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Transsynaptic virus tracing from host brain to subretinal transplants

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Abstract

The aim of this study was to establish synapses between a transplant and a degenerated retina. To tackle this difficult task, a little-known but well-established CNS method was chosen: trans-synaptic pseudorabies virus (PRV) tracing. Sheets of E19 rat retina with or without retinal pigment epithelium (RPE) were transplanted to the subretinal space in 33 Royal College of Surgeons (RCS) and transgenic s334ter-5 rats with retinal degeneration. Several months later, PRV-BaBlu (expressing *E. coli* β-galactosidase) or PRV-Bartha was injected into an area of the exposed superior colliculus (SC), topographically corresponding to the transplant placement in the retina. Twenty normal rats served as controls. After survival times of 1–5 days, retinas were examined for virus by X-gal histochemistry, immunohistochemistry and electron microscopy. In normal controls, virus was first seen in retinal ganglion cells and Müller glia after 1–1.5 days, and had spread to all retinal layers after 2–3 days. Virus-labeled cells were found in 16 of 19 transplants where the virus injection had retrogradely labeled the topographically correct transplant area of the host retina. Electron microscopically, enveloped and nonenveloped virus could clearly be detected in infected cells. Enveloped virus was found only in neurons. Infected glial cells contained only nonenveloped virus. Neurons in retinal transplants are labeled after PRV injection into the host brain, indicating synaptic connectivity between transplants and degenerated host retinas. This study provides evidence that PRV spreads in the retina as in other parts of the CNS and is useful to outline transplant–host circuitry.

Introduction

Retinal degenerative diseases affect both the photoreceptors and the retinal pigment epithelium (RPE) while leaving the inner retina relatively intact (Milam *et al*., 1998; Humayun *et al*., 1999), although remodeling occurs in late stages (Jones *et al*., 2003; Marc *et al*., 2003). It may be possible to restore visual sensitivity if the degenerated cells can be replaced with healthy cells that connect with the remaining inner retina.

One approach is to transplant fetal retinal sheets into the subretinal space of retinal degenerate recipients (review in Aramant & Seiler, 2002), using three different models: (i) light damage by blue light (Seiler *et al*., 2000); (ii) transgenic rats with a mutant human rhodopsin gene (Liu *et al*., 1999); (iii) Royal College of Surgeons (RCS) rats with dysfunctional RPE (D'Cruz *et al*., 2000).

Transplanted sheets of fetal retina develop photoreceptors with inner and outer segments in parallel layers, as long as healthy RPE (from either host or graft) is present (Seiler & Aramant, 1998; Aramant *et al*., 1999). Transplant photoreceptors show a normal shift in the distribution of phototransduction proteins in response to light, indicating functional light responses (Seiler *et al*., 1999).

Retinal transplants to rodent models of retinal degeneration restore visual responses in the superior colliculus (SC) within a small area that topographically matches the placement of the transplant in the retina (Woch *et al*., 2001; Sagdullaev *et al*., 2003; Arai *et al*., 2004; Thomas *et al*., 2004). Neuronal processes of transplant cells cross the interface between transplant and host retina (Aramant & Seiler, 1995; Ghosh *et al*., 1999; Gouras & Tanabe, 2003). Aggregate transplants placed into a retinal lesion site form synapses in the host retina (Aramant & Seiler, 1995).

However, synapse formation between a transplant in the subretinal space and a degenerated host retina has not yet been demonstrated. The present study used a tracer that is transported over synapses and, in this way, can outline a neural circuitry. The injection site on the surface of the host SC was chosen dependent on where the transplant was placed in the retina, based on the well-established topographic map between the retina and the surface of the SC (Yamadori, 1981; O'Leary *et al*., 1986; Simon & O'Leary, 1992).

The swine pathogen pseudorabies virus (PRV) has been extensively used to analyse multisynaptic circuits in the CNS (Card, 1998). Attenuated virus strains lacking certain virus membrane glycoproteins specifically infect synaptically connected neurons and migrate trans-synaptically in the retrograde direction (Strack & Loewy, 1990; Enquist *et al*., 1994; Enquist, 2002; Pickard *et al*., 2002). The double-membrane virus envelope that is necessary for spread of the virus to another cell and is lost when the virus enters a cell can only be assembled in the Golgi apparatus of neurons, and not in glial cells (Card *et al*., 1993). It has a high affinity for nerve terminals (Marchand

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& Schwab, 1986) and astrocytic membranes. Therefore, astrocytes close to a synapse will also pick up virus (Card *et al*., 1993). However, glial cells cannot assemble the virus envelope and therefore cannot release infectious virus (Card *et al*., 1993; Card, 1998).

Our aim was to demonstrate synaptic connectivity between host retina and transplant by specific trans-synaptic label of transplant neurons after PRV injection into the SC.

Materials and methods

Experimental animals

In all experimental procedures, the animals were treated according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Two normal Long-Evans and 18 Sprague-Dawley rats served as controls for the virus injections. Twenty-two albino RCS rats and 11 heterozygous albino and pigmented transgenic rats (s334ter, line 5) with retinal degeneration were used as recipients for fetal retinal transplants. Founder breeding pairs of the transgenic rats were produced by Chrysalis DNX Transgenic Sciences, Princeton, NJ, USA, and kindly provided by Dr M. M. LaVail, UCSF, San Francisco, CA, USA. Offspring were tested by PCR for the presence of the gene. Fifteen control animals and 27 of the transplant animals received injections of PRV-BaBlu, expressing E. coli β-galactosidase (Lowry *et al*., 1991) six transplanted animals received injections of PRV-Bartha (Enquist *et al*., 1994) and five control animals received injections of both PRV-BaBlu and PRV-Bartha in separate injection points.

Transplantation procedure

The procedures are described in detail elsewhere (Aramant & Seiler, 2002). Long-Evans timed-pregnant rats were anesthetized with ketamine (37.5 mg/kg) and xylazine (5 mg/kg) for C-section, and killed upon completion of the procedure. Donor tissue was obtained from embryonic day (E)17–20 pigmented Long-Evans rat fetuses, which had been prelabeled by injections of bromo-deoxyuridine (BrdU). For transplantation of retina with RPE (22 RCS and four S334ter-5 recipients), donor eyes were incubated in dispase (Collaborative Biomedical Products, Bedford, MA, USA) and the retina with its attached RPE was dissected free of surrounding tissues and embedded in 0.4% MVG alginate (Pronova, Oslo, Norway). The enzyme treatment was omitted for transplantation of retina only (eight s334ter-5 recipients). In anaesthetized rats (ketamine 37.5 mg/kg and xylazine 5 mg/kg), a small incision (≈ 1 mm) was cut behind the *pars plana* of the host eye, and the transplant (size 0.6 × 1.2 mm) was placed into the subretinal space, in the superior nasal quadrant of the left eye of the host, using a custom-made implantation tool. In one of the experiments, the transplant was placed in the temporal retinal quadrant. The placement of the transplants was evaluated after each surgery by fundus examination. RCS rats received transplants at the age of 1.2–2.1 months and s334ter-5 rats received transplants at the age of 0.9–1.7 months.

Virus strain used

Pseudorabies virus of the BaBlu (Loewy *et al*., 1991; Kim *et al*., 1999) and Bartha strains (Rinaman *et al*., 1993) was received from Dr J.P. Card, Pittsburgh, PA, USA. The viruses were originally grown in the laboratory of Dr L.W. Enquist, Princeton University, NJ, USA. The virus was shipped on dry ice, thawed on ice, immediately aliquoted and stored at −80 °C. Virus concentrations were in the range of 4×10^8 plaque-forming units (pfu)/mL.

Virus injection into the SC

Several (2.2–9.9) months after transplantation, rats were mounted in a stereotaxic apparatus and the surface of the SC was exposed by removing the overlying cortex (see Fig. 1A). In all controls and 22 transplanted rats, this was done under nembutal (38–42 mg/kg) and xylazine (3–7 mg/kg) anaesthesia, which allowed a maximum of four virus injections. In nine transplanted rats, artificial ventilation with halothane anaesthesia made it possible to make more than four injections per rat because it allowed for longer anaesthesia and speedy recovery. The rats were initially anaesthetized with ketamine and xylazine. Then a tracheotomy was performed to enable artificial ventilation. The rats were paralysed with a combination of pancuronium bromide (0.1 mg/kg/h) and curare (0.01 mg/kg/h) in saline (0.8 mL/h) and artificially ventilated with 1.0–2.0% halothane in 40% oxygen and 60% nitrous oxide. Blood pressure was maintained between 60 and 80 mmHg and the end-tidal CO_2 level between 2.8 and 3.1%. In two additional transplant experiments, rats were not intubated but breathed halothane through a rat anaesthesia mask.

Virus aliquots were thawed on ice immediately before use and handled in the fume hood on ice. Rats received 3–8 injections of the virus in different locations of the SC. A custom-made glass needle with a beveled tip (i.d. 0.07 mm) was filled with virus in a fume hood, and 100 or 200 nL was slowly injected over 10 min, at 0.3 mm depth from the surface of the exposed SC, using a micropump (World Precision Instruments). The injection sites on the surface of the SC were estimated from previous eye exams to correspond to the approximate projection area of the transplant, according to the known retina–SC topographic projections (Yamadori, 1981; Roskies & O'Leary, 1994; Simon *et al*., 1994). Because most transplants had been placed into the dorso-nasal retinal quadrants close to the optic disc, the virus was injected into the caudal SC. The needle was left in place for 5 min before it was withdrawn (see Fig. 1A).

After several injections, the surgical cavity was filled with gelfoam and the skin sewn together. Animals under halothane anaesthesia recovered after the trachea was closed with 8–0 sutures. All animals were placed in an incubator until they had recovered. The rats were kept in microisolator cages in a biohazard room in the animal facilities or in a fume–biohazard hood in the laboratory for 1–5 days.

Tissue processing for light microscopy

Most animals were killed 2–3 days after injection of virus, although survival times between 1 and 5 days were used in initial control experiments. Animals were injected i.p. with an overdose of sodium-pentobarbital (200 mg/kg) and perfused (descending aorta clamped off) through the aorta with 200 mL 0.9% saline, followed by 300 mL 4% paraformaldehyde and 0.4% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Both eyes and the brain were removed and placed in fixative. After 10–20 min in fixative, the cornea was removed and tissues were postfixed for another 60–90 min. For preparation of retinal wholemounts, the retina was dissected in phosphate buffer after 10 min fixation, flattened on a Millipore filter paper and postfixed for 60–90 min. After dissection, eyecups were either embedded in 4% agar for vibratome sectioning (80–100 μm) or infiltrated with 30% sucrose overnight, frozen in Tissue Tek on dry ice and cut at 15 μm on a cryostat. Coronal slices (3–6 mm) of the brain

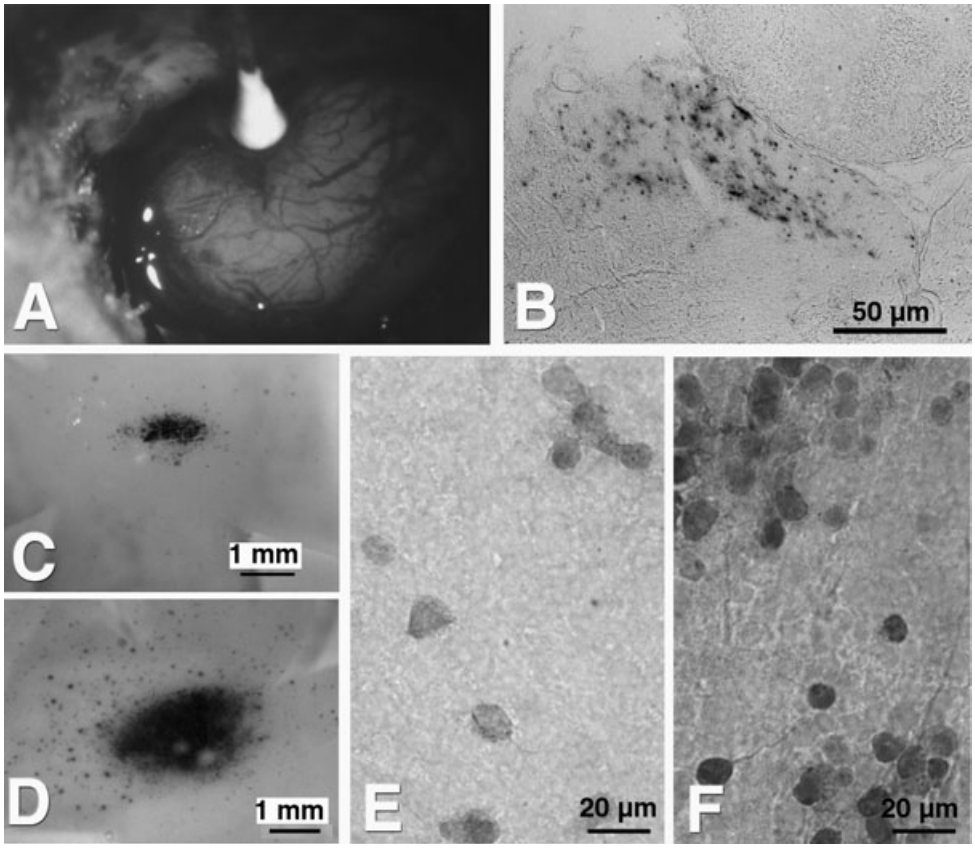

FIG. 1. BaBlu virus injection into the SC and overview of retinal label. (A) Virus injection into exposed SC. The injection needle has a white collar to prevent penetration of the SC surface beyond 0.4 mm depth. (B) Coronal cryostat section (30 µm) of SC, 70 h after injection of 400 nL virus. (C) Retinal wholemount of control retina, 40 h after injection of 150 nL of virus into SC. X-gal histochemistry. (D) Retinal wholemount of control retina, 52 h after injection of 200 nL virus into SC. Note the lateral spread of virus-infected cells. X-gal histochemistry. (E) Enlargement of retinal wholemount, 35 h after virus injection into SC. Weak label of retinal ganglion cell clusters and single cells. Immunohistochemistry for PRV. (F) Enlargement of retinal wholemount, 70 h after virus injection into SC. The labeling density is much higher, and smaller amacrine cells are labeled in addition to large ganglion cells. Cells labeled in deeper retinal layers cannot be seen. Immunohistochemistry for PRV.

that included the SC were prepared and then cut either on a vibratome (800–100 µm) or on a cryostat (30 µm; example in Fig. 1B).

Histochemistry for E.coli *β-galactosidase*

To detect the BaBlu virus, vibratome or cryostat sections or retinal wholemounts were incubated after a wash with PBS (150 mM NaCl, 15 mM sodium phosphate buffer pH 7.5) in an X-gal substrate solution in the same buffer (Sanes *et al*., 1986; $MgCl_2$, 2 mM; $K_3Fe(CN)_3$, 5 mM; $K_4Fe(CN)_6$, 5 mM; sodium deoxycholate, 0.01%; NP-40, 0.02%; and 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-gal; Sigma), 1 mg/mL, until a clear blue colour had developed, and then washed three times with PBS before being coverslipped and mounted. Selected retinal vibratome sections were cryoprotected after X-gal staining in 30% sucrose in PBS, flat-embedded and frozen in Tissue-Tek on dry ice (see above). In five control and four transplant experiments, the X-gal reaction was performed in retinal wholemounts which were then subsequently processed for paraffin, histocryl plastic or cryostat sectioning.

Immunocytochemistry

Cryostat sections or retinal wholemounts were blocked with 20% goat serum. To detect PRV-Bartha and -BaBlu virus, a polyclonal rabbit antiserum (Card *et al*., 1990; Card & Enquist, 1994), generously provided by L.W. Enquist and J.P. Card, was used at a concentration of 1 : 20 000 overnight at 4 °C. After several washes with PBS (0.1 M NaCl, 0.05 M sodium phosphate buffer, pH 7.2), a biotinylated antirabbit IgG (raised in goat) and the Elite-ABC kit with horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) was used for the detection of the primary antibody. A diaminobenzidine (DAB) kit enhanced with nickel chloride (Vector labs) was used as substrate. Alternatively, the primary antibody was used at a dilution of 1 : 5000 and detected with goat-antirabbit IgG, coupled to rhodamine-X (Molecular Probes, Eugene, OR, USA).

Tissue processing for electron microscopy

After X-gal staining or immunocytochemistry, vibratome sections or tissue pieces of three control and five transplant experiments (two RCS and three transgenic rats) were fixed with 2% OsO_4 and flat-embedded in Epon. Ultrathin sections (60–80 nm) on slotted and hex bar copper grids were examined in a Philips CM10 electron microscope. The most extensively analysed specimens were a normal retina, 3 days after virus injection (three different vibratome slices, cut at ten different levels, 5–10 µm apart; shown in Figs 3 and 4), and a retina–RPE cograft to an RCS rat, also 3 days after virus injection (cut at seven different levels, 5–10 µm apart; shown

in Figs 6 and 7). The other specimens were only cut at two or three levels.

Results

Control experiments

In pilot experiments, normal rats were killed 24 h to 5 days after injection of the virus in the SC. Because of the toxicity of the virus, two rats died at 5 and 6 days postinjection before the tissue could be harvested.

Virus label after superior colliculus (SC) injection

Virus injections produced discrete localized areas of labeled cells in the SC. The best retinal label was achieved with virus injections into the superficial layers of the SC (example in Fig. 1B). If the virus was injected too deep (> 0.4 mm below the SC surface), no retinal label was seen (data not shown). In the target area of the retina, even with injection of larger volumes of 200 nL per injection site, a very confined area was labeled at 35 h (Fig. 1C). With longer survival times, progressive spread of the virus was observed in the retina (Fig. 1D) as well as in the brain (data not shown).

Virus label in normal retina

At 1 day postinjection in the SC, only faint punctate label was seen in retinal ganglion cells (data not shown). At 35 h postinjection, isolated radial groups of cells were labeled, consisting mostly of a ganglion cell and one or several Müller glial cells in the inner nuclear layer (Figs 1E and 2A and B). Two days after injection of the virus into the SC, small radially labeled clusters of retinal cells, including photoreceptors, were labeled (Fig. 2C). Three days after virus injection, the label had spread more laterally as further projecting amacrine cells and horizontal cells had been labeled (Figs 1F, and 2D and E). Still larger areas of the retina were labeled after survival times > 3 days, and it was difficult to distinguish individual cells in

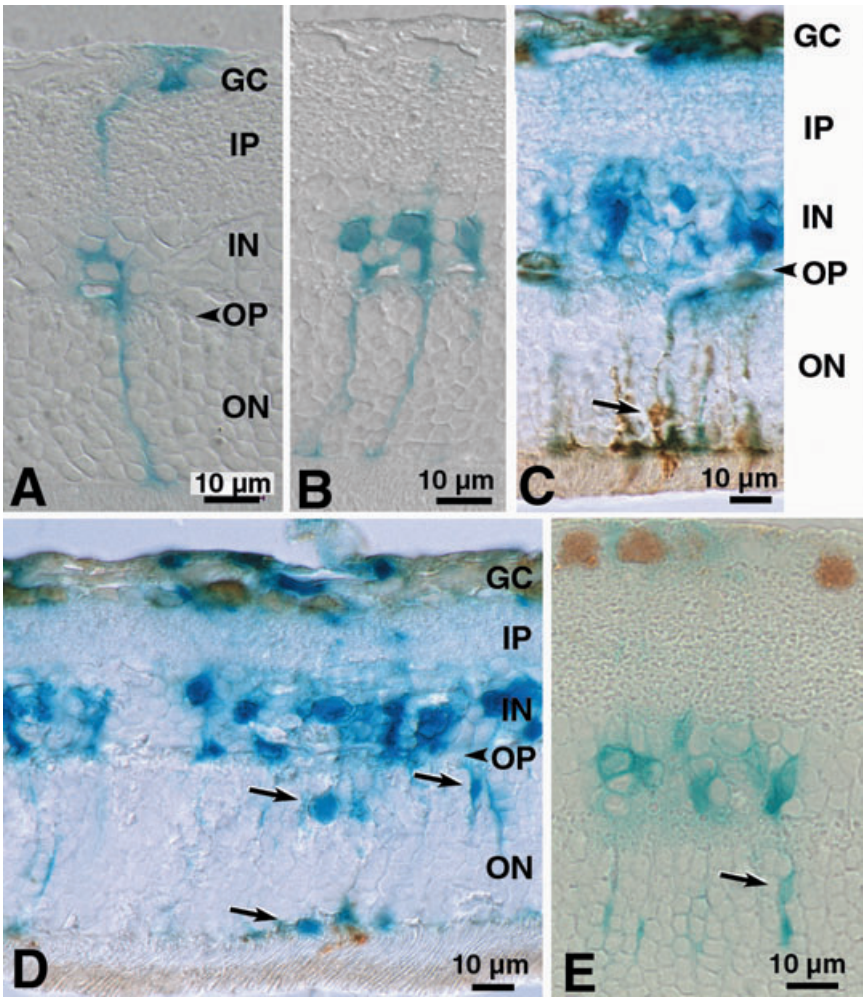

FIG. 2. Normal control retina. (A–C) Plastic sections (3 µm) through normal retina, 35 h after BaBlu virus injection into SC. X-gal histochemistry (blue). (A) Labeled ganglion cell and Müller cell. (B) Labeled Müller cells. The corresponding labeled ganglion cells are found on adjacent sections. (C–E) Immunohistochemistry for pseudorabies virus (PRV; brown) combined with X-gal histochemistry on retinal wholemounts. The PRV antibody penetrated only the surface of the retinal wholemount and labeled cells in the ganglion cell and the outer nuclear layer. Arrows in C–E indicate labeled photoreceptor cell bodies. (C) Two days (52 h) after BaBlu virus injection into SC: label of a narrow cluster of cells in all retinal layers. Note the clearly labeled photoreceptor cell bodies with inner and outer segments. Paraffin section (12 µm). X-gal histochemistry and immunohistochemistry for PRV on retinal wholemount. (D) Three days (67–68 h) after virus injection, the labeled clusters have increased in size. Cryostat section (15 µm). (E) Histocryl plastic section (3 µm) of control retina 3 days after virus injection.

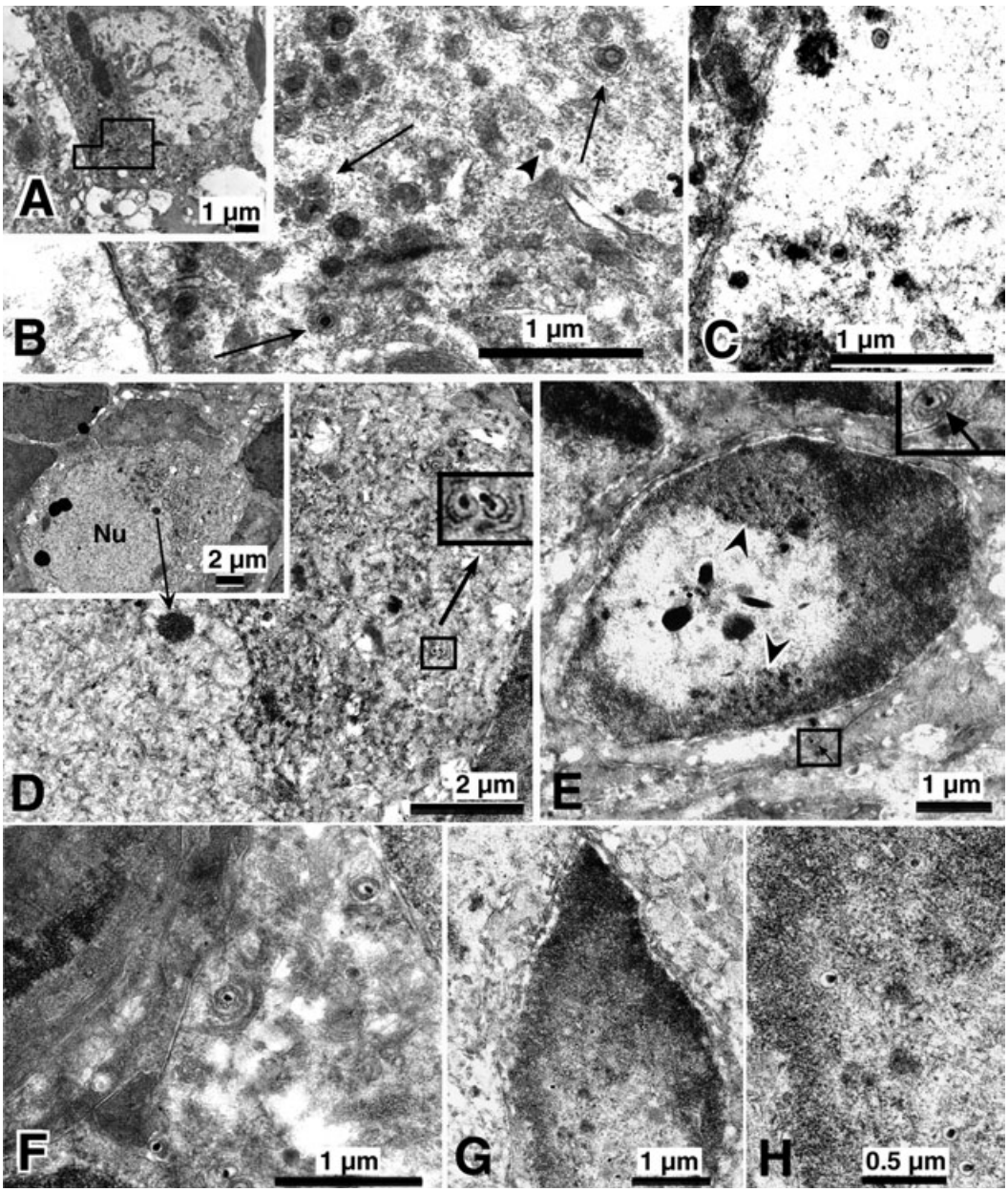

FIG. 3. Ultrastructure of normal retina 2 and 3 days after label. (A–C) Normal retina, 52 h after virus injection into the SC. The retinal wholemount had been reacted with X-gal before embedding. (A) Virus-infected ganglion cell. Box shows area enlarged in B. (B) Cytoplasm of ganglion cell containing many enveloped viruses (only three examples indicated by arrows) and some nonenveloped virus (arrowhead). (C) Nonenveloped viruses in the nucleus of another ganglion cell. (D–H) Normal retina, 67 h after virus injection. The tissue had been reacted with X-gal before embedding. (D) Large displaced ganglion cell in inner nuclear layer, containing viruses in the nucleus and cytoplasm in different stages of assembly. Left inset (white frame), overview. The assembly of two virus particles with envelopes in the Golgi can be seen in the right inset (black frame). (E) Smaller virus-infected (amacrine?) cell in the inner nuclear layer. Note the characteristic clumping of the chromatin (large blobs). The synthesis of virus DNA is indicated by regular arrangements of small dots in the nucleus (arrowheads). An enveloped virus in the cytoplasm is enlarged in the inset. (F) Cytoplasm of another neuronal cell in the inner nuclear layer, showing the assembly of the virus envelope in the Golgi. (G) Virus-infected Müller cell, enlarged in (H), showing that the cell only contains nonenveloped virus.

the originally labeled areas because of the density of the label (data not shown). Thus, the optimal time for the evaluation of the transport of the virus to the retina was between 2 and 3 days after virus injection.

Electron microscopy of normal retina 52 h after virus injection revealed virus-infected retinal ganglion cells containing both nonenveloped and enveloped virus in the cytoplasm (Fig. 3A and B) and nonenveloped virus in the nucleus (Fig. 3C). Few virus-labeled cells were found in the inner nuclear layer (data not shown). An accumulation of macrophages and microglial cells was observed in the inner plexiform and inner nuclear layer (data not shown). At 67 h after virus injection (Figs 3D–H and 4A–C), virus-infected cells could be found in all retinal layers. As reported before (Card *et al*., 1993), assembly of the virus envelope was observed in the Golgi apparatus of neurons (examples in Fig. 3D and F). Enveloped viruses were seen in the cytoplasm (Figs 3B and D–F, and 4B). Müller glial cells were infected with virus, but only nonenveloped virus was observed (Fig. 3G and H). In the outer nuclear layer, virus-infected photoreceptor cells with nonenveloped virus were observed at this stage (Fig. 4A and C).

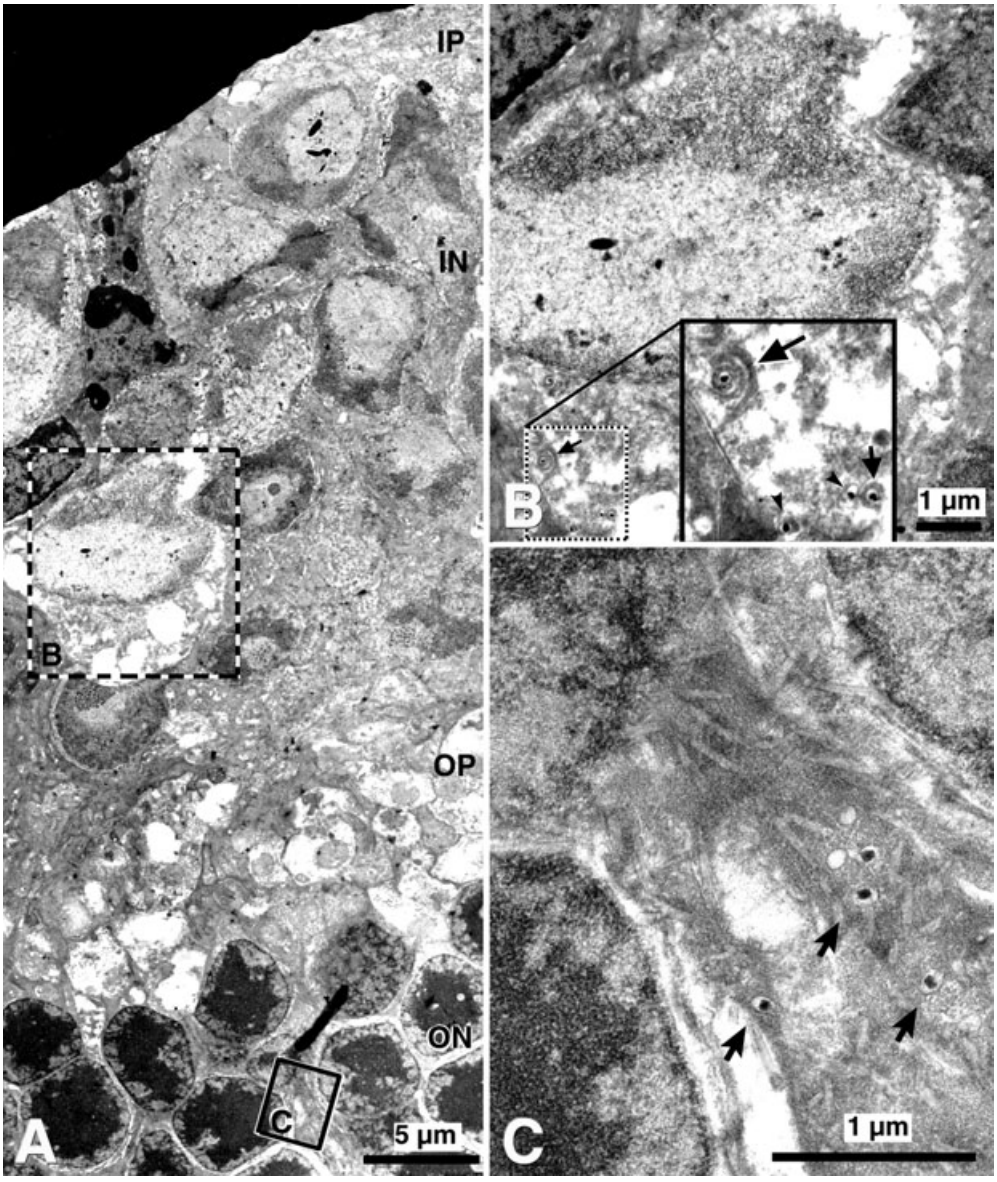

FIG. 4. Ultrastructure of normal retina 3 days after virus label. Normal retina, 67 h after virus injection into SC. The vibratome section of this retina had been reacted for X-gal before being flat-embedded in Epon. (A) Overview, showing different retinal layers. Apparently degenerated cell (left top) close to large horizontal cell (enlarged in B). Note the clumps of condensed chromatin (large blobs) in nuclei of virus-infected cells that are recognizable even at this low magnification, especially in the top cell close to the inner plexiform layer (IP). Boxes indicate enlargements shown in B and C. (B) Enlargement of horizontal cell close to outer plexiform layer. Nucleus with clumps of condensed chromatin. Arrow, enveloped virus (enlarged in inset); arrowheads, nonenveloped viruses. Area in box is enlarged in insert. (C) Photoreceptor cell with nonenveloped viruses (arrows) in cytoplasm. The virus has apparently just reached the photoreceptor cell at this stage and not yet proliferated and reassembled. The X-gal label is seen as faint irregular precipitate in cytoplasm (pale rods criss-crossing the cytoplasm).

Virus label in transplants

Transplants were easily identifiable as sheets in the subretinal space using BrdU immunohistochemistry (data not shown). In one animal with optic nerve damage, no virus label was found in the retina, only in the brain. In 13 of the 33 animals (10 at 2 days and three at 3 days postinjection), only the host retina outside the transplant site was labeled because the virus had been injected outside the topographically correct area of the SC. In three transplants (all at 2 days postinjection), no labeled cells were found in spite of virus label in the host retina above the transplant. Six transplants (five at 2 days and one at 3 days postinjection) contained few (< 25) and 10 transplants contained many labeled cells (examples in Figs 5–7). Heavily labeled transplants were seen both at 3 days postinjection ($n = 7$) and at 2 days postinjection ($n = 3$).

In most experiments, BaBlu virus was injected, followed by X-gal histochemistry. The X-gal staining (Fig. 5A–C) was more diffuse than the immunohistochemistry with the anti-PRV antibody in later experiments (Fig. 5D–G). Electron microscopy of X-gal-labeled vibratome sections showed virus-infected cells in the transplant with neuronal characteristics and the typical punctate pattern of condensed chromatin in the nucleus (Figs 6 and 7). Occasionally, enveloped virus could be seen in neurons in the transplant inner nuclear layer (Fig. 6C). Mostly nonenveloped virus could be found in the inner and

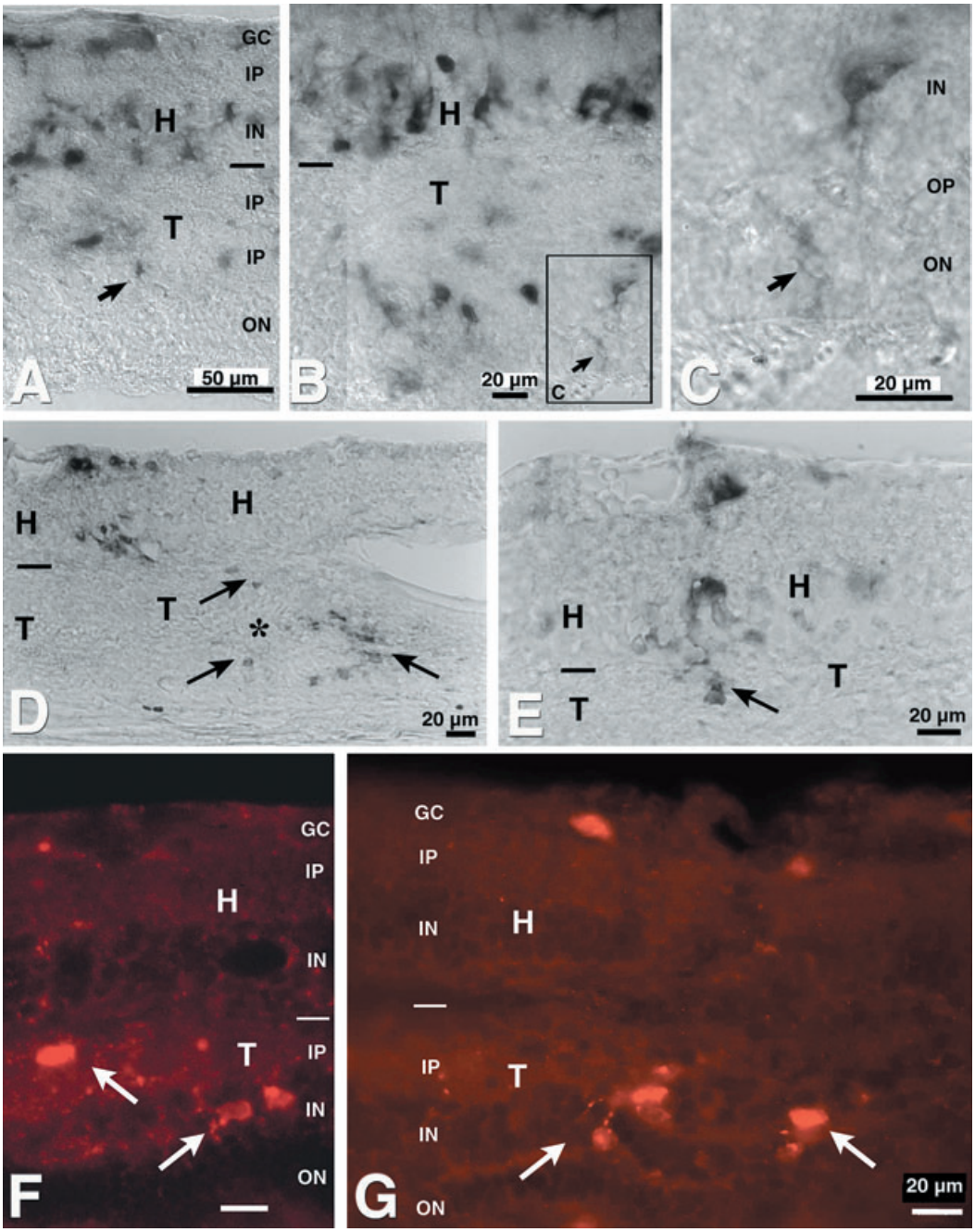

FIG. 5. Label in transplant after PRV-BaBlu or -Bartha virus injection into SC. Cryostat sections (15 µm). Line indicates approximate border between transplant and host retina. (A–C) X-gal histochemistry. Transplant to s334ter transgenic rat, 65 h after BaBlu virus injection into SC. E18 retinal transplant, 9.8 months after transplantation. Arrows point to some virus-labeled cells in transplant with apparent neuronal morphology. (A) Heavy label in the host retina (H). Underneath, in the transplant (T), several cells are labeled in the inner nuclear layer. Arrow points to cell with faintly labeled process terminating in outer plexiform layer. (B) Composite from several different focus levels. Heavy label in host retina. Label of several cells in transplant inner nuclear layer. Box indicates area enlarged in C. (C) Strong label of cell in transplant inner nuclear layer, and faint label of photoreceptor cell in transplant outer nuclear layer. (D–G) Immunohistochemistry with rabbit PRV antiserum (D–E, ABC method with DAB-labeled cells; F,G, immunofluorescence). Retinal transplant to transgenic rat, 67 h after Bartha virus injection into the SC. E19 donor, 6.7 months after transplantation. Arrows point to virus-labeled cells in transplant; asterisks indicate the lumen of rosettes (photoreceptor layer organized in a sphere, surrounded by other retinal layers). (D) Many labeled cells in a rosetted area of the transplant. (E) Transplant–host interface, showing just one transplant cell labeled by a chain of host cells. (F) Labeled cells in transplant ganglion cell and inner nuclear layers. (G) Label of two ganglion cells in host and of several cells in inner nuclear layer of transplant.

outer plexiform layers of the transplant (Fig. 7). As seen with normal retina at 3 days after virus injection, degenerated cells could be seen in the transplant inner nuclear layer in heavily labeled areas (data not shown).

Discussion

The aim of this study was to demonstrate anatomically the synaptic connectivity between retinal transplants and a degenerated host retina.

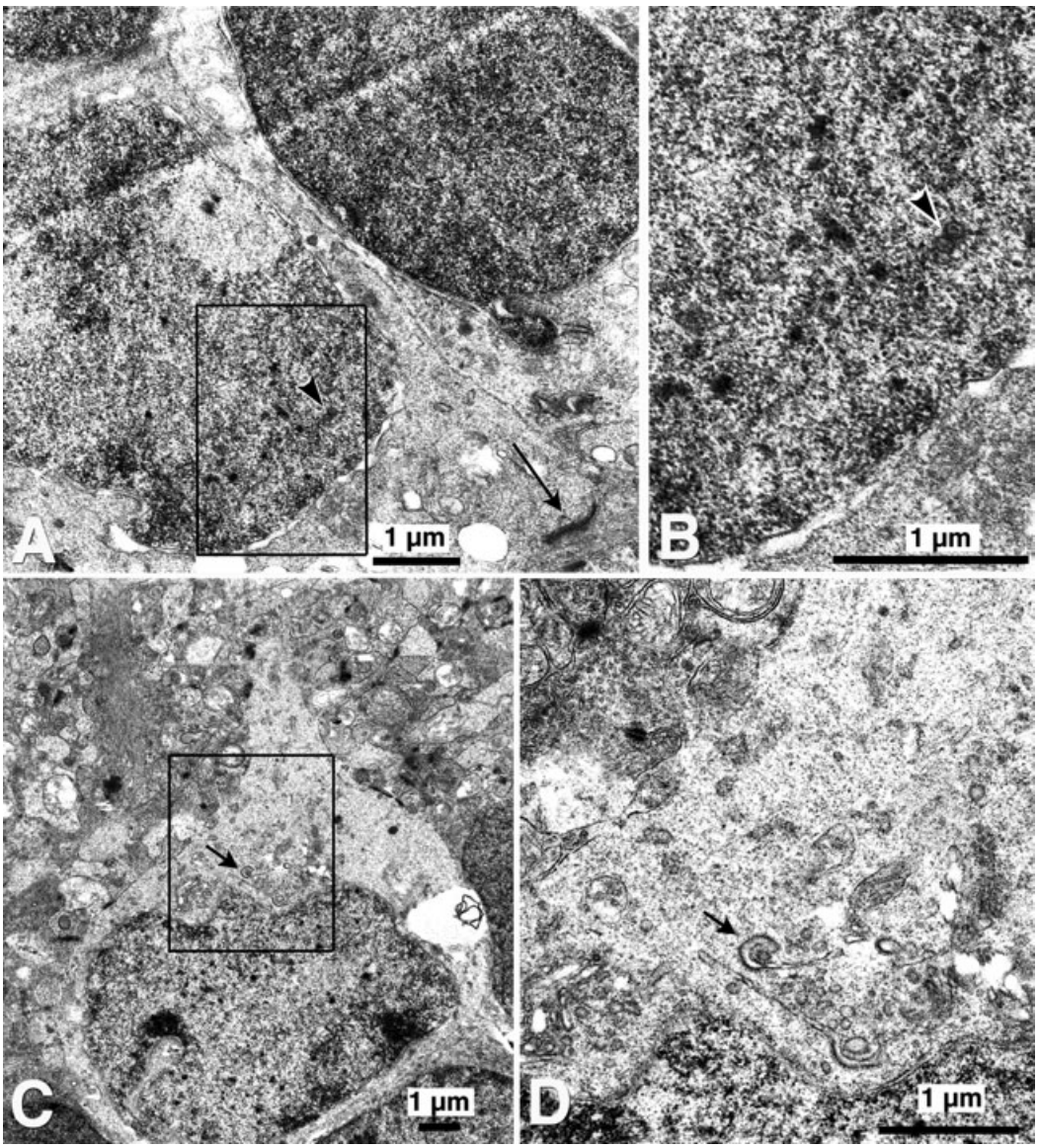

FIG. 6. Electron microscopy of virus-labeled cells in transplant. Cograft of retina with RPE (E20) to RCS rat, 4.9 months after transplantation, and 68 h after BaBlu virus injection into SC. (A) Virus-infected neuron in inner nuclear layer of transplant close to outer plexiform layer. Arrow points to synapse, arrowhead points to nonenveloped virus in nucleus. Note small dots of heterochromatin in nucleus indicating virus synthesis. (B) Enlargement of boxed area in (A). (C) Virus-infected neuron in inner nuclear layer of transplant close to inner plexiform layer. Arrow points to enveloped virus in Golgi. (D) Enlargement of box in (C).

So far, only one other study has used PRV tracing to show connectivity of intraspinal fetal grafts (Reier *et al*., 2002).

Transneuronal synaptic virus transfer and the role of glial cells

PRV-Bartha and PRV-BaBlu, the virus strains used in this study, are attenuated viruses that are mutants in the envelope glycoproteins gI and gE (Loewy *et al*., 1991; Enquist *et al*., 1994; Kim *et al*., 1999; Yang *et al*., 1999), exclusively transported in the retrograde direction (Sams *et al*., 1995; Card *et al*., 1998; Loewy, 1998; Pickard *et al*., 2002; Sollars *et al*., 2003; Smeraski *et al*., 2004), and migrate through the entire dendritic arbor (Card *et al*., 1993; Rinaman *et al*., 1993). The specific trans-synaptic passage of attenuated PRV virus has been demonstrated in different systems (Strack & Loewy, 1990; Sams *et al*., 1995; Rinaman *et al*., 1999; Pickard *et al*., 2002). Except for the initial infection, neurons can only be infected by other synaptically connected neurons because the infection is concentration-dependent (Card *et al*., 1999). It has been demonstrated that the double membrane virus envelope has a specific affinity for membranes derived from both pre- and postsynaptic nerve terminals (Marchand & Schwab, 1986; Card, 1998), but also for astrocytic membranes (discussed in Card *et al*., 1993). This affinity is specific and related to the glycoproteins of the envelope. Cells that do not have matching receptors on their membrane, such as microglia and macrophages, will not get infected. The envelope is necessary both for virus egress from the infected cell and for infection of another cell.

Virus particles are preferentially released at sites of afferent synaptic contact, but their release is not precisely at the site of the synapse; rather it is parasynaptic. Thus, released virions are close to both astrocytes and axon terminals, and can enter both cell types with equal efficiency. The virus enters a cell by binding of the virus envelope glycopoteins to specific receptors on the cell membrane and

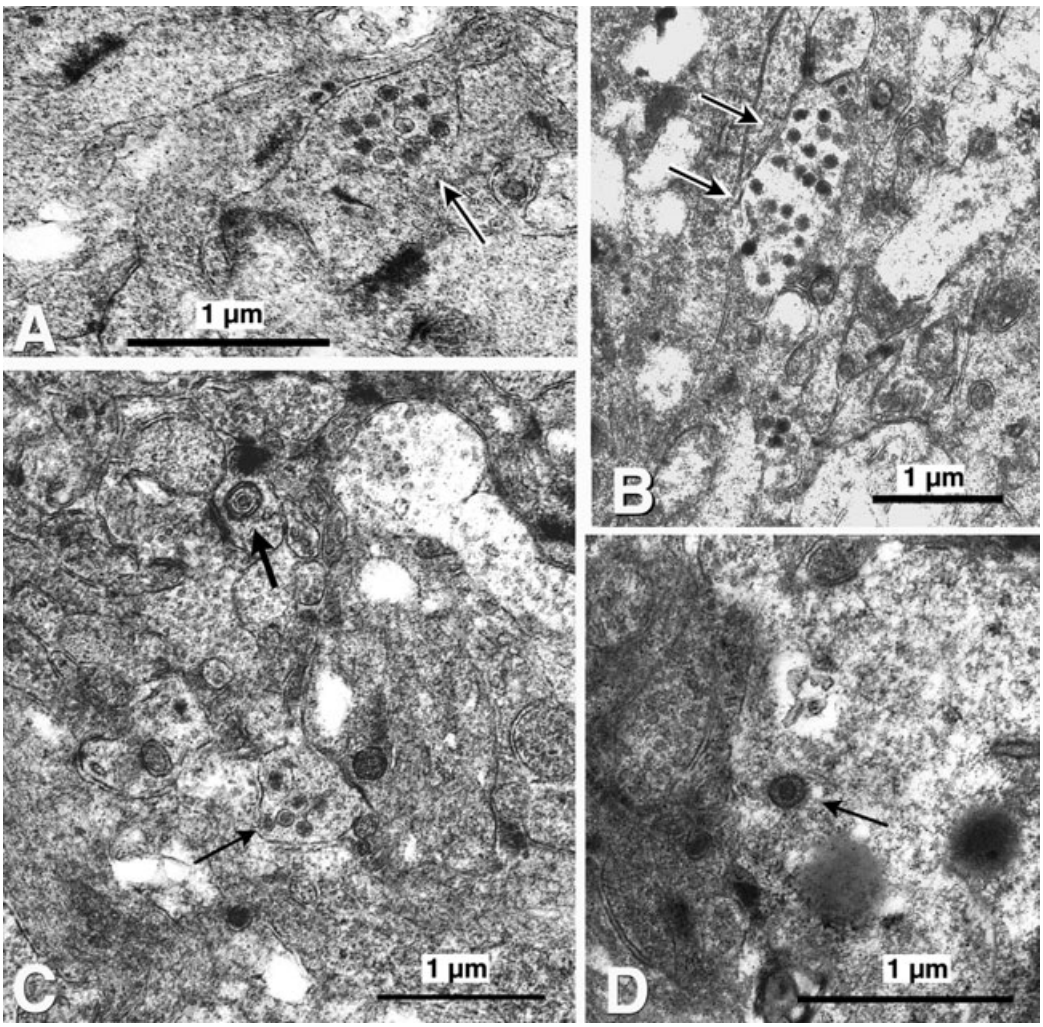

FIG. 7. Electron microscopy of virus label in transplant plexiform layers. (A–C) Inner plexiform layer of transplant. Arrows point to viruses in nerve processes (mostly nonenveloped). In (C), the thick arrow points to an enveloped virus, the thin arrow to nonenveloped viruses. (D) Enveloped virus in presumed cone pedicle in outer plexiform layer of transplant.

subsequent fusion of the envelope with the membrane. However, although PRV DNA can be replicated in various cell types, only neurons, not glial cells, can produce complete viruses with a virus envelope that can infect other cells (Card *et al*., 1993). The same principle applies to neonates (Rinaman *et al*., 1999).

The inability of astrocytes to produce infectious virus helps to prevent nonspecific extracellular spread of the virus away from the synapse (Card *et al*., 1993). Our study shows that Müller cells have the same defect as astrocytes, as no enveloped virus could be found in Müller cells.

Once PRV enters nerve terminals, the envelope is lost and the 'nude' virions migrate retrogradely to the cell body. After the virus DNA is replicated in the nucleus, it is transferred to the cytoplasm where the virus capsids are assembled. Then, the virions are packed in an envelope in the trans cisternae of the Golgi (Card *et al*., 1993). PRV cannot leave the cell and infect other cells without its envelope (Card, 1998).

There is overwhelming evidence indicating that neuron-to-neuron transmission of attenuated PRV is synaptic. The strongest proof that neurons can infect each other only via synaptic contacts comes from a developmental study (Rinaman *et al*., 2000), which showed that the transneuronal passage of PRV was directly dependent on the establishment of synapses. After virus injection into the stomach, the medullary dorsal vagal complex (DVC) was retrogradely infected in 1–8-day-old rats. However, before synapse formation between DVC neurons and CNS afferents, the virus did not spread from DVC neurons to the CNS although the CNS afferents had grown processes into the DVC.

Pseudorabies virus does not spread through the blood stream (Larsen *et al*., 1998) because the virus concentration would be too low to achieve infection (Card, 1998). The fact that no virus label in the retina was seen in a rat with optic nerve damage supports this.

With longer survival times, heavy label of neurons in one area will lead to secondary infection of glial cells and invasion of macrophages (McLean *et al*., 1989; Card *et al*., 1993; Rinaman *et al*., 1993). The reaction of non-neuronal cells occurs before the death of the infected neurons (Rinaman *et al*., 1993). This prevents the spread of infectious particles as the glial cells cannot produce the virus envelope. Microglia and macrophages cannot themselves become infected after phagocytosis of infected neurons (Card *et al*., 1993). PRV injection is always deadly as the virus eventually spreads through the whole brain and destroys infected neurons. The time to death depends on the injection site. In our setup, it took 5–6 days after injection.

Virus tracing in the visual system

For this study, pseudorabies virus tracing was done in a neuronal circuit that had not yet been characterized by this method before. One study injected different gI or gE mutant virus strains into the SC and showed retrograde transport to retinal ganglion cells, but did not investigate the subsequent virus infection of inner retinal neurons (Husak *et al*., 2000). After injection into the eye, PRV-Bartha does not spread anterogradely from retinal ganglion cells (Enquist *et al*., 1994) but is specifically transported retrogradely to the suprachiasmatic nucleus and other nuclei projecting to autonomic ganglia (Pickard *et al*., 2002; Sollars *et al*., 2003; Smeraski *et al*., 2004). The presence of functional gI and gE glycoproteins (which are missing in PRV-Bartha and -BaBlu) in the virus envelope is necessary to allow for anterograde virus transport (Husak *et al*., 2000). Enucleation of the eye 24 h after intraocular virus injection does not change the virus infection pattern in the brain, indicating that retinal ganglion cells and the optic nerve are not involved, but rather the autonomic ganglia (Pickard *et al*., 2002; Smeraski *et al*., 2004). The virus will appear in retinal ganglion cells of the contralateral eye at 96–98 h (4 days) after intraocular injection. Similar to the results in our study, Müller cell labeling is seen close to the infected ganglion cells (Sollars *et al*., 2003).

Virus label in the retina and in transplants

In our experiments, virus-labeled neuronal cells were found both in the host retina and in retinal transplants after injection into the SC. In the transplant as well as in sections from control animals, several virus-labeled nuclear layers could easily be distinguished and virus-labeled cells had neuronal characteristics at the electron microscopic level.

Label of Müller glial cells had already occurred at 35 h post-infection, after the virus had reached the somas of ganglion cells, similar to what was found by Sollars *et al*. (2003). With longer survival times it often became difficult to identify individual labeled cells, especially on thick vibratome sections. Signs of neuronal degeneration were evident at 3 days after virus infection. Müller cells were often heavily labeled in the host retina and often also in the transplants. Thus, some of the labeled cells in the transplants were infected Müller cells. Müller cells surround each retinal neuron, have mechanisms for neurotransmitter uptake (Rauen *et al*., 1998), thus regulating neurotransmission (Newman & Zahs, 1998), and express the same high affinity receptors for neurotransmitter uptake as astrocytes (review Newman, 2003).

However, our electron microscopic studies confirmed that Müller cells do not produce enveloped virus, and that enveloped virus was only found in neurons in the transplant and the host. Thus, the virus could not have spread from host or transplant Müller cells to transplant neurons.

Our study proves the presence of anatomically functional synapses between transplant and host. However, it does not show whether the synapses were physiologically functional.

Virus injections into the SC that only labeled the host retina outside the transplant and missed the target transplant area in the host retina ($n = 13$) did not result in any label of transplant cells, even if the host retina close to the transplant was labeled. Therefore, the transplant did not appear to connect to the host retina outside the transplant area.

Heavily labeled transplants were found in only 14% of the rats allowed to survive for 2 days but in 63% of the rats allowed to survive for 3 days. This indicated that the virus passed several synapses to reach the transplant, which took more time. In addition, survival times of 3 days after virus injection gave the virus a chance to spread laterally via amacrine and horizontal cells of the host retina. Thus, if the virus labeled only the edge of the transplant area of the host retina, one could still see labeled cells in the transplant, which was not the case with 2 days' survival. On the other hand, in some experiments the virus label was too heavy at 3 days after virus injection so that individual cells could not be distinguished.

Three animals did not show any label in the transplant despite the topographically correct injection of the virus. All three of these animals had a survival time of 2 days after virus injection, so it might be that the virus did not have enough time to travel into the transplant. It might also indicate that these transplants did not have any connections with the host retina in the virus-labeled area or that these connections were not sufficient to allow the virus to migrate through at the given time. Because the virus transport from the SC is restricted to a very localized retinal area, it does not exclude the possibility that such transplants had connections with the host in other areas that were not virus-labeled.

Because of the variability of transplants and methods of analysis, it is difficult to establish the extent of synaptic connectivity between transplant and host from this study, but the data indicate that synaptic connectivity exists. In most cases the intensity of virus label in the transplant was less than in the host, indicating that the virus infected the host retina first before the transplant.

In our study, the cell types in transplant and host contributing to the connections have not been identified. Solving these questions would require triple label to identify the transplant, the cell type and virus-infected cells. The recently produced PRV-Bartha strains expressing green and red fluorescent proteins (Smith *et al*., 2000; Banfield *et al*., 2003) will make the tracing much easier in future studies because no stain is necessary to detect these viruses, and they can be used in combination with different cell markers.

Significance of this study

An established method, PRV virus tracing has been proven to be useful to establish synaptic circuitry in a difficult graft–host model. This method can be of great help for researchers working with neuronal transplants to other areas of the CNS because of the specific transneuronal transfer of the virus between synapses.

In summary, this study indicates local synaptic connections between retinal transplants and a host retina with photoreceptor degeneration. Which cell types participate in these connections, and in which way the synapses are functional, need to be investigated. The transplant-mediated restoration of visual responses in blind rats (Woch *et al*., 2001; Sagdullaev *et al*., 2003; Thomas *et al*., 2004) is probably due to synaptic connections although a rescue effect of the transplant on host cones is also involved. The beneficial effect of retinal transplants indicates that retinal transplantation can be developed to a viable therapeutic treatment for retinal diseases.

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Abbreviations

E, embryonic day; H, host; GC, ganglion cell layer; IN, inner nuclear layer; IP, inner plexiform layer; ON, outer nuclear layer; OP, outer plexiform layer; PRV, pseudorabies virus; RCS, Royal College of Surgeons; RPE, retinal pigment epithelium; SC, superior colliculus; T, transplant.

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