Neuronal Differentiation and Morphological Integration of Hippocampal Progenitor Cells Transplanted to the Retina of Immature and Mature Dystrophic Rats

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Attempts to repopulate the retina with grafted neurons have been unsuccessful, in large part because donor cells prefer not to integrate with those of the host. Here we describe the first use of neural progenitor cells in the diseased adult retina. Adult rat hippocampal progenitor cells were injected into the eyes of rats with a genetic retinal degeneration. After survival times up to 16 weeks, the retinae of 1-, 4-, and 10-week-old recipients exhibited widespread incorporation of green fluorescent protein-expressing (GFP+) donor cells into the host retina. The 18-week-old recipients showed a similar pattern, but with fewer cells. Grafted cells expressed the mature neuronal markers NF-200, MAP-5, and calbindin. GFP+ cells extended numerous neurites into the host plexiform layers and these processes were intimately associated with synaptophysin+ profiles. GFP+ neurites also extended into the host optic nerve head. These results demonstrate the differentiation of substantial numbers of new neurons within the mature dystrophic retina.

INTRODUCTION

Neural progenitor cells present in the hippocampus of adult rodent (Altman and Das, 1965) have been isolated (Palmer et al., 1997), cultured (Gage et al., 1998), and transplanted to various sites within the CNS. These cells are capable of differentiating into neurons when grafted to sites where neurogenesis is known to occur (Suhonen et al., 1996; Shihabuddin et al., 1997; Gage et al., 1995). Thus far, however, there is little evidence for the neuronal repopulation of areas of pathological cell loss within the CNS of adult mammals. Moreover, attempts to transplant neurons to the eye have demonstrated very little morphological integration with the host retina (Aramant and Seiler, 1995; Silverman et al., 1992) (see Berson and Jacobiec, 1999), although recent studies suggest that limited integration between graft and host may occur (Kwan et al., 1999; Zhang et al., 1999). We have previously reported that transplanted adult hippocampal progenitor cells (AHPCs) can migrate into the retina of neonatal, normal rats (Takahashi et al., 1998). In that study, AHPC grafted into normal adult recipients did not migrate into the host retina. Here we have investigated whether grafting AHPCs into a dystrophic eye might influence the fate of the grafted cells. We show neuronal differentiation and morphological integration of AHPCs in the dystrophic retina of the adult Royal College of Surgeons (RCS) rat, an extensively studied model of retinal degeneration (Villegas-Perez et al., 1998; LaVail et al., 1975; Matthes and LaVail, 1989). Moreover, grafting of AHPCs into the normal adult eye only gives rise to neuronal integration when the retina is damaged mechanically at the time of grafting. These studies show that the integration and neuronal differentiation is enhanced, rather than mitigated, by ongoing degeneration in the host eye, and offer further support for the hypothesis that neural progenitor cells are capable of responding to injury cues in the mature CNS.

RESULTS

At 4 weeks following the injection of AHPCs into the vitreous of immature and mature dystrophic RCS rats,
at least 50% of the injected cells survived and main-
tained high levels of GFP expression in approximately
80% of the 1-, 4-, 10-, and 18-week-old recipients, while
no surviving cells were found in the 36-week-old recip-
ients. GFP+ cells were clearly evident under FITC illu-
mination and were verified to be of graft origin based
upon anti-GFP immunoreactivity, anti-BrdU immuno-
reactivity, as well as constitutive GFP expression (data
not shown). GFP labeling was readily distinguished
from autofluorescence of the host photoreceptor outer
segments based on intensity, morphology, location, and
spectral specificity. Subsequent identification of donor-
derived cells was therefore based on GFP fluorescence
alone, obviating the need for prelabeling with BrdU or
the use of anti-GFP antibodies.

Already at 1 week posttransplantation, grafted cells
could be seen migrating into the host retina and taking
up residence within the cellular retinal laminae, includ-
Table 1

Percentage of GFP-Expressing Cells Found in Each Region of the Retina

<table>
<thead>
<tr>
<th>Region</th>
<th>1-week-old recipient</th>
<th>4-week-old recipient</th>
<th>10-week-old recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 weeks post-transplant</td>
<td>8 weeks post-transplant</td>
<td></td>
</tr>
<tr>
<td>Vitreous</td>
<td>74.4 ± 18.7</td>
<td>15.3 ± 4.1</td>
<td>28.7 ± 7.9</td>
</tr>
<tr>
<td>GCL/IPL</td>
<td>5.8 ± 1.5</td>
<td>7.2 ± 4.2</td>
<td>4.9 ± 1.2</td>
</tr>
<tr>
<td>INL/OPL</td>
<td>4.9 ± 2.4</td>
<td>17.5 ± 3.4</td>
<td>17.0 ± 5.7</td>
</tr>
<tr>
<td>ONL/SRS</td>
<td>14.9 ± 4.7</td>
<td>60.0 ± 10.6</td>
<td>49.3 ± 9.7</td>
</tr>
</tbody>
</table>

Note. GCL/IPL, ganglion cell layer and inner plexiform layer; INL/OPL, inner nuclear layer and outer plexiform layer; ONL/SRS, outer nuclear layer and subretinal space.

* N.B., At this time point clearly defined outer plexiform and outer nuclear layers are not present.

ing the outer nuclear layer. No evidence of viable donor cells, or host GFP expression, was seen following injection of freeze-thawed GFP+ AHPCs, confirming our previous observations (Takahashi et al., 1998).

At 4 weeks postgrafting, widespread migration of grafted AHPCs into the host retina was seen. GFP+ cells were found within the retina of 60, 35, 48, and 60% of animals grafted at 1, 4, 10, and 18 weeks of age, respectively. At 8 weeks postgrafting, intraretinal GFP+ cells were found in 80% of the 1-week-old and 50% of the 4-week-old recipients.

In Table 1, recipients (1-, 4-, and 10-week-old) were analyzed to determine the laminar distribution of migrating AHPCs. The majority of grafted cells left the vitreous and entered the retina, where they migrated into the various laminae. Although grafted cells were also found in the ganglion cell and inner nuclear layers, they showed a predilection for the outer retina, particularly the outer nuclear layer, subretinal debris zone, and intervening layer of photoreceptor elements (collectively designated ONL/SRS). At a later time point (8 weeks postinjection), the number of cells in the ONL/SRS was greater yet. GFP+ cells appeared to gain access to the retina either by direct radial migration through the undamaged vitreal surface or, in greater numbers, by way of the peripheral injection tract with subsequent lateral migration. In the latter case, cells could be found migrating into as much as 60% of the longitudinal extent of the neuroretina. Regardless of the course taken by the migrating AHPCs, GFP+ cells were found in all layers of the host neuroretina, but not in the retinal pigment epithelium, choroid, or sclera.

In Fig. 1 (4 weeks postgrafting into 4-, 10-, or 18-week-old host, labeled with antisynaptophysin (Figs. 1a–1f) to demarcate the synaptic and cellular layers of the host retina) numerous GFP+ cells exhibiting neuronal morphologies can be seen. These cells were found in all cellular layers of the host retina, yet tended to respect the plexiform layers (particularly the inner plexiform layer) where they elaborated arborous. Moreover, the configuration of the neuritic processes extended by grafted cells often resembled those of normal retinal neurons: neurites preferentially projected either laterally (i.e., resembling those of horizontal or amacrine cells) or radially (i.e., resembling bipolar cell processes; see Fig. 1b). Whether this reflects intrinsic or extrinsic developmental factors, or is simply a consequence of restrictions imposed by the local retinal cytoarchitectural remains to be determined.

We examined a number of markers to evaluate whether grafted cells had adopted mature neuronal phenotypes. While we did find that a number of grafted cells expressed mature neuronal markers, under the conditions of these experiments, very few grafted AHPCs had high levels of expression. The percentage of grafted AHPCs that expressed high levels of a given mature marker was approximately 3–10%. This number underestimates the total number of cells expressing markers, as only one antibody was performed for each section.

A subpopulation of GFP+ cells were found to coexpress calbindin, a marker found on some retinal interneurons (Figs. 2a–2c), while others coexpressed the neuronal marker MAP-5 (Figs. 2d–2f) or NF-200 (Figs. 2g–2l). This is in contrast to our previous study, in which AHPCs grafted into the developing eye of normal animals failed to express neuronal markers (Takahashi et al., 1998). While these markers are not retina specific, they do show that hippocampal-derived progenitor cells are capable of developing mature neuronal phenotypes when grafted to a novel site such as the retina. Furthermore, the expression of these markers was regionally appropriate, with calbindin expression confined to transplanted AHPCs in the inner nuclear layer.
and NF-200 expression seen predominantly in the ganglion cell layer. We have not seen any evidence of the expression of photoreceptor-specific markers (e.g., rhodopsin or peripherin) by transplanted progenitor cells. Significantly, grafted AHPCs did not show any evidence of GFAP expression or astrocytic morphological development, suggesting a preference for neuronal differentiation in the microenvironment of the degenerating retina.

As GFP+ cells frequently developed elaborate neuronal arbors, we were interested in the relationship between donor neurites and synaptophysin expression. Although widely dispersed throughout the retina, the vast majority of synaptophysin seen was localized to the plexiform layers, consistent with host origin. From their positions in the cellular layers, grafted cells frequently extended processes into these layers, apparently in a directed manner. In Figs. 3a–3d, large GFP+ cells send neuritic processes into the host inner plexiform layer, while Figs. 3e–3h show cells with elaborate arbors intermingling with the host outer plexiform layer. Figures 3a and 3b provide one example of the way in which the configuration of GFP+ arbors frequently reflected the orientation of the host plexiform layers. One process tracks along the INL/IPL interface while another, originating from an position offset within the ILP, assumes a parallel course in the opposite direction despite the lack of a laminar interface to guide it. Confocal analysis confirmed that large numbers of GFP+ processes come into direct apposition to

FIG. 2. Confocal images of expression of neuronal markers by grafted AHPCs. (a–c) Grafted at 4 weeks, examined 4 weeks after grafting: constitutive GFP expression (a), anti-calbindin/Cy3 immunoreactivity (b), and merged image (c). Arrows indicate 2 cells coexpressing these labels. (d–f) Grafted at 10 weeks, examined 4 weeks after grafting: constitutive GFP expression (d), MAP-5/Cy3 immunoreactivity (e), merged image (f). Arrows indicate 2 cells coexpressing these labels. (g–i) Grafted at 16 weeks, examined 1 week after grafting: constitutive GFP expression (g), anti-NF-200/Cy3 immunoreactivity (h), and merged image (i). Arrows indicate 2 cells coexpressing these labels. (j–l) Grafted at 4 weeks, examined 4 week after grafting: constitutive GFP expression (j), anti-NF-200/Cy3 immunoreactivity (k), and merged image (l). Arrows indicate 2 cells coexpressing these labels.

FIG. 3. Confocal images of anti-synaptophysin/Cy3 (red) antibody, showing grafted AHPCs (green) sending processes into the inner plexiform layer (a–d), or the outer plexiform layer (e–h) (grafted at 4 weeks, examined 4 weeks after grafting). In a and b, a cell is shown merged (a), and reconstructed to show entire neuritic arbor (b). In c–h, AHPCs send neurites into the inner plexiform layer (c, higher power in d), and outer plexiform layers (e and g, higher power in f and h, respectively). These processes intermingle with, and appear to contact synaptophysin positive profiles of the host.
host synaptophysin + profiles (Figs. 3e–3h; 4 weeks postgrafting into 4-week-old hosts).

Grafted AHPCs are also capable of extending processes into the host optic nerve. Grafted cells residing in the ganglion cell layer extend neurites with large growth cones that approach, but do not cross the level of the scleral outlet at 4 weeks postgrafting into 1-week-old hosts (Figs. 4a and 4b). When examined at 8 weeks postgrafting, large numbers of growing neurites were found to cross the scleral outlet and extend long processes at least 300 μm into the optic nerve (Fig. 4c).

No integration of grafted cells was found in the normal non-dystrophic RCS recipients. We also found no integration when grafts were placed onto normal syngeneic Fischer 344 recipients. Grafted cells survived and often developed neuronal morphological features, but failed to migrate into the host retina (Figs. 5a and 5b). When small lesions were made with a micropipette at the time of grafting, widespread migration of grafted cells into the host retina were seen (Fig. 5c). These cells were found throughout the host retina, including the inner and outer plexiform layer (Fig. 5d). While in some cases grafted cells could be seen migrating directly into lesion sites, AHPCs often appeared to enter the retina through the unlesioned inner limiting membrane. Further studies are needed to fully document the migratory pattern of grafted cells in this setting.

No evidence of immunological rejection, decreased cell survival, or decreased gene expression was observed over the course of this study. The range of graft survival and incorporation obtained in different aged hosts (high level of incorporation in animals up to 10 weeks of age, lower level of incorporation seen in 18-week-old recipients, no survival in 36-week-old recipients) suggests that the progressive degeneration occurring in the RCS retina (which begins at 3 weeks of age) contributes to this variability. As the rat retina is fully developed before the end of the 3rd postnatal week, the widespread incorporation seen at 4 and 10 weeks indicates that developmental maturity is not a barrier to the acceptance of AHPCs by the diseased mammalian retina.

**DISCUSSION**

Our study shows that neural progenitor cells derived from adult hippocampus can migrate into the dystrophic neuroretina of mature animals, where they respect the local laminar organization and differentiate into neurons. We have no evidence, however, that grafted precursor cells are capable of replacing the primary cell lost in the RCS rat, the photoreceptor (Matthes and LaVail, 1989; Villegas-Perez et al., 1998). It is important to note that the previously established methods of inducing photoreceptor rescue in this animal, e.g., growth factors (Faktorovich et al., 1990), retinal pigment epithelial cell transplants (Sheedlo et al., 1989; Lopez et al., 1989), or various subretinal insults (Silverman and Hughes, 1990; Humphrey et al., 1993) are only successful when applied to 3- to 4-week-old animals. Here we demonstrate widespread neuronal incorporation after grafting into animals up to 10 weeks of age, as well as limited incorporation in 18-week-old recipients, an age when the RCS retina has degenerated severely and other interventions are ineffective. At 36 weeks of age, however, AHPCs not only fail to enter the retina but show very little survival, suggesting the loss of an important trophic influence late in the course of the dystrophy. We also show that AHPCs are capable of migration into the normal mature retina only when a lesion has been performed on that retina.

Having migrated into the retina from the vitreous, grafted AHPCs disperse within the host tissue rather than remaining adherent to each other, as is typically seen with embryonic neural grafts. After taking up residence, these cells differentiate along neuronal (as opposed to glial) lines and extend processes within the host plexiform layers. Furthermore, the orientation of many of these processes is reminiscent of the arborization pattern of retinal amacrine cells. AHPCs in the ganglion cell layer frequently extend neurites into the optic fiber layer and optic nerve. Differentiating AHPCs express some, but not all of the markers found on normal retinal neurons. Since the markers we found are also found elsewhere in the CNS, including the hippocampus, the precise neuronal phenotype of graft-derived neurons remains unresolved. Salient to this issue is the question of whether mismatches in neuronal phenotype necessarily preclude useful functional recovery, as might result from bridging a gap in a relay system. Whether hippocampal progenitor cells are capable of expressing the highly specific constellation of genes necessary for phototransduction also remains an open question. We have examined a number of photoreceptor-specific markers (rhodopsin and peripherin) and have not seen evidence of expression of the markers by grafted cells. It is worth noting that neural progenitor cells can reportedly differentiate into cells of the hematopoietic lineage ( Bjornson et al., 1999), suggesting that a hippocampal to retinal fate shift should not be dismissed. Morphologically, AHPC arborizations do appear to respond to extrinsic retinal cues in preference to any intrinsic hippocampal developmental programs.
Finally, while our finding that graft-derived neurites are intimately associated with host synaptophysin profiles does not demonstrate synapse formation, another laboratory using this same cell line has recently provided electron microscopic evidence of synapse formation in vitro, as well as excitatory postsynaptic potentials (Toda et al., 1999). These results reinforce the conclusion that the neuronal repopulation presented here represents a form of morphological integration, rather than simply cellular infiltration or random migration and neurite extension.

AHPCs readily migrate into the retina of mature, allogeneic RCS rat hosts, in contrast to nondystrophic, syngeneic Fischer 344 rat hosts (Takahashi et al., 1998), or normal nondystrophic RCS controls, where no incorporation was seen beyond the neonatal period. Furthermore, AHPC migration can occur in the normal Fischer 344 rat if a traumatic injury is induced at the time of transplantation. These results are consistent with other recent studies in which stem or progenitor cells seem to

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**FIG. 4.** Confocal images of GFP+ neurites projecting, via the host optic fiber layer, into the optic nerve head 4 weeks after grafting (a and b). These fibers have large growth cones (arrows in a, and in higher power in b), which approach, but do not cross, the scleral outlet (sc) at 4 weeks postgrafting into 1-week-old hosts. When animals were examined 8 weeks after grafting, numerous growth cone-tipped processes were found to have entered the optic nerve, extending over 300 μm beyond the scleral outlet (c).

**FIG. 5.** Images of AHPCs grafted into normal adult Fischer 344 rats. In a, an uninjured retina that received an injection of AHPC cells shows no evidence of migration, although at higher power (b), grafted cells can be seen extending neuritic processes. Following a mechanical lesion however, widespread migration and integration of grafted cells was seen (c). Confocal imaging demonstrates that cells grafted in this setting can take up residence in the nuclear layers of the retina, where they adopt morphological phenotypes strikingly similar to that of normal host retinal neurons (d).
respond to the presence of pathology, for instance, neural stem cells grafted to the bloodstream of irradiated mice repopulate the bone marrow (Bjornson et al., 1999), while similar cells grafted to the cerebral ventricles of neonatal shiverer mice replace lost oligodendrocytes (Yandava et al., 1999). What the injury cue is in the setting of these experiments is unclear. One possibility relates to changes in the state of Müller glia cells, which are known to modify expression of a number of molecules following injury (Tomita et al., 1998; Cao et al., 1997; MacLaren, 1996; de Raad et al., 1996). Alternatively, a nonspecific injury cue (e.g., IL-1) could serve as an attractant to grafted AHPCs. An alteration in the inner limiting membrane (formed by end feet of the Müller glia) might also allow migration into the retina that would otherwise be blocked. The similar pattern of migration seen in congenically dystrophic and mechanically damaged retina suggest that a similar “injury cue” may be present, and that grafted AHPCs are capable of responding to it.

Neural progenitor cells clearly possess a high degree of plasticity (Yandava et al., 1999; Johansson et al., 1999; Flax et al., 1998; Brustle et al., 1998; Morrison et al., 1999) and provide a new tool for studying mechanisms of neural development and degeneration. The data presented here provide the first definitive evidence for the survival, migration, and neuronal differentiation of a transplanted cell in the diseased, mature retina. Although much remains to be done before diseases involving neuronal loss can be reversed clinically, this study shows that neural progenitor cells can overcome many of the obstacles to neuronal integration present in the mature mammalian central nervous system.

MATERIALS AND METHODS

Donor cell line. Hippocampal progenitor cells were clonally derived from adult Fischer 344 rats as previously described (Palmer et al., 1997). Briefly, intact hippocampal formations were pooled from adult female (160–170 g) Fischer 344 rats, and the tissue dissected into small pieces and digested with protease, papain, and DNAse I. The tissue was then plated in Dulbecco’s Modified Eagles Medium/F12 (DMEM/F12, 1:1) containing 10% defined fetal bovine serum. After 24 h, the medium was replaced with DMEM/F12 containing N2 supplement (Gibco) and 20 ng/ml hFGF-2 (gift of A. Baird). These nonclonal cultures were maintained and passaged 1:3 onto polyornithine/laminin coated 100-mm dishes and used to generate the clonal cultures for these experiments.

Passage 3–4 cultures were genetically modified via a retroviral based tetracycline-responsive vector containing a CMV promoter (Hoshimaru et al., 1996) to express the modified jellyfish (Aequorea victoria) enhanced green fluorescent protein (eGFP) (Okada et al., 1999). Cultures were treated with limiting dilutions of retroviral vectors and maintained for 2–3 weeks by replacing the media with conditioned media from high density hippocampal culture, after which individual clusters of cells were harvested with glass cloning rings (Palmer et al., 1997). In some cases, the cells were pulsed with BrdU (50 ng/ml, 3 pulses over 3 days) prior to transplantation.

Recipient animals and transplantation. Pigmented dystrophic (1 week, n = 22; 4 weeks n = 41; 10 weeks, n = 6; 18 weeks, n = 4; 36 weeks, n = 9), albino dystrophic (1 week, n = 8; 10 weeks, n = 6) albino nondystrophic (4 weeks; n = 6), RCS rats received injections into the vitreous under general (ketamine/xylazine) and topical (proparacaine) anesthesia.

Injections were performed under direct observation using a binocular surgical microscope with coaxial illumination (Möller) and viewing through a dilated pupil (topical tropicamide 1%). The injections were made using a beveled glass micropipette (outside diameter 1 mm) connected to a 50-μl Hamilton microsyringe with PE tubing. The sharp tip of the micropipette allowed direct entry to the vitreous cavity through a self-sealing wound, the entry point being just vitread to the corneo–scleral junction. This approach to the vitreous avoided trauma to the ciliary body and lens, but necessarily resulted in focal perforation of the intervening uvea and peripheral retina. A total of 50,000–100,000 cells in 1–2 μl of DMEM/F12 media were injected. As a control, cells that were freeze-thawed three times were also injected (4 weeks, n = 6).

Transplants were also performed in normal Fischer 344 rats with (4 weeks, n = 4) and without (4 weeks, n = 5) mechanical lesions of the retina. In these experiments, four or five mechanical lesions were made in the neuroretina with a pulled glass micropipette immediately before transplantation. These lesions were placed in a circle around the optic nerve head, and penetrated the neuroretina, but did not damage the choroid.

Tissue preparation and histology. At 1, 4, 8, and 16 weeks posttransplantation, eyes were removed and immersion fixed with 4% paraformaldehyde, cryoprotected, and sectioned at 7–14 μm on a cryostat. Sections were processed for haematoxylin and eosin, anti-BrdU (1:400), anti-synaptophysin (1:200) and anti-GFP (1:500), anti-calbindin (1:1000), anti-rhodopsin (1:200), anti-peripherin (1:500) or anti-NF-200 (1:40), anti-MAP-5
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