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Morphological changes of short-wavelength cones in the developing S334ter-3 Transgenic rat

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Abstract

The S334ter-3 rat is a transgenic model of retinal degeneration (RD) developed to express a rhodopsin mutation similar to that found in human retinitis pigmentosa. Due to this advantage over other models of RD, a few retina transplant studies have been reported on this animal model. Currently, no information is available on cone photoreceptor changes that occur in the S334ter RD model. In this study, we investigated the effect of RD on the morphology, distribution, and synaptic connectivity of short-wavelength cones (S-cones) during development of S334ter-3 rat retinas. At P21 RD retinas, the outer-nuclear layer was significantly narrower, while S-cones showed shortening of their segments and axons compared to control retinas. From P90 onward, S-opsin-immunoreactive cells appeared at the outer margin of the inner-nuclear layer of RD retinas. Double-labelling experiments showed these cells contained recoverin and cone arrestin. Furthermore, ultra-structure study showed that synaptic ribbons are conserved in the S-cone at P180 RD retinas. Although cell density of S-cones significantly dropped after P90, survival rates depended on the retinal region. Overall, the S334ter-3 RD model shows hallmarks of cone remodelling due to photoreceptor degeneration.

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Jose R. Hombrebueno and Eun-Jin Lee contributed equally to this work.

Keywords

retinal degeneration; retinal photoreceptor; rat; retinal development

INTRODUCTION

Opsins are important proteins in the phototransduction cascade, so correct function of their mechanisms is essential to support normal vision. Genetic mutations associated with these proteins can lead to rapid loss of vision through degeneration of photoreceptors (Bird, 1995; Vugler et al., 2008).

In the mammalian retina, rod photoreceptor degeneration leads to cone photoreceptor remodelling and inner-retinal remodelling, resulting in abnormal circuitry (Marc and Jones, 2003; Marc et al., 2003; Strettoi et al., 2004; Jones and Marc, 2005; Lin et al., 2009). Neuronal remodelling in the degenerative retina has been associated with progressive morphological changes that range from neurite sprouting to atrophy and hypertrophy of cells structures (Li et al., 1995; Fei, 2002; Strettoi et al., 2003; Pow and Sullivan, 2007; Lin et al., 2009).

Therapeutic strategies that promote cone cell survival have been proposed as a possible solution for retinal degeneration (RD) (Hicks and Sahel, 1999; Delyfer et al., 2004; Wang et al., 2008). More specifically, saving cone population may allow the sustainability of photopic central vision (Wong, 1990) and subsequently avoid the remodelling of cone associated second order neurons. Hence, investigating cone remodelling in RD models is an essential step in their rescue.

A recent work by Lin et al. (2009) reported that cone photoreceptors in later stages of rd1 mouse have bipolar-cell-like morphology, with sprouting processes that contained synaptic ribbons. To try to refine this finding further, we asked whether short-wavelength cones (S-cone) undergo the same changes in the S334ter-3 rat. This transgenic model of RD express a rhodopsin mutation similar to that found in human retinitis pigmentosa. We therefore investigated the expression and cellular localization of S-opsin at various stages of S334ter-3 RD rat retina by immunocytochemistry using antibody against S-opsin.

RESULTS

S-opsin immunoreactivity in the developing S334ter-3 rat retinas

We investigated morphology of S-opsin-immunoreactive cells in development of S334ter-3 rat retinas (Fig. 1). For this purpose, we first performed immunocytochemistry in vertical sections of P15 (data not shown), P21 control (A, B), P180 control (data not shown), P15, (data not shown), P21 RD (C, D), P60 RD (data not shown), P90 RD (E, F), and P180 RD (G, H) rat retinas. In P15 and P21 control retinas, S-opsin immunoreactivity occurred in outer segments (OS), inner segments, somata in the outer nuclear layer (ONL), and axon terminals at the outer plexiform layer (OPL) (Fig. 1A, B). Similar expression of S-opsin was observed in P180 control retina (data not shown). These results on S-opsin immunoreactivity were in agreement with previous data (Rorher et al., 2005; Fujieda et al., 2009). In P15

RD retinas, no significant changes were observed in thickness of the ONL and expression of S-opsin compared to P15 and P21 control retinas. In P21 RD retinas, S-opsin immunoreactivity was observed in cells displaying disorganized segments, and axon terminals. Segments of S-opsin-positive cells showed no vertical alignment from P60 (data not shown). At P90, all S-opsin-immunoreactive cells their processes were observed at the outer part of the inner nuclear layer (INL -- Fig. 1E, F). Finally, at P180, S-opsin-immunoreactive cells exhibited more processes (Fig. 1G, H).

In whole-mount preparations of RD animals, S-opsin immunoreactive cells were distributed throughout the retinas of P90 (Fig. 2A, B) and P180 (Fig. 2C, D). In RD retina, S-opsinimmunoreactive cells showed outgrowth of new processes from the cell body and the axon terminal. Furthermore, remodelling of S-opsin-immunoreactive cells occurred with increasing age (Fig. 2C, D). In P90 retina near the optic disc, the S-opsin-immunoreactive cells had lost the OS and part of their axon terminals (Fig. 2A, arrows). Moreover, S-opsin-immunoreactive cells showed bipolar-like morphology (Fig. 2A, arrowheads). In P90 retina distant from the optic disc, the S-opsin immunoreactive cells showed numerous processes emerging from both the cell body and the remaining axon process (Fig. 2B, open arrows). Finally, in P180 retinas, some S-opsin-immunoreactive cells showed amacrine-like morphology by retracting their processes (Fig. 2C, D, open arrowheads).

Density of S-opsin-immunoreactive cells

The spatial densities of S-opsin-immunoreactive cells in the outer part of the INL were determined at P90 and P180 control, and P90 and P180 RD retinas. We counted cells from the individual optical sections that made up the reconstructed stack. In both P90 and P180 control retinas, the density of S-opsin-immunoreactive cells in the INL fell with distance from the optic disc (Fig. 3). In contrast, in P90 and P180 RD retinas, the density of S-opsin immunoreactive cells peaked at 3 mm from the optic disc. The density was lower at 2 mm from the optic disc, raising again at 1 mm from it (Fig. 3). The density of S-opsin immunoreactive cells from control and RD retinas at P90 and P180 showed significant difference near the optic disc (P90, 1 mm superior/inferior from optic disc, $p = 0.014$; 2 mm superior/inferior from optic disc, $p = 0.013$; P180, 1 mm nasal/temporal from optic disc, $p = 0.008$; 2 mm nasal/temporal from optic disc, $p = 0.004$). However, at 3 mm from the optic disc, the density of S-opsin cells in RD retinas did not show significant changes from control.

Are S-opsin-immunoreactive cells displaced cones?

The S-opsin cells in the inner RD retinas might be displaced cones or a new kind of neurons. If the S-opsin cells were cones, we would expect the former to show molecular properties of the latter. To test whether such molecular similarities exist, we performed double-labelling of S-opsin with either recoverin or cone arrestin. All S-opsin-positive cells contained recoverin (Fig. 4A-C) and cone arrestin (Fig. 4 D-F) in P90 control (data not shown) and P90 RD retinas. This result suggests that the S-opsin-immunoreactive cells are displaced S-cones.

S-opsin-immunoreactive cells still conserving normal synaptic ribbons in advanced stages of RD

We examined the synaptic terminals of distal S-opsin-immunoreactive-cell in the S334ter-3 rat retina. We performed double-labelling experiments of S-opsin (red) and bassoon (green -a marker of the arciform density of the ribbon complex - Fig. 5A). Distal S-opsin immunoreactive cells presented bassoon immunoreactivity at their synaptic terminals (Fig. 5A). In addition, ultrastructural study showed synaptic ribbons in S-opsin-immunoreactive-cell terminals at the outer part of the INL (Fig. 5B). S-opsin immunoreactivity produced an electron-dense reaction product closely associated with cytoplasmic matrices and synaptic ribbons (Fig. 5B). This result provides further support that the distal S-opsin-immunoreactive cells are displaced cones.

DISCUSSION

S-cone remodelling in the developing S334ter-3 rat retina

This study showed evidence of S-cone photoreceptor remodelling during the postnatal development of S334ter-3 rats. As RD develops with increasing age, S-cones presented retraction and outgrowth of processes. This remodelling was also accompanied by the misplacement of these cells to the outer part of the ONL. The remodelling of S-cones shared common features with previous studies in degenerative retinas. The outgrowth of new processes from cones has been reported in the rd1 mouse (Fei, 2002; Lin et al., 2009) and in human retinitis pigmentosa (Li et al., 1995). In addition, shortening and disorganization of cone segments and axons are common to both the rd10 mouse (Barhoum et al., 2008), dystrophic RCS rat (Cuenca et al., 2005) and the P23H-line-3 rat retinas (Chrysostomou et al., 2009). The reason for this remodelling may be the loss of synaptic targets in retinal neurons. Such loss modulates protein-expression pathways related with sprouting, where changes occur in cellular adhesion molecules, target derived chemoattractants, and neurotrophic factors (Heffner et al., 1990; Gallo and Letorneau, 1998; Dingwell et al., 2000; Fei, 2002). Thus, cones of all types may show common features of remodelling in RD (Fei, 2002; Lin et al., 2009).

Distribution of Remodelled S-cones in the S334ter-3 rat retina

For all the developmental stages studied, the density of S-cones in the control retina was higher near the optic disc, falling away from it. In contrast, at P90 and P180, RD retinas showed higher densities of S-opsin cells in the peripheral region compared to the area close to the optic disc. Moreover, peripheral areas showed no significant differences in S-opsin-cell density compared to age match controls. Similar to other models of photoreceptor degeneration, the RD model studied here exhibits a gradient from the optic disc outwards (Carter-Dawson et al., 1978; Garcia-Fernandez et al., 1995; Jimenez et al., 1996; La Vail et al., 1997; Strettoi et al., 2003; Cuenca et al., 2005; Barhoum et al., 2008). This delay of cone photoreceptor cell death in RD could be supported by their connections, by means of interactions with post-synaptic neurons. In the nervous system, the long-term survival of a cell may depend on the establishment of connections (Rakic, 1975; Rakic, 1988).

Synaptic terminals of S-cones in the S334ter-3 rat retina

We used electron microscopy to study the synaptic terminals of S-opsin-immunoreactive cells in the outer retina. Our finding was that they have normal synaptic-ribbon structures. This is consistent with previous study by Lin et al. (2009), showing that remodeled cones had normal synaptic ribbons in the rd1 mouse retina. We do not know what role, if any, the displaced S-cone plays in the outer retina. Our working hypothesis is that remodelled S-cone synaptic connections may remain in the degenerative retina due to the existence of normal synaptic structure of ribbons and expression of cone arrestin. Whether the contacts play a role in the processing of visual information remains to be established.

EXPERIMENTAL PROCEDURE

Animals

The third line of albino Sprague-Dawley rats homozygous for the truncated murine opsin gene (stop codon at residue 334; S334ter-3) was obtained from M.M. LaVail (University of California, San Francisco, CA). Homozygous S334ter-3 breeding pairs were mated with normal Copenhagen rats to produce offspring heterozygous for the S334ter transgene that were subsequently used in this study. S334ter-3 rats were sacrificed at post-natal (P) days 10, 15, 21, P60, P90, and P180 (n= 7 for each stage). Controls were age-matched Sprague-Dawley rats (n= 7 for each stage; Harlan, Indianapolis, IN; normal rats, N). All rats were housed under cyclic 12/12-hour light/dark conditions with free access to food and water. All procedures were in agreement with the *Guide for Care and Use of Laboratory Animals* (National Institutes of Health). The University of Southern California Institutional Animal Care and Use Committee reviewed and approved all procedures.

Tissue preparation

All rats sacrificed were deeply anesthetized by intraperitoneal injection of pentobarbital (40mg/kg body weight) and their eyes enucleated. Animals were then euthanized with an overdose of pentobarbital. Eyes were dissected to remove the anterior portions and the resulting eyecups were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4, for 2 hours. Following fixation, retinas were carefully dissected and transferred to a 30% sucrose solution in PB for 24 hours at 4°C. Retinas for vibratome sections and whole-mount were then frozen in liquid nitrogen and stored at -70°C, thawed, and transferred to 0.01M phosphate buffered saline (PBS; pH 7.4).

Immunocytochemistry

Fluorescence immunocytochemistry was carried out on 40 µm thick vibratome sections that were incubated in 10% normal donkey serum (NDS) and 1% TritonX-100 in PBS for 1 h at room temperature. This was done in order to block all the non-specific binding sites. Sections were then incubated overnight with rabbit polyclonal antibody directed against recoverin (Chemicon, Temecula, dilution 1:1500), cone arrestin (kindly provided from Dr. Cheryl Craft in Doheny Eye Institute, University of Southern California, dilution 1:1000) or goat polyclonal antibody directed against the N-terminus of human sensitive blue opsin (S-opsin - Santa Cruz,

dilution 1:500). Each antiserum was diluted in PBS containing 0.5% Triton X-100 and incubated overnight at 4°C. Retinas were washed in PBS for 30 min (3 × 10 min) and afterwards incubated for 2 h at room temperature in either carboxymethylindocyanine (Cy3)-conjugated affinity-purified donkey anti-rabbit IgG (Jackson Immuno Laboratories, West Grove, dilution 1:500) or Alexa 488 anti-goat IgG, (Molecular Probes, Eugene, dilution 1:300). The sections were washed for 30 min with 0.1M PB and coverslipped with Vectashield mounting medium (Vector Labs, Burlingame, CA). For whole-mount immunostaining, the same procedure was used. For S-opsin, the primary antibody incubation was for 4 days and the secondary antibody incubation was for 3 days.

For double-labelling, sections were incubated in a mixture of S-opsin with cone arrestin, recoverin, and bassoon (Stressgen Biotechnologies, Victoria, BC, dilution 1:500) antibodies and 0.5% Triton X-100 in PBS and incubated overnight at 4°C. Sections were then rinsed for 3 times for 10 minutes in PBS and incubated with Alexa 488 donkey anti-goat IgG and (Cy3)-conjugated donkey anti-rabbit IgG or (Cy3)-conjugated donkey anti-goat IgG and Alexa 488 donkey anti-mouse IgG for 2 hours in room temperature. Sections were washed for 3 times for 10 minutes in PB and cover-slipped with Vectashield mounting medium.

Sections and whole-mounts were then analyzed using a Zeiss LSM 510, (Zeiss, NY) confocal microscope. Alexa 488 labelling was excited using the 488nm line of an Argon ion laser. For the detection of the (Cy3) signal, the 543nm line of a green HeNe1 laser was used. Immunofluorescence images were processed in Zeiss LSM-PC software. The brightness and contrast of the images were adjusted using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA). For presentation, all Photoshop manipulations (brightness and contrast only) were carried out equally across sections.

Topography and Quantification

The topographies of the S-opsin-immunoreactive cell populations were quantified in control and RD retinas at P90 and P180 days. The density of S-opsin-immunoreactive cells was measured in four retinal whole-mount preparations from each group. Whole-mount densities were mapped systematically in 1mm steps, using a calibrated eyepiece graticule. At these locations, serial optical sections were made through the outer retina, using a confocal microscope. By following each S-opsin-immunoreactive cell throughout the sections, we ensured to count every S-opsin-immunoreactive cell in the selected region once. For the density maps, fields of 200 µm × 200 µm were sampled in 1-mm steps on the retina in superior-to-inferior and nasal-to-temporal directions, with the optic disc at the center. Cell-density measurement was expressed as mean ± standard deviation (s.d.), and one-sided Student's t-tests were used to examine the difference between two means (control and RD). All statistical tests were performed using Stat View (Abacus Concepts, Berkeley, CA, USA). A difference between the mean of separate experimental conditions was considered significant at $P < 0.05$.

Electron microscopy

For electron microscopy, three P180 RD rats were euthanized as described above. Their eyecups were fixed in a mixture of 4% paraformaldehyde and

0.2% picric acid in PB for 30 min at room temperature. The retinas were then carefully dissected out; small pieces were taken from near the optic disc and fixed for an additional 2 h at 4°C. After washing in PB, the pieces were transferred to 30% sucrose in PB for 6 h at 4°C, rapidly frozen in liquid nitrogen, thawed, and embedded in 4% agar in distilled water. The retinal pieces were sectioned at 50 µm using a vibratome, and the sections were placed in PBS. They were incubated in 10% NDS in PBS for 1 h at room temperature and then in S-opsin antibody diluted 1:500 for 12 h at 4°C. The following immunocytochemical procedures for electron microscopy were carried out at room temperature. The sections were washed in PBS for 45 min (3 × 15 min), incubated in biotin-labeled anti-goat IgG for 2 h, and then washed three times in PBS for 45 min (3 × 15 min). The sections were incubated in avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 1 h, washed in PB, and then incubated in 0.05% 3,3'-diaminobenzidine (DAB) solution containing 0.01% H₂O₂. The reaction was monitored using a low-power microscope and was stopped by replacing the DAB-H₂O₂ solution with PB. The stained sections were post-fixed in 1% glutaraldehyde in PB for 1 h and, after washing in PB containing 4.5% sucrose for 15 min (3 × 5 min), sections were post-fixed in 1% OsO₄ in PB for 1 h. They were then washed again in PB containing 4.5% sucrose and dehydrated in a graded series of alcohol. During dehydration, they were stained *en bloc* with 1% uranyl acetate in 70% alcohol for 1 h, infiltrated with propylene oxide, and flat embedded in Epon 812. After sections had been cured at 60°C for 3 days, well-stained areas were cut out and attached to an Epon support for further ultrathin sectioning using a Reichert-Jung ultratome. Ultrathin sections (70–90 nm thick) were collected on one-hole grids coated with Formvar and examined by transmission electron microscopy (JEOL 1200EX, Tokyo, Japan).

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Abbreviations

CP	cone pedicle
Cy3	Carboxymethylindocyanine
DAB	3,3'-diaminobenzidine
NDS	normal donkey serum
INL	inner nuclear layer
IPL	inner plexiform layer
ONL	outer nuclear layer
OS	outer segment
P	postnatal day
PB	phosphate buffer

PBS	phosphate buffered saline
RD	retinal degeneration
SD	standard deviation

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Fig. 1. Photomicrographs taken from 50- μ m-thick vibratome sections processed for S-opsin immunoreactivity. The micrographs are for P21 control (A and B), P21 RD (C and D), P90 RD (E and F), and P180 RD (G and H). In the RD retina, S-cone shows abnormal morphology from P21. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar = 50 μ m.

Fig. 2. Photomicrographs taken from whole mounts of RD retinas processed for S-opsin immunoreactivity. The micrographs are for P90-near-to (A), P90-far-from (B), P180-near-to (C) and P180-far-from (D) the optic disc. Whole mounts are from the outer margin of the INL. S-opsin immunoreactive cells without the OS and part of their axon terminals (arrows); S-opsin immunoreactive cells with bipolar like morphology (arrowheads); S-opsin immunoreactive cells showing numerous processes emerging from both the cell body and the remaining axon process (open arrows); S-opsin immunoreactive cells showing amacrine-like morphology (open arrow heads). Scale bar = 50 μ m.

Fig. 3. Density of S-opsin-labelled cells in P90 and P180 control, and P90 and P180 RD retinas. (A) S-opsin-immunoreactive-cell densities from inferior to superior regions. (B) S-opsin-immunoreactive-cell densities from nasal to temporal regions. RD retinas showed higher densities far from the optic disc, while areas near it were more susceptible to degeneration. OD, optic disc. Densities appear as mean $\pm$ standard deviation.

Fig. 4. Confocal micrographs of vertical sections double-labelled for S-opsin with recoverin (A-C) and cone arrestin (D-F) immunoreactivities at P90 RD retinas. Double exposure shows all S-cones at the outer margin of the INL contained recoverin and cone arrestin. Scale bar = 10 μ m.

Fig 5.

Confocal micrographs of vertical sections double-labelled for S-opsin with bassoon immunoreactivities at P180 RD retinas (A). Double exposure shows bassoon immunoreactivity in S-cone terminals (arrows). The inset rectangle shows a high-magnification view of different S-cone terminal with bassoon (arrow). Transmission electron micrographs showing vertical ultrathin sections of the outer retina of P180 RD processed for S-opsin immunoreactivity. (B) S-opsin immunoreactivity localized to the membrane of a cone-like pedicle (CP). The inset rectangle shows a high-magnification view. The cone-like pedicle synapses (arrow) onto an unlabelled process in the outer retina. Cone-pedicle profile with a synaptic ribbon are also noted. CP, cone pedicle. Scale bars, 10 μm , 5 μm (the inset -A); 0.5 μm (B).