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Foxn4 Is Required for Retinal Ganglion Cell Distal Axon Patterning

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Abstract

The regulation of retinal ganglion cell (RGC) axon growth and patterning in vivo is thought to be largely dependent on interactions with visual pathway and target cells. Here we address the hypothesis that amacrine cells, RGCs' presynaptic partners, regulate RGC axon growth or targeting. We asked whether amacrine cells play a role in RGC axon growth in vivo using Foxn4^{-/-} mice, which have fewer amacrine cells, but a normal complement of RGCs. We found that Foxn4^{-/-} mice have a similar reduction in most subtypes of amacrine cells examined. Remarkably, spontaneous retinal waves were not affected by the reduction of amacrine cells in the Foxn4^{-/-} mice. There was, however, a developmental delay in the distribution of RGC projections to the superior colliculus. Furthermore, RGC axons failed to penetrate into the retinorecipient layers in the Foxn4^{-/-} mice. Foxn4 is not expressed by RGCs and was not detectable in the superior colliculus itself. These findings suggest that amacrine cells are critical for proper RGC axon growth in vivo, and support the hypothesis that the amacrine cell-RGC interaction may contribute to the regulation of distal projections and axon patterning.

Keywords

Foxn4; retinal ganglion cell; amacrine cell; superior colliculus; axon growth; axon patterning

1. Introduction

How are the axon growth, guidance and patterning of projection neurons regulated? For example, retinal ganglion cells (RGCs) extend their axons into the nerve fiber layer of the retina, grow towards the optic nerve head attracted to netrin-1 (Holt, 1989; Leonardo et al., 1997; Serafini et al., 1996; Serafini et al., 1994) and sonic hedgehog (Kolpak et al., 2005;

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Trousse et al., 2001), are channeled down the optic nerve by semaphorin-5A (Oster et al., 2003), either cross or remain ipsilateral at the optic chiasm in response to heparan and chondroitin sulfate proteoglycans (CSPGs) (Brittis et al., 1992; Brittis and Silver, 1995), and pattern their gross connections at the superior colliculus in response to ephrins (Frisen et al., 1998; Nakagawa et al., 2000) slits (Plump et al., 2002; Thompson et al., 2006a; Thompson et al., 2006b) and CSPGs (Chung et al., 2000). Although these are clearly the properties of ligands and adhesion molecules secreted by intermediate guidepost and target cells, intrinsic properties of RGCs also determine axon targeting. For example, the RGC transcription factor Brn3b is required for normal axon guidance and mapping in the superior colliculus (Badea et al., 2009; Erkman et al., 2000) and RGC expression of the transcription factor Zic2 dictates which RGCs will remain ipsilateral at the optic chiasm (Herrera et al., 2003).

It is not well understood whether signals from presynaptic cells also regulate axon growth for proper wiring to occur, outside of the specification of retinotopic synaptic mapping conferred by electrical activity (McLaughlin et al., 2003). We previously showed that embryonic RGCs undergo a developmental loss of their intrinsic capacity for rapid axon growth (Goldberg et al., 2002b), which could likewise contribute to an intrinsic regenerative failure in adult CNS neurons (Chen et al., 1995; Chierzi et al., 2005; Dusart et al., 1997). Interestingly, we found that RGCs' developmental loss of axon growth ability could be signaled by their presynaptic retinal partners, amacrine cells. This raises the question, do amacrine cells regulate RGC axon growth *in vivo*?

In the mammalian retina, Foxn4 controls amacrine cell fate specification from retinal progenitors, and Foxn4^{-/-} mice have significantly fewer amacrine cells than their wildtype littermates (Li et al., 2004). These mice also lack horizontal cells, but the differentiation of other cell types in the retina including RGCs are not altered. Because amacrine cells can signal RGCs *in vitro* to decrease their intrinsic axon growth ability (Goldberg et al., 2002b), we hypothesized that in the developmental absence of amacrine cells, RGCs might retain their embryonic axon growth ability, or perhaps project their axons abnormally. Here we show that Foxn4 is required for proper outgrowth of RGC axons *in vivo*, suggesting a role for an amacrine cell-RGC interaction in axon growth.

2. Materials and Methods

Animal experiments were conducted in accordance with the guidelines of the University of Miami Institutional Animal Care and Use Committee (IACUC) and comply with the ARVO Statement for the Use of Animals in Research.

Foxn4^{-/-} mice and genotyping

Foxn4^{+/-} females were obtained from the Xiang laboratory (Li et al., 2004) and bred to C57/Bl6 males; heterozygotes were interbred to generate knockout mice with heterozygote and wildtype littermates. Mice were genotyped by PCR using genomic DNA from clipped tails following standard protocols. Specific primer sequences for Foxn4 and LacZ were: Foxn4: 5'-GGCCTCTCTGTCCATACCTGTA-3' (forward) and 5'-CTACTCTCTTTGATGACAGCTCCC-3' (reverse); LacZ: 5'-GGTTGTTACTCGCTCACATTTAATG-3' (forward) and 5'-CCATGCAGAGGATGATGCTCGTGAC-3' (reverse). The PCR product of wildtype (WT) mouse DNA consisted of a single band of 460 base pairs (Foxn4 only); amplification of heterozygous (HET) and knockout (KO) mouse DNA yielded either two bands of 460 base pairs (Foxn4) and 730 base pairs (LacZ) or a single band of 730 base pairs (LacZ only), respectively.

Immunofluorescence

For immunostaining of retina, animals were perfused and eyeballs were collected and fixed with 4% paraformaldehyde (PFA) for 1 hour, after which the tissues were cryoprotected overnight in 30% sucrose, snap frozen in mounting medium (OCT Tissue-Tek, Electron Microscopy Sciences, Hatfield, PA), and sectioned. Sections were postfixed in 4% paraformaldehyde and 10% trichloroacetic acid (TCA) for 10 minutes, then permeabilized with 0.2% Triton X-100 for 30 minutes, and further blocked and permeabilized with 20% normal goat or donkey serum and 0.2% Triton X-100 for 1 hour. Retinal tissues were incubated overnight with anti-Vc1.1 (1:100; Sigma, St. Louis, MO), anti-HPC-1 (1:200; Abcam, Cambridge, MA), anti-GAD65/67 (1:1000), anti-parvalbumin (1:500; Sigma, St. Louis, MO), anti-calretinin (1:5000), anti-glutamate transporter 1 (1:2000), anti-tyrosine hydroxylase (1:100; BD Biosciences, Mississauga, ON Canada), and anti-Map2 (1:150, Sigma, St. Louis, MO). Secondary detection was performed using fluorescent antibodies at a 1:500 (Alexa-488, Alexa-594) or a 1:200 dilution (Alexa-647; Invitrogen, Carlsbad, CA). Slides were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and examined in a Zeiss inverted fluorescent microscope or a Leica TCS SP5 confocal microscope.

Immunocytochemistry of purified retinal ganglion cells was performed as previously described (Wang et al., 2007). Briefly, cells were fixed with 4% PFA for 10 minutes, rinsed three times in PBS, and blocked and permeabilized for 30 minutes with 20% normal goat serum and 0.2% Triton X-100 in antibody buffer (150mM NaCl, 50mM Tris base, 1% BSA, 100mM L-Lysine, 0.04% Na azide, pH 7.4). Overnight incubation with rabbit anti-Tau (1:400, Sigma-Aldrich, St Louis, MO) was performed at 4°C. Goat anti-rabbit Alexa 647 was used at a 1:200 dilution for secondary detection and DAPI was added for nuclear staining. Cells were rinsed and kept in PBS for imaging. (See below.)

Immunofluorescence of brain tissues with Foxn4 antibodies was performed as previously described (Li et al., 2004). Briefly, P3 mice were perfused and euthanized in compliance with the University of Medicine and Dentistry of New Jersey IACUC, after which the brains were dissected and fixed for 2 hours in 4% PFA in PBS at 4°C. Following 30% sucrose infiltration and embedding in OCT (Tissue-Tek, Electron Microscopy Sciences, Hatfield, PA), the samples were sectioned and the cryosections were used for immunofluorescence. Anti-Foxn4 antibody was applied at 1:50 dilution following overnight incubation as previously reported. Images of these sections were obtained with a Nikon eclipse 80i microscope.

Imaging of retinal waves

Freshly dissected retinas from Foxn4^{-/-} mice and their wild-type littermates between the day of birth (P0) and postnatal day 5 (P5) were wicked onto filter paper with the ganglion cell layer facing up and incubated in a solution of 10 μ M Fura 2-AM (Invitrogen, Carlsbad, CA) in artificial CSF (ACSF; 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgCl₂, 1.0 mM KH₂PO₄, 2.5 mM CaCl₂, 26.2 mM NaHCO₃, and 11 mM D-glucose) containing 1% DMSO and 0.02% pluronic acid (Invitrogen, Carlsbad, CA) for 60 to 90 minutes in an oxygenated glass chamber at 33°C. After rinsing with ACSF, retinas were kept in a temperature-controlled chamber at 33°C, mounted on the stage of an inverted microscope (Axiovert 200M, Zeiss) and cultured in ACSF in a controlled environment (95% O₂/5% CO₂). Retinas were illuminated at 340 and 380 nm excitation wavelengths with a 175 Watt, ozone-free xenon lamp (Lambda DG-4, Nikon, Japan) and fluorescence intensities were detected at 500 nm. Images were collected at 5 Hz under 20 $\times$ objective lens (NA=0.4) with a cooled CCD camera (Coolsnap ES Monochrome, Photometrics). Initial single images were collected to identify neurons in the ganglion cell layer including retrograde labeled RGCs

where relevant. After data acquisition, areas of interests were selected and the files were exported to the MetaFluor software (Universal Imaging, CA, USA). Fractional changes in emission intensity, $\Delta F/F$ defined by the ratio of fluorescence intensities produced by excitation at two wavelengths, were measured from individual cell bodies and automatically exported into Excel software and also displayed as traces of $\Delta F/F$ versus time. Upward deflection indicates the increase of intracellular calcium level, induced by the propagating retinal waves. Only intensity increases above 0.003 and synchronized with others were scored as spikes. Under most circumstances, four areas per retina were selected for imaging. Data are expressed as means $\pm$ SD unless noted otherwise, and analyzed by Student's t-test, $p < 0.05$.

Anterograde and retrograde labeling

Animals were deeply anesthetized by hypothermia. 1 μ l of 1% Alexa 488- or 594-labeled cholera toxin subunit B (CTB; Molecular Probes, Invitrogen, Carlsbad, CA) was injected intravitreally using a Hamilton syringe and 33 gauge needle. Different colors were used for each eye, typically 594/red in the right eye and 488/green in the left eye. Six hours later, animals were euthanized and the brains dissected, cutting the optic nerve close to the eyes to preserve the optic chiasm. Tissues were immediately fixed in 4% PFA for 2 hours at room temperature, left on a 30% sucrose solution overnight at 4°C and frozen in OCT medium for cryosectioning. 8 μ m sections were mounted on glass slides with Vectashield and DAPI (Vector Laboratories, Burlingame, CA) and examined in a Zeiss inverted fluorescent microscope or a Leica TCS SP5 confocal microscope.

Retrograde labeling was performed on P1 pups. 1 μ l of a 1% solution of Alexa-594 (red)-conjugated CTB (Molecular Probes) was injected per animal (0.5 μ l per side) at 1 mm from bregma and 1 mm from the midline at a depth of 1 mm. Six days later, the procedure was repeated using Alexa-488 (green)-conjugated CTB and pups were euthanized the next day (at postnatal day 8). Eyeballs, optic nerves and optic chiasmata were collected and immediately fixed in 4% PFA and processed for cryosectioning and imaging as above.

RGC purification, cell culture and neurite growth analysis

RGCs from P2 and P4 mice were purified by immunopanning as previously described (Goldberg et al., 2002b; Meyer-Franke et al., 1995; Moore et al., 2009). Briefly, embryonic and postnatal rat retinas were dissociated with papain (Worthington, Lakewood, NJ) and mechanically triturated to obtain a single cell suspension. Enrichment of RGCs was achieved after sequentially depleting mouse macrophages and selecting CD90 positive cells using anti-Thy1.2 antibody (1:125, AbD Serotec, Oxfordshire, United Kingdom). Because *Foxn4*^{-/-} mice die soon after birth (Fujitani et al., 2006; Williams et al., 2004) and do not breed normally, the number of KO animals available in a litter to purify RGCs was limited. We overcame this difficulty by labeling the RGCs using different fluorophores for KO and WT or HET mice. The day before the experiment and after genotyping, we injected CTB intravitreally. The next day, dye-labeled animals were sacrificed and RGCs purified as described above, mixing the KO, WT and HET mouse-derived RGCs for the purification and plating but still able to differentiate them by color (Fig. 5A). Acutely purified RGCs were plated at clonal densities (<5 cells/mm²) on tissue culture dishes pre-coated with PDL and mouse laminin (Moore et al., 2009). At 2 days in vitro (DIV), cells were fixed in 4% PFA, processed for anti-tau immunofluorescence as described above, and automated neurite tracing was performed at 10X magnification in a KineticScan® HCS Reader (Cellomics, Pittsburgh, PA). Axon length was defined as the longest neurite per cell measured without branches, similar to our previously published technique (Goldberg et al., 2002b). The cells from KO and WT mice were identified based on their different fluorescent labels. Data

analysis was conducted in Excel (Microsoft). At least 70 cells were analyzed per condition, and the experiments were repeated at least twice.

Preparation of tissues for electron microscopy and optic nerve analysis

Optic nerves from postnatal day 4 KO and WT animals were collected and processed immediately for electron microscopy as previously described (Pearse et al., 2004) at the University of Miami Electron Microscopy Core facility. Sections were examined in a Philips CM10 electron microscope. Electron micrographs were taken from cross-sections of at least two animals per genotype. To measure the diameters of the axons, images were scanned and a grid generated by image analysis software (Image-Pro, Media Cybernetics, Bethesda, MD) was overlaid. Only the axons that were intersected by the corners of the grid were measured. At least three images per phenotype were analyzed, and they were averaged and compared by Student's t-test.

3. Results

Decreased amacrine cell number in *Foxn4*^{-/-} mice

Targeted knockout of *Foxn4* leads to a specific loss of amacrine cells and horizontal cells in the mouse retina without affecting the number of retinal ganglion cells (RGCs) (Li et al., 2004). We stained retinal cross-sections with the nuclear label DAPI and counted the number of nuclei in the ganglion cell layer (GCL) and the inner nuclear layer (INL) laying along an arbitrary horizontal line. We confirmed that *Foxn4*^{-/-} mice have fewer nuclei in the INL and GCL, where amacrine cells reside, compared to their wild-type littermates (Fig. 1A, B), but there was no difference in number of nuclei in the outer nuclear layer (ONL) where photoreceptors are found (Fig. 1C). When we immunostained for two pan-amacrine cell markers, syntaxin and Vc1.1 (Arimatsu et al., 1987; Barnstable et al., 1985), we found a greatly reduced staining in the inner aspect of the INL and the GCL (Fig. 1E), consistent with prior characterization (Li et al., 2004). Heterozygotes were indistinguishable from wildtypes (data not shown). We also observed decreased Vc1.1 and syntaxin staining of amacrine cell neurites in the inner plexiform layer (IPL) (Fig. 1E) and a reduced IPL thickness (Fig. 1D).

The number of nuclei in the INL and the IPL thickness were similarly decreased in both peripheral and central retina (Fig. 1B, C), although a comparison of the nuclei in the INL in the central retina only trended towards statistical significance ($p=0.06$; Fig. 1B). When we compared INL cell number and IPL thickness, using the number of ONL nuclei for normalization and to control for any possibly obliquely cut sections, we found that the thickness of the IPL was reduced to a greater proportional degree than the number of nuclei in the INL (Fig. 1F, G), consistent with the INL containing the nuclei of unaffected bipolar and Muller glial cells. Taken together, these results demonstrate the significantly decreased number of amacrine cells in the *Foxn4*^{-/-} mice, and also point to a decrease in amacrine cell and/or RGC neurites in the IPL.

Residual amacrine cells in *Foxn4*^{-/-} mice display normal subtype markers

In order to better characterize the amacrine cell deficit in *Foxn4*^{-/-} mice, we asked which amacrine cell subpopulations were decreased in these animals. We hypothesized that if one or more specific amacrine cell subtypes were specifically affected, we would see a reduction in the percent of one subtype, or an increase in the percent of the remaining unaffected subtypes, or both. We immunostained retinal cross-sections of P9 KO mice and their WT littermates, and we found that there was a trend towards a decreased percentage of remaining calretinin-immunoreactive amacrine cells (Fig. 2A, E), although the difference did not reach statistical significance ($p=0.12$ by Student's t-test), as there was a varying

phenotype penetrance among litters. There was also no difference in the percentage of remaining amacrine cells immunoreactive for the calcium-binding protein parvalbumin (Fig. 2B, F), tyrosine hydroxylase (Fig. 2C, G) or GAD 65/67 (Fig. 2D). Thus, the *Foxn4*^{-/-} mice demonstrate a similar reduction in all amacrine cell subtypes tested, suggesting that *Foxn4* does not play a role in the development of only one subtype, but rather is involved in amacrine cell fate determination more generally.

Retinal waves are preserved despite reduction in amacrine cells

We next asked whether the reduced amacrine cell number in the *Foxn4*^{-/-} mouse is associated with changes in early retinal activity, specifically in the generation or propagation of retinal waves of spontaneous, synchronized action potentials observed in the vertebrate retina during development (Meister et al., 1991; Wong et al., 1993). These retinal waves are only detectable before eye opening (Feller et al., 1996; Meister et al., 1991; Wong et al., 1993), and are necessary for the establishment of a precise retinotopic map (McLaughlin et al., 2003). Retinal waves are initiated from starburst amacrine cells (Zhou, 1998), a type of displaced amacrine cell in the GCL, and are propagated among neighboring RGCs and amacrine cells. To examine whether retinal waves are functional in *Foxn4*^{-/-} retinas, we analyzed the characteristics of retinal waves during development using ratiometric calcium imaging.

We recorded retinal waves in both *Foxn4*^{-/-} and wildtype retinas (Fig. 3A) and we found no differences in our ability to load retinal neurons with Fura-2 or to record retinal waves from P1 or P2 *Foxn4*^{-/-} mice and their wildtype littermates. However, by P4-P5, we were not able to detect calcium waves in *Foxn4*^{-/-} retinas and only rarely in wildtype retinas, likely due to the development of the mesh-like vasculature on the retinal surface that obscures loading and visualization of the Fura-2 dye in the GCL (data not shown). The difference in retinal wave detection between *Foxn4*^{-/-} and wildtype retinas was not significant.

To examine the properties of the retinal waves in greater detail, we individually selected all the Fura-2-loaded cells in focus within a field of view with Metafluor region-of-interest tools and quantified calcium-induced fluorescence changes on a per-cell basis. We compared the percentage of cells participating in at least one retinal wave in *Foxn4*^{-/-} versus wildtype retinas, and we found that the percentage of cells participating in retinal waves was similar in P1 and P2 retinas (Fig. 3B). We next analyzed the frequency of the retinal waves by measuring the interval between the spikes. We found no significant differences between P1 or P2 wildtype and *Foxn4*^{-/-} retinas (Fig. 3C). Finally, we examined the waves' amplitude, including the averaged amplitude of all the waves in one recording (Fig. 3D), the maximum amplitude in one field of recording (Fig. 3E), and the averaged maximum amplitude of individual cells (Fig. 3F), and found no significant differences in any of these parameters. Although we were not able to document whether starburst amacrine cells, responsible for initiating retinal waves, are disproportionately decreased in *Foxn4*^{-/-} retinas, these data suggest that they are likely present in adequate numbers. Taken together, these data demonstrate that there is no significant difference in retinal waves between *Foxn4*^{-/-} and wildtype mice, suggesting that this reduced complement of amacrine cells is sufficient to maintain electrical retinal function, at least through early postnatal development.

RGCs' intrinsic axon growth ability in vitro

We next began to investigate whether, in the context of a reduced complement of amacrine cells, RGC axon growth ability or patterning are affected. Previous experiments published by Li et al. (2004) demonstrate that *Foxn4* is not expressed in RGCs; however, it is not known whether *Foxn4* is expressed in the major target area of the rodent visual pathway, the

superior colliculus. We analyzed brain cross-sections of postnatal day 3 wildtype mice and found that *Foxn4* could not be detected in the superior colliculus (Fig. 4A), whereas the ventricle demonstrated bright immunopositive staining using the same antibody (Fig. 4B). Thus, these data suggest that *Foxn4* is not expressed in the superior colliculus, and changes in RGC axon growth or targeting are not likely to result from target-derived changes in the *Foxn4*^{-/-} mouse.

We have previously demonstrated that amacrine cells are sufficient to signal embryonic RGCs to decrease their intrinsic axon growth ability in vitro (Goldberg et al., 2002b); however, it is not known whether amacrine cells are necessary in vivo for embryonic RGCs to undergo this developmental decrease in axon growth ability. To begin to address this question in vitro, we purified labeled RGCs from age P2-P4 *Foxn4*^{-/-} mice and HET and WT littermates, and cultured them at clonal density on PDL and laminin in a serum-free defined growth media which strongly promotes RGC survival and axon growth (Fig. 5A, B) (Goldberg et al., 2002a; Goldberg et al., 2002b; Meyer-Franke et al., 1995). After 2 days, we fixed the cells and immunostained for the neurite marker Tau to measure axon growth parameters including total neurite length, length of the longest neurite and number of branch points (Fig 5B). In two independent experiments with at least 70 cells per genotype each, we found that while the rate of axon growth was consistent with previous studies (Goldberg et al., 2002b), there was no significant difference between RGCs from *Foxn4*^{-/-} mice and their wildtype or heterozygous littermates in either axon length or total neurite length (Fig. 5C). Due to difficulties in identifying genotypes at younger ages, and the rarity of knockout animals surviving much later than the first postnatal week, we were not able to characterize a time course of RGC axon growth ability (Goldberg et al., 2002b). Whether the remaining amacrine cells in the *Foxn4*^{-/-} retinas are sufficient to signal RGCs to decrease their intrinsic axon growth ability by P2-P4 as in the wildtype or heterozygous retinas could not be determined from these mice.

RGCs are delayed in reaching the superior colliculus in *Foxn4*^{-/-} mice

Although we could not detect a difference in RGC axon growth in vitro, we next asked whether, in the context of a reduced number of amacrine cells, RGC axons would have an aberrant trajectory along the optic pathway. To address this question, we examined RGC axons along the optic pathway in *Foxn4*^{-/-} mice by anterograde and retrograde tracing.

First, we injected Alexa-488 and Alexa-594-conjugated cholera toxin B (CTB) intravitreally in *Foxn4*^{-/-} mice and their wildtype littermates at four different ages (P0, P2-P3, P4 and P6). After 24 hours, we collected eyes with attached optic nerves, obtained transverse cross-sections of the immediate retrobulbar nerve, and examined the intensity of the fluorescent labels within the area of the optic nerve in each section (Fig. 6A). We found no significant differences in the mean fluorescence intensities between the *Foxn4*^{-/-} and the wildtype optic nerves between the ages of P0 and P6 (Fig. 6B), suggesting that axon number and capacity for dye transport are likely normal in the *Foxn4*^{-/-} optic nerve. We also analyzed the morphology of optic nerves by transmission electron microscopy and found that in the *Foxn4*^{-/-} mice, areas of mild tissue architecture disruption were observed (Fig. 6C, arrows). Quantification of axon diameters in these optic nerve electron micrographs revealed a statistically significant but small decrease in axon diameter in *Foxn4*^{-/-} mice (Fig. 6D). Axon crossing at the optic chiasm appeared grossly normal, with most axons crossing and a small number remaining ipsilateral (Fig. 6E). Thus even when presynaptic amacrine cells are reduced in number, there were similar numbers of RGC axons in the optic nerves and, despite mild disruptions in optic nerve structure, axon patterning at the optic chiasm appeared normal.

Next, we asked whether RGC axon targeting to the superior colliculus was disrupted. We injected superior colliculi with Alexa-594-CTB at P1 and Alexa-488-CTB at P6, where it could be taken up by any RGC axons present and retrogradely transported to the retina, lighting up both the RGC cell bodies and their dendritic processes in the IPL (Fig. 7A). First, we observed that the complexity of RGCs' dendrite morphology in the IPL was reduced in *Foxn4*^{-/-} compared to wild-type retinas (Fig. 7A). We confirmed this impression by immunostaining RGC dendrites in the retina with Map2, and again found that RGC dendrites were reduced in the context of the reduced number of amacrine cells in the *Foxn4*^{-/-} mice (Fig. 7B). Thus both RGC and amacrine cell contributions to the IPL are reduced in *Foxn4*^{-/-} mice.

We quantified the number of RGCs whose axons have reached the superior colliculus and can thus take up the retrograde dyes, and found that at P1 and P7, fewer RGCs are retrogradely labeled (Fig. 7C). This decrease in RGC axons labeled from the superior colliculus was similar in both the central and peripheral retina, suggesting that this is not a deficit confined to early (central) versus later (peripheral) retinal development. As noted above, there was variable penetrance of this phenotype; in 2 of 7 animals, there was little difference between the WT and KO RGC numbers at P7. As there are no differences in the number of RGCs between the *Foxn4*^{-/-} mice and their wildtype littermates (Fig. 1 and (Fujitani et al., 2006; Li et al., 2004)), the lower number of retrogradely labeled RGCs in the *Foxn4*^{-/-} retinas suggests that fewer RGC axons reach the superior colliculus in the *Foxn4*^{-/-} mice.

To ask whether the axons reaching the superior colliculus exhibit normal patterning at a gross level, we examined the fluorescent intensities in cross-sections of the superior colliculus after anterograde tracing of RGC axons (Fig. 8A). First, we noted that, consistent with the normal crossing phenotype in the optic chiasm, RGC axons were almost exclusively found in the contralateral superior colliculus. We found a significant decrease in RGC axon penetration into the deeper, retinorecipient layers of the superior colliculus (Fig. 8A, B). In the *Foxn4*^{-/-} mice, RGC axons at P0 were found almost exclusively along the surface of the superior colliculus (Fig. 8A, B), and even by P6 there was less targeting into the retinorecipient superior colliculi (Fig. 8A, C), although this may also reflect a decrease in axon number at the superior colliculus as found above (Fig. 7). Together with the retrograde labeling data demonstrating that fewer RGCs reached the superior colliculus, these data demonstrate that in the context of a reduced number of amacrine cells, RGC axons fail to grow and penetrate properly into their major target in early postnatal development, thus supporting a role for presynaptic amacrine cells in determining RGCs' axon growth in vivo.

4. Discussion and Conclusions

These data demonstrate that RGC axon targeting and penetration into the retinorecipient layers of the superior colliculus is disrupted in the setting of a reduced complement of amacrine cells in the *Foxn4*^{-/-} mouse. *Foxn4* is a member of the forkhead/winged helix family of transcription factors (Gouge et al., 2001). This evolutionary conserved family of genes have been implicated in a variety of developmental processes (Carlsson and Mahlapuu, 2002; Kaufmann and Knochel, 1996), including cardiac chamber formation in zebrafish (Chi et al., 2008) and neuronal cell fate determination in *Xenopus*, by regulating cell cycle (Burgering and Kops, 2002). In the mammalian retina, *Foxn4* is highly expressed in a subset of retinal precursor cells during early development (Gouge et al., 2001) and disruption of its expression leads to a decreased number of horizontal and amacrine cells (Fujitani et al., 2006; Li et al., 2004). There is no *Foxn4* expression in RGCs (Fujitani et al., 2006; Li et al., 2004) or in RGC target areas in the superior colliculus, suggesting that the

deficits seen in RGC axon outgrowth *in vivo* are attributable to their missing presynaptic partners, retinal amacrine cells.

Retinal waves are present in early postnatal *Foxn4*^{-/-} mice

Retinal activity is required for refinement of RGC synapses in the brain, but may not be required for RGC axon targeting at a gross level (Feller, 2009; Katz and Shatz, 1996). Retinal waves, recorded with calcium imaging, are still present in the *Foxn4*^{-/-} retinas at P2. Their spatiotemporal properties, including frequency and amplitude, were almost identical to their littermates, suggesting that these important developmental phenomena do not depend on the full complement of retinal amacrine cells, although it is possible that there is not a significant difference in the number of initiating starburst amacrine cells in these animals (Zheng et al., 2006). Another possibility is that the temperature at which these experiments were conducted (33°C) hindered our ability to detect a significant contribution of absent amacrine cells to retinal waves (Stafford et al., 2009). These experiments did not distinguish between RGC and amacrine cell contribution to the retinal waves, as retrograde labeling of RGCs would be inconsistent given the RGC axon projection to the superior colliculus discovered here. The lack of change in retinal waves does not preclude changes in RGCs' axon growth or patterning seen here; indeed the developmental loss of RGCs' intrinsic axon growth ability was also found to be independent of retinal activity (Goldberg et al., 2002b). Nevertheless these data demonstrate that the amacrine cell-dependent component of RGC axon targeting is independent of retinal activity, and may provide clues to differentiating aspects of retinal patterning that are activity-dependent and activity-independent (Katz and Shatz, 1996).

RGCs' intrinsic axon growth ability is not modified in the near absence of amacrine cells

One major question in neuroscience is why neurons of the central nervous system fail to regenerate their axons after injury. We previously found that amacrine cells can signal embryonic RGCs to turn off their intrinsic growth ability during development (Goldberg et al., 2002b) and thus we had initially hypothesized that in the context of a reduced number of amacrine cells, RGCs might not decrease their axon growth ability on time. We purified RGCs from postnatal *Foxn4*^{-/-} mice and found no difference in their intrinsic ability to grow axons *in vitro*, compared to RGCs from wildtype littermates. It is possible that that signal from amacrine cells that tells RGCs to turn off their intrinsic axon growth ability could have been already delivered by the residual subset of amacrine cells by P2-P4. By P0, over 95% of RGC axons have reached their targets in the superior colliculus (Hofbauer and Drager, 1985), and the decrease in RGC axon growth ability *in vivo* is largely complete by P1-P2. It will be interesting in future experiments to determine whether RGC genes important in the regulation of intrinsic axon growth ability, such as Kruppel-like family members (Moore et al., 2009), demonstrate differences in developmental regulation in the *Foxn4*^{-/-} mice.

Developmental delay in the normal projection of RGCs to the superior colliculus

To address whether RGCs would project their axons normally along the optic pathway in *Foxn4*^{-/-} mice, we studied RGCs anterogradely and retrogradely labeled by CTB. Although RGCs do not express *Foxn4* and the number of RGCs is unchanged in *Foxn4*^{-/-} mice (Fig. 1 and (Li et al., 2004), RGC axon caliber by electron microscopy, and RGC axon targeting and penetration at the superior colliculus were reduced. Both a decreased number of retrogradely labeled RGCs and decreased fluorescence intensity in superior colliculi of the *Foxn4*^{-/-} mice compared to their wildtype or heterozygous littermates, confirm that in the context of a decreased number of amacrine cells, RGCs do not project their axons to the right place at the right time. A competing hypothesis is that amacrine cells are necessary for RGC axons to properly transport the CTB dye, but this would not be likely to account for

the decreased penetration of RGC axons into the retinorecipient layers of the superior colliculus. Another possible explanation of the results shown here is that in the absence of horizontal cells (a population also altered in the *Foxn4*^{-/-} retinas), there is abnormal retinal wiring, or that the hypomorphic mouse pups have delayed development. Future characterization of the extent of the delay in RGC wiring compared to other retinal or brain circuits may help to address some of these possibilities.

Taken together, these data suggest that *Foxn4* is required for proper wiring of the retino-collicular projection during development, and suggest a novel role for a neuron's presynaptic partner in controlling the postsynaptic neurons' developmental wiring in vivo. Future experiments directed at understanding the molecular mechanisms may further enhance our understanding of this potentially novel mechanism of controlling patterning in the developing nervous system.

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Figure 1.

Decreased number of amacrine cells in the *Foxn4*^{-/-} retinas. **(A, B)** Quantitative analysis of the number of nuclei in the ganglion cell layer (GCL, A) and in the inner nuclear layer (INL, B) of *Foxn4*^{-/-} mice and their wildtype littermates showed a significant decrease in the number of DAPI-labeled nuclei along the lines traced in these layers both in central and peripheral retina. **(C)** The number of nuclei in the outer nuclear layer (ONL) was similar. **(D)** The thickness of the inner plexiform layer (IPL) was decreased in *Foxn4*^{-/-} retinas. **(E)** Retinal cross-sections from postnatal day 6 mice were immunostained with two amacrine cell markers, syntaxin and VC1.1, demonstrating the decrease in amacrine cells and their neurites. **(F)** Quantification of the IPL thickness in relationship to the number of nuclei in the ONL. **(G)** Quantification of the number of nuclei in the INL in relationship to the number of nuclei in the ONL. Mean $\pm$ SEM shown; # $p=0.06$; * $p<0.05$; ** $p<0.01$; *** $p<0.005$, Student's t-test; N=4 retinas (P8-P9), 3 measurements per retina. Scale bar: 60 μ m.

Figure 2.

Characterization of amacrine cell subtypes in the $Foxn4^{-/-}$ retinas. (A–D) P9 retinas were immunostained with antibodies as labeled. The magnifications are equal, but the $Foxn4^{-/-}$ retinas appear smaller due to the reduced IPL/INL thicknesses. (E–G) Quantification of the percentage of immunopositive amacrine cells of the Vc1.1+ population. No significant differences were detected between $Foxn4^{-/-}$ mice and their wild-type littermates. The high expression of GAD precluded accurate quantification. Mean $\pm$ SEM shown. Scale bar: 120 μ m.

Figure 3. Retinal waves do not differ between *Foxn4*^{-/-} and wildtype mice. **(A)** Probability of recording waves in both WT and KO retinas across different postnatal retinal ages. Each circle represents one retina. **(B–F)** There was no statistically significant difference by Student's t-test between WT and KO for the different parameters measured: fraction of cells participating in at least one calcium wave **(B)**, retinal waves interval **(C)** and amplitude **(D–F)**, including the averaged amplitude of all the waves in one recording **(D)**, the maximum amplitude in one field of recording **(E)**, and the averaged maximum amplitude of individual cells **(F)**. (Mean $\pm$ SD shown.)

Figure 4. Foxn4 immunostaining in the superior colliculus. **(A)** Foxn4 expression is not detectable in the superior colliculus of P3 wildtype mice. **(B)** Foxn4 immunoreactivity was detected in the ventricle (arrow).

Figure 5. Analysis of RGC axon growth in vitro. (A) The day before the experiment and after genotyping, RGCs were labeled by intravitreal injection of CTB, using different fluorophores for KO (green) and WT or HET (red) mice. Scale bar: 40 μm. (B) The following day, dye-labeled animals (P2 and P4) were sacrificed and RGCs were purified, cultured in serum-free media and immunostained after 2DIV with anti-Tau. Automated tracing was performed on >900 wildtype RGCs and >70 KO RGCs at each age. Scale bar: 60 μm. (C) Axon length and total neurite length were quantified in two independent experiments with similar results. Mean ± SEM shown.

Figure 6.

Optic nerve axon morphology and number. **(A)** Postnatal mice of different ages ranging from P0 to P6 were injected with CTB in the vitreous. 6 hours post-injection, animals were euthanized and optic nerves were collected and cryosectioned. Shown are example cross-sections of P4 optic nerves; DAPI (blue) counterstains the glial nuclei. The fluorescence intensity of the axon transported dye (red) was quantified and normalized to the area of the nerve **(B)**. **(C)** Electron micrograph of optic nerve shows mild disruption and vacuolization in optic nerve architecture (arrows) in $Foxn4^{-/-}$ mice compared to their wildtype littermates. **(D)** Quantification of axon diameters in optic nerve electron micrographs revealed a decreased axon diameter in $Foxn4^{-/-}$ mice compared to their wildtype littermates. (Mean $\pm$ SEM; $14 < n < 55$; * $p < 0.05$, Student's t-test.) **(E)** Axon crossing at the optic chiasm appeared grossly normal in $Foxn4^{-/-}$ mice, with most axons crossing contralaterally and a small percentage remaining ipsilateral (arrow).

Figure 7.

RGC axon and dendrite patterning in vivo. **(A)** RGCs from P1 and P6 mice were retrogradely labeled with CTB-594 (red) and -488 (green), respectively, and the animals were euthanized 24 hours later. Retinal cross sections demonstrating RGC cell body and dendrite fluorescence are shown (brackets and arrows); a small amount of background autofluorescence from the rest of the retina is also seen. **(B)** Postnatal day 7 *Foxn4*^{-/-} and heterozygous mice were injected 1% CTB in the superior colliculus. Animals were sacrificed the next day and the retinas were collected to perform immunostaining with Map2 antibody. Qualitative analysis of the retinal cross-sections shows a decrease in the number of labeled RGCs in the *Foxn4*^{-/-} retinas. **(C)** Quantitative analysis of the P7 retina confirms the decrease in the number of RGC axons reaching the superior colliculus by P1 and by P6-P7, (n=7, ±SEM). Small scale bar: 40 μm; large scale bar: 80 μm.

Figure 8.

RGC axons fail to penetrate the superior colliculus in $\text{Foxn4}^{-/-}$ mice. **(A)** The brains of P0 or P6 anterogradely labeled animals were collected, sectioned and mounted on glass slides, and counterstained with DAPI to show nuclei (blue). At both P0 and P6, a reduction in the penetration of RGC axons into the retinorecipient layers of the superior colliculus is apparent. **(B, C)** Quantification of the mean fluorescence intensity as a function of distance from the surface of the superior colliculus (arrow labeled “Depth” in A) revealed more axons remaining over the surface of the superior colliculus at P0 (difference marked * in B), and a decrease in the labeled projections penetrating into the retinorecipient layers of the superior colliculus in the $\text{Foxn4}^{-/-}$ mice at P0 and P6 (difference marked # in B and C, respectively).