Influence of adult Schwann cells and olfactory ensheathing glia on axon–target cell interactions in the CNS: a comparative analysis using a retinotectal co-graft model

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We used an in vivo transplant approach to examine how adult Schwann cells and olfactory ensheathing glia (OEG) influence the specificity of axon–target cell interactions when they are introduced into the CNS. Populations of either Schwann cells or OEG were mixed with dissociated fetal tectal cells (presumptive superior colliculus) and, after re-aggregation, pieces were grafted onto newborn rat superior colliculus. Both glial types were prelabeled with lentiviral vectors encoding green fluorescent protein. Grafts rapidly established fiber connections with the host and retinal projections into co-grafts were assessed 6–56 days post-transplantation by injecting cholera toxin B into host eyes. In control rats that received pure dissociated–reaggregated tectal grafts, retinal ganglion cell (RGC) axons selectively innervated defined target areas, corresponding to the retinorecipient layer in normal superior colliculus. The pattern of RGC axon ingrowth into OEG containing co-grafts was similar to that in control grafts. However, in Schwann cell co-grafts there was reduced host retinal input into presumptive target areas and many RGC axons were scattered throughout the neuropil. Given that OEG in co-grafts had minimal impact on axon–target cell recognition, OEG might be an appropriate cell type for direct transplantation into injured neuropil when attempting to stimulate specific pathway reconstruction.

Keywords: Glia, regeneration, axonal guidance, transplantation, retinal ganglion cells

INTRODUCTION

To re-establish functionally effective synaptic connections in the injured adult CNS, axons must be induced to regrow either through or around an area of injury; they then also must re-enter undamaged CNS tissue to terminate in relevant regions on appropriate target neurons. When used as bridging substrates, transplanted Schwann cells and OEGs, or a combination of the two, have been reported to promote the regrowth of injured CNS axons (e.g. David and Aguayo, 1981; Xu *et al.*, 1995; Li *et al.*, 1997; Ramon-Cueto *et al.*, 1998; Dezawa and Adachi-Usami, 2000; Ramon-Cueto *et al.*, 2000; Bamber *et al.*, 2001; Takami *et al.*, 2002; Plant *et al.*, 2003; Pearse *et al.*, 2007). However, a comparison of how these two cell types influence the way regenerating axons recognize and re-connect with deafferented target regions/neurons in CNS neuropil has not yet been made.

Previous peripheral nerve graft experiments have offered insights into the influence of Schwann cells on innervation of the superior colliculus (SC) by regenerating adult rodent retinal ganglion cells (RGCs). Ultrastructural and functional synaptic features resembled the retinal innervation in normal SC, but misplaced terminals were seen below the retinorecipient target layer, and only imprecise topographic maps were formed (Carter *et al.*, 1991; Carter *et al.*, 1994; Sauvé *et al.*,

1995; Thanos *et al.*, 1997; Sauvé *et al.*, 2001). Furthermore, RGC axons that have regrown through a peripheral nerve graft environment can form aberrant contacts with non-target tissues such as cerebellum and skeletal muscle (Zwimpfer *et al.*, 1992; Tan and Harvey, 1999).

To study the specificity of axon innervation of CNS target regions *in vivo*, a graft model has been developed previously involving the transplantation of fetal tectal tissue onto the midbrain of newborn host rats (Lund and Hauschka, 1976; Lund and Harvey, 1981; Harvey and Lund, 1984) (Fig. 1). After transplantation, fetal tectal grafts establish fiber interconnections with the underlying host midbrain. These initial connections are retained into adulthood despite the fact that, as the host brain matures, the grafted tissue is usually displaced caudally by the rostrocaudal growth of the maturing cerebral cortex. This physical displacement of the graft tissue frequently results in the formation of discrete fiber tracts between the host and graft (Fig. 1). Mature grafts possess features that are characteristic of the normal SC. For example, they contain localized areas or patches that morphologically (Lund and Harvey, 1981; Harvey and Warton, 1986; Harvey *et al.*, 1993) and neurochemically (Harvey and MacDonald, 1985; Tan and Harvey, 1987; Harvey, 1994; Harvey *et al.*, 1994) resemble the retinorecipient superficial gray layer (stratum griseum superficiale, SGS).

Fig. 1. Schematic representation of the experimental fetal tectal co-graft model. (A) Dorsal view of a rat superior colliculus (SC) after removal of the overlying cerebral cortex, 50 days after fetal tectal transplantation. The limits of the mature graft located on the dorsal midbrain surface are indicated by the dotted line. A fiber tract (arrows) maintains a connection between the host SC and the graft. (B) Schematic representation of typical RGC innervation of the graft tissue. Retinal axons from the neonatal host eyes innervate their normal target, the SC (1), and also form localized connections with the graft (2), specifically within retino-recipient (AChE-positive) target areas near the graft surface. In the presence of neonatal Schwann cells (co-grafting experiments), many RGC axons become scattered throughout the graft neuropil (3), no longer restricted to specific superficial target regions (Harvey and Plant, 1995). G, fetal tectal graft; IC, inferior colliculus; C, cerebellum.

Ingrowing host RGC axons specifically terminate in these localized areas, which, like the SGS, are intensely acetylcholinesterase (AChE)-positive, but only patches located close to the graft surface are innervated (Lund and Harvey, 1981; Harvey and MacDonald, 1985). This retinal input is functional (Dyson *et al.*, 1988; Golden *et al.*, 1989; Girman, 1993). Characteristic AChE-positive ‘target’ areas form even after the fetal tectal tissue is dissociated and reaggregated *in vitro* prior to transplantation (Bairstow and Harvey, 1992).

Based on these observations, this model was previously used in a co-graft study designed to assess the influence of neonatal Schwann cells on host retinal innervation of transplanted target cells (Harvey and Plant, 1995). Purified Schwann cells were mixed with fetal tectal cells before transplantation into neonatal host rats. The presence of these peripheral glia was found to be detrimental to target selection in that many RGC axons tracked Schwann cells into deeper parts of the graft, away from presumptive retino-recipient target areas.

Although transplanted OEG are reported to promote regeneration in spinal cord and optic nerve, the influence of these glia on target selection by regenerating CNS axons is unknown. In their normal environment OEG are associated with the axons of olfactory sensory neurons, which are being turned over continuously throughout adult life. OEG are thought to chaperone newly growing axons into their CNS target, the olfactory bulb, where they synapse on the dendrites of mitral and tufted cells in the glomerular layer. Because the olfactory pathways are continuously being rebuilt, we hypothesized that OEG might have only minimal

impact on the growth of RGC axons towards appropriate target areas in CNS tissue.

OBJECTIVE

Using the tectal co-graft model, we set out to determine whether RGC axons in the presence of adult OEG continue to form appropriate connections with target regions in the transplants. Normal grafts as well as co-grafts of fetal tectal cells and Schwann cells were used for comparison (Bairstow and Harvey, 1992; Harvey and Plant, 1995). To identify Schwann cells and OEG, these cells were prelabeled by *ex vivo* transduction with a lentiviral vector encoding the gene for green fluorescent protein (GFP) (Ruitenber *et al.*, 2002; Hu *et al.*, 2005; Pearce *et al.*, 2007). The labeled cells were mixed with dissociated fetal tectum before reaggregation and were then transplanted onto the midbrain of newborn host rats. Survival of GFP-labeled Schwann cells and OEG was monitored and host retinal projections into each graft type examined up to 56 days post-transplantation by injecting both host eyes with the anterograde tracer cholera toxin B (CTB). Retinal input into putative target areas and the pattern and distribution of host retinal innervation was then analyzed.

METHODS

A total of 56 male or female newborn (Po) Fischer F344 rats in seven litters (Animal Resources Centre, Perth, WA) were

used as hosts in this study. Seven time-mated Fischer rats (E15, day after mating = E0) were used to provide donor tectal tissue and a further 20 rats (8–10-weeks old, female) were used to provide adult Schwann cell and OEG culture preparations. Rats that received tectal grafts were weaned at P21. Animals had free access to water and food and were maintained at 12 hour dark–light cycle. Experiments were conducted in accordance with NHMRC guidelines and approved by the UWA Animal Ethics Committee.

Primary cell cultures

Schwann cells

Methods used to isolate adult Schwann cells have been described previously (Morrissey *et al.*, 1991). In brief, Schwann cells were harvested from sciatic nerves of euthanized (Sodium Pentobarbitone; 150 mg kg⁻¹ i.p.) adult rats. After removal of the perineurium, nerves were chopped and placed in dishes containing 2 ml of Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹) (Gibco), i.e. D-10S. Fibroblasts were allowed to grow out of explants for three weeks. Nerve explants were transferred to a new dish each week. Nerve explants were then dissociated with dispase (1.25 U ml⁻¹; Roche) and collagenase (0.05%; Worthington) in DMEM containing 15% FCS and incubated overnight at 37°C in 5% CO₂. After centrifugation and resuspension cells were plated onto poly-L-lysine (PLL, 100 µg ml⁻¹; Sigma) coated dishes in D-10S medium with added mitogens (20 µg ml⁻¹ bovine pituitary extract and 2 µM forskolin) (both Sigma).

Olfactory ensheathing glia

Primary adult OEG cultures were prepared as previously described (Yan *et al.*, 2001). After removal of sciatic nerves for Schwann cell preparations (see above), the ventral portion of olfactory bulbs was dissected out, finely chopped and incubated in 0.25% trypsin and 50 µg ml⁻¹ DNase (Gibco) in 2 ml Hank's balanced salt solution (HBBS; Sigma) at 37°C for 1 hour with continual shaking. After addition of 2 ml of a 1:1 mixture of DMEM and Ham's F-12 medium (DF; Sigma) containing 10% FCS, 2 mM L-glutamine, 50 µg ml⁻¹ gentomycin (Gibco), 20 µg ml⁻¹ bovine pituitary extract, 2 µM forskolin (DF-10S plus mitogens), tissues were further dissociated by trituration. After centrifugation and resuspension, cells were plated on PLL-coated dishes (100 µg ml⁻¹) and grown for 1 week before purification by immunopanning against the p75 receptor. For this, mouse monoclonal anti-p75 antibody (clone 192; Gift of Dr. Patrick Wood, University of Miami) was bound to non-tissue-culture-treated dishes as described by Yan *et al.* (Yan *et al.*, 2001). Next, primary olfactory bulb cultures were dislodged by trypsinization, triturated with a narrow bore glass pipette to ensure single cell suspension before being added to anti-p75-coated dishes. Cells were allowed to adhere for 30 minutes at 4°C. Any unbound cells were removed via several rounds of washing with L-15 medium (Gibco, Invitrogen). The p75-selected OEG were then replated onto PLL-coated dishes and fed every 3 days with DF-10S plus mitogens. Routinely these preparations yield 95–98% pure Schwann cell and OEG preparations, determined by the ratio of p75-positive cells versus total cell number (Hoechst 33342 nuclear dye, Sigma) (Fig. 2A,B).

Lentiviral transduction of primary cell cultures

Transplanted Schwann cells and OEG are difficult to identify in CNS tissues because they lack unique phenotypic markers. To overcome this problem, primary cell cultures were transduced before mixing with fetal tectal cells with replication-deficient LV vector encoding GFP. The production, purification and transduction methods of Schwann cells and OEG have been described in detail elsewhere (Ruitenber *et al.*, 2002; Hu *et al.*, 2005; Pearse *et al.*, 2007). Twenty-four hours before infection, the medium was changed to DF-10S with reduced mitogen concentration. Cultures were subsequently transduced by overnight incubation with LV-GFP (multiplicity of infection of 50). The following day, the medium was replaced and the cells left for a further 2 days to allow for initiation of transgene expression.

After three washes with HBBS, cells were detached by trypsinization, centrifuged (300×g) and washed with serum-free DF medium. Cells were resuspended in DF, counted and pelleted by low-speed centrifugation. Pelleted cells were resuspended in an appropriate volume of DF. The efficiency of LV-GFP transduction of glial cells was determined by comparing the ratio of the number of cells fluorescing green under ultraviolet light (expressing GFP) with the total number of cells identified by Hoechst 33342 dye. Both cell types expressed the phenotypic marker p75 (Ramon-Cueto and Nieto-Sampedro, 1992; Brook *et al.*, 1994; Li *et al.*, 1998; Mosahebi *et al.*, 2000).

Preparation and implantation of the graft

Fetal cells were obtained from E15 tecta using procedures described previously (Bairstow and Harvey, 1992). Dissected tecta were incubated in 5 ml Ca²⁺- and Mg²⁺-free Hank's medium (Gibco) at 37°C. After 10 minutes, tecta were trypsinized in 5 ml of Hank's solution and 0.05% trypsin for 25 minutes at 37°C. Cold Hank's medium was added and after centrifugation the pellet was washed and resuspended in 1 ml of cold D-10 medium. Cells were dissociated by trituration and counted. About 2 × 10⁶ cells were obtained per whole tectum (two SCs).

Fetal tectal grafts were reaggregated by low-speed centrifugation (5 minutes, 300×g). The control group received pure dissociated–reaggregated fetal tectal graft whereas the test grafts were composed of tectal tissue mixed with either adult LV-GFP-transduced Schwann cells or OEG. To achieve this, the number of dissociated fetal cells was estimated under a bright field microscope using a hemocytometer and an appropriate number of glial cells was thoroughly mixed into the tectal suspension. The proportion of adult Schwann cells to tectal cells in two litters was 12.5% and 15%, for OEG the proportions for different litters were 12%, 12%, 13% and 15%. These minor variations resulted from small differences in total LV-transduced glial cell numbers available at the time of co-graft preparation. Importantly there was no correlation between initial number of glial cells and retinal innervation in mature co-grafts. After reaggregation of control or tectal co-grafts the pellet was drawn up into a pipette and slowly extruded into media. The aggregated material was then cut into pieces, equivalent to one superior colliculus or ~10⁶ cells (i.e. containing ~10⁵ Schwann cells or

Fig. 2. See following page for legend.

OEG), and each piece was grafted onto the left SC of ether-anesthetized newborn (Po) rats (Lund and Harvey, 1981).

Tissue processing

Most host animals ($n = 37$) survived for 48–56 days (long-term or mature grafts). Two mature Schwann cell co-grafts were analyzed slightly earlier, 35 days after transplantation. Six animals with immature OEG/tectal co-grafts and three with immature Schwann cell co-grafts survived for 6 days (P6) and another eight animals (three OEG and five Schwann cell co-grafts) were euthanized 15 days (P15) after transplantation. At these times, host rats were anesthetized with halothane (induction 5%, maintenance 2% in 1:3 O₂/N₂O mixture) and both eyes were injected with 0.5–0.6 μ l of cholera toxin B (CTB, 0.5%; Sigma) to trace retino-tectal and retino-graft connections. CTB was injected intravitreally via a glass micropipette. Twenty-four hours later, rats were deeply anesthetized with sodium pentobarbitone (150 mg kg⁻¹, i.p.) and transcardially perfused with heparinized (1 U ml⁻¹) phosphate buffered saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and post-fixed for 2–3 hours in 4% paraformaldehyde, cryoprotected in 30% sucrose overnight, followed by freezing and coronal sectioning (40 μ m) through the graft area and underlying host brainstem.

Immunohistochemistry

Traced RGC axons were visualized using standard immunohistochemical procedures. Endogenous peroxidase activity was quenched with hydrogen peroxide and sections were incubated overnight at 4°C with an anti-CTB antibody (List Biological Laboratories). The antibody was diluted 1:8000 in phosphate buffered saline containing 2% normal horse serum, 2.5% bovine serum albumin (Sigma) and 0.2% Triton X-100 (Sigma). After several washes, secondary antibody (1:800 biotinylated anti-goat IgG; Vector Laboratories) was applied for 2 hours at room temperature. The signal was amplified via avidin–biotin horseradish peroxidase complex (1:200, Vectastain Elite ABC kit; Vector Laboratories). Peroxidase conjugate was reacted with 0.05% 3,3-diaminobenzidine (DAB; Pierce) and enhanced with nickel ammonium sulphate in the presence of hydrogen peroxide, resulting in a black precipitate.

AChE-containing regions (presumptive RGC targets) in tectal grafts were identified using a direct coloring histochemical method (Harvey and MacDonald, 1985). Free-floating sections were washed in 0.1 M sodium maleate (pH 6.0) and incubated for 2 hours in a solution containing the reaction substrate acetylthiocholine iodide (0.5 mg ml⁻¹; Sigma) and an inhibitor of butyrylcholinesterase (10⁻⁴ M

Astra; alpha-dimethylaminopropionyl phenothiazine-HCl). Sections were then washed, mounted onto slides, dehydrated and coverslipped in Permount (Fisher Scientific).

Some tissue sections were immunostained with an antibody to GFP (Chemicon; MAB3580), diluted 1:200 in PBS containing 10% normal goat serum and 0.2% Triton-X-100. Secondary antibody, CYTM3 anti-mouse (1:500; Vector Labs) was applied for 30 minutes. Sections were mounted with Citifluor containing Hoechst 33342 dye (Sigma). Laminin in grafts was visualized using a pan-laminin antibody (1:200; ICN Biomedicals), diluted in PBS containing 10% normal goat serum and 0.2% Triton-X-100. Secondary antibody, CYTM3 anti-rabbit (1:500) was applied for 30 minutes. Sections were mounted with Citifluor containing Hoechst 33342 dye. Immunostaining and GFP fluorescence were visualized using conventional light and fluorescence microscopy as well as confocal laser scanning imaging.

Analysis and quantification

An estimate of the number of GFP-labeled Schwann cells and OEG at different survival times was obtained by counting the total number of GFP-labeled cells in grafted tissue in every sixth section from an adjacent series of sections. Next, the area of grafted tissue in the sections (1 in 6 series) was determined using Image Pro-plus software (Media Cybernetics) and multiplied by 6 $\times$ 40 μ m to obtain an approximate volume of the graft. The number of scattered axons and GFP-labeled cells was expressed per mm³ of graft.

To obtain semi-quantitative data on the specificity of host RGC axonal innervation of identified target regions in the different types of grafts, the number of CTB-labeled RGC axons in non-target areas was counted in 1 in 6 series throughout the whole of each graft. Non-target areas were defined as regions that were not AChE dense and at least 100 μ m from the surface of the graft. Axons found in these areas were termed ‘scattered’. The 100 μ m band from the surface of the graft was chosen because axons close to the surface, although not in obvious AChE-dense regions might nonetheless have been en route towards target regions in nearby sections (Harvey and Lund, 1984). In addition, the number of clearly defined, superficially located AChE-dense target areas was determined for each of the grafts and compared to the number of target areas that were innervated by host CTB-labeled RGC axons.

The innervation density of these AChE-dense target areas by CTB-traced RGC axons was also measured using ImageJ software. A total of 31 target patches in CTB-immunostained sections were randomly selected from 15 grafts ($n = 6$ from control graft; $n = 10$ from OEG co-grafts and $n = 15$ from Schwann cell co-grafts). To control for variations in intensity of immunolabeling between runs, gray value measurements

Fig. 2. Morphology of glial cells *in vitro* and *in vivo*. (A,B) Purified cultures of OEG (A) and Schwann cells (B) labeled with p75 (green), GFAP (red) and Hoechst nuclear dye (blue). (C,D) LV-GFP transduced OEG (C) and Schwann cells (D) before co-grafting with tectal cells. Both cell types expressed GFP (green) *in vitro*. There was some variability in transgene expression and a proportion of cells lacked detectable GFP label. Both cell types also stained for the phenotypic marker p75 (red); nuclei are labeled with Hoechst 33342 (blue). (E,F) GFP-labeled Schwann cells (E) and OEG (F) in tectal co-grafts 6 days after transplantation. Note early process extension by both cell types and the characteristic elongated morphology of Schwann cells. (G,H) Morphology of GFP-positive Schwann cells in 15-day-old tectal co-grafts. Note the bipolar or spindle shape of the majority of cells (G) and the formation of an aligned nerve-like tract (arrows) between two adjacent pieces of transplant in H (T₁ and T₂). (I–M) In mature co-grafts, surviving Schwann cells tended to cluster in small groups; OEG were more dispersed throughout the graft. Schwann cells were mainly bipolar in shape (I) whereas OEG exhibited a wide range of morphologies and sizes, some of them branching extensively (J,K; confocal images). (L,M) Same field from a Schwann cell co-graft showing intrinsic GFP fluorescence in Schwann cell bodies and processes (L) and visualization of GFP using GFP antibodies and indirect immunofluorescence (M). H, host midbrain. Scale bars: A–D,G,H,I,K, 100 μ m; E,F,I, 50 μ m; L,M, 40 μ m.

were normalized against the mean gray value in an adjacent non-innervated tissue region.

Cell counts and RGC innervation data from the different groups were statistically analyzed using one-way ANOVA and parametric (Bonferroni's post-test) or non-parametric tests (Mann-Whitney test or Kruskal-Wallis test with Dunn's post-test).

RESULTS

In vitro and *in vivo* characterization of Schwann cells and OEG

In cell culture, 60–80% of Schwann cells and ~60% of OEG expressed clearly detectable amounts of GFP and, thus, were unequivocally transduced with the LV vector. Immunolabeling with a GFP antibody did not appreciably increase these proportions. Both purified adult OEG (Fig. 2C) and Schwann cells (Fig. 2D) expressed high levels of p75. There was good intermixing of Schwann cells or OEG within the larger population of tectal cells, evidenced by the fact that in each host litter all co-grafts contained GFP-positive glia (see also Harvey and Plant, 1995).

In vivo, the morphology of GFP-labeled Schwann cells in tectal co-grafts was mainly unipolar or bipolar, with only ~5% of cells exhibiting a more branched morphology. A typical example of GFP-labeled Schwann cells in a graft 6 days after transplantation is shown in Fig. 2E. Note at this early time-point the expression of processes by these cells, many of which appeared aligned. By P15, fewer labeled cells were detected but many of them displayed typical spindle shape of Schwann cells (Fig. 2G). Cells sometimes formed coherent structures in tectal grafts; in Fig. 2H an example is shown of two grafts (T_1 and T_2) and GFP-labeled Schwann cells forming a tract (arrows) between the two transplants. In mature grafts, the number of Schwann cells appeared to be reduced further and the few remaining cells were often found clustered together (Fig. 2I,L).

The typical morphology of GFP-labeled OEG in a P6 co-graft is shown in Fig. 2F. At this short survival time, GFP-positive cells already displayed numerous branches and, often, irregular processes. In contrast to Schwann cells, OEG did not align but were dispersed in a more random manner throughout the graft. OEG were also seen in grafted material that had become situated more ventrally and laterally, around the side of the host midbrain. In mature tectal co-grafts fewer cells were seen, but identified GFP-labeled OEG exhibited a wide variety of morphologies ranging from small, unipolar and bipolar cells (75% of cells) to large, stellate-like cells with feathered and flattened processes (Fig. 2J,K). Extensive branched morphology was observed in 25% of grafted OEG. Compared to Schwann cells, in both immature and mature co-grafts GFP-labeled OEG were dispersed more evenly throughout the transplanted tissue. Immunostaining of sections with an antibody to GFP did not reveal obviously greater numbers of LV-transduced cells in mature co-grafts (Fig. 2L,M). In fact, intrinsic fluorescence in GFP-transduced cells often appeared brighter, with clearly identifiable glial cell bodies and nuclei (Fig. 2L). With GFP immunohistochemistry the Schwann cell nuclei were not labeled, perhaps because of lack of antibody penetration (Fig. 2M).

Some GFP-cells were located within the meninges that covered the co-grafts. On average, 12% of GFP-labeled Schwann cells and 31% of GFP-labeled OEG were found either in or in graft tissue just below these meninges, where they co-localized with p75 expression and high levels of laminin immunoreactivity (not shown). Immunostaining for laminin was greatest in Schwann cell co-grafts and lowest in control grafts, but in all cases was higher than in adjacent host tissue. No GFP-labeled glial cells were seen within the host neuropil at any time post-transplantation.

Analysis of co-graft volume and number of GFP-labeled Schwann cells and OEG

Qualitative inspection of co-grafts at P6, P15 and in mature grafts indicated a substantial decline in the number of both GFP-labeled Schwann cells and OEG. In 43 grafts (22 Schwann cell and 21 OEG co-grafts) the number of GFP-labeled glia was therefore counted in a 1:6 series of 40- μ m sections through the co-grafts, and an estimate obtained of the number of glia per mm³ in Schwann cell and OEG co-grafts at different times post-transplantation. The volume of graft tissue was also estimated. Non-parametric statistical analyses of these quantitative data revealed that average GFP-positive Schwann cell and OEG numbers per mm³ did indeed decline in the weeks following co-transplantation with fetal tectal tissue (Fig. 3A). There was substantial variance in the P6 and P15 data ($n = 3-5$ each group) but the number of Schwann cells and OEG were not significantly different from each other at these two early time-points. However, in

Fig. 3. Quantitative analysis of cell survival and graft volume. (A) Estimated number of GFP-labeled Schwann cells and OEG per unit graft volume at different times after transplantation. Note the decrease in number of both cell types over time; this decrease was significant when comparing P6 with long-term grafts. Pairwise comparisons (non-parametric Mann-Whitney test) showed no significant difference between Schwann cell and OEG numbers at P6 and P15, during the period of host retinal innervation. Schwann cell numbers were slightly higher in long-term grafts. (B) Estimated graft volume at different times post-transplantation. Data are mean $\pm$ s.e.m.

mature grafts, estimated numbers of Schwann cell were slightly higher than OEG ($P < 0.05$, Mann-Whitney test). Surviving GFP-labeled Schwann cells and OEG were often found to be located towards the surface of the grafted tissue.

In agreement with a previous report (Harvey and Lund, 1984), there was a trend towards a slight increase in graft volume in the days following transplantation (Fig. 3B) although in the present study changes in average graft volumes were not significant (Kruskal-Wallis comparisons). These findings do however demonstrate that the substantial fall in Schwann cell and OEG numbers with graft maturation does not merely reflect a decrease in encounter rates because of dilution of transplanted glia in an increased volume of neuropil.

Host retinal innervation of co-grafts

After injection onto the newborn host SC, by 3–4 days post-grafting almost all fetal tectal grafts have begun to receive ingrowth of RGC fibers (Lund and Harvey, 1981; Harvey and Lund, 1984). This innervation therefore occurs at a time when Schwann cell and OEG numbers are maximal in co-grafts and not significantly different from each other (Fig. 3). Host CTB-labeled RGCs axons were already present in co-grafts at P6 but the pattern of ingrowth was different between Schwann cell versus OEG co-grafts (Fig. 4). In developing Schwann cell co-grafts, ingrowing RGC axons were not restricted to the graft surface but penetrated into the core of the grafts, often in close association with GFP-labeled Schwann cells (Fig. 4A,B). In OEG co-grafts, initial retinal innervation was restricted mainly to the surface, even when OEGs were located nearby (Fig. 4C), similar to the pattern of RGC axonal ingrowth described in normal tectal grafts (Harvey and Lund, 1984). By P15, although occasional RGC axons were seen throughout the graft neuropil, host retinal innervation of OEG co-grafts was essentially target selective (data not shown).

The pattern of RGC axon innervation in P6 and P15 co-grafts was recapitulated in the mature co-grafts. Animals with 'pure' tectal grafts served as controls for the effect of the dissociation-reaggregation procedure. Seven of the 10 mature control grafts, 10 of the 14 Schwann cell co-grafts and 12 of the 15 OEG co-grafts had clear tissue continuity with the underlying host SC. However, in four of the connected grafts (two control and two OEG co-grafts) there was relatively sparse CTB labeling in host SC at the site of the interconnection, indicating incomplete eye injections; these grafts were not included in subsequent analysis of host retinal innervation.

In control grafts, background AChE staining was relatively uniform, but there were clearly defined AChE-dense regions (Fig. 5A). It is well-established that these AChE patches contain presumptive superficial layer neurons and when located close to the surface of tectal grafts these patches are target areas for retinal axons (Lund and Harvey, 1981; Harvey and MacDonald, 1985; Tan and Harvey, 1987; Dyson *et al.*, 1988). Anterograde CTB label was seen in the superficial retinorecipient layers of the host SC, with small numbers of axons also observed coursing just below the surface of the host inferior colliculus (not shown). In control grafts, CTB-immunoreactive RGC axons (Fig. 5B) were almost always located within AChE-dense areas that were situated either at

Fig. 4. Pattern of retinal innervation in 6-day old tectal co-grafts. (A,B) In P6 Schwann cell co-grafts, host CTB-labeled retinal axons had already grown into the grafted tissue (arrows). Axons were not confined to the surface of the grafts and were often seen in close association with GFP-labeled Schwann cells deep inside the graft (A and B are combined fluorescent and transmitted light images). (C) Many OEG were also seen in P6 tectal co-grafts. Host retinal input to the co-grafts usually remained unaffected by OEG presence [i.e. at the surface of the grafts (arrows) even though were OEG nearby]. T, transplant; H, host midbrain. Scale bars: A,C, 100 μ m; B, 50 μ m.

or just below the graft surface (Fig. 5A). Ramified arbors of retinal axons, bearing numerous varicosities, were seen within AChE patches to a depth of about 100–150 μ m from the graft surface. Only in one instance did we observe a central AChE patch that contained CTB-labeled retinal axons.

In Schwann cell co-grafts, numerous host CTB-labeled retinal axons were found in presumptive target (AChE patches) and, most importantly, non-target areas (i.e. no AChE label). In most of these grafts substantial numbers of RGC axons were scattered throughout the co-graft neuropil (Fig. 5C). Scattered RGC axons were sometimes found in close proximity to GFP-labeled Schwann cells (Fig. 5C₁,C₂).

Fig. 5. Pattern of retinal innervation in mature tectal co-grafts. (A) Typical example of a normal AChE-stained tectal (control) graft with small, localized, AChE-dense regions (e.g. arrow). (B) Host retinal innervation is confined to these regions (arrow). (C) In mature Schwann cell co-grafts, CTB-labeled RGC axons were often found dispersed throughout the graft in non-target areas (arrows). The boxed areas in C are shown at higher magnification in C₁ and C₂ where some of the scattered RGC axons can be seen associated with GFP-labeled Schwann cells (combined fluorescent and transmitted light image). (D) AChE distribution is relatively uniform in an OEG co-graft with distinct AChE-dense target areas (arrow and asterisk). (E) Both regions are selectively innervated by CTB-labeled RGC axons, with either no or few scattered fibers. In this example axons in E denoted by the asterisk were superficial because the section was cut close to the rostral border of the graft and, thus, *en face*. Scale bars: A, C, D, E, 500 µm; B, 100 µm; C₁, C₂, 50 µm.

On occasion, Schwann cells were located near RGC axons that had nonetheless appropriately innervated an AChE-dense target area. In co-grafts containing OEG, the innervation pattern resembled that in control grafts. The major sites of retinal axonal innervation were associated with AChE-dense patches (arrows in Fig. 5D,E). Most RGC axons not associated with AChE-dense target areas were found within 100 µm of the surface, but some axons were occasionally seen that were apparently located deeper within the neuropil

(asterisk, Fig. 5E). However, in this example the axons were quite superficial because the sections were cut close to the rostral boundary of the grafts and, thus, were *en face*.

The relative number of CTB-labeled RGC axons in non-target areas of Schwann cell and OEG co-grafts was extrapolated from counts from a 1:6 series of sections through mature grafts. Non-target areas were defined as regions that were not AChE dense and were at least 100 µm away from the surface of the graft. Axons in these areas were

termed scattered. Analysis was confined to grafts that were clearly connected to a host Schwann cell that contained good CTB label at the site of the host-graft connection ($n = 5$ control, $n = 7$ Schwann cell co-graft, $n = 10$ OEG co-graft). The number of scattered axons per mm^3 in each graft, and the mean $\pm$ s.e.m. for each graft type, are shown in Fig. 6A. These data reveal greatly increased scatter of retinal axons in Schwann cell co-grafts compared to control and OEG co-grafts. Parametric comparisons (Bonferroni) revealed that this increase of scatter in the presence of Schwann cells was significant ($P < 0.05$) although significance was not attained using non-parametric tests (Kruskal-Wallis). The difference between control and OEG co-grafts was not significant with either test. Importantly, within each co-graft group there was no discernible relationship between the number of surviving, GFP-labeled Schwann cells or OEG and the number of scattered axons, supporting the suggestion that mispatterning of RGC axons took place at a very early stage when there was no significant difference between the number of Schwann cells and OEG.

As a corollary of this increased axonal scatter in Schwann cell/tecal co-grafts it might be expected that presumptive target areas in such grafts would be either less frequently or

less densely innervated. We therefore also analyzed the number of clearly defined, superficially located AChE-dense target areas for each graft type and compared the number of target areas that were innervated by host CTB-labeled RGC axons. In the 1:6 series of AChE-stained sections from control grafts, 19 AChE-dense, superficially located target areas were identified. Eight of these patches (42%) were innervated. In OEG co-grafts, 68 superficial target areas were identified of which 27 (40%) were innervated and in Schwann cell co-grafts 48 AChE-rich areas were identified, of which only 16 (33%) were innervated. Furthermore, semi-quantitative analysis revealed that the density of retinal innervation of AChE-rich regions was significantly reduced ($P < 0.05$, Bonferroni, Kruskal-Wallis tests) in the Schwann cell co-grafts compared to target patches in control and OEG co-grafts (Fig. 6B). Thus, there was an inverse relationship between the proportion of target areas innervated and the number of scattered axons in co-grafts.

CONCLUSIONS

- Purified adult Schwann cells and OEG can be intermixed with dissociated fetal tectal tissue and, after reaggregation and transplantation to the neonatal brain, their fate and phenotype can be monitored in co-grafts by transducing the glial cells *ex vivo* with LV encoding GFP.
- There was loss of both glial cell types over time, but some survived and differentiated within the mature CNS graft neuropil.
- The presence of adult Schwann cells but not OEG in tectal co-grafts altered the pattern of host RGC axonal innervation, Schwann cells apparently misrouting axons away from presumptive target areas in the graft neuropil.

DISCUSSION

Crucial to the goal of attaining effective functional recovery in the damaged brain and spinal cord is the ability of regenerating axons to recognize relevant target areas and then reconnect with appropriate target neurons. Adult Schwann cells and OEG have both been shown to promote sparing and regrowth of injured axons in adult CNS, but their impact on the specificity of reinnervation is unknown. The purpose of this study was to evaluate how the introduction of adult Schwann cells and OEG into CNS tissue affected axon-target cell interactions. Specifically, we examined how these glial cells influenced the ability of growing rat RGC axons to selectively innervate their appropriate target areas within developing CNS neuropil. To do this, we took advantage of a unique co-graft model in which either adult Schwann cells or OEG were mixed with fetal tectal cells that were then grafted onto the neonatal rat brain, providing a new, ectopic target for host RGC axons. The pattern of host retinal innervation of these grafts was then assessed.

Survival of Schwann cells and OEG in tectal co-grafts

The use of LV-GFP to prelabel Schwann cells and OEG allowed us to identify transplanted glia within tectal co-grafts

Fig. 6. Quantification of Schwann cell and OEG influence on axonal innervation in tectal co-grafts. (A) Average number of scattered axons per unit graft volume (mm^3) in mature control tectal grafts and in Schwann cell and OEG co-grafts. Points represent data obtained from individual grafts (median values for these three graft types were 3.7, 243.9 and 19.9, respectively). (B) Semi-quantitative analysis of the density of RGC axons in localized target areas in grafts. The presence of Schwann cells impacts negatively on target innervation in this co-graft model. Data are mean $\pm$ s.e.m.

at different survival times and to monitor their long-term viability. No migration of GFP-labeled cells into host neuropil was observed at any time point. Comparisons revealed similar numbers of GFP-labeled Schwann cells and OEG in 6- and 15-day-old co-grafts, but there were slightly more GFP-labeled Schwann cells in mature grafts. In both types of co-graft there was a significant decline in the number of GFP-labeled Schwann cells and OEG in the weeks after transplantation. We have shown in other graft models that LV-GFP vectors stably transduce adult Schwann cells and OEG, and that these GFP-labeled cells survive for months after transplantation (Ruitenberg *et al.*, 2002; Hu *et al.*, 2005); however, to reduce the possibility that transduced glia were still present in co-grafts but their intrinsic GFP fluorescence was below detectable levels, we also probed sections with an antibody to GFP. This approach did not reveal increased numbers of transduced glial cells, either in culture or *in vivo*, thus, it is most likely that the observed loss of GFP label in tectal co-grafts was due to glial cell death, although silencing of transgene expression in at least some surviving glia may have occurred. Substantial loss of both adult Schwann cells and OEG has also been documented in the days following transplantation into injured spinal cord (Pearse *et al.*, 2007).

Integration and morphology of Schwann cells and OEG within tectal co-grafts

Although Schwann cells and OEG were intermixed with fetal tectal cells at the time of transplantation, and were dispersed within P6 grafts, Schwann cells were often found clustered and aligned in small groups in P15 and mature grafts. Most GFP-labeled Schwann cells displayed a bipolar configuration, which is typical of their appearance *in vitro* and *in vivo* (Ghirnikar and Eng, 1994; Guenard *et al.*, 1994; Xu *et al.*, 1995). More rarely, processes of Schwann cells located either in or close to the pia exhibited a more complex branching pattern. In contrast, GFP-labeled OEG were distributed more evenly throughout the grafts and exhibited a greater range of shapes and sizes. The majority were small unipolar and bipolar cells but an extensive branched morphology was observed in 25% of grafted OEG, with cells extending numerous fine processes. This morphology has been seen occasionally in culture as well as in the natural OEG environment, the olfactory nerve (Doucette, 1991; Plant *et al.*, 2002; Field *et al.*, 2003; van den Pol and Santarelli, 2003; Vincent *et al.*, 2003). OEG phenotype varies depending on the source, age and density of cells as well as the *in vitro* and *in vivo* conditions in which they reside or are placed (e.g. Harvey and Plant, 2006; Wewetzer and Brandes, 2007) (see also Fig. 2A,B). The present results indicate that elements within the developing tectal graft environment provided competent signals that either induced or allowed morphological differentiation of some co-grafted adult OEG.

Target specificity within tectal grafts

Control grafts of fetal tectal cells were used to confirm target-specific innervation. Our observations confirmed that there was selective host retinal innervation of superficial AChE-dense target regions in dissociated–reaggregated grafts (Bairstow and Harvey, 1992). As in normal development of retinofugal pathways, the vast majority of host retinal axons

that enter tectal grafts have a preference for near the tissue surface rather than within the graft neuropil (Harvey and Lund 1984). Consistent with this and similar to earlier studies (Lund and Harvey, 1981; Harvey and MacDonald, 1985), CTB tracing revealed that most RGC axons were located adjacent to the graft surface, with only occasional axons growing deeper than 100 μm into the graft neuropil. This mode of ingrowth does not appear to be caused by mechanical barriers because other host afferents, such as those from visual cortex, grow into and innervate the whole depth of grafted tectal tissue (Lund and Harvey, 1981).

Target specificity within Schwann cell and OEG tectal co-grafts

The major aim of the present study was to undertake a comparative analysis of the interactions between host retinal axons and presumptive target areas within tectal grafts in the presence of either adult Schwann cells or OEG. Compared to control grafts, adult Schwann cell co-grafts contained significantly more RGC axons scattered throughout the neuropil. Innervation of presumptive target (AChE-dense) areas was still seen in Schwann cell co-grafts, but the frequency of such interactions was reduced and these AChE-positive areas were innervated less densely. In P6 transplants, ingrowing host retinal axons were already seen to be closely associated with GFP-labeled Schwann cells deep within the graft neuropil. This observation is consistent with previous research in which bundles of RGC axons were diverted either past or away from target areas in the presence of neonatal Hoechst-labeled Schwann cells (Harvey and Plant, 1995). In mature grafts (>5 weeks post-implantation), abnormal innervation was still present and, even though fewer GFP-positive Schwann cells were detected, they were usually found in proximity to scattered RGC axons.

In OEG co-grafts, the pattern of retinal innervation resembled that in control tectal grafts. Relatively few scattered axons were seen and most RGC axons grew into superficial AChE-dense areas known to be homologous to the normal retinorecipient SC layers. The density of this innervation was not significantly different from controls. It is important to reiterate that in newborn hosts retinal axons grow into tectal grafts within 3–4 days after transplantation (Harvey and Lund, 1984). This is crucial to the interpretation of the present data because ingrowth occurred at a time when large, comparable numbers of viable Schwann cells and OEG were present in the immature co-grafted tissue. Despite this, in OEG tectal co-grafts, host RGC axons were mostly found at the graft surface and retinal innervation attained a mature configuration without passing through a transitional phase of non-specific graft innervation.

Schwann cells and OEG support the growth of RGC axons *in vitro* (Hopkins and Bunge, 1991; Kumar *et al.*, 2005; Leaver *et al.*, 2006; Pastrana *et al.*, 2006) and *in vivo* (Harvey *et al.*, 1995; Li *et al.*, 2003; Harvey *et al.*, 2006). Both glial populations synthesize similar types of diffusible growth factors (Mirsky and Jessen, 1999; Woodhall *et al.*, 2001; Lipson *et al.*, 2003) and a recent study has implicated brain-derived growth factor in OEG-mediated promotion of neurite extension from adult retinal neurons (Pastrana *et al.*, 2007). Nonetheless, our comparative co-graft data indicate that Schwann cells but not OEG are tropic for RGC axons

and either mask or compete with signals released by the target neurons themselves (see also Cho and So, 1989; Harvey and Plant, 1995). Schwann cell-conditioned medium promotes retinal neurite extension (McLoon and McLoon, 1988) but OEG-conditioned medium is less effective than the OEG themselves (Sonigra *et al.*, 1999; Leaver *et al.*, 2006) indicating that enhanced RGC axonal growth in the presence of OEG is not related to diffusible trophic factors but is primarily a contact-mediated effect. This is consistent with other work showing that the major neurotrophic effects of OEG are 'exerted at close range' and that factors produced by these cells might be present in low concentrations or are not secreted unless the cells are injured (Lipson *et al.*, 2003).

Significance for CNS repair: bridges versus direct transplantation into neuropil

Schwann cells and OEG have been transplanted directly into adult CNS neuropil in experimental studies aimed at promoting sparing and regeneration of damaged or severed CNS axons (e.g. Brook *et al.*, 1994; Li *et al.*, 1997; Li *et al.*, 1998; Ramon-Cueto *et al.*, 1998; Ramon-Cueto *et al.*, 2000; Takami *et al.*, 2002; Plant *et al.*, 2003; García-Álías *et al.*, 2004; Ruitenber *et al.*, 2005; Pearse *et al.*, 2007). Schwann cells (either purified or in peripheral nerve grafts) have also been used to promote axonal regrowth via external tissue bridges that bypass injured CNS areas (e.g. David and Aguayo, 1981; Xu *et al.*, 1995; Sims *et al.*, 1999; Dezawa and Adachi-Usami, 2000; Bamber *et al.*, 2001; Harvey *et al.*, 2006). Although these bridges promote regeneration, axonal outgrowth into host tissue can be limited, perhaps by trapping of axons in the neurotrophin-rich environment of the Schwann cell grafts.

Outgrowth might also be restricted by scarring at the Schwann cell-astrocyte interface. Schwann cells and OEG interact in different ways with mature astrocytes. *In vitro* and *in vivo*, Schwann cells tend to segregate from astrocytes (Sims *et al.*, 1999; Lakatos *et al.*, 2000; Plant *et al.*, 2001; Lakatos *et al.*, 2003) and, upon contact with Schwann cells, astrocytes become reactive, upregulating molecules such as proteoglycans that inhibit axonal growth (Lakatos *et al.*, 2000; Plant *et al.*, 2001; Takami *et al.*, 2002; Lakatos *et al.*, 2003; García-Álías *et al.*, 2004; Grimpe *et al.*, 2005; Leaver *et al.*, 2006). By contrast, OEG normally co-exist with astrocytes (Doucette, 1993) and reportedly have a greater capacity to intermingle with these glia elsewhere in the CNS (Ramon-Cueto *et al.*, 1998; Lakatos *et al.*, 2000; Lakatos *et al.*, 2003; García-Álías *et al.*, 2004; Fairless *et al.*, 2005; Santos-Silva *et al.*, 2007). These observations are not inconsistent with the more dispersed distribution of OEG with diverse and complex cell morphologies seen in our mature tectal co-grafts.

When using Schwann cell bridges, axonal outgrowth can be enhanced by co-grafting OEG to the distal end of the bridges (Ramon-Cueto *et al.*, 1998; Fouad *et al.*, 2005), grafting Schwann cells, supplying growth factors to distal neuropil (Bamber *et al.*, 2001), and reducing inhibitory components in the glial scar (Fouad *et al.*, 2005). However, the present results, together with evidence for example of synaptic loss in CNS neuropil occupied by transplanted Schwann cells (Sims and Gilmore, 2000), indicates that the direct introduction of Schwann cells into CNS neuropil will elicit significant axonal elongation, but via strong tropic influences these cells might interfere with the capacity of

regenerating axons to recognize and re-establish contact with appropriate target sites and the neurons therein. By contrast, in our co-graft model at least, OEG seem to have a relatively benign influence on CNS axon-target cell interactions, perhaps because of their normal role of guiding newly grown sensory axons into appropriate regions in the olfactory bulb. Thus OEG might be an appropriate cell type for transplantation directly into CNS neuropil, where they can promote sparing and facilitate axonal regrowth but have minimal impact on the specificity of axonal innervation of appropriate target structures.

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