Differential Expression of DSCAM Guides the Patterning of Retinal Axons Along Their Path and at Their Target in the Developing *Xenopus* Visual System

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Abstract

Background

The *Xenopus* retinotectal circuit is organized topographically, where the dorsal-ventral axis of the retina maps respectively on to the ventral-dorsal axis of the tectum; axons from the nasal-temporal axis of the retina project respectively to the caudal-rostral axis of the tectum. Studies throughout the last two decades have shown that mechanisms involving molecular recognition of proper termination domains are at work guiding topographic organization. Such studies have shown that graded distribution of molecular cues is important for topographic mapping. However, the molecular cues organizing topography along the developing optic nerve, and as retinal axons cross the chiasm and navigate towards their target in the tectum, remain unknown. Down syndrome cell adhesion molecule (DSCAM) has been characterized as a key molecule in axon guidance, making it a strong candidate involved in the topographic organization of retinal fibers along the optic path.

Methods

Using a combination of whole-brain clearing and immunohistochemistry staining techniques we characterized DSCAM expression and the projection of ventral and dorsal retinal fibers starting from the eye, followed to the optic nerve into the chiasm, and into the terminal target in the optic tectum in *Xenopus laevis* tadpoles. We also assessed the effects of DSCAM on the establishment of retinotopic maps through spatially and temporally targeted DSCAM knockdown on retinal ganglion cells (RGCs) with axons innervating the optic tectum.

Results

Highest expression of DSCAM was localized to the ventral posterior region of the optic nerve and chiasm; this expression pattern coincides with ventral fibers derived from ventral RGCs. Downregulating DSCAM levels affected the segregation and proper sorting of medial axon fibers, derived from ventral RGCs, within the tectal neuropil, indicating that DSCAM plays a role in retinotopic organization.

Conclusion

These findings together with the observation that DSCAM immunoreactivity accumulates on the primary dendrites of tectal neurons indicates that DSCAM exerts multiple roles in coordinating retinotopic order and connectivity in the developing vertebrate visual system.

Background

During embryonic eye development, connections from the retina to the brain are carefully arranged in a preserved spatial manner that creates a topographic map of the visual world. In the amphibian visual system, retinal ganglion cell (RGC) axons project to the tectum in a manner that mirrors the relative positioning of RGCs across the retina – effectively constructing a point-to-point representation of visual
space in the brain (1-3). The formation of precise topographic maps requires active molecular cues guiding specific axon targeting and establishing selective synaptic connections. For example, in the developing embryonic *Xenopus* visual system, dorsal retinal axons expressing high levels of Ephrin-B ligands specifically target ventral tectal regions with high EphB receptor expression via an attractive guidance mechanism (4). Such studies demonstrate that molecular recognition of proper termination domains, often organized in matching gradient distribution, are important for topographically organizing neuronal circuits during development. Likewise, in mouse models, topographic mapping of retinal axons along the anterior-posterior axis of the superior colliculus (equivalent to the tectum in lower vertebrates) relies heavily on repulsive-mediated signaling between EphA receptors and their Ephrin-A ligands (5-7). Disrupting the signaling gradient either by knocking out the receptor or the ligand affects topographic ordering, but not entirely (5-7). Disruption of ephrin signaling, only to a certain extent, shifts axonal fibers posteriorly and others anteriorly (8). Furthermore, prior to reaching the tectum, retinal axon fibers are already topographically sorted along the optic nerve where graded ephrin signaling has not been reported (9-13). These findings suggest that ephrin signaling does not exclusively shape topography and that additional key molecules are involved. The molecular cues organizing topography along the developing optic nerve and as retinal axons cross the chiasm, remain unknown.

Down Syndrome Cell Adhesion Molecule (DSCAM) has been implicated in multiple aspects of neural circuit development, modulating dendrite and axon growth in both the vertebrate and invertebrate nervous systems (14). A specific role for DSCAM in axon growth, fasciculation and guidance is supported by a number of studies (15-19), but whether the molecule is involved in the topographic organization of retinal fibers had yet to be investigated. Multiple studies have confirmed that DSCAM is expressed by RGCs and in retinal projections along the developing mouse optic nerve (15, 20-22). Erskine and colleagues showed that DSCAM knock out disrupted the timing at which mouse retinal axons arrived at the thalamus, suggesting that DSCAM acts as a permissive signal and mediates growth-promoting interactions that help facilitate retinal axon growth towards their target (15). DSCAM was also shown to be involved in segregating contralateral retinal projections from ipsilateral fibers in the dLGN (20). While these studies did not directly test DSCAM’s involvement in organizing retinal topography, they indicate that DSCAM may contribute to the specificity of axonal wiring within the target. Previous work from our laboratory showed that in *Xenopus*, DSCAM acts as a permissive signal that facilitates axon arbor growth once RGC axons reach their target in the optic tectum. Here, we used the *Xenopus* tadpole visual system to further examine potential roles for DSCAM in establishing retinotopic order as axons travel towards and establish synaptic connections at their target. We observed differential DSCAM expression along the ventral and posterior regions of the optic nerve and chiasma, indicating that a subpopulation of retinal fibers express DSCAM as they navigate the optic path. By tracing the projection of ventral and dorsal retinal fibers as they exit the eye into the optic nerve and chiasm in fixed tadpoles, and imaging in real time retinal axon arbors in the *Xenopus* optic tectum with altered DSCAM expression, we provide evidence that DSCAM affects the segregation and proper sorting of axon fibers derived from ventral RGCs, both along the optic nerve and within the tectal neuropil, indicating that DSCAM plays a role in retinotopic
organization of RGC axons. Our work, for the first time, shows DSCAM having multiple direct roles in coordinating retinotopic order in the developing vertebrate central nervous system.

**Methods**

**Animals**

*Xenopus laevis* embryos were obtained via natural mating between adult male and female frogs. Adult frogs of both sexes were primed with human chorionic gonadotropin (10,000 units; Millipore Sigma) before natural mating. Embryos and tadpoles were raised in rearing solution (60 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO$_3$)$_2$, 0.83 mM MgSO$_4$, 10 mM HEPES, pH 7.4, and 40 mg/L gentamycin) supplemented with 0.001% phenylthiocarbamide (PTU) to prevent melanocyte pigmentation. All embryos were anesthetized during experimental manipulations with 0.05% tricane methanesulfonate (Finquel; Argent Laboratories, Redmond, WA). Staging of embryos was performed according to Nieuwkoop and Faber (23). Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine (Animal Welfare Assurance Number A341601).

**Immunohistochemistry**

Stage 45-46 *Xenopus laevis* tadpoles were euthanized with tricaine methanesulfonate (Finquel MS-222) and fixed in 4% paraformaldehyde in 1x PBS, pH 7.5, for 4 hrs. Tadpoles were cryoprotected in 30% sucrose for 1 hr at room temperature, and embedded in OCT compound (Sakura Finetek, Torrance, CA, USA). 40-μm coronal and horizontal cryostat sections were obtained and washed with phosphate buffered saline + 0.01% Tween-20 (1x PBST) 3 times, 5 minutes each. Sections were then blocked, for 1 hr, using 10% normal goat serum (Antibodies Incorporated) in 1x PBST. Blocking solution was removed and sections were incubated overnight with an antibody against the middle region of human DSCAM (rabbit polyclonal, 1:1000 dilution; Aviva System, San Diego, CA, USA) and 3A10 mouse anti-neurofilament-associated protein antibody (1:2000; Developmental Studies Hybridoma Bank) in blocking solution (2% normal goat serum in PBST). Brain tissues were washed then incubated in goat anti-rabbit Alexa 568 secondary antibodies (1:500 dilution; Invitrogen, Eugene, OR, USA). Sections were washed prior to being coated with DAPI. The mean fluorescence intensity of DSCAM and 3A10 antibody staining was measured using a circular region tool on the program ImageJ (National Institutes of Health). Repeated measurements were made using a defined circular region of interest (ROI; 3.5 mm diameter) tool at various anatomical locations of the sample being analyzed.

**Whole Brain Clearing**

A *Xenopus*-Fast Clearing Technique (X-FaCT) was performed as described in the protocol by Affaticati and colleagues (24) to reduce light scattering throughout the brain tissue. In brief, stage 45 to 46 tadpoles were euthanized with Finquel and fixed in 4% paraformaldehyde in 1x PBST overnight. Tadpoles were washed in 1x PBST and whole heads were dissected. Tissues were first placed in pre-incubation solution 0.5× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.2), 0.1% Tween 20, and were then incubated in
depigmentation solution (5% formamide, 0.5× SSC, 3% H₂O₂) to remove melanocyte pigmentation. Samples were transferred into a 2 mL glass vial and were blocked for 4 hrs at room temp. To visualize both DSCAM immunoreactivity and axon bundles in the tadpole’s head, tissues were incubated in DSCAM rabbit polyclonal (1:500; Aviva System) and 3A10 mouse anti-neurofilament-associated protein antibody (1:500; Developmental Studies Hybridoma Bank) in 10% DMSO, 1% Triton X-100 in 1x PBST. Goat anti-rabbit Alexa 568 and goat anti-mouse Alexa 488 antibodies (both at 1:500; Invitrogen) were used as secondary antibodies, respectively. To reduce light scattering throughout brain and head tissues, samples were submerged in a fructose–based high–refractive index solution (1.45) at room temp overnight. Cleared samples were imaged using a LSM780 confocal microscope (Zeiss).

Labeling Retinal Ganglion Cell Axons

To visualize retinotopic organization, ventral and dorsal RGCs axons were labeled by direct retinal electroporation following a similar protocol developed by Haas and colleagues (25). Tadpoles at stage 45 were anesthetized in diluted tricaine methanesulfonate. A custom-made trench, to hold the head of a stage 45 tadpole, was carved out in sylgard (Silicone Elastomer Kit). In the trench, a single embryo was placed laterally on their side and a standard size harp slice grid (ALA Scientific Instruments) was used to hold the embryo in place. The tadpole’s right eye was positioned and made available for electroporation. Lissamine-tagged standard control morpholinos oligonucleotides (Gene Tools) were electroporated into the ventral quadrant of the retina to label ventral retinal axons, followed by electroporation of fluorescein-tagged control morpholinos or Alexa Fluor 488 fixable dextran (10,000 MW, Invitrogen) into the dorsal quadrant of the retina to label dorsal axon fibers. Fluorescein-tagged control morpholinos were used for histology because they labeled axon fibers better; Alexa 488 fixable dextran labeled axon arbors better for in vivo imaging. To alter DSCAM levels in ventral RGCs, a morpholino antisense oligonucleotide (MO) targeting *Xenopus laevis Dscam* mRNA was designed with the sequence 5’-ACATAAGACCTTCGACAGACGT-3’. Individual reagents were loaded into an aluminosilicate glass electrode (with filament; AF100–64-10, 1.00 mm, 0.64 mm, 10 cm) equipped with a silver wire connected to a Grass SD9 electrical stimulator. An external ground wire, connected to the stimulator, was placed in the sylgard trench dish holding the anesthetized tadpole. For lissamine-tagged MO electroporation, repeated currents were delivered at 200 Hz, 2 ms delay, 2 ms duration, 20 V until ventral RGCs were labeled. Fluorescein-tagged control MOs or Alexa 488 fixable dextran was delivered at 200 Hz, 4 ms delay, 4 ms duration, 40 V until dorsal RGCs were stained. Tadpoles with axons that were labeled properly were used for histology or in vivo imaging. We quantified the territory occupied by laterally-projecting arbors (derived from dorsal RGCs) and the medially-projecting arbors (derived from ventral RGCs) using MetaMorph (Universal Imaging, West Chester, PA). Medial and lateral arbor width was measured at the center point of both groups of arbors, respectively. Unpaired t-tests were used for statistical analysis as described previously (26). Data results were considered significant in comparison to control as follows: *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.001, unless otherwise indicated on the graph with bars marking additional significant comparisons.
Results

Specific Expression of DSCAM in the Developing Xenopus Optic Nerve, Chiasm, and Tectum

Previous work from our laboratory showed that DSCAM immunoreactivity localizes to the plasma membrane surface of both RGCs within the retina and neurons in the optic tectum in *Xenopus* tadpoles at stage 45 (27). Without permeabilization, punctate DSCAM immunoreactivity was localized to the tectal neuropil where retinotectal axons and dendrites establish functional synaptic connections at this stage. These observations led us to further characterize DSCAM expression patterns across the *Xenopus* visual system as a means to inform us about its roles in the structural development of retinotectal circuits. For these experiments, we permeabilized tissues and co-immunostained sections of stage 45 to 46 tadpoles with antibodies to DSCAM (as in our previous study (27)) together with the anti-neurolament protein antibody (Mab 3A10) that has been shown to label a subset of retinal axons (28). As observed on coronal tissue sections, DSCAM was highly expressed at the ventral region of the optic nerve (Fig 1a, b). The mean fluorescence intensity of both DSCAM and 3A10 immunoreactivity were measured across the ventral-dorsal axis of the optic nerve bundle (measurements started at the ventral side of the optic nerve then continued to the dorsal side as marked by the white dotted line on Fig 1b). DSCAM immunofluorescent signal progressively decreased as measurements were obtained along the dorsal regions of the optic nerve. We plotted the mean fluorescence intensity on a graph with the x-axis representing regions along the ventral to dorsal portions of the optic nerve (Fig 1d). This analysis quantitatively confirmed a high-ventral to low-dorsal graded pattern of DSCAM expression. In contrast, 3A10 labeled axons were strongly localized to the dorsal region of the optic nerve (border noted by white dotted line on Fig 1b) with lower intensity signal along the ventral side. 3A10 immunofluorescence exhibited a low-ventral to high-dorsal distribution (Fig 1d) in what appeared to be an inverse of the high-ventral to low-dorsal DSCAM immunoreactivity pattern. Analysis of retinal axons as they crossed the midline at the optic chiasm (white arrowhead, Fig 1c) showed that DSCAM immunoreactivity strongly localized at the ventral base of the chiasm. Here again, the intensity of DSCAM immunostaining was gradually decreased at more dorsal areas of the optic chiasm (white dotted line, Fig 1c, e). 3A10 immunoreactivity in axonal fibers continued to show a low-ventral to high-dorsal fluorescence intensity along the optic chiasm (Fig 1c, e).

Having encountered differential DSCAM expression along the ventrodorsal axis of the optic nerve and chiasm, we further characterized the distribution of DSCAM along the posteroanterior axis. In horizontal tissue sections, we found higher DSCAM immunofluorescence intensity specifically at the posterior region of the optic nerve bundle (Fig 2a) and optic chiasm (white arrowhead, Fig 2b). DSCAM immunofluorescence intensity was lower at the anterior portions of the optic nerve and chiasm compared to the posterior side, indicating a high-posterior to low-anterior pattern of DSCAM expression (Fig 2 c, d). Immunostaining with the 3A10 antibody showed an inverse pattern, where the fluorescence distribution of 3A10 staining appeared lower posterior and higher anterior at both the optic nerve and chiasm (Fig 2 a-d). It is important to note that we did observe DSCAM expression along a number of fibers stained with 3A10 (white arrows, Fig 2a), confirming that the DSCAM immunostaining identified RGC axon fibers.
These results suggest that a subpopulation of RGC axon fibers rely on DSCAM as a potential mechanism to navigate the optic nerve pathway and cross the optic chiasm.

To further determine if axonal arbors terminating and branching in the optic tectum express DSCAM in a pattern similar to the high-ventral to low-dorsal pattern found along the optic nerve and chiasm, we performed immunostaining of whole brain cleared tissues to preserve the structural layout of axonal tracts and arbors innervating the tectum. Compared to brain sectioning, brain clearing is a powerful technique that permits obtaining a novel three-dimensional perspective of any potential gradient pattern of DSCAM expression within the intact tectum (24). Cleared tissue samples of stage 45 to 46 tadpoles were immunostained with DSCAM and 3A10 antibodies (Fig 3a). Our results revealed that the optic nerve (solid white and yellow arrowhead, Fig 3a), as well as sensory and motor cranial nerves throughout the tadpole head were stained by the 3A10 antibody (Fig 3a). Even at low magnification, we were able to observe DSCAM immunoreactivity along the optic nerve throughout its full extent up to the optic chiasm (solid white and yellow arrowhead, Fig 3a, b). At the optic tectal neuropil, strongest DSCAM immunoreactivity coincided with axon terminals labeled with the 3A10 antibody (empty white and yellow arrowheads at the tectum, Fig 3a). When we examined individual horizontal z-stacks at the level of the neuropil, the fluorescence intensity of DSCAM immunoreactivity was higher along axon terminals as revealed by the 3A10 co-immunostaining (solid white and yellow arrowheads, Fig 3b) and lower at fasciculated axon bundle tracts as they enter the neuropil (empty white and yellow arrowheads, Fig 3b). To better outline DSCAM expression patterns, whole intact brain tissues were dissected, cleared, and immunostained with the DSCAM and 3A10 antibodies. When viewing the orthogonal planes of the confocal images of cleared brain tissues, we found that DSCAM immunoreactivity colocalized with the 3A10 retinal axon marker both at the x- and y-orthogonal planes (solid white and yellow arrows, Fig 3c). The mean average fluorescent intensity of DSCAM expression along axon terminals were more substantially elevated compared to the DSCAM signal along axon fiber tracts (Fig 3d).

It is important to note that DSCAM expression along the optic tectum is distinct compared to other brain regions of the *Xenopus* CNS. Noticeably, strong uniform DSCAM expression was found in the olfactory bulb and telencephalic regions of the tadpole brain (white and yellow arrowhead at OB and TEL, Fig 4a, b) where spiny neurons are located (29). A prominent pattern of DSCAM immunoreactivity was detected along axonal tracts at the lateral regions of the hindbrain (white and yellow arrowhead at the HB, Fig 4a, d). We quantitatively measured the mean fluorescent intensity of DSCAM of two intact tadpole heads at various regions of the *Xenopus* brain. DSCAM immunoreactivity at the olfactory bulb (Fig 4a, b, e) was higher in intensity than in the telencephalic brain region, the hindbrain, and the optic tectum. Similar to the patterns observed in dissected brain tissues (Fig 3c), the intensity of DSCAM immunostaining was higher in RGC axons at their axon terminals and lower at axonal tracts entering the neuropil (Fig 4c, e). These results suggest that DSCAM is differentially expressed or transported throughout the CNS.

To determine whether the patterns of DSCAM expression in the optic nerve, chiasma and in the optic tectum correspond with differential DSCAM expression within the retina, we analyzed retinas of
stage 45 tadpoles in cleared intact tissues and cryostat sections immunostained with DSCAM and the 3A10 antibodies (Fig 5a, b). As previously shown, DSCAM immunoreactivity was observed in the ganglion cell layer (GCL), inner plexiform layer (IPL) and inner nuclear layer (INL) of the Xenopus retina (27), with punctate DSCAM expression found around cell bodies within the GCL (Fig 5b). The majority of cell bodies immunostained with the 3A10 antibody localized to the GCL, adjacent to the IPL. However, as observed both in coronal sections and in cleared intact eyes, not all RGCs were immunopositive for 3A10 (Fig 5a, c) indicating that only a subset of RGCs express neurofilament-associated proteins recognized by the 3A10 antibody. Analysis of coronal sections of retinas co-stained with DSCAM and 3A10 antibodies showed that some axon fibers exiting the eye along the optic fiber layer (Fig 5d) and the optic nerve head were immunopositive for both DSCAM and 3A10 (Fig 5e; empty arrowheads), although a number of 3A10 positive fibers did not express DSCAM (white arrowheads; Figs 5d, e). These observations reveal a differential pattern of expression of DSCAM by RGC axons as they exit the eye. Moreover, different subsets of RGCs, including those that differentially express DSCAM and neurofilament-associated proteins recognized by the 3A10 antibody, appear to organize in distinct topographic order as they navigate along their pathway to their target in the optic tectum.

Dorsoventral Axon Sorting in the Xenopus Retinotectal System and DSCAM Effects on Topographic Segregation at the Optic Tectum

A graded distribution of molecular cues has largely been implicated in topographic mapping. Based on its differential distribution, it is likely that DSCAM collaborates with other guidance and cell adhesion molecules in the topographic organization of axon retinal fibers at multiple points along their path (30). Indeed, analysis of a mouse model of Down syndrome showed that DSCAM regulates eye-specific segregation of retinogeniculate projections at the target, in the dorsal lateral geniculate nucleus (20). Thus, to explore whether DSCAM is directly involved in retinotopic organization in the Xenopus optic tectum, we first characterized the projection and ordering of ventral and dorsal retinal fibers as they travel from the eye through the chiasm and into the brain (as depicted schematically in Fig 6a). A scrambled control fluorescein-tagged MO (to serve as a green fluorescent marker) and a control lissamine-tagged MO (red fluorescent marker) were electroporated separately to label dorsal and ventral RGCs, respectively (Fig 6b, c). Our results show that ventral RGCs project axon fibers that are positioned along the ventral portion of the optic nerve, while dorsal RGCs send axon fibers along the dorsal region of the optic nerve (Fig 6d, e). As axons of both ventral and dorsal RGCs enter and cross the chiasm and turn contralaterally into the tectum, we observed a shifting of fiber arrangement, with lissamine MO-labeled axon fibers that were originally positioned on the ventral side of the optic nerve intermixing and positioning more dorsally in the optic chiasm (Fig 6d, f). This inverted projection was also observed for the fluorescein MO-labeled axon fibers that originate in the dorsal portion of the retina, shifting more ventrally (Fig 6f). A complete inverted arrangement was observed for axons as they innervate the tectum, with ventral RGC axons entering the tectum through the dorsal branch and dorsal RGC axons projecting ventrally within the tectum (Fig 6g) in agreement with previous studies (31, 32). Based on these results, our immunohistochemical data indicates that specific DSCAM expression along the ventral portion of the
optic nerve would coincide with axon fibers traveling on the ventral side of the optic nerve pathway prior to crossing at the tectum (Fig 1a and Fig 3b).

Analysis of axon terminals along the lateral-medial axis (as depicted schematically in Fig 7a), showed that ventral RGC axons (labeled with lissamine-tagged MO) innervate the tectum laterally, while dorsal RGC axons (labeled with Alexa 488 dextran) travel more medially, as shown for other species (9, 33). Indeed, ventral and dorsal RGC axons from tadpoles injected at stage 46 and imaged 48 hrs later showed correct topographic mapping but with a consistent degree of arbor overlap (average 20 μm) as shown in Fig 7b. When retinal neurons were labeled few days later, at stage 47, and imaged 48 hrs after, medial arbors were visibly separated from lateral arbors (data not shown). This separation between lateral and medial arbors in the Xenopus tadpole is consistent with observations in zebrafish larvae at 5 days postfertilization, when the optic tectum is first fully innervated (9). Thus these in vivo imaging studies confirm that in Xenopus, dorsal RGC axons projecting through the lateral branch initially overlap with ventral RGC axons traveling through the medial branch; then, as the tectum expands and arbors become more complex, laterally and medially projecting arbors remodel and clearly separate along the Xenopus neuropil.

To identify specific cellular actions of DSCAM in directing retinotopy in the tectum, we targeted the population of RGCs that preferentially express DSCAM to manipulate its expression when a majority of axons have already arborized in the tectum, but when medially and laterally projecting axons still overlap. For this, we electroporated a morpholino (MO) targeting Xenopus laevis Dscam mRNA to block translation and downregulate endogenous DSCAM levels in axons of ventral RGCs in tadpoles at stage 46, while also labeling dorsal axons with Alexa 488 dextran. This strategy allowed us to manipulate and visualize the innervation patterns and topographic organization of axon arbors in the neuropil rather than interfere with axon pathfinding or initial axon branching (27). As shown for control tadpoles, axons derived from ventral and dorsal RGCs were correctly sorted along the medial-lateral axis (Fig 7a, b), with ventral RGC axons predominantly arborizing in the medial portion of the neuropil and dorsal RGCs axons arborizing laterally. However, 48 hours after DSCAM MO injection, ventral RGC axon arbors were positioned more medially compared to controls (Fig 7b). To quantify this effect, we measured the area occupied by the axon arbors within the tectal neuropil; total arbor width from ventral RGCs injected with DSCAM MO was compared to that from ventral RGCs in tadpoles injected with control MO. The average arbor spread of axons positioned medially in tadpoles with DSCAM MO knockdown was significantly larger than controls (Controls 117.5 ± 9.73 μm, n = 6; DSCAM MO 150.2 ± 7.81 μm, n = 6, p = 0.0257, Fig 7c). Dorsal RGC axons labeled with Alexa 488 dextran projecting laterally within the tectum in either control MO or DSCAM MO treated tadpoles had the same arbor width independent of ventral RGC treatment (Controls 75.03 ± 8.723 μm, n = 6; DSCAM MO 78.85 ± 2.95 μm, n = 6, p = 0.6875, not significant, Fig 7d). As shown above, in stage 46 tadpoles there is a degree of overlap between medially-projecting ventral RGC axons (red marker) and laterally-projecting dorsal RGC axons (green marker) within the tectal neuropil (Fig 7b, e). At this stage, axons from ventral RGCs with DSCAM MO knockdown showed a significant reduction in lateral and medial arbor overlap compared to arbors in control MO treated tadpoles (Controls 22.02 ± 3.915 μm, n = 6; DSCAM MO 9.185 ± 2.193 μm, n = 6, p = 0.0169, Fig
When measuring the entire spread of the arbors along the medial to lateral axis for each group, tadpoles treated with DSCAM MO had RGC axons that occupied a larger territory within the neuropil (Controls 117.5 ± 9.733 μm width, n = 6; DSCAM MO 150.2 ± 7.808 μm, n = 6, *p = 0.0257, Fig 7f). When expressed as percentage overlap per total arbor territory in width the reduction in overlap for ventral axons with DSCAM MO knockdown was also significantly different from controls (Controls 18.82 ± 2.685 %, n = 6; DSCAM MO 6.135 ± 1.482 %, n = 6, **p = 0.0019, Fig 7g). Together, these findings suggest that changes in DSCAM expression in ventral RGC axons affect their projection patterns acting at the target where an increase in segregation of medial and lateral axons is observed in response to lowered endogenous DSCAM levels.

**Dendritic Localization of DSCAM in Post-synaptic Tectal Neurons**

Our previous work showed that downregulation of DSCAM expression in single RGCs interferes with axon growth and branching at the target, indicating that endogenous DSCAM acts as permissive cue that facilitates RGC axon growth. In contrast, single-cell downregulation or overexpression of DSCAM in tectal neurons showed that DSCAM acts as a restrictive cue to regulate the size and complexity of their dendritic arbors (27). Thus, in addition to RGCs, DSCAM can differentially influence postsynaptic neurons in the *Xenopus* visual system. Indeed, punctate DSCAM immunoreactivity can be detected not only within the *Xenopus* retina but also surrounding cell bodies in the tectum as well as in the tectal neuropil in unpermeabilized tissues (Fig 8a, b). To further differentiate DSCAM expression in tectal neurons, we electroporated embryos with a GFP plasmid, and at stage 45, tadpoles with isolated or small clusters of GFP-expressing neurons were fixed and immunostained for DSCAM. We found that DSCAM is not only expressed along the cell body surface of tectal neurons (white circle, Fig 8a), as previously shown (27), but is also expressed along primary dendrites and dendritic branches of tectal neurons (white arrows Fig 8a). Analysis of DSCAM immunostained tissues further revealed a unique pattern of expression, strongly labeling thin processes within the tectal neuropil (Figs 8 a, b; arrowheads). In some tadpoles, the random transfection and expression of GFP within brain neurons revealed strong DSCAM immunoreactivity along the primary processes of GFP-expressing cells that were positioned within the neuropil (Fig 8b). The identity of these cells in *Xenopus* is unknown, but they share similar morphology and features to tegmental projection neurons characterized in id2b transgenic zebrafish larvae that are found exclusively in the neuropil and have a prominent primary process that protrudes apically (34). Further experiments are needed to confirm their identity in *Xenopus* and potential roles for DSCAM in these neurons.

**Discussion**

Our previous studies explored cell-autonomous roles for DSCAM during the development of pre- and postsynaptic structural and functional connectivity in the developing *Xenopus* retinotectal circuit. We found that DSCAM primarily acts as a neuronal brake to limit and guide postsynaptic dendrite growth of tectal neurons while it also facilitates arborization of presynaptic RGC axons cell autonomously (27). In that study, we targeted ventral RGCs for our analysis since their axons are easier to visualize *in vivo* with confocal imaging as they project to the most dorsal part of the tectal neuropil (35). For this study, we
characterized the expression of DSCAM along the ventrodorsal axis of the optic nerve, and we followed the navigation of ventral and dorsal retinal axons corresponding to this expression. We found a specific pattern of DSCAM expression along the *Xenopus* optic nerve that correlated with how optic nerve fibers are topographically organized. Fasciculated bundles of ventral fibers derived from ventral RGCs normally navigate the optic nerve along its ventral side, which coincided with strong DSCAM expression. As fibers crossed the optic chiasm, we observed that DSCAM expression was decreased. This coincides with ventral and dorsal retinal axons rearranging topographically as fibers pass the chiasm and project contralaterally into the optic tract and optic tectum. DSCAM has been well characterized as a homophilic binding molecule mediating intracellular adhesion and the fasciculation of axon bundles (15, 36). The site and timing of expression suggests that DSCAM is involved, to some degree, in maintaining the ventrodorsal topography of optic nerve fibers and the spatial arrangement that mirrors how axons exit the optic nerve head. It is possible that through its adhesive properties and homophilic interactions, DSCAM serves to anchor ventral fibers together, preventing any rearrangement or interchange with dorsal axons as fibers navigate the optic pathway from the optic nerve head to the chiasm. Differential fasciculation of fibers along the optic nerve may be an underlying mechanism to traffic axons in an orderly manner to the chiasm. Organized arrival of axons at the site of the chiasm would allow axons to respond to the next set of guidance cues, including ephrins, other chemoattractant cues, and neurotrophic factors (28, 37-40), which all prepare for the subsequent stage of morphological trajectory into the tectum. We showed preferential DSCAM expression on ventral RGC axons and along the posterior region of the *Xenopus* optic chiasm as well (Fig 2a), a finding that is in agreement with observations of DSCAM expression in the posterior region of the mouse optic chiasm (15).

In addition to mechanisms organizing the topography and spatial arrangement of axon fibers, it is important to note that there are also time-based mechanisms involved that indirectly contribute to the topographic wiring of circuits. During *Xenopus* eye development, new retinal ganglion cells are generated at the ciliary margin located at the periphery of the eye (35, 41). Older cells are pushed towards the central portion of the retina and a gradient of maturing cells is created along the retina radius. Because of the temporal pattern of early eye development, the deployment of emerging RGC axons along the optic pathway is set to a defined temporal sequence. Dorsal retinal fibers exit the eye first, navigate the optic pathway, and reach the tectum six hours ahead of ventral retinal axons. The newer set of axon fibers exiting the eye travel along the most ventral portion of the optic nerve as innervation takes place (35, 42). It is possible that fasciculation of retinal fibers by DSCAM indirectly modulates the pacing of “younger” ventral axons along the optic nerve – perpetuating a difference in timing at which ventral and dorsal axons reach their target sites. In our previous work using real-time imaging of RGC axons as they innervate the optic tectum, we showed that DSCAM is important in promoting the branching rate of retinal axons *in vivo* (27), which supports the idea that DSCAM is involved in distinct temporal aspects of RGC axon development and differentiation.

Differential timing of retinotectal projections was initially thought to be the mechanism that generates topographic mapping in the optic tectum, with the argument that pioneering dorsal fibers innervate ventral areas in the tectum simply for arriving first at the available sites. This hypothesis stated
that ventral fibers of the retina would later follow and would be forced to occupy the next available sites at the dorsal area of the tectum, due to the constraints of existing dorsal axons (35). Studies, however, have shown that disrupting the timing of retinal axon deployment, by heterochronic transplantation of early age RGCs into older embryos, does not seem to affect the topographic mapping formed during development, indicating that other mechanisms are at work (35). It is becoming increasingly evident, based on a number of studies, that sub-populations of RGCs employ different molecular and cellular strategies to achieve axon-target specificity (43, 44). For example, sub-populations of RGCs heavily rely on repellant and attractive cues for precise axon targeting. In amphibians, populations of RGCs differentially express ephrin-Bs in a high dorsal to low ventral gradient in the retina (45, 46). This gradient pattern in the retina complements EphB1 receptors expression along the *Xenopus* tectum which is distributed in a high ventral to low dorsal gradient. Signaling between EphB1 receptors and ephrin-B ligands have been suggested to be the underlying mechanism that attracts dorsal retinal axons into the ventral portion of the tectum (45). The work we present in this study adds DSCAM to a growing of list of molecular strategies that retinal axons use to self-organize topographically along the optic nerve and within the target.

We observed that DSCAM expression diminishes after axons cross the optic chiasm and enter the optic tract, but then reemerges gradually at the tectal neuropil where retinal axons arborize and form connections with post-synaptic tectal partners. At this spatial gap where DSCAM expression is decreased, axons fibers are rearranged, most likely by pre- and post-synaptic molecular interactions mediated by Ephs/ephrin signaling, to reorient the topography of axons in an inverted manner, while also distributing their projection along the mediolateral axis in the neuropil. Here we tested specifically whether DSCAM, plays a role in sorting the arrangement of arbors across the mediolateral plane of the tectal neuropil independent of its effect on axon branching. We examined effects of DSCAM on the same population of RGCs (ventral) at a distinct time of development by timing the DSCAM MO knockdown by targeting the electroporation of tadpoles at different stages. We found that downregulating DSCAM expression in ventral RGCs with axons already terminating medially within the tectum caused an aberrant shift and extension of their terminal arbors away from those of dorsal RGCs, suggesting that DSCAM guides remodeling and topographic organization of arbors derived from ventral RGCs. During zebrafish development (which closely resembles *Xenopus* development), dorsal retinal fibers normally reach the optic tectum via the lateral branch, while ventral axons project via the medial branch (9). Disrupting mechanisms dependent on RNA-binding proteins, such as Hermes, causes an aberrant shift in topographic ordering and results in lateral dorsal axons projecting ectopically into the medial branch arbor (9). Thus, our studies indicate that DSCAM, together with other signaling molecules including Hermes, participate in the medio-lateral topographic mapping at the target.

During RGC axon arborization, coordinated addition and retraction of axonal branches and of dendrites of tectal neurons allows for a gradual recognition between pre- and postsynaptic partners which allows for new synaptic connections to be formed (47, 48). Additionally, bi-directional communication at the molecular level is also thought to be at work facilitating synaptogenesis. For example, neurotrophins, including brain-derived neurotrophic factor (BDNF), can act as a retrograde
signal to influence presynaptic neurons, while also acting as an anterograde factor on postsynaptic cells (47, 49). This type of bi-directional signaling can generally induce the development and maturation of synapses, or even modify the structure of existing synapses. Unpublished work from our lab shows that DSCAM localizes to only a sub-set of retinotectal synapses, suggesting that endogenous DSCAM, localized post-synaptically may be implicated in the stability and/or maintenance of synapses (R.A. Santos and S. Cohen-Cory, unpublished). Studies have shown that topographic arrangement of axons is also precisely organized at the synapse level. Studies both in mouse and in *C. elegans* indicate that graded inhibitory cues for synapse formation and maintenance are also used to restrict synapse distribution and create synapse topographic maps (50). Homophilic binding between DSCAM proteins in rodents mediates neurite adhesion, which helps facilitate precise synaptic targeting within a specific sub-lamina in the retina (51). DSCAM can also functionally interact with other cell-adhesion molecules, specifically cadherins and protocadherins, to “mask” their adhesive properties and consequently prevent neurite collision and fasciculation (52). In Aplysia, DSCAM acts trans-synaptically and in collaboration with AMPA-like receptors promotes synapse formation (53). In the developing *Xenopus* tadpole, visually driven *Ca*²⁺ signals are topographically organized at the subcellular dendritic scale in tectal neurons (33). Characterizing the spatial distribution of molecules, such as DSCAM, on both pre- and post-synaptic arbors to match their anatomical location along synapses remains open to investigation.

**Conclusion**

In the *Xenopus*, endogenous DSCAM acts at multiple levels along the visual circuit, independently modulating dendrite and axon arborization, where cell-autonomous roles vary depending on the cell type. Our current work demonstrates that DSCAM is also involved in retinotopic organization at distinct points along the retinotectal pathway, directing the topographic organization of retinal fibers as they travel along the optic nerve, in sorting and remodeling of axon arbors along the mediolateral axis within the neuropil, and in maintaining pre- and postsynaptic retinotectal arbors. The finding of selective localization of DSCAM protein in primary dendrites of a subpopulations of neurons in the neuropil opens the possibility of additional roles for DSCAM during neural circuit development.

**Abbreviations**

*CNS*: central nervous system; *DSCAM*: Down syndrome cell-adhesion molecule; *GCL*: ganglion cell layer; *INL*: inner nuclear layer; *IPL*: inner plexiform layer; *MO*: morpholino antisense oligonucleotide; *RGC*: Retinal ganglion cell.

**Declarations**

**Ethics approval and consent to participate**

All procedures were authorized by the University of California, Irvine Institutional Animal Care and Use Committee (IACUC) (Animal Welfare Assurance Number A3416-01) and were performed in accordance
with national and international standards for humane animal research as set forth by the National Institutes of Health, Institute of Laboratory Animal Research, USDA, and Assessment and Accreditation of Laboratory Animal Care, International.

**Consent for Publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

RAS carried out *in vivo* experiments and immunohistochemical studies and coordinated the experiments. RAS and SCC conceived of the study, participated in its design, analysis and figure preparation, and drafted the manuscript. RDR Jr, ADA, GB and BZV contributed to *in vivo* imaging experiments and analysis. All authors read and approved the final manuscript.

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**Figures**

**Figure 1**

**Distribution of DSCAM in a ventral-to-dorsal gradient along the optic nerve and chiasm of stage 45/46 *Xenopus* tadpoles.** (a) Coronal section of the *Xenopus* tectum and optic nerve immunostained with DSCAM (red; right and left panels) and 3A10 anti-neurofilament (green; left panel overlay) antibodies. (b) Higher magnification image of the optic nerve shows high DSCAM immunoreactivity ventrally along the optic nerve bundle, while 3A10 antibody preferentially stains fibers along the dorsal region of the optic nerve (overlay image; white dotted line). (c) Coronal section at the level of the optic chiasm (white arrowhead), shows DSCAM immunoreactivity localized to fibers at the ventral base of the chiasm (white arrowhead), while most 3A10 immunopositive retinal fibers cross the optic chiasm more dorsally (white dotted line). (d, e) The average fluorescence intensity of DSCAM and 3A10 immunoreactivity was measured along the ventrodorsal axis of the optic nerve and optic chiasm using a 3.5 μm diameter ROI using ImageJ. Seven measurements using the circular region tool were obtained along the ventrodorsal axis, with five sets of measurements obtained at distinct locations along the optic nerve or chiasm. (d) Plotting mean fluorescence intensity along the optic nerve revealed a high-ventral to low-dorsal distribution of DSCAM immunoreactivity while 3A10 immunoreactivity was higher medially and dorsally. (e) Quantification of the mean fluorescence intensity shows a high-ventral to low-dorsal distribution of DSCAM immunoreactivity at the optic chiasm. Scale bars: 40 μm for a; 20 μm for b, 50 μm c.

**Figure 2**

**Distribution of DSCAM in the optic nerve and chiasm along the posterior-to-anterior axis of stage 45/46 *Xenopus* tadpoles.** (a) High magnification horizontal section at the level of the optic nerve shows differential distribution of DSCAM immunoreactivity (red) along retinal axon fibers. A gradual distribution of DSCAM immunopositive fibers is observed from posterior to anterior while most 3A10 immunopositive fibers localize more anteriorly. White arrows identify a subset of axon fibers within the optic nerve that are immunopositive for both DSCAM and 3A10. (b) Horizontal section at the level of the optic chiasm shows DSCAM immunoreactivity in fibers that organize caudally (posterior; empty white arrowhead) to most 3A10 immunopositive fibers. (c, d) Fluorescence intensity of DSCAM (red) and 3A10 (green) immunoreactivity was measured using a 3.5 μm ROI at six positions along the posterior to anterior axis.
tool as for Fig 1, with ten sets of measurements made at distinct locations along the optic nerve or optic chiasm. Mean fluorescence intensity is shown with the x-axis designating the optic nerve or optic chiasm from the posterior to anterior regions. (c) A high-posterior to low-anterior distribution of DSCAM fluorescent signal is found along the width of the optic nerve, while 3A10 fluorescent signal is higher in the anterior portion of the optic nerve. (d) As for the optic nerve, measuring DSCAM and 3A10 fluorescence showed a high-posterior to low-anterior distribution of DSCAM immunopositive signals within the optic chiasm, while 3A10 fluorescent signal was low posterior and increased anteriorly. Scale bars: 10 μm for a; 50 μm c.

Figure 3

Visualizing DSCAM expression in cleared *Xenopus* brain tissues (a, b) Whole tissue clearing followed by immunostaining was used to further characterize DSCAM expression in intact *Xenopus laevis* tadpoles. (a) DSCAM immunoreactivity (red, DSCAM only; right panel) along the optic nerve (solid yellow arrowhead) and within the midbrain (empty yellow arrowheads) co-localized with 3A10 antibody staining (green, 3A10 and DSCAM overlay) of optic nerve fibers (solid white arrowhead) and RGC axon terminals within the tectal neuropil (empty white arrowheads). In addition to the optic nerve, the 3A10 antibody stains axonal fibers in sensory and motor cranial nerves. (b) Individual confocal planes from horizontal z-stacks further illustrate co-localization of DSCAM and 3A10 immunoreactivity in the midbrain neuropil (from dorsal-left to ventral-right). Stronger DSCAM immunoreactivity is observed on the dorsal-most portion of the tectum, where axon terminals extensively branch (solid white and yellow arrows). The 3A10 antibody staining also reveals RGC axon fibers as they enter the midbrain more ventrally (empty arrowheads) that show weaker DSCAM immunostaining. (c) Higher magnification confocal images of dissected brains further illustrate DSCAM and 3A10 neurofilament-associated protein co-localization RGC axon terminals identified by the 3A10 antibody that are also immunopositive for DSCAM (solid white and yellow arrowheads; yellow lines indicate location of x-z and y-z orthogonal planes, thickness of sample imaged was 85 μm). (d) The fluorescence intensity of DSCAM and 3A10 immunostaining was measured in whole brain tissues using a 25 μm circular ROI tool and analyzed using ImageJ with ten measurements obtained across ten regions each at the level of the axon terminals and axon tracts within the midbrain neuropil. Measurements were taken from both brain hemispheres equally. Scale bars: 100 μm for a and b; 25 μm for c.

Figure 4

Comparing DSCAM immunoreactivity across CNS regions in *Xenopus laevis* tadpoles. (a) A maximum projection image of a cleared *Xenopus* tadpole illustrates the distribution of DSCAM immunoreactivity (red) across several structures within the CNS. The 3A10 anti-neurofilament antibody (green) is used to visualize axonal tracts. (b, c, d) Single plane images show specific patterns of DSCAM expression in
multiple brain structures including the olfactory bulb (OB), telencephalon (TEL), optic tectum (TEC), and hindbrain (HB). Note the absence of 3A10 staining from axonal fibers in the olfactory nerve, OB and TEL. (e) DSCAM and 3A10 immunofluorescent signals were measured in two tadpoles using a 25 µm ROI tool, with 10 measurements obtained per anatomical structure and across 10 regions for each brain structure (OB, TEL, and HB). Note that the TEC was subdivided into axon terminals or axonal tracts. Measurements were taken from both brain hemispheres equally. Fluorescence intensity for DSCAM at the OB was higher than in other brain structures. Scale bars: 200 µm for a; 100 µm b, c, d.

Figure 5

Differential expression of DSCAM by RGC axon fibers in retina. (a) Coronal section of a stage 45 tadpole eye immunostained with DSCAM (red) and 3A10 anti-neurofilament antibodies (green) shows strong DSCAM immunoreactivity in the ganglion cell layer (GCL), inner plexiform layer (IPL) and Inner nuclear layer (INL). 3A10 immunopositive cell bodies are confined to the GCL, adjacent to the IPL. (b) Punctate DSCAM immunoreactivity in the GCL is further revealed by the confocal image of a second stage 45 tadpole eye immunostained with DSCAM only. (c) Frontal and orthogonally views of an eye in a cleared Xenopus tadpole head immunostained with DSCAM (red) and 3A10 anti-neurofilament (green) antibodies shows strong DSCAM immunoreactivity in the GCL and IPL and differential distribution of 3A10 immunoreactivity in a subset of cells in the GCL (yellow lines indicate location of x-z and y-z orthogonal planes, thickness of sample imaged was 296 µm). (d, e) Confocal images of coronal sections of a tadpole eye illustrate DSCAM and 3A10 immunoreactivity at the level of (d) the optic fiber layer (OFL) within the retina and (e) optic nerve head. The magnified images show a subset of 3A10-immunopositive axon fibers (white arrowheads; middle panel, 3A10 and DSCAM overlap; right panel, DSCAM only) that do not express DSCAM. (e) Coincident immunostaining for DSCAM and 3A10 (empty arrowheads) is seen in ventral fibers along the optic nerve head and optic nerve (ON). Scale bars are as shown for each image.

Figure 6

Topographic organization of retinal axon fibers along the developing Xenopus retinotectal path. (a) Schematic representation of the developing tadpole visual system and of experimental design. (b) Coronal section of a stage 46 tadpole eye shows localization of a lissamine-tagged control MO (red) after electroporation into the ventral half of the retina and fluorescein-tagged control MO into the dorsal half (green). (c, d, e, f) High magnification confocal images show the trajectories of lissamine-tagged RGCs axon fibers and fluorescein-tagged control MO labeled axon fibers as they exit the eye (c, d), and along the optic nerve, chiasm, and optic tract (d). Note the topography of RGCs in the eye and their spatial arrangement along the optic nerve and chiasm, where axon fibers from ventral RGCs labeled by the lissamine tag travel along the ventral side of the optic nerve and chiasm while axons of RGCs labeled fluorescein-tagged control MO travel along the dorsal side of the optic nerve. (d, e, f) Lissamine MO-
labeled axon fibers that were originally positioned on the ventral side of the optic nerve are positioned more dorsally in the optic chiasm. In contrast, fluorescein MO-labeled axon fibers that originate in the dorsal portion of the retina shift more ventrally. (g) At the optic tectum, the lissamine-labeled RGC axon fibers localize to the dorsal branch while the fluorescein-tagged control MO-labeled axon fibers localize ventrally. Scale bars: 100 μm for b; 50 μm for c, d, and g; 20 μm for e; 25 μm for f.

**Figure 7**

**DSCAM impacts the topographic organization of ventral RGC axon fibers branching at the target.** (a) Schematic illustrates the sequential electroporation of ventral RGCs with lissamine-tagged control or DSCAM MO, and dorsal RGCs with Alexa Fluor 488-dextran. (b) Confocal projection of axon terminals imaged *in vivo* 48 hrs after transfection illustrates the organization of retinal fibers along the medial to lateral axis. Control MO lissamine-tagged axons from ventral RGC terminate medially (M) within the tectal neuropil while Alexa Fluor 488 dextran-labeled axons from dorsal RGCs terminate more laterally (L) with some degree of overlap. Transfection of DSCAM MO in ventral RGCs resulted in wider spread of their axons medially (red, right panel) with less overlap. (c, d, e, f, g) Quantitative analysis of territory occupied by dorsal and ventral RGCs axons within the tectal neuropil in tadpoles transfected with either DSCAM or Control MO and imaged 48 hours later. The midpoint between the first branch point and the terminal tips of the arbors was used to measure the width of the labeled arbors. (c) A significant difference in arbor width is observed when comparing axon arbors from ventral RGCs that project medially in DSCAM vs Control MO treated tadpoles (*p = 0.0377), while (d) no difference in widths is observed for arbors from dorsal RGCs that project laterally (*p = 0.6875). (e) Quantitative analysis shows a significant difference in the degree of overlap (mm) at the midpoint between medial and lateral RGC axon arbors in DSCAM vs Control MO treated tadpoles (*p = 0.0169). (f) A significant difference is also observed when measuring the total territory occupied by axons (width in mm) projecting both medially (ventral RGCs) and laterally (dorsal RGCs) between DSCAM MO- and Control MO-treated animals (*p = 0.0257), and (g) when calculating the percent in overlap over total arbor width (lissamine + Alexa 488 dextran-labeled axons) (**p = 0.0019). Statistical analysis was by unpaired, two-tailed t test with equal sample sizes (*n = 6) for control and DSCAM MO. (*p ≤ 0.05, **p ≤ 0.005). Scale bars: 40 μm for b.

**Figure 8**

**Localized DSCAM immunoreactivity to primary process of cells with bodies within the tectal neuropil.** Electroporation with a CMV-driven GFP expression plasmid was used to randomly label neurons with GFP in young embryos. Coronal sections from stage 46 tadpoles with GFP-positive cells were fixed and immunostained with a DSCAM antibody. (a) *Left panel:* A maximum confocal projection at the level of the midbrain shows of GFP positive neurons immunostained for DSCAM. *Right panel:* A large magnification of a single confocal plane (white box; left panel), shows DSCAM immunoreactivity
localized in a punctate manner on the membrane surface of tectal cells (white dotted circles) as well as on primary dendrites and dendritic branches (white arrows). Note strongly DSCAM labeled fibers near dendrites of GFP labeled neurons in the low magnification image (arrowhead). (b) Random expression of GFP in a subset of cells within the tectal neuropil reveals in DSCAM immunostaining within the primary processes of those cells (arrowhead). The white dotted line indicates the boundary between the cell body layer and the neuropil. Scale bars: 20 µm for a and b; 10 µm for the magnified view of panel a.