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Article

Conserved Role of the *Vsx* Genes Supports a Monophyletic Origin for Bilaterian Visual Systems

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Summary

Background: Components of the genetic network specifying eye development are conserved from flies to humans, but homologies between individual neuronal cell types have been difficult to identify. In the vertebrate retina, the homeodomain-containing transcription factor Chx10 is required for both progenitor cell proliferation and the development of the bipolar interneurons, which transmit visual signals from photoreceptors to ganglion cells.

Results: We show that *dVsx1* and *dVsx2*, the two *Drosophila* homologs of *Chx10*, play a conserved role in visual-system development. DVSX1 is expressed in optic-lobe progenitor cells, and, in *dVsx1* mutants, progenitor cell proliferation is defective, leading to hypocellularity. Subsequently, DVSX1 and DVSX2 are coexpressed in a subset of neurons in the medulla, including the transmedullary neurons that transmit visual information from photoreceptors to deeper layers of the visual system. In *dVsx* mutant adults, the optic lobe is reduced in size, and the medulla is small or absent. These results suggest that the progenitor cells and photoreceptor target neurons of the vertebrate retina and fly optic lobe are ancestrally related. Genetic and functional homology may extend to the neurons directly downstream of the bipolar and transmedullary neurons, the vertebrate ganglion cells and fly lobula projection neurons. Both cell types project to visual-processing centers in the brain, and both sequentially express the Math5/ATO and Brn3b/ACJ6 transcription factors during their development.

Conclusions: Our findings support a monophyletic origin for the bilaterian visual system in which the last common ancestor of flies and vertebrates already contained a primordial visual system with photoreceptors, interneurons, and projection neurons.

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Introduction

The eyes of vertebrates and flies, despite their very different structures, rely on a shared regulatory network of genes (such as *Pax6/eyeless*) for their specification [1, 2]. This led to the proposal that invertebrate and vertebrate eyes are derived from a common ancestor. It is not known, however, whether homologies exist between the cell types that make up the neural circuit required for vision, although it has been suggested that the vertebrate eye is a composite structure made up of distinct types of photoreceptive cells of independent evolutionary origins [3].

In the vertebrate retina, transmission of the signal from photoreceptors to ganglion cells is mediated by bipolar interneurons [4]. The neurons of the retina develop from a pool of proliferating and multipotent neuroepithelial cells known as the retinal progenitor cells (RPCs) [5]. A suite of transcription factors regulates retinal proliferation and neurogenesis [5–7]. One of these—the homeodomain- and CVC-domain-containing transcription factor Chx10—is required for both RPC proliferation and bipolar cell specification [8].

In the mouse retina, *Chx10* is expressed in the RPCs as well as in developing and mature bipolar cells [9]. *Chx10* mutant retinas are hypocellular because of a marked decrease in the rate of RPC proliferation, and bipolar cells are completely absent [8]. Additionally, *Chx10* mutant eyes are micropthalmic, suggesting that *Chx10* nonautonomously controls the growth of the entire eye. The role of *Chx10* in eye development is conserved in zebrafish and in humans; null mutations in *Chx10* have been identified in families with congenital micropthalmia [10]. Vertebrates contain a second *Vsx*-family gene, *Vsx1*, and in the mouse, retina expression of *Vsx1* is restricted to a subset of differentiating and mature cone bipolar cells, where it is required for their late differentiation and function [11, 12]. In *C. elegans*, the *Vsx* homolog *ceh-10* is required for the development of the AIY thermoregulatory interneuron [13]. Because the AIY neuron may be directly targeted by a photoreceptive cell in some nematodes [14], it has been suggested that the *Vsx* genes play a conserved role in photoreceptor target-interneuron development that extends from humans to worms [13].

Here, we characterize the role of the two *Drosophila* homologs of *Chx10* in visual-system development. The *Drosophila* visual system is made up of two major structures: the compound eye and the underlying optic lobe, which is located in the brain [15]. The photoreceptors in the compound eye project to either the first or second ganglion of the optic lobe, respectively the lamina and the medulla [16]. Thus, in contrast to vertebrates, in which the photoreceptors and their target interneurons are located together in the retina, in the fly, the target neurons of the photoreceptors are found in the brain. Transmedullary neurons then relay visual information to the third optic ganglion, the lobula complex [17]. In turn, neurons in the lobula complex project from the optic lobe to the higher-order visual-processing centers of the brain [18].

The compound eye and optic lobe develop from distinct epithelial structures in the larva. The photoreceptor cells are derived from the eye imaginal disc [19], and the optic lobe develops from two groups of neuroepithelial progenitor cells,

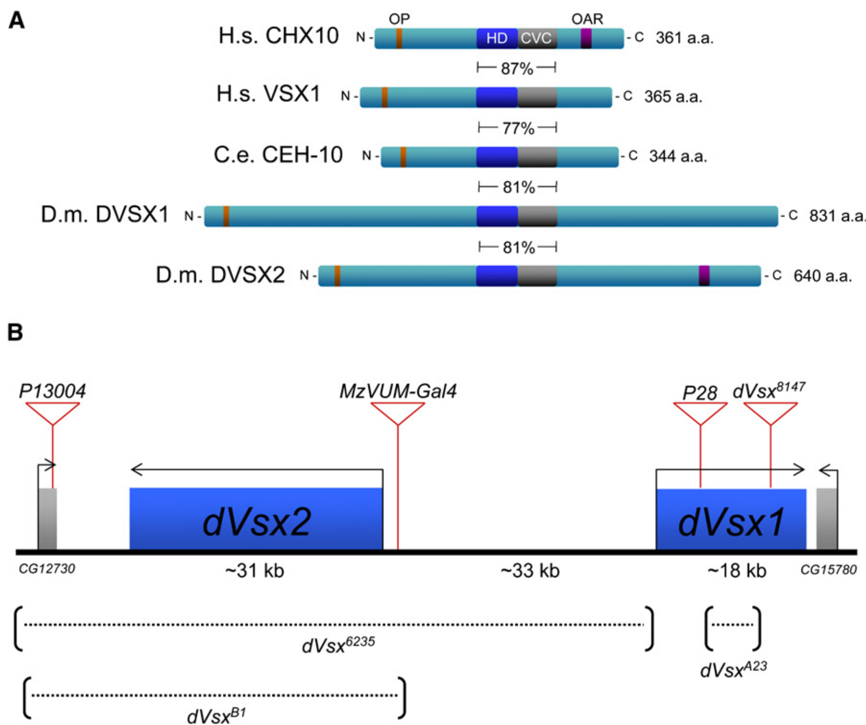


Figure 1. *dVsx1* and *dVsx2* Are *Drosophila Chx10* Orthologs Located 33 kb Apart on the X Chromosome

(A) The fly (D.m.) proteins DVSX1 and DVSX2 share a conserved domain structure with CVC orthologs in humans (H.s.) and worms (C.e.). HD, homeodomain; CVC, CVC domain; OP, octapeptide motif; OAR, OAR motif. The percentage of aa identity to Chx10 across the homeodomains and CVC domains is listed above each Chx10 ortholog.

(B) Schematic representation of the *dVsx* genomic locus and the molecular lesions associated with mutations mapped therein. *dVsx1* and *dVsx2* are located at cytological position 5A3–7 of the X chromosome. Gray boxes represent the genes closest to the *dVsx* locus, and red triangles represent *P* element insertions. *MzVUM-Gal4* is a *P* element inserted 886 bp upstream of *dVsx2* that drives *Gal4* in *dVsx*-expressing cells. *P13004* and *P28* are *P* elements used for *dVsx* mutagenesis. Dashed lines surrounded by closed brackets represent DNA removed by deletions. *dVsx* mutants: $dVsx^{8147}$ is the product of a P-element insertion (*P8147*) into the second intron of *dVsx1*; in these mutants, the DVSX1 protein is restricted to the cytoplasm. $dVsx^{A23}$ contains a deletion and inversion caused by the mobilization of *P28*, which removes the nuclear-localization signal of DVSX1. $dVsx^{6235}$ contains a 69932 bp deletion generated by Exelixis, which removes all of *dVsx2* and *CG12730* and 32.5 kb of intergenic DNA. The deletion ends 334 bp before the start of the *dVsx1* 5′UTR. In $dVsx^{6235}$ mutants, DVSX1 remained expressed in a few cells of the brain and pharynx (data not shown), indicating that the transcription start site of *dVsx1* is intact. The loss of DVSX1 expression in many tissues, including the VNC, PI, OOA, and medulla, is probably a consequence of the removal of enhancer elements located in the intergenic region. $dVsx^{B1}$ contains a 43604 bp deletion generated by an imprecise excision of *P13004*, which removes all of *dVsx2* and *CG12730* and 1.5 kb of intergenic DNA. The lethality associated with this allele maps to the *dVsx* locus because deletions that remove only *CG12730*, a gene of unknown function, are viable.

termed the inner and outer optic anlagen (the IOA and OOA, respectively) [20, 21]. Whereas the genetic control of neurogenesis has been intensively studied in the eye disc, comparatively little is known about the genes required for progenitor proliferation and neuronal cell-type specification in the developing optic lobes.

We demonstrate here that the two *Drosophila* homologs of *Chx10*, *dVsx1* and *dVsx2*, are required for optic-lobe development. DVSX1 is expressed in a population of multipotent and proliferating OOA progenitor cells, and both DVSX1 and DVSX2 are expressed in the developing and mature medulla, which derives from the OOA. *dVsx*-positive neurons in the medulla include both local neurons and transmedullary neurons that directly connect the inner photoreceptors, R7 and R8, to the lobula complex. In *dVsx* mutants, the optic lobes are very small, the medulla being most strongly affected. We show that the hypocellularity observed in the adult optic lobes results from an early defect in OOA progenitor proliferation. The expression pattern and mutant phenotypes observed for the *dVsx* genes in the fly visual system are similar to those previously described for *Chx10* in the vertebrate retina. On the basis of several functional and molecular similarities, we propose that (1) the progenitors of the OOA are evolutionarily related to the retinal progenitors of vertebrates, and (2) the photoreceptor target interneurons and visual-projection neurons of flies and vertebrates share several homologous features.

Results

dVsx1 and *dVsx2* Are the *Drosophila* Homologs of *Chx10*

Full-length cDNAs encoding two *Drosophila Chx10* homologs, *dVsx1* and *dVsx2*, were cloned from embryonic and adult head cDNA libraries, respectively. Their predicted proteins possess a domain structure characteristic of all *Vsx* homologs [22] (Figure 1A and Figure S1 available online): a paired-like homeodomain and an adjacent CVC domain (a putative DNA-binding domain, which defines the family). DVSX1 and DVSX2 each share 81% amino acid identity with Chx10 across the homeodomains and CVC domains (Figure 1A and Figure S1). Unlike DVSX1, DVSX2 also possesses a C-terminal OAR domain (Figure S1D), which is believed to function in transcriptional repression and which is present in a subset of Vsx homologs [23].

Sequence alignment and phylogenetic analyses suggest that DVSX1, DVSX2, and the *C.elegans* Vsx protein, CEH-10, are ancestrally more closely related to Chx10 than the second vertebrate Vsx protein, Vsx1 [22]. This finding suggests that the *dVsx* genes are the product of a duplication event from an ancestral *Vsx* gene, which occurred after the split of chordates, nematodes, and arthropods. Consistent with the theory of a duplication event, *dVsx1* and *dVsx2* are located at the same locus on the X chromosome (polytene bands 5A3–7), separated by a 33 kb intergenic region (Figure 1B).

DVSX1 Is Expressed in the Central Domain of the Outer Optic Anlage, which Comprises Multipotent Neuroprogenitor Cells

DVSX1, but not DVSX2, is embryonically expressed (Figures S2 and S3) in the pars intercerebralis [24], ventral nerve cord [25], pharynx, optic anlage, and isolated cells throughout the brain. Because *Chx10* in vertebrates is expressed in, and required for, the proliferation of retinal progenitor cells, we focused on the expression pattern of DVSX1 in the optic-lobe progenitors. In the embryo, DVSX1 expression in the visual system was first observed in the preinvagination optic placode

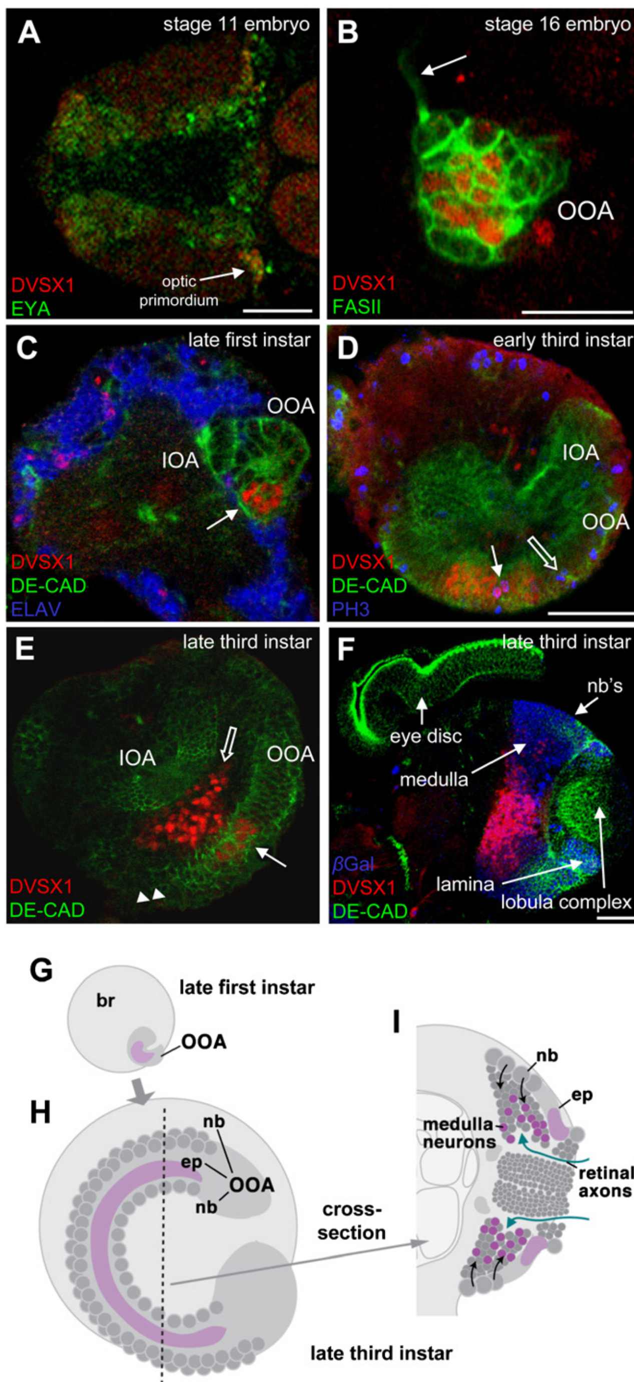


Figure 2. DVSX1 Is Expressed in the Central Domain of the OOA, which Comprises Uncommitted Progenitor Cells

Immunostains with anti-DVSX1 in embryos and larvae.

(A) DVSX1 is expressed in the optic primordium of the stage 11 embryo, which is marked with EYES ABSENT (EYA).

(B) DVSX1 is expressed in the central domain of the FASII-labeled OOA of a stage 16 embryo. The arrow denotes Bolwig's nerve.

(C–F) Anti-DE-CADHERIN (DE-CAD) labels the membranes of cells in the larval OOA and IOA.

(C) DVSX1 is expressed in the central domain of the OOA (denoted by the arrow) in the late-first-instar larva. Absence of anti-ELAV staining, which labels the nuclei of neurons, indicates that the cells of the anlagen are not neuronal.

(D) DVSX1 continues to be expressed in the central domain of the OOA in the early-third-instar larva. Cells both outside (denoted by the open arrow) and within (denoted by the other arrow) the DVSX1 domain of the OOA are PH3 positive.

(E) In the late-third-instar larval brain, DVSX1 expression is maintained in the central domain of the OOA (denoted by the arrow). DVSX1 expression is absent from the neuroblasts (denoted by double arrowheads). DVSX1 is then expressed in nascent medulla neurons (denoted by the open arrow).

(F) *MzVUM-Gal4*-based cell-lineage tracing in the third-instar larval brain. The *MzVUM-Gal4* driver was used to permanently mark all *dVsx*-expressing cells with *lacZ*. β-Gal expression is observed throughout the lamina and medulla and in the OOA neuroblasts (nb's) but not in the lobula complex or the eye disc.

(G–I) Schematic representation of DVSX1 expression during larval optic-lobe development.

(G) Lateral view of a late-first-instar larval brain. DVSX1 (in magenta) is expressed in a central domain of the crescent-shaped OOA; br, brain.

(H) Lateral view of a late-third-instar larval brain. The OOA has grown and transformed into neuroblasts (nb) at its medial and lateral edges. DVSX1 expression (in magenta) remains restricted to a central population of epithelial progenitors (ep).

(I) Cross-section of (H). Medial OOA neuroblasts generate the neurons of the medulla (denoted by black arrows). DVSX1 is expressed in the epithelial progenitors of the OOA (in magenta), is downregulated as they transform into neuroblasts and is upregulated in a subset of medulla neurons (in purple). Incoming retinal axons are shown in green. Scale bars represent 50 μm in (A), (D), and (F) and 20 μm in (B).

(Figure 2A) [26]. After optic-placode invagination, DVSX1 expression was detected in approximately 12 cells in the center of each optic anlage (Figure 2B). The progenitors of the optic anlagen remain quiescent until midway through the first larval instar, when they segregate into two morphologically distinct columnar epithelia, the IOA and OOA, and commence symmetric divisions [15, 20, 21]. Shortly after the resumption of proliferation, DVSX1 expression was detected in approximately nine cells in a central region of the OOA (Figures 2C and 2G), and colabeling with a phosphohistone H3 (PH3) antibody indicated that these cells are mitotically active (Figure 2D). In the third-instar larva, when the majority of optic progenitors convert to asymmetrically dividing neuroblasts that generate the neurons of the adult optic lobe, DVSX1 expression remained restricted to a small central population of OOA epithelial progenitors (Figures 2E, 2H, and 2I). A second domain of DVSX1 expression also became evident in a subset of cells in the nascent medulla cortex.

To establish the DVSX1-positive progenitor cells' contribution to the optic lobe, we performed a cell-lineage-tracing experiment with the *MzVUM-Gal4* driver (Figure 1B), which faithfully reports for DVSX1 expression (Figure 1B and Figure S4). In this experiment, DVSX1-expressing cells and their progeny are permanently marked with *lacZ* expression. In the third-instar larval brain, β-Galactosidase (β-Gal) expression was detected throughout the outer optic lobe, including in cells of the OOA, outer neuroblasts, lamina, and medulla (Figure 2F), but not in the lobula complex or eye disc. Thus, DVSX1-positive cells in the OOA are competent to give rise to cells throughout the outer optic lobe.

The above data demonstrate that DVSX1 is (1) expressed in proliferating and uncommitted OOA progenitors, (2) downregulated as OOA progenitors become neuroblasts, and (3) subsequently upregulated in the medulla cortex (Figures 2E and 2I). This expression pattern strongly resembles that of *Chx10* in vertebrates; *Chx10* is expressed in multipotent and proliferating neuroretinal progenitors, downregulated before neurogenesis, and then upregulated in bipolar interneurons [9, 27]. Of note, one significant difference between the visual-system expression patterns for the two genes is that whereas

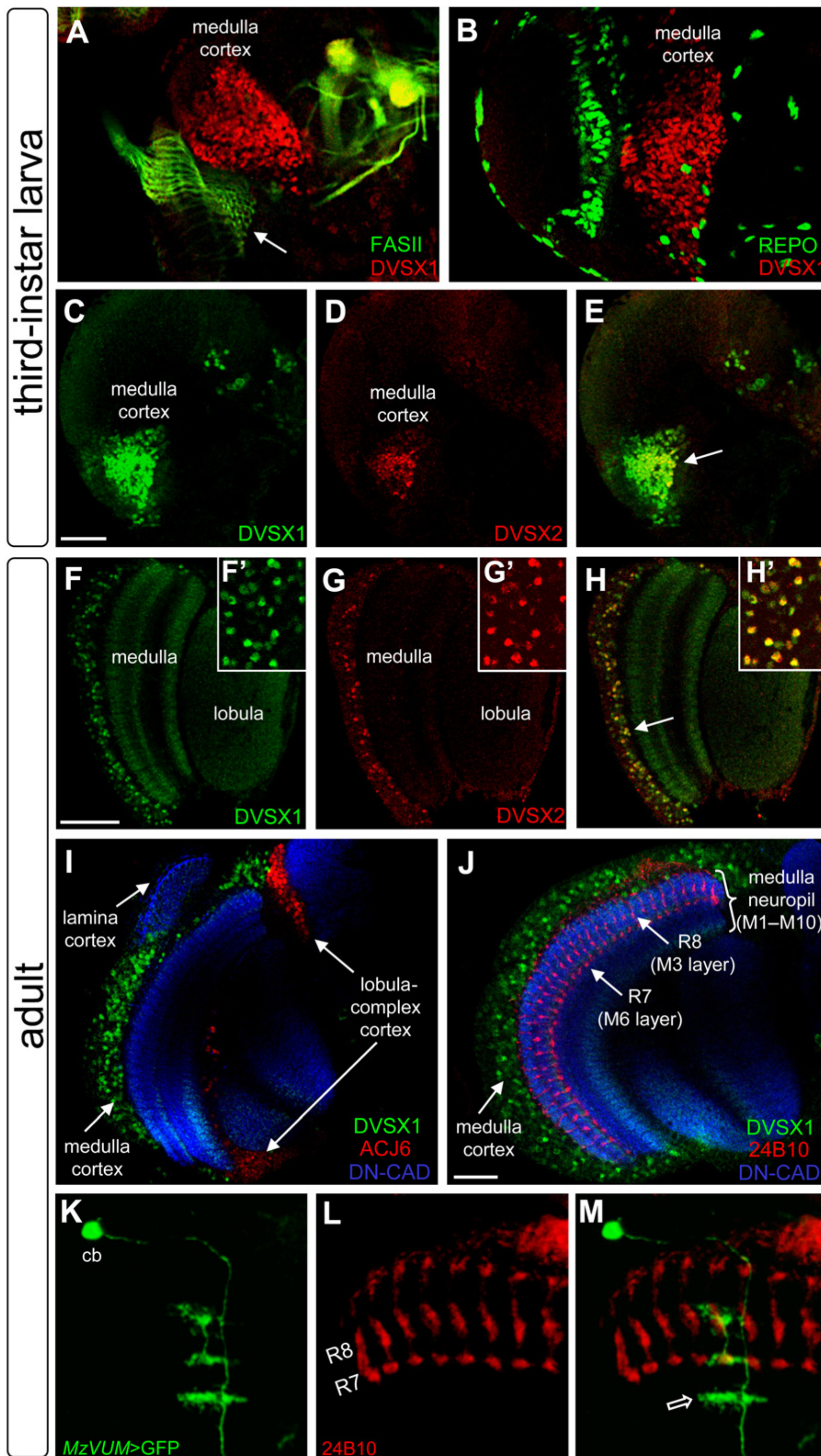


Figure 3. DVSX1 and DVSX2 Are Coexpressed in a Subset of Neurons in the Medulla Cortex, Including R7 and R8 Target Neurons

(A–E) Immunostains of third-instar larval brains.

(A) FASII marks the incoming photoreceptors that project to the lamina neuropil and medulla neuropil (denoted by the arrow). DVSX1 is expressed in a subset of cells in the medulla cortex region which lies anterior to the neuropil.

(B) DVSX1 is not expressed in glial cells, labeled with anti-REPO.

(C–E) DVSX2 is expressed in a subset of DVSX1 expressing cells (denoted by the arrow).

(F–M) Immunostains of adult brains.

(F–H) DVSX1 and DVSX2 are coexpressed in the adult medulla (denoted by the arrow).

(F′–H′) High-magnification views of the medulla cortex.

(I and J) Neuropil regions are marked with anti-DN-CADHERIN.

(I) DVSX1 is expressed in the medulla cortex, but not in the lobula-complex cortex (marked with anti-ACJ6) or the lamina cortex (unlabeled).

(J) The open bracket denotes the medulla neuropil. R7 and R8 photoreceptor axons (labeled with anti-MAb24B10) terminate in the M6 and M3 layers, respectively. DVSX1 is expressed in the medulla cortex, which houses the cell bodies of neurons that contribute to the medulla neuropil.

(K–M) The MARCM system was used to positively label *dVsx*-expressing neurons in the adult medulla with membrane-bound GFP driven by *MzVUM-Gal4*. A *dVsx*-positive neuron arborizes in the R7 and R8 target layers and the M8 layer (denoted by the open arrow) and projects to the lobula complex. This neuron's morphology is identical to that of the previously described transmedullary neuron TM5; cb, cell body. Scale bars represent 50 μm in (C)–(H) and 25 μm in (J).

DVSX1 expression is restricted to a central subset of OOA progenitors, Chx10 is expressed in all RPCs.

DVSX-Expressing Medulla Neurons Are Directly Targeted by the Inner Photoreceptors R7 and R8

We next investigated whether the DVSX1-expressing cells in the medulla cortex include the neurons that directly contact the inner photoreceptors R7 and R8. In the late-third-instar larva, DVSX1 was expressed in a subset of neurons in the medulla cortex (Figures 3A and 3B), and DVSX2 was expressed—for the first time—in a subset of these DVSX1-positive cells (Figures 3C–3E). DVSX1 and DVSX2 continued to be coexpressed in the medulla cortex at pupal stages (data not shown) and in the adult (Figures 3F–3I) in ∼20% of the cells in the medulla cortex.

The adult medulla is made up of ten synaptic layers (M1–M10) and more than 60 neuronal cell types, a subset of which act as the direct target interneurons for R7 and R8 [17, 28]. R7 and R8 form synapses in the M6 and M3 layers, respectively (Figure 3J). To determine whether the *dVsx* genes are expressed in the target interneurons of the inner photoreceptors, we labeled the membranes of *dVsx*-expressing cells with GFP using the MARCM system and the *MzVUM-Gal4* driver. Arborizations of MARCM-labeled and *dVsx*-positive neurons were detected in several layers of the medulla, including the R7 and R8 target layers (Figures S5A–S5A″) comprising amacrine, intrinsic, projection, and transmedullary neurons (Figures S5B–S5G′). A notable *dVsx*-positive neuron was TM5, previously identified in classic Golgi impregnation studies

as a transmedullary interneuron (Figures 3K–3M) [17, 29]. This neuron makes synapses in the M3, M6, and M8 layers of the medulla, terminates in layers Lo4–6 of the lobula, and has been proposed to be a direct target of the inner photoreceptors. Thus, like *Chx10* in vertebrates, the *dVsx* genes are expressed in photoreceptor target neurons that transmit visual signals to deeper layers of the visual system. One significant difference between the *Chx10* and *dVsx* expression patterns is that whereas *Chx10* expression is never observed in local retinal neurons (amacrine or horizontal cells), the *dVsx* genes are expressed in local medullary neurons.

dVsx Mutants Die as Pharate Adults and Exhibit Developmental Delays

We isolated four lethal mutations in the *dVsx* locus: $dVsx^{A23}$, $dVsx^{8147}$, $dVsx^{B1}$, and $dVsx^{6235}$ (Figure 1B and Figure S6A). $dVsx^{A23}$ and $dVsx^{8147}$ represent strongly hypomorphic or null alleles of *dVsx1* (Figures S6B–S6E). $dVsx^{B1}$ is a null allele of *dVsx2* and $dVsx^{6235}$ is a null allele of both *dVsx1* and *dVsx2* in the OOA and medulla.

In $dVsx^{A23}$, $dVsx^{8147}$, and $dVsx^{6235}$ mutants, lethality was observed at all postembryonic stages, whereas $dVsx^{B1}$ mutants died at the pharate adult stage. Pharate adults for each of the four *dVsx* alleles exhibited phenotypically normal external morphology and possessed no gross abnormalities in eye shape or size (data not shown). Thus, unlike *Chx10* in vertebrates, the *dVsx* genes are not required for the formation of the eye.

Although the external morphology of *dVsx1* adults appeared normal, mutants exhibited developmental defects as early as the first larval instar; the time taken to reach the molt to second instar from hatching was ~27 hr in heterozygous controls, ~35 hr in $dVsx^{6235}$ mutants, and ~42 hr in $dVsx^{A23}$ mutants. This phenotype is specific to *dVsx1* because $dVsx^{B1}$ mutants did not exhibit developmental delays.

In *dVsx1* Mutants, the Adult Optic Lobes Are Small and Disorganized

To determine whether *dVsx* function is required for optic-lobe development, we analyzed the brains of *dVsx* pharate adults. In $dVsx^{B1}$ mutants, which lack the *dVsx2* and *CG12730* genes, no gross abnormalities were detected in optic-lobe size or organization (Figures 4A and 4B). In contrast, in $dVsx^{A23}$, $dVsx^{8147}$, and $dVsx^{6235}$ mutants (which lack *dVsx1* function), the optic lobes were greatly reduced in size or even absent (Figures 4C–4F). To quantify the defects, we measured the relative size of the optic lobes as a normalized ratio of optic-lobe area over medial-brain area. Whereas no significant difference was observed between the ratios of wild-type (1.00) and $dVsx^{B1}$ (0.95 ± 0.10) brains, the $dVsx^{A23}$ (0.64 ± 0.22), $dVsx^{8147}$ (0.52 ± 0.22), and $dVsx^{6235}$ (0.43 ± 0.27) optic-lobe ratios were significantly smaller (Figures 4A–4F).

In addition to a decrease in size, we observed a high level of disorganization in *dVsx1* mutant optic lobes. In $dVsx^{B1}$ optic lobes, photoreceptor projections to the medulla were normal, indicating that *dVsx2* is not required for proper R7 and R8 targeting (Figure 4G). In contrast, in $dVsx^{6235}$ adult brains, the incoming photoreceptor axons exhibited marked projection errors in both the lamina and deeper layers of the optic lobe (Figures 4H and 4I). In the most severe cases, projections terminated deep in the brain (indicated by the arrow in Figure 4I), probably because of an extensive disruption in outer-optic-lobe organization. To assess the inner optic lobe, we performed immunostaining with the lobula-complex-cortex marker, anti-ACJ6. In $dVsx^{B1}$ control brains, ACJ6 expression was observed medial to the medulla in the lobula complex (Figure 4J). In $dVsx^{6235}$ mutant brains, ACJ6 expression was often shifted laterally and found adjacent to the lamina (Figures 4K and 4L), where the medulla is located in the wild-type brain. These data suggest that the lamina and lobula complex are present (though smaller and disorganized) but that the medulla is absent in these mutants.

The disorganization and hypocellularity observed in the optic lobes of *dVsx1* mutants—particularly in the medulla, which houses the inner photoreceptor target neurons—are phenotypes that are similar to those seen in the retina of vertebrates carrying null alleles of *Chx10*. These retinas are hypocellular and disorganized, and they lack the bipolar cells onto which photoreceptors synapse [8].

dVsx1 Is Required for OOA Progenitor Proliferation

We next investigated whether the reduced size of *dVsx1* mutant brains was due to a requirement for *dVsx1* in OOA progenitor proliferation. In $dVsx^{6235}$ mutants, the third-instar larval OOA was dramatically reduced in size (compare Figures 5B and 5D with Figures 5A and 5C), and, in some brains, was not visible at all (denoted with asterisks). In contrast, the IOA appeared grossly normal (compare Figures 5B and 5D with Figures 5A and 5C). These phenotypes are concordant with those observed in the adult (Figure 4).

In $dVsx^{6235}$ stage 16 embryos, the OOA appeared normal in shape and size, suggesting that *dVsx1* is not required for the specification, invagination, or early development of the optic primordium (Figures 5E and 5F). However, soon after the resumption of optic-progenitor proliferation in early-second-instar larvae, defects were observed in the OOA, which was hypoplastic and disorganized, with the arms of the outer anlagen failing to form the U-shaped structure seen in controls (compare Figure 5G with Figure 5H). This hypocellularity could be a consequence of decreased proliferation, increased apoptosis, and/or precocious neuronal differentiation of the outer optic progenitors. Apoptosis was excluded because cleaved-Caspase-3 expression was not detected in the OOA of $dVsx^{6235}$ mutants (Figure S7). Moreover, the expression of the neuronal marker ELAV in and around the optic anlagen was similar to that seen in sibling controls (Figure S8), indicating that the outer progenitors were not differentiating prematurely.

To investigate whether a defect in proliferation underlay the hypocellular OOA phenotype, mutant and control brains were immunostained with the mitotic marker anti-phospho-histone H3 (PH3). The number of PH3-positive cells per OOA was decreased more than 5-fold in $dVsx^{6235}$ mutants compared to heterozygous controls (Figures 5I and 5J, and Table S1). The OOA cells that continue to proliferate in *dVsx* mutants may be the subset of progenitors that do not express DVSX1 in the wild-type OOA. PH3 levels were also decreased in the IOA and the more medial region of the brain of $dVsx^{6235}$ mutants, although to a lesser degree (~2.5-fold), consistent with the observation that these structures were slightly smaller in $dVsx^{6235}$ mutant adults.

We conclude that *dVsx1* is required for OOA progenitor proliferation, consistent with the role of *Chx10* in the neuroretinal progenitors of vertebrates. This finding, taken together with the observation that the progenitors in both flies and vertebrates first undergo symmetric divisions and then, later, switch to asymmetric divisions to generate photoreceptor target neurons [5, 20, 30], suggests that the OOA progenitors of flies and the neuroretinal progenitors of vertebrates may be evolutionarily related. It is worth noting, however, that a key

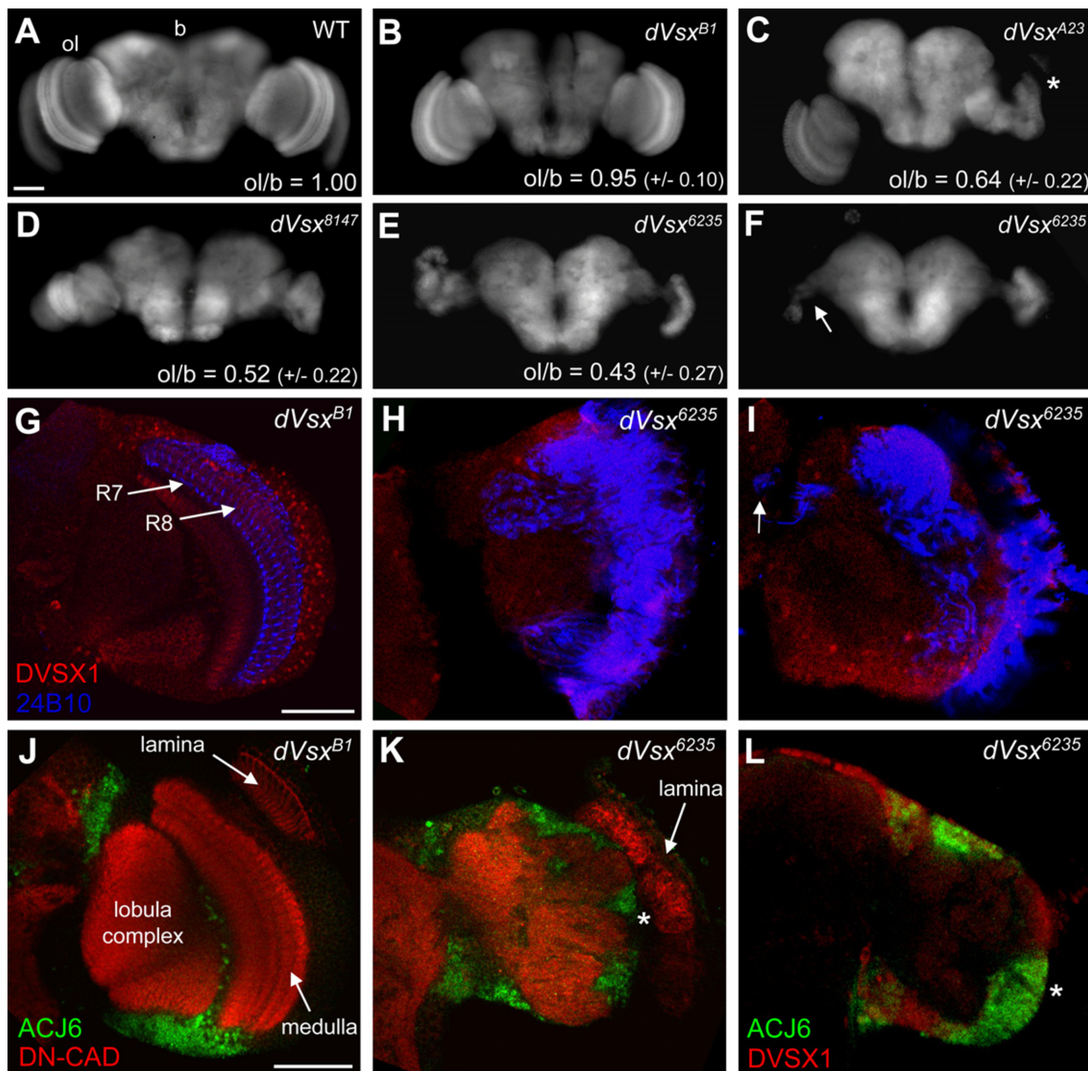


Figure 4. The Adult Optic Lobe Is Small and Disorganized in *dVsx* Mutants

Immunostains of adult brains.

(A–F) *dVsx1* mutant optic lobes are small. Representative fluorescence micrographs of brains immunostained with the neuropil marker anti-DN-CADHERIN.

(A) Wild-type (WT) control. The ratio of the area of the optic lobe neuropil (ol) over the area of the non-optic brain neuropil (b) is normalized to 1.00 for the control (n = 35).

(B) *dVsx*^{B1} optic lobes are normal in size, ratio = 0.95 (n = 19). The standard deviation is given in brackets.

(C) *dVsx*^{A23} optic lobes are reduced in size, ratio = 0.64 (n = 10). Note that within the same brain one optic lobe appears normal, whereas the other is very small (denoted by the asterisk).

(D) *dVsx*^{8147} optic lobes are reduced in size, ratio = 0.52 (n = 13).

(E and F) *dVsx*^{6235} optic lobes are very small, ratio = 0.43 (n = 24). In some cases, the optic lobe is completely absent (denoted by the arrow in [F]).

(G–I) Photoreceptor targeting is severely disrupted in *dVsx*^{6235} mutant optic lobes. Immunostains with anti-DVSX1 and the photoreceptor axon marker, anti-MAb24B10.

(G) In the control (*dVsx*^{B1}) medulla, R7 and R8 photoreceptors target the M6 and M3 layers, respectively.

(H and I) Two confocal slices of a *dVsx*^{6235} mutant optic lobe. Photoreceptors exhibit severe projection errors. Some axons project to the deeper layers of the brain (denoted by the arrow).

(J–L) The medulla is small or absent in some *dVsx*^{6235} mutant optic lobes. Immunostains with anti-DVSX1 and anti-ACJ6, a marker for the lobula complex.

(J) In control (*dVsx*^{B1}) optic lobes, ACJ6 marks the lobula-complex cortex, which lies medial to the medulla.

(K and L) Examples of *dVsx*^{6235} mutant optic lobes with (K) and without (L) the lamina attached. ACJ6 staining shifts to the lateral edge of the optic lobe in these mutants (denoted by asterisks), suggesting that the lobula complex is present but the medulla is small or absent. Note that the lamina is disorganized in (K). Scale bars represent 50 μm.

difference between these vertebrate and fly progenitor populations is that the vertebrate RPCs give rise to all of the neurons of the retina, whereas the OOA of the fly does not generate the photoreceptors in the compound eye or the neurons of the lobula complex.

Photoreceptor Target and Projection Neurons of the Vertebrate Retina and Fly Optic Lobe Share the Expression of Key Regulatory Genes

We next searched for additional regulatory genes that may be expressed in both bipolar cells and transmedullary neurons.

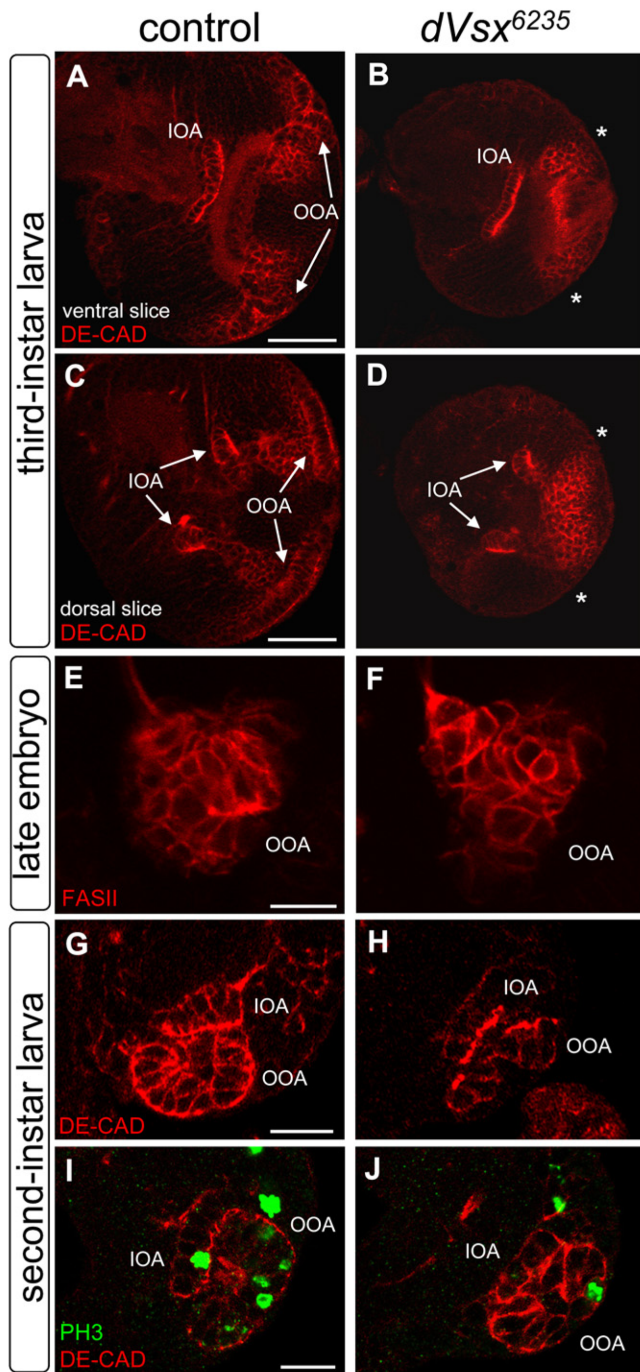


Figure 5. The OOA Is Hypoplastic and Exhibits Proliferation Defects in *dVsx*^{6235} Mutants

(A–D) The late-third-instar OOA is dramatically reduced in size in *dVsx* mutants.

(A and C) Ventral (A) and dorsal (C) slices of a control (*dVsx*^{B1}) brain. In (A), the base of the IOA arc is visible, as is the OOA at the lateral edges. In (C), the arms of the IOA are visible as are the lateral arms of the OOA.

(B and D) Ventral (B) and dorsal (D) slices of a *dVsx*^{6235} mutant brain. The IOA appears normal in shape and size, whereas the OOA is not visible in either the ventral or dorsal slices (asterisks denote where the OOA should be seen). Also, note that the entire brain is smaller in *dVsx*^{6235} mutants.

(E–J) Heterozygous controls (E, G, and I) and *dVsx*^{6235} mutants (F, H, and J) were analyzed.

(E and F) Anti-FASII immunostains of stage 16 embryos. The OOA is normal in size and shape in *dVsx*^{6235} mutants.

(G and H) Larval brains from the beginning of the second instar, immunostained with anti-DE-CAD. The *dVsx*^{6235} mutant OOA is hypocellular and fails to form the characteristic U shape seen in the wild-type brain.

(I and J) Larval brains from the beginning of the second instar, immunostained with anti-DE-CAD and the mitotic marker anti-PH3. The number of PH3-positive cells is decreased in the OOA, IOA, and remainder of the brain of *dVsx*^{6235} mutants. Scale bars represent 44 μm in (A) and (C), 10 μm in (E), and 20 μm in (G) and (I).

Lhx3 and *Lhx4* encode LIM-homeodomain transcription factors that are expressed in developing vertebrate bipolar cells [31]. The *Drosophila* ortholog of the *Lhx* genes is *apterous*, and, in late-third-instar brains carrying a *lacZ* reporter for the *apterous* gene, *apterous*-driven β-Galactosidase expression was observed in the medulla cortex (Figure 6A). Nascent *apterous*-positive cells did not express DVSX1, whereas older cells did, suggesting that only maturing cells coexpress both proteins. In the adult medulla, DVSX1 continued to be expressed in ∼30% of *apterous*-positive cells (n = 386), and ∼67% of DVSX1-positive neurons were *apterous*-positive (n = 173) (Figures 6B and 6B′). DVSX1-positive neurons that coexpressed *apterous* included the photoreceptor-targeted Tm5 transmedullary neuron [28]. Coexpression of the two proteins in bipolar cells and transmedullary neurons further supports the argument that these neurons are evolutionarily related cell types.

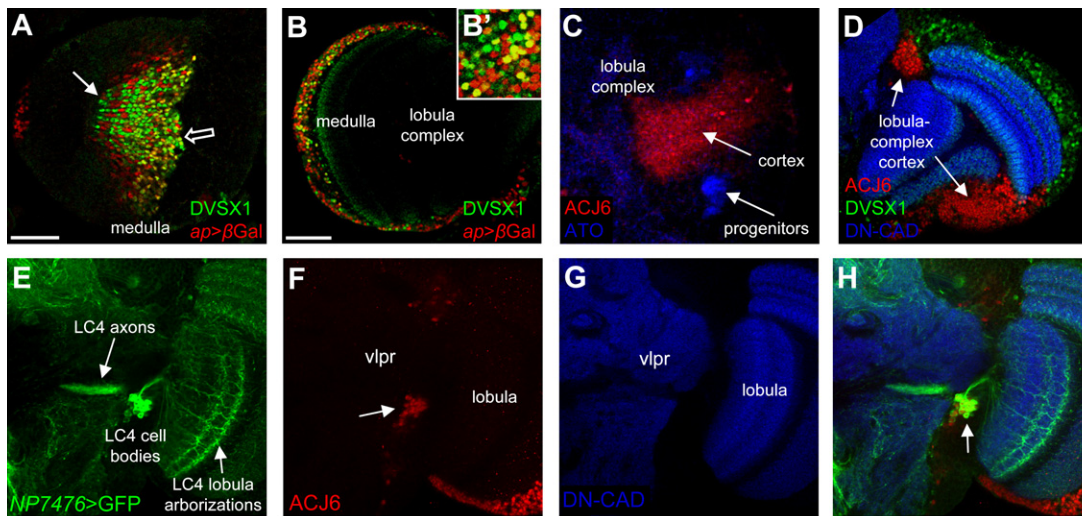
We next investigated the possibility that shared cell-type homologies in the visual system's neuronal circuitry may also extend to the ganglion cells and lobula projection neurons; both cell types receive synaptic input from photoreceptor target interneurons, and both project to the higher-order visual-processing center in the brain [4, 18]. In vertebrates, ganglion cell specification and differentiation requires the activity of the *Math5* and *Brn3b* genes, respectively [7]. *Math5* expression is restricted to the progenitors of the ganglion cells, whereas *Brn3b* is expressed in differentiating and adult cells. The *Drosophila* homologs of *Math5* and *Brn3b* are *atonal* (*ato*) and *acj6*, respectively. In the third-instar larva, ATO was expressed in the inner optic lobe's progenitor cells, which generate the neurons of the lobula complex (Figure 6C) [32]. ATO expression was restricted to these progenitors and was not observed in their developing or mature neuronal progeny (Figure 6C and data not shown). ACJ6 was expressed in the developing and mature lobula-complex cortex, but not in its progenitor cells (Figures 6C and 6D). Contrary to a previous report [33], ACJ6 expression was not observed in the lamina or the medulla. The widespread expression of ACJ6 in the lobula complex makes it likely that it is expressed in both local and projection neurons. An analysis of reporters for lobula-complex projection neurons indicated that ACJ6 is expressed in the cell bodies of LC4 projection neurons [18], which collect visual information from layers Lo2 and Lo4 of the lobula and project to the ventrolateral protocerebrum, a higher-order visual-processing center in the brain (Figures 6E–6H). Thus, like Brn3b in vertebrate ganglion cells, ACJ6 is expressed in projection neurons that transmit visual signals to the deeper regions of the brain.

The sequential expression of Math5/ATO and Brn3b/ACJ6 during the development of vertebrate ganglion cells and fly lobula-complex projection neurons suggests that these cell types are ancestrally related.

Discussion

The *dVsx* Genes Are Required for Optic-Lobe Development

DVSX1 is expressed in a central region of the OOA, where it is required for progenitor cell proliferation. The spatial restriction



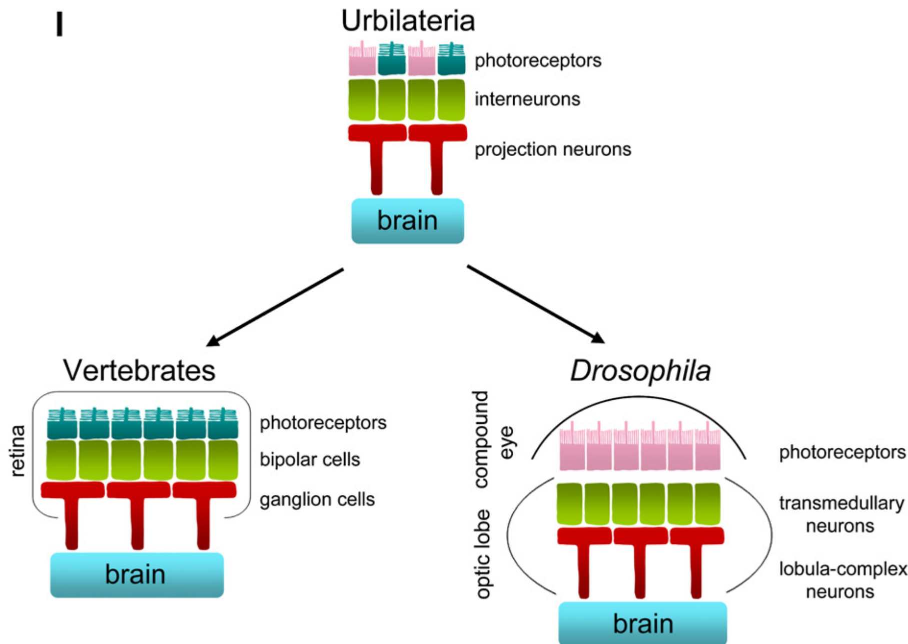


Figure 6. Vertebrate Retinal Bipolar and Ganglion Cells May Be Ancestrally Related to Neurons in the *Drosophila* Optic Lobe

(A, B, and B′) DVSX1 and *apterous*-driven β-Gal are coexpressed in a subset of cells in the medulla. *apterous* is the *Drosophila* homolog of the vertebrate *Lhx* genes.

(A) Late-third-instar larval brain. In nascent cells, there is no colocalization (denoted by the arrow) of β-Gal and DVSX1, but as cells mature, they express both proteins (denoted by the open arrow).

(B and B′) Adult brain. DVSX1 is expressed in ~30% of *apterous*-driven β-Gal-positive cells.

(B′) High-magnification view of the medulla cortex.

(C) Third-instar larval brain. ATONAL, the *Drosophila* homolog of Math5, is expressed in lobula-complex progenitor cells. ACJ6, the *Drosophila* homolog of Brn3b, is expressed in cells of the lobula-complex cortex.

(D) Adult brain. ACJ6 is expressed in cells of the lobula-complex cortex.

(E–H) ACJ6 is expressed in visual projection neurons that connect the lobula with the ventrolateral protocerebrum (vlpr). Adult brain carrying the *NP7476*-Gal4 and UAS-CD8-GFP transgenes.

(E and G) *NP7476*-Gal4 drives expression of GFP in the LC4 projection neurons that receive visual information from the lobula and target the vlpr.

(F) The arrow denotes ACJ6 expression in a cluster of cells located between the lobula and the vlpr.

(H) ACJ6 is expressed in the cell bodies of the LC4 projection neurons (denoted by the arrow). Scale bars represent 50 μm.

(I) Model for the evolution of the vertebrate and fly visual systems. Urbilateria contained ciliary and rhabdomeric photoreceptors, which targeted *Chx*- and *Lhx*-positive interneurons. These interneurons synapsed onto *Math5*- and *Brn3b*-positive projection neurons that targeted the brain. In the evolutionary line leading to vertebrates, the rhabdomeric photoreceptors were lost and the ciliary photoreceptors and their targets developed together in the retina. In the line leading to arthropods, the ciliary photoreceptors were lost. Rhabdomeric photoreceptors developed in the ectoderm and their neuronal targets developed in the brain.

of DVSX1 to the center of the OOA is unexpected because it has been assumed that the cells of the OOA constitute a homogeneous population of symmetrically proliferating progenitors [21]. Our data indicate that molecular and, perhaps, functional differences are present among OOA progenitor cells as early as the stage 16 embryo. It is possible that the DVSX1-positive central OOA cells and the DVSX1-negative peripheral OOA cells differ with respect to their cell-cycle kinetics and/or the neuronal populations that they generate. In vertebrate RPCs, *Chx10* regulates the expression of the cyclin-dependent kinase inhibitor, *p27Kip1*, and *CyclinD1* [34]. It will be important to determine whether *dVsx1* regulates the expression of the *Drosophila* homologs *dacapo* and *Cyclin D* in the progenitors of the OOA.

dVsx expression was observed in the transmedullary neurons that arborize in the M3 and M6 layers (Figure 3M), as well as those that arborize in layers targeted by the lamina monopolar neurons (Figure S5G), suggesting that the *dVsx* genes may function in both the color-vision and motion-detection pathways. A complete list of *dVsx*-positive transmedullary neurons will be required to determine whether they act as a bridge for all visual signals, as *Chx10*-positive bipolar cells do in the vertebrate retina. It is notable that not all *dVsx*-positive neurons project out of the medulla. Some are amacrine or intrinsic neurons that make only local connections within the medulla. These neurons are functionally analogous to the horizontal and amacrine neurons of the vertebrate retina, cell types in which *Chx10* is not expressed [8, 9]. We propose that the *dVsx*-positive amacrine and intrinsic cells may have evolved from an ancestral population of *dVsx*-positive transmedullary neurons. The observation that sets of transmedullary and local neurons exhibit highly similar arborization patterns [28] supports the hypothesis that these neurons may be ancestrally related.

Expression of the *dVsx* genes is absent in the lamina, which houses the target neurons of the outer photoreceptors R1–R6. The lack of *dVsx* expression in the lamina may be a product of the evolutionary history of the *Drosophila* photoreceptors. Phylogenetic and ontogenic considerations suggest that R8 represents the ancestral photoreceptor [35]. Because R8 terminates in the medulla, the fly neurons that are most closely related to the ancestral photoreceptor target neurons may be found in the medulla, not the lamina.

A Monophyletic Origin for Bilaterian Visual Systems

Debate regarding the phylogenetic origins of the bilaterian visual system is ongoing. The traditional view has held that eyes have evolved multiple times, an assertion that is supported by the existence of eyes with different morphologies, photoreceptor types, and embryonic origins [36, 37]. This view has been challenged by the surprising observation that morphologically disparate eyes rely on a conserved network of genes for their formation [1]. Recently, the polyphyletic view has been argued with data from *Platynereis*, a model for the last common ancestor of bilateria [3]. It has been proposed that the insect eye evolved from the rhabdomeric photoreceptors found in the eye of the last common ancestor, whereas the vertebrate eye evolved from the population of ciliary photoreceptors found in its brain. The rhabdomeric photoreceptors were then assimilated into the evolving retina, where they became ganglion cells.

Our identification of cell-type homologies between the vertebrate and fly visual systems supports a monophyletic origin for bilaterian visual systems (Figure 6I). We propose that the last common ancestor of flies and vertebrates, Urbilateria, contained *Vsx*-positive interneurons that relayed information from a mixture of ciliary and rhabdomeric photoreceptors to projection neurons that targeted the brain. These neuronal populations developed from progenitor cells dependent on the *Vsx* genes for their proliferation. In the evolutionary line leading to vertebrates, the rhabdomeric photoreceptors were lost, and the remaining ciliary photoreceptors and their neuronal targets developed together in the retina. In contrast, in the line leading to arthropods, the ciliary photoreceptors were lost, and the progenitors for the rhabdomeric photoreceptors and the deeper neuronal layers were separated during development. Consequently, the photoreceptors in arthropods develop in the ectoderm, whereas the progenitors for their target neurons invaginate and develop in the brain. The progenitors for the visual system interneurons remained *Vsx* dependent, whereas those for the photoreceptors and the projection neurons became *Vsx* independent.

The eyes of several extant organisms contain a mixture of ciliary and rhabdomeric photoreceptors, including the *Branchiostoma* cerebral vesicle and the larval eye of *Pseudoceros* [38]. Thus, it is possible that the eyes of Urbilateria contained a mixture of both photoreceptor types and that the situation in *Platynereis* is derived rather than ancestral. Our proposal that genetic and functional homology extends to the lobula projection neurons and ganglion cells is at odds with the suggestion, based partly on the shared expression of melanopsin, that vertebrate ganglion cells and fly rhabdomeric photoreceptors are ancestrally related cell types [3]. Because only a very small subset of ganglion cells express melanopsin, which is also expressed in several other retinal cell types [36, 39], expression of this photopigment in rhabdomeric photoreceptors and ganglion cells may be coincidental. It will be important to determine where the homologs of the *Vsx*, *Lhx*, *Math5*, and *Brn3b* genes are expressed in extant models of Urbilateria, such as *Platynereis* or the flatworm *Macrostomum* sp [40].

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, eight figures, and one table and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/17/1278/DC1/.

Acknowledgments

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