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Retinal Ganglion Cell Death and Optic Nerve Degeneration by Genetic Ablation in Adult Mice

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Abstract

Despite the magnitude of the problem, no effective treatments exist to prevent retinal ganglion cell (RGC) death and optic nerve degeneration from occurring in diseases affecting the human eye. Animal models currently available for developing treatment strategies suffer from cumbersome procedures required to induce RGC death or rely on mutations that induce defects in developing retinas rather than in mature retinas of adults. Our objective was to develop a robust genetically engineered adult mouse model for RGC loss and optic nerve degeneration based on genetic ablation. To achieve this, we took advantage of *Pou4f2* (*Brn3b*), a gene activated immediately as RGCs begin to differentiate and expressed throughout life. We generated adult mice whose genomes harbored a conditional *Pou4f2* allele containing a *floxed-lacZ-stop-diphtheria toxin A* cassette and a *CAGG-Cre-ERTM* transgene. In this bigenic model, Cre recombinase is fused to a modified estrogen nuclear receptor in which the estrogen-binding domain binds preferentially to the estrogen agonist tamoxifen rather than to endogenous estradiol. Upon binding to the estrogen-binding domain, tamoxifen derepresses Cre recombinase, leading to the efficient genomic deletion of the floxed *lacZ-stop* DNA sequence and expression of diphtheria toxin A. Tamoxifen administered to adult mice at different ages by intraperitoneal injection led to rapid RGC loss, reactive gliosis, progressive degradation of the optic nerve over a period of several months, and visual impairment. Perhaps more reflective of human disease, partial loss of RGCs was achieved by modulating the tamoxifen treatment. Especially relevant for RGC death and optic nerve degeneration in human retinal pathologies, RGC-ablated retinas maintained their structural integrity, and other retinal neurons and their connections in the inner and outer plexiform layers appeared unaffected by RGC ablation. These events are hallmarks of progressive optic nerve degeneration observed in human retinal pathologies and demonstrate the validity of this model for use in developing stem cell therapies for replacing dead RGCs with healthy ones.

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Keywords

Retinal ganglion cells; optic nerve degeneration; Pou4f2/Brn3b; diphtheria toxin A; genetic ablation; retinal disease mouse model

Introduction

A number of mouse models for retinal ganglion cell (RGC) death and optic nerve degeneration have been described in the ocular research literature. These models have proven useful for investigating various retinal pathologies, including glaucoma induced by elevated intraocular pressure (IOP), optic neuritis, and ischemic optic neuropathy. For example, DBA/2J inbred mice develop chronic hereditary glaucoma and are an important model to study the molecular mechanisms underlying this disease (Jakobs et al., 2005; Schlamp et al., 2006; Bosco et al., 2008). A mouse model for autosomal dominant optic atrophy relying on a disabling mutation in the *Opa1* gene locus has been recently generated (Davies et al., 2007). These mice show impaired optic nerve structure and visual dysfunction. In non-genetic rodent models, invasive surgical and pharmacological procedures are routinely used to elevate IOP or damage optic nerves (Porciatti et al., 1996; Gross et al., 2003).

Despite their usefulness, these models are far from ideal and are limited in their ability to address many of the crucial issues that face researchers who are attempting to restore healthy RGCs to damaged retinas. Notably, a gene knockout model is available for specifically investigating RGC loss; retinas of *Math5*-knockout mice lack virtually all RGCs (Brown et al., 2001; Wang et al., 2001). However, as with many gene knockouts, the absence of *Math5* results in deficiencies in RGC development that occur during embryogenesis rather than a loss of mature functional RGCs in postnatal life. Developmental defects that prevent RGCs from forming are not likely to reflect the events that occur following the death of fully differentiated RGCs in adult retinas. Adult life is when most human retinal diseases associated with RGC death occur (Levin and Gordon, 2002; Margalit and Sadda, 2003). It would therefore be advantageous to create a genetic model in which mature RGCs could be efficiently removed and the consequent effects on retinal integrity and optic nerve degeneration determined.

To specifically ablate RGCs in adult life, we took advantage of *Pou4f2* (*Brn3b*), a gene that is activated immediately in RGCs as they begin to differentiate. *Pou4f2* encodes a class IV Pou domain transcription factor that is required for RGC differentiation and survival (Gan et al., 1996; 1999). We generated adult mice whose genomes contain a *Pou4f2* allele with an inserted *floxed-lacZ-stop-diphtheria toxin a (dta)* cassette and a *CAGG-Cre-ERTM* transgene (Hayashi and McMahon, 2002). Tamoxifen administration by intraperitoneal injection resulted in the efficient ablation of RGCs and a suite of characteristic properties representative of degenerative retinas. Notably, RGC-ablated retinas maintained their structural integrity. In addition, by modulating tamoxifen treatment, we could produce retinas with varying degrees of RGC loss and optic nerve degeneration. The progressive degeneration in this novel genetic model shares features with RGC death and optic nerve degeneration that are observed in most human retinal pathologies. The model should therefore be useful for experimental approaches in which attempts are made to replace damaged host retinas with healthy donor RGC progenitors to repair the degenerating optic nerve.

Materials and Methods

Mouse handling and tamoxifen administration

Mice heterozygous for the *Pou4f2^{lacZ-dta}* allele (Mu et al., 2005) and heterozygous for the *CAGG-Cre-ERTM* transgene (Hayashi and McMahon, 2002) were maintained as stock lines. The mice were bred and maintained on a C57BL6/129SvEv mixed background. These mice are available to all interested investigators upon request. All genotypes described were confirmed by polymerase chain reaction. *Pou4f2^{lacZ-dta};CAGG-Cre-ERTM* double-heterozygous mice were used for the analyses. Wild-type, *Pou4f2^{lacZ-dta}* heterozygous, and *CAGG-Cre-ERTM* heterozygous mice served as controls. Tamoxifen (Sigma, St. Louis, MO) was administered to one-month or four-month old mice by intraperitoneal injection once a day over 1 to 5 consecutive days at a concentration of 5 mg/40 g of body weight in corn oil (Sigma). Non-tamoxifen-treated control mice were injected with the same volume of corn oil.

All animal procedures in this study followed the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at The University of Texas M. D. Anderson Cancer Center.

Immunohistochemical analysis

Twelve μ m cryostat sections were obtained from 10% buffered formalin-fixed eye samples from embryonic day 16.5 (E16.5) to 11 months of age. Endogenous peroxidase activity and nonspecific antibody binding was blocked with 3% hydrogen peroxide in methanol for 25 minutes followed by 3% normal serum with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.2–7.4) for 1 hour. Goat anti-Brn3b/Pou4f2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:200 dilution. Rabbit polyclonal cleaved anti-caspase 3 antibody (Asp170, Cell Signaling Technology, Beverly, MA) was used at 1:200 dilution. Rabbit anti-neurofilament light chain (Santa Cruz), anti-Thy1.1 (Biolegend, San Diego, CA), anti-Brn3a/Pou4f1 (Chemicon, San Antonio, TX), and anti-Pax6 (Chemicon), antibodies were used at 1:200, 1:50, 1:200, and 1:1,000 dilutions, respectively. All antibodies were diluted in 3% normal serum in PBS containing 3% BSA, and samples were incubated with antibodies at 4° C for 16 hours. Immunoreactivity was visualized using an ABC kit (Vector Laboratories, Burlingame, CA) for colorimetric visualization (brown) and counterstained with methyl green (green). For immunofluorescence staining, donkey cy3-conjugated (red) anti-rabbit, anti-mouse, and donkey fluorescein isothiocyanate-conjugated (green) anti-goat antibodies were used at 1:400 dilutions (Molecular Probes, Eugene, OR) and counterstained with 4',6-diamidino-2-phenylindole (DAPI).

β -galactosidase detection by X-gal staining

Retinas from two month old mice were dissected from eyes and fixed in 2% paraformaldehyde in PBS for 2 hours, rinsed three times with PBS, and incubated in 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) solution (2 mM MgCl₂, 0.01% deoxycholate, 0.02% NP-40, 100 mM PBS, pH 7.4, 5 mM potassium ferrocyanide (K₄Fe(CN)₆), 5 mM potassium ferricyanide (K₃Fe(CN)₆), and 1 mg/ml X-gal) at room temperature for 8–16 hours. X-gal-stained retinas were post-fixed with 2% paraformaldehyde for 8–16 hours and embedded in Tissue-Tek® O.C.T. compound (Sakura, Japan). 12 μ m cryosections were counter-stained with nuclear fast red.

Retrograde labeling with 1, 1'-dioctadecyl-3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI)

Four-month old mice were sacrificed by overdose anesthesia and cervical dislocation. The palatine bone was carefully removed to expose the optic chiasm. The orbital bones and surrounding tissue were then carefully removed to expose the optic nerve. Special effort was made to avoid any physical damage or pressure to the eyeball and optic nerve. During the dissection process, 4% paraformaldehyde in PBS was frequently applied onto the gaps between the bone and nervous tissue to ensure no breakage of the optic nerve or damage to the eyeball. The optic nerve was cut immediately adjacent to the optic chiasm. The eyeball with attached optic nerve was further fixed for 1 hour in 4% paraformaldehyde in PBS and washed 3 times with 0.5× PBS. Approximately 2 mm of the optic nerve was trimmed away to expose axons that were possibly retracted during fixation. The optic nerve was inserted into a glass capsule filled with 5% DiI in dimethyl sulfoxide (DMSO). The opening of the glass capsule was then sealed with gap filling glue (M&M Hobby Center, Instra-Cure+). The glued DiI capsule-eyeball component was transferred into a 1.5 ml microtube filled with 0.5× PBS and stored in the dark at room temperature. On day 9, the eyeball was separated from the rest of the component and the cornea and lens were removed. The eyecup was post-fixed for 30 minutes in 4% paraformaldehyde in PBS and counterstained with TOTO-3 (Molecular Probe) before the final flat mounting procedure. Images were collected using an Olympus FV 1000 laser scanning confocal microscope.

Quantification of cell numbers in the ganglion cell layer and determination of cross-sectional areas of optic nerves

The total number of cells in a selected area of the ganglion cell layer was estimated by counting cells within a 0.15 mm² area from an average of three to five sections from at least three independently prepared retinas. Mean ± S.D. values were evaluated for control and *Pou4f2^{lacZ-dta};CACG-Cre-ERTM* animal groups. To determine the cross-sectional area of the optic nerve, sections were stained with cresyl violet, traced and calculated with NIH Image J (version 1.38×), and averages were determined. Statistical significance was determined by Student's *t*-test. A *p* value < 0.01, 99% confidence level, was considered statistically significant.

Transmission Electron Microscopy

Optic nerves were dissected from euthanized 4-month-old mice, fixed, and embedded in EPON medium as described in Mu et al. (2005). Ultra-thin sections across the optic nerve were cut 3 mm from the optic disk, stained with uranyl acetate and lead citrate, and examined in a JEM 1010 transmission electron microscope.

Vision acuity test

Mice were placed into a light-dark cardboard box chamber (dimensions 440 × 260 × 250 mm) with a 20-watt bulb mounted on the light side of the chamber and a flap allowing access to both sides of the chamber. The time that mice spent in each side of the chamber over a five-minute interval was determined manually. One *Pou4f2^{lacZ-dta};CACG-Cre-ERTM* mouse and one control mouse were placed together in the dark side of the chamber for 1–2 hours before allowing access to the light side of the chamber. Five-minute intervals for each pair of mice were repeated 3 times and 4 pairs of mice were used in the analysis. Mean ± S.D. values were calculated and statistical significance was determined by Student's *t*-test. A *p* value < 0.01 was considered statistically significant (a 99% confidence level).

Photography

Micrographs were obtained with a digital camera (Axiovision 3.1) mounted on an Axioskop2 microscope (Carl Zeiss, Thornwood, NY) or a confocal laser scanning microscope (Olympus Fluoview laser microscope, Olympus Optical Co., Japan). Cranial images were obtained with a Canon Rebel XT digital camera (Canon, Japan). Photographs were assembled in Adobe Photoshop (Adobe Systems, San Jose, CA). Contrast, brightness, and color balance were adjusted to obtain optimum images.

Results

Generation of *Pou4f2-dta*; *Cre-ER*TM mice

In an earlier study that was aimed at determining the effects of RGC ablation during retinogenesis, we inserted a *floxed-lacZ-stop-dta* cassette into the *Pou4f2* locus to create a *Pou4f2^{lacZ-dta}* allele (Mu et al., 2005). Excision of *lacZ* by Cre recombinase restores the open reading frame of *dta* (Grieshammer et al., 1998). In order to activate *dta* in adult retinas, we used a transgene containing a conditional *Cre* in which Cre recombinase is fused to a modified estrogen receptor ligand-binding domain (*Cre-ER*TM) that has high affinity for the estrogen antagonist tamoxifen (Hayashi and McMahon, 2002). The expression of the *Cre-ER*TM transgene is under the control of a ubiquitous *actin* gene promoter (*CAGG*). We bred *CAGG-Cre-ER*TM transgenic mice to mice harboring the *Pou4f2^{lacZ-dta}* allele to generate a line of *Pou4f2^{lacZ-dta}*; *CAGG-Cre-ER*TM (*Pou4f2^{Z-dta}*; *Cre-ER*TM) mice.

Expression of *Pou4f2* in developing and postnatal retinas

To ensure that the *Pou4f2* locus could be used effectively for RGC ablation throughout life, we confirmed and extended previous studies on the spatiotemporal expression pattern of *Pou4f2* by immunostaining with an anti-*Pou4f2*/Brn3b antibody (Xiang et al., 1995; Fig. 1). As previously demonstrated, *Pou4f2* was first detected at E12.5 in a scattering of newly forming RGCs found mainly in the central retina and extending to the periphery (data not shown; Gan et al., 1999). At E16.5, most *Pou4f2*-positive cells were localized in the developing ganglion cell layer, although a few cells still remained in the neuroblast layer (Fig. 1A). By E18.5, all *Pou4f2*-positive cells were in the developing ganglion cell layer (Fig. 1B). At birth (P0) and postnatal day 3 (P3), *Pou4f2*-positive cells were localized to the still forming ganglion cell layer (Fig. 1C, D), and by P15, when the ganglion cell layer was fully formed, all *Pou4f2*-positive cells were localized to this layer (Fig. 1E). In the adult, *Pou4f2*-expressing cells represented approximately 40% of the cells in the ganglion cell layer; only a few RGCs did not express *Pou4f2* in the mature ganglion cell layer (see below) and non-*Pou4f2*-expressing cells were mostly but not entirely displaced amacrine cells. The expression of *Pou4f2* was maintained at constant levels in the ganglion cell layer throughout adult life (Fig. 1F). To ablate RGCs, we selected one month of age as an appropriate time to administer tamoxifen, when the retina is fully functional and mice are sexually mature.

Effective ablation of RGCs by tamoxifen administration

Initially, we administrated a single dose of tamoxifen (5 mg/40 g of body weight) into the peritoneum of P30 *Pou4f2^{Z-dta}*; *Cre-ER*TM mice and *Pou4f2^{Z-dta}* control mice and determined the extent to which RGCs underwent DTA-induced apoptosis using a cleaved form of anti-Caspase-3 antibody to detect apoptotic cells (Fig. 2). In *Pou4f2^{Z-dta}*; *Cre-ER*TM mice, many apoptotic cells were observed exclusively in the ganglion cell layer, as expected, but no positive cells were observed in the retinas of *Pou4f2^{Z-dta}* or wild-type control mice (Fig. 2). By this qualitative measure, tamoxifen treatment by IP injection appeared to be an effective mode of delivery.

The dosage dependency of tamoxifen was determined more quantitatively by monitoring the loss of *lacZ* expression in retinas of two-month-old *Pou4f2^{Z-dta};Cre-ERTM* mice after a 1-, 3-, or 5-consecutive day regime of tamoxifen administration to one-month-old mice (Fig 3). Loss of *lacZ* expression is a direct measure of the loss of *Pou4f2*-expressing RGCs. In the retinas of mock-treated *Pou4f2^{Z-dta};Cre-ERTM* mice, *lacZ* expression was readily observed in RGCs within the ganglion cell layer (Fig. 3A, E). *LacZ* expression was considerably reduced by 1-day administration of tamoxifen, but there were still many *lacZ*-expressing cells in the ganglion cell layer (Fig. 3B, F). Tamoxifen administered for 3 consecutive days further reduced the number of *lacZ*-expressing cells, although a few RGCs still remained (Fig. 3C, G). When tamoxifen was administered over a 5-consecutive day regime, no *lacZ*-expressing cells were detected in the retina (Fig. 3D, H). Furthermore, the total number of cells in the ganglion cell layer of *Pou4f2^{Z-dta};Cre-ERTM* mice was reduced to 60% (29.3 ± 4.3 cells/150 μm^2) at one month after tamoxifen administration when compared to the total cell number in the ganglion cell layer of wild-type littermate retinas (51.7 ± 12.0 cells/150 μm^2). Based on these results, we chose to administer tamoxifen with a daily dose of 5 mg/40 g of body weight for 5 consecutive days. Importantly, the results demonstrate that modulating the tamoxifen dosage in this manner results in varying degrees of RGC ablation.

To further characterize the cells that remained in the ganglion cell layer following tamoxifen-mediated DTA ablation, we used antibodies representing RGC and amacrine cell markers. For this analysis, we used retinas from mice one month after tamoxifen treatment (Fig. 4). *Pou4f2*-immunoreactive cells were detected in the ganglion cell layer of retinas from *Pou4f2^{Z-dta}* mice, but no *Pou4f2*-labeled cells were detected in the ganglion cell layer of retinas from *Pou4f2^{Z-dta};Cre-ERTM* mice (Fig. 4A, B). The total number of cells in the ganglion cell layer decreased in *Pou4f2^{Z-dta};Cre-ERTM* retinas while the other nuclear layers exhibited no differences between two genotypes (Fig. 4A, B; Supplemental Fig. S1A, B). Immunostaining with either anti-neurofilament light chain (NFL) or a anti-Thy1.1 antibodies, which label axons and dendrites of RGCs, respectively, was significantly decreased and remained at low levels in *Pou4f2^{Z-dta};Cre-ERTM* retinas compared with the corresponding immunostaining in *Pou4f2^{Z-dta}* control retinas (Fig. 4C, D, E, F).

The observation that no *Pou4f2*-expressing cells were found in DTA-ablated retinas while a few NFL- and Thy1.1-expressing cells were found, suggested the presence of non-*Pou4f2*-expressing RGCs in adult retinas. We therefore used an anti-*Pou4f1* (Brn3a) antibody to detect other RGCs in the ganglion cell layer. *Pou4f1* expression partially overlaps with *Pou4f2* expression (Xiang et al., 1995; Xiang, 1998). *Pou4f1*-expressing cells were detected in the ganglion cell layer from retinas of *Pou4f2^{Z-dta}* and *Pou4f2^{Z-dta};Cre-ERTM* mice, although the number of *Pou4f1*-expressing cells was reduced in *Pou4f2^{Z-dta};Cre-ERTM* retinas, reflecting RGCs expressing both *Pou4f1* and *Pou4f2* (Fig. 4G, H). DTA ablation of *Pou4f2*-expressing RGCs resulted in no significant differences in the number of displaced amacrine cells in the ganglion cell layer, as determined by immunostaining with anti-Pax6 antibody, in retinas of *Pou4f2^{Z-dta}* versus *Pou4f2^{Z-dta};Cre-ERTM* mice (Fig. 4I, J).

Aside from the loss of *Pou4f2*-expressing RGCs, the overall integrity of DTA-ablated retinas was remarkably intact (e.g., Fig. 3; Fig. 4A, B). Immunostaining with antibodies representing markers for photoreceptor cells in the outer nuclear layer, and horizontal, bipolar, and amacrine cells in the inner nuclear layer showed no differences in these neurons in retinas of *Pou4f2^{Z-dta}* and *Pou4f2^{Z-dta};Cre-ERTM* mice (Supplemental Fig. S2). DTA-ablated retinas showed no significant morphological defects in mice up to one year of age (Supplemental Fig. S1A, B).

Increased reactive gliosis in DTA-ablated retinas

Retinal injury or disease triggers reactive gliosis, which is defined by characteristic changes in the morphology of astrocytes and Müller cells and by an increased production of the intermediate filament proteins, glial fibrillary acidic protein (GFAP) and vimentin (Lewis and Fisher, 2003; Bringmann et al., 2006). To determine whether the loss of RGCs in the retinas of *Pou4f2^{Z-dta};Cre-ERTM* mice induced reactive gliosis, we performed immunohistochemistry with an antibody against GFAP (Fig. 5A, B). GFAP-immunoreactive cells, representing astrocytes, were found at the vitreal surface underneath the ganglion cell layer of *Pou4f2^{Z-dta}* retinas as expected (Fig. 5A), but were found not only at the vitreal surface but also in other retinal layers in *Pou4f2^{Z-dta};Cre-ERTM* retinas (Fig. 5B). These GFAP-expressing cells represented Müller glia activated by retinal injury. Reactive gliosis is known to involve the induction of the EGF signal transduction pathway and to increase the levels of phospho-MAP kinase (p-MAPK) (Bringmann et al., 2006). Immunostaining with an anti-p-MAPK antibody showed a strong upregulation of p-MAPK in retinas of *Pou4f2^{Z-dta};Cre-ERTM* mice in Müller cells compared to littermate controls (Fig. 5C, D). We confirm that increased levels of p-MAPK in the inner nuclear layer were restricted to Müller cells by double immunostaining for a Müller cell marker, cellular retinaldehyde binding protein (CRALBP), and p-MAPK (Fig. S3). Increased levels of p-MAPK were also found in astrocytes underneath the ganglion cell layer of *Pou4f2^{Z-dta};Cre-ERTM* retinas compared with *Pou4f2^{Z-dta}* littermate control retinas (Fig. 5C, D). These results indicate that RGC death by tamoxifen-mediated DTA ablation induces a typical reactive gliosis response in the damaged retina.

Progressive optic nerve degeneration in *Pou4f2^{Z-dta};Cre-ERTM* mice

RGC death induced by DTA should result in the subsequent degeneration of the axons in the optic nerve. Therefore, we examined the morphology of the optic nerve at the skull base at 1-, 3-, and 10-months after tamoxifen administration (Fig. 6). Tamoxifen was administered for 5 consecutive days to *Pou4f2^{Z-dta}* and *Pou4f2^{Z-dta};Cre-ERTM* mice at one month of age. Mock-administered *Pou4f2^{Z-dta}* and *Pou4f2^{Z-dta};Cre-ERTM* mice were used as negative controls; no optic nerve defects were observed in these mice one month after tamoxifen treatment (Fig. 6A, B). In contrast, tamoxifen-treated *Pou4f2^{Z-dta};Cre-ERTM* mice showed a progressive degeneration in their optic nerves over a ten month period when compared to *Pou4f2^{Z-dta}* littermate control mice (Fig 6A). Optic nerve degeneration was readily observable as a gross atrophy (Fig. 6A, left panels) as well as a reduction in cross-sectional area (Fig. 6A, right panels). In the skull base of the head, no other nerve fibers including the trigeminal nerve were affected by tamoxifen administration of *Pou4f2^{Z-dta};Cre-ERTM* mice (Fig. 6A, left panels), indicating that the effects of DTA ablation were highly restricted to RGCs and their axons.

As early as one month after tamoxifen administration, there was already a significant difference in the cross-sectional area between the optic nerves of *Pou4f2^{Z-dta}* and *Pou4f2^{Z-dta};Cre-ERTM* mice (Fig. 6B). Three months following tamoxifen administration, the optic nerves in DTA-ablated mice had decreased in area by 48.8%, and by ten months, optic nerve areas were only 19.7% of those of control mice (Fig. 6B). Similar results were also observed in eight-month old mice, three months after tamoxifen administration (Fig. S4).

To obtain a more detailed picture of the optic nerve defects caused by DTA-mediated RGC death, we examined the ultrastructure of optic nerve in mice three months after tamoxifen treatment (Fig. 7). In *Pou4f2^{Z-dta}* mice, RGC axons within the optic nerve were tightly packed and highly myelinated, characteristically exhibiting multiple folds of myelin sheath (Fig. 7A, C). In contrast, as a result of massive RGC death in the DTA-ablated retina,

residual axons were disorganized and showed features of severe degeneration with vacuolization, glial hypertrophy, and empty or abnormal myelin profiles (Fig. 7B, D). In addition, the thickness of myelin sheaths surrounding the axons was significantly decreased when compared to that of controls (Fig. 7C, D). However, a few intact axons were also found in the optic nerves of *Pou4f2^{Z-dta};Cre-ERTM* mice, suggesting that non *Pou4f2*-expressing RGCs were still able to project their axons into the brain (Fig. 7C, D, arrows).

For further confirmation that the observed optic nerve degeneration occurs exclusively in the RGCs of the retina, and that the few remaining axons in the optic nerve of tamoxifen-treated *Pou4f2^{Z-dta};Cre-ERTM* mice were still viable, we performed an ex vivo retrograde labeling procedure using dissected optic chiasm-optic nerve-eyeball preparations and the tracer DiI. To perform the analysis, we used four-month old *Pou4f2^{Z-dta};Cre-ERTM* mice with and without tamoxifen treatment, and as an additional control, tamoxifen-treated *Pou4f2^{Z-dta}* mice (Fig. 8). DiI successfully diffused out of the optic chiasm side of the optic nerve and labeled all RGCs as well as nerve fibers in the ganglion cell layer of control mice (Fig. 8A, B, D, E). However, in the retinas of tamoxifen-treated *Pou4f2^{Z-dta};Cre-ERTM* mice, very few DiI-labeled RGCs were observed and no nerve fibers or nerve bundles were detected in the ganglion cell layer (Fig. 8C, F). In control retinas, DiI-labeled cells and nerve fibers were only observed in the ganglion cell layer and there were no histological or DiI labeling differences in any of the other retinal layers when tamoxifen-treated *Pou4f2^{Z-dta};Cre-ERTM* mice were compared with those of the control mice (data not shown). These results indicate that DTA-induced RGC ablation is specifically restricted to RGCs in the ganglion cell layer. In addition, the few remaining axons that were observed in the degenerated optic nerve appeared viable because a corresponding small number of DiI-labeled axons were observed in the ganglion cell layer of *Pou4f2^{Z-dta};Cre-ERTM* mice (Fig. 8F).

Abnormal visual acuity in *Pou4f2^{Z-dta};Cre-ERTM* mice

To determine the visual acuity of *Pou4f2^{Z-dta};Cre-ERTM* mice, we performed a simple behavioral test relying on light avoidance (Fig. 9). A dark-light chamber was constructed as shown in Figure 9A, and we determined the time that mice spend in the light side of the chamber over a 5 minute interval (Fig. 9A, B). *Pou4f2^{Z-dta};Cre-ERTM* mice at four months of age and their *Pou4f2^{Z-dta}* littermate controls were dark-adapted for 1–2 hours and then provided access to the light side of the chamber. Control mice showed strong light avoidance and resided in the light side of the chamber for only 6% of the five-minute interval (Fig. 9B). In contrast, *Pou4f2^{Z-dta};Cre-ERTM* mice spend significantly more time in the light side of the chamber and resided there for 33% of the timed interval (Fig. 9B). These results suggested that the tamoxifen-treated *Pou4f2^{Z-dta};Cre-ERTM* mice were visually impaired, although additional behavioral experiments are required to demonstrate that the observations are specific to RGC loss.

Discussion

In this study, we successfully induced optic nerve degeneration in the adult mouse retina by selectively depleting RGCs using genetic ablation. We used an inducible *Cre-ERTM* transgene coupled with a *Pou4f2^{lacZ-dta}* allele to deplete RGCs in the ganglion cell layer of the mature retina. Tamoxifen-mediated DTA ablation of RGCs led to a slow, progressive degeneration of the optic nerve over a period of ten months with a corresponding loss of visual acuity.

A new animal model for RGC death and optic nerve degeneration

We generated our *Pou4f2^{Z-dta};Cre-ERTM* mice with the goal of establishing a robust animal model to develop stem cell or progenitor cell replacement therapy for RGC loss and optic

nerve degeneration. This model should be useful to investigators seeking new approaches for repairing damaged optic nerves. Manipulating the survival to death ratio of RGCs in the ganglion cell layer was a critical aspect of our study since any realistic animal model should reflect the slow, progressive degeneration of the optic nerve as is observed in human retinal diseases. We were able to control the amount of RGC death by modulating the exposure of *Pou4f2^{Z-dta};Cre-ERTM* mice to tamoxifen. Varying the dose of tamoxifen appeared to correlate closely with the number of RGCs whose genomes underwent Cre recombinase-mediated activation of DTA. Notably, we found that gross optic nerve defects were not observed in DTA-ablated retinas during the first three months after tamoxifen treatment, despite the fact that RGCs underwent cell death within the first few days. This delayed axon degeneration is commonly observed in many human neurodegenerative diseases (Coleman and Perry, 2002; Whitmore et al., 2005). The ability to induce RGC death in older mice is particularly relevant for human retinal pathologies, since optic nerve degeneration in humans commonly occurs in older individuals (Levin and Gordon, 2002; Margalit and Sadda, 2003).

Depleting RGCs in adult and embryonic retinas

Depleting RGCs during retinogenesis results in pronounced alterations in proliferating retinal progenitor cells in the neuroblast layer of E14.5 embryos in the absence of differentiating RGCs (Mu et al., 2005). The proliferation rate of progenitor cells is substantially decrease and the expression of *Math5*, a key regulator of RGC specification, is strongly upregulated. RGCs in developing wild-type retinas secrete signaling molecules like Sonic hedgehog, Vegf, GDF8, and GDF11, which control a delicate balance between progenitor cells remaining in the cell cycle and progenitor cells exiting the cell cycle and committing to a RGC fate (Mu and Klein, 2008). The strong upregulation of *Math5* expression indicates that abnormal numbers of retinal progenitor cells are being pushed to commit to a RGC fate because the retinal microenvironment senses a need for additional RGCs.

In contrast to developing retinas in embryos, mature retinas in adult mice do not have a neuroblast layer or actively dividing progenitor cells, although quiescent progenitor cells may reside at the ciliary margins of adult retinas (Trobepe et al., 2000). We did not detect an upregulation of *Math5* or any signs of reactivation of quiescent progenitor cells in DTA ablated adult retinas (J.H.C. and W.H.K., unpublished results), suggesting that the mature retina is not a readily available source of progenitor cells, at least in vivo. The microenvironment of the adult retina is therefore clearly different from the developing retina as it is not adapted for promoting progenitor cell proliferation. However, studies from other investigators indicate that the adult rodent retina is not refractory to exogenously introduced progenitor cells (Takahashi et al., 1998). In fact, the adult mouse retina can support the proliferation and differentiation of donor progenitor rod photoreceptors, provided the donor cells are isolated at the correct stage in their development (MacLaren et al., 2006).

We observed that complete loss of RGCs had no major effects on the rest of the retina, even in retinas of one-year old mice. The outer and inner nuclear layers were intact and the full complement of non-RGC neurons appeared to be present. Moreover, we did not detect any morphological differences in the outer and inner plexiform layers between ablated and non-ablated retinas, suggesting that synaptic contacts were being made. Since RGC dendrites make synaptic connections with amacrine and bipolar cell processes in the inner plexiform layer, the normal appearance of the inner plexiform layer suggests that fortuitous connections are made with the residual non-*Pou4f2*-expressing RGC processes or with processes from displaced amacrine cells within the ganglion cell layer. The maintenance of the overall integrity of the DTA ablated retina is an important aspect of our *Pou4f2^{Z-dta};Cre-ERTM* mouse model, particularly when considering its use for stem cell or progenitor cell

replacement strategies, because repairing the damaged retina would be limited solely to replacing dead RGCs. Notably, minor damage to a host retina helps to facilitate the incorporation and migration of grafted cells (Das et al., 2006).

Reactive gliosis in the retinas of *Pou4f2^{Z-dta};Cre-ERTM* mice

As RGC loss increased following a series of daily tamoxifen treatment, we observed increased gliosis in Müller glia within the inner nuclear layer and astrocytes underneath the ganglion cell layer of ablated retinas. GFAP expression in both of these glial cell types is known to be substantially increased by the induction of gliosis (Dyer and Cepko, 2000). Retinal injury often induces reactive gliosis, which is a neuroprotective response that has both positive and negative effects on damaged retinas; gliotic Müller cells protect neurons via release of neurotrophic factors but also display a dysregulation of various supportive functions (Bringmann et al., 2006). The gliotic pathway operates through the activation of a signal transduction pathway that includes the activation of MAP kinase. We did not detect an increased level of Müller cell proliferation (data not shown), but p-MAPK expression was strongly upregulated in astrocytes and Müller glia in RGC-ablated retina. Controlling the reactive gliosis in the damaged retina could therefore be an important issue for successful approaches using retinal tissue transplantation. Recently, reduced reactive gliosis activation with minocycline treatment has been demonstrated in the DBA/2J mouse model for glaucoma (Bosco et al., 2008)

Animal models for RGC death and optic nerve degeneration

In addition to its potential value as a host for cell replacement therapy, DTA-ablated retinas readily offer the opportunity to examine in detail the events that follow RGC death in a controlled, well-defined model. Other rodent models have been used to gain a better understanding of the mechanisms that result in optic nerve degeneration, including those involving optic nerve axotomy, intoxicification of neurotoxins, and genetic manipulation (Berkelaar et al., 1994; Jakobs et al., 2005; Kitaoka et al., 2006; Schlamp et al., 2006).

Optic nerve axotomy prevents neurotrophic factors, such as BDNF, from reaching RGC soma and results in the apoptotic RGCs death by 14 days following the transection (Berkelaar et al., 1994). In addition, many neurotoxins have been used to injure retinal neurons. For example, intoxicification of neurotoxins like ouabain and domoic acid induce rapid reactive gliosis and result in retinal dysplasia in the outer segment as well as vascular abnormalities (Dyer and Cepko et al., 2000). Intraocular administrated TNF- α , which is released from glial cells in the central nervous system, and acrylamide result in progressive retinal and optic nerve degeneration (Godin et al., 2000; Kitaoka et al., 2006). Although these methods induce injury at any age of adult life, they rely on cumbersome procedures and often lead to massive glial cell invasion at the site of injury, causing secondary responses by drastically altering the internal environment of the retina (Nitzan et al., 2006).

To overcome the disadvantages of surgical procedures or intoxicification, many studies induce RGC degeneration using predisposed inbred strains, transgenic, or gene targeted models, including DBA/2J inbred mice as a model for glaucoma, and *Opa1* knockout mice and mice deficient for the glutamate transporter *GLAST/EAAC1* as more general models for optic nerve degenerative diseases (Jakobs et al., 2005; Schlamp et al., 2006; Harada et al., 2007; Bosco et al., 2008). These models have varying degrees of usefulness, but all depend on the nature of the genetic modification. In the case of DBA/2J inbred mice, the basis of elevated IOP is ill-defined although it appears to be the result of multiple genetic changes (Bosco et al., 2008; Soto et al., 2008). In contrast, transgenic and targeted gene knockout models rely on single gene mutations that may exert their effects in the embryo rather than the adult. The *Pou4f2^{Z-dta};Cre-ERTM* mouse model described here, has the advantage of inducing varying

degrees of RGC death anytime in adult life with high specificity. However, a disadvantage of genetic ablation is that it depends on an artificial mechanism to induce cell death. Nonetheless, the *Pou4f2^{Z-dta};Cre-ERTTM* mice represent a useful tool for investigating the mechanisms of optic nerve degeneration caused by RGC death, and should prove valuable as a host for transplantation of healthy neuronal progenitors into damaged retinas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Spatiotemporal expression of Pou4f2/Brn3b in the developing and adult retina. (A–F) Retinal sections from time points indicated at the top left of each panel were immunostained with anti-Brn3b/Pou4f2 antibody (brown) and counterstained with methyl green (blue-green). Arrows in panel A point to immature RGCs that are in the neuroblast layer (NL) in the process of migrating to the ganglion cell layer (GCL). By P15, the inner nuclear layer (INL) and outer nuclear layer (ONL) have formed. Scale bar in panel A: 100 μ m.

Figure 2.

Induction of RGC death following tamoxifen administration. (A–F) Retinal sections from *Pou4f2^{Z-dta}* (A, C, E) or *Pou4f2^{Z-dta};Cre-ERTM* one-month-old mice (B, D, F) treated with tamoxifen. (A, B) Fluorescence staining to detect nuclei with the DNA stain DAPI. (C, D) Immunofluorescence staining with a cleaved form of anti-caspase-3 antibody to detect cell death. (E, F) Merged images of DAPI and anti-caspase-3. Arrows point to apoptotic cells. Scale bar in panel A: 50 μ m.

Figure 3.

Dose-dependent depletion of RGCs by multi-day tamoxifen administration. Tamoxifen was administered to *Pou4f2^{Z-dta}; Cre-ERTM* one-month-old mice by intraperitoneal injection for 0 (non-treated), 1, 3, or 5 consecutive days as indicated above the panels. (A–D) Whole-mounted retinas. (E–H) retinal sections counterstained with nuclear fast red. *LacZ* expression in RGCs is visualized by X-gal staining (blue). Scale bars in panels A–D: 1 mm; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars in panels E–H: 100 μ m

Figure 4.

DTA-mediated ablation of *Pou4f2*-expressing RGCs in the ganglion cell layer of *Pou4f2^{Z-dta};Cre-ERTM* mice. Retinal sections from *Pou4f2^{Z-dta}* (A, C, E, G, I) or *Pou4f2^{Z-dta};Cre-ERTM* (B, D, F, H, J) two-month-old mice, one month after tamoxifen administration, were immunostained with anti-Brn3b/Pou4f2 for *Pou4f2*-expressing RGCs (A, B, green), anti-NFL for all RGC axons (C, D, red), anti-Thy1.1 for all RGC dendrites (E, F, red), anti-Brn3a/Pou4f1 for *Pou4f1*-expressing RGCs (G, H, red) or anti-Pax6 for amacrine cells (I, J, red). Sections were counterstained with DAPI to visualize nuclei (A, B, I, J, blue). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A: 100 μm.

Figure 5.

Increased reactive gliosis in the DTA-ablated retinas. Retinal sections from *Pou4f2^{Z-dta}* (A, C) or *Pou4f2^{Z-dta};Cre-ERTM* (B, D) two-month-old mice, one month after tamoxifen administration, were immunostained with anti-Brn3b/Pou4f2 (A, B, green), anti-GFAP (A, B, red), or anti-p-MAPK (C, D, red) antibody. Sections in panels C and D are counterstained with DAPI (blue). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A: 100 μ m.

B **Figure 6.**

Optic nerve degeneration in DTA-ablated mice. (A) morphology of optic nerves of *Pou4f2^{Z-dta}* (first and third panels) or *Pou4f2^{Z-dta};Cre-ERTM* (second and fourth panels) in the skull base at 1-, 3-, and 10-months after tamoxifen administration. The left-most two panels are gross cranial images in which the skull has been removed to visualize optic nerves (arrows) and trigeminal ganglia (arrowheads). The right-most two panels are cross-sectional areas of optic nerves counterstained with cresyl violet. The first row shows a mock-injected (Non TM) control. Scale bars in the left-most panels: 5 mm; scale bars in the right-most panels: 100 μ m. (B) Quantification of cross-sectional areas (mm²) from *Pou4f2^{Z-dta}* (black bars) and *Pou4f2^{Z-dta};Cre-ERTM* (white bars) at 1-, 3-, and 10 months after tamoxifen administration. The left-most two bars represent mock-injected controls (Non TM). Error bars are two standard deviations from the mean. Asterisks indicate *p* < 0.01 (n = 4).

Figure 7.

Ultrastructural analysis of RGC axons in the optic nerves of DTA-ablated mice. Optic nerve cross-sections from *Pou4f2^{Z-dta}* (A, C) and *Pou4f2^{Z-dta};Cre-ERTM* (B, D) mice 4 months after tamoxifen treatment. (A, B) low magnification electron micrographs. Inserts show entire optic nerve cross-section. Red boxes indicate regions depicted at higher magnification in panels C and D. In panels B and D, arrows point to normal appearing axons and asterisks indicate empty axonal sheaths. “v” indicates vacuolization and “a” indicates abnormal myelin profiles. Scale bars in panels A and B: 4 μ m; scale bars in panels C and D: 1 μ m.

Figure 8.

Decrease in number of optic nerve fibers in RGC-ablated retinas in retrograde DiI-labeled ex vivo eye-optic nerve preparations. Flat mount retinas from *Pou4f2^{Z-dta}* (A) and *Pou4f2^{Z-dta};Cre-ERTM* (B, C) mice three months after treatment with tamoxifen (A, C) or no tamoxifen treatment (B). White boxes indicate regions depicted at higher magnifications in panels D–F. In panel F, arrows point to normal appearing axons labeled with DiI (red). TOTO-5 (blue nuclei) was used for counterstaining. Scale bars in panels A–C: 200 μm; in panels D–F: 50 μm

Figure 9. Lowered visual acuity in DTA-ablated mice. (A) Schematic showing experimental light-dark chambered box. (B) Quantification of time in seconds over a 5 minute interval spent in the light side of the chamber for control (black bars) and DTA-ablated (white bars) 4-month-old mice, three months after tamoxifen administration. Error bars are two standard deviations from the mean. Asterisks indicate $p < 0.01$ ($n = 4$).