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Visual restoration and transplant connectivity in degenerate rats implanted with retinal progenitor sheets

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Abstract

The aim of this study was to determine whether retinal progenitor layer transplants form synaptic connections with the host and restore vision. Donor retinal sheets, isolated from E19 rat fetuses expressing human alkaline phosphatase (hPAP), were transplanted to the subretinal space of thirteen S334ter-3 rats with fast retinal degeneration at the age of 0.8 to 1.3 months. Recipients were sacrificed at the age of 1.6 to 11.8 months. Frozen sections were analyzed by confocal immunohistochemistry for the donor cell label hPAP and synaptic markers. Vibratome slices were stained for hPAP, and processed for EM. Visual responses were recorded by electrophysiology from the superior colliculus (SC) in 8 rats at the age of 5.3 to 11.8 months. - All recorded transplanted rats had restored or preserved visual responses in the SC corresponding to the transplant location in the retina, with thresholds between -2.8 and -3.4 log cd/m². No such responses were found in age-matched S334ter-3 rats without transplant, or in sham surgeries. Donor cells and processes were identified in the host by light and electron microscopy. Transplant processes penetrated the inner host retina in spite of occasional glial barriers between transplant and host. Labeled neuronal processes were found in the host inner plexiform layer, and formed apparent synapses with unlabeled cells presumably of host origin. Conclusions: Synaptic connections between graft and host cells, together with visual responses from corresponding locations in the brain, support the hypothesis that functional connections develop following transplantation of retinal layers into rodent models of retinal degeneration.

Keywords

Retinal degeneration; retinal transplantation; retinal progenitor cells; superior colliculus; human placental alkaline phosphatase; electron microscopy

Introduction

Diseases of the outer retina, such as age-related macular degeneration (ARMD) (Zarbin, 2004; Jager *et al.*, 2008) and retinitis pigmentosa (Kalloniatis & Fletcher, 2004; Kennan *et*

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et al., 2005) affect over 12 million people in the United States alone. In these diseases, photoreceptors and/or RPE are dysfunctional and degenerate. However, the remaining inner neural retina that connects to the brain can still remain functional (Papermaster & Windle, 1995; Santos *et al.*, 1997; Milam *et al.*, 1998; Humayun *et al.*, 1999), although significant remodeling occurs over time (Strettoi *et al.*, 2003; Marc *et al.*, 2007). If the diseased cells can be replaced with new cells that can make appropriate and functional connections with the host retina, a degenerated retina might be repaired and eyesight restored.

Transplantation of freshly isolated retinal progenitor sheets improves and preserves visual responses in the superior colliculus (SC) in different retinal degeneration models which cannot be seen in sham surgeries or age-matched controls (Woch *et al.*, 2001; Sagdullaev *et al.*, 2003; Arai *et al.*, 2004; Thomas *et al.*, 2004; Thomas *et al.*, 2006). Synaptic connections between the transplant and the degenerated host retina have been indicated by trans-synaptic tracing studies (Seiler *et al.*, 2005). In addition, it has been demonstrated that visual responses in the superior colliculus can be traced back to retinal transplants in the subretinal space (Seiler *et al.*, 2008b). However, direct synaptic connections have not been conclusively demonstrated ultrastructurally.

This study investigates transplant-host connectivity of retinal sheet transplants in a rat model of retinal degeneration. Our data indicate that transplanted rats had restored or preserved visual responses in the SC. Importantly, visual restoration correlated with the presence of donor cells and processes that penetrated the inner host plexiform layer, and formed synapses with the host.

Material and Methods

Experimental Animals

For all experimental procedures, animals were treated in accordance with the NIH guidelines for the care and use of laboratory animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and under a protocol approved by the Institutional Animal Care and Use Committee of the Doheny Eye Institute, University of Southern California. All efforts were made to minimize animal suffering and to use only the minimum number of animals necessary to provide an adequate sample size. Thirteen transgenic pigmented S334ter-line-3 retinal degenerate rats expressing a mutated human rhodopsin protein (Sagdullaev *et al.*, 2003) received retinal sheet transplants in one eye at the age of P24 to 38. Of these, eight animals were selected for recording of visual responses in the superior colliculus (see Table 1).

Most of the procedures used in these experiments have been described in detail elsewhere (Woch *et al.*, 2001; Sagdullaev *et al.*, 2003; Thomas *et al.*, 2004).

Donor Tissue

Transgenic rats carrying the human alkaline phosphatase gene (hPAP) (Kisseberth *et al.*, 1999) were used as the source of donor tissue. Rats expressing both GFP and hPAP were used as donors for 8 of the 18 experiments, and were derived from a cross between hPAP and GFP transgenic rats (Hakamata *et al.*, 2001). Rat mating was confirmed by vaginal smears. At day 19 of gestation (day of conception = day 0), fetuses were removed by Cesarean section. A small piece of the fetuses' tails or limbs were tested by histochemistry for hPAP (Kisseberth *et al.*, 1999) to identify transgenic fetuses. Embryos were stored in Hibernate E medium (Brainbits, Springfield, IL) with B-27 supplements (Invitrogen, Carlsbad, CA) for up to 6 hrs. The retinal tissue was flattened in a drop of medium. Retinal progenitor sheets were cut into rectangular pieces of $1\text{--}1.5 \times 0.6$ mm to fit into a previously described custom-made implantation tool (Seiler & Aramant, 1998; Aramant & Seiler,

2002). Immediately before implantation, the tissue was taken up in the correct orientation (ganglion cell side up) into the flat nozzle of the implantation tool.

In 9 of 18 experiments (see table 1), retinal sheets were incubated in BDNF microspheres for at least 1 hour before transplantation (Mahoney & Saltzman, 2001; Seiler *et al.*, 2008a).

Transplant Recipients

Transgenic pigmented S334ter-line 3 rhodopsin mutant rats were used as graft recipients at the age of 26–38 d. The rats were originally produced by Xenogen Biosciences (formerly Chrysalis DNX Transgenic Sciences, Princeton, NJ), and developed and supplied with the support of the National Eye Institute by Dr. Matthew LaVail, University of California San Francisco (<http://www.ucsfeye.net/mlavailRDratmodels.shtml>). Recipients were the F1 generation of a cross between albino homozygous S334ter-line 3 and pigmented Copenhagen rats (Harlan, Indianapolis, IN).

Transplantation Surgery

S334ter-3 rats were anesthetized by intraperitoneal injection of a mixture of 37.5 mg/kg ketamine and 5 mg/kg xylazine in sterile saline and their pupils dilated by 1% atropine sulfate. A small incision (approximately 1 mm) was cut behind the pars plana. A custom-made implantation tool (US patent #6 159 218) was loaded with a retinal progenitor sheet and placed into the subretinal space in the superior nasal quadrant of the host retina. Then the donor tissue was slowly and gently released. The scleral incision was closed with 10-0 sutures. Immediately following the surgery, the fundus of the rat was examined by a contact lens on the cornea to identify the transplant placement. The eyes were treated with gentamycin ointment. Rats recovered from anesthesia in an incubator before they were returned to their cage. As a control, other rats received injections of medium into the subretinal space, using the same instrument and nozzle (sham surgery).

Superior Colliculus Recording

Electrophysiological assessment of visual responses in the SC was performed in 15 of the 21 rats according to the method described previously (Thomas *et al.*, 2005). Rats were dark-adapted overnight. Animals were initially anaesthetized by intraperitoneal injection of ketamine/xylazine (37.5 mg/kg ketamine and 5 mg/kg xylazine) and subsequently by a gas inhalant anesthetic (1.0–2.0% halothane in 40% O₂/60% N₂O) administered via an anesthetic mask (Stoelting Company, Wood Dale, IL). The surface of the right superior colliculus was exposed by suction. Eyes were covered with a custom-made eye-cap to prevent bleaching of the photoreceptors during the surgery, and the eye-cap was removed for visual stimulation. Using nail polish coated tungsten microelectrodes, multi-unit visual responses were recorded extracellularly from the superficial laminae of the exposed SC. At each recording location, up to 16 presentations of a full-field illumination (controlled by a camera shutter) were projected onto a white plexiglas screen placed 10 cm in front of the contralateral eye. The intensity of the light stimulus at the beginning of the recording was $-6.46 \log \text{ cd/m}^2$ and gradually increased (controlled by neutral density filters) until the visual threshold was measured. An interstimulus interval of 6 seconds was used. All electrical activity was recorded using a digital data acquisition system (Powerlab; ADI Instruments, Mountain View, CA, USA) and responses (8–16 sweeps) at each SC site were averaged using Matlab software (R2006b). Blank trials, in which the illumination of the eye was blocked with an opaque filter, were also recorded. As a control, age-matched non-transplanted S334ter rats, sham surgeries and normal pigmented Long-Evans rats underwent the same recording protocol as transplanted rats.

Tissue Processing

Rats were perfusion fixed through the ascending aorta with a mixture of 4% paraformaldehyde and 0.1 – 0.4% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2). The eyes were enucleated, the anterior segment was removed and the posterior eyecup was postfixed in the same fixative overnight at 4°C. After washing the eyecups several times with 0.1M sodium phosphate buffer, the eye cup area containing the transplant was dissected along the dorso-ventral axis and either infiltrated with 30% sucrose overnight, embedded in Optimal Cutting compound (OCT, Sakura Finetek USA, Torrance, CA), and frozen in isopentane on dry ice to cut 10 µm cryostat sections; or embedded in 4% agarose in 0.1M phosphate buffer for vibratome sectioning at 80µm.

Confocal Double-label Immunohistochemistry for Human Placental Alkaline Phosphatase (hPAP) and Synaptic Markers

Cryostat sections were processed for immunohistochemistry using established methods (e.g. Seiler *et al.*, 2008a). After a wash with PBS (phosphate-buffered saline), sections were incubated in 10% goat serum for at least one hour for blocking, followed by incubation in a mixture of primary antibodies overnight at 4°C. After several PBS washes, sections were incubated for 30 – 60 minutes with an appropriate mixture of secondary goat antibodies directed against mouse or rabbit IgG, diluted 1:200 in blocking serum, tagged with either AF488 or Rhodamine Red X (Molecular Probes). After further washes, slides were mounted with DAPI-containing mounting medium (Vectashield, Vector Labs, Burlingame CA).

For detection of the hPAP donor tissue, a monoclonal mouse antibody 8B6 (Chemicon, Temecula, CA) was used at a dilution of 1:400 – 1:500 in combination with the following rabbit antisera: anti-mGluR 6 (metabotropic glutamate receptor 6), 1:200 (Neuromics, Edina, MN); and anti-synapsin 1, 1:500 (Chemicon). Alternatively, a rabbit monoclonal antibody against hPAP was used (clone SP15, Epitomics, Burlingame, CA; dilution 1:50) which required antigen retrieval of dried frozen sections for 20 min. at 70°C using HistoVT One (Nacalai USA Inc., San Diego CA), followed by 3 washes in PBS and incubation in blocking serum. The following monoclonal mouse antibodies were used in combination with the rabbit SP15 antibody: anti synaptophysin, 1:5000 (Sigma, St. Louis, MO); anti-bassoon, 1:600 (Stressgen, Ann Arbor MI), anti PSD-95, 1:500 (Stressgen), and anti-syntaxin 1 (HPC-1), 1:500 (Barnstable *et al.*, 1985) (gift of Dr. Barnstable, now at Penn State College of Medicine, Hershey, CA). As control, primary antibodies were omitted.

Sections were imaged using a Zeiss LSM710 confocal microscope. Stacks were created from 7–12 slices at different focal levels (0.36 µm apart), and analyzed with ZEN software (Zen 2008 light edition, Carl Zeiss MicroImaging, Inc., Thornwood, NY). Images (separate for each color channel) were exported and combined in Adobe Photoshop CS.

Immunohistochemistry for Human Placental Alkaline Phosphatase on Vibratome Slices

Selected vibratome sections were washed five times for 10 min in 0.1M-phosphate buffer (PB) and then were incubated for 15 min. in 1% sodium borohydride (NaBH₄) in 0.1M PB. After washing sections five times for 10 min, the sections were treated for 30 min. in 50% ethanol in PBS followed by three 10 min PB washes. Vibratome sections were washed with phosphate-buffered saline (PBS) (0.01 M Na-phosphate buffer pH 7.2, and incubated for 2 hours in 10% goat serum in PBS/1 % BSA. The sections were incubated at 4°C with different mouse monoclonal antibodies against hPAP-antigen, diluted in blocking serum, under continuous rotation: clones MAB102 (1:5000) (Chemicon, Temecula, CA) and 8B6 (1:1000, 1:2000) (Chemicon, Temecula, CA; Sigma, St. Louis, MO; Cymbus Biotech, Eastleigh, U.K.). After 72 hours, sections were incubated in a 1:200 dilution of biotin-conjugated goat anti-mouse IgG (Chemicon, Temecula, CA, USA) for 18–24 hours at 4°C.

Controls included slices that were incubated without primary antibody, and slices that did not contain any transplant (either from non-surgery eyes or outside the transplant area) that were incubated with hPAP antibodies. After washing 5 times for 10 min. with PBS, slices were incubated overnight at 4°C in Elite ABC conjugated to horse radish peroxidase (Vector Labs, Burlingame CA). After five 10 min. washes with PBS, sections were stained for peroxidase activity by incubation in diaminobenzidine tetrahydrochloride (DAB) substrate kit (Vector Labs). The DAB reaction was stopped at 5–7 min by five 10 min. washes in PBS, followed by 0.05M Tris-HCl buffers. Sections were then exposed to silver-gold toning according to the protocol of Teclariam-Mesbah et al. (Teclariam-Mesbah *et al.*, 1997): (1) 3×10 min sodium acetate 2%; (2) incubation in a freshly made solution consisting of 150 ml 3% methenamine, 20 ml 5% silver nitrate and 20 ml 1% sodium tetraborate for 5 min at 60° C; (3) 3×10 min sodium acetate 2%; (4) 5 min 0.1% gold chloride; (5) 3×10 min sodium acetate 2%; (6) 5 min sodium thiosulfate 3%; (7) 3×10 min sodium acetate 2%; (8) 3×10 min rinse in PBS.

Electron Microscopy

Selected slices were washed 5× with 0.1M cacodylate buffer, postfixed with 3% glutaraldehyde with 0.1M cacodylate buffer, washed 5× with 0.1M cacodylate buffer again, osmicated (1% Osmium in 0.1 M cacodylate buffer), stained en bloc in 1 % uranyl acetate in 50 mM sodium acetate buffer pH 5.2 overnight, and then processed for Epon embedding and electron microscopy. Semithin and ultrathin sections (70nm thick) were cut with a diamond knife. For electron microscopy, semithin sections were cut until the transplant-host interface was clearly identifiable, and then ultrathin sections were cut. Sections were not counterstained. Sections were viewed either in a Philips CM10, Zeiss EM10 or a JEOL1400 electron microscope. Synapses were identified at magnifications above 7500× by presence of synaptic vesicles on the presynaptic side and presence of postsynaptic density on the postsynaptic side.

Quantification of Electron Microscopic Staining

In selected images of the host inner plexiform layer (magnification range between 3000× and 11,500×; 21 hPAP-stained images of 7 animals and 11 control images of 3 animals) silver grains were counted by two independent observers. EM images of immunostained slices were selected based on their content of stained regions; control images were selected randomly from control samples (controls included omission of primary antibody, host retina outside transplant on stained slice, and stained slice of no-surgery eye). Silver grain densities (silver grains/μm²) were statistically compared using Graphpad Version 3.05 software (San Diego, CA) (unpaired t-test with Welch correction).

Results

Visual Responses in the Superior Colliculus (SC) (Table 1, Figure 1)

Fifteen transplanted rats with clear corneas and lenses were selected for electrophysiological evaluation of the visual responses. All rats showed visual responses to low light stimulation (−3.42 to −2.8 log cd/m²) in a small area of the SC corresponding to the placement of the transplant in the retina (see Table 1). Representative examples of traces recorded at different light intensities from the SC of a normal pigmented Copenhagen rat, a 3-month-old non-surgery S334ter-3 rat, and 5-months-old sham surgery and transplanted S334ter-3 rats are shown in Figure 1. Responses recorded from the transplanted rats had comparatively longer latencies than that of normal pigmented rats, and they had a higher background activity than normal rats. No responses were found in age-matched or younger S334ter rats without surgery or with sham surgery at or below a light intensity of −1.0 log cd/m².

Transplant Organization (Light Microscopy)

Eight of the 18 transplants contained laminated areas (examples in Figures 2; 3; and 4) with photoreceptor outer segments in the correct orientation in contact with the host RPE (Figure 4C). All 18 transplants contained areas with photoreceptors in rosettes (arranged in spheres with outer segments in the center of the rosette, surrounded by inner retinal layers) (example in Figure 3 B2; 4 B).

Confocal Analysis of Immunostaining for hPAP and Synaptic Markers (Figures 2, 3)

Figures 2 and 3 show examples of staining of rat #17, using a combination of mouse or rabbit antibodies with rabbit anti synapsin 1, mouse anti-syntaxin (HPC-1), -Synaptophysin (Figure 2), rabbit-mGluR6, mouse-Bassoon and -PSD95 (Figure 3). hPAP staining with either the mouse antibody 8B6 or the rabbit antibody SP15 consistently showed extension of transplant processes past remnants of host cones into the host inner nuclear and inner plexiform layers. Most of these processes were neuronal as they showed double staining for synaptic markers. Processes in the transplant-host interface partially colocalized with the synaptic markers synapsin I (Figure 2A), syntaxin (HPC-1) (Figure 2B), synaptophysin (Figure 2C), and mGluR6 (Figure 3A), indicating that they contained pre- and post-synaptic elements. Transplant processes at the border between host inner nuclear and inner plexiform layer appeared to be post-synaptic as seen with Bassoon staining (Figure 3 B2, B3). PSD-95 staining showed several transplant processes pre-synaptic adjacent to PSD 95 immunoreactive processes in the host outer plexiform layer (Fig 3C). Because of the dense staining of synapses in the host inner plexiform layer with the antibodies synapsin, syntaxin, synaptophysin and bassoon, it was impossible to determine whether transplant processes in the host IPL were pre- or post-synaptic. Similar results were seen in rats #18 –21 (data not shown).

Controls where primary antibodies were omitted did not show any staining (data not shown).

hPAP Staining of Vibratome Slices (Light Microscopy) (Figure 4)

hPAP immunoreactivity was found up to 10 μ m from both surfaces of the vibratome slices, clearly showing the cytoplasm of the donor tissue (examples in Figure 4 A, C1, C2, D). Thus, when the surface of the slice was unevenly oriented, only partial staining was visible (example in Figures 4 C, D). Transplants could be observed extending processes into the host inner nuclear layer, and many hPAP-immunoreactive processes could be observed in the inner plexiform layer overlying the transplant (examples in Figure 4 D). No comparable staining was observed in controls where the primary antibody was omitted (Figure 4 E), or in eyes without a transplant (Figure 2 F), although there was some non-specific marginal staining in the ganglion cell layer close to the retinal surface. Outside the transplant area, no hPAP immunoreactivity was observed in the host inner plexiform layer (Figure 4 G).

hPAP Staining (Electron Microscopy)(Figures 5–7

After gold-silver toning, hPAP immunoreactivity could be identified as silver grains of varying sizes (Figures 6–7) which were much easier to clearly identify in the electron microscope than the DAB precipitate in experiments without silver-gold toning (data not shown; see Peng *et al.*, 2007).

In control slices where the primary antibody was omitted (Figure 5 A–C), or in non-transplanted eyes that had been stained for hPAP (Figure 5 D–I), only a low concentration of silver grains were found. Unspecific silver grains were mainly observable as edge staining outside the tissue (Figure 5A), or as random accumulations inside the tissue (Figure 5 D–I). The unspecific stain was clearly different from the specific stain shown in Figures 6–7. A

significant difference in silver grain densities was observed between stained images and controls (Figure 5 J).

Examples of staining at the transplant-host interface are shown in Figure 6. Donor-derived processes could be observed penetrating the inner nuclear layer (INL) of the host retina (Figures 6 A–D), sometimes forming close, potentially synaptic, contacts with unlabeled cells, presumably belonging to the host (Figures 6 D–F).

In the inner plexiform layer (IPL) of the host retina, many hPAP-immunoreactive neuronal processes could be observed in all experiments (Figure 7). The extent and density of processes varied between experiments (Figure 7). In many instances, labeled processes made apparent synapses with unlabeled host cells (Figures 7 A–F). Most synapses appeared to be conventional; ribbon synapses could be observed rarely (Figure 7A). Labeled processes were found both on the presynaptic (Figures 7 A, D, E) and postsynaptic side (Figures 7 B, C, F) of synapses. The hPAP label obscured some of the synaptic structures. The resolution of membranes was not comparable to tissue processed for conventional electron microscopy because of the difference in fixation and the lack of counterstain with lead citrate.

Discussion

This study documents for the first time direct evidence on the ultrastructural level for synaptic connectivity between retinal progenitor sheet transplants and degenerating host retinas, and provide further evidence that synaptic connectivity between graft and host plays an important role in transplant-mediated visual restoration. These findings confirm and extend our previous observations of restored visual function (Woch *et al.*, 2001; Sagdullaev *et al.*, 2003; Thomas *et al.*, 2004) and synaptic connectivity shown by trans-synaptic tracing (Seiler *et al.*, 2005; Seiler *et al.*, 2008b) of retinal sheet transplants in rodent models of retinal degeneration.

In all cases, retinal sheet transplants restored responses to mesopic light stimulation (-2.8 to -3.4 log cd/m²) in an area of the superior colliculus corresponding to the placement of the transplant in the retina (age up to 12 months, up to 10.5 months after transplantation). The transplant responses had longer latencies than those from normal rats. Non-transplanted or sham surgery S334ter-3 rats only responded to much stronger light intensities (0 to -1.0 log cd/m²) with much longer latencies and lower amplitudes at the age of 2.4 – 3 months and not later (Thomas *et al.*, 2006). It has been proposed that visual responses of transplanted rats are solely due to a rescue effect of the transplant on host cells (MacLaren & Pearson, 2007). However, we have shown that in rats with visual responses in the superior colliculus, retinal sheet transplants do not rescue host cones (Seiler *et al.*, 2008a). In that study, the degenerating host retina overlying the transplant did not contain more red-green opsin immunoreactive cones than the host retina outside the graft. In one experimental group, significantly lesser cones were found in the host retinas over the transplant. In all experiments, the visual responses in the superior colliculus were limited to areas corresponding to the position of the transplant in the host retina (Seiler *et al.*, 2008a). This was contrary to results of another group using rod sheet transplants in the rd mouse model which found a rescue effect of the transplant on host cones (Mohand-Said *et al.*, 2000), similar to our results in the same mouse model using retinal progenitor sheet transplants (Arai *et al.*, 2004).

The present study is the first in combining superior colliculus recordings with ultrastructural analysis of donor cell label. Kwan and colleagues (Kwan *et al.*, 1999) transplanted retinal microaggregates of neonates to old rd mice, and demonstrated that transplantation affected the light-dark preference of the recipient mice. They found evidence for the formation of an

additional synaptic layer at the transplant-host interface at 2.5 weeks after transplantation, but lacked a label for donor cells. Gouras and Tanabe (Gouras & Tanabe, 2003) transplanted microaggregates of newborn retinal cells from a donor mouse strain expressing X-gal in rod photoreceptors to a rd mouse strain expressing X-gal in rod bipolar cells, and analyzed their results by electron microscopy. They observed close non-synaptic contacts between donor rods and recipient bipolar cells, and extensions of glial, rather than neuronal processes between transplant and host. They suggested that those close membrane contacts could provide an indirect means of communication between transplant and host neurons, which could be mediated through the retina to the brain, and which according to them could explain the “weak and delayed” visual responses seen in different transplant models (Radner *et al.*, 2001; Woch *et al.*, 2001). However, with our retinal sheet transplant model, we have seen robust - although delayed - visual responses in different retinal degeneration models. In the S334ter line 3 rat model, we have demonstrated that synapses are involved in the visual responses from the superior colliculus of transplanted rats and that the degree of trans-synaptic labeling of the transplant from the host SC is correlated with the visual threshold in SC recordings (Seiler *et al.*, 2008b). The current study is very different both in design and methodology than the previous trans-synaptic studies (Seiler *et al.*, 2005) (Seiler *et al.*, 2008b) which showed indirect evidence of synapses by injection of pseudorabies virus into the visual responsive site of superior colliculus. The current study shows direct evidence of transplant processes penetrating into the host retina and label on the electron microscopic level.

The ability of retinal progenitor cell transplants to connect and integrate into a degenerative host retina has been questioned. MacLaren and colleagues demonstrated that only postnatal stages of dissociated retinal progenitor cells, expressing the photoreceptor precursor marker Nrl, integrated into the retina to a very limited extent (0.1–0.3% of transplanted cells) following transplantation (MacLaren *et al.*, 2006; MacLaren & Pearson, 2007). However, their study was not comparable because it was conducted using dissociated cells, and the mouse models MacLaren used were different from our rat model. In our study, we found evidence of numerous neuronal processes from the transplant penetrating the inner plexiform layer of the host retina. No such staining was found in slices taken far away from the transplant, or in non-surgery eyes that were stained with the same antibodies. Supporting previous trans-synaptic tracing studies (Seiler *et al.*, 2005; Seiler *et al.*, 2008b), our current results indicate that the connectivity with the host occurs via the inner retinal cells of the transplanted fetal retinal sheet (bipolar cells, horizontal cells, amacrine cells etc.), and not through direct connections of transplant photoreceptors with host cells. However, only very few ribbon synapses could be demonstrated that would indicate synapses of photoreceptors or bipolar cells. Preliminary data in another study indicate that any major communication between host and transplant is likely to involve amacrine cells, both glycinergic and GABAergic. Such synapses would not involve classic ribbon synapses.

One shortfall of our study is that we did not use the hPAP antibody in combination with synaptic markers at the EM level. We have stained previously for synaptic markers at the light microscopic level where we used synaptic markers on sections adjacent to hPAP-stained sections (e.g., Seiler *et al.*, 2008a), but it would not have been compatible with the tissue processing for electron microscopy in this study. Therefore, a parallel set of animals was processed for immunohistochemistry and confocal analysis of a combination of donor cell and synaptic markers. Most of the markers used label presynaptic structures. E.g., synapsin 1, syntaxin and synaptophysin are all elements of synaptic vesicles. Syntaxin 1 is a SNAP (synaptosome-associated protein) receptor (SNARE) protein that is expressed in conventional synapses and along amacrine cell processes and cell bodies (Barnstable *et al.*, 1985; Sherry *et al.*, 2006). Synapsins regulate the reserve pool of synaptic vesicles (Gitler *et al.*, 2004). mGluR6 is a specific receptor in on-bipolar cells (review: Duvoisin *et al.*, 2005;

Snellman *et al.*, 2008). Bassoon is a cytomatrix component in the active synaptic zone, and found in the retina in photoreceptor ribbon and amacrine conventional synapses, not in bipolar ribbon synapses (Brandstatter *et al.*, 1999; tom Dieck *et al.*, 2005). Photoreceptor synaptic transmission is severely disturbed in bassoon knockout mice (Dick *et al.*, 2003).

The confocal and electron microscopic data indicate that transplant processes were both pre- and post-synaptic to hPAP-negative, presumable host cells. At the transplant-host interface in the host outer plexiform layer, there was only partial colocalization of transplant processes with synapsin and synaptophysin. Syntaxin immunoreactivity appeared to overlap much more suggesting that many of the extending transplant processes were derived from amacrine cells. This fits with the observation that few hPAP-labeled ribbon synapses were found at the transplant-host interface by electron microscopy. Transplant processes were seen directly adjacent to synaptophysin and mGluR 6 labeled structures indicating that they were post-synaptic, but also directly adjacent to PSD-95 labeled structures indicating that they were presynaptic.

During the progression of photoreceptor degeneration in the host retina, other retinal cell types are also becoming affected. The inner retina responds with progressive remodeling, formation of new synaptic circuits, rewiring and cell death (Jones *et al.*, 2003; Strettoi *et al.*, 2003; Jones & Marc, 2005) (for review, see Marc *et al.*, 2007). As our transplants were performed in an early stage of this process, it is conceivable that the recipient's cells were particularly receptive for new contacts from the healthy, actively differentiating transplant cells.

In summary, our data indicate that visual responses in the SC were restored in a rodent model of retinal degeneration following subretinal transplantation of retinal progenitor cell layers. Importantly, visual restoration correlated with the presence of donor cells and donor processes that penetrated the inner host plexiform layer. This is the first indisputable demonstration of synapse formation between the host and transplant in this system, evidenced by confocal and electron microscopy.

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List of Abbreviations in Text and Figures

BDNF	Brain-derived neurotrophic factor
CNS	central nervous system
DAB	diaminobenzidine
DAPI	4'6'-diamidino-2-phenylindole hydrochloride
GC	ganglion cell layer
H	host retina
hPAP	human placental alkaline phosphatase

IN	inner nuclear layer
IP	inner plexiform layer
mGluR6	metabotropic glutamate receptor 6
ms	milliseconds
ON	outer nuclear layer
OP	outer plexiform layer
OS	outer segments
PB	phosphate buffer
PBS	phosphate-buffered saline
RPC	retinal progenitor cells
SC	superior colliculus
RCS	Royal College of Surgeons
RPE	retinal pigment epithelium
T	transplant

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Figure 1. Examples of SC recordings at different light intensities

normal pigmented rat; S334ter rat without surgery (age 3 months); sham surgery rat #16 (age 5.4 months); retinal transplants rat #3 (age 5.3 months), #17 and #20 (age 3.0 – 3.1 months). The light stimulus (duration 85 ms) is indicated at the bottom of each panel. The onset of light responses is indicated by arrows. **A**) At a light intensity $-0.4 \log \text{cd/m}^2$, faint responses with low amplitudes and long latencies can be recorded in the non-surgery and sham surgery S334ter-3 rat, whereas the transplanted rats (#3 and #20) show a robust response in one area that is only slightly delayed compared to normal. Transplanted rats show a more noisy background activity. **B**) At the slightly reduced light intensity of $-1.0 \log \text{cd/m}^2$, no responses can be observed in the non-surgery and sham surgery S334ter-3 rat whereas the response from the transplant rats (#3 and #20) remain robust. **C**) At a much reduced light intensity of $-3.4 \log \text{cd/m}^2$, there is no response in the sham surgery rat. In the transplant rats (#3 and #17), there are still clear responses, although with a much longer latency than in the normal rat. [Rat #20 had a response threshold of $-2.8 \log \text{cd/m}^2$ (not shown) and did not respond at this light intensity.]

Figure 2. Combination of donor cell label (hPAP) with the pre-synaptic markers synapsin, syntaxin (HPC-1), and synaptophysin (confocal imaging)

All images are of rat # 17. Similar images were obtained from rats #18–21. All images are oriented with the host ganglion cell layer up. White asterisks (*) indicate nuclei of remnant host cones (containing clumped chromatin). The cytoplasm, including processes of all transplant cells (not the nuclei) are labeled by hPAP (green).

(A1–2) Combination of mouse anti hPAP (8B6, green) in combination with rabbit anti synapsin (red, marker for synaptic vesicles and synaptic terminals), and DAPI nuclear label (blue). Both images are three-dimensional (3D) stacks. (A1) Overview. Transplant processes extend past remnants of host cones to the outer plexiform layer of the host. In addition, there

are numerous hPAP stained processes in the inner plexiform layer of the host, mostly obscured by the synapsin stain. Arrowhead points to a group of processes that is visible at this level. The white dashed box indicates enlargement in A2. **(A2)** Enlargement of transplant-host interface. Examples of areas with potential transplant-host synaptic interactions (transplant processes close to red stained synaptic structures outside transplant) are indicated by arrows. The black space in the transplant-host interface is a cutting artifact. **(B1–3)** Rabbit anti hPAP (SP15) (green) in combination with mouse anti syntaxin (HPC-1) (red). Syntaxin stains synaptic layers and somas of amacrine cells. **(B1)** Overview of transplant. Composition of two single slices at the same focus level. Note the overlap of red and green channels in the transplant-host interface. Box with green dashes indicates area shown in B2; box with red dashes indicates area shown in B3. **(B2)** 3D stack of transplant-host interface, showing hPAP staining (green channel). Transplant processes are extending into the host inner nuclear layer. Numerous fine processes can be seen in the host inner plexiform layer (arrow heads). **(B3)** higher enlargement of transplant-host interface, 3D stack of red and green channel. Arrow points to colocalization of hPAP and syntaxin in transplant process.

(C1–4) Rabbit anti hPAP (SP15) (green) in combination with mouse anti synaptophysin (red, marker for synaptic vesicles) (slide adjacent to Fig. 2B). **(C1)** Overview of transplant. Projection of stack at several focus levels. Box with green dashes indicates area shown in C2; box with white dashes indicates area shown in C4. Arrow points to area enlarged in C3. **(C2)** 3D stack of transplant-host interface, hPAP staining (green channel). Arrowheads point to transplant processes close to host ganglion cell layer. Note the transplant process extending into the host inner nuclear layer on the right side. **(C3)** Enlargement of transplant-host interface in C1, single slice. Arrow points to transplant process in contact with synaptophysin immunoreactive process, presumably from host. **(C4)** 3D stack of same area at higher magnification, making it clear that the transplant process is indeed contacting a host-derived process (arrow). Bars = 20 μm (A1, A2, B1, C1), 10 μm (B2, B3, C2–C4).

Figure 3. Combination of donor cell label (hPAP) with the synaptic markers mGluR6, bassoon, and PSD95 (confocal imaging)

All images are of rat # 17. Similar images were obtained from rats #18–21. All images are oriented with the host ganglion cell layer up. White asterisks (*) indicate remnants of host cone nuclei with clumped chromatin. Black spaces at the transplant edge towards the host are a cutting artifact.

(A1–3) Combination of mouse anti hPAP (8B6, green) with rabbit anti mGluR6 (marker of synaptic terminals of on-bipolar cells; red); 3D stack images (section adjacent to Fig. 2A).

(A1) Overview. mGluR6 stains the dendritic tips of host and graft on-bipolar cells in the outer plexiform layer. Transplant processes extending past host cones, and penetrating host

inner nuclear layer. There are also numerous processes in the host inner plexiform layer close to the inner nuclear layer (arrow heads). White dashed box indicates area of enlargement in A2. **(A2)** Enlargement, showing interaction between transplant processes and host on-bipolar cell dendritic tips. White dashed box shows area of enlargement in A3. **(A3)** Transplant process penetrating host inner nuclear layer. Arrow points to process double-stained for hPAP and mGluR6.

(B1–3) Rabbit anti hPAP (SP15) (green) in combination with mouse anti Bassoon (marker for ribbon synapses, red) (section adjacent to Fig. 2B). **(B1)** Overview of same area as in Fig. 2B. DAPI nuclear counterstain. Arrows point to transplant-host interface in host outer plexiform layer. Note row of red dots indicating ribbon synapses in outer plexiform layer of host. Some of the dots are just outside, but close to transplant processes. **(B2)** Adjacent area on same section (red and green channel only) at a more disorganized transplant area where photoreceptors have rolled up into a rosette. Note numerous transplant processes in the host inner plexiform layer (arrow heads). Box indicates area of enlargement in B3. **(B3)** Arrow head points to thick process at border of host inner nuclear and inner plexiform layer.

(C1–2) Rabbit anti hPAP (SP15) (green) in combination with mouse anti PSD95 (post-synaptic marker; red) (section adjacent to Fig. 2B and Fig. 3B). **(C1)** Overview, single slice. Arrow heads point to transplant processes in contact with PSD95 immunoreactive processes in host inner plexiform layer. **(C2)** 3D stack of area in white dashed box in C1. Bars = 20 μm (A1, A2, B1, B2, C1), 10 μm (A3, B3, C2).

Figure 4. Identification of the transplant by immunohistochemistry for human placental alkaline phosphatase (hPAP) – light microscopy

Dashes indicate approximate transplant-host border, where applicable. **A)** Vibratome slice, thickness 100 μm (rat #3), stained with monoclonal antibody 8B6 against hPAP (dilution 1:1000). The transplant inner plexiform and outer plexiform layers appear dark brown. **B)** Control slice (rat #7), incubated with blocking serum instead of primary antibody, shows even light brown stain. **C)** Example of stained vibratome slice (Rat #5). This transplant has developed an area with photoreceptors in normal orientation and outer segments (indicated by asterisks). The DAB stain penetrated the surface of the 80–100 μm -thick vibratome slices up to a depth of approximately 10 μm . Thus, if the surface of the tissue is unevenly oriented

and sectioned, as seen in A) and B), only partial staining is visible at a given sectioning level. **C1)** DAB stain at transplant-host interface. **C2)** Stain of transplant photoreceptors was seen in previous sections of this slice. **D)** Rat #1. DAB stain of transplant photoreceptors and in host inner plexiform layer. The approximate border between transplant and host is indicated by dashes. Infiltration of the inner plexiform layer of the host retina by many labeled graft processes (examples indicated by arrow heads). The stained transplant-host interface had been seen in previous sections because the slice was not embedded perfectly flat. – **E)-G) Controls.** **E)** Section through control slice of rat # 7 that had been incubated with blocking serum instead of primary antibody. Non-specific edge staining in the ganglion cell layer and blood vessels is apparent, but no stain exists in the transplant. **F)** Section through non-surgery fellow eye (degenerate retina without photoreceptor layer) of rat#13 without transplant. No stain. The RPE cells (arrowhead) contain black melanin granules. **G)** Section of host retina outside transplant area (rat #9). No stain. Bars = 100 μm (A), 50 μm (B, C2), 20 μm (C1, E–G).

Figure 5. Examples of controls in the electron microscope

Unspecific silver grains can be seen as edge stain in A), and are indicated by arrows in (D–I). This unspecific stain is clearly different from the specific stain shown in Figures 5–8. (A–C) Omission of primary antibody (blocking serum control, rat #11). (D–F) Host retina outside transplant area, stained with hPAP antibody 8B6 (rat #9). (G–I) Non-surgery fellow eye, stained with hPAP antibody MAB102 (rat #12). **J**) Diagram: Silver grains in selected images of the host inner plexiform layer of transplants and controls were counted by 2 independent observers. The results were averaged and expressed as silver grains/μm². Error bars indicate S.E.M.

Figure 6. Processes at transplant – host interface (EM)

The hPAP immunolabel can be recognized as darkness at lower magnification (A, C) and is confirmed by silver grains (dark dots) at high magnification (B, D, E–F). Note transplant processes extending into the inner nuclear layer of the host retina (A–D). Boxes in A, and C indicate areas of enlargement in B and D, respectively. E, F) Labeled transplant processes in close contact with unlabeled processes of presumable host cells. Box in E) shows enlarged process in insert. – (A) Low power overview (rat #10). (B) Enlargement. Absence of silver grains in the host tissue. (C, D) Rat #13. (E, F) Rat #11. Bars = 1 μ m (A, C), 0.5 μ m (E), and 0.2 μ m (B, D, F).

Figure 7. Transplant processes and synapses in inner plexiform layer of the host retina
 Immunohistochemistry for hPAP, recognizable as silver grains. Arrows indicate a presynaptic element of an apparent synapse between transplant and host cell. A) Labeled ribbon synapse. Long synaptic ribbon indicated by asterisks. Labeled processes are pre-synaptic in A, D, E, and post-synaptic in B, C, F. (A, E) Rat # 6. (B) Rat #13. (C) Rat #10. (D,F) Rat # 8. Bars = 0.2 μm.

Table 1

Overview of experiments

Rat #	Donor tissue	Age at surgery (mo.)	Age at sacrifice (mo)	Time after surgery (mo)	Visual threshold at SC recording (log cd/m ²)	Method of analysis
1	E19 retina + BDNF	0.9	1.6	0.7	n.a.	EM
2 *	E19 retina + BDNF	0.8	2.8	2.0	n.a.	EM
3	E19 retina + BDNF	1.2	5.3	4.1	- 3.4	EM
4	E19 retina	1.1	5.4	4.3	- 2.8	EM
5 *	E19 retina	1.1	5.4	4.3	- 2.8	EM
6	E19 retina + BDNF	0.9	6.9	6.1	- 2.8	EM
7	E19 retina + BDNF	0.9	7.6	6.7	- 2.8	EM
8	E19 retina	1.0	8.9	8.0	n.a.	EM
9	E19 retina	1.0	8.9	8.0	n.a.	EM
10	E19 retina	1.0	10.1	9.1	n.a.	EM
11	E19 retina + BDNF	1.3	11.6	10.3	- 2.8	EM
12	E19 retina + BDNF	1.3	11.7	10.5	- 2.8	EM
13	E19 retina + BDNF	1.3	11.8	10.5	- 2.2	EM
14	Sham surgery	1.0	2.7	1.6	No response	Light microscopy
15	Sham surgery	0.8	3.1	2.3	No response	Light microscopy
16	Sham surgery	1.1	5.4	4.2	-0.4	Light microscopy
17	E19 retina	0.9	3.0	2.1	- 3.4	confocal
18	E19 retina	0.9	3.0	2.1	- 2.8	confocal
19	E19 retina + BDNF	0.9	3.0	2.1	- 2.8	confocal
20	E19 retina	0.9	3.1	2.2	- 2.8	confocal
21	E19 retina	0.8	3.5	2.7	Not recorded	confocal
21 rats	9 retina + BDNF 9 retina only 3 sham surgery	0.8- 1.3	1.6-11.8	0.7 - 10.5 mo.	15 of 21 recorded	

Experiments are ordered according to age at sacrifice and time post-surgery. Slices of rats # 6 - 13 were processed for gold-silver toning after hPAP immunostaining. "+ BDNF" indicates that donor tissue was incubated with BDNF containing microspheres before transplantation. Rats # 14-16 (sham surgery), and # 17 - 21 were only processed for light microscopy (rats # 17 - 21 were studied by confocal immunohistochemistry).

* Histology of rat #2 and 5 was shown in Peng *et al.*, 2007 without silver-gold toning of hPAP immunostaining. For the current paper, additional slices were immunostained for hPAP with silver-gold toning and processed for EM.