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Retinotopic order in the absence of axon competition

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Abstract

The retinotectal projection has long been studied experimentally and theoretically, as a model for the formation of topographic brain maps^{1–3}. Neighbouring retinal ganglion cells (RGCs) project their axons to neighbouring positions in the optic tectum, thus reestablishing a continuous neural representation of visual space. Mapping along this axis requires chemorepellent signalling from tectal cells, expressing ephrin-A ligands, to retinal growth cones, expressing EphA receptors⁴. High concentrations of ephrin A, increasing from anterior to posterior, prevent temporal axons from invading the posterior tectum. However, the force that drives nasal axons to extend past the anterior tectum and terminate in posterior regions remains to be identified. We tested whether axon–axon interactions, such as competition, are required for posterior tectum innervation. By transplanting blastomeres from a wild-type (WT) zebrafish into a *lakritz* (*lak*) mutant, which lacks all RGCs⁵, we created chimaeras with eyes that contained single RGCs. These solitary RGCs often extended axons into the tectum, where they branched to form a terminal arbor. Here we show that the distal tips of these arbors were positioned at retinotopically appropriate positions, ruling out an essential role for competition in innervation of the ephrin-A-rich posterior tectum. However, solitary arbors were larger and more complex than under normal, crowded conditions, owing to a lack of pruning of proximal branches during refinement of the retinotectal projection. We conclude that dense innervation is not required for targeting of retinal axons within the zebrafish tectum but serves to restrict arbor size and shape.

Axons originating in the temporal retina form connections with the anterior (rostral) part of the tectum, whereas nasally located RGCs project to posterior (caudal) tectum (Fig. 1a). Although a single gradient of repulsive ephrin A (Fig. 1b) is sufficient to explain the projection of temporal axons to anterior tectum, the preference of nasal axons for posterior tectum, where ephrin A molecules are most highly concentrated, has remained unexplained. Nasal axons carry fewer EphA receptors and are less sensitive to ephrin A^{6,7}, but they still avoid high concentrations of ephrin A *in vitro*⁸. Our current understanding of retinotectal mapping therefore relies on a postulated second gradient of activity. In principle, such a gradient could either be presented by the target, independently of axonal input, or be produced by interactions between axons.

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Author Contributions N.J.G., L.M.N. and H.B. devised experiments. N.J.G. performed all transplantation experiments and quantified images. L.M.N. conducted single-cell labelling studies in the tectum. N.J.G. and H.B. wrote the manuscript.

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To distinguish between these possibilities it is useful to consider the case of a single axon terminating in the tectum in the absence of all other axons (Fig. 1c, d). If the map is formed by one-to-one matching of retinal and tectal markers (chemoaffinity)^{9,10}, then the projection of this solitary axon should be indistinguishable from that of an axon originating from the same retinal position under normal, crowded conditions (Fig. 1c). If, alternatively, interactions between RGC axons are responsible for the production of a second, posterior-directing activity, then retinotopy should be altered when no other axons are present (Fig. 1d shows one possible outcome). Recent studies have proposed that individual axons determine their spatial order by comparing their own ephrin-A/EphA signalling levels with that of their neighbours or with that of all other axons¹¹⁻¹⁴. These models have been very successful in explaining the plasticity of the map in response to surgical and genetic manipulations^{3,15}, although pure chemoaffinity theories have also accomplished this¹⁶. Competition for a limiting supply of target-derived factors (such as neurotrophins) has been proposed as a cellular mechanism for spreading retinal input over the available tectal territory^{12,15,17}. If competition is the force driving nasal axons into repellent territory, then in the absence of other axons, a single nasal axon should always terminate preferentially in the anterior tectum (Fig. 1d).

We have now tested this prediction by creating mosaic zebrafish eyes with only one RGC. Cells were transplanted at the blastula stage from WT donors into *lak* mutant host embryos (Fig. 2a). *lak* mutants fail to develop any RGCs as a result of disruption of the proneural transcription factor *Ath5* (*Atoh7*)⁵. WT cells in the mutant environment may undergo the full RGC differentiation programme^{18,19}. The *lak* mutant has no known defects outside the retina, and *ath5* mRNA is only found in the retina. To reveal single RGCs and their axons, WT donor embryos carried the *Brn3c:mGFP* transgene, which is expressed in roughly half of RGCs projecting to the tectum²⁰. We then selected for analysis those chimaeras in which single green fluorescent protein (GFP)-labelled axons were resolvable in the tectum.

In chimaeras consisting of a WT host that had received cells from a WT donor (*WT^{Brn3c:mGFP}→WT*; *n* = 30 axons), donor-derived, GFP-labelled RGCs showed stereotyped retinotectal projections (Fig. 2b). Their axons exited from the retina, crossed the midline and innervated the contralateral optic tectum, forming a branched terminal arbor. In *lak* hosts (*WT^{Brn3c:mGFP}→lak*; *n* = 19 axons), solitary axons remained capable of the multiple pathfinding steps required for innervation of the contralateral tectum (Fig. 2c). Many of these axons projected well beyond the anterior tectum. A minority of solitary RGCs showed pathfinding errors within the retina that were not seen under normal conditions, when their cell body was in a peripheral region, at a distance from the optic fissure (Supplementary Video 1). Our results clearly show that RGC–RGC interactions are not absolutely required for innervation of the posterior tectum.

To analyse possible changes in retinotopy, we compiled summary mapping functions at 7 days after fertilization (d.p.f.) for single axons that had developed under either crowded or solitary conditions. We therefore determined the locations of the RGC soma along the nasal–temporal axis in the retina and its axonal arbor in the tectum (see Supplementary Fig. 2 for an example of such an analysis). In WT zebrafish, axons enter the tectum at its anterior pole, extend only in a posterior direction, and do not overshoot their target. Because single terminal arbors in larval zebrafish cover about 5–10% of the tectal surface²¹⁻²³, we derived two separate mapping functions for each experimental condition: one for the most distal branch, the other for the most proximal branch of each arbor.

As predicted by earlier axon-tracing studies in zebrafish^{21,24}, crowded axons formed a continuous map. Both distal and proximal branch positions conformed to roughly linear and parallel mapping functions (Fig. 2d, e). A very similar relationship was seen for the distal branches in solitary axons (Fig. 2d). In fact, slope and absolute values of the mapping function

were indistinguishable from the crowded condition (F -test, $P = 0.630$), suggesting that posterior targeting of RGC axons occurred in the absence of axon–axon interactions.

However, a strong difference was seen for the proximal branches (Fig. 2e; F -test, $P < 0.00001$). Although retinotopic order was retained, the slope of the mapping function was shallower, indicating that solitary axons formed (or maintained) branches more anteriorly than crowded axons and that this shift was more pronounced for the nasal axons than for temporal axons.

We examined whether the observed anterior shift in proximal branching could be caused by altered retinal or tectal positional cues. The expression of *epha4b*, a marker for temporal retina, was unchanged in the *lak* mutant retina (Fig. 3a, b). Furthermore, the mRNA gradient of *efna5b*, the gene encoding ephrin A5b, was maintained in the mutant tectum (Fig. 3c, d). Together, these findings suggest that the nasal–temporal axis of the retina is normally patterned in *lak* mutants and that tectal guidance cues are unaffected by the absence of retinal innervation.

Although molecular patterning appears normal in *lak* mutants, it was possible that retinal input might be necessary for tectal cell differentiation. By imaging individual tectal cells labelled with membrane-bound GFP (see Methods), we found morphologically normal neuronal and glial-like cells in the *lak* mutant tectum (Fig. 3c–h). Additionally, a quantitative survey revealed no difference between WT and *lak* in relative abundance of five distinct morphological cell types (Supplementary Fig. 3).

To verify that our transplantations did not disrupt NT patterning of the retina and that solitary RGCs adopt appropriate positional fates, we analysed *efna5b* expression as a specific marker for nasal RGCs (Fig. 3k, l; see Methods for details). In the $WT^{Brn3c:mGFP} \rightarrow WT$ retina, graded *efna5b* expression persisted (Fig. 3m). In $WT^{Brn3c:mGFP} \rightarrow lak$ chimaeras, small numbers of donor-derived RGCs visibly expressed *efna5b* in nasal locations but not in the temporal retina ($n=6$; Fig. 3n), which is consistent with previous findings that retinal positional identity is specified before, and independently of, RGC genesis^{18,25,26}.

Finally, we ruled out a delay of tectal innervation in $WT^{Brn3c:mGFP} \rightarrow lak$ chimaeras by imaging chimaeras at 80 h after fertilization, an early stage of RGC tectal innervation. RGC axons, tipped with growth cones, were seen in the tecta of both WT and *lak* hosts (Fig. 3o, p). The axon arbor phenotype is therefore unlikely to be a consequence of developmental differences of the tectum in $WT^{Brn3c:mGFP} \rightarrow lak$ chimaeras, but rather is attributable to the difference in the density of RGC axons.

We predicted from the mapping functions of solitary axons (see Fig. 2d, e) that their arbors would be larger than those under crowded conditions. Indeed, morphometric analysis of arbor shapes revealed that solitary arbors covered a larger territory and had an increased number and length of branches (Fig. 4a, b). This increase in arbor size and complexity was already evident at 4 d.p.f. and became more pronounced by 7 d.p.f., through the net addition of branches (Fig. 4c–e). These findings suggest that axonal arbors are normally restricted in their initial size by the presence of other axons. This is consistent with the view that competition for target territory refines the map by suppressing or eliminating branches in retinotopically inappropriate territory². Solitary axons in zebrafish showed a positional bias: excessive branching occurred largely on the proximal side of the arbor (Fig. 4f).

Our results indicate that, at least under our experimental conditions, RGC axon–axon interactions, including competition, are not required for retinotopic targeting along the anterior–posterior axis. Although this does not rule out the possibility that competition can profoundly influence the map in a densely innervated tectum^{2,3,13}, our findings support a mapping mechanism that requires a second tectum-derived gradient, balancing the repellent signal provided by ephrin A molecules. This gradient, whether provided by the growth-

promoting effect of ephrin A on nasal axons²⁷, by reverse chemorepellent signalling from tectally expressed EphA to retinal axons²⁸ or by a different mechanism^{29,30}, is sufficient to guide axons to the posterior tectum. Within the termination zone, axon–axon interactions then sculpt the axonal arbor and restrict branching along the length of the axon.

METHODS SUMMARY

Generation of chimaeras

About 5,000 blastomere transplantations were performed, with the use of WT donor embryos carrying the *Brn3c:mGFP* transgene and WT or *lak* host embryos. Chimaeric embryos with donor-derived clones in the neural retina were selected at 30–36 h after fertilization, then raised for subsequent analysis of RGC axon mapping and arbor morphology.

Immunostaining, imaging and quantification

Larvae with small numbers of RGC axons were selected at appropriate time points and imaged live, or fixed and immunostained for GFP and monoclonal antibody zn-5 (Zebrafish International Resource Center). RGC soma position was calculated as the percentage of distance from the nasal pole of the retina along the nasal–temporal axis. Tectal positions were measured as the percentage of the distance between the anterior and posterior poles of the tectum. Individual axon arbors were imaged at 4 or 7 d.p.f. and analysed with Object-Image, blind to host genotype, as described previously²³.

In situ hybridization

Whole-mount *in situ* hybridization was performed as described previously¹⁸.

Single cell electroporation

Small numbers of tectal cells were transfected with GFP in live larvae at 4 or 5 d.p.f. and imaged at 6 or 7 d.p.f.

METHODS

Generation of chimaeras

WT donor embryos carrying the *Brn3c:mGFP* transgene²⁰ were injected with a solution of 1–4% tetramethylrhodamine-conjugated dextran and 1–4% biotin-conjugated dextran (Invitrogen). Host embryos were collected from heterozygous *lak* carrier incrosses, or heterozygous male carriers mated to homozygous *lak* mutant females. Donor and host embryos were dechorionated enzymatically by incubation for 5–8 min in a solution of 0.5 mg ml^{−1} pronase (Sigma) in water. Chimaeric embryos with rhodamine-positive clones in the neural retina were selected at 30–36 h after fertilization, and raised in 0.2 mM phenylthiocarbamide to inhibit melanin synthesis.

Smaller donor-derived clones, which are more likely to give rise to single RGCs, were found in locations biased towards the central retina, probably as a result of the proliferation of cells in the post-embryonic peripheral retina.

Immunohistochemistry

Larvae carrying fewer than four RGC axons were selected at 3, 4 or 7 d.p.f. and stained for GFP and zn-5 as described previously²⁰, with the following modifications: larvae at 80 h after fertilization and 7 d.p.f. were permeabilized in 1 mg ml^{−1} collagenase in PBS for 1.5 and 2.5–3.0 h, respectively. Anti-GFP and zn-5 antibodies were used at 1:2,000 and 1:400 dilutions,

and donor-derived cells in larvae at 80 h after fertilization and 7 d.p.f. were identified by reaction with Alexa-fluor-conjugated avidin (Invitrogen).

Imaging and quantification

Larvae at 4 d.p.f. were anaesthetized and immobilized in 1% low-melting-point agarose. Whole-mount stained 7 d.p.f. larvae were mounted in ~90% glycerol. Dorsal confocal stacks were collected with a Bio-Rad MRC 1024 or Zeiss LSM Pascal microscope.

Custom-designed macros in Object-Image (available at <http://simon.bio.uva.nl/Object-Image/1-Introduction.html>) were used for image analysis. Mapping functions were obtained blind to host genotype and retinal cell body position.

RGC soma position (percentage NT) was calculated as the percentage of distance from the nasal pole of the retina along the nasotemporal axis (Supplementary Fig. 2a–c):

$$\text{Percentage NT} = 100 \times [(NT_{\max} - NT_{DV}) / 2 + NT_{\text{cell}}] / NT_{\max}$$

where $NT_{\max}$ is the maximum distance from nasal to temporal locations in the ganglion cell layer, NT_{DV} is the distance from nasal to temporal poles at the dorsal–ventral position of the cell, and NT_{cell} is the distance from the nasal pole to the cell body. (All distances were measured as curved lines along the inner plexiform layer, as revealed with zn-5 reactivity.)

Tectal positions were measured as the percentage of the distance between the anterior and posterior poles of the tectum, demarcated by extent of zn-5 label (Supplementary Fig. 2d–f).

Summary retinotectal mapping functions were assembled for 7 d.p.f. larvae with 30 RGCs in 27 $WT^{Brm3c:mGFP} \rightarrow WT$ chimaera eyes, and 19 RGCs in 18 $WT^{Brm3c:mGFP} \rightarrow lak$ chimaera eyes.

Individual axon arbors were imaged at 4 or 7 d.p.f. and analysed with Object-Image, blind to host genotype, as described previously²³. Axon arbor stickfigures were exported directly from morphometric analysis and prepared with Adobe Photoshop.

In situ hybridization

Digoxigenin-labelled *epha4b* antisense probes were synthesized *in vitro* as published²⁶. Whole-mount *in situ* hybridization was performed as described previously¹⁸. For sections, larvae were mounted in gelatin/albumin and 25- μm slices were cut with a Leica vibratome. Stained embryos were imaged with a SPOT charge-coupled device camera mounted on a Leica dissecting microscope or Zeiss compound microscope with differential interference contrast optics, and prepared with Adobe Photoshop.

Single-cell electroporation and morphological analysis

Small numbers of neurons were labelled in live larvae by using a method adapted from ref.³¹. Tectal cells were transfected with either $\alpha\text{-tubulin}:GFP$, $eF1\beta:GFP$ or mixed $\beta\text{-tubulin}:gal4;UAS:GFP$ plasmids at a DNA concentration of $1.5 \mu\text{g} \mu\text{l}^{-1}$. WT and *lak* mutant larvae were electroporated at 4 or 5 d.p.f. and imaged as above at 6 or 7 d.p.f.

In ‘palm cells’, cell bodies are located near the tectal midline, neurites in the tectal neuropil. In ‘vine cells’, cell bodies are located in the tectum, arbors extend to more ventral brain structures. In glial-like ‘giant kelp cells’, cell bodies are directly apposed to the tectal midline; endfeet are at the dorsal surface of the brain, superficial to RGC input layers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Two potential mechanisms for formation of the retinotectal map

a, Graphical representation of the normal retinotectal mapping function. Increasingly nasal (N) RGCs project to increasingly posterior (P) regions of the optic tectum. A, anterior; T, temporal.

b, Gradients of tectal ephrin-A ligands and retinal EphA receptors repel axons from the posterior tectum. Additionally, retina and tectum express countergradients of ephrin A and EphA, respectively. **c**, **d**, Hypothetical maps formed by solitary axons. If chemoaffinity (**c**) between axons and targets guides retinotectal mapping, then solitary nasal axons retain projections to posterior tectum. If axon-axon competition (**d**) drives nasal axons to posterior tectum, then solitary axons should terminate in the anterior tectum, where ephrin-A signalling is lowest.

Figure 2. Retinotectal mapping functions in the presence or absence of axon-axon interactions
a, Cell transplantation from WT into *lak* mutants generates chimaeras with single RGC axons.
b, c, Chimaeric larvae at 7 d.p.f. with GFP-labelled RGC axon arbors (green) and tectal neuropil (red, stained with zn-5). Scale bar, 200 μ m. **b**, $WT^{Brn3c:mGFP} \rightarrow WT$ chimaera with single labelled arbor. **c**, $WT^{Brn3c:mGFP} \rightarrow lak$ chimaera, with single arbor on the left tectum and two fainter arbors on the right tectum. **d, e**, Retinotectal mapping functions for chimaeras at 7 d.p.f.; 0–100% anteroposterior (AP) is 180 μ m. The most distal extension of RGC arbors is not different between WT and *lak* hosts (**d**). The most proximal branchpoint is shifted anteriorly in *lak* hosts (**e**).

Figure 3. Evidence for normal patterning of retina and tectum in *lak* mutants

a, b, Temporal retinal *epha4b* expression in WT (**a**) and *lak* (**b**) at 28 h after fertilization. Optic fissure is at lower left. **c, d**, Tectal expression of *efna5b* is unchanged between WT (**c**) and *lak* (**d**). **e–j**, Tectal cellular composition in WT (**e, g, i**) and *lak* (**f, h, j**). Scale bar, 100 μ m. ‘Palm cells’ (**e, f**), ‘vine cells’ (**g, h**), and ‘giant kelp cells’ (radial glia, **i, j**; also present here, ‘palm cells’ labelled P) are present in normal numbers. **k–n**, *efna5b* expression in larvae at 80 h after fertilization. Insets show magnifications of the red boxes. Scale bar, 100 μ m. *efna5b* is present in nasal RGCs in WT (**k**), but completely absent in *lak* (**l**). Expression is unaffected in WT hosts (**m**). Donor-derived nasal RGCs express *efna5b* (**n**, arrowheads) in *lak* hosts. **o, p**, A single GFP-labelled axon extends into the tectum of WT (**o**) and *lak* (**p**) host at 80 h after fertilization. Scale bar, 50 μ m.

Figure 4. Axon competition restricts axon arbor size and complexity

a, b, Examples of RGC axon arbors at 7 d.p.f. in WT (**a**) and *lak* (**b**) hosts. Arrowheads indicate positions of the most proximal branch. Scale bar, 50 μ m. **c–e**, At both 4 and 7 d.p.f., arbors have a greater number of branches (**c**), total branch length (**d**) and area (**e**) in *lak* hosts (filled columns) than in WT hosts (open columns). Results are shown as means $\pm$ s.e.m. For all 4 d.p.f., $P < 0.01$ (two-tailed t -test, $n = 17$ WT, $n = 8$ *lak*). For all 7 d.p.f., $P < 0.01$ (two-tailed t -test, $n = 19$ WT, $n = 19$ *lak*). **f**, Summary interpretation. RGC axons project retinotopically, but are elongated, in the absence of axon-axon interactions. Proximal branches are normally pruned (triangle tips) by competition with other axons, but persist in solitary arbors (right).