Fig. 2 In models of RP where rods and cones die simultaneously, bipolar cells lose dendrites and all iGluR/mGluR responsivity. In phase 0–1, rod bipolar cells downregulate GluR expression in the dendrites. In phase 1–2, rod and cone photoreceptor stress and death occur while dendritic modules are lost. In phase 3, wider retinal remodeling ensues, resulting in sprouting and formation of new axonal modules
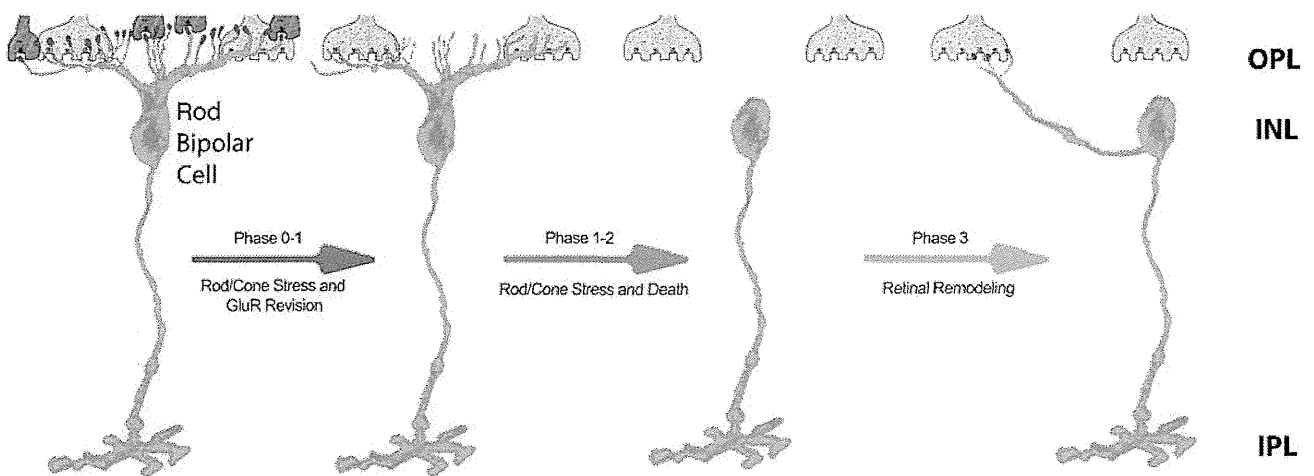

Fig. 3 In models of RP where cones outlive rods, some bipolar cell dendrites switch targets. In normal rod bipolar cell architecture, dendrites from rod bipolar cells bypass cone pedicles. In phase 0–1, as in rod/cone dystrophies, rod bipolar cells downregulate GluR expression in the dendrites. In phase 1–2 with rod stress and death, dendritic modules are lost. In late phase 2/phase 3, some rod bipolar cells form sprouts that contact cone pedicles, making peripheral contacts on cone pedicles

N-methyl-D-aspartate (NMDA) receptors on amacrine cells from PND 15 animals [76].

Retinitis pigmentosa disorders occur in both cone-sparing and cone-decimating forms. In models of RP where rods and cones die within similar time domains, retinas undergo large-scale remodeling events where bipolar cells lose dendrites and all iGluR and mGluR responsivity. Glial seal formation occurs, as does large-scale neuritogenesis leading to microneuroma formation, as well as neuronal migration [24]. In RP models where cones outlive rods (Fig. 3), some bipolar cell dendrites become involved in target switching, as shown by the fact that rod bipolar cells receive ectopic synapses from cones in the absence of rods

[77], though cones may persist for some time, with Müller cells engaging in seal formation around them [24, 74]. As long as cones or remnant cone cell bodies are present, bipolar cells underneath the cone photoreceptors appear to persist and progression of the neural retina into phase 3 appears to be arrested or dramatically slowed. Other retinal changes in cone-sparing retinal degenerations in phase 2 involve the initiation of anomalous sprouting, which often coalesces into structures called microneuromas, with contributions from not only bipolar cells but also other neuronal cell classes, including amacrine cells and horizontal cells, which can be observed to extend processes down into the inner plexiform layer [20–22, 74, 78].

Phase 3 is characterized by persistent remodeling, which further revises the fundamental topology of the retina via bidirectional migration of neurons through the vertical axis of the retina, as evinced by the migration of surviving bipolar and amacrine cells into the ganglion cell layer. Conversely, in many advanced degenerate retinas in phase 3, ganglion cells can be observed migrating into the inner nuclear layer [7, 9, 11, 12, 24]. The evolution of processes from all remaining neuron types in the retina continues to occur in phase 3, with some processes forming fascicles that can run in bundles for great distances within the neural retina (>100 microns) [24], while others run together forming tangles or tufts of novel neuropils termed microneuromas, which form outside the normal lamination of the inner plexiform layer [7, 8, 11, 12, 24, 26, 79]. Pigmented bone spicules (Fig. 4) are a common finding in patients with RP, and are often translocated into the neural retina [80]. Pigmented bone spicules appear to co-segregate with Müller cell columns, which appear to mediate much of the gross topological restructuring and are responsible for the translocation of RPE invasion of the neural retina (Fig. 4), as well as the formation of the aforementioned pigmented bone spicules.

As phase 3 remodeling progresses, breaks in Bruch's membrane provide opportunities for some neurons to emigrate out of the neural retina proper and into the membrane choriocapillaris complex [13] (Fig. 5). Whether or not similar emigration events out of the neural retina appear in human diseases like AMD is not clear, though it seems likely in AMD and AMD-like disorders. Certainly in the late forms of AMD with vascular involvement, there are breaches of Bruch's membrane, but other evidence indicates that Bruch's membrane may become calcified

following the breakdown of elastin and collagen in non-vascular or dry AMD [81].

Remodeling process variation in the presence of cones

While total loss of all photoreceptors leads to the complete loss of functional iGluR expression in ON and OFF bipolar cells, patches of cones may persist in cone-sparing forms, but they are substantially altered and embedded in the Müller cell seal. These altered cones still possess synaptic ribbons [82], but a more recent work [26] revealed that rod bipolar cells can withdraw their dendrites from rod photoreceptor terminals, subsequently elaborating transient ectopic non-ribbon contacts with surviving cone photoreceptors. Bipolar cells initiate these changes by retracting dendrites upon the loss of rod and cone contact [20–22], followed by a loss of signaling capacity in response to glutamate in regions of photoreceptor loss [75] (Figs. 2, 3). While RP disorders occur in both cone-sparing and cone-decimating forms, in cone-sparing RP, patches or isolated cones can persist for some time and appear to prolong the onset of gross topographic retinal remodeling. It is important to note that changes to network topology and iGluR/mGluR6 expression do occur in response to cone loss. Specifically, the retina underneath any remaining cone pedicles appears to be relatively preserved topologically, along with the seeming preservation of circuitry and iGluR expression in bipolar cell dendrites. However, proximal cones within 50 μm are fundamentally altered, with no iGluR signaling capacity remaining [26].

Neural information processing in the retina is immediately impacted by retinal photoreceptor degeneration and

Fig. 4 Retina from a human patient with advanced RP, illustrating pigmented bone spicules—accumulations of RPE pigment granules that derive from translocations of Müller cells, which alter the topology of the neural retina and cause the accumulation of pigment along clumps, lines, and grooves in the vertical axis of the neural retina. Scale bar 200 μm
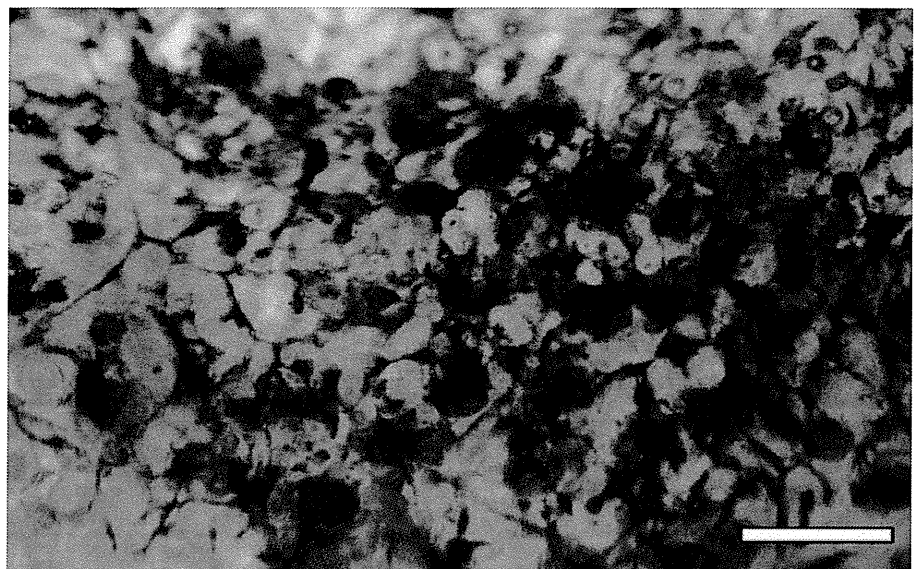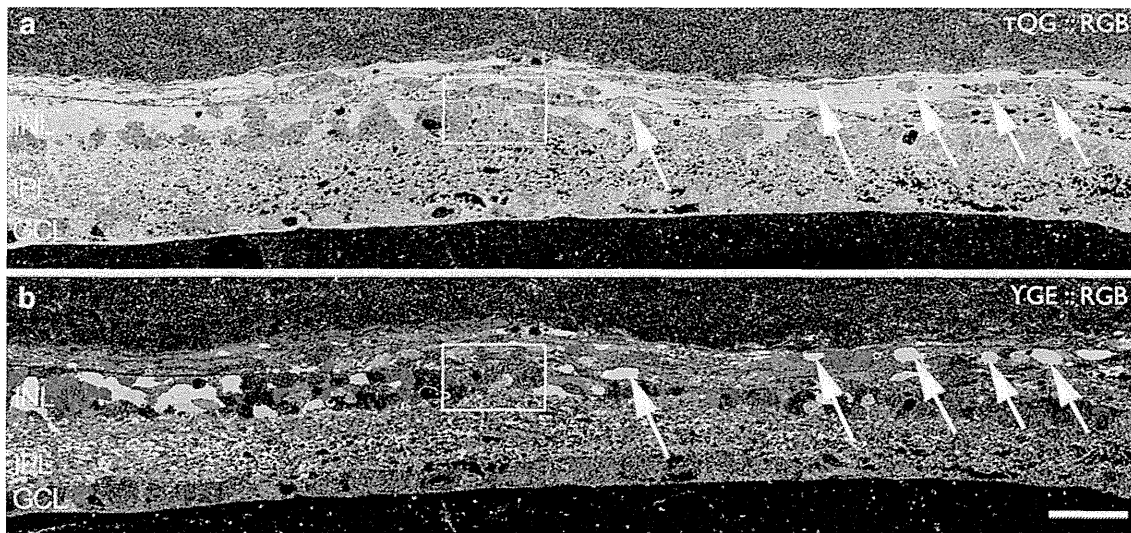
Fig. 5 Neural emigration in a light damage model of retinal degeneration in rat. Taurine, glutamine, glycine::r, g, b mapping in **a** demonstrates Müller glia with glycinergic amacrine cells embedded in them passing outside of the retina through a break (*box*) in Bruch's membrane. GABA, glycine, glutamate::r, g, b mapping in **b** demonstrates both GABAergic and glycinergic amacrine cells in addition to bipolar cells escaping from the neural retina into the choroid (*arrows*). *Scale bar* 90 μm

subsequent remodeling. The structural, biological, and information processing consequences of rod bipolar cell rewiring alone are substantial (Figs. 2, 6). The normal flow of information in the retina begins in the photoreceptors, where rod photoreceptor terminals bypass cone photoreceptor pedicles to make synapses on rod bipolar cells at their terminal spherules. Normally, hyperpolarizing signals from rod photoreceptors depolarize rod bipolar cells via sign-inverting mGluR6 receptors. Rod bipolar cells then "piggyback" onto the ON cone bipolar cell pathway through synaptic contact onto glycinergic amacrine cells that mediate signaling through to the ON cone bipolar cells via gap junctions, resulting in a sign-conserving signal, and to the OFF cone bipolar cell pathway via sign-inverting glycine receptors (Fig. 6). In cone-sparing RP, bipolar cell recordings reveal wholesale phenotype class switching from rod bipolar cells to OFF bipolar cell populations through alterations in the iGluR populations, effectively reprogramming the retina by inverting rod bipolar cell to amacrine cell to cone bipolar cell pathways, creating networks that are unable to maintain coherent signaling out of the retina [26] (Fig. 6). Additionally, from a circuit topology perspective, problems are obvious. In cone-sparing RP, the remaining cone pedicles are not numerous enough to accommodate all remaining rod bipolar cells. Furthermore, there aren't enough ribbons to accommodate rod BC dendrites, and the ON cone BCs already occupy the existing ribbon contacts. Those ectopic contacts that are formed are too far away from the ribbon to activate the existing mGluR6 receptors. Therefore, each cell type expresses receptors that are matched to concentrations that

prevail at a given distance from the ribbon in normal retinas at least. If rod BC mGluR6 receptors are not activated, then rod BCs should be persistently depolarized, which presents an additional problem for visual processing.

Human diseases and animal models of retinal degeneration that show remodeling

Human tissues from patients with late-stage RP show dramatic changes to the normal architecture of the retina (Fig. 7a, b). These samples demonstrate complete loss of rod and cone photoreceptors, bipolar cell loss, and subsequent topological restructuring of the retina. YGE > rgb imaging (Fig. 7a) reveals novel tufts of neuropils, termed microneuromas [24], with amacrine cells abutting the choroid and no barrier in-between due to the absent RPE. TQE > rgb signals demonstrate the formation and elaboration of the Müller cell seal isolating the neural retina (Fig. 7b). The subretinal space in these tissues is absent and impacts potential rescue strategies. In fact, because of the adherent nature of the glial seal, experimental retinal detachments of retinas in the regions of a Müller cell seal are almost impossible without some degree of trauma to the neural retina.

One might presume that since photoreceptors are lost in retinal degenerative diseases, the neural retina would be inactive in the absence of any afferent input. However, the sprouting of the neuronal processes and the alteration of the topology of the retina beg the question of function. It turns out that, even though photoreceptors are lost in regions of
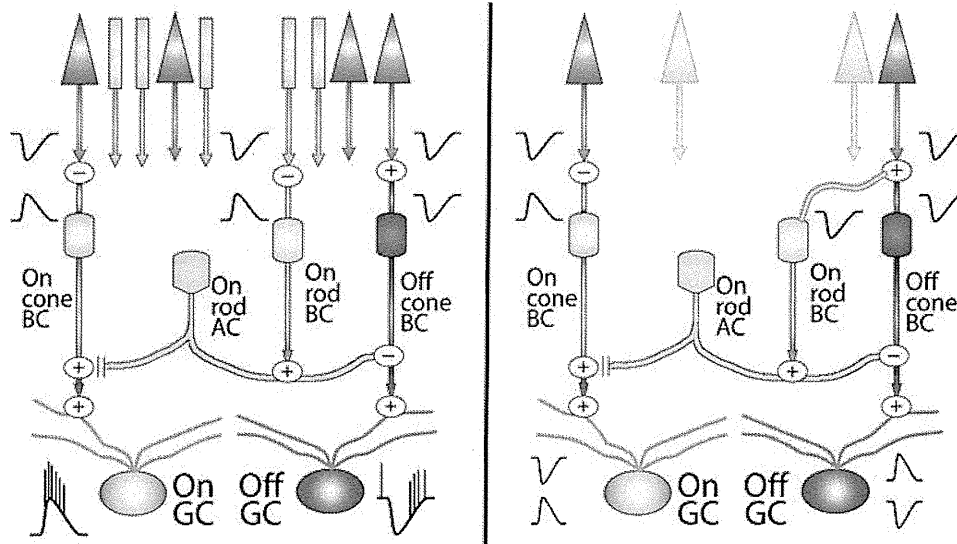
Fig. 6 Circuit diagram: rods provide sign-inverting input to ON bipolar cells via mGluR6-mediated synaptic connections. OFF bipolar cells are driven by cones through sign-conserving KA-mediated iGluRs, while ON bipolar cells are driven by cones through sign-inverting mGluR6-mediated synapses. Cone bipolar cells also synapse upon ganglion cells with sign-conserving AMPA-mediated iGluRs that define ON and OFF channels of information flow out of the retina. Information flow through the retina in humans during scotopic light levels is driven by rods and rod bipolar cells that piggyback onto intermediary amacrine cells, which shunt the flow of information to ON cone bipolar cells via sign-conserving gap junctions. Signaling to OFF cone bipolar cells occurs through sign-inverting glycinergic conventional synapses. In sum, a flash of light hyperpolarizes rod photoreceptors, and the network preserves the ON and OFF channel polarities. However, in cone-sparing RP seen in human and animal models, network flows are compromised through the formation of pathological networks that generate conflicting signaling driven by the remaining cones. With cone degeneration, this cone signaling is eventually lost, and retinas are driven by signaling intrinsic to the remnant neural retinal amacrine and ganglion cells

Fig. 7 Imaging of human retina from late-stage RP. GABA, glycine, glutamate::r, g, b mapping in **a** reveals novel tufts of neuropil, termed microneuromas (*box*), with amacrine cells abutting Bruch's membrane and the choroid. **b** Taurine, glutamine, glycine::r, g, b mapping of the same region demonstrates Müller cell revision and Müller cell seal formation (*arrows*), walling off the neural retina. *Scale bar* 90 μm
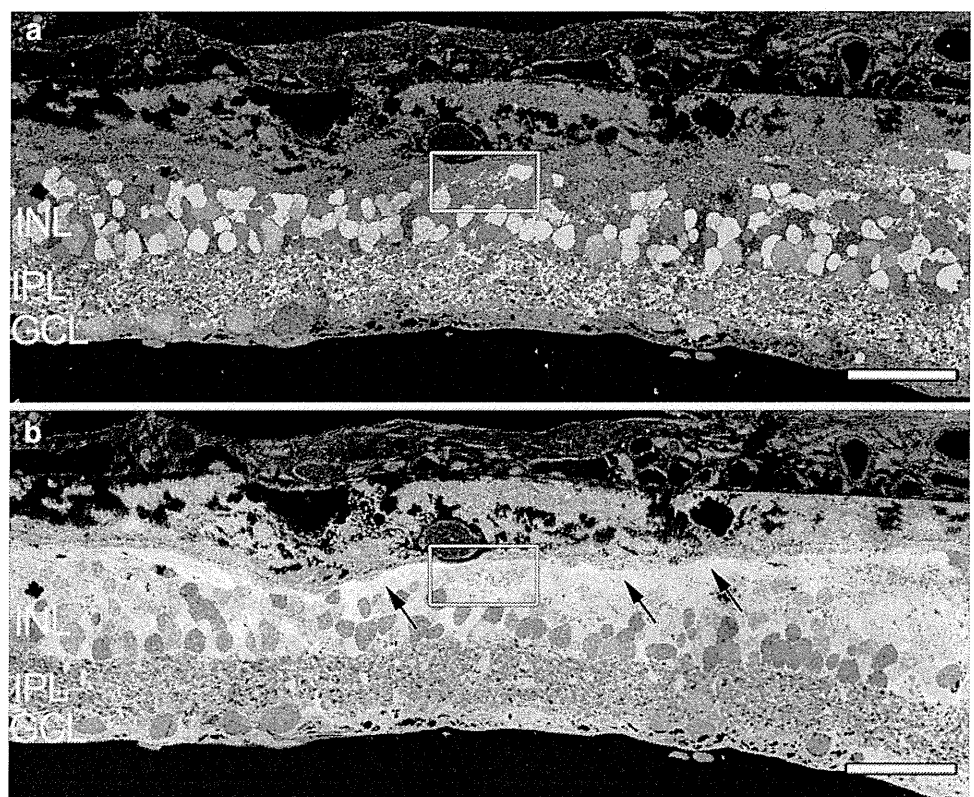
retinal atrophy, the neurons that are left in the retina continue to signal. Previous work examining the intrinsic activity of the *rdcl* mouse revealed dramatic levels of activity in the amacrine and ganglion cells, even though the bipolar cells were silent [26], while traditional electrophysiologic methods by Margolis and Detwiler [83] in the *rd1* mouse reveal substantial increases in oscillatory spike activity in the ganglion cells. Prior work by Pu et al. [17] in the RCS rat as well as Stasheff [19] in the *rd1* mouse and Sekirnjak et al. [84] in the P23H rat using single unit recording also shows substantial increases in the numbers of spontaneous ganglion cell spikes, despite the loss of photoreceptors to drive retinal activity. While results from single unit recording have yet to be correlated to retinas from human patients with RP, other recording methodologies were able to correlate retinal, specifically bipolar cell activity in both human and animal models of RP, which revealed identical disease mechanisms in both human and models of cone-sparing RP.

By combining excitation mapping with computational molecular phenotyping (CMP) [10], activated neurons (whether by ligands or light pathways) can be visualized ex vivo using small molecular probes. Simultaneous imaging of an activity probe, 1-amino-4-guanidobutane (AGB, functioning as a nonselective marker of ionotropic glutamatergic activity), combined with the visualization of other intrinsic small molecular signals [10], allows for the interrogation of neuronal cell classes that have been activated by either endogenous light drive or by pharmacologic activation (Fig. 8). Using CMP to segment images based upon their intrinsic small molecular signals allows the simultaneous extraction of excitation histograms revealed by AGB permeation for each class of bipolar cell, allowing the discrimination of OFF bipolar cell classes from ON bipolar cell classes. Additionally, ON bipolar cell classes can be segmented into rod and cone classes, allowing the elucidation of response profiles to AMPA [26, 74].

In remodeling retinas, amacrine and ganglion cells continue to encode signals via iGluR receptor complexes. This is an important finding, as identical findings in the mouse, rabbit, and human [26, 74] in relation to cone-sparing RPs demonstrate that after CMP segmentation, the incidence of OFF-like iGluR-expressing bipolar cells doubles in cone-sparing RP, while the incidence of remnant rod bipolar cells drops nearly tenfold. However, there appears to be no detectable bipolar cell death, leading to the conclusion that most rod bipolar cells make new contacts and begin to express iGluRs, leading to significant implications for retinal wiring. In these cases where cones outlive rods, the rod BCs actively down-regulate the expression of dendritic glutamate receptor modules and retarget them to cone pedicles. The outcome is mGluR6 to iGluR phenotype switching and substantial corruption of cone pathway signaling. Conversely, in RP disorders where both rod and cones die concurrently, all BCs engage in the disassembly of iGluR modules in the dendrites, repress GluR expression, and form supernumerary axons in combination with substantial neuronal death. Mixed cone-decimating and cone-sparing zones are also possible in both human RP and animal models [26, 74].

The spectrum of diseases in humans that include retinal degeneration and subsequent remodeling is broader than widely considered. Studies show that RP and animal models of RP demonstrate and duplicate features of human retinal degeneration and remodeling. Mouse, rat, pig, dog, cat, and rabbit models of retinal degeneration are extensively documented [7–9, 11–13, 20–22, 24, 26, 74, 85–90]. Porcine P23H models of RP show a progressive loss of photoreceptors (Fig. 9a–d) with a concomitant loss of visual percepts as measured by ERG [91]. Additionally, these porcine models demonstrate aberrant Müller cell signatures (Fig. 9a) identical to those observed in human (Figs. 7b, 12b, inset), rodent, and rabbit (Fig. 10b) [74].

Fig. 8 Excitation recording with KA and AGB in horizontal sections through the bipolar cell layer of a 12-week old TgP347L rabbit retina visualized with glycine, AGB, glutamate::r, g, b mapping, showing that most (~82 %) of the bipolar cells have phenotypically switched from rod (*diamonds*) and ON-like BC (*squares*) responses to OFF-like BC (*triangles*) responses, revealing a fundamental molecular reprogramming of response states. *Scale bar* 60 μm
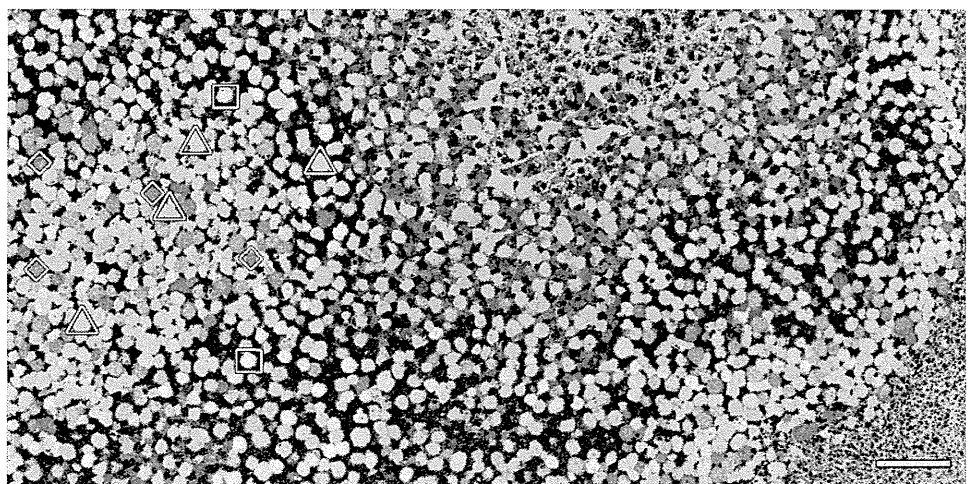
During the relatively early stages of degeneration, porcine tissues also demonstrate early aberrant neuritic sprouting in the glycinergic and GABAergic amacrine cell populations (Fig. 9c, d), identical to that observed in rodent models [24] and human RP [26, 74]. Some of the more dramatic retinal remodeling revisions are held at bay so long as cone photoreceptors appear to be present. By the time cone photoreceptors are decreasing in number, Müller cell hypertrophy begins and forms the Müller cell seal, walling off the neural retina from the remnant RPE and choroid (Fig. 10b). During the later stages, when the cone photoreceptors have gone, the retina begins more dramatic revisions, including the formation of microneuromas and neuronal translocation through the axis of the retina (Fig. 11), duplicating findings observed in human (Fig. 7) [26, 74].

The obvious question is whether other retinal degenerative diseases also show retinal remodeling, even though the principal mechanisms of retinal degeneration are different. Does retinal remodeling happen in age-related macular degeneration (AMD) for instance? Could retinal

remodeling also exist in glaucoma, even though the retina is not deafferented in a top-down fashion?

The documentation on negative plasticity or aberrant remodeling in other retinal degenerative diseases is sparse, though some labs have demonstrated bipolar cell sprouting and synaptic abnormalities in human AMD [23] and in potential animal models of AMD [13]. Additional preliminary work in our lab revealed that remodeling events in both glycinergic and GABAergic amacrine cells in human geographic atrophy (GA), as shown in Fig. 12a, b, also reveal the earliest histologically observable signs of retinal remodeling. YGE > rgb imaging (Fig. 12a) also shows aberrant sprouting in both the glycine signals as well as in GABA signals (inset). TQE > rgb signals show some early indications of Müller cell variation in metabolism (inset), but no evidence at this time point of gross morphological alterations or responses in Müller cell populations.

These cells are tertiary network cells in the retina, representing critical interneurons with a complex network topology [92] that is fundamental to visual processing. Alterations of connectivities in these populations
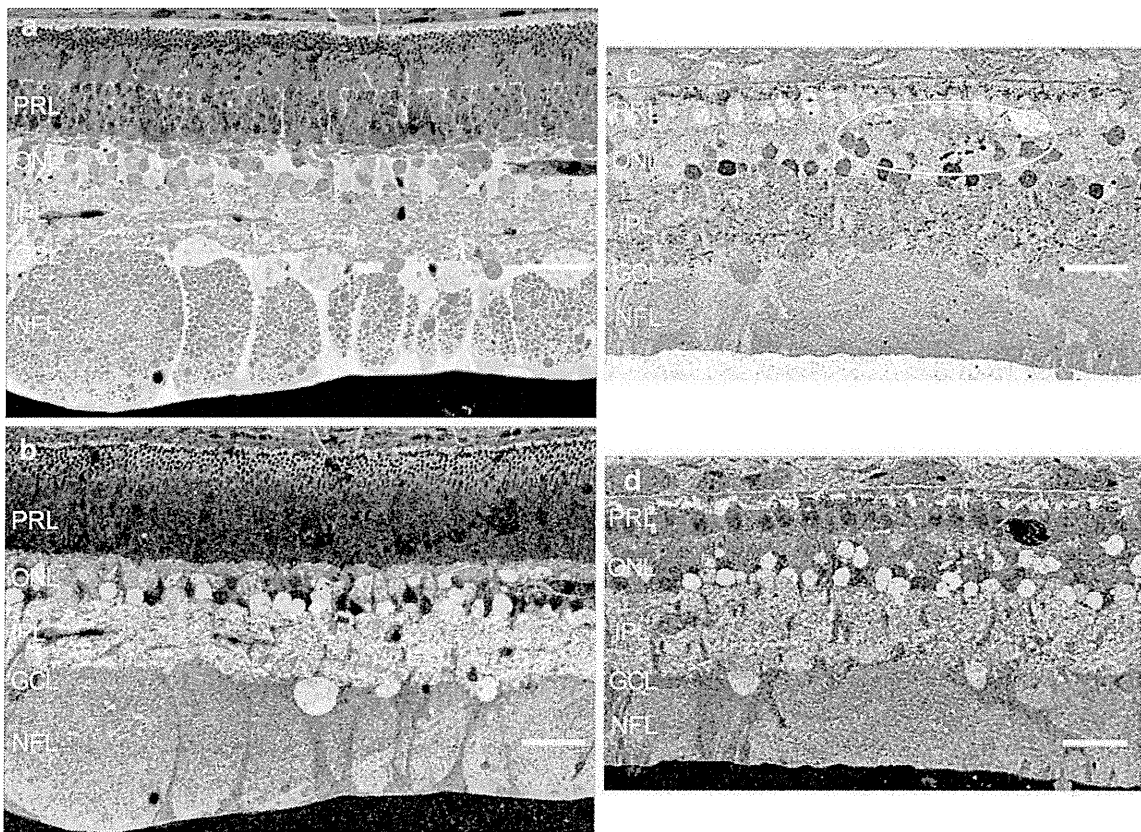

Fig. 9 Early-stage porcine P23H model of retinal degeneration. **a** Taurine, glutamine, glycine::r, g, b showing reduced photoreceptor outer segment length and early stages of Müller cell stress and alteration of molecular signatures (*green/yellow*). **b** GABA, glycine, glutamate::r, g, b of the same region showing normal-appearing OPL, IPL, and neuronal signatures. **c** Glycine immunohistochemistry

demonstrating early retinal remodeling/sprouting in the glycinergic amacrine cell populations. **d** GABA, glycine, glutamate::r, g, b mapping shows dramatically truncated photoreceptor outer and inner photoreceptor segments with the glycine signal shown in **c** in the *green* channel, demonstrating remodeling events. *Scale bar* 30 μm

Fig. 10 a GABA, glycine, glutamate::r, g, b mapping of a 746-day-old GHL rabbit, showing a glial column with migration of amacrine and bipolar cells into the ganglion cell layer. Microneuroma (*rectangle*) has also formed distal to the heavily depleted inner nuclear layer. **b** Taurine, glutamine, glycine::r, g, b mapping of the same P347L rabbit tissue, revealing normal and abnormal Müller cells (*box*) in the mid-stage degenerate retina. *Scale bar* 30 μm
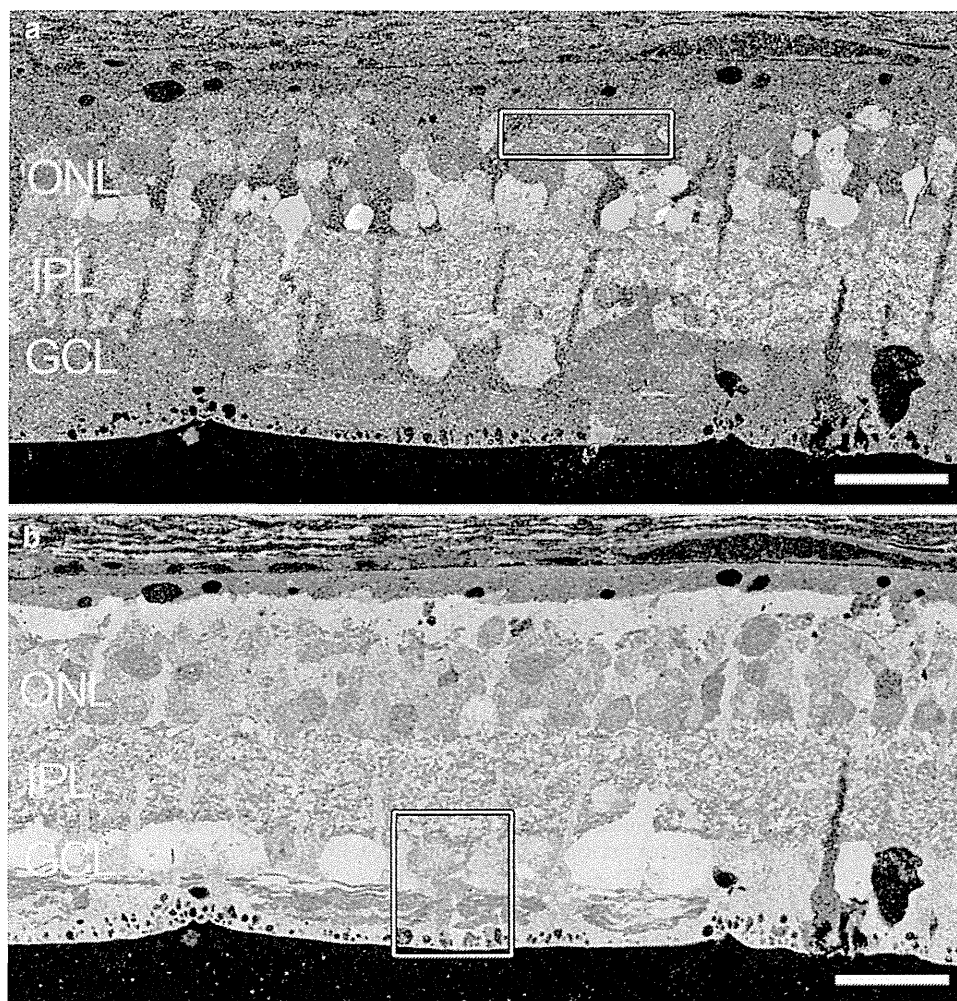
presumably dramatically disrupt visual processing, even in the presence of surviving afferent photoreceptors.

Retinal negative plasticity in glaucoma is also sparsely represented in the literature, though with a notable exception. Nico Cuenca demonstrated remodeling events in ON rod bipolar cells and horizontal cells in a model of glaucoma [93]. Work in our lab in collaboration with Monica Vetter and Alejandra Bosco demonstrated dramatic remodeling events of GABAergic amacrine cell processes in the DBA/2J mouse model of glaucoma (Fig. 13). Additional work with different disease models is beginning to show evidence for retinal remodeling in a number of animal models not traditionally associated with retinal disease, including spinocerebellar ataxia [94].

Mechanisms

Many mechanisms may be responsible for the various observed pathologies identified in retinal remodeling, including alterations in glutamate channel expression,

changes in integrin expression/signaling, and other disparate molecular pathways that ultimately result in retinal circuit reprogramming and restructuring or topological revision of the surviving retina. In this review, we present three potential mechanisms that may be responsible for some of the sequelae identified in retinal remodeling: retinal cell reprogramming mediated by alterations in GluR expression, topological restructuring mediated by integrin expression or alterations in integrin expression, and neuritic sprouting found in remodeling retina mediated by retinoic acid receptors (RARs).

Retinal cell reprogramming (alterations in iGluR expression)

Retinal degenerative diseases result in functional reprogramming of the retina in at least three distinct ways. (1) Defined bipolar cell pathways of mGluR6- and iGluR-mediated ON and OFF responses may become corrupted. (2) Alternatively, in the absence of externally mediated drive, intrinsic drive from specific cell populations in the
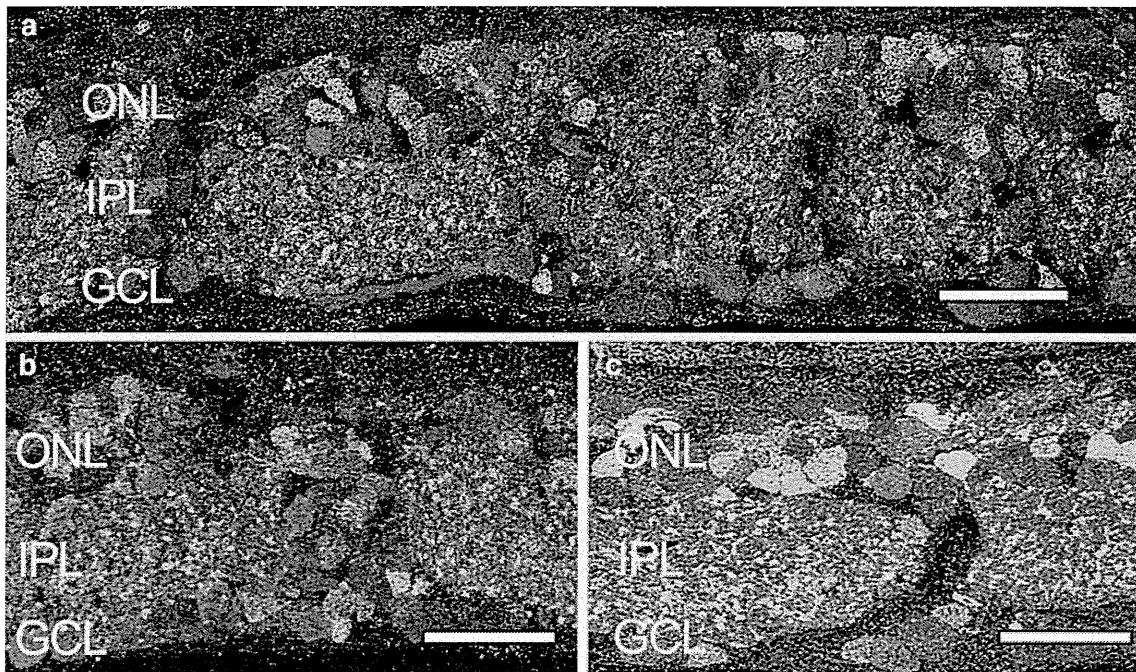
Fig. 11 a GABA, glycine, glutamate::r, g, b mapping of a 900-day-old RCS rat retina. This image shows three columns of neuronal translocation from ONL to GCL in which bipolar and amacrine cells are migrating through the retinal axis. **b** GABA, glycine, glutamate::r, g, b mapping of a 630-day-old rd1 mouse, demonstrating a column of neurons bridging the depleted inner nuclear layer with bidirectionally migrating amacrine and bipolar cells. **c** GABA, glycine, glutamate::r, g, b mapping of a 746-day-old GHL mouse, showing a glial column with migration of amacrine and bipolar cells into the ganglion cell layer. A microneuroma has also formed distal to the heavily depleted inner nuclear layer. *Scale bar* 60 μm

retina occurs, resulting in self signaling. (3) Cone-sparing forms of retinal degeneration alter iGluR expression, thus functionally changing phenotypic response profiles [26].

Retinal bipolar cell reprogramming is a universal feature in retinal degenerative diseases in humans, rodents, and rabbits [26, 74]. In retinal degenerative diseases and animal models of the same diseases that ablate both rods and cones, all evidence indicates that bipolar cell dendrites are lost [20–22], as are any responses mediated by glutamate [75]. Additionally, experiments with AGB (Fig. 8) demonstrate an absence of a cation current in these tissues [26], implying that the mGluR6-mediated signal transduction is defective through potential alteration or elimination of the receptors, or due to alterations in mGluR6 trafficking to other regions of the neuron [20–22].

Most notably, the presence of cones partially rescues the overall retinal structure, and the remaining bipolar cells underneath the surviving cones alter their response profiles so that the ratios of OFF cone to ON cone and ON rod bipolar cells shift from roughly 40:30:30 to ∼80:15:05 [26, 74]. Normally, in primates, rodents, and rabbit, ON and OFF cone bipolar cells and rod bipolar cells each comprise about 33 % of all bipolar cells.

Unmasking of autoexcitatory retinal signaling also occurs in retinas bereft of photoreceptor input [26], perhaps through variations in intrinsic calcium levels that result in

alterations of amacrine cell potentials [95]. These variations in responsivity or excitation may be due to oscillating inhibitory feedback from remaining glycinergic or GABAergic inputs. An alternative mechanism could arise from an existing excitatory cell type in the retina that is spontaneously active and has extensive input to both ON and OFF pathways. The dopaminergic amacrine cell has considerable input into AII amacrine cell pathways [96, 97], demonstrates spontaneous spike activity in the absence of synaptic input [98], and exhibits glutamatergic signatures. Additionally, dopaminergic amacrine cells are strongly activated by release from inhibition, and perhaps function by increasing tonic excitatory drive via synaptic glutamate release [99].

Light-induced retinal degeneration (LIRD) represents an effective tool for coherent photoreceptor loss that results in retinal remodeling and reprogramming [13]. Though the overall anatomy of the neural retina, and especially the inner nuclear layer, inner plexiform layer, and ganglion cell layer seem normal early in LIRD, key synaptic markers in the inner retina demonstrate rapid inner retina responses to photoreceptor stress that lead to fundamental reprogramming of neuronal responses and may represent an attempt to prevent excitotoxic damage and/or cell death.

In LIRD, GluR2 subunits of AMPA receptors predominantly associated with inner retinal processing display

Fig. 12 a GABA, glycine, glutamate::r, g, b mapping in human geographic atrophy/ AMD tissue demonstrates processes arising from both glycinergic and GABAergic amacrine cells (GABAergic processes extending into the outer plexiform layer in *inset*). These processes are the beginnings of microneuroma formation. **b** Taurine, glutamine, glutamate::r, g, b mapping demonstrates alterations in Müller cell signatures, notably an increase in the amount of taurine in subsets of Müller cells indicative of Müller cell stress (*inset*). *Scale bar* 90 μm
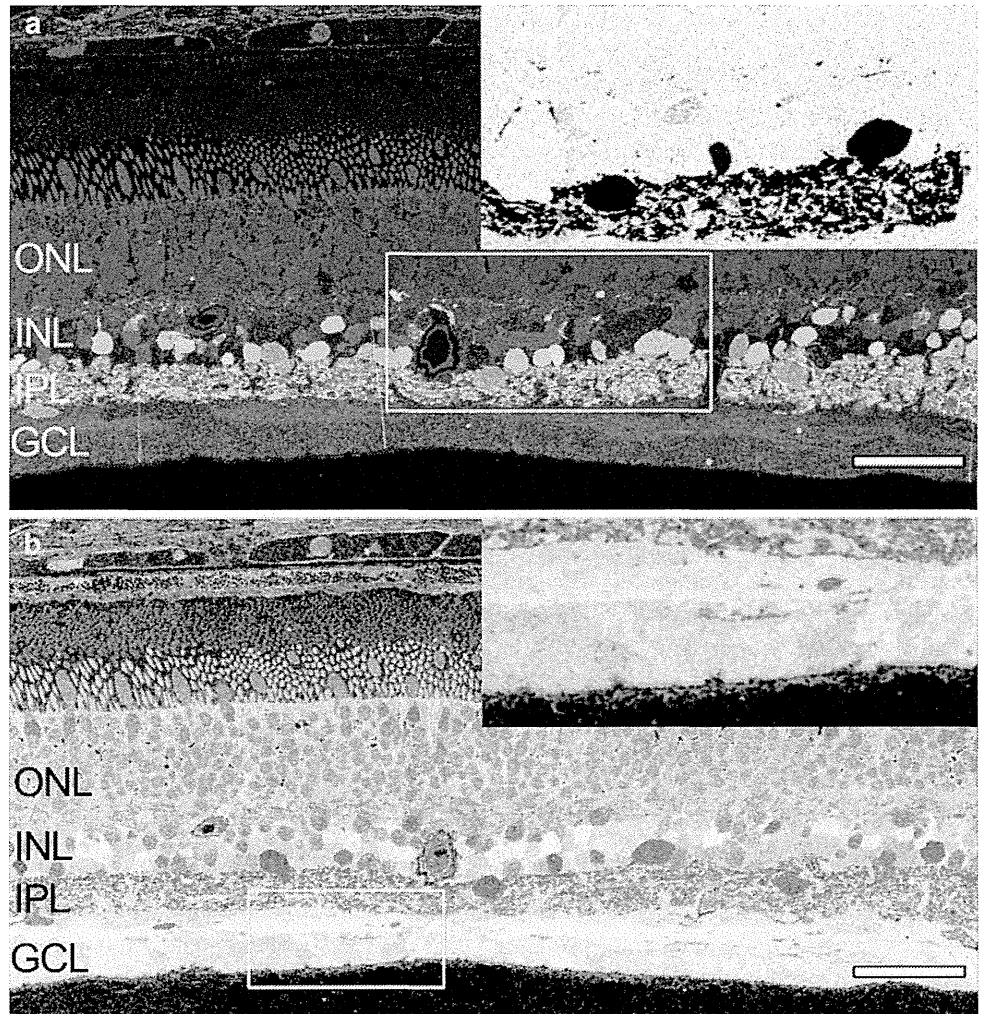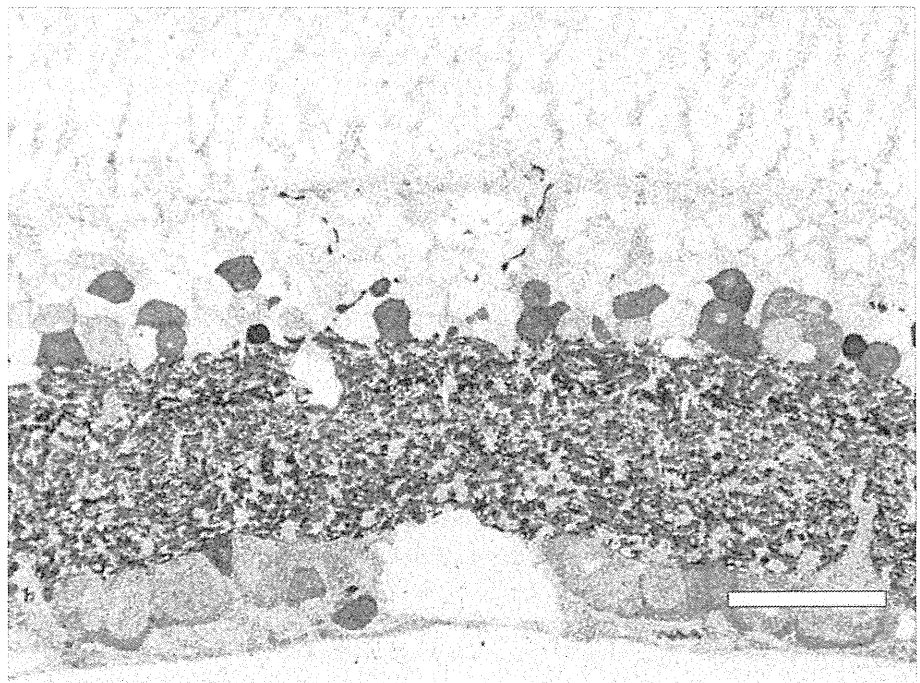

Fig. 13 Retina from a 23-month-old male DBA/2J mouse labeled for GABA, demonstrating aberrant GABAergic amacrine cell remodeling with new neurites projecting upwards into the outer plexiform layer. *Scale bar* 30 μm

rapid and significant alterations in protein levels and may reflect the same pathways used in CNS for neuroprotection [100]. Examination of GluR expression in LIRD demonstrates alterations in GluR expression over 60 days post light damage. Specifically, low-conductance AMPA receptor GluR2 subunits increase by 65 % (measured by protein level), high-conductance KA receptor GluR5 subunits decrease by 50 %, while AMPA receptor GluR1 subunits show no significant change in expression (Lin et al., submitted). A 65 % increase in GluR2 subunit availability is sufficient to stochastically add one subunit to every AMPA receptor that does not already express one, potentially performing three protective actions: (1) decreasing the Ca^{2+} permeability of the entire channel unitary conductance by 10×, (2) decreasing the conductance of the entire channel by over 50 %, and (3) preventing GluR1 phosphorylation-dependent increases in channel conductance. Combined, these effects potentially play a powerful role in neuroprotection by decreasing Ca^{2+} loads in neurons (Lin et al., submitted).

Topological restructuring via alterations in integrin expression

Other potential mechanisms involved in the observed topological restructuring of degenerate retina lie in alterations of integrin-mediated signaling. It is thought that virtually all adult tissue remodeling and cell migration involves integrin–integrin receptor signaling [101, 102]. It could be argued that the activation or reconfiguration of integrins might change cell–cell recognition or contact programming without substantial alterations in gene expression. If this is the case, clinical interventions that are designed to modulate integrin arginyl-glycyl-aspartic acid (RGD) motif binding in the remnant retina may work to modify or attenuate retinal remodeling without the need to resort to gene therapies. These studies in vivo are complicated by the slow progression in many models of retinal degeneration, particularly those that mimic human retinal degenerative diseases more precisely in their inhomogeneity or patchy degeneration. One possibility is to perform the modeling of integrin-mediated alterations in in vitro systems, but substantial evidence is present to suggest that cell–cell adhesion mechanisms are very different in in vitro systems as opposed to in vivo systems [103].

Evidence from Usher syndrome studies reveals links between the Usher protein complex and cadherins/catenins in junction-associated complexes which may play substantial roles in specific static or developmental cell polarity and tissue organization [104]. It also begs the question of whether these pathways and other integrin-mediated pathways are altered in retinal degenerative diseases.

### Neuritic sprouting mediated by retinoic acid receptors (RARs)

Other pathways, including retinoic acid (RA) mediated signaling pathways, are likely involved in retinal remodeling in the adult retina. RA-mediated signaling is activated in the LIRD model, where coherent photoreceptor loss and early dendritic remodeling occur. Additionally, exogenous application of RA leads to robust neuritic growth in primary cultured rod bipolar cells [105]. We believe that RA signaling displays large alterations early in retinal degeneration, suggesting that RA signaling pathways may be responsible for survival-related neuritogenesis and subsequent plasticity. Deficiencies in RA signaling may contribute to neurite degeneration, and retinoic acid (RA) and RA/retinoid receptor (RAR/RXR)-dependent pathways in retina incur the activation of neuritogenesis, resulting in the formation and elaboration of complex circuits and structures comprising all of the remaining retinal neuronal classes.

We demonstrated that RAR/RXR signaling is a mechanism driving pathological neuritogenesis. Even more compellingly, RAR/RXR antagonists appear to attenuate neuritogenesis from BC populations, while CaMKII inhibitors block neuritogenesis [105–107]. RAR/RXR signaling in phase 3 remodeling appears to induce anomalous neuronal sprouting and outgrowth, forming fascicles and microneuromas. This sometimes leads to retinas becoming so completely topologically restructured that the tissue no longer resembles retina. The model is that loss of photoreceptors eliminates retinoid buffering capacity (opsins) and increases retinoid precursor availability, leading to the generation of RA. RAR/RXR transcription subsequently induces sprouting and neuritogenesis. This occurs simultaneously with the loss of photoreceptors, which reduces glutamate-mediated neurotransmission and, by extension, decreases Ca^{2+}-mediated signaling. βCaMKII then detects the decrease in Ca^{2+} flux and contributes to RAR/RXR-mediated neuritogenesis through RXR binding.

Circuit outcomes of retinal remodeling

By hybridizing light microscopy with TEM analysis, phenotypic identity can be established for neurons, glia, and processes in ultrastructural datasets (Fig. 14) [24, 108], and circuitry can be defined for those phenotyped neurons [92, 109]. Analysis of over 30 animal models of RP and human RP samples indicates that, as remodeling progresses, the retinal circuitry substrate is altered. Beginning with bipolar cell dendritic truncation and polyaxonal growth early in the disease process [26] through to amacrine and ganglion cell neuritogenesis [24], evolving into anomalous fascicles and

Fig. 14 GABA, taurine,
glutamate::r, g, b overlay on a
TEM image of a peptidergic
GABAergic amacrine cell
(*asterisk*) adjacent to a forming
microneuroma in the RCS rat
retina. Overlay CMP/TEM
imaging enables connectomics
and pathoconnectomics projects
that elucidate precise,
ultrastructural reconstruction of
neuronal circuitry. *Scale bar*
6 μm
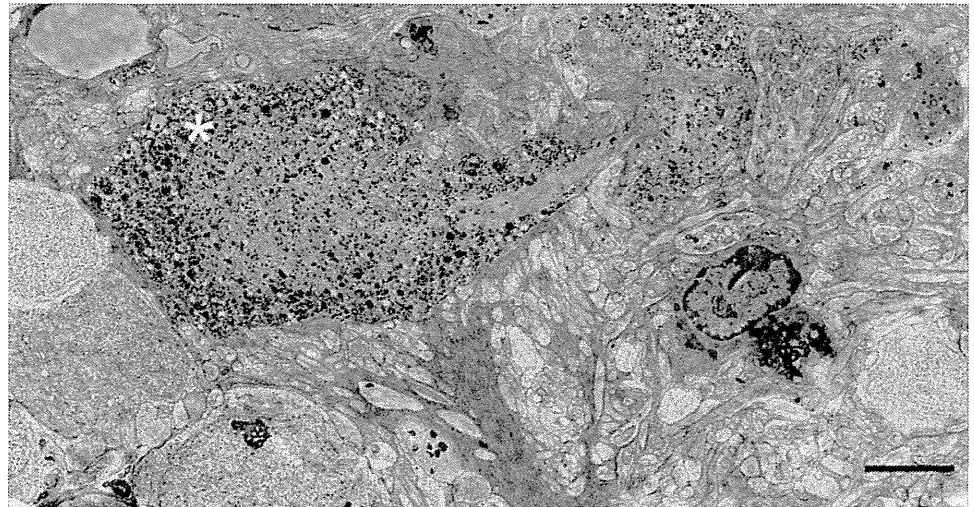
microneuromas, the retina is restructuring its connectivities. Whether or not those connectivities are proper or improper is currently unknown and will only be answered through complete connectomic approaches [92, 109]. This is work that is underway in the P347L rabbit [110], the RCS rat [111], and the light-induced retinal degeneration mouse model [26], and it is so far demonstrating that these novel structures are not synaptically silent. Microneuromas exhibit numerous synaptic structures from both ribbon and conventional synapses, but it is still too early to judge their viability as functional or nonfunctional visual circuits.

Implications for retinal vision rescue

Retinal remodeling as a phenomenon introduces substantial barriers to any therapy designed to recover vision loss. Interventions in the remaining populations of neurons are complicated by the loss of BCs during the remodeling process [26]. More importantly, as discussed above, the initial response of the neural retina likely induces a substantial change to the retinal circuitry prior to cell loss, with initial alterations in BC connectivities and programming eliminating targets for photoreceptor progenitor cell or fetal retina transplants.

Retinas are reactive when deafferented and demonstrate enhanced remodeling in response to direct intervention. Retinal transplants, for example, remodel more aggressively than the host degenerate retina [112]. Prevention of neurite/dendritic loss appears to be critical to holding off phase 3 remodeling events, yet that loss appears to be one of the earliest indicators of neural disease in retina [20], cortex [113], and midbrain [114], with dendrites appearing to be far more sensitive to pathophysiological alterations than axons. Early changes include the aforementioned

mislocalization of mGluR6 receptors [20–22], followed by bipolar cell regression and loss of iGluR expression in the dendrites, which effectively reprograms the physiologic response profiles of these bipolar cells and—one would presume—all subsequent downstream elements. Bipolar cells do not recover iGluR expression and thus the ability to signal through iGluR-mediated circuitry [26], making them poor targets for intervention strategies. Once downstream circuit revision occurs, there is persistent loss of iGluR expression in other cell populations as well [26], which leads to irrevocable alterations of the topology and circuitry of the retina [7–9, 11–13, 24, 26, 79].

Failing the prevention of dendritic loss of bipolar cells, secondary targets might prove successful by preventing horizontal, amacrine, and ganglion cells from sprouting aberrantly. A number of approaches aimed at reducing aberrant neuronal sprouting have been attempted, including electrical stimulation, which appears to enhance sprouting (data not published), tetrodotoxin (TTX) exposure in post-traumatic epilepsy models [115], and manipulation of integrins and retinoid X receptors [105], as discussed above. These approaches appear promising, as the loss of photoreceptors removes retinoid binding pools, thus increasing the availability of retinoid precursors. RA becomes available [106, 116], which subsequently triggers neuritogenesis through RAR/RXR transcriptional activity [117]. Simultaneously, photoreceptor loss reduces neural Ca^{2+} influx, activating the βCaMKII and driving neuritogenesis [107, 118]. This opens the door to therapeutic interventions that are designed to antagonize CaMKII, among other therapies.

Another approach we are working on in collaboration with Wolfgang Baehr is an early intervention with AAV-rRho-shRNA vectors to suppress rod opsin synthesis while patients still have photoreceptors, in order to arrest or delay photoreceptor cell death and prolong vision preservation by

decreasing the continued retinal stress and bystander kill-ing effects of dying rods on cones. If one were to presume that rod opsin mediated toxicity, either via misfolding or mistrafficked opsins, is responsible for rod photoreceptor stress and subsequent cell death, it might be appropriate to reduce total overall rod opsin expression levels. The fun-damental idea is to prolong rod photoreceptor life and limit the bystander killing effect on cone photoreceptors. This strategy has shown promise in GCAP1-Y99C transgenic mice, delaying vision loss through the expression of a GCAP1 shRNA by recombinant scAAV2/8 [119].

Other molecular changes occur in degenerate retina, and there is no evidence to suggest that retinal remodeling has an end point or arrest point [7, 24, 26]. More needs to be done in these areas, and possibly also in controlling or manipulating integrin-mediated contact on cell surfaces, which brings up the possibility of other forms of repro-gramming in neural systems that have been deafferented. Other molecular changes are unquestionably occurring in the degenerate retina, and substantial evidence indicates that remodeling does not have an arrest point [7, 24, 26]. Fundamentally, these approaches are designed to slow the progression of retinal degeneration and not to recover vision that has already been lost.

A number of approaches are proposed that are designed to recover lost vision, but these approaches all have the same number of problems. The fundamental problem in rescuing vision is to define windows of opportunity or when to intervene. Current therapeutic interventions are designed for those patients that have already lost some, if not all, of their vision. These patients often present at a late stage with advanced retinal degeneration (Fig. 4), and are likely to exhibit early profound alterations to the retinal circuitry that corrupt any surrogate inputs. Any visual prosthetic design will depend upon the stage of retinal degeneration of the patient. Specifically, intervening in a highly remodeled retina to recover vision will represent an entirely different set of bioengineering or bionic engineering hurdles than intervening in a retina that has most or all of its circuitry still intact. It might be supposed that unless one actually knows what the fundamental circuitry is in the normal retina, one cannot predict what the output of the neural retina will be. Additionally, early evidence indicates that synaptic contacts in the remodeling retina are inappropriate, with some models [26] predicting that circuits may generate oscilla-tory ringing, which is incompatible with normal visual processing. Therefore, predicting what the ganglion cell output of the retina will be is difficult at best.

Other approaches, including stem cell transplants or embryonic sheet transplants, will have to overcome a number of issues, such as (and not limited to) improper wiring, cell fusion, and the rather scary possibility of transplant "rescues" being co-opted into Frankenstein-like

assemblies of defective or nonfunctional cell populations by resident neurons and glia. A number of reports of transplanted stem cells that co-opt the phenotypes of host cells are documented as instances of cell fusion [120]. In addition to the documented circuit revision that occurs in retinal remodeling, there is a question of patterning, as remodeling retinas likely have altered spacing and visual functions that are related to alterations in cell–cell spacing. Cell populations that are introduced into an existing retina, albeit remodeled, do not have a history of normal devel-opmental structuring such as that which occurs during retinal maturation, and no proposed research has ever discussed re-patterning the retina to recapitulate spacing rules. So, if introduced cell populations are to be success-ful, properly phenotyping transplanted cells [121] becomes a critical task in order to track identity and any positional issues that might impact visual field processing, though most efforts to date document a rejection of transplant cells [122] or a loss of the transplant cells' own mature pheno-types at various time points post-transplantation.

Fundamentally, most bionic or biological transplant/ implant approaches presume or are engineered on the belief that the underlying neural retina is normal. The degenerate retina, on the other hand, is not normal, and most of its cell types show abnormality. The basic assumptions of trans-plant technologies (intactness, receptivity, and capacity of the host neural retina to carry the signals offered by the implant/transplant) are false.

However, we believe that there is a way forward using approaches pioneered in the optogenetics community, uti-lizing existing, remnant cell populations with intervention prior to large-scale restructuring of the retina. These genetic, therapeutic approaches using AAV-mediated chan-nelrhodopsins/halorhodopsin vectors that target appropriate classes of retinal neurons could conceivably happen as a relatively late intervention when patients have lost func-tional photoreceptors [123–125]. This approach has been used before in ON bipolar cells in mouse to deliver chan-nelrhodopsin2 [125]. The key here will be to target the right classes of neurons, as photoreceptor loss in RP effectively deafferents approximately 20 different ganglion cell pathways [10, 126]. Targeting broad ganglion cell classes with channelrhodopsins [127] is tricky, as separate ganglion cell outflow channels will then be generating the same signals to the cortex and other regions simulta-neously. This is likely to set up errors in higher visual signal processing, so the decision about which specific retinal cell classes to target will be critical.

Human tissues

Tissue from patients with advanced RP and early GA/AMD were obtained from the Lion's Eye Bank donor pool at the

University of Utah within 3 h of death. The Lion's Eye Bank donor procurement and distribution complies with the Declaration of Helsinki. All data were de-identified in accordance with the HIPAA Privacy Rule.

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Transcorneal Electrical Stimulation Promotes Survival of Photoreceptors and Improves Retinal Function in Rhodopsin P347L Transgenic Rabbits

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Purpose. To determine whether transcorneal electrical stimulation (TES) has neuroprotective effects on the photoreceptors, and whether it slows the rate of decrease of the electroretinogram (ERG) in rhodopsin P347L transgenic (Tg) rabbits.

Methods. Six-week-old Tg rabbits received TES through a contact lens electrode on the left eye weekly for 6 weeks. The right eyes received sham stimulation on the same days. Electroretinograms (ERGs) were recorded before and at 12 weeks after the TES. After the last ERG recordings, the animals were euthanized for morphologic analysis of the retinas. Immunohistochemical (IHC) analysis was performed to detect the immunostaining by peanut agglutinin (PNA) and rhodopsin antibodies in the retinas.

Results. The a- and b-wave amplitudes of the photopic ERGs and the b-wave amplitudes of the scotopic ERGs at higher stimulus intensities were significantly larger in the TES eyes than in the sham stimulated eyes ($P < 0.05$, respectively). Morphologic analyses showed that the mean thickness of the outer nuclear layer (ONL) in the visual streak at 12 weeks was significantly thicker in TES eyes than in sham-stimulated eyes ($P < 0.05$). IHC showed that the immunostaining by PNA and rhodopsin antibody in the TES-treated retinas was stronger than that in the sham-stimulated retinas.

Conclusions. TES promotes the survival of photoreceptors and preserves the ERGs in Tg rabbits. Although further investigations are necessary before using TES on patients, these findings indicate that TES should be considered for therapeutic treatment for RP patients with a P347L mutation of rhodopsin. (*Invest Ophthalmol Vis Sci.* 2012;53:4254–4261) DOI: 10.1167/iovs.11-9067

Patients with RP have a progressive loss of rod and cone photoreceptors that leads to a severe decrease in the visual acuity and a severe constriction of the visual field.[1,2] The worldwide prevalence of RP is approximately 1 in 4000,

meaning that more than 1 million individuals are affected worldwide.[3] As such, RP is one of the leading causes of blindness in the world.

Many promising treatments to save or restore vision in RP patients are being investigated clinically and experimentally.[4-9] Electrical stimulation (ES) of the retina is one of the methods that is being tried because it is less invasive than other treatments and has been shown to have neuroprotective properties on the visual system.[10-18] ES of the transected optic nerve stump in rats promoted the survival of axotomized retinal ganglion cells (RGCs) in vivo.[10] Transcorneal electrical stimulation (TES) in rats was reported to rescue axotomized RGCs[11,12] and promote axonal regeneration of injured RGCs.[13,14] TES was also shown to improve the visual function of patients with traumatic optic neuropathy and nonarteritic ischemic optic neuropathy.[15]

We have demonstrated that TES promoted the survival of photoreceptors and preserved the retinal function of Royal College of Surgeons (RCS) rats, an animal model of RP.[16] Ni et al.[17] also reported that TES had neuroprotective effects on the photoreceptors after phototoxicity in rats. In a preliminary clinical trial, Schatz et al.[18] demonstrated that TES improved the visual function in RP patients.

However, RP is a genetically heterogeneous disease, and mutations in several photoreceptor-specific and some non-specific genes are known to cause RP.[19] Therefore, it is necessary to examine the neuroprotective effect of TES on the photoreceptors in the retinas of various RP animal models to determine which genetic type of RP is responsive to TES.

Rhodopsin Pro 347 Leu (P347L) transgenic (Tg) rabbits have been generated by Kondo et al.[20] This sequence of alterations is similar to those in human patients with autosomal dominant RP (adRP) with the rhodopsin P347L mutation.[21,22] This animal model has a rod-dominated, progressive photoreceptor degeneration with regional variations in the pattern of photoreceptor loss.[20,23]

The purpose of this study was to determine whether TES has a neuroprotective effect on the photoreceptors and improves the amplitudes of the electroretinogram (ERG) in Tg rabbits. Our morphologic and electrophysiological analyses showed that TES had a neuroprotective effect on the photoreceptors and improved the amplitudes of the ERG of Tg rabbits.

MATERIALS AND METHODS

Animals

All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision

Research, and the procedures were approved by the Animal Research Committee, Osaka University Graduate School of Medicine. Five Tg rabbits were purchased from the Kitayama Labes Co. (Ina, Japan). They were raised on a 12-hour dark 12-hour light cycle with an ambient light intensity of 100 lux.

Transcorneal Electrical Stimulation

The rabbits were anesthetized intramuscularly with a mixture of medetomidine (0.3 mg/kg, Domitor; Orion Corporation, Espoo, Finland), midazolam (4 mg/kg, Dormicum, Astellas Pharma Inc., Tokyo, Japan), and butorphanol (5 mg/kg, Betorphal; Meiji Seika Pharma, Co., Ltd., Tokyo, Japan). For the electrical stimulation, the corneas were also anesthetized with a drop of 0.4% oxybuprocaine HCl, and a contact lens electrode with inner and outer concentric electrodes (Mayo Corporation, Nagoya, Japan) was placed on the cornea with a drop of 2.5% methylcellulose to maintain good electrical contact and prevent corneal drying. Biphasic rectangular current pulses (700 μA, 10 ms/phase duration) were delivered at a frequency of 20 Hz from an electrical stimulation system (Stimulator: SEN-7320, Nihon Kohden, Tokyo, Japan; Isolator: WPI, Sarasota, FL) through the contact lens electrode.

TES was given to 6-week-old rabbits for 1 hour once a week until the animals were 12 weeks old. Only the left eye was electrically stimulated. The same type of contact lens electrode was placed on the right eyes but no electrical current was delivered (sham stimulation).

Electroretinograms

ERGs were recorded from the animals at 6 weeks of age just before the beginning of the TES and after the end of the TES treatments at 12 weeks of age. For the TES, animals were anesthetized intramuscularly with a mixture of medetomidine (0.3 mg/kg), midazolam (1 mg/kg), and butorphanol (1 mg/kg). The pupils were dilated with 2.5% phenylephrine hydrochloride and 0.5 % tropicamide.

After 1 hour of dark adaptation, the animals were restrained in a box and were prepared for the recordings under dim red light. ERGs were recorded from both eyes simultaneously with a corneal electrode carrying LEDs creating a mini-Ganzfeld stimulator (WLS-20, Mayo Corporation). A 2.5% hydroxypropyl methylcellulose ophthalmic solution was used with the corneal contact lens electrode. The reference electrode and a ground electrode were inserted subcutaneously into the left ear and the nose, respectively.

The luminance of the scotopic ERG stimuli was increased from −5.0 to 1.48 log cd·s/m^2 in 0.5 or 1.0 log unit steps. After the scotopic ERG recordings, animals were light-adapted for 30 minutes, and the photopic ERGs were recorded. The luminance of photopic ERG stimuli was increased from −1.0 to 1.95 log cd·s/m^2, and the stimuli were presented on a white background of 25 cd/m^2.

The responses were amplified, band pass filtered from 0.3 to 1000 Hz, and digitized at 3.3 kHz. A computational ERG recording system (Neuropack μ; Nihon Kohden, Tokyo, Japan) was used to average the ERG responses. Five to 20 responses were averaged with interstimulus intervals from 1 to 10 seconds depending on the intensity of the stimulus.

ERG Analysis

The scotopic (dark-adapted) and photopic (light-adapted) a-wave amplitudes were measured from the prestimulus baseline to the peak of the a-wave, and the b-wave amplitude was measured from the trough of the a-wave to the peak of b-wave.

To determine the significance of differences in the ERG amplitudes between TES electrically stimulated eyes and sham-stimulated eyes for the full intensity range, we plotted the average ratio of the TES-treated to the sham-stimulated eyes at all intensities and performed statistical analyses.[22-24]

Histological Analysis

Immediately after the final ERG recordings, the rabbits were euthanized with an overdose of pentobarbital sodium. The eyes were removed and placed in a mixture of 10% neutral buffered formalin and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) for 30 minutes at room temperature. Then eyes were trimmed, and part of the eye cups, including the optic nerve, were postfixed in 4% glutaraldehyde in 0.1 M PB at 4°C. The tissues were trimmed, embedded in paraffin, sectioned vertically, and stained with hematoxylin and eosin for light microscopy. All sections were cut along the vertical meridian of the eye passing through the optic nerve. Five serial sections of each eye were analyzed for each experimental animal.

The degree of retinal degeneration was assessed by measuring the thickness of the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). Photographs were taken of the superior and inferior hemispheres at 10 defined points with a camera attached to a light microscope (E800; Nikon, Tokyo, Japan). The first photograph was taken at approximately 2 mm from the center of the optic nerve head, and subsequent photographs were taken every 2 mm more peripherally. The thickness of ONL, INL, and GCL were measured on the photographs (Scion Image analyzer; Scion Corp., Frederick, MD). Each eye was coded so that the investigator making the measurements was masked to treatment of the eye.

Immunohistochemistry

The paraffin-embedded sections (5 μm) were processed for immuno-fluorescence staining with antirhodopsin antibody (1:100; RET-P1; Santa Cruz Biotechnology, Santa Cruz, CA), followed by Cy3-conjugated anti-mouse IgG (1:200), and FITC-conjugated peanut agglutinin (1:100) (PNA; Invitrogen, Carlsbad, CA), a lectin that binds specifically to rabbit cone photoreceptors. The TES-treated and sham-stimulated sections were observed with a fluorescence microscope (E800; Nikon).

Statistical Analysis

Data were analyzed with a commercial software (JMP8; SAS Institute Japan, Tokyo, Japan). The data were expressed as the means ± SDs or SEMs. Comparisons between two groups were made by Student's *t*-tests when the data were normally distributed or by the Mann-Whitney rank-sum test when the data were not normally distributed. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of TES on Survival of Photoreceptors in Tg Rabbits

Representative retinal sections in the area of the visual streak from 12-week-old Tg rabbits that had TES (left eye) or sham stimulation (right eye) are shown in Figures 1A and 1B. The number of rows of nuclei in the ONL at the visual streak was two to three and the nuclei were closely packed in the retina receiving TES (Fig. 1A). In the sham-stimulated retina, only one row of nuclei was found in the ONL at the visual streak and they were loosely packed (Fig. 1B). In contrast, there was no difference in the structure and thickness of the ONL in other areas of the retina away from the visual streak between the TES-treated and sham-stimulated retinas (Figs. 1C, 1D). The architecture and thickness of the middle and inner retinal layers were well preserved in both TES-treated and sham-stimulated retinas (Figs. 1A-D).

Quantitative analyses showed that the thickness of the ONL in the visual streak in the TES-treated eyes was 13.9 ± 3.3 μm
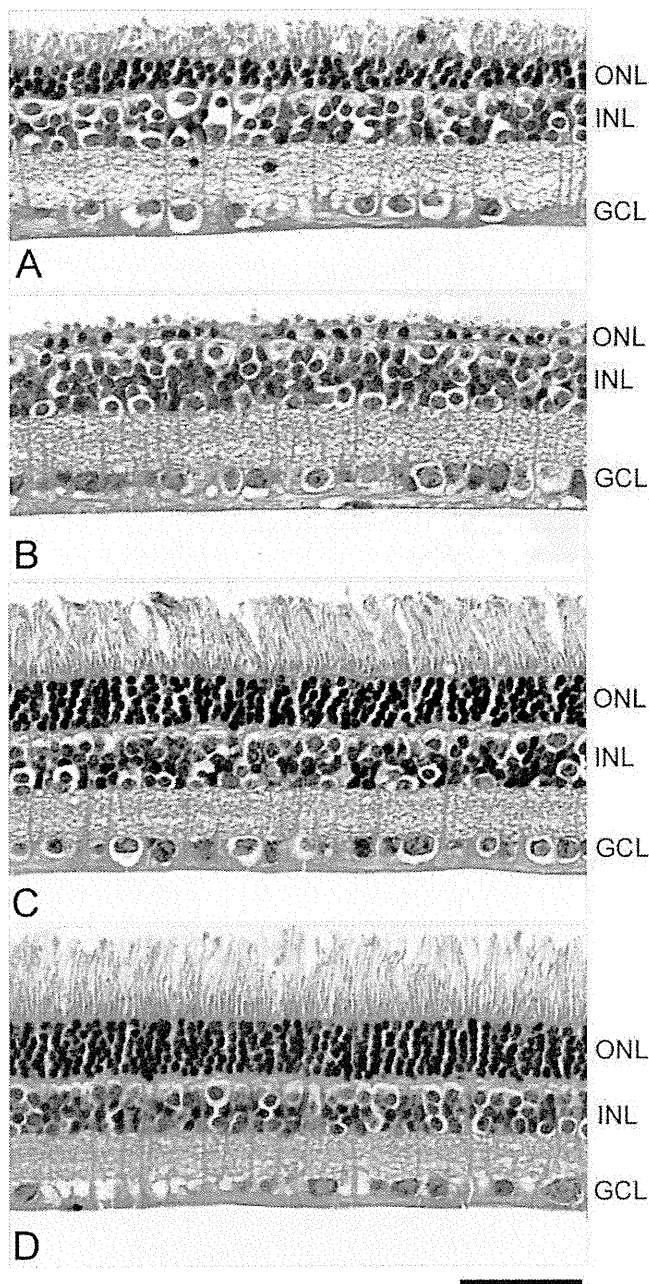
FIGURE 1. Photomicrographs of TES-treated and sham-stimulated retinas from 12-week-old Tg rabbits. Retinal sections of the visual streak from TES-treated retina (A) and sham-stimulated retina (B). Peripheral retinas at 6 mm superior to the optic nerve head from TES-treated retina (C) and sham-stimulated retina (D). Scale bar = 50 μm.

(mean ± SD, $n = 5$) which was significantly thicker than that in the sham-stimulated eyes (8.8 ± 2.8 μm, $n = 5$, $P < 0.05$) (inferior hemisphere 1). In contrast, there was no significant difference in the mean ONL thickness outside the area of the visual streak (Fig. 2A). Thus, TES promoted the survival of photoreceptors in the area of the visual streak at 12 weeks of age.

To determine whether TES affected other layers of the retina, we measured the thickness of the INL and GCL. There were no significant differences of the mean thickness of INL and GCL between the TES retinas and in the sham retinas ($n = 5$ each; Figs. 2B, 2C).
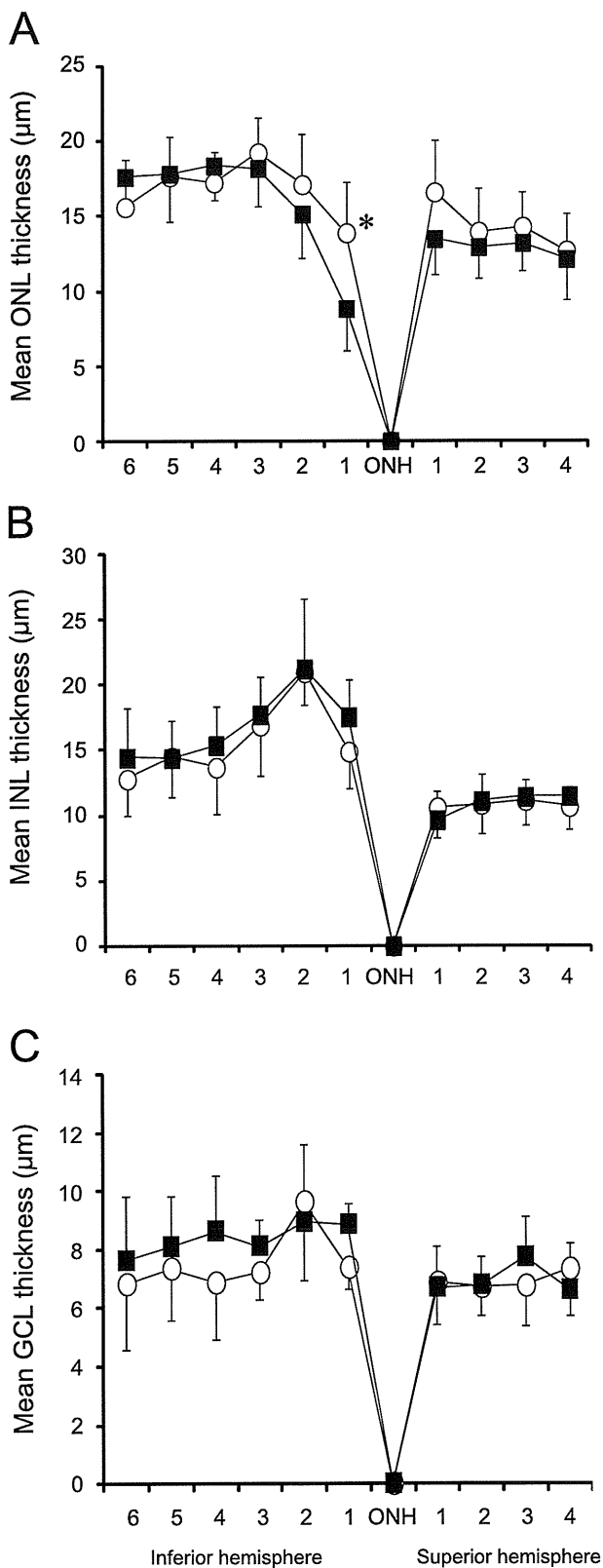

FIGURE 2. Thickness of the ONL (A), the INL (B), and the GCL (C) along the vertical meridian measured at 10 retinal locations at 2-mm intervals. Mean ± SD of five Tg rabbits are plotted. There was a significant difference of the mean ONL thickness between TES-treated retinas (○) and sham-stimulated retina (■) at the visual streak (Student's t-test for two groups; $^*P < 0.05$).
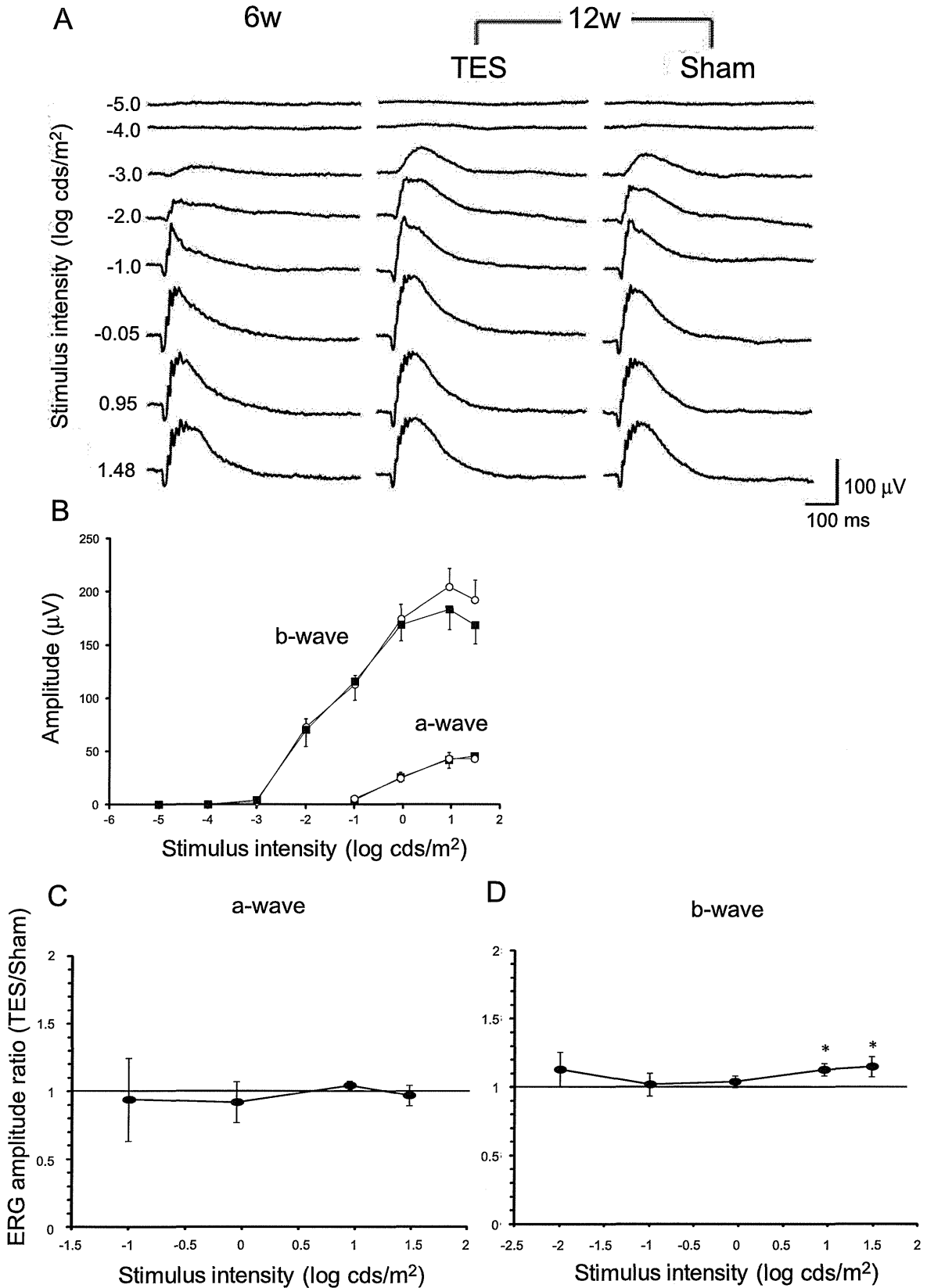
FIGURE 3. Scotopic ERGs recorded from 6- and 12-week-old rhodopsin P347L Tg rabbits. (A) Scotopic ERGs elicited by eight different stimulus intensities. (B) Scotopic ERG mean amplitude versus flash intensity for the a- and b-waves in the TES-treated (○) and sham-stimulated eyes (■) ($n =$ 5, each, mean ± SEM). Average ratio (TES/sham) of the a- (C) and b-wave (D) amplitudes at 12 weeks of age ($n = 5$, each, mean ± SEM). Pointwise comparison indicated a significant difference in b-wave amplitudes at 1.48 and 0.95 log cd·s/m² (Student's *t*-tests for two groups; *$P < 0.05$).