Engraftment of Adult Neural Progenitor Cells Transplanted to Rat Retina Injured by Transient Ischemia

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PURPOSE. To optimize delivery parameters for achieving engraftment, migration, and differentiation of adult neural progenitor cells transplanted to the retinas of rats after transient retinal ischemia.

METHODS. Retinal ischemia was induced by transiently raising the intraocular pressure. Some animals then received transplantation of green fluorescent protein (GFP)-expressing cells derived from the adult rat hippocampus and were allowed to recover for 6 hours to 9 weeks. Retinal cryosections were prepared for TUNEL analysis to determine the time course of ischemia-induced cell death, and some sections were prepared for immunohistochemistry for retinal neuronal antigens.

RESULTS. TUNEL analysis revealed that ischemia-induced cell death peaked at 24 hours. By 96 hours, the inner nuclear (INL) and ganglion cell (GCL) layers were largely obliterated in the central retina, sparing peripheral regions. By 2 weeks after transplantation, numerous GFP-expressing cells had engrafted into the host retina, migrated to the inner retina, and extended processes. At 4 weeks, many GFP-labeled cells were present throughout the INL and displayed horizontal-, bipolar-, and amacrine cell-like morphologies. GFP-expressing cells were also present in the GCL with fibers extending into the nerve fiber layer. At 5 weeks, many GFP-expressing cells were present at the optic nerve head, and some GFP-labeled fibers were present in the optic nerve, occasionally passing through the full extent of the lamina cribrosa. Only rarely were GFP-expressing cells found that coexisted retinal phenotypic markers at any time point examined.

CONCLUSIONS. Adult hippocampus-derived neural progenitor cells transplanted to the subretinal space readily engrafted into a host retina that has undergone ischemic injury. Many cells migrate to specific retinal cellular layers and undergo limited morphologic differentiation reminiscent of retinal neurons, including extension of processes into the optic nerve. Concurrent control studies demonstrate that optimal engraftment is achieved by subretinal delivery within a specific temporal window. These results imply that certain inductive cues may be regulated after injury, and they demonstrate the potential for adult neural progenitor cell transplantation for the treatment of retinal neurodegenerative diseases. (Invest Ophthalmol Vis Sci. 2003;44:5194–5201) DOI:10.1167/iovs.02-04075

Neurodegenerative diseases of the inner retina are major causes of blindness worldwide. These include the glaucomas, which primarily affect retinal ganglion cells, and ischemic retinopathies (including diabetic and hypertensive retinopathies) that affect other populations of inner retinal neurons as well.1,2 Current therapies for inner retinal degenerations are largely retardant and are not sufficient to restore visual function after severe impairment of inner retinal circuitry.

Numerous neuroprotective strategies have been used in an effort to prolong the survival of inner retinal neurons damaged in rodent models of inner retinal degeneration.3–13 However, many neurodegenerative diseases of the inner retina, such as primary open-angle glaucoma (POAG) are quite protracted, extending over many months to years. Experimental models for these diseases have, by necessity, compressed this time course dramatically. The mechanisms of ganglion cell death (and potential neuroprotection) have been examined extensively in models of optic nerve transection or crush,14–17 and in models using either acute ischemia followed by reperfusion18–20 or chronic elevation of intraocular pressure (IOP).21–24 Therapeutic strategies in these models have included delivery of growth factors or cytokines to retard ganglion cell loss5–10 or transplantation of retinal or optic nerve glial cells to enhance optic nerve axon regrowth or myelination.12,13 These experimental models also often result in degeneration of cells in the inner nuclear layer (INL). However, preservation of these cells and inner retinal circuitry is still problematic.

Recent advances in stem cell biology have invigorated the potential for achieving partial restoration of visual function after inner retinal neurodegeneration by augmenting the remaining inner retinal circuitry. It has been demonstrated that neural progenitor cells can be isolated from near the ventricular wall of the adult brain, as well as from the adult hippocampus,25–30 Retinal neural progenitor cells have also been isolated from embryonic retina31 and from the adult pigmented ciliary margin.32–35 Recent studies on transplantation of cultured retinal progenitor cells have yielded varied results. Trister et al.34 found that two distinct quail neuroretinal cell lines (one amacrine/ganglion cell; the other, Müller cell), injected into the vitreous chamber of embryonic chicks, became incorporated into the developing retina, where they migrated to the appropriate layer and extended processes. In contrast, Chacko et al.31 showed that transplantation of embryonic rat retinal progenitor cells into the subretinal space of 2-week-old rats resulted in survival of donor cells at the site of the transplant and the expression of a photoreceptor-specific antigen (RET-1), but failure to integrate into the host retina. However, transplantation of neural progenitor cells derived from the adult hippocampus into the vitreous cavity of neonatal rats resulted in only limited engraftment and phenotypic morphologic differentiation.35,36 Recently, cells from a similar source were transplanted into the vitreous cavities of both normal adult rats and rats with mechanical injury to the retina.37 After 4 weeks, no cells had become engrafted into the normal retinas, whereas in injured retinas many donor cells displayed cytoskeletal characteristics of neurons and glia (microtubule associated proteins, (Invest Ophthalmol Vis Sci. 2003;44:5194–5201) DOI:10.1167/iovs.02-04075

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MAP2ab and MAP5, and glial fibrillary acidic protein, GFAP), respectively. Expression of mature retinal phenotypic markers was not observed, but there were some indications of synaptic-like contact between some donor and host cells. More recently, it has been demonstrated that cells derived from the adult rat iris can be induced to display immunoreactivity for the 200-kDa neural filament (NFP-200) protein in vitro. Furthermore, these cells can acquire a photoreceptor phenotype (i.e., rhodopsin expression) when infected with a recombinant viral gene construct expressing the homeodomain transcription factor, Crx.

Overall, an implication of these studies is that expression of certain inductive cues (e.g., the elaboration of specific tropic and trophic factors) may be recapitulated after injury and perhaps in neurodegenerative diseases. Herein, in an effort to gain a better understanding of the behavior of transplanted neural progenitor cells, we used a model of transient ischemia-reperfusion and transplantation of neural progenitor cells derived from the adult hippocampus.

Materials

Preparation of Neural Stem Cells for Grafting

For these studies, we used a well-characterized source of neuronal progenitor cells derived from the adult rat hippocampus and expressing green fluorescent protein (GFP; a generous gift from Fred H. Gage). The cells were cultured on laminin/polyornithine–coated flask containing Dulbecco’s modified Eagle’s medium-Ham’s F12 (DMEM-F12) supplemented with N2 and 20 ng/ml basic-fibroblast growth factor (bFGF) and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. The culture media were changed every 2 to 3 days. The cells were passaged when they reached 80% to 90% confluence. Before transplantation, the cells were trypsinized with 0.05% trypsin-EDTA, washed with DMEM-F12, and suspended at a density of 3 to 7.5 × 10⁴ cells/μL in high-glucose Dulbecco’s phosphate-buffered saline (PBS).

Retinal Ischemia by Transient Elevation of IOP

All procedures for the care and handling of animals were approved by Duke University Institutional Animal Care and Use Committee and are in accordance with the guidelines established by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

To induce retinal ischemia, we used modifications of a previously established technique. Eight to 12-week-old female Fischer 344 rats weighing 180 to 200 g were anesthetized with a mixture of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (5 mg/kg) administered intramuscularly. The corneas were anesthetized with topical application of 0.5% proparacaine hydrochloride, and the pupils were dilated with 2.5% phenylephrine eye drops. The anterior chamber of the left eye was cannulated gently through the cornea near the corneoscleral limbus with a 30-gauge needle connected to a reservoir of lactated Ringer’s solution by intravenous (IV) drip line under visualization with an ophthalmic microscope. The IV line was then opened, and IOP was raised to 110 mm Hg for 60 to 90 minutes by elevating the reservoir. Retinal ischemia was confirmed by direct microscopic observation of the total occlusion of retinal vessels and subsequent whitening of the retina. The whitening of the iris and total occlusion of iris vessels were additional indirect signs. At the end of the procedure, the needle was gently withdrawn from the anterior chamber, and reperfusion was visually confirmed. The animals were then prepared for subretinal transplantation of neural progenitor cells.

Subretinal Transplantation of Neural Progenitor Cells

Dissociated neural progenitor cells were transplanted into the subretinal space immediately after transient ischemia (unless otherwise described), using a transscleral approach as previously described, with a few modifications. Briefly, under ophthalmic microscopic observation, the temporal conjunctiva was dissected at the limbus to expose the sclera. A sclerotomy was performed tangentially between the two varicose veins approximately 1 mm beyond the limbus and directed toward the posterior pole with a 30-gauge needle. A 30-gauge blunt needle attached to a 10-μL syringe (Hamilton, Reno, NV) was introduced tangentially through the sclerotomy site in the subretinal region, (paraformaldehyde for an additional 2 hours, washed with PBS, and transferred to 30% sucrose in PBS overnight before cryosectioning. Retinal sections (7 μm) were mounted onto clean, subbed slides and stored at −20°C until processing.

Some eyecups were postfixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde overnight, followed by dehydration through a graded series of ethanol. Then the eyecups were embedded in Spurr’s low-viscosity epoxy resin. Semithin (1 μm) sections were obtained with an ultramicrotome (Leica, Heidelberg, Germany). The sections were attached to glass slides, stained with methylene blue and coverslipped for histologic analysis.

TUNEL Analysis

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP terminal nick-end labeling (TUNEL) assay was performed to detect inter-nucleosomal DNA fragmentation (Fluorescein Apoptosis Detection System; Promega, Madison, WI). Slides were washed two times by immersion in fresh PBS for 5 minutes at room temperature. After incubation with 1 μg/ml protein kinase K (Sigma-Aldrich, St. Louis, MO), the sections were immersed in equilibration buffer (200 mM potassium cacodylate [pH 6.6] at 25°C, 25 mM Tris-HCl [pH 6.6] at 25°C, 0.2 mM dithiothreitol [DTT], 0.25 mg/mL BSA, and 2.5 mM cobalt chloride). TdT incubation buffer was prepared by mixing 45 μL equilibration buffer, 5 μL nucleotide mix (50 μM fluorescein-12-dUTP, 100 μM dATP, 10 mM Tris-HCl [pH 7.6], and 1 mM EDTA), and 1 μL TdT enzyme (25 U/μL) and applied to the sections. Then the sections were covered with paraformaldehyde for an additional 60 minutes inside a humidified chamber protected from direct light. The reactions were terminated by immersing the slides in 2× SSC for 15 minutes at room temperature. The slides were then stained with freshly diluted propidium iodide solution (1 μg/mL) for 5 minutes at ambient temperature in the dark. Finally, slides were mounted with glass coverslips and analyzed immediately under a fluorescence microscope (Optiphot; Nikon, Garden City, NY).
**Histologic Analysis and Immunohistochemistry**

For histologic analysis, cryosectioned slides were washed in PBS and coverslipped with a glycerin-PBS mixture. Some sections were counterstained with propidium iodide to enhance the contrast of the background cellularity.

The primary antibodies used for immunohistochemical studies (and their dilutions) included anti-rhodopsin (a generous gift from Jeremy Nathans, Johns Hopkins University, Baltimore, MD; 1:400), anti-recoverin (a generous gift from James Hurley, University of Washington, Seattle, WA; 1:1000), anti-αPKC (1:10; Amersham, Arlington Heights, IL), anti-calbindin (C-8666, 1:200; Sigma-Aldrich), anti-calretinin (AB149, 1:200; Chemicon, Temecula, CA), anti-parvalbumin (1:1000; Sigma-Aldrich), anti-synaptophysin (1:1000; Sigma-Aldrich), anti-NFP-200 (1:400; Sigma-Aldrich), anti-growth-associated protein (GAP)-43 (1:100; Chemicon, Piscataway, NJ) and anti-GFP (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Detailed procedures for immunohistochemistry have been published elsewhere.\(^{42,43,44}\) Briefly, cryosections from some eyes were washed in several changes of PBS. For immunofluorescence, tissue sections were incubated in primary antibody diluted in 0.1 M PBS + 0.5% Triton X-100 + 10% normal goat serum (NGS) for 12 to 16 hours at 4°C. Sections were then washed in PBS (three times for 10 minutes each) and incubated in the appropriate secondary antibody (1:100–1:200; Jackson ImmunoResearch, West Grove, PA) conjugated to carboxymethylindocyanine (Cy3) for 1 hour at room temperature. Finally, some sections were counterstained with the nuclear stain, 4′, 6′-diamidino-2-phenylindole hydrochloride (DAPI). Slides were then washed thoroughly in PBS and coverslipped with a glycerin-PBS mixture. Sections were analyzed using a microscope equipped with epifluorescence (Optiphot; Nikon) and appropriate filter cubes or a laser-scanning confocal microscope (model 410; Carl Zeiss Meditec, Thornwood, NY).

Alternatively, slides were incubated in H$_2$O$_2$ (150 µL/100 mL) and thoroughly washed in PBS before incubation in GFP antibody. Slides were washed in PBS and transferred to goat anti-rabbit biotinylated secondary antibody (1:50; Jackson ImmunoResearch). After incubation and washing, sections were incubated in an avidin-biotin-peroxidase mixture (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Slides were then incubated in 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) for 10 minutes followed by brief (1–2 minutes) treatment with DAB with 0.03% H$_2$O$_2$ for completion of the chromogen reaction, then washed thoroughly in buffer, dehydrated through graded ethanol to xylene, and coverslipped. Sections were analyzed microscopically (Optiphot; Nikon) with differential interference contrast (DIC) optics.

**RESULTS**

After transient ischemia-reperfusion, we observed gross atrophic changes in retinal morphology by 96 hours after reperfusion. Most damage was confined to the central retina, where both the inner nuclear (INL) and ganglion cell (GCL) layers were largely obliterated (Figs. 1A, 1B). DNA fragmentation assay revealed that TUNEL-positive cells were present 6 hours after reperfusion (not shown). Their number increased by 12 hours (not shown) and sharply peaked at 24 hours, mostly confined to the GCL and INL (Fig. 1C). Likewise, the number of TUNEL-positive cells decreased precipitously by 48 hours (not shown). Notably, however, at 24 hours TUNEL-positive cells, as well as the retina itself, still presented clear lamination. Then, as the number of TUNEL-positive cells decreased, the lamination of the inner retina disappeared. By 96 hours, the INL and GCL were largely obliterated in the central retina, sparing far peripheral regions. These findings implied a specific temporal window for the rescue of dying cells. Thus, the initial time points for transplantation of adult hippocampal stem cells in the present study were grounded on these results.

**Figure 1.** Morphologic changes after ischemia-reperfusion. (A) One-micrometer plastic-embedded section of normal adult rat retina. Note the distinct lamination. (B) The contralateral retina displaying gross atrophic changes in retinal morphology at 96 hours after ischemia. Damage was confined primarily to the central retina, where the boundaries between the inner retinal layers were obscured. (C) TUNEL assay for DNA fragmentation demonstrates that inner retinal cell death peaked at twenty-four hours after reperfusion. TUNEL-positive cells (bright white cells) were mostly confined to the INL and GCL. Scale bars, 50 µm.

**Engraftment, Migration, and Differentiation of Hippocampus-Derived Neural Progenitor Cells**

In eyes transplanted with GFP-expressing neural progenitor cells immediately after 90 minutes of ischemia, as early as 2
weeks after transplantation, widespread incorporation of GFP-expressing cells was detected within the ischemia-damaged retina (Figs. 2A, 2B). GFP-expressing cells were concentrated near the site of delivery with few labeled cells remaining in the subretinal space. Furthermore, many labeled cells had migrated to the inner limiting membrane, where they formed a near-continuous layer adjacent to the GCL, whereas numerous others were observed throughout the INL. At later time points (3–5 weeks), GFP-expressing cells in the INL displayed obvious signs of limited morphologic differentiation. Some cells in the distal INL extended processes laterally into the outer plexiform layer (OPL) and resembled horizontal cells (Fig. 2C). Other cells, in more proximal strata of the INL, extended processes into both the OPL, where they came into close apposition with synaptophysin-immunostained presynaptic elements of the host retina and into the inner plexiform layer (IPL), where they intermingled with immunostained processes (Fig. 2D). Some of these cells displayed morphologies similar to bipolar cells observed in retinas undergoing synaptic remodeling after photoreceptor degeneration. In addition to prominent apically and basally oriented processes, these cells often extended fine lateral processes as well. These lateral processes are believed to be present only transiently during remodeling (Peng Y.-W., personal communication, 2002). Some synaptophysin-immunoreactive profiles in the OPL appeared to contain GFP. These double-labeled terminal processes probably arose from fine, descending processes that were most visible in the ONL (Fig. 2C).

At or near the border of the INL and IPL many GFP-expressing cells displayed amacrines cell–like morphologies, extending processes into, and ramifying in, the IPL (Figs. 2D, 2E). Occasionally, cells with amacrine-like morphology were observed to be “misplaced” in the IPL (Figs. 2F). Finally, by 5 weeks after transplantation, many GFP-expressing cells in the GCL extended processes into the nerve fiber layer (NFL), where they appeared to intertwine with NFP-200–immunostained axons of host retinal ganglion cells (Fig. 2G). In addition, occasional GFP-containing processes were observed in the NFL that were also NFP-200–immunoreactive (Fig. 2H).

In other double-label immunofluorescence studies using markers for specific retinal cellular phenotypes, only rare GFP-expressing cells were observed to express parvalbumin, calcitomin, and calbindin (data not shown). However, transplanted GFP-expressing cells were not generally observed to express retinal phenotypic markers at the time points examined in our model.

Extension of Processes of Engrafted Cells in GCL

The rat anterior optic nerve is divided into three regions: optic nerve head (the neck), the conical transitional zone, and the optic nerve proper. By 5 weeks after transplantation, some GFP-expressing cells had migrated to the optic nerve head, and many GFP-labeled processes were observed streaming into the optic nerve and passing through the neck and transitional zone (Fig. 3A). These fibers, some with apparent growth cones, were intermeshed with host optic nerve axons, as identified by NFP-200 immunoreactivity (Fig. 3B). The presence of GFP-labeled growth cones was confirmed by double-label studies with GAP-43 (Fig. 3C). GAP-43–positive fibers not containing GFP were also observed. These, presumably, arose from cells of the host retina. Occasionally, processes were observed that had penetrated the entire thickness of the dense, multilayered lamina cribrosa and reached the optic nerve proper. These profiles were best demonstrated using an antibody directed to GFP and the avidin-biotin method (Fig. 3D).

Limited Engraftments of Transplanted Neural Progenitor Cells in Control Eyes

Engraftment of transplanted neural progenitor cells was analyzed in three distinct control models: subretinal delivery of cells to the normal retina, intravitreal delivery of cells to the ischemic retina, and subretinal delivery of cells 72 hours or 1 week after ischemia. In control animals (i.e., without ischemia), by 2 weeks most transplanted cells remained in the subretinal space, whereas only a few cells had engrafted into the outer retina near the injection site (Fig. 3E). No GFP-expressing cells were observed in the inner retina. In control animals receiving 90 minutes of ischemia with intravitreal transplants, most GFP-expressing cells remained in the vitreous cavity or lined the outer surface of the inner limiting membrane 2 weeks after transplantation. Only rare GFP-expressing cells were seen within the inner retina (Fig. 3F).

Finally, in animals that received GFP-expressing cells transplanted to the subretinal space either 72 hours or 1 week after 90 minutes of ischemia (time points at which many host inner retinal neurons have been eliminated), only limited incorporation of GFP-expressing cells into the outer retina, but not inner retina, was observed 2 weeks after transplantation (Fig. 3G).

DISCUSSION

Adult Neural Progenitor Cells in a Heterotopic Retinal Environment

Recently, it has been demonstrated that neural progenitor cells from the adult hippocampus retain the potential to develop into functional central nervous system (CNS) neurons when provided a permissive environment. Previous studies have shown that after homotopic transplantation, neural progenitor cells retained the capacity to survive and generate well-differentiated neurons. Subsequently, heterotopic transplantation of adult hippocampus-derived progenitors into the rostral migratory pathway generated mature olfactory bulb neurons. Several studies have now shown that these cells also have the capacity to survive and differentiate in a heterotopic retinal environment.

To demonstrate a similar ability in the developing retina, in preliminary studies we seeded adult neural progenitor cells adjacent to the germinal layer of postnatal day 0 retinal explants and maintained the cultures for up to 2 weeks. After 2 weeks, neural progenitor cells had incorporated into the explants and displayed limited neuronal morphologic differentiation (Rickman DW, Saloupis P, Shaw SJ, Guo Y, ARVO Abstract 3692, 2002). These results provided the basis for the present study.

In this study, we used transient ischemia-reperfusion as a model to determine the extent of engraftment, migration, and differentiation of adult neural progenitor cells in the injured adult retina. Our results demonstrate that these cells have the capacity to engraft into the host retina and migrate to the inner retina, where the injury was localized. Furthermore, engrafted cells extended processes and displayed morphologic differentiation often resembling known retinal phenotypes. GFP-expressing cells in the GCL even extended processes into the NFL and proximal optic nerve. These results demonstrate that heterologous neural progenitor cells are competent to adapt to a new inductive (retinal) environment.

Fate of Engrafted Cells after Transient Retinal Ischemia-Reperfusion

Previous studies have shown that neural progenitor cells can incorporate into injured retina. However, those studies showed the ubiquitous incorporation of the cells into the retina. We observed in our ischemia-reperfusion model that...
FIGURE 2. Engraftment and differentiation of GFP-expressing neural progenitor cells in the ischemic retina. (A) Two weeks after subretinal transplantation, widespread incorporation of GFP-expressing cells was detected throughout the ischemia-damaged retina. GFP-labeled cells were concentrated in the retina, near the site of delivery; few remained in the subretinal space (✱). Many labeled cells had migrated to the inner limiting membrane, where they formed a near-continuous layer. (B) Higher magnification of (A) showing GFP-expressing cells in the INL and GCL. Some cells in the INL extended processes into the IPL. (C, D) Confocal images showing engraftment in the 7 weeks after transplantation retina stained with an antibody to synaptophysin (red). GFP-expressing cells (green) were present in the INL and GCL and extended processes into the OPL and/or IPL, where they appeared to intermingle with processes of the host retina. Some GFP-expressing cells in the INL displayed a bipolar cell-like morphology (D). Processes of transplanted cells were in close apposition to presynaptic processes of the host retina in the OPL. Some presynaptic terminals in the OPL contained GFP (arrows). (E–G) Amacrine cell-like morphologic differentiation of GFP-expressing neural progenitor cells at the border of the INL and IPL 5 weeks after transplantation. Retinas were counterstained with propidium iodide. (H) Anti-200 kDa neurofilament protein immunostaining of retina 9 weeks after transplantation. GFP-expressing cells (green) were present in the INL and GCL. Cells in the distal INL extended processes into the OPL, where they intermingled with immunostained process of host horizontal cells (red). GFP-expressing cells in the GCL extended processes to the NFL, where they joined axonal processes of host ganglion cells (red). Some double-labeled processes were present (arrows). Scale bars: (A–D, H) 50 μm; (E–G) 10 μm.
subretinally transplanted cells were delivered to the intended target region, the inner retina, where they displayed morphologic differentiation and clear lamination. GFP-expressing cell bodies contributed to the cellular layers of the inner retina, whereas orderly oriented GFP-labeled processes contributed to the OPL, IPL, and NFL, retaining the clear lamination of the host retina. The pattern of engrafted cells was similar to the pattern of TUNEL-positive cells in the INL and GCL (Fig. 1C).

Therefore, we postulate that specific, local inductive cues are produced in these layers that influence the migration and differentiation of neural progenitor cells. These may include bFGF,50,51,52 or glial derived neurotrophic factor (GDNF)51 produced by Müller cells or nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF) released by activated resident microglia51—substances that have been shown to be upregulated after retinal injury. Thus, an ischemia-reperfusion insult may elicit specific instructive cues that facilitate the engraftment and differentiation of neural progenitor cells transplanted to the subretinal space. Furthermore, Björklund and Lindvall53 postulated that host cells that have lost their normal inputs may release factors that stimulate axon outgrowth, thereby promoting their reinnervation by grafted cells. Concurrently, transplanted cells may, themselves, contribute trophic support that helps to maintain the integrity of the host retinal microenvironment. This is supported by our observations that at least partial inner retinal structural integrity appears to be maintained in transplanted retinas.

These hypotheses raise a number of questions: (1) Will neural progenitor cells engraffe into the normal adult retina (i.e., a presumably noninductive environment)? (2) Will neural progenitor cells engraffe into the ischemic retina when transplanted into the vitreous cavity (thus penetrating the physical barrier formed by the inner limiting membrane)? (3) Is there a specific temporal window for successful engraftment of neural progenitor cells after injury? That is to say, should transplantation be performed before any presumptive inductive cues disappear? The results of our current studies demonstrate that in the normal rat retina, GFP-expressing cells transplanted to the subretinal space underwent very limited engraftment, most cells remaining in the subretinal space, where they had been delivered. In eyes that had undergone ischemia-reperfusion injury, when neural progenitor cells were transplanted into the vitreous cavity, no significant engraftment of neural progenitor cells into the retina was observed. Presumably, the cells could not penetrate the barrier of the inner limiting membrane. This is consistent with the results of a study by Kurimoto et al.54 that also showed very limited engraftment using this delivery approach.

We also transplanted cells into the subretinal space 72 hours or 1 week after ischemic insult, at which times the INL and GCL had been virtually obliterated centrally, and inductive cues may have dissipated. We observed limited engraftment in the outer retina, whereas only rare cells migrated to the inner retina. However, the limited engraftment observed in the outer retina may be attributable to the retinal detachment injury created by the delivery procedure itself. Taken together, the findings in these control studies suggest that specific spatial and temporal inductive cues in the inner retina contribute to the fate of engrafted cells in transient retinal ischemia-reperfu-
sion, including migration, cell positioning and morphologic phenotypic differentiation.

**Limited Differentiation of Engrafted Neural Progenitor Cells into Retinal Neurons**

In the present study, engrafted neural progenitor cells displayed morphologic characteristics of inner retinal neurons, such as horizontal cells, bipolar cells, amacrine cells, and ganglion cells. Also, strikingly, many cells extended processes into the NFL and optic nerve, sometimes penetrating the lamina cribrosa. However, as late as 9 weeks after transplantation, only occasional GFP-labeled engrafted cells were observed to express retinal phenotypic neurochemical markers. The successful acquisition of specific phenotypes of neural progenitor cells depends on the interplay between the intrinsic potential of donor elements and specific inductive properties of the recipient environment.55 Our results suggest that inductive cues develop after transient ischemia-reperfusion, and that neural progenitor cells are competent to respond to these inductive cues. Hence, the limited differentiation of engrafted cells is presumably attributable to two possibilities. In the first, insufficient (or inappropriate) inductive cues were elicited in the injured retina (i.e., the recipient environment). Alternatively, heterotopic neural progenitor cells have a limited capacity to respond to novel inductive cues. Strategies to enhance the expression of mature retinal neuronal markers can either enhance the intrinsic competence of neural progenitor cells by genetic manipulation and in vitro induction with certain instructive molecules before transplantation or, alternatively, enhance the in vivo instructive signals of the recipient retinas by agents such as neuroprotectants.

**Rewiring of the Retinal Circuitry after Adult Progenitor Cell Engraftment**

Successful transplantation requires both selective replacement of lost phenotypes and the re-establishment of the original patterns of connection with both local and distant host elements.55 Our results demonstrate the potential of selective repopulation of degenerated cells of the inner retina after transient ischemia-reperfusion by heterologously transplanted cells. Transplanted neural progenitor cells migrate to specific, appropriate cellular layers, where they undergo limited morphologic differentiation, including the extension of processes that appear to come into close apposition with host dendrites within the plexiform layers. However, conclusive evidence of synaptogenesis awaits electron microscopic analysis. It has yet to be demonstrated that these morphologically similar but unauthentic retinal phenotypes contribute to functional recovery. Functional analysis from brain studies showed that conditionally immortalized neuroepithelial progenitor cell grafts reversed age-associated memory impairment in rats, and grafting neural progenitor cells improved the impaired spatial recognition in ischemic rats, although only small portions of grafted cells presented site-specific differentiation.57 These improvements may have been achieved by the compensation of local circuits by differentiated neurons or, alternatively, by glial differentiation through matrix support or release of neurotrophins and neurotransmitters to supplement the host and grafted neurons.56

**Conclusions and Future Directions**

In conclusion, adult hippocampus-derived neuronal progenitor cells transplanted to the subretinal space are readily engrafted into a host retina that has undergone ischemic injury when transplanted within a specific temporal window. In addition, many cells migrate to specific retinal layers, where they undergo morphologic differentiation to resemble known retinal neuronal phenotypes. These studies suggest that certain inductive cues are produced after injury, and they demonstrate the potential utility of adult stem cell transplantation for the treatment of retinal neurodegenerative diseases.

Given the heterogeneity of inner retinal cellular phenotypes, the complexity of their interconnecting circuitry, and their ultimate connection to the brain, restoration of the function of the injured inner retina is challenging. However, our present data show that retinal neuronal repopulation is possible. Enhanced integration and differentiation of transplanted cells, leading to functional restoration, may benefit from the following two strategies: the ex vivo modification of transplanted cells to express retina-specific proteins or neuroprotection to further maintain the integrity of the host retina.

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