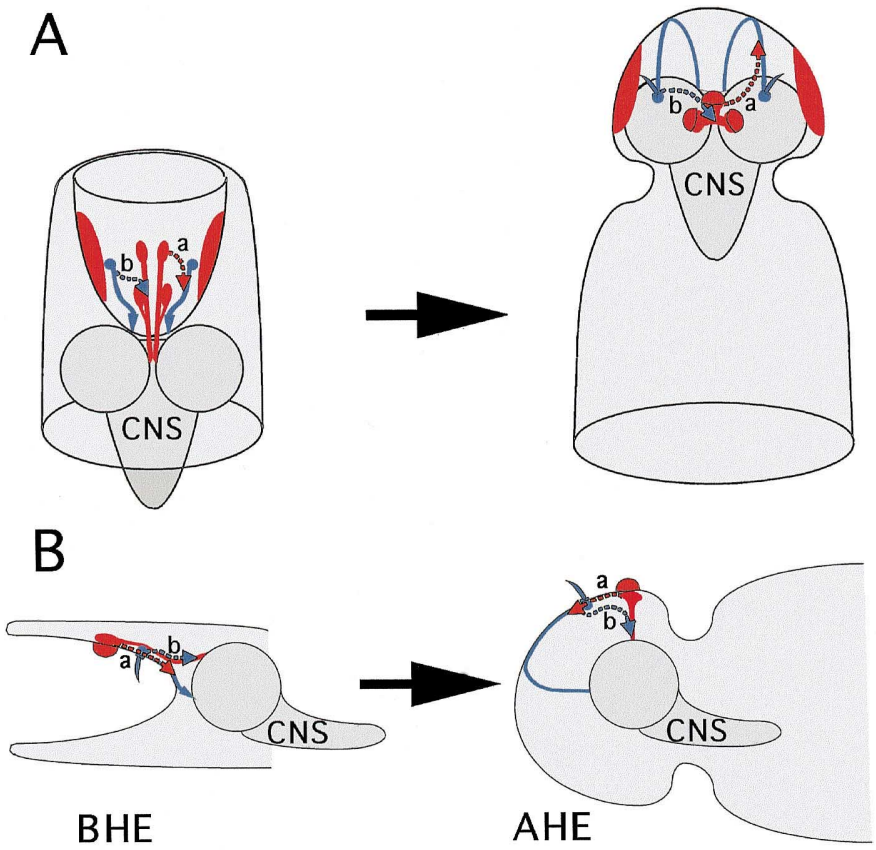

Figure 4. Ocellar pioneer and bristle axon projections in the developing adult head. Two different planes of view are drawn: horizontal, anterior is up (*A*), lateral, anterior is left (*B*). Two classes of phenotypes are represented before (BHE) and after head eversion (AHE) by small letters: (a) ocellar pioneer axons attach to the underlaying epithelium and project along bristle axons in *lamA* and some *nrt* mutant conditions; (b) bristle axons detach from the epidermis and project along the ocellar pioneer nerve in ectopic expression conditions for *nrt*. See text for further details. Red: ocellar pioneers and compound eye; blue: bristle cells.

tions, either because this molecule does not have any influence over the guidance of these axons or because the hypomorphic nature of these alleles is not enough to reveal a requirement.

Another molecule involved in OP axon extension is neurotactin (Nrt) [21]. Nrt is expressed by OP axons but not by bristle axons or epidermis. *nrt* null mutants show a high penetrance of defasciculation of the OP axon bundles. In addition, *nrt* null mutants exhibit, with a very low penetrance, OP axons attached to the epidermis where they show the same tendency to fasciculate with the bristle axons and stall as in the case of *lamA* mutants. The penetrance of this phenotype is low even in the *nrt* null and may represent an indirect consequence of the highly penetrant defasciculation phenotype. This could be the case if guidance of individual OP axons within the ECM were not a completely efficient process. This, in turn, might also help explain why there are so many pioneers. Coupling the guidance of roughly 50 axons simultaneously may serve to build a robust process. Selective fasciculation and directional growth cone guidance seem to be separable processes in many experimental situations [76]. However, in the OP case, selective fasciculation may be involved in directional guidance.

The fact that Nrt can mediate directional guidance is revealed in its ectopic expression condition. Expression of Nrt in bristle axons makes them abandon their normal pathway of extension and follow the OP axons [21] (see fig. 4). Ectopic expression of Nrt in the head epithelium causes OP axons to adhere to the epidermis (a lamA-like phenotype). Again, OP axons attached to the epidermis eventually stall. Since the association of OP axons with the epidermis would be mediated by Nrt itself, this result indicates that the epidermis has a specific inhibitory influence over OP axon extension.

After head eversion, glial cells become associated with and enwrap the four OP fascicles. The OP axons undergo degeneration around the end of the second day of pupal life. These axons are replaced by those of around 12 giant interneurons that grow from the brain along the OP nerve [23]. The adult photoreceptors are also born around this time. Thus, the OP nerve serves as a bridge between the ocelli and the brain for the growth of the giant interneuron axons. The OP bridge appears when the relative positions of the head capsule and the brain are perhaps more favorable for axon extension. At later stages, after head eversion, when the adult photoreceptors and the giant interneurons extend axons, there might not be another physical substrate suitable to cross from ocelli to brain in the absence of the OP nerve. Indeed, in the absence of this pioneer nerve (as in *lamA* mutants), giant interneuron axons are not found between the ocelli and brain which remain unconnected [23].

Summary

Studies on the wing and eye show that sensory axons can use local, short-range cues in the epithelium or ECM to project along the imaginal discs. Less extensively investigated sensory systems like the antenna, leg or the ocellar system also produced results compatible with this idea. Of course, this does not imply that during normal development, long-range diffusible cues do not play any role in guidance. In fact, both long-range diffusible and short-range (probably contact) cues are likely to operate simultaneously in many instances. For example, in the grasshopper limb bud, axon guidance depends on both contact and diffusible signals [77]. The presence of long-range diffusible cues can also help explain cases in which axons can accomodate to dramatic perturbations. For example, in the moth, experimentally misrouted olfactory axons can find their way to their targets in the CNS from distant ectopic locations [78].

Larval nerves (eye, leg, antenna) have been shown not to be essential for sensory axon guidance within the imaginal discs. Then, either axons do not rely on them as guiding substrate, or growth cones can accommodate to the perturbation caused by their loss by using other alternative substrates. Thus, sensory axons from the legs normally project along the corresponding larval sensory nerves. However, in their absence, sensory axons can project along the imaginal disc epithelium. Thus, the absence of the normal guidance substrate uncovers the presence of alternatives for guidance. During the formation of PNS nerves in the embryo, a similar scenario has been reported [79]. In the first optic chiasm, the lack of IrreC in the newly projecting axons causes R and lamina axons to fasciculate with C&T axons and follow their course reaching, nevertheless, the correct targets (albeit through an abnormal pathway). In all these cases, alternative substrates seem to function for the guidance of axons to their correct targets. Similar scenarios occur in other organisms, such as the cockroach developing limb [80].

In the majority of cases, in the embryonic or adult PNS, the basement membrane, the epidermis, the mesoderm, and the trachea are arranged in relative configurations that do not change over time and could be used indifferently to guide axons towards the same target. Therefore, axons may have evolved to use multiple and/or alternative cues within these substrates. In experimental conditions, mutations which alter the guidance in one substrate may cause an association with another substrate but the phenotype might be difficult to see due to the arrangement of the tissues. For example, a change of substratum for extension by the sensory axons of the wing from the ECM to attachment to the epidermis would be very difficult to visualize. In this respect, the phenomenology in the ocellar sensory system is illuminating. The OP axons follow an alternative substrate in the absence of the normal cues within the ECM; however, in this case, the OP axons do not reach their normal targets. Since the process of head eversion changes the relative configuration of epidermis, ECM, and CNS after the OP pathway has been formed, there is no possible redundancy as substrate between epithelium and ECM in this case. On the other hand, head eversion also helps reveal OP axon phenotypes which would have been much more difficult to see if the original arrangement of tissues and structures had remained unchanged. Indeed, it is difficult to distinguish whether or not OP axons are attached to the epidermis before head eversion but very easy once head eversion has taken place.

Even though it is clear that larval nerves are not essential for sensory axon guidance within the imaginal discs, these nerves can have an essential role as bridges from the appendages to the CNS. The case of the compound eye is special. Since the bridge function between the imaginal disc and the CNS is fulfilled by the OS, the larval optic nerve may have become dispensable. In the ocellar sensory system there is no larval nerve; however, a transient population of pioneer neurons forms the bridge between the head capsule and the brain when the configuration of tissues seems most favorable for axon extension. Once within the CNS, ingrowing adult sensory axons seem to use persisting larval axon tracts as cues in some cases while they could pioneer new pathways in others.

It is possible that much of the canalization during growth cone guidance could be the result of growth cone regulative capacities. Since axons seem able to use

multiple cues simultaneously to increase the accuracy of the guidance process, it seems also possible that the growth cone could regulate for a more efficient use of the remaining cues (in the normal or an alternative substrate) in the absence of one of them. If this is so, the different mechanisms functioning in growth cone guidance would not operate as independent units, but would be linked by cross-regulatory interactions. The regulatory capacities that help correct axon projection abnormalities (as in the reported case of wing margin sensory axons in cell polarity mutants [81]) could be based on the use of the same cross-regulatory interactions. Indeed, even during normal development, errors in growth cone guidance are made which are subsequently corrected [82].

Axon guidance in the adult PNS of *Drosophila* shows the same remarkable degree of flexibility displayed in other developmental stages and organisms. It is a general theme from insects to vertebrates that pathfinding axons can often accommodate to perturbation (genetic or enviromental) and generate normal projections. This behavior has lead to the realization that axons extending on their natural substrate simultaneously use multiple cues for pathfinding. In addition, in perturbed situations, axons can often use alternative substrates to reach their normal targets. Finally, the existence of error correction mechanisms during axon guidance suggests, additionally, the presence of molecular regulatory interactions that help check the correctness of projections and might strongly contribute to generate the high canalization shown by pathfinding axons.

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