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Disruption of gradient expression of Zic3 resulted in abnormal intra-retinal axon projection

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Summary

The targeting of retinal ganglion axons toward the optic disc is the first step in axon pathfinding in the visual system. The molecular mechanisms involved in guiding the retinal axons to project towards the optic disc are not well understood. We report that a gene encoding a zinc-finger transcription factor, Zic3, is expressed in a periphery-high and center-low gradient in the retina at the stages of active axon extension inside the retina. The gradient expression of Zic3 recedes towards the periphery over the course of development, correlating with the progression of retinal cell differentiation and axogenosis. Disruption of gradient expression of Zic3 by retroviral overexpression resulted in mis-targeting of retinal axons and some axons misrouted to the sub-retinal space at the photoreceptor side of the retina. Misexpression of Zic3 did not affect neurogenesis or differentiation inside the retina, or grossly alter retinal lamination. By stripe assay, we show that misexpression of Zic3 may induce the expression of an inhibitory factor to the retinal axons. Zic3 appears to play a role in intra-retinal axon targeting, possibly through regulation of the expression of specific downstream genes involved in axon guidance.

Key words: Zic3, Gradient expression, Intra-retinal axon projection, Chick

Introduction

During development of the visual system, ganglion cell axons from all radial positions within the cup-shaped retina project to optic disc, a small opening in the center of the retina. From optic disc, the axons exit the eye to form the optic nerve (Fig. 1) (Halfter, 1985; Thanos and Mey, 2001). Ganglion cells located at central positions of the retina differentiate and extend their axons first, followed by cells at more peripheral positions, in a wave-like fashion. In chicken, the first ganglion axons appear in the dorsal central retina shortly after secondary invagination of the optic vesicles at Hamburger-Hamilton stage 15 (E2 to E3) (Goldberg and Coulombre, 1972; Horder and Mashkas, 1982; Kahn, 1973; Rager et al., 1993). By E8, most of the retinal axons have reached the optic disc except those at the very periphery of the retina.

Despite the recent discovery of a number of conserved families of axon guidance cues, the molecular mechanisms underlying the process of axon projection toward the optic disc remain unclear (Oster and Sretavan, 2003). By intraretinal grafting experiment, it has been shown that the optic disc does not provide detectable chemotrophic activity to direct the retinal axon projection (Halfter, 1996). In netrin1/DCD or the EphB ligand-deficient mouse embryos, RGC axon pathfinding defects have been observed near the optic disc (Birgbauer et al., 2000; Deiner et al., 1997), suggesting that these molecules may not be involved in long-range intra-retinal guidance (Deiner et al., 1997).

A number of molecules have been implicated to be involved in centrally directed projection of the retinal axons toward the optic disc. Chondroitin sulfate proteoglycans (CSPGs), a major component of the ECM, are suggested to act as inhibitory molecules to prevent the growth of retinal axons toward the periphery of the rat retina (Brittis et al., 1992; Snow et al., 1991). The CSPGs recognized by a monoclonal antibody CS-56 are expressed in a gradient in the rat retina, low in the center and high in the periphery (Brittis et al., 1992). However, the distribution of CSPGs in the retina of chick, quail and cat embryos has been shown to colocalize with the growing optic axons, suggesting a different role in retinal axon guidance (McAdams and McLoon, 1995; Ring et al., 1995).

Axonal growth toward the optic fissure also requires expression of cell adhesion molecules (CAM) of the immunoglobulin superfamily like L1, neural cell adhesion molecule (NCAM) and neurolin (DM-GRASP). Intravitreal injection of the antibodies to NCAM disrupted the orderly formation of the fissure and resulted in massive overshooting of axons which failed to exit at the optic disc (Thanos and Bonhoeffer, 1984). Injection of Fab fragments to L1 resulted in defasciculation of axons (Giordano et al., 1997), whereas injection of Fab fragments to neurolin caused both defasciculation and aberrant intraretinal axonal trajectory (Ott et al., 1998). In addition, a number of receptor tyrosine phosphatases have been shown to play a role in intraretinal axon guidance (Ledig et al., 1999).

We report that a gene encoding a zinc-finger transcription factor, Zic3, is involved in patterning the retina for intraretinal...
axon guidance. Zic genes are the vertebrate homologues of the Drosophila pair-rule gene, odd-paired (opa) and are key regulators of neural and neural crest development (Aruga et al., 1998; Brewster et al., 1998; Nakata et al., 1997). Recently, Zic2 has been shown to pattern binocular vision that it is both necessary and sufficient to regulate RGC axon repulsion by cues at the optic chiasm (Herrera et al., 2003). Mutations in Zic genes are known to cause holoprosencephaly and situs abnormalities in human (Brown et al., 1998; Gebbia et al., 1997).

By in situ hybridization, we have shown that Zic3 is expressed in a periphery-high, center-low gradient in the developing retina, and the gradient expression of Zic3 recedes toward the periphery of the retina correlating with the progression of retinal cell differentiation and axonogenesis. Disruption of gradient expression of Zic3 by retroviral overexpression resulted in mis-targeting of the retinal axons and some axons misrouted to the subretinal space at the photoreceptor side of the retina. Misexpression of Zic3 did not affect neurogenesis or differentiation inside the retina, or grossly alter retinal lamination. By stripe assay, we have further shown that Zic3 may play a role in intra-retinal axon guidance by regulation of the expression of an inhibitory factor(s) to drive the axons to project to the optic disc.

Materials and methods

In situ hybridization and immunofluorescent staining

The procedures of in situ hybridization on cryosections, whole-mount and flat-mount samples were essentially as described previously (Bao and Cepko, 1997; Jin et al., 2003). The chicken Zic3 and Netrin-1 probes were kindly provided by Dr Cepko (Lin and Cepko, 1998) and Dr Tabin (K. Vogan and C. Tabin, unpublished) at Harvard Medical School.

Immunofluorescent staining on flat-mounts or sections of retina was essentially as described previously (Jin et al., 2003). Axons were stained with monoclonal antibody 270.7, a generous gift from Dr Virginia Lee (University of Pennsylvania Medical School). Viral infection was confirmed by immunofluorescence staining with either a monoclonal antibody 3C2 (diluted 1:5) or a polyclonal antibody p27 (SPAFAS, Norwich CT, diluted 1:10,000). Mouse monoclonal antibodies against Islet-1 (39.4D5), collagen IX (2C2) and visinin were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), and used at 1:50, 1:5 and 1:10, respectively. The monoclonal antibody CS-56 was obtained from Sigma (St Louis, MO) and used at 1:200 dilutions.

Retrovirus and chick embryo injection

A 1.5 kb fragment of cDNA containing the entire coding region of the human ZIC3 gene (Gebbia et al., 1997) was cloned into the ClaI site of an avian replication-competent retroviral vector (RCAS) (Hughes et al., 1987; Morgan and Fekete, 1996). RCAS-Zic3 virus was prepared by transfection of a chicken fibroblast line, DF1, as previously described (Bao et al., 1999). All viral stocks were prepared by transfection of a chicken fibroblast line, DF1, as previously described (Bao et al., 1999). RCAS-Zic3 virus was prepared by transfection of a chicken fibroblast line, DF1, as previously described (Bao et al., 1999).

TUNEL assay

Cryosections (20 μm) were prepared from the E7 or E10 retinas injected with either the control RCAS-GFP or RCAS-Zic3 virus. The DeadEnd Colorimetric TUNEL system from Promega was used to detect programmed cell death. The assay was carried out according to the manufacturer’s instruction except that the treatment of proteinase K was changed to 3 μg/ml of concentration for 10 minutes at room temperature to preserve tissue integrity. After TUNEL assay was completed, the tissue sections were further co-stained with anti-neurofilament antibody 270.7 and anti-P27 antibodies, to visualize the axons and the viral antigens, respectively.

Stripe assay

The stripe assay was carried out essentially as described previously (Walter et al., 1987). For some experiments, uninjected E8 chick eyecups were dissected out and cut along at one half radial distance to the center, in order to obtain the central and peripheral halves of the retina. For other experiments, optic vesicles were injected with either the RCAS-Zic3 or a control RCAS-GFP virus at HH stage 10 and incubated until E8. Only the central halves of the virus-injected retinas were collected and homogenized by pressing through 1 ml syringe needles multiple times in a buffer containing 10 mM Tris-HCl, pH 7.4, 1.5 mM CaCl2, 1 mM spermidine and protease inhibitor cocktail (Roche). Nuclei and cytoplasm were removed by centrifugation in step sucrose gradient for 10 minutes at 50,000 g.

The membrane fragments were collected and washed in PBS and vacuumed onto a polycarbonate filter (Waterman, pore size 0.1 μm) following the manufacturer’s instruction (Max-Planck-Institute at Tubingen). One type of membrane fragments were vacuumed on first to form the first stripes and the second type of membrane fragments mixed with green fluorescent beads (505/515) (Molecular Probes) were filled in to form the second stripes. After the stripes were formed, the membranes were coated with 50 μg/ml of laminin (BD Biosciences) for 1-4 hours at room temperature.

E6 chick retinas were dissected and flat-mounted with the ganglion cell side up onto a nitrocellulose filter (Sartorius). Thin strips of retina were cut and laid onto the polycarbonate filter with the ganglion cell side facing the polycarbonate filter. Retinal explants were cultured in DME/F-12 medium (1:1) containing 10% fetal calf serum, 2% chicken serum and 0.4% methyl cellulose, 40-44 hours later, the explants with the membranes were fixed in 4% paraformaldehyde and stained with an anti-neurofilament antibody (270.7) followed by a Cy3-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories).

The fluorescent images of axons and stripes were photographed with a digital camera (SPOT camera). Green images of stripes and red images of anti-neurofilament staining were merged using the SPOT Advanced software. To quantify the results, axons were traced and scanned to obtain digital images. We quantified only those patches that had axons covering eight or more stripes. The number of black pixels of the tracings on the stripes with the fluorescent beads (second stripes) and those on the stripes with no beads (first stripes) was calculated by a public domain NIH Image program. The ‘preference’
of axonal growth was calculated based on the ratio (s/f) of the number of black pixels on the second stripes to that on the first stripes and normalized to the width of the stripes. Control stripe assay experiments were performed to assess the variation of the experimental data by laying down same membrane preps from the central halves of the uninjected E8 retinas in both stripes. Axonal growth preference between two membrane fragments (ratio s/f) was compared with the control stripe assay data (ratio s/f) and statistical significance was analyzed by using the unpaired Student’s t-test.

Results

Expression of Zic3 mRNA in the embryonic chicken eye

By in situ hybridization, we have analyzed the expression of the Zic3 gene in embryonic chicken eye. At HH stage 10 (E1.5), Zic3 is already expressed in the lateral regions of the diencephalon which are designated to form optic vesicles (Fig. 2A). Zic3 expression is retained in the optic vesicle and optic cup at later stages (Fig. 2B,C). On flat-mount retinas (Fig. 2D-G), we observed that Zic3 is expressed in a graded fashion with higher expression at the periphery than the center. The graded expression recedes toward the retinal periphery over the course of development. At E5.5, high level of Zic3 mRNA is detected at the periphery, although some expression at the center remains (Fig. 2D). At E7, the expression is much decreased in the center, while at E9, the expression of Zic3 is mostly confined to the ventral periphery of the retina (Fig. 2E,F). In addition, Zic3 expression is not completely even in the four quadrants of the retina. At E5.5, higher expression was detected in the anterior side of the retina than the posterior side (Fig. 2D). At E7 and E9, Zic3 expression was detected at higher levels in the ventral than in the dorsal retina (Fig. 2E,F).

The dynamic changes in expression patterns of the Zic3 gene appear to correlate with the progression of neurogenesis and axonogenesis inside the retina. It is known that the ganglion cells at the center of the retina differentiate and extend their axons first, followed by cells at more peripheral locations. The earliest axonal extension is in the dorsal central region of the retina around E3 in chicken, whereas the axons at the peripheral ventral retina initiate the extension at E7-E8. The process of axonal extension is also more advanced in dorsal than in ventral, and in nasal than in temporal retina (Goldberg and Coulombre, 1972; Fig. 2).

Fig. 2. Expression of Zic3 mRNA in whole-mount chicken embryos (A-C) and flat-mount retinas (D-G) by in situ hybridization. (A) HH stage 10; (B) HH stage 12; (C) HH stage 17. Zic3 is expressed in early optic vesicle (arrows in A and B) and optic cup (arrow in C). (D) E5.5 retina; (E) E7 retina; (F) E9 retina; (G) high-magnification view of E7 retina. Zic3 is expressed in a periphery-to-center gradient with higher expression at the periphery. All the flat-mount retinas were shown with the ganglion cell side upwards, oriented with the anterior side (A) towards the left, the posterior side (P) towards the right, the dorsal side (D) upwards and the ventral side (V) downwards.

Fig. 3. Expression of Zic3 mRNA on cross sections of embryonic chick eye. In situ hybridization experiments were performed on the cryosections of embryonic chick eye at various stages: (A) E4.5; (B) E6; (C,D) E9; (E,F) E16. Note that Zic3 mRNA expression is in a periphery-high, center-low gradient at E4.5 and E6 (A,B). At E9, most of Zic3 expression is confined to the retinal periphery (labeled ‘P’ in C,D). Zic3 is also highly expressed in the ciliary margin area (C,E,F). At E16, Zic3 expression can be barely detected inside the retina while at a high level at the ciliary margin (E,F). C, center of the retina; P, periphery of the retina; R, retina; CM, ciliary margin. Arrowhead in C indicates the remaining undifferentiated cells located in the inner nuclear layer that are weakly expressing Zic3 mRNA at the retinal center. Scale bars: 100 μm in A,B,D-F; 400 μm in C.
The graded expression pattern of the Zic3 gene was confirmed by in situ hybridization on the cross sections of the embryonic chicken eye (Fig. 3). In addition, we found that Zic3 is highly expressed in the ciliary margin region (Fig. 3C,E,F), similar as reported in mouse (Nagai et al., 1997). Within the retina, Zic3 appears to be expressed in the undifferentiated progenitor cell population. The graded expression of Zic3 is consistently periphery-high and center-low in the undifferentiated cell population at all developmental stages analyzed. At E16, Zic3 expression is no longer detected inside the retina and is only retained in the ciliary margin (Fig. 3E,F).

**Misexpression of Zic3 resulted in abnormal intraretinal axon targeting**

To understand the role of Zic3 gene in eye development, we subcloned the full-length Zic3 gene into a retroviral vector, RCAS (Fig. 4A). This viral construct can produce replication-competent viral particles that propagate among the proliferating cells and express Zic3 protein in the infected cells. A similar viral construct with a GFP gene (RCAS-GFP) was used as a control. Injected at HH stage 10-11 (~E1.5) with the viral stocks, the infected eyes were analyzed at various ages including E6, E7, E10, E12, E16 and E18. Because Zic3 is highly expressed in the ciliary margin region, we examined the structure of the anterior chamber of the eye. We did not detect any obvious defect in eye anatomy, including in the ciliary margin area (data not shown).

Because the retinal axons project from periphery to retinal center, in the same direction as the graded expression of Zic3, we analyzed the axonal projection in the retinas infected with the RCAS-Zic3 virus. The infected retinas were flat-mounted and co-stained with an anti-neurofilament antibody (270.7) and an anti-viral GAG antibody (P27) for visualizing the axons and the infected area, respectively. In the retinas injected with a control virus RCAS-GFP, the axons appeared completely normal as in the uninjected wild-type samples, projecting straight toward the center of the retina where the optic disc was located (Fig. 4B). In the RCAS-Zic3 injected samples, however, the axons appeared abnormal in some areas. Most of the areas with axonal phenotype appeared somewhat long and thin in shape and were strongly stained by anti-viral GAG antibody (Fig. 4F,G).

First observed at E6, the number of areas with axonal abnormality increased over time but peaked around E10 (Table 1). In two cases, we observed that a small group of axons turned back 180° to project toward the periphery of the retina (Fig. 4D). In another case, the axons appeared to go around in a circle (Fig. 4C). More commonly (>150 areas), however, the axons had the appearance of being blocked from projecting toward the center of the retina and piled up into a line (Fig. 4E,F). By adjusting the focal plane of the microscope, we found that some axons penetrated the entire thickness of the retina and misrouted to the sub-retinal space (Fig. 5A-F). There, the axons initially followed the same aberrant trajectories as in the ganglion cell side (Fig. 5A,B). Farther away from the site where the axons crossed over, the axons appeared much more disorganized (arrowhead, Fig. 5D).

<table>
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<tr>
<th>Age</th>
<th>E6</th>
<th>E7</th>
<th>E8</th>
<th>E10</th>
<th>E12</th>
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<tr>
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<td>13</td>
<td>24</td>
<td>108</td>
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<tr>
<td>Number of retinas</td>
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<td>6</td>
<td>7</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>Affected areas per retina</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>5</td>
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The misrouted axons did not project to the optic disc, indicating that the molecular cues for directing the axons to the center of the retina are not present at the photoreceptor side.
Misexpression of Zic3 did not interfere with neurogenesis in the retina

To verify that the axonal phenotype was not secondary because Zic3 altered the normal course of cell differentiation and maturation, we examined retinal cell marker expression in the samples infected with RCAS-Zic3 virus. Incomplete infection allowed us to compare marker expression in the infected areas with that in the adjacent wild-type areas (Jin et al., 2003; Schulte and Cepko, 2000). Expressed by differentiated retinal neurons, islet 1 is mostly expressed by ganglion cells at early stages such as E7. We did not observe any difference in either the number or distribution of the islet 1-expressing cells in the areas infected with the RCAS-Zic3 virus compared with the uninfected areas (Fig. 6A,B). The expression of the marker for amacrine cell (Pax6) and photoreceptor cell (visinin) also remained unchanged (data not shown). These results suggest that misexpression of Zic3 did not grossly alter the course of neurogenesis or cell differentiation in the retina.

Next, we examined whether the axonal phenotypes of Zic3 misexpression was indirectly caused by induction of ciliary margin formation inside the retina. Morphologically, we did not observe any change in the RCAS-Zic3-infected retinas that might suggest ectopic formation of ciliary margin. Furthermore, a marker of the ciliary margin, collagen IX (Liu et al., 1993), showed identical patterns in the RCAS-Zic3-infected retinas as in the uninjected wild-type retinas (Fig. 6C-F). These results suggest that the axonal phenotype was not due to ectopic formation of ciliary margin inside the neural retina.

Misexpression of Zic3 did not grossly affect retinal lamination

To examine whether Zic3 misexpression affects retinal lamination, E10 retinas infected with the RCAS-Zic3 virus were sectioned and stained with various cell type-specific antibodies. Similar to what is observed in flat-mount retinas, the axons crossed over to the sub-retinal space at the photoreceptor side (Fig. 7A-C). However, the optic fiber layer appeared largely normal, although some protrusions of the axons were observed (arrows, Fig. 7A,B). By contrast, the photoreceptor layer often appeared broken, possibly by misrouted axons (Fig. 7D,G). After examining all sections
from two infected retinas containing 15 areas of abnormal axonal crossings, we did not observe any gross defect in lamination. However, some disturbances of retinal layers were observed, at the immediate vicinity of axon misrouting. Cells in GCL and inner nuclear layer (INL) were seen to disperse into the deeper layers of the retina toward the photoreceptor side (Fig. 7H,I). Within the sites of axon crossing over, the antiviral GAG antibody staining appeared much brighter than the adjacent infected areas (Fig. 7F). Consistent with the results obtained on flat-mount retina, we also did not observe any significant change in the number of cells expressing islet 1, Pax6 or visinin in retinal sections. These results support the idea that Zic3 misexpression appears to affect intra-retinal axon targeting specifically without grossly altering cell differentiation and lamination in the retina.

To understand the cause of the increased anti-GAG staining at the crossing-over sites, we next analyzed programmed cell death by TUNEL assay followed by staining with anti-neurofilament and anti-GAG antibodies. As shown in Fig. 8, these sites had a substantial increase of cell death compared with the adjacent tissues that were also infected. The increase of TUNEL-positive cells coincided with the increase of intensity of anti-GAG staining, suggesting that the apoptotic cells may release a higher concentration of viral proteins in the area. However, the increased cell death coincided only with the sites of axon crossing over, not viral infection. Although the samples used for the TUNEL assays were infected in over 90% of the total area, increased cell death was restricted to the vicinity of axon crossing over. In addition, the apoptotic cells tended to accumulate at the photoreceptor side, not the ganglion side (Fig. 8A,D-F).

Results of stripe assay suggest that Zic3 may activate the expression of an inhibitory factor to the retinal axons

Because Zic3 encodes a zinc-finger transcription factor, its role in axon guidance is most probably indirect, through regulation of the expression of secreted or membrane-bound molecules. To test whether the axonal phenotype by Zic3 misexpression is caused by activation of the expression of a negative factor, we performed stripe assays. Membrane fragments were prepared from the E8 central retinas (one half radial distance to the center) injected with either the RCAS-Zic3 or the control RCAS-GFP virus. We chose to use only the central retina because it has little endogenous Zic3 gene expression and may have a relatively low level of other negative guidance factors.

As shown in Fig. 9B, axons appeared to ‘prefer’ the stripes prepared from the control RCAS-GFP-injected retinas to the stripes prepared from the RCAS-Zic3-injected samples. To quantify the results, the distribution of the axons on the stripes was digitized and calculated by a NIH Image software and the preference of the axons were assessed by the ratio of axonal growth on the second stripes (RCAS-GFP-injected) to that on the first stripes (RCAS-Zic3-injected) (s/f) normalized to the width of the stripes (see Materials and methods). A total of 15 sample areas (from five independent experiments) were quantified and a mean ratio (s/f) was 5.74.

**Fig. 7.** Misexpression of Zic3 did not grossly alter retinal lamination. Cryosections of E10 retinas infected with RCAS-Zic3 were stained with anti-neurofilament (A-C) and various cell type-specific antibodies including: anti-visinin (G), anti-islet 1 (H) and anti-Pax6 (I). (C-F) Same RCAS-Zic3-infected retinal section was shown stained with anti-neurofilament (C), DAPI (D), anti-viral GAG antibody (F) and in phase contrast (E). Note some axons in the RCAS-Zic3-infected retinas misrouted to the subretinal spaces at the photoreceptor side of the retina (arrowheads, A-C). However, the laminated structure of the retina was largely normal (D-I), except some dispersion of cells towards the photoreceptor side (arrowheads, H and I). The optic fiber layer was largely normal, except for some protrusions of the axons (arrows in A, B). Frequently, however, the photoreceptor layer was broken (arrowheads, D and G). Note that the anti-viral GAG antibody staining was much more intense at the sites of axon crossing-over than the nearby infected region (arrowhead in F). PR, photoreceptor layer; OFL, optic fiber layer; GCL, ganglion cell layer; INL, inner nuclear layer.
To assess the variation in the assay, we laid down the membrane fragments from the E8 uninjected central retinas both as the first and as the second stripes and similarly analyzed the results. As shown in Fig. 9A, the axons appeared to grow randomly on the stripes and the mean ratio of the axonal growth (s/f) was 0.953 (n=15, three independent experiments). Student’s unpaired t-test was performed to compare the ratio (s/f) in the Zic3/GFP experiments with the ratio (s/f) in the control central/central experiments with P<0.001, indicating that the RCAS-Zic3-injected membrane preps was significantly less preferable by the retinal axons than the RCAS-GFP-injected membrane preps.

To determine whether an endogenous inhibitory factor is present at higher concentration in the periphery than in the retinal center, we performed stripe assays by laying down the membrane fragments prepared from the peripheral E8 retina as the first stripes and those from the central E8 retina as the second stripes. As shown in Fig. 9C, the axons appeared to grow preferentially on the stripes prepared from the central retina. The results were similarly quantified (mean ratio s/f=5.13, n=15, from five independent experiments). Statistical analysis by the Student’s unpaired t-test indicates that the stripes prepared from the central membrane fragments were significantly more preferable to the retinal axons than those from the peripheral membrane fragments (P<0.001). These results suggest that an endogenous negative factor(s) is present at a higher concentration in the peripheral than in the central retina, and Zic3 misexpression may activate the expression of an inhibitory factor to the retinal axons.

Fig. 8. Apoptosis was increased at the sites of axon crossing-over. E10 retinas infected with RCAS-Zic3 were analyzed by TUNEL assay (A,D-F), followed by staining with anti-neurofilament (B) and anti-viral GAG (P27) antibodies (C). At the sites with axon crossing-over (arrows in B), there was an increase in the number of apoptotic cells (arrows in A) and intensity of anti-viral GAG staining (arrows in C). Note that the adjacent area was also completely infected (marked with *), and some of the fluorescent signals were blocked by the DAB precipitates from the TUNEL assay. In addition, the apoptotic cells tended to concentrate at the photoreceptor side, not the ganglion side (A,D-F). PR, photoreceptor layer; G, ganglion cell layer. Scale bar: 50 µm.

Fig. 9. Stripe assays. The membrane fragments prepared from various retinal tissues were compared for their abilities to support axonal growth by stripe assays. Green fluorescent beads were mixed in to mark the second stripes. (A) The membrane fragments prepared from the central halves of the uninjected retinas were laid down both in the first and second stripes. (B) The membrane fragments prepared from the RCAS-Zic3-injected retinas were laid down in the first stripes, whereas those from the RCAS-GFP-injected retinas were filled in as the second stripes. (C) Membrane fragments prepared from the peripheral E8 retinas were laid down in the first stripes, whereas those from the central E8 retinas were filled in as the second stripes. Note that the membrane fragments prepared from the RCAS-GFP-injected retina were more preferable than those from the RCAS-Zic3-injected retina, and the membrane preps from the central retinas were more preferable than those from the peripheral retinas. (D) Quantification of the stripe assay results. Scale bar: 100 µm.
Misexpression of Zic3 may activate the expression of a currently unknown guidance factor

We next tested a few candidate molecules that might be downstream of Zic3. In chicken, Netrin1 is also expressed in the optic fissure and optic disc. No change in Netrin1 expression was observed in the retinas infected with the RCAS-Zic3 virus compared with the control-uninjected retinas (Fig. 10A,B). Similar to previous reports (McAdams and McLoon, 1995; Ring et al., 1995), we also observed that the staining of an anti-chondroitin sulfate proteoglycans (CSPGs) antibody (CS-56) persisted in the center of the retina in chicken, albeit at a much lower level than in the periphery. This suggests that CSPGs may colocalize with the growing optic axons and play a different role in retinal axon guidance. Furthermore, we did not detect any obvious changes in CS-56 staining, in the areas infected with the RCAS-Zic3 virus compared with that in the uninfected wild-type areas, suggesting that the expression of CSPGs is not subjected to the regulation by Zic3 (Fig. 10C-F).

Further experiments have shown that the expression of neither Slit1 nor Slit2 was altered in the samples with misexpression of Zic3 (data not shown). Ephb5 (cek11) mRNA is reported to be expressed in the center-high and periphery-low patterns inside the retina (Sefton et al., 1997). However, we found that the expression of Ephb5 mRNA was not subjected to the regulation by Zic3 by in situ hybridization (data not shown). These results confirm the notion that Zic3 misexpression does not alter retinal cell differentiation grossly and further suggest that Zic3 may regulate a currently uncharacterized pathway to influence intra-retinal axon pathfinding.

Discussion

In this paper, we have shown that disruption of Zic3 gradient expression by retroviral misexpression resulted in aberrant intraretinal axon projection. Zic3 is expressed in a periphery-to-center gradient inside the retina with higher expression at the periphery, correlating with the directed retinal axon projection from periphery to retinal center. The gradient expression of Zic3 appears to be important for intraretinal axon targeting. Misexpression of Zic3 caused specific axonal phenotypes, possibly through regulation of the expression of specific downstream genes involved in axon guidance.

Because cell differentiation and axonogenesis initiates from the cells at the center of the retina followed by cells at the more peripheral positions in a wave-like fashion, many genes involved in cell growth and differentiation exhibit dynamic expression patterns from center to periphery. However, the expression of these genes usually begins at the central retina, where the retinal cells are actively differentiating at the early stages such as E4.5. The expression of these genes gradually moves toward retinal periphery correlating with the wave of retinal differentiation. By contrast, Zic3 gene is consistently expressed at higher levels at the periphery, even at E4.5. Zic3 did not appear to affect cell differentiation grossly because misexpression did not alter the expression of islet 1, Pax6 and visinin. Furthermore, Zic3 misexpression did not alter the expression of many axon guidance molecules expressed in the retinal cells, including Slit1, Slit2, Robo1, Ephb5 and CSPGs, suggesting that Zic3 misexpression did not change the properties of the retinal cells substantially. The axonal phenotype caused by Zic3 may, therefore, be due to more specific changes in the expression of molecules involved in axon guidance.

Members of the Eph family of receptor protein-tyrosine kinases, together with their ephrin ligands have been shown as graded molecular tags in establishment of retinotectal map (Flanagan and Vanderhaeghen, 1998; O’Leary and Wilkinson, 1999). A number of studies have also shown that the graded expression of Eph family molecules and ephrins is set up by graded expression of various transcription factors. Expressed in opposing gradient to Epha3, two homeobox genes, Soho1 and Gh6 are shown to specifically repress Epha3 expression in the retina (Schulte and Cepko, 2000). At the D/V axis, a homeobox gene Vax/Vax2 expressed in a gradient in the ventral retina has been shown to specify the D/V axis of the retina to influence retinotectal mapping (Mui et al., 2002; Schulte and Cepko, 2000). In mesencephalon, a gradient of engrailed gene expression defines the rostral/caudal axis of the tectum and set up the gradient expression of the axon guidance cues, Eif1 and Rags (Friedman and O’Leary, 1996; Itasaki and Nakamura,

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Fig. 10. Zic3 misexpression did not alter the expression of netrin 1 and CSPGs. The RCAS-Zic3-infected samples were hybridized with the netrin 1 probe (A) and then immunostained with an anti-GAG antibody (OD, in A) and optic fissure, similar to wild-type uninjected retinas. The RCAS-Zic3 virus-infected retinas were also double stained with an anti-chondroitin sulfate antibody (CS-56) (C,E) and an anti-viral GAG antibody (P27) (D,F). CS-56 staining is intense at the periphery of a currently unknown guidance factor.
and with the fact that axon guidance cue. This is consistent with the axonal misexpression of Zic3 upregulates the expression of a negative axon guidance cue. This is consistent with the previous findings that staining of CS-56 antibody persists in the center of the retina in chicken (McAdams and McLoon, 1995; Ring et al., 1995), we cannot rule out that a particular constituent of the CSPGs may still be involved in intraretinal axon targeting or subjected to the regulation by Zic3. The identities of the core proteins of the CSPGs have remained undefined.

At the sites of axon crossing-over to the photoreceptor side in the RCAS-Zic3-infected retinas, we have shown that there were disturbances in cell distribution and increased cell death. It is possible that the misrouting of axons is due to physical anomalies generated by increased cell death. However, it is also possible that the abnormal trajectories of the axons disrupted normal organization of the retina, which has resulted in increased cell death. Although we currently do not have definitive evidence to distinguish these models, several lines of evidence appear to support the latter. First, the results of stripe assay demonstrate that a negative guidance cue is induced by misexpression of Zic3. The induction of an inhibitory molecule in vivo can inhibit the normal centrally directed axon projection and cause misrouting of the axons. Results of stripe assays comparing the membrane fragments prepared from the central retina with the peripheral retina further suggest that an endogenous negative guidance factor is present in higher concentration in the periphery than in the center. Second, the increase in cell death did not coincide with viral infection, hence Zic3 misexpression. Despite the fact that the retinas were infected in over 90% of the total area, the areas with increased cell death were restricted to the immediate vicinity of the sites where axons crossed over. If increased cell death were resulted directly from Zic3 misexpression, a much wider spread of cell death would be expected. Third, similar phenotypes of axon misrouting to sub-retinal spaces have been reported for netrin 1 and sonic hedgehog mutant mice (Dakubo et al., 2003; Deiner et al., 1997). Because both netrin 1 and sonic hedgehog proteins are known to act as axon guidance cues (Charron et al., 2003; Deiner et al., 1997), the misrouting of axons to sub-retinal spaces are likely resulted directly from mis-targeting of the axons. Further study is needed to determine whether there is a common molecular basis for these similar phenotypes.

It is also intriguing that the phenotype with the axons turning around constituted only a minor fraction of the total number of the phenotypes. One possibility is that increased anti-GAG staining intensity was used in most of the experiments to identify the sites with axonal phenotypes. A small fraction of the sites with axons turning but no crossing-over might remain undetected. Second, it is also possible that additional factors are present to keep the axons in the peripheral-central pathways. It has been shown previously by intraretinal grafting experiments that the axons are unlikely to grow in the directions other than the peripheral-central pathways (Halfter, 1996).

We have previously shown that Slit1 is involved in definition of retinal axon trajectories by providing intermediate targets to the retinal axons. In the low Slit1 expression area resulted from overexpression of Irx4, the axons appeared to turn to avoid the low Slit1 area and were excessively fasciculated (Jin et al., 2003). However, the axons still managed to project to the optic disc, suggesting that Slit1 does not provide overall direction for the retinal axons to project to the optic disc. In this paper, we have further shown that Slit1 expression is not under the control of Zic3 regulation, suggesting that Slit1 and Zic3 are in two separate pathways.

Interestingly, it has recently been reported that Zic2 patterns binocular vision by specifying the uncrossed retinal projection (Herrera et al., 2003). It is common that transcription factors within one gene family play distinct roles in biological processes by regulating different downstream target genes. In human, mutations in Zic2 gene have been shown to cause holoprosencephaly, whereas mutations in Zic3 have been associated with X-linked situs abnormalities (Brown et al., 1998; Gebbia et al., 1997). Moreover, Zic2 and Zic3 are expressed in overlapping but distinct patterns during embryonic development (Nagai et al., 1997). In contrast to Zic2, which is expressed specifically in postmitotic ganglion cells in ventrotemporal mouse retina but not in chick (Herrera et al., 2003), Zic3 is found only in undifferentiated progenitor cells in chick retina. Future experiments aimed at identification of downstream targets of the Zic family transcription factors may thus provide further insights in the molecular mechanisms of the retinal axon guidance.

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