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Genetic analysis of *Laminin A* in *Drosophila*: extracellular matrix containing laminin A is required for ocellar axon pathfinding

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SUMMARY

Genetic analysis of the *Laminin A* (*LamA*) gene in *Drosophila* reveals that distinct classes of sensory axons have different requirements for extracellular matrix (ECM) containing laminin A versus epithelial cell surfaces. In the eye-antenna imaginal disc, the nerve from the three simple eyes (ocelli) to the brain is pioneered by a population of transient ocellar neurons whose axons extend on an ECM that covers and connects the disc epithelium and brain. Axons from neighboring mechanosensory (bristle) neurons extend under the ECM in direct contact with the surface of the disc cells, and pioneer a different axon pathway that enters the brain in a different location. In *LamA* mutants, the ocellar pioneer axons display striking pathfinding defects, while neighboring bristle axons appear normal; the ocellar pioneers usually extend in the proper direction, adhering to the epithelium and sometimes fasciculating with mechanosensory axons, but they invariably fail to reach the brain.

Key words: *Drosophila*, *Laminin A*, extracellular matrix, ocellar axons, growth cone guidance, axon pathfinding, pioneer neurons

INTRODUCTION

Laminin, a multisubunit glycoprotein complex of the extracellular matrix (ECM), has been shown to be a potent promoter and substratum for neurite outgrowth in vitro (reviewed by Timpl and Brown, 1994; e.g., Gomez and Letourneau, 1994; Kuhn et al., 1995). These in vitro results, coupled with the abundance of laminin in regions of axon pathway formation (e.g., Rogers et al., 1986; McLoon et al., 1988; Letourneau et al., 1988), have led to the hypothesis that neurons in vivo use laminin as a neurite outgrowth-promoting substratum (e.g. reviews by Sanes, 1989; Bixby and Harris, 1991; Reichardt and Tomaselli, 1991; Hynes and Lander, 1992).

Although there is a great wealth of knowledge about the function of laminin in vitro, and its expression in vivo, little is actually known about the function of laminin in growth cone extension and guidance in the developing organism. For example, does laminin itself, or other components of the ECM that bind to laminin, provide a necessary substratum for growth cones? Moreover, do all peripheral growth cones use laminin, or ECM containing laminin, as a primary substratum for axon pathfinding, or do only a subset of growth cones rely on this ECM substratum?

Compounding the problem of laminin function in vivo is the discovery that laminin in vertebrates is not a single molecular complex, but rather a family of at least seven different complexes using alternative subunits (at least 3 αs, 3 βs, and 2 γs; see Burgeson et al., 1994, for revised vertebrate nomenclature) interacting with a variety of other ECM molecules. The best characterized form in vertebrates, laminin 1, exists as a heterotrimer of subunits α1 (400×10^3 M_r; formerly A), β1(220×10^3 M_r; B1), and γ1(200×10^3 M_r; B2). Other laminin complexes use different subunits (e.g., Engvall et al., 1990) including, for example, β2 (S-laminin; Hunter et al., 1989), a subunit enriched at synaptic junctions, and α2 (merosin; Ehrig et al., 1990), a subunit enriched in striated muscle and peripheral nerve. The different forms of laminin play different roles for different cell types in terms of adhesion versus anti-adhesion (Calof and Lander, 1991) and growth-promoting versus stop signals for neuronal growth cones (Hunter et al., 1989, 1992; Porter et al., 1995). In mouse, a mutation in the gene encoding α2 (merosin) results in a form of muscular dystrophy (Xu et al., 1994), while a mutation in the gene encoding β2 (S-laminin) leads to defects at the neuromuscular junction (Noakes et al., 1995).

To begin to unravel the in vivo functions of laminin during growth cone guidance, we have taken a genetic approach using *Drosophila*. Insects have ECM and basement membranes with laminin and other components similar to those in vertebrates (Blumberg et al., 1988; Fessler et al., 1987; Mirre et al., 1988; Montell and Goodman, 1988). The ECM is associated with the formation of some PNS axon pathways (e.g. Nardi, 1983; Berlot and Goodman, 1984; Anderson and Tucker, 1988; Condic and Bentley, 1989).

Thus far, only one form of laminin has been extensively characterized in *Drosophila*. Therefore, in this paper, we

continue to use the A, B1 and B2 nomenclature. As a first step toward a genetic analysis of laminin function in *Drosophila*, we previously cloned the three genes encoding the B1, B2 and A subunits in *Drosophila* (Montell and Goodman, 1988). We next characterized their sequence and expression (Montell and Goodman, 1988, 1989; Henchcliffe et al., 1993), as did others (Chi and Hui, 1989; Kusche-Gullberg et al., 1992; MacKrell et al., 1993), and then went on to generate and characterize mutations in the *Laminin A* (*LamA*) gene (Henchcliffe et al., 1993). Here we use the analysis of these different *LamA* mutant alleles to reveal the function of ECM containing laminin A in axon pathfinding. The same mutants have been used to analyze laminin A function in the development of heart, somatic muscle and gut (Yarnitzky and Volk, 1995).

Two different classes of recessive *LamA* mutations were generated (Henchcliffe et al., 1993). The first class comprises null alleles that are embryonic lethal. The second class comprises hypomorphic (partial loss-of-function) alleles that, in combination with alleles of either the first or second class, can give rise to viable pupae and some viable adults (called escapers). These adult escapers show abnormalities in the shape of legs and in the organization of ommatidia in the compound eye (Henchcliffe et al., 1993). In accordance, laminin A is strongly expressed in basement membranes covering most organs, including the imaginal discs that generate a large part of adult structures (Fessler et al., 1987; Montell and Goodman, 1989; Kusche-Gullberg et al., 1992).

In the present study, we examined pathfinding by both photoreceptor and mechanosensory axons as they extend from the eye-antenna imaginal disc to the brain in *LamA* mutant larvae and pupae. Null conditions of *LamA* are embryonic lethal (without obvious pathfinding defects). Laminin A function is non-cell autonomous, precluding the generation of a null condition in mosaic territories of imaginal discs (Henchcliffe et al., 1993). Thus, we have relied on the study of hypomorphic conditions that produce adult escapers. We focused our analysis on the larval and pupal development of the axons that pioneer pathways from the head to the brain for different types of sensory neurons. Our results indicate that ECM containing laminin A is directly required for normal axon pathfinding by ocellar pioneer axons. In contrast, axons from neighboring mechanosensory neurons extend along the epithelial surface and appear not to require an ECM containing laminin A.

MATERIALS AND METHODS

Genetics

The different classes of *LamA* mutant alleles have been previously described (Henchcliffe et al., 1993). Mutant chromosomes were balanced with the balancer *TM6B*, which carries the dominant *Tubby* (*Tb*) mutation that allows us to recognize heterozygous *TM6B* larvae and pupae (Lindsley and Zinn, 1992). All flies were raised at 25°C.

Staging

Pupae were staged as either before or after head eversion based on the appearance of the larval mouth hooks. The period before the larval-pupal apolysis (at around 4 to 6 hours after puparium formation) is called 'pre-pupal', but, for simplicity, we have referred to all stages within the pupal case as being pupal. Pupae with everted mouth hooks have proceeded through the head eversion stage (around 12 to 13 hours after puparium formation; Bainbridge and Bownes, 1981). Pupae with mouth hooks everted were sorted as either before or after expansion and folding of their wings. Pupae in the stage after head eversion and before wing expansion have unpigmented eyes and proceed through the pupal-adult apolysis (roughly 28 to 30 hour after puparium formation). Whether the molt has occurred is easily distinguished by the appearance of a space between the cuticle and the body.

Staining

Larvae were cut about 2/3 of their length in 4% paraformaldehyde.

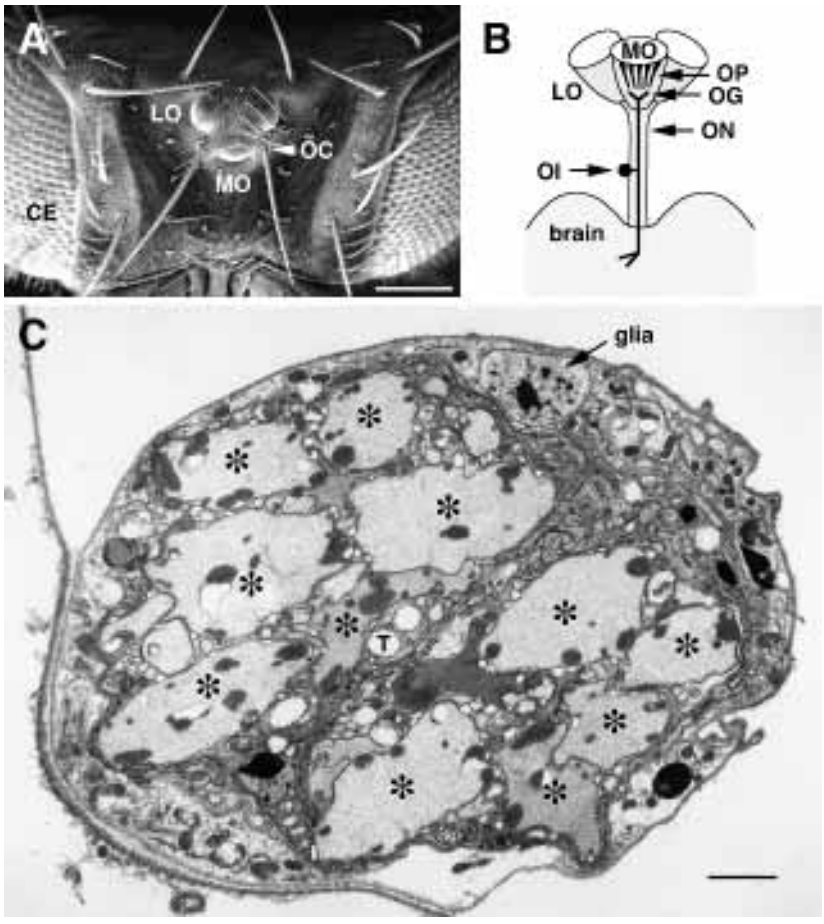

Fig. 1. The three ocelli and ocellar nerve of the adult fruitfly. (A) Scanning electron micrograph showing a dorsal view of the adult head, with the two large compound eyes (CE), the three ocelli, including the two lateral ocelli (LO) and the median ocellus (MO), and the various large bristles, including the ocellar (OC) bristles near the MO. Scale bar: 100 μm. (B) Schematic diagram showing the three ocelli, the ocellar nerve (ON) and the brain. The ocellar photoreceptors (OP) synapse on the dendrites of the ocellar interneurons (OI) in the ocellar ganglion (OG). (C) Transmission electron micrograph showing a cross section of the adult ocellar nerve. The nerve shown here is surrounded by glia, includes tracheal (T) branches, and contains 11 large diameter axons (asterisks) from the giant ocellar interneurons, and a small number of medium and small diameter axons of unknown original. Scale bar, 2 μm.

After fixation overnight at 4°C, a piece containing the CNS, eye-antenna imaginal discs, and mouth hooks was dissected. Pupae were fixed in 4% paraformaldehyde for 24 hours. To allow penetration of the fixative, a hole was made through the pupal case and body. Head capsules with attached CNS from pupae before head eversion were dissected by cutting the epidermis connecting the thorax and the head capsule contained within it. Heads of pupae after head eversion and pupal-adult apolysis were cut and the pupal cuticle peeled off. Fixed and dissected pieces from the different stages were washed several times in PBT and incubated for 1 hour in 5% normal goat serum in PBT. Antibodies were added at 1:100 (mAb 24B10) and 1:5 (mAb 22C10), incubated overnight at 4°C, and washed with PBT for 2 hours at 25°C. Normal goat serum in PBT (5%) was added for 1 hour. An HRP-conjugated secondary antibody was used at 1:300, incubated at room temperature for 2 hours and washed with PBT for 2 hours. The pieces of tissue were incubated for 5 minutes in diaminobenzidine/PBT (0.7 mg/ml) and reacted with hydrogen peroxide (0.003%). After several washes with PBS, tissues were mounted in 50% glycerol/PBS or Aqua-Mount (Lerner Laboratories).

BrdU injections and staining

Labeling of adult ocellar photoreceptors with BrdU was carried out using the protocol of Truman and Bate (1988) with modifications. Pupae of stages P5 and P6 (12 to 46 hours after puparium formation) were recognized by an everted head and the lack of eye pigmentation. They were brushed with 70% ethanol to clean and sterilize the pupal case. Pupae were injected with a BrdU solution in injection buffer (1 mg/ml). We used a microinjection needle to deliver approximately 0.1 or 0.2 μl to each pupae, injecting at roughly the position of the posterior thorax or first abdominal segment. Injected pupae were allowed to develop in an humidified atmosphere at 25°C until eclosion or complete pigmentation of the cuticle was evident. Adults or terminal pupae were fixed in 4% paraformaldehyde as above. Once fixed, they were removed from the pupal case and a small piece of cuticle bearing the ocelli was dissected. Pieces of cuticle with ocelli were washed with PBT, treated with 2 N HCl in PBT for 30 minutes, washed several times with PBT and then processed like the younger pupae for antibody staining. Signal was visualized using anti-BrdU antibody (Becton-Dickinson) at 1:200 and the Elite ABC kit (Vector laboratories) plus Ni.

Electron microscopy

Third instar larvae, or pupae before head eversion, were opened and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hour. After rinsing with buffer, the brains and attached eye-antennal discs were removed, and further processed, sectioned and observed by EM as previously described (Auld et al., 1995).

RESULTS

The ocellar nerve is prefigured by a scaffold of pioneer axons

In addition to the pair of large compound eyes, adult fruitflies (like most insects) have three simple eyes (ocelli) located near the midline on the dorsal surface of the head (Fig. 1A). In *Drosophila*, the left and right ocelli derive from the left and right eye-antenna imaginal discs, while the median ocellus derives equally from both discs once they fuse together after puparium formation (Fig. 2A).

In the adult, the ocellar photoreceptors (~80 per ocellus) have short axons that synapse on the dendrites of the ocellar giant interneurons (~4 per ocellus) in the neuropil of the ocellar ganglion, which lies just below the ocelli. The axons of the ocellar giant interneurons project to the brain in the ocellar nerve, which in the adult, contains ~12 giant interneuron axons

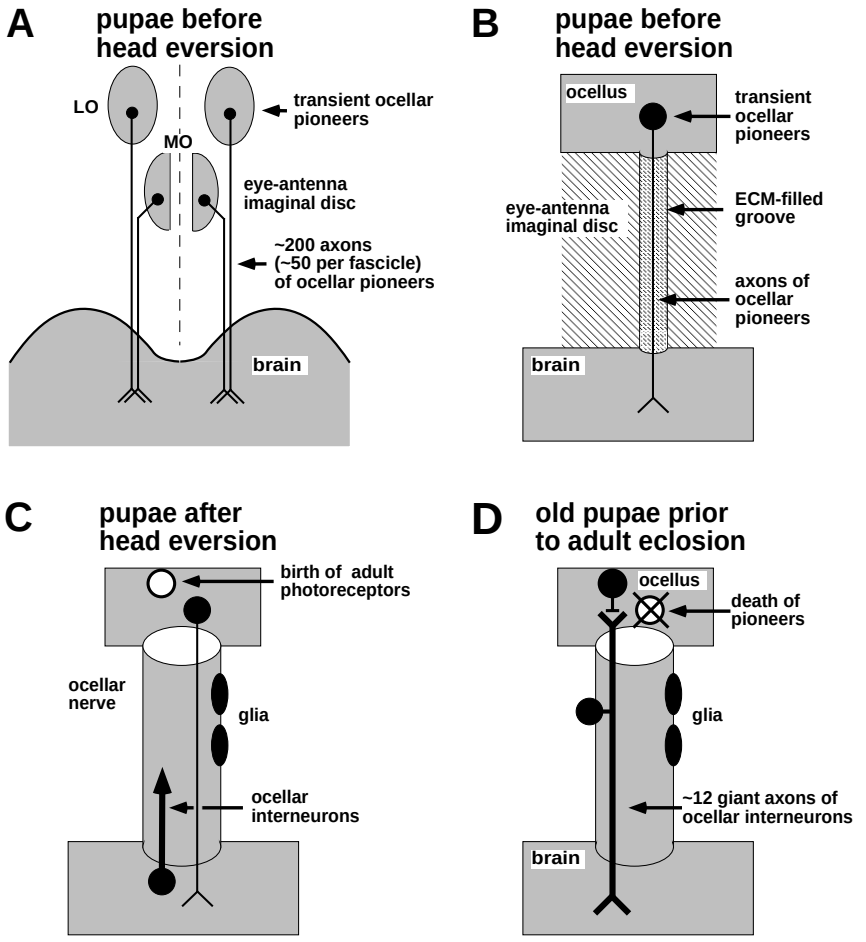

Fig. 2. The axons from a transient population of ocellar neurons pioneer the ocellar nerve. Schematic diagrams showing the development of the ocellar nerve (A,B) in pupae before head eversion (which occurs at 12-13 hours), (C) after head eversion in 30 hour pupae and (D) in 72 hour or older pupae prior to adult eclosion. In the adult (similar to D; see Fig. 1), the ocellar photoreceptors (~80 per ocellus) have short axons that synapse on the dendrites of giant interneurons (4 per ocellus) in the neuropil of the ocellar ganglion which lies just below the epidermis and photoreceptors; the axons of the ocellar interneurons project to the brain. In the adult, the ocellar nerve contains ~12 giant interneuron axons (11 giant axons are seen in the example shown in Fig. 1C). This nerve is pioneered during pupal development by a different, transient population of axons. The single ocellar nerve begins before head eversion (A,B) as a set of four separate axon fascicles that are each initially pioneered by the axons of a transient population of ~50 ocellar pioneer neurons that project from the ocelli to brain. The axons of the giant ocellar interneurons extend out the nerve pathway from the brain to the ocelli much later (C). The adult photoreceptors are born and differentiate, and the ocellar pioneers eventually die (D). By the time of adult eclosion, the ~200 ocellar pioneer axons are gone, and only the ~12 giant axons of the ocellar interneurons remain in the ocellar nerve.

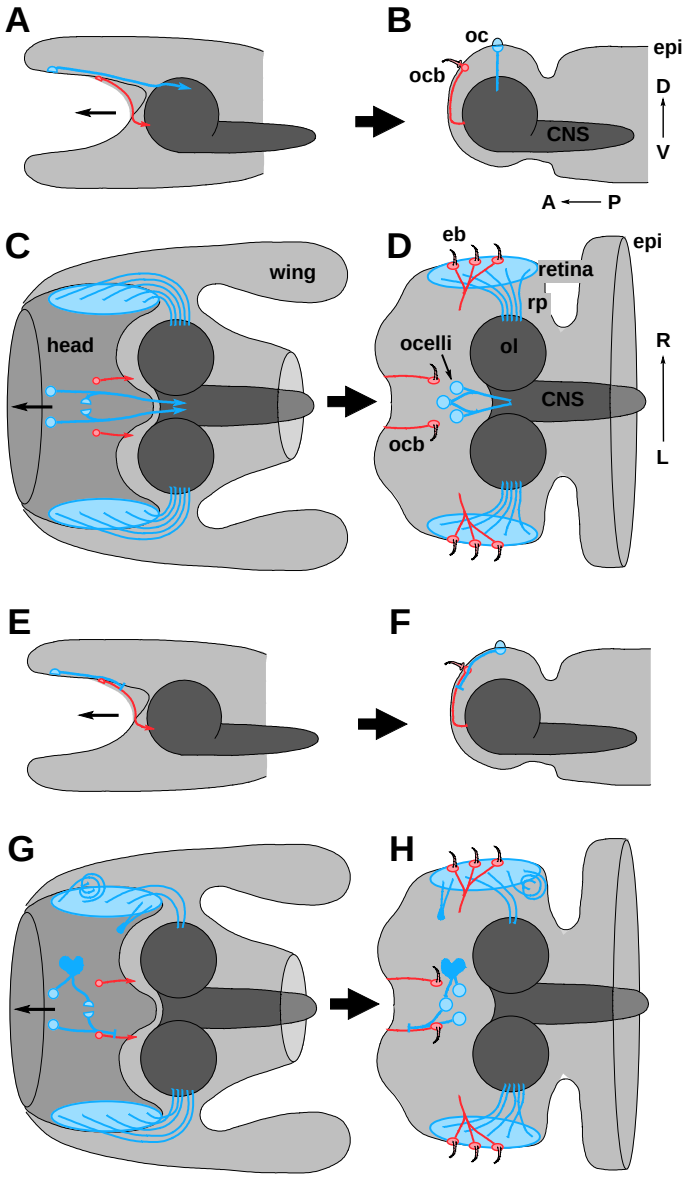

Fig. 3. Development of ocellar pioneer and mechanosensory axons in the *Drosophila* head. Development of the photoreceptor axon pathways from the three simple eyes called ocelli (oc), and from the retina of the two large compound eyes, as compared to mechanosensory axon pathways from the ocellar bristles (ocb) and eye bristles (eb), in wild-type and *LamA* mutant pupae. These schematic diagrams show side views (from the left side; A,B,E,F) and dorsal views (from above; C,D,G,H) of wild-type (A-D) and *LamA* mutant (E-H) pupal heads before (A,C,E,G) or after (B,D,F,H) head eversion. Antenna and other structures of the head capsule have been omitted for clarity. During the initial stages of pupal development, the fused eye-antenna imaginal discs (labeled head) are anterior to the brain, have an inside-out configuration, and are inside the thoracic capsule (part labeled wing). During head eversion, the head capsule pops out of the thorax (in the direction of the arrows in A,C) and the brain comes to sit inside. (A,C) Before head eversion, the ocellar pioneer axons normally project posteriorly, straight towards the dorsal surface of the medial region of the brain. The ocellar pioneer axons tightly fasciculate with one another, forming two bundles each with two fascicles. Each fascicle is derived from either the left or the right ocellus or from the left or right halves of the median ocellus. The bristle axons, on the other hand, extend posteriorly but follow the contour of the epidermis. During larval development, the photoreceptor axons from the retina of the compound eye have begun to project inside the optic stalk (in pupae, called the retinal projection or rp) towards the optic lobe (ol) of the brain. (B,D) After head eversion, the ocellar nerve projects across the head capsule to a dorsal region of the brain, whereas the bristle nerve projects along the epidermis of the head and then to a more ventral region of the brain. (E, F, G, H) *LamA* mutant pupae display defects in pathfinding by photoreceptor axons; in contrast, neighboring mechanosensory axons usually appear normal. In *LamA* mutants, the ocellar pioneer axons instead extend a short distance along the epidermis, often stalling, sometimes fasciculating with mechanosensory axons, but typically failing to reach the brain. Similarly, in *LamA* mutants, photoreceptor axons of the compound eye often form aberrant whirls, in many cases do not properly enter the optic stalk, and in some cases extend into inappropriate regions of the epidermis.

and very few other axons (Fig. 1C) (Strausfield, 1976; Stark et al., 1989). While, in some insects such as grasshopper, the cell bodies of the ocellar interneurons are located within the brain (Goodman, 1974, 1976), in Dipterans, these cell bodies lie along the peripheral nerve (Strausfield, 1976).

We observed that the ocellar nerve frequently does not form in *LamA* mutants. In order to determine the cause of this phenotype, we examined which axons pioneer this nerve, and how they behave in *LamA* mutants. We discovered that, although the ocellar nerve in the adult contains ~12 giant axons of the ocellar interneurons, these axons are followers and not pioneers. Rather, the ocellar nerve is pioneered by ~200 axons from a transient population of ocellar pioneers that appear around puparium formation and die prior to adult eclosion (Fig. 2B-D). The single ocellar nerve in the adult begins in the pupae as a set of four separate axon fascicles that are pioneered by the axons from four separate transient populations of ~50 ocellar pioneer neurons that project to the brain (Fig. 2A). At a later pupal stage, the adult ocellar photoreceptors are born and differentiate concentrically outside the cluster of pioneers, and the pioneers then die (Fig. 2C). The axons of the giant ocellar interneurons extend out along the nerve pathway from the brain to the ocelli (Fig. 2C). The cell bodies of the giant ocellar interneurons appear to be born in the brain and migrate out along the ocellar nerve. A few of these features of ocellar nerve development have been reported previously for grasshopper and cockroach (Mobbs, 1976, 1979; Toh and Yokohari, 1988).

After the formation of the puparium, major morphogenetic movements occur (Fig. 3A-D). Imaginal discs evaginate, spread and fuse to form the adult epidermis. During the initial stages after puparium formation (prepupa and pupa prior to head eversion), the eye-antenna imaginal discs are anterior to the brain, fused to each other medially and have an inside-out configuration (Fig. 3A,C). At these stages prior to head eversion, both the fused eye-antenna imaginal discs and the brain are inside the thoracic capsule. The first axons start projecting from the four ocellar rudiments (Fig. 2A) just after the eye-antenna imaginal discs fuse. These axons project posteriorly on top of the fused discs and towards the brain. The projection between ocelli and brain is established well before head eversion occurs. These ocellar pioneers express the photoreceptor-specific epitope recognized by mAb 24B10 and the

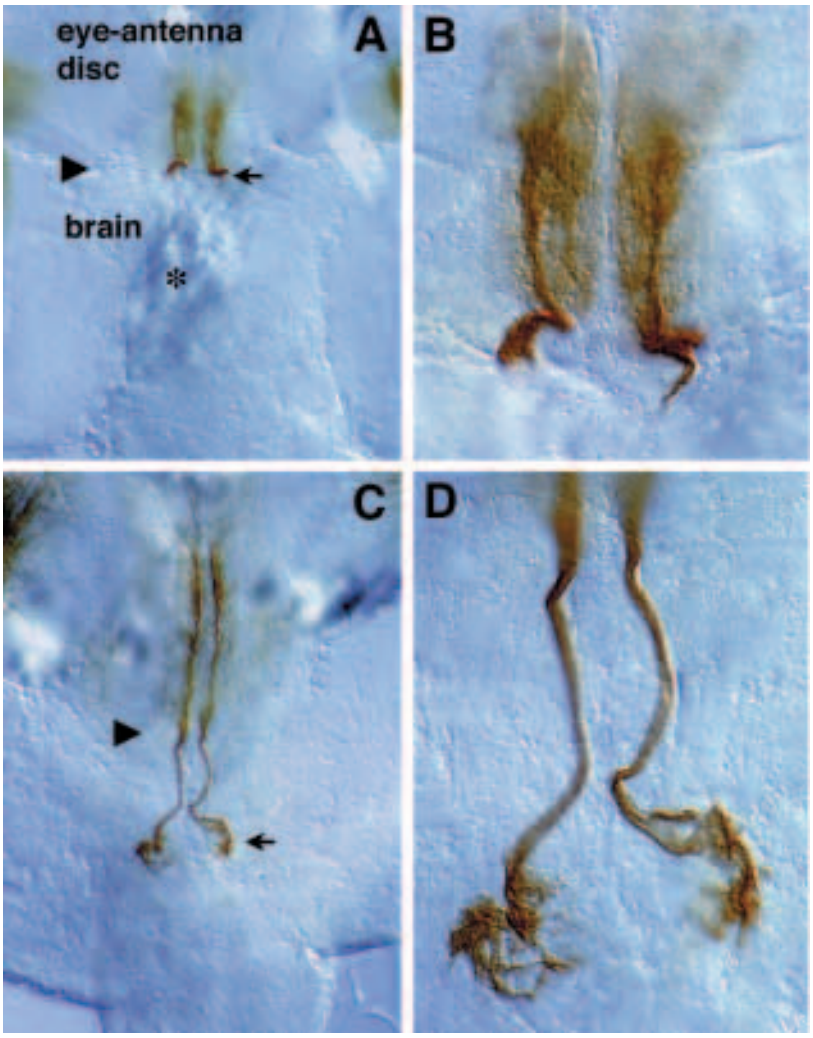

Fig. 4. Pathfinding by ocellar pioneer axons in wild-type pupae. Axons of the ocellar pioneers extend and arborize in their target regions in the brain before head eversion at 13 hours of pupal development. Staining with mAb 24B10 (anti-chaoptin) reveals that in wild-type pupae, two axon bundles composed of axons from the right or left ocellus plus half of the median ocellus pioneer the two ocellar nerve pathways towards the dorsal surface of the brain. EM analysis reveals that within each bundle, the groups of axons from the lateral versus median ocellus are in separate fascicles (see Fig. 6B). (A,B) In wild type, ocellar axons (arrow) have reached the ridge between the disc (future head capsule epidermis) and the brain. The asterisk shows the location on the dorsal surface of the brain where these axons will ultimately project in wild-type pupae. The arrowhead marks the boundary between the fused eye-antenna imaginal disc and the brain; (B) close-up of A. (C,D) Later in wild-type pupae, the ocellar axons have reached their target region in the brain (asterisk in A); (D) close-up of the arborization region in C. As in A, the arrowhead marks the boundary of the brain.

epitope recognized by mAb 22C10 (Zipursky et al., 1984) (Fig. 4).

In wild-type pupae, the ocellar pioneer axons project posteriorly towards the dorsal surface of the medial region of the brain connecting the two brain lobes (Figs 2A, 3A,C). These axons fasciculate with one another, forming a left and right nerve of two fascicles each. Of the four fascicles, one each is derived from the left or right ocellus, and one each from either the left or right rudiments of the median ocellus. The ocellar pioneer axons extend towards the brain along a non-cellular substratum. Electron microscopy (EM) analysis shows that these axons extend along the ECM without contacting the underlying epidermal cell surfaces (Figs 5A,B, 6A). For most of their journey along the fused disc, they extend along the ECM in a groove in the epithelium that forms just opposite a tracheal branch (Fig. 5A,B); the groove precedes the pioneer growth cones (Fig. 5A).

During the same stages (pupae before head eversion), the axons of neighboring mechanosensory neurons in the fused eye-antenna imaginal discs (e.g., from the ocellar and orbital bristles) also extend posteriorly, but they do not grow on the ECM in the groove, but rather they grow in direct contact with the cell surfaces of the disc epithelium and follow an epithelial pathway to a more ventral region of the brain.

Well after the ocellar pioneer axons have finished projecting into the brain, head eversion occurs, which brings the brain inside the head capsule, and the head anterior and out of the thorax (Fig. 3B,D). Before head eversion, the ocellar pioneer axons are apposed to the ECM along the internal side of the prospective head epidermis. During head eversion, the head capsule pops out of the thorax and the brain comes to lie inside. This movement displaces the ocellar pioneer projections from their initial position parallel to the epidermis to the final position of the ocellar nerve perpendicular to it (Fig. 3A,B), and thus no longer in contact with any physical substratum.

Before head eversion, EM analysis shows no glia around the ocellar axons (Figs 5A,B, 6A). Well after head eversion in 30 hour pupae, the ocellar nerve still contains four fascicles of ocellar pioneer axons but they are now surrounded by two sheets of glial cells, an inner sheath around the individual axon fascicles and the outer one around the entire nerve (Fig. 6B).

In contrast to the projections of the ocellar pioneers, the neighboring mechanosensory axons remain attached to the inside surface of the epidermis after head eversion (data not shown) and thus, in later pupal and adult stages, these axons follow the contour of the head epidermis (Fig. 3A,B).

Beginning at ~48 hours after puparium formation, the

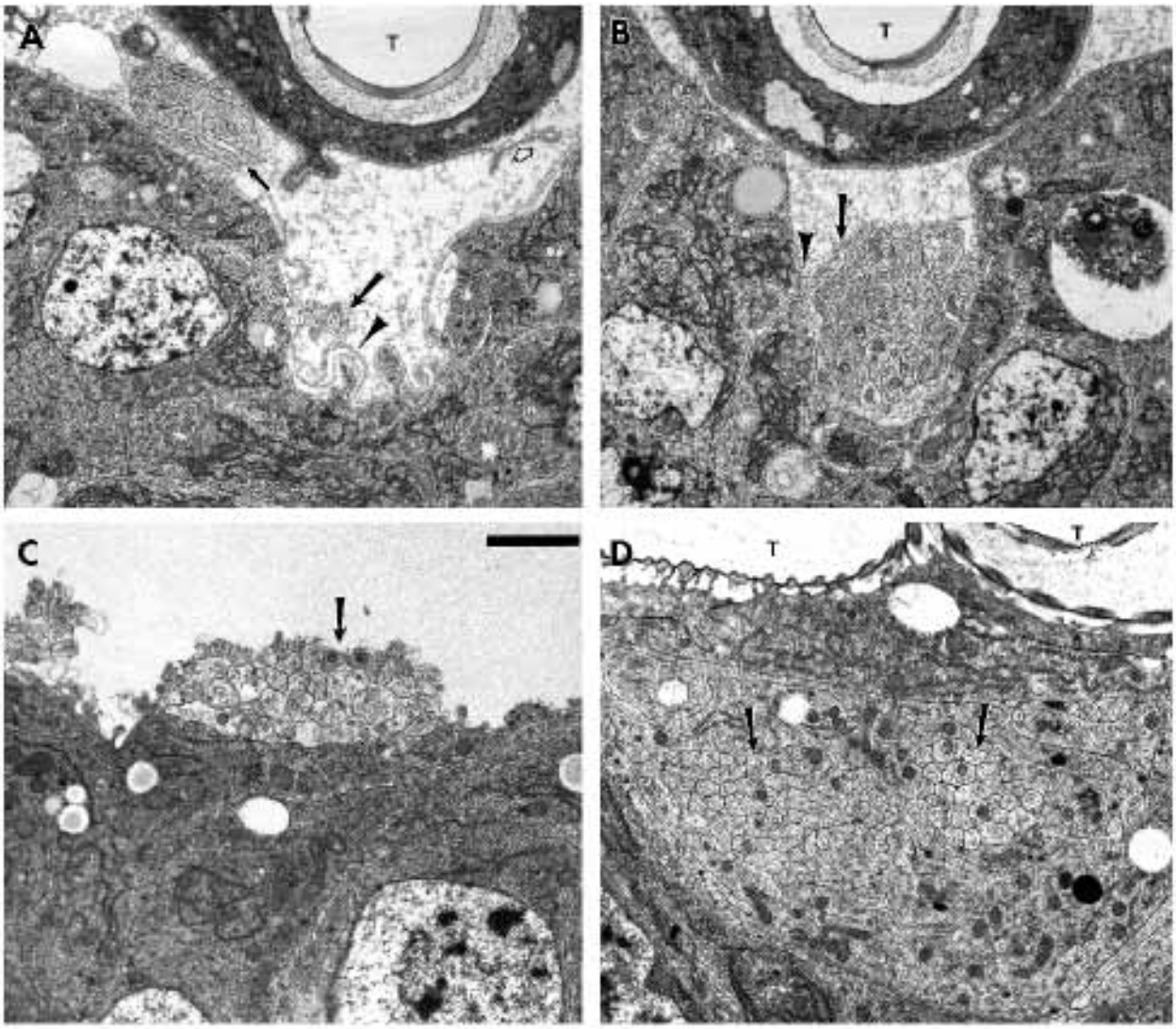

Fig. 5. The ocellar pioneer axons contact the ECM in wild type and the epidermis in *LamA* mutant pupae. Transmission electron micrographs of ocellar pioneer axon fascicles extending across the fused eye-antenna imaginal disc in wild-type (A,B) and *LamA* mutant (C,D) pupae prior to head eversion. (A) In wild type, a groove forms in the dis epithelium just opposite a tracheal (T) branch. The groove is filled with extracellular matrix (ECM) material (arrowhead in groove), just as the entire disc epithelium and trachea (open arrow) are surrounded by ECM. In this photograph, two of the pioneer axons have already entered the groove (arrow in groove), while a large group of pioneer axons are just lateral to the groove and have not yet entered it (arrow to the left of the groove). (B) In a section further posterior to A, all of the ocellar pioneer axons (arrow) have entered the groove, and are in contact with ECM material (arrowhead) but not in direct contact with the surface of disc epithelial cells. (C,D) We observe two classes of ultrastructural phenotypes in *LamA* mutants. Note that in this region of the disc, the ECM appears to be reduced (while in other regions of larave and pupae, we observe either normal or increased levels of ECM; see text). This difference in the relative thickness of the ECM in different regions of *LamA* mutant pupae may either reflect real differences in the levels of ECM, or alternatively, a decrease in the stability of the mutant ECM during the processing for EM. Sometimes (C) the pioneer axons are in direct contact with the external surface of the epithelial cells, while in other cases (D), the pioneer axons become surrounded by epithelial cells. Scale bar, 2 μm.

structure and composition of the ocellar nerve begins to change dramatically, giving rise to the pattern of axons in the adult. The ocellar pioneers are a transient population that die during later stages of pupal development and are replaced by the adult photoreceptors and giant interneurons. The ocellar pioneers are all born prior to head eversion. After head eversion, the pioneers die, as revealed by both EM analysis and acridine orange staining. BrdU injections into pupae after head eversion leads to the labeling of all of the adult ocellar photoreceptors, revealing a round of DNA synthesis which might reflect their cell birth. EM analysis at this stage confirms this hypothesis; many cell divisions are observed concentric to and just outside the clusters of ocellar pioneers. These new born cells appear to be the adult photoreceptors labeled by BrdU. Taken together, the EM and BrdU analysis demonstrate that the adult photoreceptors are born after the ocellar nerve pathway is formed by ocellar pioneer axons, and that the ocellar pioneers die prior to adult eclosion.

EM analysis of the ocellar nerve at ~48 hours after puparium formation shows the degeneration of the axons of the ocellar

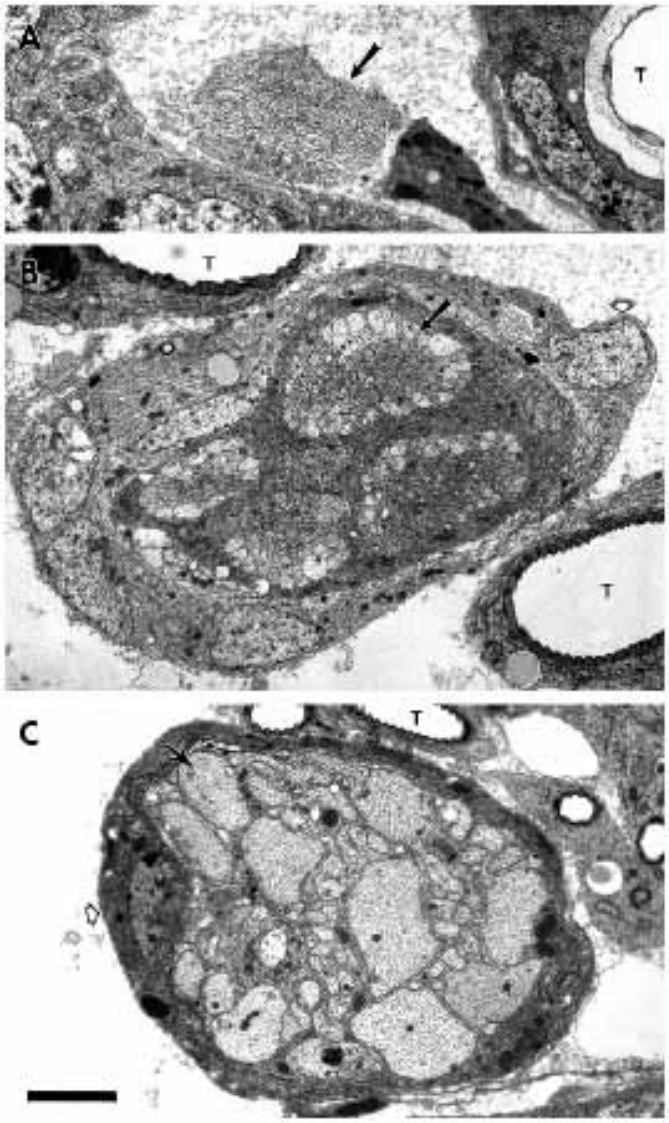

Fig. 6. The composition of the ocellar nerve changes after pupal head eversion. Transmission electron micrographs of one of the ocellar pioneer axon fascicles prior to head eversion (A) and the changing ocellar nerve either 48 hours (B) or 72 hours (C) after head eversion. (A) Fascicle of ocellar pioneer axons (arrow) prior to head eversion is in contact with ECM material just adjacent to the brain (trachea, T). Note the absence of glia around the axons. (B) After head eversion, the nerve contains four fascicles of ocellar pioneer axons (arrow); each fascicle consists of ~50 axons. The four fascicles are surrounded by an inner glial sheath (closed arrowhead), and this structure is then ensheathed by a second external layer of glia (open arrowhead). Note that the inner sheath of glia and many (but not all) of the pioneer axons stain darkly at this stage, suggesting that they are undergoing degeneration. (C) Later in pupal development, the inner sheath of glial cells and most of the ocellar pioneer axons, all of which stained darkly at an earlier stage (see B), now have degenerated and disappeared. The outer layer of glia (open arrowhead) remains. The nerve now contains the large diameter axons of the giant ocellar interneurons whose growth cones are extending from the CNS out to the ocellar ganglion. There still remains some medium and small diameter axons. Note that in the adult (Fig. 1C), most of the small diameter axons are gone. Scale bar, (A,C) 2 μm, (B) 3 μm.

pioneers. By this stage, the small diameter axons of the ocellar pioneers stain darkly, a sign that they are beginning to undergo degeneration (Fig. 6B). EM analysis of 48-72 hour pupae shows the progressive replacement of the ~200 small diameter axons of the ocellar pioneers (which are dying and disappearing) by a small number (~12) of very large axons of the ocellar interneurons which are progressively extending out towards the ocelli (Fig. 6C). At intermediate stages, we observe more large axons closer to the brain, and fewer out near the ocelli, indicating that these axons are growing out from the brain. By the time of adult eclosion, the ~12 giant axons of the ocellar interneurons are the predominant axon profiles in the ocellar nerve (Fig. 1C), and their cell bodies have migrated from the CNS out along the nerve.

LamA is required for the guidance of the ocellar pioneer axons

We used the following *LamA* mutant alleles for our analysis: the EMS-induced alleles $LamA^{160}$, $LamA^{25}$, $LamA^{216}$ and $LamA^{81L}$; and the protein null (P-element imprecise excision-induced) allele $LamA^{6-36}$ (Henchcliffe et al., 1993). The same types of axon pathfinding defects were observed in the following different *LamA* allelic combinations: $LamA^{160}/LamA^{25}$, $LamA^{160}/LamA^{216}$, $LamA^{160}/LamA^{81L}$, $LamA^{160}/LamA^{6-36}$ and $LamA^{216}/LamA^{6-36}$. As controls, we examined wild type and in addition the P-element precise excision (line *1-30*, a wild-type stock of the same genetic background as the protein null alleles) over a hypomorphic *LamA* allele ($1\text{-}30/LamA^{216}$). We observed no ocellar or compound eye axon pathfinding defects in 18 control pupae examined of the $1\text{-}30/LamA^{216}$ genotype. Staining with an anti-laminin A antibody (Henchcliffe et al., 1993) reveals that laminin A is a component of the ECM and basement membranes surrounding the eye-antenna imaginal discs and the optic stalk during larval and pupal stages.

Before head eversion, we observed several different types of aberrant pathfinding phenotypes in the projections of the ocellar pioneer axons in *LamA* mutants. In some mutant pupae, these axons had not grown as far as in wild type, or even as far as ocellar pioneer axons on the other side of the same mutant (Fig. 7A-D, right side). Occasionally some ocellar pioneer axons extended away from the brain (Fig. 7B). In other *LamA* pupae, the axons did not form the characteristic pair of ocellar pioneer axon bundles, but rather formed multiple axon fascicles, some of which entered the brain at abnormal positions (Fig. 7C,D).

EM analysis of *LamA* mutants showed that the ocellar pioneer axons grew in close contact with the surface of epidermal cells (Fig. 5C), in some cases completely surrounded by them (Fig. 5D). In *LamA* mutants, we observed either normal or increased levels of ECM and basement membranes around many structures, including the wing disc and the optic stalk. However, in the region of the eye-antenna disc over which the ocellar pioneers normally extend, EM analysis revealed what appeared to be reduced ECM material. In other regions of the same mutant discs, we observed an increase in ECM material. This difference in the relative thickness of the ECM in different regions of *LamA* mutant pupae may either reflect real differences in the levels of ECM, or alternatively, a difference in the stability of the mutant ECM during the processing for EM. Immunocytochemistry using laminin antibodies of whole-mount mutant pupae revealed laminin

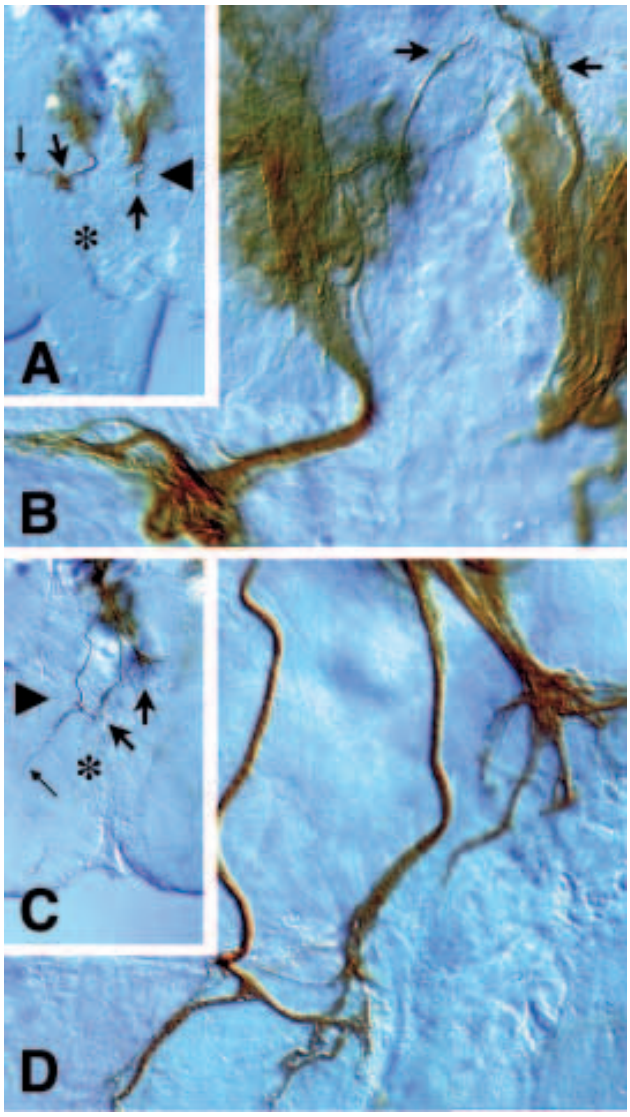

Fig. 7. Abnormal pathfinding by ocellar pioneer axons in *LamA* mutant pupae before head eversion. In *LamA* mutant pupae ($LamA^{216}/LamA^{6-36}$), the ocellar pioneer axons sometimes extend abnormally along the ridge between the disc and the brain (arrowhead in A and C) or in an abnormal direction (arrows in A and B), either laterally (left arrow in A) or anteriorly (arrows in B). Ocellar axons can also form multiple, abnormal branches that extend in different abnormal directions. Right arrow in C shows ocellar axons forming multiple fascicles and stalling in the disc epithelium. Other axons (left arrows in C) can enter the brain in abnormal locations and project to ectopic positions within the brain (see enlargement in D). All views are dorsal of dissected pupae before head eversion. The asterisk in A and C shows the location on the dorsal surface of the brain where these axons project in wild-type pupae.

expression covering all disc surfaces. Thus, ECM still forms in the absence of laminin A, but its quantity or stability is changed as prepared and viewed for EM.

We next examined the trajectory and substratum of the ocellar pioneer axons in *LamA* versus wild-type pupae after head eversion (but before the differentiation of the adult photoreceptors and death of the pioneers). We observed ocellar pathfinding defects in 44% (34/78) of all mutant pupae examined (see below). Although we observed a range of pathfinding defects, they can be described as falling into two major classes. In the more extreme class of defects (9/78 = 9%), the ocellar pioneer axons did not form the normal projection that traverses the head capsule from the epidermis to the brain, but rather extended for a short distance in the epidermis and then stalled, forming large fasciculated masses, and occasional whirls, of axons (Fig. 8B,E,F). These stalled axons remained attached to the head epidermis.

In 27/78 (35%) of *LamA* mutant pupae, the ocellar pioneer axons extended in multiple fasciculating bundles for a short distance in the epidermis, and then some of them clustered together forming one or two large axon bundles that followed the contour of the epidermis towards the antennae (Fig. 8B-D). Staining with mAb 22C10 (which stains both photosensory and mechanosensory axons) revealed that these large bundles of ocellar pioneer axons have joined the normal axon fascicles of the ocellar and orbital bristles, something they never do in wild type. The fascicles of ocellar pioneer axons joining the mechanosensory pathways can be very different in size, ranging from only one or two axons to large bundles containing many ocellar pioneer axons (Fig. 8B-D). Whenever the ocellar pioneer axons grew for any distance, they appeared to have joined one of the bristle axon pathways.

Staining with mAb 24B10 showed that all of the ocellar pioneer axons eventually stalled and did not follow the bristle axons the entire distance into the brain. Although the ocellar axons usually extended anterior along the head epidermis and/or bristle axons (after head eversion, equivalent to extending posterior along the disc epithelium prior to head eversion), occasionally some extended posteriorly.

The vast majority of bristle axons (orbitals and ocellars) project in a normal fashion in *LamA* mutant pupae. We observed occasional defects in bristle axons in 9% of pupae stained with mAb 22C10 (3/34) after head eversion. In each case, however, the defect was minor and corresponded to only a single bristle axon that was misrouted but then joined one of the other bristle nerves.

In *LamA* adult escapers, ectopic ocellar axons can be detected under the cuticle although, at this stage, the epidermis has already degenerated. These axons probably represent projections from the adult photoreceptors that have followed the abnormal projection by the ocellar pioneers. Two results support this notion. First, EM analysis of *LamA* escapers showed that the normal ocellar nerve frequently had not formed between ocelli and brain. Second, BrdU injection of *LamA* pupae after head eversion labeled the ocellar adult photoreceptors of these escapers, suggesting that the ocellar pioneers had died and the adult photoreceptors had been born on schedule, even though the ocellar pioneer axons never reached their targets in the brain and the axons of the ocellar interneurons never extended out the ocellar nerve. We cannot rule out the possibility that some ectopic axons correspond to a small percentage of ocellar pioneers that avoided cell death.

We observed the following percentages of ocellar pioneer defects (pooling mAb 24B10 and mAb 22C10 stainings) in the various *LamA* genotypes examined after head eversion:
$LamA^{160}/LamA^{25}$ = 9/15 (60%) with defects, 2 were extreme;
$LamA^{6-36}/LamA^{216}$ = 10/16 (62%) defects, 2 extreme;
$LamA^{6-36}/LamA^{160}$ = 6/18 (33%) defects, 0 extreme;

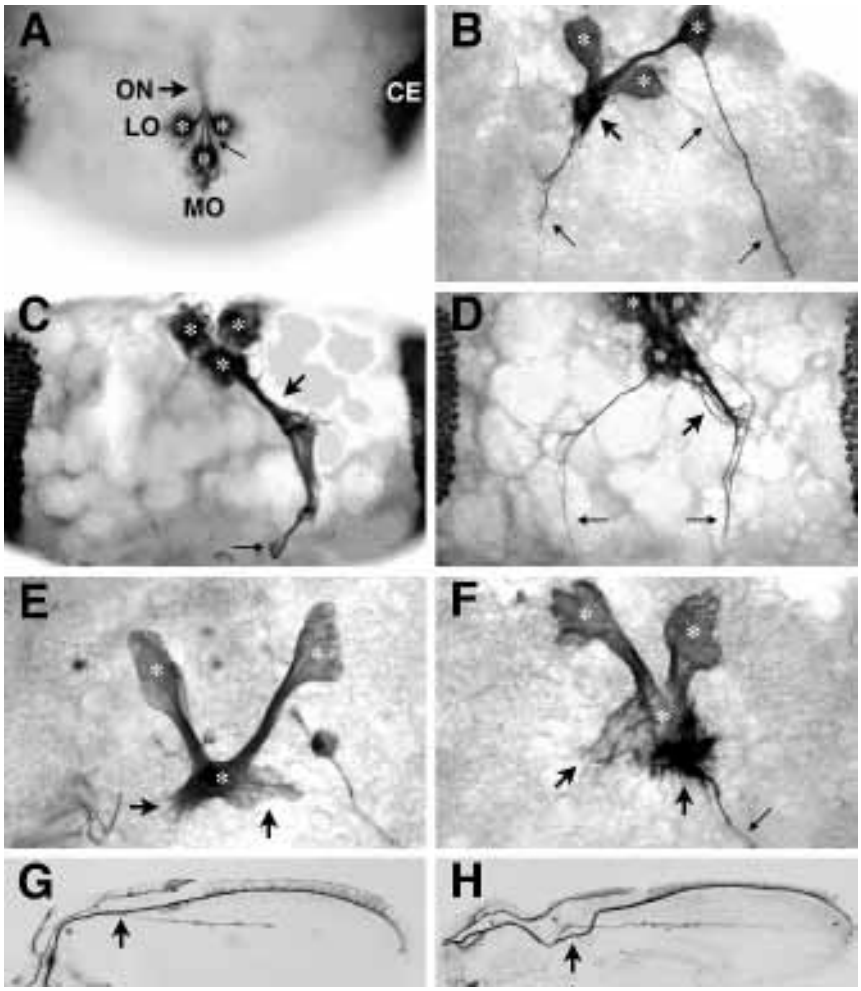

Fig. 8. Axon pathfinding by ocellar pioneer axons and wing axons in *LamA* mutant pupae after head eversion. (A) In wild-type pupae after head eversion, the ocellar pioneer axons become oriented perpendicular to the epidermal surface in the ocellar nerve (thin arrow), projecting ventrally across the head capsule towards the brain. Asterisks in this and all other panels mark ocelli; LO, lateral ocelli; MO, median ocellus; ON, ocellar nerve; CE, compound eye. (B-D) In *LamA* mutant pupae after head eversion, staining with mAb 24B10 reveals that the ocellar axons remain attached to the head epidermis, projecting anteriorly in one or two bundles (arrows). Staining with mAb 22C10 reveals that the ocellar axons in these cases have fasciculated with mechanosensory (bristle) axons (data not shown). These ocellar axons do not reach the brain, but rather stall somewhere along the bristle nerves. (E,F) Extreme examples of *LamA* mutant phenotypes of ocellar pioneer axons. These axons extend in the epidermis and then stall, remaining close to the ocelli and branching profusely in a disorganized fashion (arrows). (G,H) *LamA* mutant wing discs with wild type (G) and abnormal pathfinding defects (H). We observed a defect in the pattern of axon projections in only 2/41 *LamA* mutant wings examined (one is shown here; arrow marks defect). In both cases, the axons appeared to be misrouted for a short distance but then corrected their trajectories and eventually exited the wing in the appropriate nerve location. All views are dorsal. Genotypes: (A) wild type; (B) $LamA^{160}/LamA^{6-36}$; (C,D) $LamA^{216}/LamA^{6-36}$; (E,G,H) $LamA^{160}/LamA^{25}$ and (F) $LamA^{81L}/LamA^{160}$. (A-D,F) Stained with mAb 24B10; (E,G,H) stained with mAb 22C10.

$LamA^{160}/LamA^{216}$ = 4/11 (36%) defects, 1 extreme; $LamA^{81L}/LamA^{160}$ = 5/18 (28%) defects, 2 extreme.

Compound eye photoreceptor axons also display pathfinding defects in *LamA* mutants

We also used mAb 22C10 and mAb24B10 to examine the projections of photoreceptor axons from the compound eye in 3rd instar larvae and in pupae both before and after head eversion. We observed major axon pathfinding defects in the photoreceptor axons of the compound eye in around 50% (40/79) of *LamA* mutant larvae and pupae. In the most penetrant genotype for this phenotype, $LamA^{160}/LamA^{25}$, defects were observed in 62% of pupae (16/26). We detected defects in the projection of photoreceptor axons of the compound eye as early as 3rd instar larvae. The optic stalk was often very broad in shape and the axons had a loose and disorganized appearance. Rather than the normal single tight nerve, in 9 of 10 *LamA* mutant 3rd instar larvae examined, the optic nerve was split into two or more bundles. Moreover, in several 3rd instar discs, we detected whirls of retinal axons within the retina, a phenotype that is more pronounced during pupal stages.

In *LamA* pupae before head eversion, the projections of retinal axons were often highly aberrant. In those pupae with the strongest mutant phenotype, the axons did not extend into the optic stalk but rather formed whirls of axons within the retina. We also observed growth cones within the retina extending in abnormal directions and failing to enter the optic stalk.

In *LamA* pupae after head eversion, these abnormal whirls of retinal axons remained prominent. In addition, the axon projection from the retina to the lamina was sometimes split into dorsal and ventral zones. Most common was a general disorganization of retinal axons as they entered the retinal projection. Some retinal axons also projected outside of the retina just beneath the head epidermis surrounding the eye, usually in fasciculated bundles of axons. These axons usually stalled in the epidermis.

Although we observed striking defects in compound eye retinal axon pathfinding in *LamA* mutants, we also observed an abnormal distribution of glial cells. Thus, we do not know whether these pathfinding defects are a primary consequence of an axonal requirement for laminin A, or alternatively, whether the glia are primarily disrupted and the axon defects secondary.

Sensory axons in the wing only rarely display pathfinding defects in *LamA* mutants

We examined axon pathfinding in the pupal wing using three combinations of *LamA* alleles: $LamA^{160}/LamA^{25}$, $LamA^{160}/LamA^{81L}$ and $LamA^{6-36}/LamA^{160}$. We observed a defect in axon projections in only 2 of 41 *LamA* mutant wings. In both cases, the axons were misrouted for only a short distance and

then corrected their trajectories and entered the appropriate nerve (Fig. 8H).

DISCUSSION

The genetic analysis presented here shows that extracellular matrix (ECM) containing laminin A is required for the normal pathfinding by the ocellar pioneer axons in *Drosophila*. Dramatic pathfinding defects are observed in the ocellar pioneer projections in 44% of *LamA* mutant pupae examined. This probably represents an underestimate of the penetrance of *LamA* pathfinding defects. Null conditions of *LamA* are embryonic lethal. Laminin A function is non-cell autonomous and *LamA*$^{-}$ cell clones produce weaker phenotypes than do hypomorphic mutations (Henchcliffe et al., 1993), precluding the study of the null condition in mosaic territories of imaginal discs. Thus, we have studied hypomorphic (partial loss-of-function) conditions for *LamA*. We do not know what the penetrance and expressivity of pathfinding defects would be in a complete absence of laminin A function.

For ocellar photoreceptor axons, an ECM containing laminin A is required for the establishment of normal axon pathways. In contrast, an ECM containing laminin A is not required for pathfinding by neighboring mechanosensory (bristle) axons in the head, or by bristle axons in the wing. It may be that bristle axons rely predominantly on guidance cues on epidermal cell surfaces, the surface over which they normally extend. The projections of retinal axons from the compound eye are also disrupted in *LamA* mutants, although we do not know whether this defect is primary or secondary.

In most parts of the developing PNS in *Drosophila* (such as the embryonic body wall or pupal wing disc), neighboring sensory axons fasciculate with one another and follow the same pathway into the CNS (e.g. Blair et al., 1987; Ghysen et al., 1986). In these cases, a multitude of guidance cues in the ECM and on various cell surfaces might guide sensory axons along the same pathway. Thus the absence of any one cue, such as occurs in the *LamA* mutant, might not have a very dramatic effect on axon pathfinding. For example, in the developing limb bud of the grasshopper embryo, the Ti1 growth cones pioneer a PNS pathway followed by mechanosensory axons (e.g. Bentley and O'Connor, 1992). During their initial proximal extension, these growth cones appear to use the basement membrane as a substratum (Anderson and Tucker, 1988), and enzymatic removal of the basement membrane leads to growth cone retraction (Condic and Bentley, 1989). However, if the axons are allowed to re-extend, a second enzymatic digestion has no effect, suggesting that these growth cones can also extend on the surfaces of epidermal cells.

A very different situation is observed in the fly head. Different classes of neighboring sensory neurons extend axons that follow different substrata, selectively fasciculate in different modality-specific pathways and enter the brain in very different locations. In wild type, the ocellar pioneer axons extend on the ECM. In the absence of laminin A, the ocellar pioneer axons adhere to and extend on an alternative substratum – the epidermis – but only for a short distance. They fasciculate with one another, and often aggregate into large masses and whirls of ocellar axons. In *LamA* mutants, the ocellar pioneer axons often contact and fasciculate with mechanosensory axons (something they never do in wild type), although here too they eventually stall and do not extend the entire distance to the brain. These results suggest that ECM containing laminin A is necessary for the proper extension and guidance of ocellar pioneer axons towards and into the brain.

In the absence of laminin A, the ocellar pioneer axons adhere to and can extend a modest distance along both epidermal cells and mechanosensory axons but, in both cases, they eventually stall and cease their extension. Evidently, these two substrata – the epidermis and the other class of sensory axons – can provide adhesive surfaces for ocellar pioneer growth cones but do not provide the necessary growth-promoting signals for their complete extension into the brain (or alternatively, do provide specific growth-inhibiting signals). Even though the ocellar axons fasciculate with mechanosensory axons, they do not extend all the way into the brain. We interpret these in vivo results as revealing a fundamental distinction between the adhesiveness of a substratum versus its ability to promote axonal extension. In vitro studies on NCAM function have led to a similar conclusion (e.g. Doherty and Walsh, 1992).

Clearly, ECM containing laminin A cannot be the entire story for the guidance of ocellar axons towards the brain. In *LamA* pupae, the ocellar axons usually (but not always) extend in the appropriate direction along the epidermis. This suggests that, in the absence of laminin A, the ocellar axons can still read directional cues pointing them towards the brain. Thus, while the laminin-rich ECM provides the appropriate growth-promoting substratum, some other signal must provide the directional cue.

We draw two major conclusions from our genetic analysis of *LamA* function during axon pathfinding. First, either laminin A or ECM containing laminin A does indeed function in axon guidance in the developing organism. We have shown that laminin A plays a major role in regulating the normal guidance of ocellar axons. This genetic analysis does not allows us to distinguish, however, between a direct role for laminin A in axon guidance versus an indirect role for laminin A in organizing some other guidance component that binds laminin. However, given the extensive literature showing that laminin is a potent promoter of neurite outgrowth in vitro (see Introduction), we suggest that laminin may be the ECM component required for the guidance of ocellar pioneer axons.

Second, the differential requirement of different classes of sensory axons for laminin A in part controls their divergent pathway choices: one class of sensory axons follows the laminin-rich ECM towards one brain entry point, while another neighboring class of sensory axons follows the contour of the epidermis towards a different entry point. These results suggest that the differential expression of ECM (possibly laminin) receptors by different classes of sensory neuron growth cones may regulate the formation and choice of specific axon pathways in *Drosophila*.

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