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BDNF-treated retinal progenitor sheets transplanted to degenerate rats: Improved restoration of visual function

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Abstract

The aim of this study was to evaluate the functional efficacy of retinal progenitor cell (RPC) containing sheets with BDNF microspheres following subretinal transplantation in a rat model of retinal degeneration. Sheets of E19 RPCs derived from human placental alkaline phosphatase (hPAP) expressing transgenic rats were coated with poly-lactide-co-glycolide (PLGA) microspheres containing brain-derived neurotrophic factor (BDNF) and transplanted into the subretinal space of *S334ter* line 3 rhodopsin retinal degenerate rats. Controls received transplants without BDNF or BDNF microspheres alone. Visual function was monitored using optokinetic head-tracking behavior. Visually evoked responses to varying light intensities were recorded from the superior colliculus (SC) by electrophysiology at 60 days after surgery. Frozen sections were studied by immunohistochemistry for photoreceptor and synaptic markers. Visual head tracking was significantly improved in rats that received BDNF-coated RPC sheets. Relatively more BDNF-treated transplanted rats (80%) compared to non-BDNF transplants (57%) responded to a “low light” intensity of 1 cd/m² in a confined SC area. With bright light, the onset latency of SC responses was restored to a nearly normal level in BDNF-treated transplants. No significant improvement was observed in the BDNF-only and no surgery transgenic control rats. The bipolar synaptic markers mGluR6 and PSD-95 showed normal distribution in transplants and abnormal distribution of the host retina, both with or without BDNF treatment. Red-green cones were significantly reduced in the host retina overlying the transplant in the BDNF-treated group. In summary, BDNF coating improved the functional efficacy of RPC grafts. The mechanism of the BDNF effects—either promoting functional integration between the transplant and the host retina and/or synergistic action with other putative humoral factors released by the RPCs—still needs to be elucidated.

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Keywords: retinal transplantation; superior colliculus; head-tracking behavior; BDNF microsphere; retinal progenitor cells; *S334ter*

Abbreviations: BDNF, brain-derived neurotrophic factor; DAPI, 4'6'-diamidino-2-phenylindole hydrochloride; CNS, central nervous system; 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 (synaptic receptor on bipolar cells); ms, milliseconds; ON, outer nuclear layer; OP, outer plexiform layer; OS, outer segments; PBS, phosphate-buffered saline; PKC, protein kinase C (marker for rod bipolar cells); PLGA, poly-lactide-co-glycolide; PSD-95, post-synaptic density protein 95 (post-synaptic marker on bipolar cell dendrites); RG-opsin, red-green opsin; RPC, retinal progenitor cells; SC, superior colliculus; RCS, Royal College of Surgeons; RPE, retinal pigment epithelium; T, transplant.

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1. Introduction

Retinal degenerations such as age-related macular degeneration and retinitis pigmentosa lead to irreversible vision loss. Animal models of retinal degeneration have allowed investigators to better elucidate the sequence of physiological and biochemical changes associated with these diseases (Jones and Marc, 2005; Marc et al., 2003). These studies have also provided investigators with information to assist in optimizing therapeutic strategies aimed at preventing visual loss and or restoring lost vision. Therapeutic interventions in animal models, including growth factor treatment, gene therapy, retinal prosthetics and retinal cell transplantation, have been presumed to improve visual sensitivity (Aramant and Seiler, 2002, 2004; Chaum, 2003; Delyfer et al., 2004; Loewenstein et al., 2004; Lund et al., 2001) by delaying the progression of the disease and/or by rescuing the remaining host photoreceptors. Some studies of retinal sheet transplants have suggested that transplanted photoreceptors may also contribute directly to the visual restoration (Aramant and Seiler, 2004; Seiler et al., 2005a). After injecting freshly harvested retinal progenitor cells in the rho^{-/-} mouse, increased ganglion cell responses and pupillary reflexes have been interpreted as visual improvements (MacLaren et al., 2006), similar to previous results by other groups (Kwan et al., 1999; Radner et al., 2002). Clinical retinal sheet transplantation studies also demonstrated an improvement in visual sensitivity in an RP patient (Radtke et al., 2004) that is still maintained after 5 years (Radtke et al., 2007; unpublished observations).

Our group has developed a specially designed instrument and procedure to deliver sheets of fetal retinal neuroblastic progenitor cells into the subretinal space (Aramant and Seiler, 2002, 2004). Such transplants develop both inner and outer segments (Seiler and Aramant, 1998; Seiler et al., 1999), show a shift in the distribution of phototransduction proteins according to the light cycle (Seiler et al., 1999) and remain healthy for many months. In four different retinal degeneration models, such transplants have been shown to restore visual responses in the superior colliculus (SC) after long survival times of 2–8 months (Arai et al., 2004; Sagdullaev et al., 2003; Thomas et al., 2004a; Woch et al., 2001). Synaptic connectivity between transplants and host retina has been demonstrated by trans-synaptic virus tracing (Seiler et al., 2005a). The visually responsive site in the SC can be traced back to the retinal transplant by trans-synaptic virus tracing (Seiler et al., 2005b; manuscript submitted), indicating that the area of transplant in the retina is the origin of visual restoration in the SC. However, in most transplantation experiments, a considerable level of visual restoration was apparent only in a very small SC area indicating that the area of integration between the transplant and the host retina is limited.

Among the various neurotrophins involved in the development and integration of the central nervous system (CNS), the modulatory influence of BDNF is well established, especially during the development of the visual sensory system (Berardi and Maffei, 2004). Studies performed in normal and retinal degenerate rat models demonstrate that BDNF has a neuro-

protective role (Chaum, 2003; Gauthier et al., 2005; Ikeda et al., 2003; Keegan et al., 2003; Lawrence et al., 2004; Nakazawa et al., 2002; Paskowitz et al., 2004). Therefore, the present investigation evaluates the possible role of BDNF in promoting the functional efficacy of the retinal neuroblastic sheets transplanted into the subretinal space of retinal degenerative rats.

2. Materials and methods

2.1. 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, 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 for statistically meaningful scientific conclusions. Twenty-nine transgenic pigmented *S334ter* line 3 retinal degenerate rats expressing a mutated human rhodopsin protein (Sagdullaev et al., 2003) were light-damaged for 5–6 days with blue light (Seiler et al., 2000) to accelerate photoreceptor degeneration, starting at postnatal day (P) 22–26, and then received retinal sheet transplants in one eye shortly afterwards at the age of P24–37.

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

2.2. BDNF microspheres

BDNF microspheres (average diameter $1 \pm 0.5 \mu\text{m}$) were prepared using a previously described methodology (Mahoney and Saltzman, 2001). Briefly, 300 mg of (50:50) PLGA ($M_n = 54,100$ Birmingham Polymers) was dissolved in 2 ml of methylene chloride. 100 μl of a solution containing 500 μg BDNF was added to the dissolved PLGA on ice. The solution was sonicated for 10 s at 40% amplitude to yield a homogeneous mixture. Four milliliters of aqueous 1% polyvinyl alcohol (PVA, $M_w = 25,000$, Polysciences) was added to this emulsion and vortexed for 10 s. This double-emulsion was poured into a beaker with 100 ml of aqueous 0.3% PVA and stirred for 3 h to achieve microsphere formation. Microspheres were collected by centrifugation at 2000 rpm for 10 min and washed three times with deionized water before they were frozen in liquid nitrogen and then lyophilized for 24 h. Microspheres were stored at 4 °C until use. The BDNF release rate (based on 1 mg microparticles) is shown in Fig. 1.

Immediately before transplantation, a suspension of 5 mg BDNF microspheres (corresponding to 8.3 μg BDNF) was incubated for 1 h in 10 $\mu\text{g}/\text{ml}$ poly-DL-lysine in PBS to give the microspheres a positive charge to enhance tissue adherence.

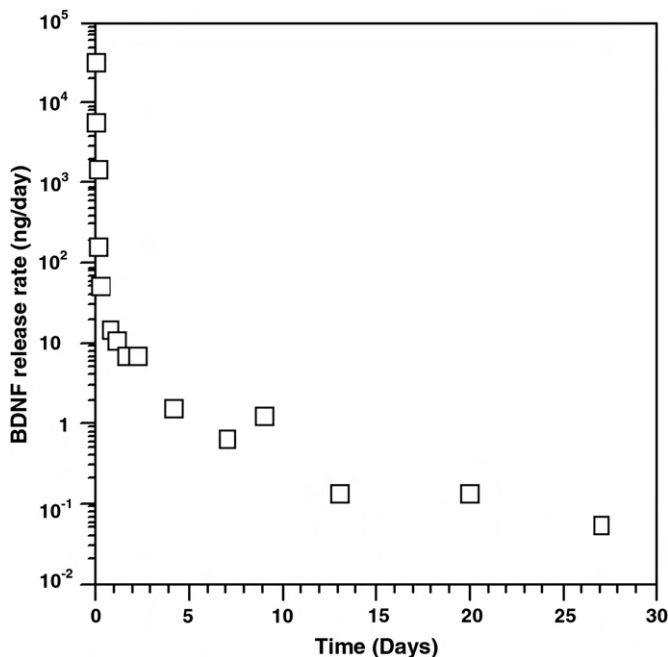


Fig. 1. Release rate of BDNF, based on 1 mg of nanoparticles, containing 1.66 μ g of BDNF.

After washing three times with Hibernate E medium (Brain-Bits, Springfield, IL) with B-27 supplements (Gibco BRL, Baltimore MD), the microspheres were resuspended in Hibernate E medium at a concentration of 5 mg microspheres/ml (8.3 μ g BDNF/ml).

2.3. Donor tissue

Transgenic rats carrying the human placental alkaline phosphatase gene (hPAP) (Kisseberth et al., 1999) were used as the source of donor tissue for these studies. 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 with B-27 supplements for up to 6 h. Hibernate E medium is specially formulated to maintain embryonic tissue alive when refrigerated without oxygen or CO₂. The retinal tissue was flattened in a drop of medium. For BDNF microsphere treatment, a drop of a freshly prepared BDNF microsphere solution (50 μ l, corresponding to 41.5 ng BDNF) was added to the retinal tissue which was then incubated for at least 2 h on ice with gentle agitation to attach the microspheres to the donor tissue. Retinal progenitor sheets were cut into rectangular pieces of 1–1.5 \times 0.6 mm to fit into the previously described custom-made implantation tool (Aramant and Seiler, 2002; Seiler and Aramant, 1998). Immediately before implantation, the tissue was taken up in the correct orientation (ganglion cell side up) into the flat nozzle of the implantation tool. The orientation of the donor tissue could easily be observed in the dissection microscope. It was difficult to estimate the total volume of microspheres (i.e. the concentration of BDNF) that

attached to the donor tissue, but it was in any case less than 0.5 μ l of the microsphere solution (i.e. less than 8 ng BDNF).

2.4. Transplant recipients

Transgenic pigmented *S334ter* line 3 rhodopsin mutant rats were used (age at surgery 24–37 days). 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).

Ten rats received transplants coated with BDNF microspheres, and seven rats received transplants without microsphere coating. Six rats received injections of BDNF microspheres only (ca. 1 μ l = 8.3 ng BDNF). Six age-matched rats without surgery were tested as negative controls.

2.5. Transplantation procedure

The transplantation procedure was performed according to previously described methods (Aramant and Seiler, 2002). Rats were anaesthetized by intraperitoneal injection of ketamine/xylazine (37.5 mg/kg ketamine and 5 mg/kg xylazine). A small incision (0.5–1 mm) was made just posterior to the pars plana, parallel to the limbus. The implantation instrument was inserted with extreme care to minimize disturbance of the host RPE. The graft was released into the subretinal space posteriorly near the optic disc. Transplants were placed into only one eye, leaving the other eye as a control. The incision was closed with 10–0 sutures and the eyes were treated with gentamycin eyedrops and artificial tears. The rats were placed in an incubator for recovery.

2.6. Optokinetic testing

The testing apparatus (Thomas et al., 2004b) consisted of interchangeable circular drums with black and white stripes rotating at a velocity of 2 turns/min around a stationary holder in which the rat sits unrestrained. Rats were tested at a spatial frequency of 0.25 cycles/degree (medium stripes). Three light sources were used to evenly illuminate one half of the drum. The optokinetic testing was done under photopic light conditions. The other half of the drum was concealed from the rat by positioning a stationary black wall to block the light path. The rat holder was positioned in the apparatus to expose only one eye to the rotating stripes, thereby permitting unilateral optokinetic testing. Each rat was tested for 4 min during one session, 2 min for each eye: 1 min in one direction of drum rotation and 1 min in the other direction. Videotapes of the rat's head movements were subsequently analyzed. The amount of time the rat spent head tracking was calculated separately for each eye in a masked fashion (the examiner was

not aware if the animal was a transplanted or control animal). Rats were tested at 5, 8 and 11 weeks of age.

2.7. Electrophysiology

Electrophysiological recordings were made in the SC of transgenic rats with retinal transplants ($n = 17$), non-surgery transgenic controls ($n = 6$) or normal pigmented rats ($n = 4$) from 11 to 14 weeks of age, ca. 60 days post-surgery. For electrophysiological assessment of visual responses in the SC (Thomas et al., 2004a), rats were dark-adapted overnight and then the eyes were covered with a custom-made eye cap. After ketamine/xylazine anesthesia (see above), the gas inhalant anesthetic (1.0–2.0% halothane in 40% O₂/60% N₂O) was administered via an anesthetic mask (Stoelting Company, Wood Dale, IL). Rats were mounted in a stereotaxic apparatus, a craniotomy was performed and the SC was exposed. Multi-unit visual responses were recorded extracellularly from the superficial laminae of the SC using nail polish-coated custom-made tungsten microelectrodes. Recording sites (200–400 μm apart) covered the full extent of the SC with the exception of its medial area, which was located just under the superior sagittal sinus. At each recording location, up to 16 presentations of a full-field strobe flash (1.0–1300 cd/m^2 , Grass model PS 33 Photic stimulator, W. Warwick, RI), positioned 30 cm in front of the rat's eye, were delivered to the contralateral eye. The luminance level of the flash was controlled using neutral density filters. An interstimulus interval of 6 s was used. All electrical activity was recorded using a digital data acquisition system (Powerlab; ADI Instruments, Mountain View, CA) 100 ms before and 500 ms after the onset of the stimulus and all responses at each site were averaged. Blank trials, in which the illumination of the eye was blocked with an opaque filter, were also recorded at each site.

The following characteristics of visual responses were analyzed: (1) response onset latency, defined as the point at which a clear, prolonged (>20 ms) increase in the light evoked activity could be measured above background (which was determined using the 100 ms of activity preceding the light flash) and (2) peak response amplitude, defined as the largest excursion peak to peak in the averaged response.

2.8. Histology

At the end of the recording session, animals were euthanized with an overdose of halothane, eyes were immersion fixed in 4% paraformaldehyde in 0.1 M Na-phosphate buffer for 2–4 h and washed three times with 0.1 M Na-phosphate buffer. Dissected eye cups, oriented along the dorso-ventral axis, were infiltrated with 30% sucrose overnight, and frozen in Tissue Tek on dry ice. Transverse sections of the retina were cut at 10 μm thickness on a cryostat and mounted on to slides. Every 5th slide was stained by histochemistry for hPAP (Kisseberth et al., 1999; Mujtaba et al., 2002). A series of sections through the full extent of each transplant were evaluated at the light microscopic level by immunohistochemistry for various markers (see below).

Frozen sections were washed with phosphate-buffered saline (PBS) and blocked for at least 30 min in 20% goat serum. Sections were incubated in primary antibodies overnight at 4 °C, followed by five PBS washes. The binding of the primary antibody was detected using a 1:100 dilution of AF546 anti rabbit IgG, or a combination of AF488-anti-rabbit IgG and Rhodamine-X anti mouse-IgG, respectively (Molecular Probes, Eugene, OR), and coverslipped with DAPI (4',6'-diamidino-2-phenylindole hydrochloride)-containing Vectashield mounting medium (Vector Labs). The following primary antibodies were used: rabbit antiserum against recoverin (gifts of Alexander Dizhoor (Dizhoor et al., 1991) and McGinnis (McGinnis et al., 1997); dilution 1:500); rabbit antiserum against red-green opsin (Chemicon, Temecula CA, dilution of 1:2000), or a rabbit antiserum against the metabotropic glutamate receptor mGluR6 (Neuromics, Minneapolis MN, 1:200) in combination with various mouse monoclonal antibodies: antibody against human placental alkaline phosphatase (hPAP) (Chemicon, clone MAB102; 1:200); antibody against post-synaptic density protein PSD-95 (Ghosh et al., 2001) (Stressgen, Victoria BC, Canada; 1:500), protein kinase C (PKC) (Biodesign, Saco, ME; 1:100), or rhodopsin (rho1D4, 1:50 (Molday, 1983)).

Of the three sections on a slide, one section was always double-stained with the antibody against hPAP in combination with one of the above mentioned rabbit antisera, and the other two sections with other combinations. Sections were imaged using a Zeiss LSM510 confocal microscope. Five to seven confocal slices were combined in a projection image.

Immunoreactivity for red-green opsin was used to identify the residual cone photoreceptors in the host retina as well as cone photoreceptors in the transplant in selected experiments. Red-green cones of the host retina of six transplant experiments without BDNF, seven transplant experiments with BDNF, and of four of the six BDNF-only treated rats, were counted by two to three observers (two independent counts), and the results expressed as cones/100 μm . Confocal images were magnified as much as possible on a computer monitor for counting purposes. The observers were masked during counting. The counts from different observers were averaged to one value. Because of the varying orientation of the transplant photoreceptors (laminated areas versus rosettes), cones in the transplants were not counted.

2.9. Statistics

Statistical comparisons were made using the Fisher exact probability test and a one-way analysis of variance (ANOVA) with subsequent post hoc tests, using a statistics package of GraphPad Software, Inc., San Diego, CA. The varying N of different experimental groups was taken into account.

3. Results

3.1. Optokinetic testing

Transplantation of BDNF coated retinal progenitor sheets significantly improved the optokinetic head tracking behavior

in the transplanted eye ($p < 0.05$, Fig. 2), compared with transplants that were not coated with BDNF. The effect of transplantation persisted throughout the testing period (up to 11 weeks following transplantation). Although some initial improvement in the head-tracking behavior was observed in the BDNF control group (treated with BDNF microspheres only), the effect of the treatment was attenuated when animals were tested at a later age (4 weeks and 7 weeks after transplantation, Fig. 2C).

3.2. SC recording

Electrophysiological evaluation of the visual responses from the SC revealed that in 80% (8/10) of the rats that received BDNF-coated transplants (Fig. 3A), visual responses could be recorded with stimulation at low light levels (1 cd/m²). In the non-BDNF transplant group, only 57% of the rats (4/7) showed visual sensitivity to such low level light stimulation, and none of the control rats (treated with BDNF microspheres alone, $n = 6$) showed this effect.

The area with visual restoration in the SC corresponded to the area of the placement of the graft under the host retina (Fig. 3B).

Rats that received BDNF microspheres (BDNF alone as well as BDNF coated transplants) showed visual response to bright light stimulation (1300 cd/m²) in a large SC area (Fig. 3B) suggesting a more global effect caused by the BDNF treatment. However, responses from transplanted rats covered a larger additional area where no responses were found in BDNF-only rats. BDNF-only and non-surgery rats showed no responses with low intensity light (Fig. 3B).

Detailed evaluation of the characteristics of the SC visual responses further elucidated the effect of BDNF in improving the functional efficacy of the fetal retinal sheet transplants (Table 1). When comparing responses to bright light in the SC area corresponding to the location of the transplant in the host retina, the response onset latency was considerably shorter among rats that received BDNF-coated transplants ($p < 0.005$ vs. transplant only group) when tested with bright

light. A quantitative comparison of the peak response amplitude did not reveal any significant difference between the various treatment groups.

3.3. Histological evaluation

Transplant organization was variable within both groups (see Table 2). Several transplants contained laminated areas with photoreceptor outer segments contacting host RPE in the center. All transplants also contained areas with rosettes (photoreceptors with inner and outer segments arranged in spheres), and some transplants were composed mostly of disorganized areas. There was no apparent correlation between transplant organization and BDNF treatment as evidenced by recoverin staining of photoreceptors and cone bipolar cells in transplants and host retina (data not shown).

In contrast to red-green opsin immunoreactivity in the normal retina which was concentrated in the outer segments (Fig. 4A), cones in the degenerate *S334ter* line 3 retinas showed immunoreactivity in the cell soma, cell processes, and sometimes in very short outer segments (Figs. 4B, 5C,D,G,H and 6C,F). In well-laminated areas, transplant cones showed normal red-green opsin immunoreactivity (mainly in the cone outer segments) (see Fig. 5A,E and 6A,D), whereas abnormal staining of cone somas was seen in more disorganized areas (Figs. 5B,F and 6B,E). PSD-95 staining was much stronger and often almost normal in the outer plexiform layers of the transplants even when the photoreceptors were arranged in rosettes (see Fig. 6) whereas PSD-95 immunoreactivity was patchy in the host retina independent of the presence of a transplant. In normal retina, mGluR6 immunoreactivity was seen as punctate staining in the outer plexiform layer (Fig. 7A). The immunostaining pattern for mGluR6 was abnormal in degenerated retinas, independent of the presence of a transplant or BDNF treatment (Figs. 7B,C and 8).

Cone counts of red-green opsin immunoreactive cones showed a significant reduction of cones in the host retina over the transplant versus the rest of the host retina in the BDNF-treated groups (Fig. 9). Although there were fewer

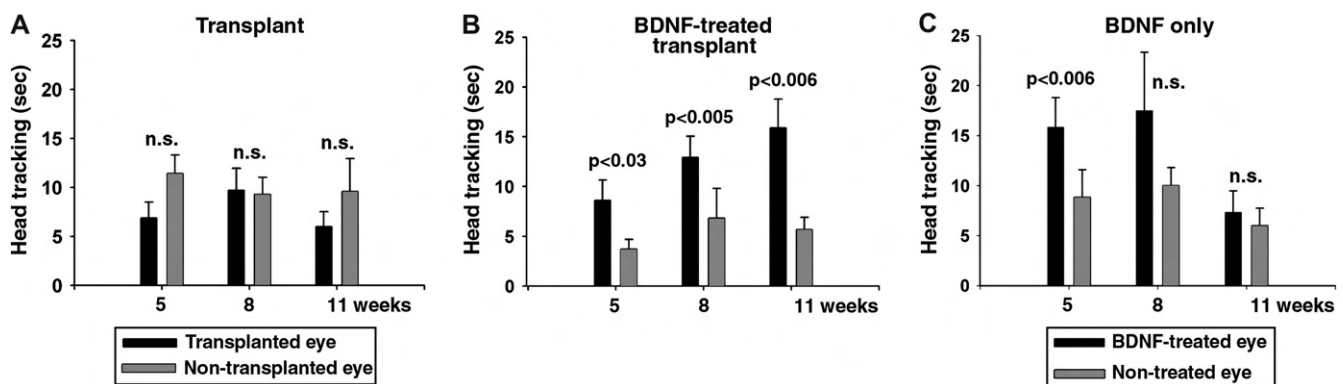


Fig. 2. Optokinetic head tracking: comparison between treated and non-treated control eyes. (A) Transplant only ($n = 7$); (B) transplant with BDNF ($n = 10$); (C) BDNF only ($n = 6$). BDNF-treated transplanted rats exhibited a significant increase in optokinetic head tracking (spatial frequency 0.25 cycles/degree). With progression of age (tests at 5, 8 and 11 weeks), the rats with BDNF-treated transplants show an increasing and statistically significant difference between the transplanted and non-transplanted eye.

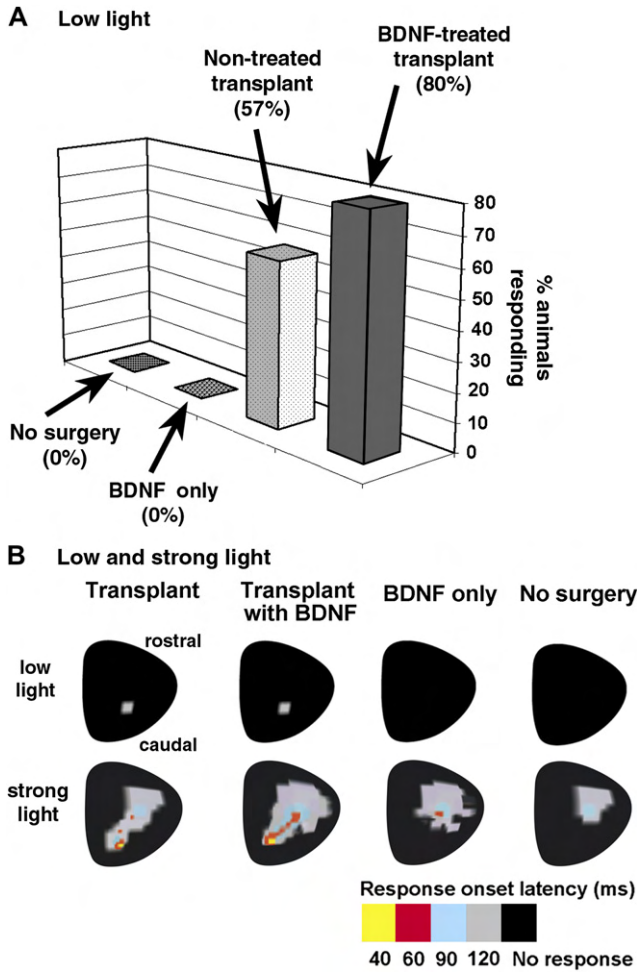


Fig. 3. Superior colliculus recording. (A) Percentage of animals responding to low light. Responses to low light (1 cd/m²) were only found in the transplanted groups. BDNF treatment increased the percentage of transplants responding to low light. BDNF-only treatment without transplant gave no response. (B) Schematic representation of response latencies in the superior colliculus to low and bright light in different experimental groups. At low light (1 cd/m²), responses are only found in transplanted rats in an area of the superior colliculus corresponding to the placement of the transplant in the host retina. BDNF-treated transplants and BDNF-only treated controls show increased responses to strong light (1300 cd/m²) in the superior colliculus, with a shorter latency. Note that the responses in transplanted rats are found in an additional area of the SC, not seen with BDNF only.

host cones in the transplant area in the non-treated transplant group, this difference was not significant. Both BDNF-treated and non-treated transplant areas contained significantly less host cones than the BDNF-only treated retinas. The cones in the transplant could not be counted reliably because of difficulties presented by their varying orientation within rosettes. There was no significant difference in the number of cones between the corresponding retinal locations of BDNF-treated and no treatment control eyes.

4. Discussion

This study demonstrates that treatment with BDNF microspheres enhances the functional efficacy of retinal progenitor

Table 1
Visual responses to bright light (1300 cd/m²) recorded from SC

Groups	No. of animals used (n)	Response onset latency (RL)	Peak response amplitude (RA)
Normal pigmented rat (control)	4	37.2 ± 7.0	89.5 ± 3.2
Transgenic (RD control)	6	No response	No response
BDNF only	6	No response	No response
Transplant only	7	68.4 ± 8.9	51.57 ± 3.69
Transplant + BDNF	10	41.9 ± 2.8	64.8 ± 4.55

Recordings were made from an area of the SC that corresponds to the area where the transplants are generally placed inside the eye. Transplants treated with BDNF microspheres had a shorter response onset latency than untreated transplants (*p* < 0.005, transplant + BDNF vs transplant only). See also Fig. 2.

sheet transplants in *S334ter* line 3 rats. This is evidenced by visual head-tracking behavior as well as recording of visual responses in the brain (SC) by electrophysiology.

In a previous study (Thomas et al., 2004b), it was shown that retinal transplants (without BDNF) delay the deterioration of optokinetic responses to large stripes (0.125 cycles/degree). In the current study, more narrow stripes were used (0.25 cycles/degree). When the transplants were coated with BDNF microspheres, head-tracking behavior of the transplanted eye was consistently better. Although BDNF-treated control rats (without transplants) also showed some initial improvement in the head-tracking behavior, this effect was not apparent when tested at a later age, suggesting that the initial visual improvement observed in the BDNF control group (BDNF microspheres only) may be due to a temporary delay in the progression of the disease. In contrast, the more persistent improvement in visual head tracking behavior observed in rats that received BDNF coated transplants suggests that the transplanted cells contributed to the increased function. Alternatively, if combining BDNF spheres with the transplant results in prolonged activity of the biological effect of BDNF (e.g., via decreased clearance rate of the microspheres from the eye), then one way to interpret Fig. 2B and C is that the restoration of head tracking is due to BDNF and not the transplant.

Functional improvement following BDNF-coated retinal sheet transplantation was further demonstrated by electrophysiological recording from the SC. Previous electrophysiology

Table 2
Overview of histological organization of transplants

Experimental group	N	Laminated areas + rosettes	Rosettes only	Rosettes + disorganized
Transplants	7	3	4	—
Good response in SC to low light	4	1	3	—
No or weak response to low light	3	2	1	—
Transplants with BDNF	10	2	6	2
Good response in SC to low light	8	2	5	1
No or weak response to low light	2	—	1	1

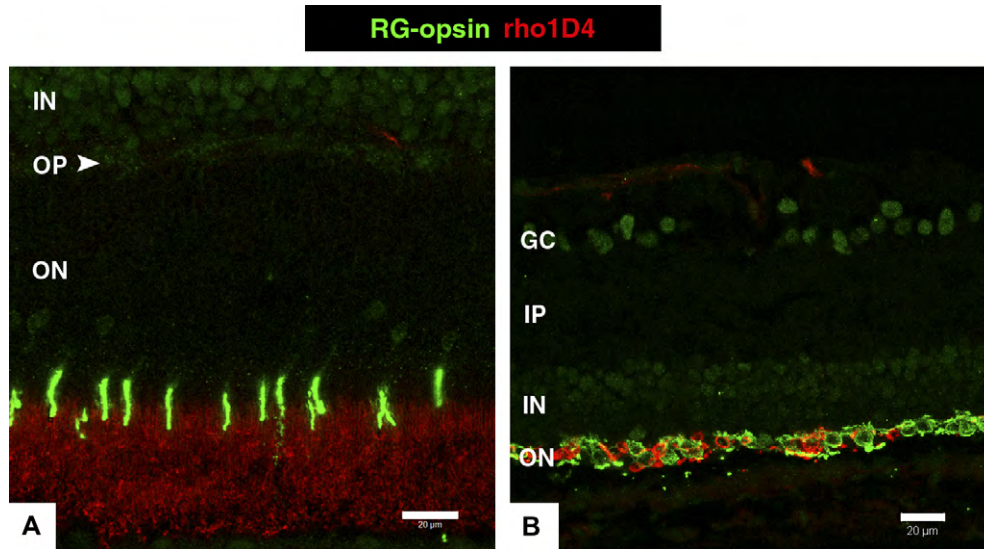


Fig. 4. Comparison between normal and retinal degenerate transgenic retina: red-green opsin and rhodopsin immunoreactivity. (A) Normal retina. Red-green opsin (RG opsin) (green) and rhodopsin (rho1D4) (red) immunoreactivity in outer segments of cones and rods, respectively. (B) Transgenic *S334ter* line 3 retina, age 35 days, 5 days light damage (postnatal day 23–28). Cell bodies and processes of residual photoreceptors stained with RG-opsin and rhodopsin. No rhodopsin immunoreactivity was seen in older *S334ter* line 3 rats (data not shown). Bars: 20 μm .

data obtained from three different retinal degenerate rat models demonstrated that retinal transplantation has a restorative effect even at many months after transplantation, as recorded in the SC (Sagdullaev et al., 2003; Thomas et al., 2004a; Woch et al., 2001). Since visual responses in these prior studies were evaluated using a very intense light stimulus (1300 cd/m^2), the possible contributions from the transplanted photoreceptors (primarily rods) could not be distinguished from responses derived from the residual host cones. It is important to note that in *S334ter* line 3 rats at the time of transplantation (24–37 days of age), most of the host rod photoreceptors are degenerated, leaving only a few cones and rare surviving rods (Sagdullaev et al., 2003) (Fig. 4B). No rods can be detected by immunohistochemistry for rhodopsin at an age of 50 days or later (unpublished data). The only rods in the eye present at the time of SC recording (72–97 days) are localized in the transplant as evidenced by histological examination (unpublished data). Thus, the present study is different in design from other studies that are looking at photoreceptor rescue since in this study most photoreceptors have degenerated at the time of transplantation. For example, McGee-Sanftner et al. (2001) showed that GDNF overexpression rescued photoreceptors both morphologically and functionally—as determined by ERG—in the *S334ter* line 4 rat model which has a slower time course of retinal degeneration than the *S334ter* line 3 rat used in the current study. In the present investigation, low intensity mesopic light (1 cd/m^2) (which stimulates both rods and cones) was used to record the visual contribution from the transplanted rods in addition to cones. BDNF treatment increased the percentage of transplanted rats responding to this low level of light. The location of the visual responses to low light in the SC was restricted to an area corresponding to the position of the graft. This suggests that the photoreceptors of the transplant contributed to the light

sensitivity. No improvement in the visual sensitivity to low light was observed when BDNF treatment alone was administered (without any graft).

When a strong light stimulus was used (1300 cd/m^2), a comparatively large SC area showed robust visual activity from both groups of BDNF-treated rats (BDNF-only and transplant with BDNF). However, in the BDNF-treated transplanted rats, this highly responsive area included the SC area corresponding to the placement of the graft in the eye, whereas, in the BDNF-only treated group, the improvement was mainly localized to the SC area where some residual visual activity could be detected even in non-treated control *S334ter* line 3 rats. The restored visual activity in the SC of the BDNF-treated transplant group was characterized by a shorter response onset latency. One possible mechanism responsible for this shorter onset latency could be a beneficial effect on host cones by the BDNF. The visual electrical output from the retina may be higher in the presence of a large number of surviving cones, and this higher output may enhance the transmission speed of the visual signals, thus shortening the response onset latency. A relatively normal response onset latency was correlated with photoreceptor rescue by RPE transplants in the Royal College of Surgeons (RCS) rat (Sauve et al., 2002). In addition, BDNF may have an effect on the inner host retina (Harada et al., 2005; Pinzon-Duarte et al., 2004; Thanos and Emerich, 2005). Although previous retinal progenitor sheet transplantation studies (without BDNF coating) demonstrated some improvement in the response latency (Sagdullaev et al., 2003; Thomas et al., 2004a; Woch et al., 2001), the present investigation of BDNF-treated transplants demonstrates a much faster response latency, approaching that of normal (non-transgenic) rats.

Using ganglion cell recordings and pupillary responses, MacLaren et al. concluded that transplants of retinal progenitor

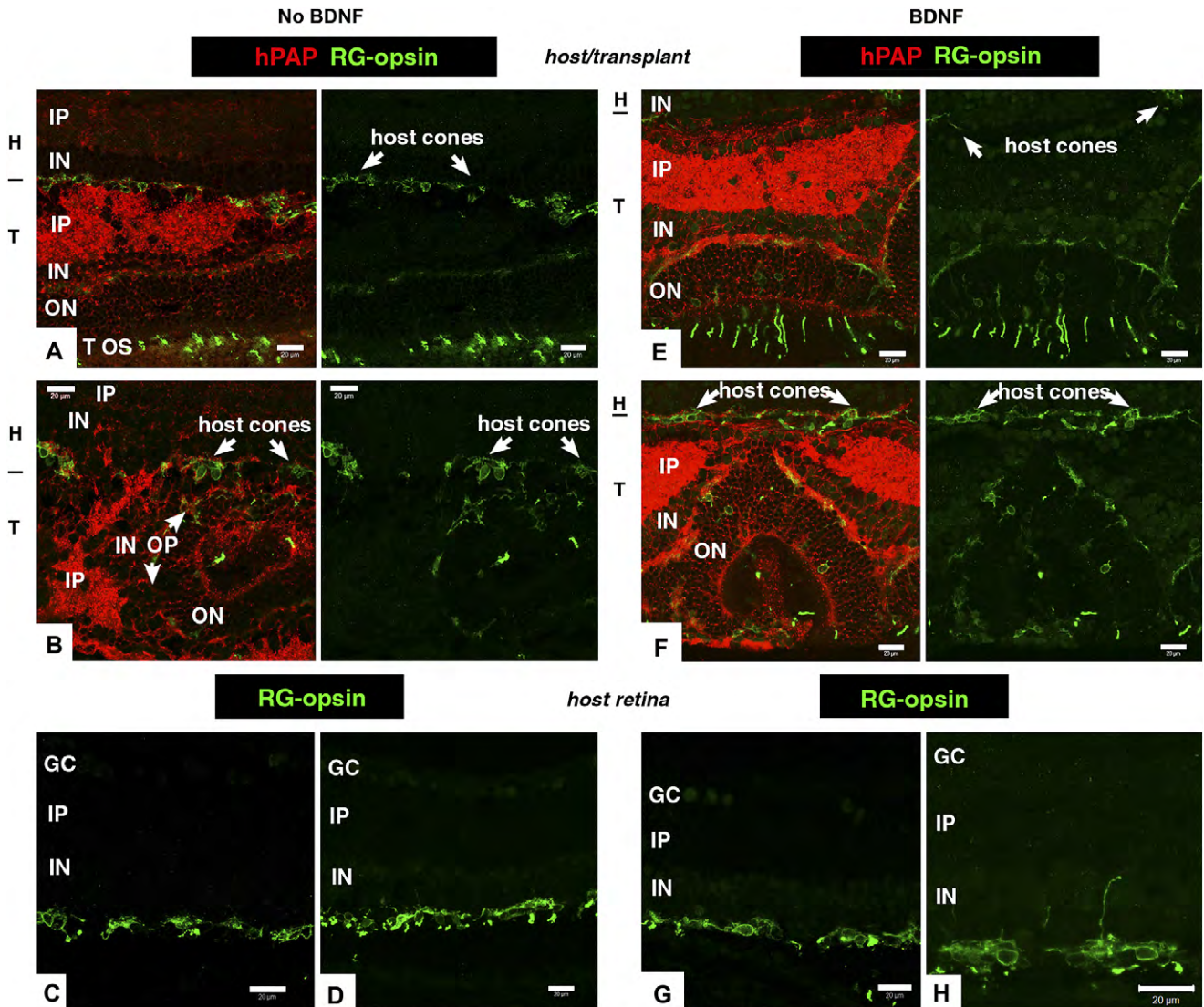


Fig. 5. Red-green opsin immunoreactivity. Host cones variable and abnormal, laminated transplant areas normal. This figure shows that the number of host cones overlying the transplants is variable, and sometimes reduced (see results of counts in Fig. 8), that the immunoreactivity of host cones is abnormal, and that transplants have normal cone opsin immunoreactivity in cone outer segments in laminated areas. (A,B,E,F) show double staining for RG-opsin (green) and the transplant label human alkaline phosphatase (red) on the left, and single RG-opsin staining of the same field on the right. (C,D,G,H) show only RG-opsin staining. (A) Transplant without BDNF, age 91 days, 60 days post-surgery. Normal RG cone-opsin distribution in transplant cones. Abnormal cone opsin in the overlying host retina. (B) Transplant without BDNF, age 90 days, 58 days post-surgery. Abnormal RG cone-opsin immunoreactivity in the cell somas of host cones. The transplant contains a photoreceptor rosette on the right with few cone outer segments. (C,D) Representative RG cone opsin staining of central ventral host retina of two different transplant experiments (age 96 days (C), age 83 days (D)). RG-opsin staining of cone cell somas, processes, and few short outer segments. (E) Transplant with BDNF, age 78 days, 54 days post surgery. Normal immunoreactivity in transplant, almost no cone opsin in host. (F) Different area of transplant in (E). Cell processes of cones in overlying host retina immunoreactive for RG-opsin. Staining of some outer segments in transplant rosette. (G,H) Representative RG cone opsin staining of central ventral host retina of two different transplant experiments with BDNF treatment (age 91 days (G), age 78 days (H)). Note the abnormal sprouting of a cone process into the inner plexiform layer in (H). Bars: 20 μ m.

cells caused visual improvement in different mouse models (MacLaren et al., 2006). Previous studies have demonstrated restoration of pupillary responses (Silverman et al., 1992) and ganglion cell responses (Radner et al., 2002) due to retinal transplants, but the reliability of these testing methods to show visual improvement has been questioned (An et al., 2002; Kovalevsky et al., 1995).

However, if host cone rescue was the sole reason for this effect, one should also expect a corresponding increase in the peak response amplitude, which was not apparent in any of the

experimental groups. Also, in the BDNF-only treated group, no rescue effect was apparent in the area of the SC corresponding to the transplant area of the eye. This suggests that cone rescue cannot explain the visual improvement observed in the BDNF coated transplant group. The underlying mechanism may involve not only BDNF, but other factors related to the transplanted cells. Indeed, our cone counts showed that the transplants did not rescue host cones; fewer host cones were found in the transplant area than in the rest of the host retina whereas numerous rod and cone photoreceptors could be found in the transplants.

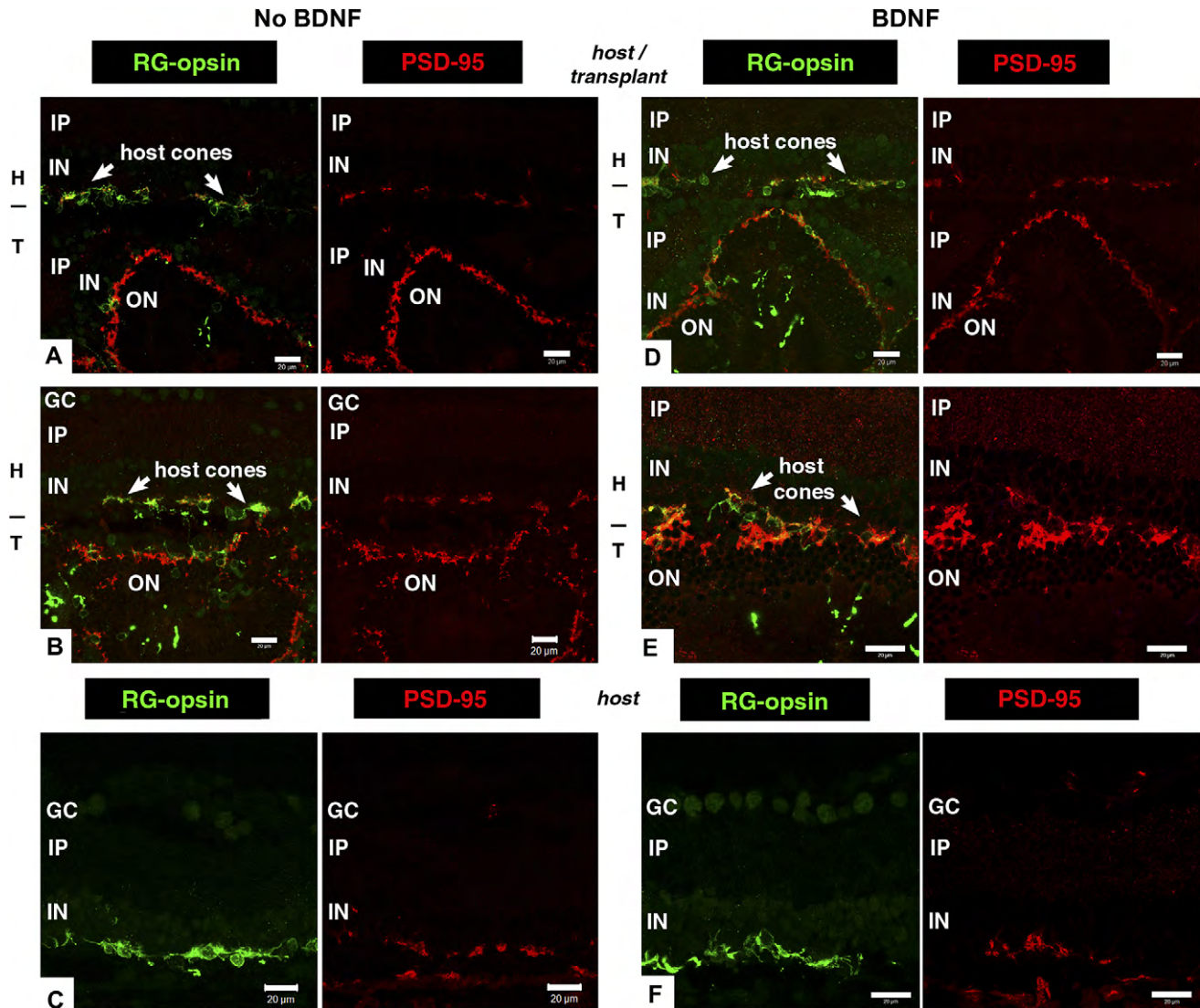


Fig. 6. Strong PSD-95 staining in outer plexiform layer of transplant, reduced in host. All figures show double staining for RG-opsin (green) and post-synaptic marker PSD-95 (marker for bipolar cell synapses with photoreceptors) (red) on the left, and single PSD-95 staining (red) of the same field on the right. The area of the transplant was determined by hPAP staining of adjacent sections (see Fig. 5). (A) Transplant without BDNF, age 96 days, 65 days post surgery. Intense PSD-95 staining of outer plexiform layer of laminated transplant. Only patchy PSD-95 staining in overlying host retina. (B) Another area of the same transplant. Outer plexiform layer in rosettes shows intense PSD95 staining. Only patchy PSD95 in host close to terminals of remaining cones. (C) Central ventral host retina of same experiment. Only patchy PSD-95 staining close to terminals of remaining cones. (D,E) Transplants with BDNF treatment. Strong PSD-95 staining of outer plexiform layer of transplant rosettes. Abnormal RG-opsin in host cones while the transplant exhibits more normal outer segment staining. (D) Same transplant as in Fig. 5E). (E) Different transplant, age 93 days (63 days post-surgery). (F) Central host retina of BDNF-treated transplant, age 79 days (42 days post-surgery), similar in appearance to non-BDNF-treated host retina with patchy PSD-95 (C). Bars: 20 μ m.

Various physiological and biochemical processes may be involved in this BDNF-induced visual restorative mechanism. It is well documented that progressive changes take place in the retinal neural circuitry following the degeneration of the photoreceptors (Jones and Marc, 2005; Marc et al., 2003; Strettoi et al., 2004). The immunohistochemical studies of the bipolar synaptic markers mGluR6 and PSD-95 was consistent with abnormal rewiring of the inner host retina after photoreceptor loss. These markers were chosen because of their abnormal distribution shown in other retinal degeneration models (Blackmon et al., 2000; Cuenca et al., 2004, 2005; Peng et al., 2003). There was no apparent effect of the

transplant or of BDNF treatment on the abnormal distribution of these markers in the host retina of rats with visual responses. An important finding was that the distribution of these synaptic markers was almost normal in laminated areas of the transplant. Some of these anomalies that occurred in the host neural circuitry may influence the normal propagation of the retinal neural signals, resulting in a longer response onset latency that has been reported among different retinal degenerative rat models (Sagdullaev et al., 2003; Thomas et al., 2004a; Woch et al., 2001). The present study demonstrates that a more or less normal response latency level could be restored by transplantation with retinal progenitor sheets coated

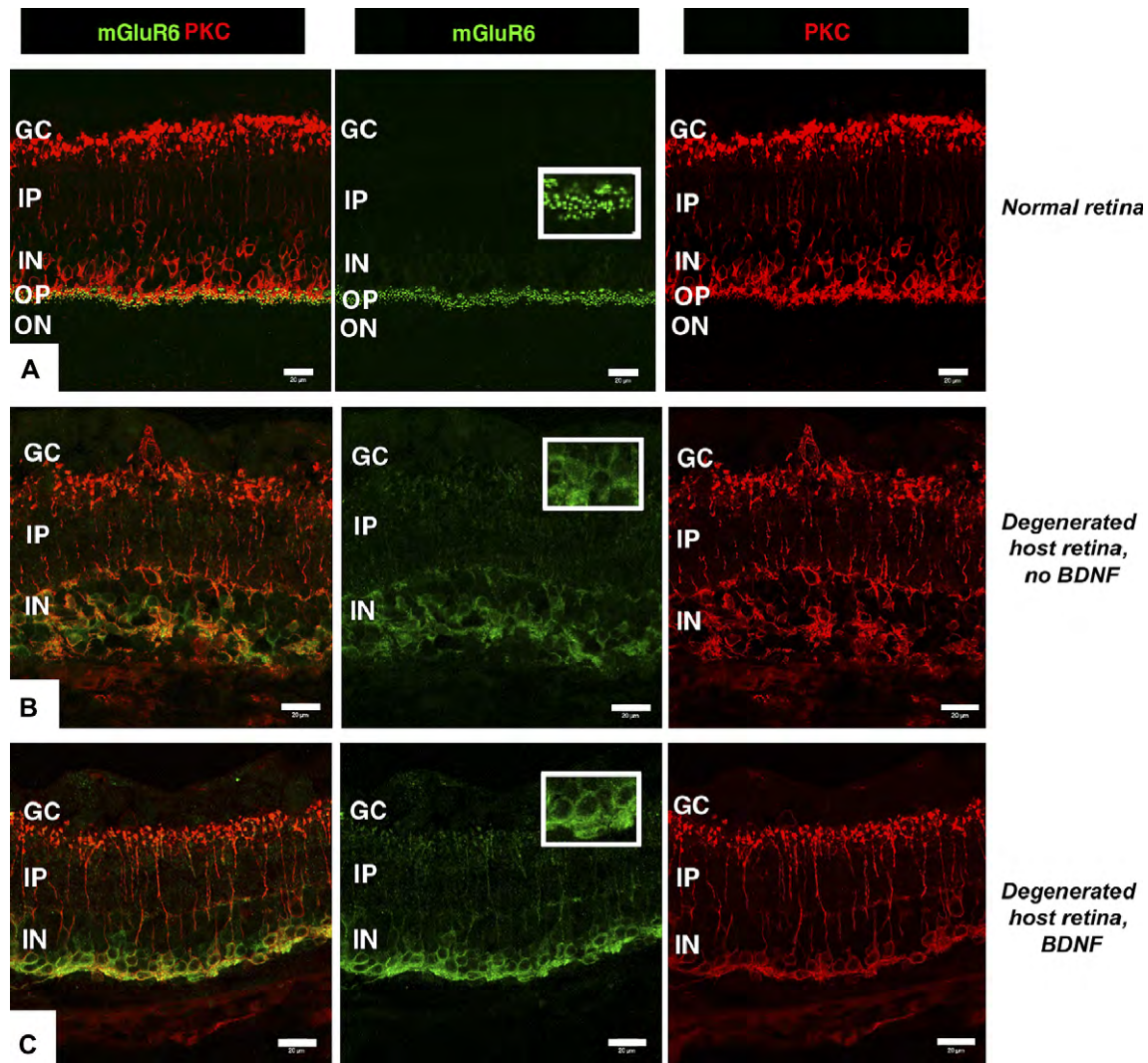


Fig. 7. Comparison between normal and retinal degenerate transgenic host retina: mGluR6 (marker for bipolar cell synaptic dendrites) in combination with protein kinase C (PKC; marker for rod bipolar cells). Enlargements of the outer plexiform layer staining are shown in white framed insets. (A) Normal retina. mGluR6 (green) stains small spots in the outer plexiform layer where rod bipolar cells stained by PKC (red) receive synaptic input from rods. (B) Central ventral *S334ter* line 3 host retina outside transplant without BDNF treatment, age 91 days (same animal as in Fig. 5A). mGluR6 has an abnormal staining pattern of bipolar cell processes and cell bodies verified by double staining with PKC. (C) Central ventral *S334ter* line 3 host retina of BDNF-treated transplant, age 78 days (same animals as in Fig. 5E). Abnormal distribution of mGluR6 (similar to (B)). Bars: 20 μ m.

with BDNF microspheres. Treatment with BDNF alone (without a graft) had no effect on the response latency which suggests that BDNF acts synergistically with other ‘factors’ released by the transplanted tissue. This combined effect may result in some restoration of the disturbed neural circuitry of the degenerating host retina which is supported by data from other investigators demonstrating neuro-protective effects of BDNF (Chaum, 2003; Ikeda et al., 2003; Keegan et al., 2003; Lawrence et al., 2004; Nakazawa et al., 2002; Paskowitz et al., 2004).

A major limitation in most retinal transplantation studies is the unknown level of functional integration between the transplant and the host retina, despite recent trans-synaptic tracing studies demonstrating synaptic connections at the host–transplant interface (Seiler et al., 2005a). Deficits in the functional integration could be one among many factors responsible for

a limited level of visual restoration in transplanted rats (Sagdullaev et al., 2003; Thomas et al., 2004a; Woch et al., 2001).

Although various techniques have been performed to promote the neuronal integration between the transplant and the host retina (Kinouchi et al., 2003; Lavik et al., 2005), no clear evidence of functional synaptic connectivity has yet been published. However, preliminary data from our group have shown that neurons in the transplant can be trans-synaptically labeled from the visually responsive site in the SC (Seiler et al., 2005b; manuscript submitted). The visual improvement described in the present study may also be due to an improvement in the quality of neuronal connections established between the transplant and the host retina.

It is remarkable that only a low BDNF dose during a very short term period markedly enhances tissue transplantation

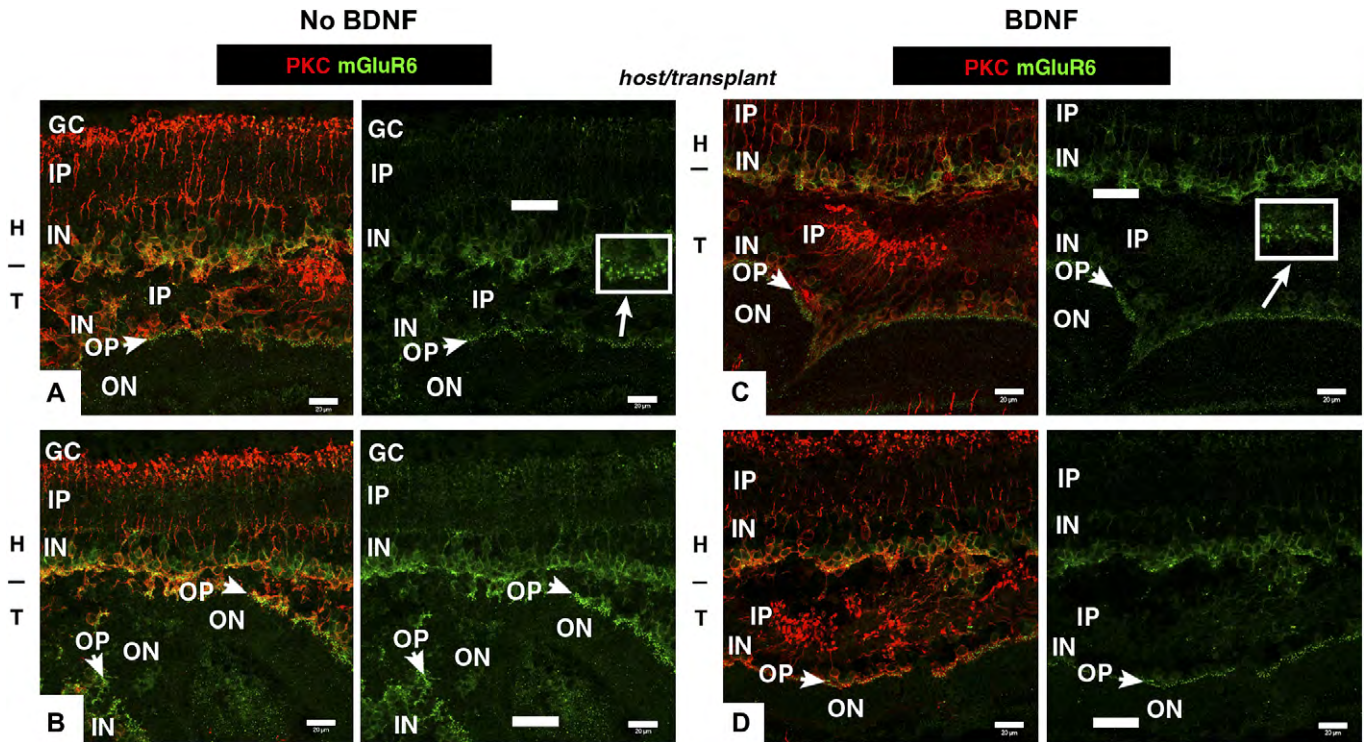


Fig. 8. Near normal bipolar cells in transplant, but abnormal in host: mGluR6 in combination with protein kinase C. Enlargements of the outer plexiform layer staining are shown in white framed insets. The approximate border between transplant and host is indicated by a black line on the side. The area of the transplant was determined by hPAP staining of adjacent sections (see Fig. 5). (A) Transplant without BDNF, age 89 days (57 days post-surgery). Laminated transplant area. The bipolar cells receiving input from transplant rods show close to normal small spot mGluR6 staining in the outer plexiform layer of the transplant. Abnormal interactions between host bipolar cells and host cones indicated by irregular staining of cell bodies and processes of bipolar cells with mGluR6. (B) Rosetted area of transplant without BDNF (same transplant as in Figs. 5A and 6A,B). (C,D) Transplants with BDNF treatment. Normal punctate staining of mGluR6 in transplant OPL, abnormal staining in host retina. (C) Strong PKC immunoreactivity of transplant bipolar cell terminals and of cone outer segments. (D) Laminated transplant area (same transplant as in Figs. 5D,E and 6D). (D) Transplant with BDNF treatment, age 79 days (42 days post-surgery). Bars: 20 μ m.

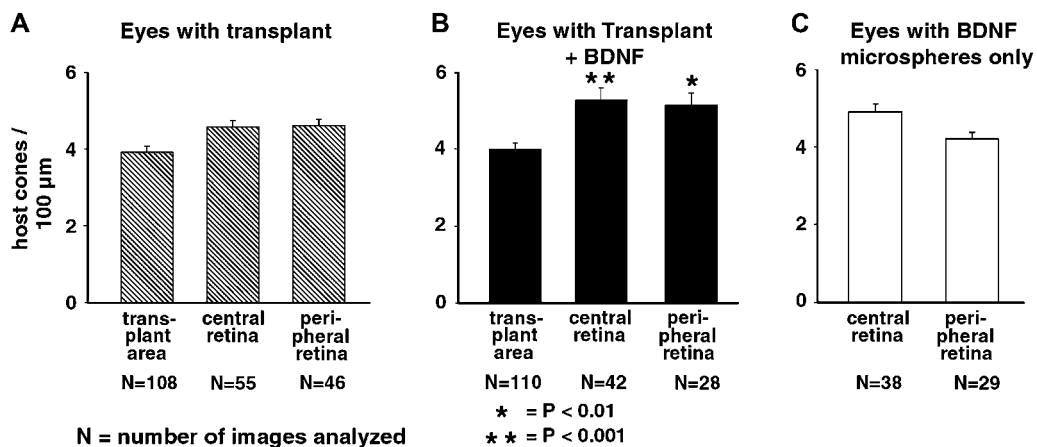


Fig. 9. Quantification of host cones. This figure implicates that visual responses are independent of the density of host cones. Red-green opsin immunoreactive host cones were counted in the transplant area, and host central and peripheral retina outside the transplant. Counts were done on enlarged images. (A) Transplanted eyes without BDNF treatment, (B) transplanted eyes with BDNF treatment, and (C) eyes with injection of BDNF microspheres only. *N* indicates the number of images evaluated. Values shown are mean \pm SEM cones per 100 μ m. In the BDNF-treated groups, significantly less host cones were found in the transplant area than in the surrounding host retina ($p < 0.001$ BDNF-treated transplant area vs. BDNF-treated central host retina; $p < 0.01$ BDNF-treated transplant areas vs. BDNF-treated peripheral host retina). Less host cones were also found in the transplant area in the non-treated group vs. the host retina outside the transplant. This difference was not significant, however. Both BDNF-treated and non-treated transplant areas contained significantly less host cones than the BDNF-only treated retinas. The cones in the transplant were not counted. There was no significant difference between BDNF treatment and non-treatment in the corresponding locations.

efficacy. A maximum of 8 ng BDNF was administered into the subretinal space and around 2000-fold less BDNF was measured 24 h after an *in vitro* incubation test. The maximum volume that can be injected reliably into the subretinal space is about 3 μl (Dureau et al., 2000); thus, the total volume of the subretinal space is likely only about 5 μl . However, the microspheres and the transplant were confined to a small area (about 1 mm^2) so the local concentration of BDNF could be much higher.

In summary, BDNF enhanced the functional efficacy of the retinal progenitor sheet transplants as evidenced by behavioral and electrophysiology data. The effect was not significant with BDNF treatment alone (without transplants) suggesting a synergistic effect between BDNF and the graft. BDNF may have acted in conjunction with other ‘factors’ released from the transplanted cells and helped to restore some of the retinal environment and circuitry, thereby accounting for the apparent normalization of the response latency. Given the enhancement in responsiveness to low light stimulation, BDNF may have also promoted neuronal connections between the transplant and the host retina which still requires further study.

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