# Limitation of Anatomical Integration between Subretinal Transplants and the Host Retina

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**Purpose.** In previous studies of subretinal transplantation in rabbits, the host photoreceptor layer seemed to prevent the bridging of neuronal fibers between the graft and the host retina. The current study was undertaken to determine whether the same phenomenon occurs in transplants to the subretinal space of the vascularized retina of rats. Bridging of fibers was examined in transplants to animals of different genetic backgrounds (normal versus dystrophic rats), of different ages, and after different survival times.

**Methods.** Sprague-Dawley (SD) rat retinal tissue from embryonic day (E)18 was subretinally grafted to adult (60-day-old) normal SD rats, to RCS rats (32 and 73 days old), and to adult (60-day-old) transgenic P23H rats. After various survival times (28–183 days), transplanted retinas were processed for routine histology and immunocytochemistry. Antibodies against calbindin, neuronal nitric oxide synthase (NOS), and protein kinase C (PKC) were used to identify specific retinal cell types and their processes.

**Results.** The shape and position of the immunoreactive cell bodies indicated that the expected neuronal populations were labeled within the grafts and in the host retina. Labeled neuronal processes were also observed. In each case, NOS-, calbindin-, and PKC-immunolabeled fibers formed bridges between the graft and the host tissues. However, regardless of the extent of host photoreceptor cell loss, the age of the recipient, or the genetic background, bridging fibers were observed only in areas where the host photoreceptor layer was discontinuous or completely missing.

**Conclusions.** The present study demonstrates that the host photoreceptor layer plays a role in limiting graft–host anatomical integration. (*Invest Ophthalmol Vis Sci.* 2003;44:324–331) DOI:10.1167/iovs.02-0132

The conditions for successful long-term survival of various types of retinal transplants have been investigated extensively in experimental animals. It has been demonstrated that when embryonic, neonatal, and even adult neuroretina are transplanted intraocularly, a large number of the transplanted cells survive.[1–5] The intrinsic neurochemical development of these transplants has also been evaluated by using specific neuronal markers, establishing that the differentiation and development of the grafted retinal cells relies largely on genetically determined programs. Thus, even in grafts exhibiting poor morphologic organization (transplantation of retinal fragments), all retinal cell types (except ganglion cells) and proteins essential for phototransduction and neurotransmission are detected even several months after transplantation.[6–9] These results support the notion that, in general, retinal transplants not only survive but also acquire and maintain characteristic morphologic and neurochemical parameters.

However, data about the reconstruction of specific circuits between a subretinal graft and host are scarce. For such a reconstruction to occur, it is necessary that grafted cells establish contacts not only with their neighboring cells within the graft but also that they be guided toward the host retina. There are some anatomic and functional data showing that subretinal retinal grafts establish contacts with the host retina.[10–19] It is apparent from these studies that neuronal fibers can grow from subretinal grafts into the host retina. However, the number of bridging fibers is always low, regardless of the age of the transplanted cells or the morphologic organization of the graft. It is conceivable, then, that factors associated with the host retina also play a role.

We have shown that neuronal nitric oxide synthase (NOS)-containing cells of rabbit-to-rabbit subretinal transplants can send long fibers into the target site of the host retina.[17] However, such bridging is not observed in areas where one or more cell rows of host photoreceptor cells are present at the graft–host border. This inability of graft-derived neurites to penetrate the host's outer nuclear layer (ONL) is intriguing, because even adult retinal cells have been shown to retain a certain degree of plasticity when challenged (by injury or disease), allowing them to sprout neurites that run through various retinal layers.[20–22] In the current study, we therefore further explored the issue of graft–host integration in several rat-to-rat transplantations in which both normal rats and different strains of dystrophic rats (RCS rats and the transgenic rhodopsin mutant rats, strain P23H) of different ages (thus exhibiting different degrees of photoreceptor cell loss at the time of transplantation) were used as recipients. Using different specific cell markers, we found that bridging of fibers between a retinal transplant and the host retina indeed occurred only through discontinuities in the host ONL or in areas where photoreceptor cells of the host were absent.

## Materials and Methods

### Experimental Animals

The experiments were conducted with the approval of the local animal experimentation ethics committee. Animals were handled according to the guidelines on care and use of experimental animals set by the Government Committee on Animal Experimentation at the University of Lund and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Embryonic Sprague-Dawley rats (SD; BK Universal, Sollentuna, Sweden; embryonic day [E]18, normal gestation 21 days) provided donor tissue. Four groups of animals were the recipients: adult (60-day-old, postnatal day [P]60) Sprague-Dawley rats (SD-SD); young (32-day-old, P32) pink-eyed, tan-hooded RCS rats (SD-RCS/

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**TABLE 1.** Survival Times of Rats that Underwent Retinal Cell Transplantation

| Survival Time (Days after Transplantation) | SD-SD (P60)* | SD-RCS (P32)* | SD-RCS (P73)* | SD-P23H (P60)* |
|---|---|---|---|---|
| 28 | 5 | 9 | 6 | 3 |
| 48 | 6 | 8 | 4 | 3 |
| 97 | 6 | 9 | 3 | 3 |
| 183 | — | 3 | 7 | — |

* Recipient age.

Data are the number of animals in each of the experimental groups that survived for the times shown.

32); adult (73-day-old, P73) pink-eyed, tan-hooded RCS rats (SD-RCS/73); and adult (60-day-old, P60) P23H-1 rats (SD-P23H). The extent of photoreceptor cell loss at the time of transplantation was used as the criteria for choosing the age of the recipients. In RCS rats, a mutation in the gene encoding the receptor tyrosine kinase MERTK[23] results in defective phagocytosis of shed rod outer segment disc membranes by the retinal pigment epithelium, ultimately leading to photoreceptor cell degeneration.[24,25] At P32, three to four photoreceptor cell rows are normally seen in the superior retina of these animals, whereas at P73, only a few photoreceptor cells remain. The RCS rats were kindly provided by Dr. Olaf Strauss, Freie University Berlin (Berlin, Germany). In P23H rats, the photoreceptor degeneration results from a rhodopsin mutation (a proline-to-histidine substitution at amino acid position 23). Homozygous breeders of P23H rats (produced by Chrysalis DNX Transgenic Sciences, Princeton, NJ) were developed and kindly provided by Matthew M. LaVail (University of California, San Francisco, CA). Transplants were performed in heterozygous animals, which were obtained by mating with wild-type Sprague-Dawley rats in our laboratory. In the line used, P23H-1, five to six photoreceptor cell rows were still present at the time of transplantation (P60).

## Transplantation Procedure

The transplantation surgical technique used in this study is based on a method described previously.[26] Briefly, timed-pregnant female SD rats were anesthetized with carbon dioxide and decapitated. The embryos were obtained by cesarean section and placed in cold 4°C Ames medium (Sigma, Diesenhofen, Germany). Eyes were enucleated and the neural retinas dissected, transferred to fresh cold Ames medium, and cut into small pieces. The retinal pieces did not include the RPE or the optic nerve head region. Recipients were anesthetized with Equitisin (pentobarbital 9.72 mg/mL, chloral hydrate 42.5 mg/mL, magnesium sulfate 86.25 mM, 10% vol/vol ethanol, 40% vol/vol propylene glycol, 0.3 mL/100 g body weight, intraperitoneally)[26] and were locally anesthetized with 1% amethocaine hydrochloride. Donor retinal tissue (2 $\mu$L total volume) was drawn into a plastic (polyethylene) pipette tip (GELoader Tip; Eppendorf, Hamburg, Germany) connected to a precision microsyringe (Hamilton, Reno, NV) and injected through the sclera into the superior subretinal space of the recipients. The survival time varied from 28 to 183 days after transplantation, corresponding to transplant ages between P25 to P180. These data are summarized in Table 1. All animals were maintained on a 12 hour light–dark cycle with free access to food and water. No immunosuppression was used.

## Tissue Preparation and Analysis

After the different survival times (Table 1), the transplant-recipient eyes were quickly enucleated and immersed in a freshly prepared solution of 4% paraformaldehyde in Sörensen buffer (0.1 mM; pH 7.2). The eyes were hemisected in the fixation medium, and the anterior segment, lens, and vitreous body were removed. The posterior segments containing the transplants were transferred to fresh fixation medium and kept at RT for 2 hours. The tissue was subsequently rinsed, cryoprotected in Sörensen buffer containing increasing concentrations of sucrose, embedded in an albumin-gelatin medium (30 g egg albumin, 3 g gelatin, 100 mL distilled water) and frozen. Sections were obtained on a cryostat (12 $\mu$m), collected on gelatin-chrome, alum-coated glass slides, air dried, and stored at −20°C until further processing.

Some sections were stained with hematoxylin and eosin. Others were processed by immunocytochemistry with antibodies against specific and well-established retinal neuronal cell markers. These included the calcium-binding protein calbindin, which in rats labels horizontal and amacrine cells[27]; protein kinase C (PKC), which labels rod bipolar cells[28]; and the neuronal form of NOS, which labels a subpopulation of amacrine cells.[29]

The cryostat sections were preincubated for 90 minutes with 0.1 M PBS containing 1% BSA, 0.25% Triton X-100 (PBTX), and 5% normal serum. This was followed by overnight incubation at 4°C with the following primary antibodies: mouse monoclonal anti-calbindin-D (28 kDa, 1:200; Sigma), or rabbit anti-human PKC (1:1000; Chemicon, Temecula, CA), or sheep anti-neuronal NOS (1:4000; gift from Ian G. Charles and Piers C. Emson, Medical Research Council, Cambridge, UK), or mouse monoclonal anti-rhodopsin (Rho-1D4; 1:400; gift from Robert S. Molday, University of British Columbia, Vancouver, British Columbia, Canada). All primary antisera were diluted in PBTX containing 2% normal serum. After a rinse, sections were incubated for 90 minutes with Texas red sulfonyl chloride conjugated to either donkey anti-mouse, donkey anti-rabbit, or donkey anti-sheep (1:100; Jackson ImmunoResearch, Hamburg, Germany). After completing the staining procedure, sections were rinsed, coverslipped with buffered glycerol containing the antifade agent phenylenediamine, and viewed by light microscope equipped for fluorescence microscopy. Most micrographs depicted in each of the figures correspond to different specimens, to illustrate the consistency of the observations.

# RESULTS

After the grafting of the small retinal pieces, typical rosettes were observed in all transplants with hematoxylin and eosin staining. A certain degree of lamination was observed within the rosettes, with photoreceptors surrounded by an equivalent to inner retinal layers (see Figs. 1 to 5). Distinct rosettes were still present in old transplants (see Fig. 3). After short survival times and in transplantation into normal animals, the presence of the host photoreceptor layer allowed us to determine the graft–host interface. In cases in which the host photoreceptor layer was missing, the host retina was still readily identifiable, thanks to its regular and laminated organization, demonstrable with different neuron-specific labels (calbindin, NOS, and PKC), even after long survival times.

## Transplantation of SD Embryonic Tissue to Adult SD Rats: SD-SD Group

The morphology of host SD retinas was well preserved except in adjacency to the grafts, where the number of photoreceptor rows was slightly reduced. In addition, in some areas, the host ONL was folded, probably as a result of mechanical damage produced during the surgery. Large folds at times produced discontinuities in the host ONL, and cells belonging to inner retinal layers were present between the folds (Fig. 1A).

Cellular and fiber labeling were detected in both host and grafts at all time points examined and with all neuronal markers used. In the host SD retina, NOS-immunolabeled amacrine cell bodies were identified in characteristic positions next to the inner plexiform layer (IPL), and their neurites formed regular stratified profiles within the IPL (Fig. 1B). Some more weakly labeled bipolar cells were also present. In the retinal grafts, NOS-immunolabeled cells projected toward zones within the graft that by all criteria were equivalent to an IPL. NOS-immunoreactive fibers ran also alongside the graft–host interface in the areas where host photoreceptor cells were

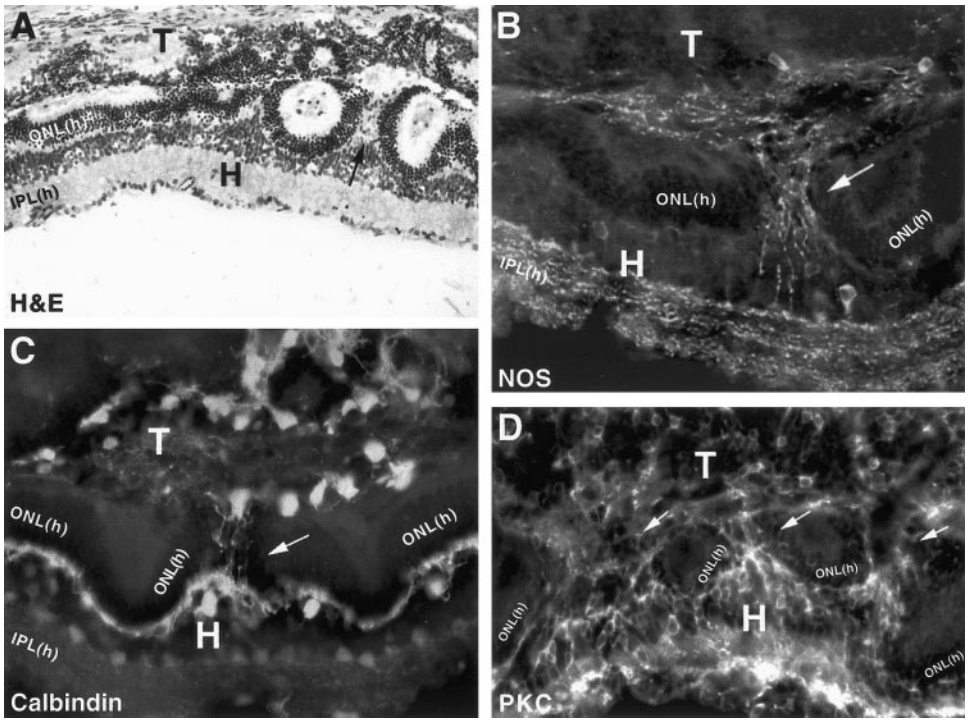


**FIGURE 1.** Transplantation to adult SD rats (SD-SD group) 28 days after surgery. (**A**) H&E staining: the ONL of the host (H) retina, ONL(h), appeared slightly thinner next to the transplant (T). *Arrow*: an area between two rosette-like structures that formed in the host ONL. (**B**) NOS-immunolabeled cells and fibers in host and transplant. In the transplant, fibers ran alongside the graft–host interface in areas adjacent to host photoreceptors (ONL[h]). Bridging fibers and cells ran perpendicularly between the ONL folds (*arrows*). (**C**, **D**) The same phenomenon for calbindin- and PKC-labeled fibers, respectively (*arrows*). (**B**, **C**) Host immunolabeled cells sprouted outward (toward the transplant) exclusively in association with the folds (*arrows*). H&E, hematoxylin and eosin.

present (Fig. 1B). However, in areas in which the host ONL was discontinuous, labeled fibers ran perpendicularly between the folds, mingling with homologous fibers in the graft and in the host retina (Fig. 1B).

Calbindin-immunoreactive horizontal cells (strongly labeled) and amacrine cells (weakly labeled) were present in the host SD retinas (Fig. 1C). Immunolabeled fibers in the host were associated with horizontal cells at the level of the outer plexiform layer (OPL). Labeled cells were also scattered in the transplants. Calbindin-immunoreactive fibers bridged the graft and the host retina, exclusively in areas where the host ONL was folded (Fig. 1C). Bridging fibers seemed to be associated with horizontal cells on both sides of the graft–host interface, and no preferred direction was observed.

Intense PKC immunolabeling was evident among bipolar cells and in the IPL in the host retinas. In the grafts, labeled cell bodies and fibers were present throughout. Similar to that observed with calbindin and NOS labeling, bridging was observed only in areas where the host photoreceptor cell layer was disrupted (Fig. 1D). It was difficult to distinguish individual fibers and to determine their directions, but it was clear that no crossover occurred except through the gaps created by the folds.

The same observations were made at longer survival times (48 and 97 days after transplantation), except that at these time points the overall thickness of the host ONL was reduced adjacent to the graft (data not shown).

## Transplantation of SD Embryonic Tissue to Young and Adult RCS Rats: SD-RCS Group

As opposed to SD recipients, more than half of the photoreceptors (at P32) and practically all of them (at P73) were lost in the host RCS retina at the time of transplantation. In transplants to the young RCS rats (P32), hematoxylin and eosin staining of sections through the transplanted retina revealed that, in contrast with intact littermates and with the control contralateral eye, a number of photoreceptor cells were present in the host ONL. Remaining photoreceptors were present at most survival times, but only adjacent to the grafts, indicating partial and local photoreceptor cell rescue by the transplantation procedure and/or the grafts themselves (examples illustrated in Figs. 2A, 2D, 3A). The overall distribution of calbindin-, NOS-, and PKC-immunolabeled structures in the host RCS retinas and in the grafts was similar to that observed in SD-SD transplants (as described in the prior section). However, it should be noted that a few calbindin- and PKC-immunolabeled fibers were at times found to have grown for short distances into the rescued ONL, which was particularly discernible at survival times shorter than 28 days (not illustrated). With longer survival times and in transplantation to the older animals (P73), the organization of the immunoreactive structures (somata and fibers) appeared further distorted in the host retinas (described later). This was most evident next to the grafts, but was observed also in the remainder of the host retina.

## P32 RCS Recipients: SD-RCS/32 Group

Figures 2A–C illustrate the observations made in this experimental setup at 28 days after transplantation. In the P60 RCS host retina (P32 + 28 days' survival time), one to two rows of photoreceptor cells were observed next to the grafts, allowing us to define the graft–host interface (Fig. 2A). The organization of the inner retina was also quite well preserved in these specimens. Individual NOS immunopositive fibers in the grafts ran alongside the graft–host interface without traversing the two rows of host photoreceptor cells (Fig. 2B). Similarly, no bridging was observed between the transplants and the host retinas in tissue processed for calbindin (not illustrated) or PKC (Fig. 2C), where, consistent with the observations on NOS, fibers seemed unable to traverse the remaining photoreceptors in the host retina.

Figures 2D–F illustrate the observations made in P32 RCS animals, 48 days after transplantation. In the P80 host retina, one to two rows of photoreceptor cells still remained but these rows were discontinuous in some places (Fig. 2D). In the graft, NOS immunopositive fibers were again observed to run alongside the graft–host interface in areas where photoreceptor cell rows could be detected in the host retina. NOS-immunolabeled

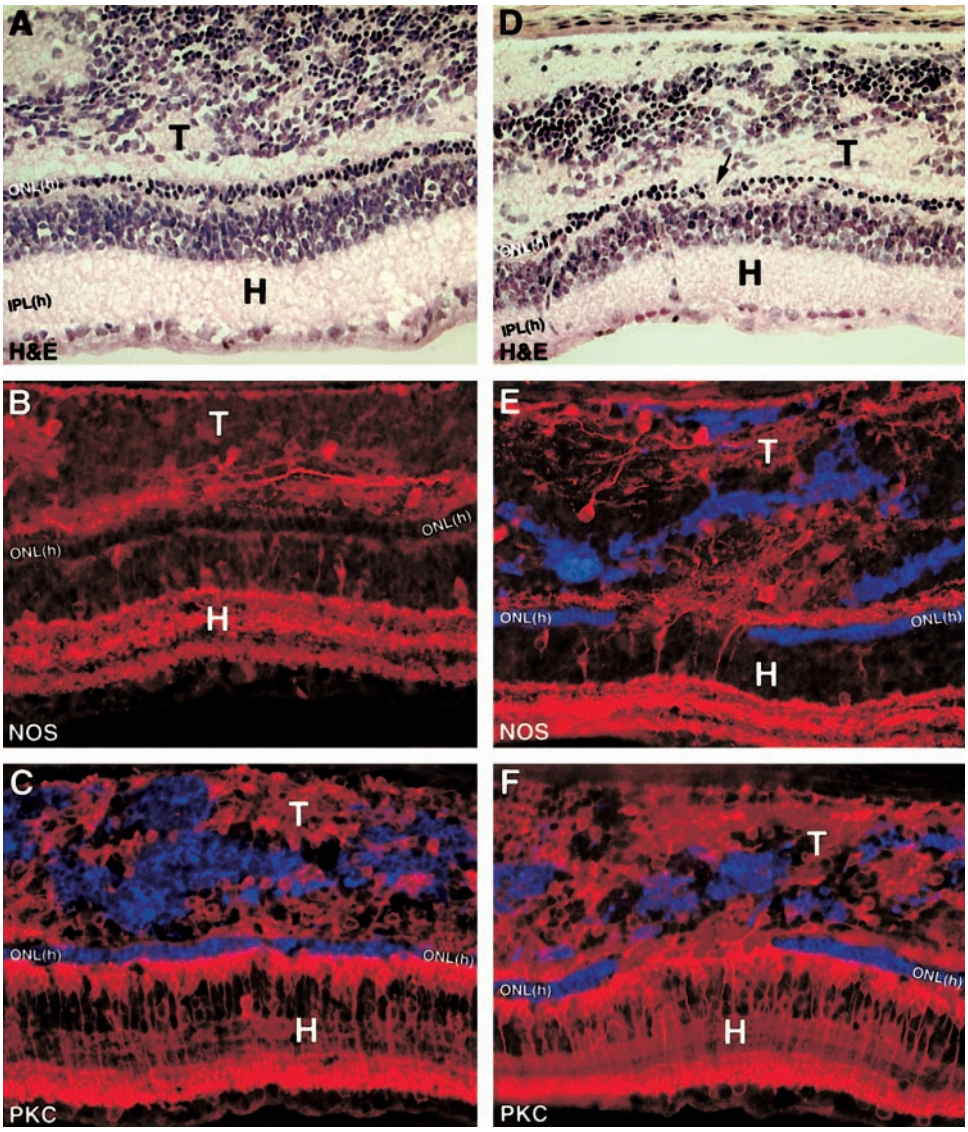


**FIGURE 2.** Transplantation to young RCS rats (SD-RCS/32 group). (**A–C**) Twenty-eight days after surgery (60-day-old host). (**A**) H&E staining showed one to two cell rows of photoreceptors in the host retina (H) next to the transplant (T). (**B**, **C**) Immunolabeled structures in the inner host retina appeared symmetrical and undistorted. No bridging fibers were present between the transplant and the host retina with NOS (**B**) or PKC (**C**) immunostaining. (**D–F**) Forty-eight days after surgery (80-day-old host). (**D**) H&E staining showed one to two cell rows of photoreceptor cells remaining in the host retina (H) next to the transplant (T). In small areas, the host ONL, ONL(h), was discontinuous (*arrow*). Bridging fibers were visible only through the breaks in the host ONL with NOS (**E**) and PKC (**F**) immunostaining. (**C**, **E**, **F**) Photoreceptor cells in the host retina ONL(h) and in the graft were labeled for rhodopsin (*blue*).

fibers were also visible through the breaks in the host photoreceptor layer (Fig. 2E), some of which appeared to form bridges between immunolabeled plexuses in the graft and in the host retina. A similar observation was made after PKC staining. Labeling in host bipolar cells was in these cases separated from labeling in cells and fibers of the graft in areas where rows of host photoreceptor cells were detected, whereas no clear delimitation was observed at the breaks in the host photoreceptor layer (Fig. 2F). In the latter, PKC-labeled fibers, presumed to belong to graft cells, at times projected toward the host OPL.

Figure 3 illustrates the observations made in P32 host animals, 97 days after retinal transplantation. In the host (P129) retina, some fragments of ONL were still present (Fig. 3A). Fusion between graft and host immunoreactive profiles was generally observed at this time point in larger areas with all markers, and a continuous host photoreceptor cell layer was never observed in the fused regions (Fig. 3B–D).

In specimens killed 183 days after transplantation, rosettes with viable photoreceptor cells were still present in the grafts (Fig. 3E). In the host (P215) retina, no photoreceptors were observed and more disruption was noted in the inner nuclear layer (INL) than in the younger specimens (NOS immunolabeling in the IPL was less intense, calbindin immunoreactivity among horizontal cells and in the OPL was reduced, PKC-labeled bipolar cells appeared shorter and distorted; Figs. 3F–H). At this survival time, immunoreactive fibers (NOS, calbindin, and PKC), the origin of which could not be determined, bridged the graft and the host tissues throughout (Figs. 3F–H).

## P73 RCS Recipients: SD-RCS/73 Group

A photoreceptor cell layer was not observed in the host retinas at any of the survival times examined (28–183 days after transplantation). Figure 4 illustrates the observations made 48 days after transplantation in P73 RCS rats. In these animals, the host ONL had completely degenerated, and some disorganization of the inner nuclear layer was visible. Similar to what was observed at the longest survival times in SD-RCS/32 transplants, graft–host fusion was observed to occur in all areas at all survival times, with NOS- calbindin-, and PKC-immunolabeled fibers bridging between the graft and the host retina (Figs. 4B–D).

## Transplantation of SD Embryonic Tissue to Adult Mutant Rhodopsin Transgenic Rats: SD-P23H Group

Five to six cell rows were present in the superior retina of the P60 recipients at the time of transplantation. Of these, two to

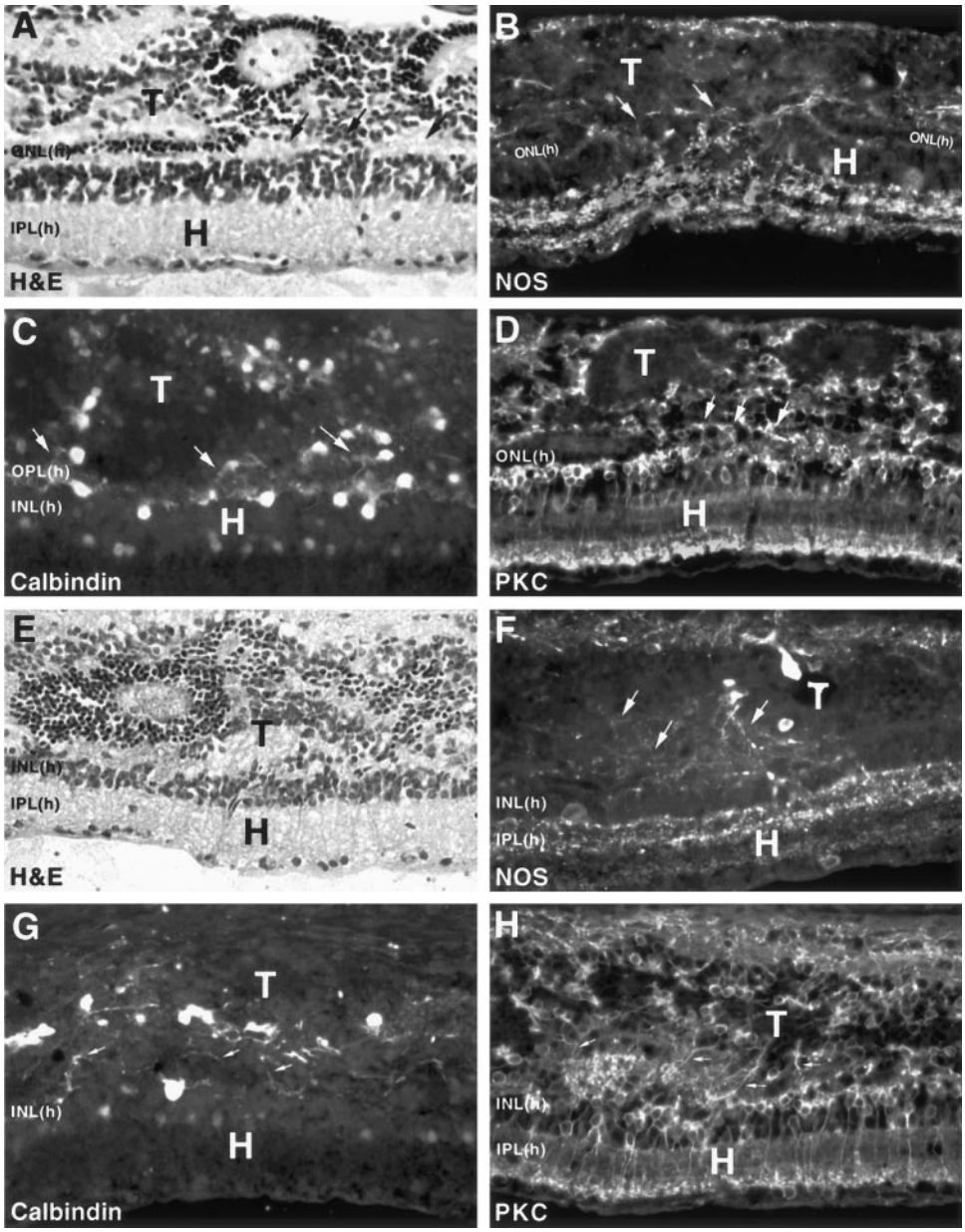


**FIGURE 3.** Transplantation to young RCS rats (SD-RCS/32 group). (**A–D**) Ninety-seven days after surgery (129-day-old host). (**A**) H&E staining showed larger areas of the host ONL, ONL(h), to be disrupted, compared with shorter survival times (*arrows*). Bridging fibers through such areas were visible with NOS (**B**), calbindin (**C**), and PKC (**D**) immunostaining (*arrows*). (**E–H**) One hundred eighty-three days after surgery (215-day-old host). (**E**) H&E staining showed no photoreceptor cells in the host retina. Bridging fibers were visible in wide areas with NOS (**F**), calbindin (**G**), and PKC (**H**) immunostaining (*arrows*). OPL(h), host OPL; INL(h), host INL; IPL(h), host IPL.

three rows were still present 28 days after transplantation in areas away from the grafts and in the contralateral intact eye (not illustrated). At the grafts, one cell row (in some places two) was present (Figs. 5A–D), indicating that, similar to what was observed in normal host animals, the transplantation procedure locally accelerated cell loss. Analogous to our observation with transplantation in SD rats and young RCS rats, no immunolabeled fibers were observed to run between the graft and the host tissues in areas where continuous rows of photoreceptor cells were visible in the host (Figs. 5B–D).

Forty-eight days after transplantation, one photoreceptor cell row was occasionally identified in some places in the host retina (Fig. 5F). By 97 days after transplantation, only a few scattered photoreceptor cells were present next to the grafts. At both time points, NOS-, calbindin-, and PKC-immunolabeled bridging fibers were again found only in areas where the host photoreceptor cell layer was missing (Figs. 5F–H).

It should be noted, however, that in this study we did not compare the degree of fusion in transplantation to young versus old dystrophic animals or how it was affected over time (at long posttransplantation periods). The size of the grafts, the position of the labeled grafted cells in relation to the host retina, the cellular markers used, all influence the number of bridging fibers visible. As described earlier, at early survival times, bridging fibers coursed through small areas which, as expected, varied in size as we screened throughout all sections from the same specimen and between different specimens. At later survival times, or in transplantation to older animals, again a highly variable number of bridging fibers traversed larger areas. Further, it was not always possible to establish with certainty the origin of the bridging fibers. Thus, also host-derived fibers may have contributed to the number of bridging fibers, but again to a variable degree.

## DISCUSSION

The transplants produced in this work always formed rosettes but viable grafts were nevertheless observed, even 6 months after transplantation. Photoreceptors as well as other cell types

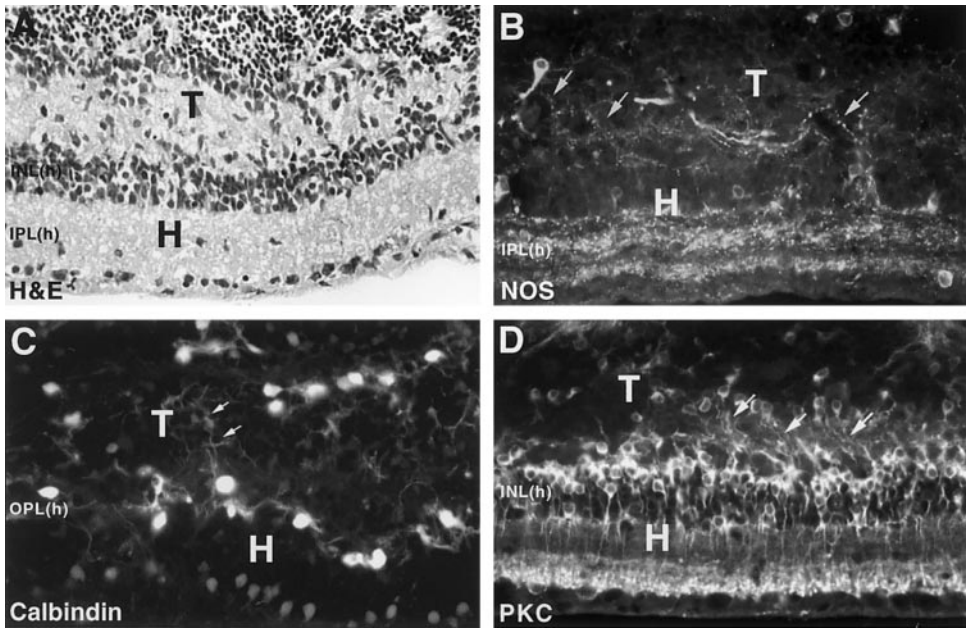


**FIGURE 4.** Transplantation to older RCS rats (SD-RCS/73 group) 48 days after surgery (121-day-old host). (**A**) H&E staining showed no photoreceptor cells in the host retina. Bridging fibers were observed throughout with NOS (**B**), calbindin (**C**), and PKC (**D**) immunostaining (*arrows*). OPL(h), host OPL; INL(h), host INL; IPL(h), host IPL.

were present, as demonstrated by the expression of the several neuronal markers used. These observations indicate that the differentiation and at least the short-term survival of the grafted cells is not primarily influenced by the structure of the graft.[26] The latter is likely to influence long-term survival and the functional capacity of the grafts—requirements that should be favored by the improved surgical techniques that now produce large sheets of well laminated transplants.[30,31] Despite these advances, however, only a few reports have demonstrated outgrowth of neuronal fibers from subretinal grafts into the host retina, and when observed, the number of bridging fibers has been low, regardless of the morphologic organization of the grafts.

As shown in the present study, the trajectory followed by the graft neurites indicates that factors associated with the host retina might prevent widespread penetration of the graft processes. The consistent and main finding of this study is that no, or only poor, integration occurred between a subretinal graft and the host retina in areas where one or more rows of host photoreceptor cells were present at the border. Particularly with the NOS antibody, it was possible to observe that many graft-derived neurites ran parallel to the host photoreceptor layer, at times for long distances, without innervating the latter.

After transplantation, a localized loss of photoreceptor cells is normally induced in the host retina as a result of the mechanical trauma and the permanent detachment created by the surgical procedure. The cell loss observed in the current study in transplantation to normal rats was not as severe as that previously seen in transplantation to normal rabbits, in which we[17] and others[32] have observed a rapid loss of host photoreceptor cells and the presence of bridging fibers even at short survival times. This difference may partly be due to the fact that the host rat retina is vascularized, rendering it relatively more resistant to the effect of detachment. As expected, the photoreceptor cell layer of normal, dystrophic RCS and P23H transgenic host rats degenerated at various speeds. Yet, independent of the number of cell rows remaining, bridging fibers were observed only in hosts without photoreceptors (in transplantation to older dystrophic animals or at longer survival times), through patches of complete photoreceptor cell loss (occasionally present in younger dystrophic animals or in shorter survival times), or between folds produced in the host photoreceptor cell layer (observed e.g., in normal hosts). Observations we have made previously in rabbit-to-rabbit subretinal transplants also suggest that the photoreceptor cell layer of the host retina somehow poses a barrier to anatomic integration.[17] It should be noted also that in none of the previous studies in which graft–host bridging fibers have been demonstrated, host photoreceptor cells are visible in the areas of integration.[13,15,17]

It seems remarkable that neurites would not run across the photoreceptor cell layer. Under normal conditions, developing retinal cell processes extend toward the plexiform layers. However, under pathologic conditions, retinal cell neurites grow ectopically. After retinal detachment, calbindin-positive horizontal cells and PKC-positive rod bipolar cells extend neurites into the photoreceptor cell layer.[21] Calbindin- and PKC-positive fibers were also observed in the present study growing into the photoreceptor cell layer in RCS rats, in agreement with previous studies.[33] These observations indicate that the layer of photoreceptor cells, as such, is not averse to the passage of neurites. It is possible that the signals triggering and guiding the reactive sprouting of mature retinal fibers through the photoreceptor cell layer are not the same as those directing the growth of graft-derived fibers through this same layer. However, we did not observe bridging fibers, even when only one row of photoreceptor cells remained. This finding suggests that not the photoreceptor cells themselves, but the molecular environment and/or structural specializations at the outer margin of the photoreceptor cell layer prevent fusion between the graft and the host retina.

Gouras et al.[13] have shown by electron microscopy that graft-derived neurites extend into the host retina through what they term the pseudoexternal limiting membrane in old normal mouse hosts (in which only photoreceptor cell debris was present at the graft–host border) and in transplants to *rd* mice (in which no photoreceptor cells remained at the border). Similar integration was not described or shown through areas with only partial photoreceptor cell loss. A normal external (or outer) limiting membrane (OLM) consists of unique heterotypic (involving Müller cells and photoreceptors) and homotypic (between Müller cells) adherens junctions.[34,35] We found bridging fibers in normal rat recipients between displaced photoreceptor cells that had arranged as rosettes that may have been caused by (or resulted in) severe disruption of the host OLM. We also found bridging fibers wherever host photoreceptor cells were missing in dystrophic hosts. Our observa-

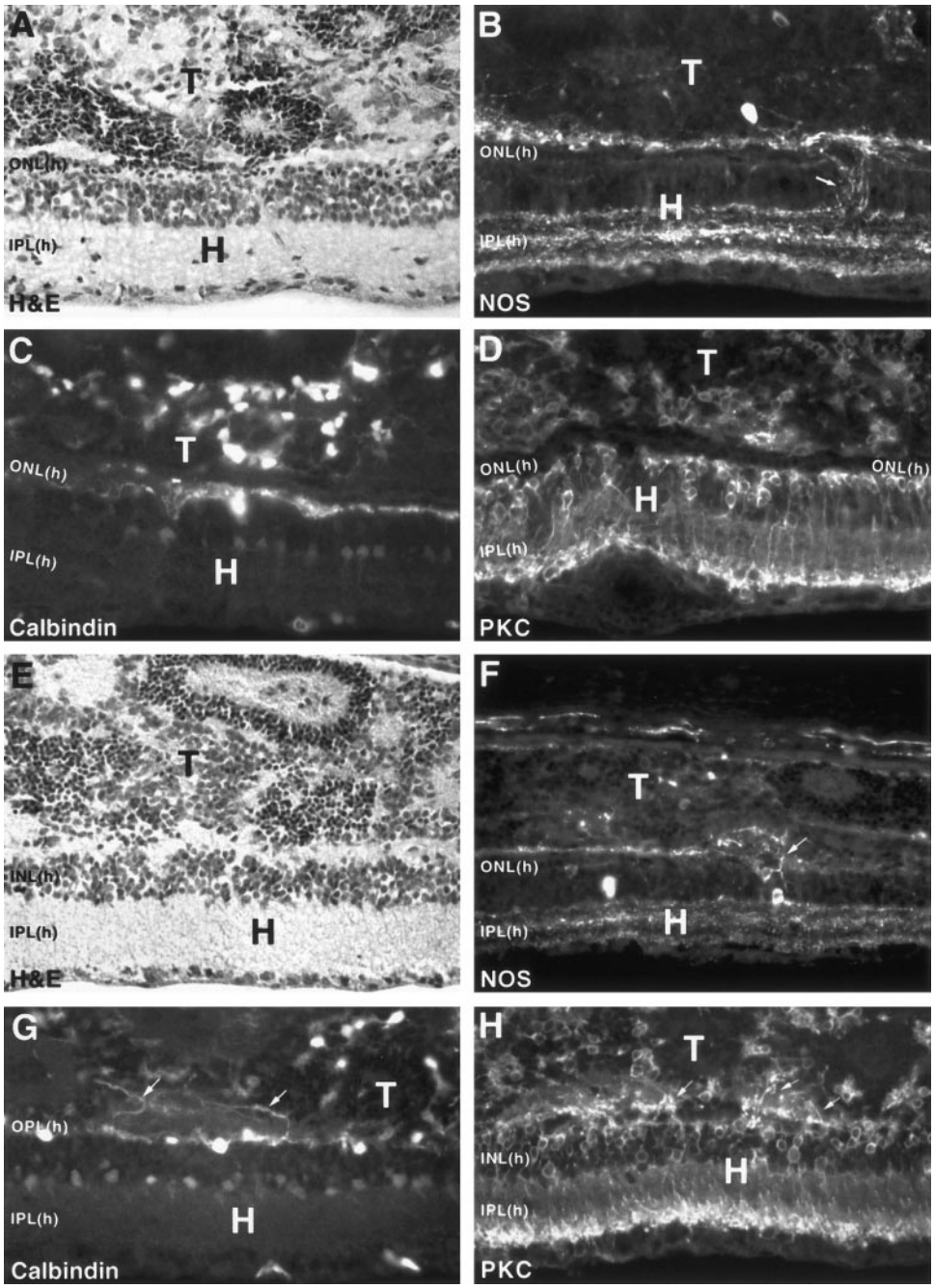


**FIGURE 5.** (**A–D**) Transplantation to P23H rats (SD-P23H) 28 days after surgery (88-day-old host). (**A**) H&E staining showed one to two cell rows of photoreceptors in the host retina (H) next to the transplant (T). Bridging fibers coursed through small areas where the host ONL, ONL(h), was discontinuous (**B**, *arrow*), but not where host photoreceptor cells were present at the graft–host interface. (**B**) NOS, (**C**) calbindin, and (**D**) PKC. (**E–H**) Transplantation to P23H rats (SD-P23H) 48 days after surgery (108-day-old host). (**E**) H&E staining showed only a few scattered photoreceptor cells in the host retina (H). (**F**) In some specimens, one cell row of photoreceptor cells remained in some places in the host retina, ONL(h), and bridging fibers were present only in small areas (NOS immunostaining). Bridging fibers were otherwise generally found traversing larger areas than at the earlier survival time (**G**, **H**, *arrows*). (**G**) Calbindin (**H**) PKC immunostaining. OPL(h), host OPL; INL(h), host INL; IPL(h), host IPL.

tions thus seem to extend those of Gouras et al.,[13] in that breaks in the OLM or a loss of the photoreceptor component of the OLM (which would change its physical properties and/or molecular composition) appear to be necessary for fibers to form bridges.

Further, we observed also in the present study that bridging fibers were present at long survival times after transplantation to young dystrophic animals (in which host photoreceptors were still present at the time of transplantation) and in transplantation to older dystrophic animals (in which most or all photoreceptor cells were lost at the time of transplantation). Degeneration-induced loss of neuronal cells causes activation of glial cells, resulting in cytoskeletal changes, migration, and upregulation of a number of molecules, some of which inhibit neurite outgrowth.[36–38] It is thus conceivable that increased expression of such molecules in a degenerating retina creates a less-permissive host environment for incoming graft-derived fibers. The observations in this and previous studies[13] appear to indicate that some long-term integration can occur, despite the gliotic reaction. However, whether and how integration is affected over time has yet to be determined.

In conclusion, the present study demonstrates that bridging of fibers between a subretinal graft and the host retina occurs only when the host photoreceptor layer is missing or severely damaged, regardless of the genetic background, the age of the host, or the survival time. Further studies are needed to elucidate the mechanisms underlying this phenomenon.

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