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Long-term Vision Rescue by Human Neural Progenitors in a Rat Model of Photoreceptor Degeneration

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Abstract

Purpose—As a follow-up to previous studies showing that human cortical neural progenitor cells (hNPC^{ctx}) can sustain vision for at least 70 days after injection into the subretinal space of Royal College of Surgeons (RCS) rats, the authors examined how functional rescue is preserved over long periods and how this relates to retinal integrity and donor cell survival.

Methods—Pigmented dystrophic RCS rats ($n = 15$) received unilateral subretinal injections of hNPC^{ctx} at postnatal day (P) 21; control rats ($n = 10$) received medium alone and were untreated. All animals were maintained on oral cyclosporine A. Function was monitored serially by measuring acuity (using an optomotor test) and luminance thresholds (recording from the superior colliculus) at approximately P90, P150, and P280. Eyes were processed for histologic study after functional tests.

Results—Acuity and luminance thresholds were significantly better in hNPC^{ctx}-treated animals than in controls ($P < 0.001$) at all time points studied. Acuity was greater than 90%, 82%, and 37% of normal at P90, P150, and P270, whereas luminance thresholds in the area of best rescue remained similar the whole time. Histologic studies revealed substantial photoreceptor rescue, even up to P280, despite progressive deterioration in rod and cone morphology. Donor cells were still present at P280, and no sign of donor cell overgrowth was seen.

Conclusions—Long-term rescue of function and associated morphologic substrates was seen, together with donor cell survival even in the xenograft paradigm. This is encouraging when exploring further the potential for the application of hNPC^{ctx} in treating retinal disease. (*Invest Ophthalmol Vis Sci.* 2008;49: 3201–3206)

The photoreceptor degeneration seen in age-related macular degeneration (AMD) and retinitis pigmentosa (RP) represents the major cause of blindness in developed countries, for which no cure is available.^{1–3} Cell-based therapies have been shown effective in rescuing vision in animal models of retinal degeneration.^{4–9} For donor cells to be suitable in a clinical setting they should be human derived, must be effective in reversing or slowing the

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degenerative events, must be readily renewable and not senescent, and must be effective over a long period.

In a series of studies, we have used the Royal College of Surgeons (RCS) rat to explore the efficacy of cell-based therapies in rescuing vision. In this animal, the retinal pigment epithelial cell (RPE) fails to phagocytose shed outer segment material at a normal rate because of a mutation in the *Mertk* gene.¹⁰ This results in an accumulation of outer segment debris and subsequently leads to photoreceptor cell loss.^{11,12} Although comparable defects have been seen in a cohort of patients with retinitis pigmentosa,¹³ the animal does serve to some degree as a model for photoreceptor loss caused by defective or dysfunctional RPE and for retinitis pigmentosa in general.

In previous work,¹⁴ we showed that human cortical neural progenitor cells (hNPC^{ctx}) rescued vision at near normal levels when injected into the subretinal space of the RCS rat. Some-what unexpectedly, they formed an RPE-like layer between photoreceptors and the host RPE layer and migrated to the retina. Given the potential for such cells to provide a therapy for degenerative diseases throughout the central nervous system,¹⁵ including the retina, we felt it important to evaluate the long-term effects of grafting. Critical questions that remained centered on the duration of cell survival and vision rescue and on the long-term behavior of and host response to the transplanted cells. To answer these questions, we observed a new group of rats that underwent transplantation at postnatal day (P) 21; functional tests were conducted at approximately P90, P150, and P280. A crucial part of the study was to observe the performance of individual rats at three times. Some rats were observed separately for validation of the previous morphologic results at the earlier times and to provide a context for the later data.

Materials and Methods

Pigmented dystrophic RCS rats ($n = 15$) received unilateral subretinal injections of human neural progenitor cells derived from prenatal cortical tissue (hNPC^{ctx}) at P21, and control rats ($n = 10$) received medium alone. The other eye provided untreated controls. All animals were maintained on cyclosporine A (CyA; Novartis, Basel, Switzerland), administered in the drinking water (210 mg/L; resultant blood concentration, approximately 300 $\mu\text{g/L}$)¹⁶ from 1 day before transplantation until they were killed. These studies were conducted with the approval and under the supervision of the Institutional Animal Care Committee at the University of Utah; all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Transplantation

Human fetal cortex was dissected from postmortem brain tissue at 21 weeks of gestation. The method of collection conformed to the National Institutes of Health guidelines for the collection of such tissues and the institutional review board requirements for the University of Wisconsin. Human cortical neural progenitor cells (hNPC^{ctx}) were cultured as neurospheres and passaged by chopping. After 4 weeks in culture, FGF-2, heparin, and B27 were removed, and N2 (1:100; Invitrogen, Carlsbad, CA) was added. After 10 passages (10–15 weeks in culture), 10 ng/mL leukemia inhibitory factor (Chemicon, Temecula, CA) was also added (for details, see Gamm et al.¹⁴). hNPC^{ctx} neurospheres were washed and dissociated into cell suspension; 2×10^4 cells in 3 μL DMEM/F12 (Invitrogen) carrying medium were delivered into the subretinal space through a small scleral incision using a fine glass pipette (internal diameter, 75–150 μm) attached by tubing to a 25- μL Hamilton syringe. The cornea was punctured to reduce the intraocular pressure and to limit the efflux of cells. The sham-operated group was treated similarly except that carrying medium alone was injected. Immediately after injection, the fundus was examined to check for retinal

damage or signs of vascular distress. Any animals showing such problems were removed from the study and were not included in the animal counts.

Trypan blue dye exclusion was performed on cell suspensions before and immediately after transplantation and revealed greater than 95% and 90% cell survival, respectively.

Spatial Resolution Records

Animals were tested at P90, P150, and P270 using an optometry testing apparatus (Cerebra Mechanics, Lethbridge, CA)¹⁷ composed of a rotating cylinder displaying a vertical sine wave grating presented in virtual three-dimensional space on four computer monitors arranged in a square. Rats standing unrestrained on a platform in the center of the square tracked the grating with reflexive head movements. The center of the virtual cylinder was clamped at the viewing position by repeated recentering on the head of the test subject. Acuity was quantified by increasing the spatial frequency of the grating until an optomotor response could no longer be elicited.

Luminance Threshold Responses

To assess luminance thresholds, single and multiunit activity in the superficial layers of the superior colliculus (SC) was recorded with a modified version of a procedure we developed in previous work.¹⁸ Recordings were made from the superficial layers of the SC to a depth of 100 to 300 μm using glass-coated tungsten electrodes (resistance, 0.5 M Ω ; bandpass, 500 Hz–5 kHz). Small craniotomies of approximately 100 μm in diameter were made to access the brain. Such small holes were necessary for the serial recordings made here to be effective. A uniform background of 0.2 to 0.4 log units was sustained on a hemisphere with an opaque surface. The brightness of a spot projected onto the hemisphere was systematically reduced using neutral density filters (minimal steps of 0.1 log unit) until a response was obtained that was double that of the background activity. This gave the threshold level for that point on the visual field, calculated as 5.2 log units minus the value of the filter. Sixteen to 20 positions were recorded. All rats were tested at P90, but three were recorded at P106, P160, and P280 to see functional changes in single animals with time.

Histology

At the end of testing, rats were overdosed with sodium pentobarbital (Sigma) and perfused with phosphate-buffered saline (PBS). The eyes were removed and immersed in 2% paraformaldehyde for 1 hour. Eyes were infiltrated with sucrose and embedded in optimum cutting temperature compound, and horizontal sections (10 μm) were cut on a cryostat. Sections were stained with cresyl violet for assessment of the injection site and retinal lamination and with antibodies, according to previous protocols.⁵ Retinal sections were examined by regular and confocal microscopy.

Results

Functional Assessments

Spatial Frequency—At P90, the visual threshold measured from eyes receiving hNPC^{ctx} ($n = 15$) injections gave a figure of 0.54 ± 0.04 cyc/deg (90% of normal value, 0.6 cyc/deg), significantly better than in sham-operated eyes (0.27 ± 0.03 cyc/deg; $n = 10$) and untreated control eyes (0.16 ± 0.05 cyc/deg; $n = 10$). Even at P150, when sham and untreated controls were no longer responsive to the optomotor stimuli, hNPC^{ctx}-injected rats still gave a figure of 0.49 ± 0.05 cyc/deg (82% of normal value; Fig. 1A). From P150, the visual threshold dropped gradually, and a figure of 0.22 ± 0.02 cyc/deg was recorded at P270 (37% of

normal; Fig. 1B). Further analysis revealed that the visual threshold of cell-injected eyes even at P270 was still significantly better than untreated dystrophic eyes measured at P90 (0.16 ± 0.05 cyc/deg), indicating that hNPC^{ctx} cells delayed the progress of vision loss for more than 6 months.

Luminance Threshold Testing—Luminance threshold recordings from the SC measure functional sensitivity across the visual field, which in turn provides a geographic indication of the magnitude and area of rescue across the retina. In dystrophic RCS rats, threshold levels at approximately P90 were greater than 3.0 log units above the background level of 0.02 log candela (cd)/m², whereas in nondystrophic rats the threshold was less than 0.6 log units above background. For this study, rats selected ($n = 8$) according to optomotor test results were recorded at P90; cell-injected eyes performed significantly better than untreated eyes ($n = 6$) or eyes given sham injections ($n = 6$; Fig. 1C). Specifically, nearly 80% of the SC area of cell-injected eyes produced thresholds lower than 2 log units, with best test points falling within the normal, nondystrophic range. This is in contrast to sham and untreated controls, in which 0% of the SC area gave figures better than 2 log units. It should be noted, however, that because of the selection procedure, luminance results gave best-case performances, as opposed to the acuity tests in which all rats were measured.

Three rats with cell injection were subsequently recorded at P106, P160, and P280, and the luminance thresholds were presented in Figure 1D. Thresholds across the range recorded were significantly better at P106 than at P160; more than 60% of the SC area gave thresholds lower than 1.5 log units at P106 compared with 28% of the SC area at P160. However, at the lower threshold range of better than 1.2 log units, they showed no difference, and this was sustained to P280, when the overall threshold curve at P280 and P160 was essentially identical with that at P160. Records of an individual animal from this group over the three time points attest to the sustained performance (Fig. 1D).

Morphologic Examination

General Retinal Organization—Retinas from hNPC^{ctx}-injected, sham-operated, and nonoperated controls were examined in cresyl violet–stained sections. At P90 (Fig. 2A), a data point not examined in our previous study, a well-preserved outer nuclear layer (ONL), 9 to 10 cells deep with an almost diminished debris zone, was present in the hNPC^{ctx}-protected area, gradually reduced to one cell thick outside the graft; the ectopic RPE-like layer seen previously at P150 was already present (Fig. 2A, arrows). By contrast, the ONL was reduced to a single layer in untreated eyes (Fig. 2B) and to a layer of two cells immediately adjacent to the injection site in sham-operated eyes. Even at P150, the ONL was still 8 to 10 cells deep in the graft-protected area (Fig 2C), as was seen previously. Notably the inner retina showed no abnormalities, but distant from the graft, as in untreated or sham controls, only scattered photoreceptors were left, and the secondary pathologic changes described previously, including laminar irregularities in the INL, and invasion of RPE cells into the retina and associated abnormal vessels, were becoming evident (Fig. 2D). At P280, the ONL was reduced to three to four cells deep (Fig 2E), but the inner retina was still well organized. Distant from the graft (Fig. 2F), as in untreated retinas, the secondary pathologic changes described above were more prominent than at P150.

Antibody Staining—The human-specific nuclear marker MAB1281 was used to examine donor cell distribution and survival. The donor cells were found in two distinct locations, as previously reported,¹⁴ as a separate, nearly continuous sub-retinal layer between the host RPE and photoreceptors and containing pigment granules (Figs. 2G, 2I) and as individual nonpigmented cells distributed throughout the inner retina (Figs. 2G, 2H). However, at P280, there was a dramatic reduction in the number of donor cells: the RPE-like layer was

no longer seen, but a clump of cells around the injection site was still positively stained with MAB1281 (data not shown). Although cells were still distributed within the inner retina, they were also greatly reduced in number (Fig. 2H). In the area with the highest density of positive nuclear marker staining, 20 cells/300 μm were counted at P150 against 8 cells/300 μm at P280.

Rhodopsin—In dystrophic RCS retinas, the outer segments and debris zone were rhodopsin positive. Even at P150, rhodopsin staining was restricted to the outer segment area in the graft-protected retina (Fig. 3A, down arrows), and rod bipolar cells had fairly normal dendrites (Fig. 3A, up arrows). In contrast, there was some rhodopsin-positive material in the untreated retina, and the dendrites of rod bipolar cells underwent sprouting at the same time (Fig. 3B). At P280, cell bodies and outer segment material were all stained with rhodopsin (Fig. 3D), and some rod bipolar cells lost their dendrites. In the untreated retina, there was no rhodopsin-positive staining, and rod bipolar cells were distorted (Fig. 3E, left arrows).

Cone Arrestin—Cone antibody arrestin reveals the whole profile of cone photoreceptors. In the graft-protected area, cone density was similar to that of nondystrophic rats at P150 (30 cells/300 μm ; Fig. 3C), and structural elements of rescued cones were well preserved, though cone processes were clearly shorter than normal and were poorly organized. Cone photoreceptors underwent further abnormal morphologic changes with time so that at P280, the outer segments were no longer lined up, and they were less clearly delineated even in graft-protected areas (Fig. 3F). In sham-operated and untreated eyes at P280, cone photoreceptors were essentially absent (Fig. 3G).

Donor Cell Identity—To study whether donor cells, especially those migrating to the inner retina, expressed retinal cell markers, sections with grafts were double-stained with human nuclear marker and retinal cell antibodies, as follows: anti-recoverin for photoreceptors and ON cone bipolar cells, anti-PKC α for rod bipolar cells, anti-parvalbumin for AII amacrine cells, anti-calbindin for horizontal cells, and anti-calretinin for amacrine cells. No double-labeled cells were found (data not shown).

Proliferating Cell Nuclear Antigen—To examine whether donor cells continued to divide as shown in our previous study,¹⁴ antibody against human proliferating cell nuclear antigen (PCNA) was used, along with sections from P150, as positive controls. There were no PCNA-positive cells in sections from P280, whereas sections from P150 gave consistently positive staining. Therefore, though donor cells do continue to divide up to at least P150, this capability is lost by P280, by which time there is also a considerable reduction in donor cell numbers.

Human-Specific Nestin—Our previous study showed that transplanted hNPC^{ctx} cells continued to express nestin for 5 months. Here we found that hNPC^{ctx} cells still stained positively for human nestin at P280, indicating that hNPC^{ctx} cells remained undifferentiated throughout this prolonged period in vivo.

Discussion

The present study enlarges on the previous work in showing that hNPC^{ctx} cells, when injected into the subretinal space of the RCS rat at P21, can sustain visual function for at least 280 days, when normally severe retinal deterioration is encountered^{5,19,20} and rats are visually nonresponsive. The acuity measured by the optomotor test continued undiminished between P90 and P150 and was reduced but still present by P280, when sham-injected animals, like untreated controls, had long become nonresponsive. For luminance threshold

responses, the level of best performance was unchanged between P90 and P280, though there was a decrease in the area adjacent to that of maximum sensitivity

In this study, in contrast to previous work using this methodology to assess the effects of retinal degeneration and rescue, individual animals were followed up longitudinally over three extended time points. These results show that for each individual, visual function changed surprisingly little over time. Morphologically, the overall thickness of the outer nuclear layer showed little change at P150 compared with P90, but the layer was reduced by more than 50% at P280. Given that the functional tests used here are likely to emphasize cone function,¹⁸ we also examined the morphology and presence of cone and rod photoreceptors. Cone photoreceptors showed some deterioration over this period, with reductions in the length of outer segments, but cone density in the best rescued area at P150 remained similar. However, between P150 and P280, cones became significantly distorted such that accurate counts could not be obtained. Rhodopsin staining patterns also showed significant changes, such as cell body staining, in the same period. One important observation here was that although the outer retina showed considerable diminution in thickness by P280, the inner retina did not manifest the extreme changes normally seen by this age in RCS rats,^{5,19,20} among them laminar disarray, vascular abnormality, abnormal migration of RPE cells, and retinal neurons. This raises the possibility that an intact inner retina is critical for the long-term function of resilient photoreceptors that, though disrupted, retain the capacity to detect light and relay visual information in late-stage disease. In this scenario, the long-term rescue effects of hNPCctx on inner retinal cells and circuitry may be at least as important for the preservation of visual function as the direct rescue effects of hNPCctx on the photoreceptors themselves. The fact that only a small number of functional photo-receptors is required to significantly impact vision was shown by MacLaren et al.,⁶ who noted the restoration of certain visual functions after the integration of only a few hundred photoreceptors.

The morphologic change in general correlated well with the optomotor results in that there was sustained morphologic normality and optomotor activity up to P150 but reduction by P280. The area with low threshold response in the SC correlated with the best rescue area in the retina, and the threshold responses increased gradually outside the most light-sensitive area. The continued maintenance of luminance threshold responses is surprising because in a previous study,²¹ it was found that a perturbation that caused a small change in the length of outer segments led to a corresponding reduction in luminance thresholds. The deterioration in morphology at the longest time point was not reflected in the luminance responses even of individual animals, where it had not changed significantly between P160 and P280. Whether this reflected changes in gain in the inner retina or the central visual structures or changes in patterns of circuitry—including the possibility that the remaining rods contributed to the response drive in an anomalous way through new pathways—remains to be seen. Furthermore, the different responses of the two functional measures over time might have reflected the likelihood that they are driven by different neural pathways. The results emphasize at least that even with a poorly ordered outer retina, a remarkably good level of vision can be sustained.

Finally, donor cells continued to be present in considerable numbers up to P150 as a sheet and appeared as individual cells embedded in the retina. By P280, the numbers of cells had diminished considerably, and the typical sheet of RPE-like cells was no longer evident. Although PCNA staining showed that the donor cells continued to divide up to at least P150, such cell division was no longer seen at P280. It should be noted that while cell division continued after transplantation, it did not lead to unregulated growth or tumor-like formations. However, the sustained performance over the prolonged period of this study might have suggested that cell renewal was continuing and that cells lost for various reasons

were actually replaced until advanced ages. One possible reason for the cell loss was that the immunosuppressive therapy, oral cyclosporine, was inadequate or inappropriate for xenografting as used here. Other studies investigating survival without immunosuppressive therapy in syngeneic and allogeneic grafts^{8,22} have shown that photoreceptor survival and visual function deteriorate with time in allogeneic grafts whereas syngeneic grafts allow good photoreceptor survival and sustained visual function. Noticeably, in a number of studies, even when donor cells are lost, there is no indication of a florid immune response. The immune-provocative nature of the cortex-derived neural progenitor cells has not been explored systematically.

The longevity of effect in the present studies certainly point to the value of exploring further the potential for hNPC^{ctx} cells in treating retinal disease. The mechanism by which hNPC^{ctx} rescue vision is unclear. Although hNPC^{ctx} form an extra RPE-like layer, they do not express characteristic RPE antigens, such as RPE65 and bestrophin. One important observation is that the almost diminished debris zone in the grafted protected area—whether the donor cells took on some of the RPE function or made defected host RPE cells work better, or both—warrants further study for an examination of phagocytes in donor and host RPE cells. With respect to factor production, which is at least in part the mechanism by which hNPC^{ctx} rescue vision, a previous study¹⁴ has detailed at least two factors hNPC^{ctx} produce in vitro that may be effective in promoting photoreceptor rescue. In the central nervous system, unmodified hNPC^{ctx} had no effect on host neuron survival and function, but cells transfected to produce glial cell–derived neurotrophic factor are effective in sustaining function.^{23,24} Clearly, region-specific and possibly disease-specific factors influence the host response to this versatile cell type; it may be that a particular growth factor specificity comes into play. Previous analysis¹⁴ showed that nontransfected cells produced such molecules as insulin-like growth factor type I and fibroblast growth factor type II; it is possible that these and other factors thus far not isolated sustain the retinal cells.

These results indicate that transplanted hNPC^{ctx} survive and delay the progression of vision loss for more than 8 months after transplantation, along with concomitant rescue of the inner and outer retina. Despite continued donor cell division up to at least P150, there was no evidence of untoward growth or tumor formation even at P280. Although deterioration occurred in morphologic appearance with time, the luminance threshold response showed little change during the time studied, even when individual rats were studied longitudinally. However, there was some reduction between P150 and P280 in the acuity response measured here, though the deterioration in cone morphology seemed more severe than would be predicted from the behavioral measures. The loss of donor cells and the concomitant morphologic changes at the longest time point may be a product of the immune mismatch in this xenograft.

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Figure 1.

Spatial resolution (**A**) as measured by the optomotor test at P90 showed that the hNPC^{ctx}-treated eyes ($n = 15$) performed significantly better than sham ($n = 10$) and untreated ($n = 10$) eyes ($P < 0.001$; t -test). At P150, when sham and untreated eyes were no longer responsive, hNPC^{ctx}-treated eyes still gave visual thresholds close to 0.5 cyc/deg. (**B**) Optomotor responses were tested at three time points on the same rats (cells, $n = 6$; sham, $n = 3$; untreated, $n = 3$) showing functional deterioration with time. Even at P270, however, the visual threshold (0.2 cyc/deg) was still better than in the untreated eye at P90 (0.16 cyc/deg). (**C**) Luminance threshold responses recorded across the SC at P90 (cells, $n = 8$), compared with sham ($n = 6$) and untreated controls ($n = 6$). Each curve (mean $\pm$ SEM) shows the percentage of retinal area (y -axis), where the visual threshold is equal to or less than the corresponding value on the x -axis (log units, relative to background illumination, 0.02 cd/m²). *Points at which the curves for grafted and control eyes are statistically different ($P < 0.05$; t -test). (**D**) Luminance threshold responses recorded on the same rat (cells, $n = 3$; sham, $n = 3$) at three time points: P106, P160, and P280. Again, each curve (mean $\pm$ SEM) was presented. Although there are clearly morphologic changes between P150 and P280, the luminance threshold was hardly changed; the two curves run almost parallel.

Figure 2. (A–F) RCS retinas with hNPC^{ctx} grafts (A, C, E, I) and untreated (B, D, F) at three time points. At P90, the ONL was 9 to 10 cells deep in hNPC^{ctx}-grafted retina compared with a single layer of ONL in untreated retina (compare A and B). There was a layer of cells (RPE-L; A, arrows) between host RPE layer and ONL in hNPC^{ctx}-grafted retina. At P150, the hNPC^{ctx}-grafted retina was 8 to 10 cells deep with ONL, and the RPE-L was still evident (C, arrows), whereas in the untreated RCS retina, almost no photoreceptor remained, host RPE cells migrated to the inner retina (D, arrows), and the INL became uneven. At P280, the ONL was reduced to 3 to 4 cells deep in the hNPC^{ctx}-grafted area, and retinal lamination remained orderly (E). In the untreated retina, typical secondary abnormalities including RPE cells (F, right arrows) and inner retinal neurons (F, left arrow) migrating along blood vessels were clearly evident. (G–I) Human nuclear marker staining showing hNPC^{ctx} at P150 (G, I) and P280 (H). Donor cells formed an RPE-L (G, up arrows) layer and distributed within the inner retina (left arrows). (I) High-power image taken from hNPC^{ctx}-grafted retina at P150. Up arrows: hNPC^{ctx}-positive for antinuclear marker. Left arrows: pigment granules. Scale bars, 20 μ m (A–H); 5 μ m (I).

Figure 3.

Confocal images of RCS retinas. (A) hNPC^{ctx}-grafted retina stained with rhodopsin (red) and PKC α (green) at P150. *Down arrows*: confined rhodopsin-positive staining. *Up arrows*: bipolar cell dendrites. In untreated retina, rhodopsin-positive material was mixed with sprouting dendrites of bipolar cells (B, *arrows*). (D) At P280, rhodopsin-stained cell body (ONL) and outer segment materials (D, *up arrows*) in hNPC^{ctx}-grafted retina. Dendrites of bipolar cells were also reduced. In untreated retina (E), there was no rhodopsin staining, and bipolar cells formed lumps and were disorganized (*left arrows*). (C, F, G) Cone arrestin staining at P150 (C) and P280 (F, G). At P150, cone segments were shorter than normal (data not shown), and cone density in the best-rescued area was similar to normal. Dramatic morphologic deterioration was observed at P280. Cone axons were shorter, and segments were even shorter and more disorganized (F, *arrow*) than at P150. In untreated retina, there was no recognizable cone morphology (G, *arrows*). Scale bars, 20 μ m.