

# Radial Glial Cell Line C6-R Integrates Preferentially in Adult White Matter and Facilitates Migration of Coimplanted Neurons *in Vivo*

Adilia Hormigo,\* Maria McCarthy,† Jean Manuel Nothias,‡ Koichi Hasegawa,§ Wencheng Huang,§ David R. Friedlander,\* § Itzhak Fischer,‡ Gord Fishell,† and Martin Grumet\* §

§W. M. Keck Center for Collaborative Neuroscience, Rutgers, State University of New Jersey, 604 Allison Road, Piscataway, New Jersey 08854-8082; \*Department of Pharmacology and †Skirball Developmental Genetics Program, NYU Medical Center, 550 First Avenue, New York, New York 10016; ‡Department of Neurobiology and Anatomy, Medical College of Pennsylvania, Hahnemann University, Philadelphia, Pennsylvania 19129

Received July 6, 2000; accepted November 27, 2000

**C6-R is a cell line derived from C6 glioma cells that exhibits key properties of radial glia including the ability to support neuronal migration in culture. To explore its potential use in promoting neuronal migration *in vivo*, we analyzed the behavior of C6-R cells in the intact and injured adult rat CNS. At 6–11 days postimplantation at the splenium of the corpus callosum, green fluorescent protein-labeled C6-R cells were observed primarily in either the corpus callosum or the hippocampus in the brain, and in the spinal cord they migrated more extensively in the white matter than in the grey matter. To determine whether C6-R cells retain their ability to promote neuronal migration *in vivo*, they were coinjected with labeled neurons into adult brain. When rat embryonic neurons were coimplanted with C6-R cells, the neurons and C6-R cells comigrated through a much larger volume than neurons alone or neurons coimplanted with fibroblasts. In brains preinjured with ibotenic acid, C6-R cells as well as coimplanted neurons distributed widely within the lesion site and migrated into adjacent brain tissue, while transplants with neurons alone were restricted primarily to the lesion site. The results suggest that radial glial cell lines can serve as a scaffold for neuronal migration that may facilitate development of experimental models for neural transplantation and regeneration.** © 2001 Academic Press

## INTRODUCTION

Transplantation has been used to restore function to cells and tissues that have a limited capacity for regeneration. This therapeutic modality has successfully rescued people from life threatening diseases, such as end-stage cardiac, liver, lung, or kidney disease, but its use in the nervous system is still at a relatively early stage. One major application in the brain has been in Parkinson's disease where transplants of autologous

adrenal medullary tissue and fetal dopaminergic allografts and xenografts have survived in the human brain (5, 9, 21, 22, 32). Transplantation therapies are actively being investigated for other nervous system conditions including ischemic cerebrovascular disease, degenerative dementias, cerebellar degeneration, and traumatic injuries of the head and spine.

Strategies for experimental transplantation in the central nervous system have attempted to compensate for the defect using embryonic neurons (30, 43, 44, 46), grafts of neural tissue (6, 45), and immortalized neural precursors (3, 28). Progress has been also made in transplanting various types of genetically modified immortalized cells to introduce neurotrophic factors, neurotransmitters, and metabolic enzymes *in vivo*, in attempts to correct deficiencies or make the environment more favorable for regeneration (28). The potential differentiation of stem cells in the adult brain (2–4, 11, 12, 40) and spinal cord (3) raises exciting new possibilities. It is becoming increasingly clear that nonneuronal cells can contribute to creating a more favorable environment *in vivo* for neuronal regeneration. Schwann cells, and, more recently, olfactory ensheathing glial cells, have been used to facilitate regeneration of axons (24, 25, 34, 35).

Interactions among neuronal and glial cells are critical factors for neural development and play additional roles in graft survival and integration in the brain. In the developing central nervous system (CNS), radial glia cells act as a scaffold and substrate for the migration of neurons (15, 33). In most neural regions radial glia cells are found only transiently, although they may give rise to other cells including mature astrocytes and Bergmann glia. While there is some evidence that this glial maturation process may be somewhat reversible, the adult CNS does not appear to contain the appropriate factors necessary for maintenance of radial glia. Radial glia are a major factor in promoting neural cell

migration during development, and their absence in the mature nervous system contributes to making it a relatively unfavorable environment for neuronal migration. Moreover, mature astrocytes do not provide a good environment to promote migration of neurons and their processes (41).

Given that mature glia have limited abilities to promote neuronal migration in the adult CNS, it is interesting to consider the use of glial cell lines to promote neuronal migration *in vivo*. Recently, we characterized a radial-like glial cell line, called C6-R, with phenotypic properties of radial glia that are maintained for extended periods of time *in vivo* (10). C6-R was derived by transfection of C6 glioma cells, with a construct encoding a mutant form of the human receptor protein tyrosine phosphatase  $\beta$  (RPTP $\beta$ ). The radial morphology of C6-R cells does not seem to be due directly to expression of the mutant receptor, rather it is likely to result from expression of genes in the cells driven by the CMV promoter that was stably introduced in the transfection plasmid (17). C6-R cells express cytoskeletal proteins found in radial glia including RC1 and promote both neuronal migration and process extension in culture (10). To visualize the cells *in vivo*, C6-R cells were transfected to express green fluorescent protein (GFP). These cells were found to adopt a radial morphology, often extending from the ventricle to the pial surface, when implanted in embryonic rat forebrain (10).

In the present study, we found that C6-R cells can migrate in the adult rat brain and spinal cord and exhibit a preference for white matter. These radial glial cells promoted neuronal migration for homografts and xenografts of embryonic neurons in the brain. Furthermore, C6-R cells facilitated migration of embryonic neurons when coimplanted in the injured brain. These observations suggest that radial glial cell lines may facilitate neural transplantation and should be explored for their potential application in promoting nerve regeneration.

## MATERIAL AND METHODS

### *Cell Lines*

The rat glioma cell line C6 was obtained from the American Type Culture Collection (Rockville, MD). The C6-R cell line was generated by transfection of C6 glioma cells with a construct encoding a mutant of the human RPTP $\beta$ , inserted into the pCDNA3 vector (Invitrogen, San Diego, CA) as previously described (10). It lacks the phosphatase domains and contains an HA tag in place of the two cytoplasmic domains. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 50  $\mu$ g/ml gentamycin

(GIBCO BRL, Grand Island, NY). C6 and C6-R cells were intrinsically labeled with the GFP by cotransfection with the plasmid pEGFP-N<sub>1</sub> (Clontech, Palo Alto, CA) which codes for GFP, and plasmids conferring resistance to hygromycin or neomycin were used for selection (10).

To prepare for implantation, cells were grown to ~75% confluence. They were detached by a 3-min incubation in 0.25% trypsin/1 mM EDTA, and DMEM/FCS was added to quench trypsinization. Cells were dissociated by vigorous pipetting, washed twice with Ham's F12, and counted using a hemocytometer.

### *Embryonic Neurons*

Neurons were obtained from the ventricular zone of lateral ganglionic eminence (LGE) of E16.5 embryos removed from pregnant Sprague-Dawley (SD rats) or CD1 mice (E14.5) via caesarian section. The tissue was dissociated by trituration in calcium-magnesium-free phosphate-buffered saline containing 1 mM EDTA (CMF-PBS, 1 mM EDTA) with 0.1% DNase (Worthington). The resulting single cell suspension was labeled with the fluorescent membrane-bound lipophilic dye PKH-26-GL (Sigma, St. Louis, MO) following the manufacturer's instructions. In brief, neurons were washed with Ham's F12, resuspended in 250  $\mu$ l of diluent C, mixed with diluent C containing 5  $\mu$ l of PKH dye, and incubated with gentle rocking for 5 min at 37°C. Staining was stopped by adding 500  $\mu$ l of DMEM/10% FCS. Cells were washed twice with Ham's F12, once with 3.5% BSA/Hanks, and twice with Ham's F12. They were resuspended at 10<sup>7</sup> cells/ml in DMEM/F12 supplemented with 2% B27, 1% glutamine, and 1% penicillin (GIBCO Life Technologies). The neuron cell suspension was diluted 1:1 with Ham's F12 or other cell suspensions.

### *Primary Fibroblasts*

Rat or mouse embryos were placed in Hank's balanced salt solution (BSS), where they were decapitated and eviscerated under a dissecting microscope. The embryos were then washed three times in Hank's BSS with agitation in order to minimize the presence of blood and proteins. The embryos were transferred to a flask containing 0.25% trypsin/1 mM EDTA and they were agitated at 150 rpm for 8 min at 37°C. DMEM/10% FCS was added to the flask and the trypsinized tissue was allowed to settle. The supernatant was collected into a tube and DNase was added to a final concentration of 100  $\mu$ g/ml. After centrifugation, the pellet was triturated to obtain a suspension of dissociated cells that was washed twice by centrifugation and once through a 3.5% BSA step gradient. The resulting single cell suspension was labeled with the fluorescent membrane-bound lipophilic DiO (Molecular Probes,

Eugene, OR) using the method described for staining neurons, except that the PKH26-GL dye was substituted by 15  $\mu\text{l}$  of a 3 mg/ml solution of DiO in 90% ethanol/10% DMSO.

### *Implantation of Cells in Rat Brain*

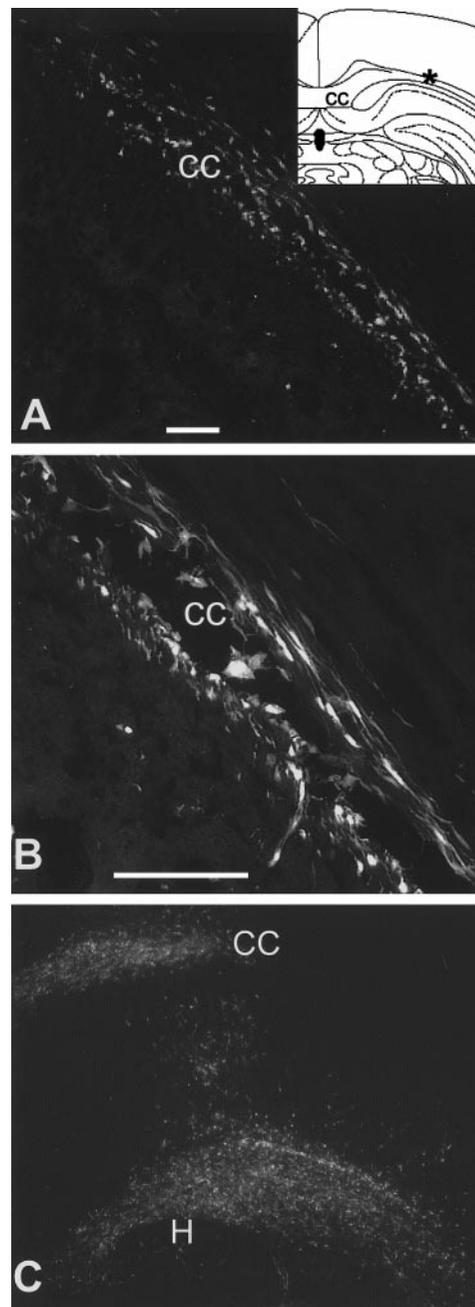
Adult female Sprague–Dawley rats (250–300 g) (Taconic Farms, Germantown, NY) were anesthetized (ip) with ketamine and xylazine at doses of 80 and 10 mg/kg, respectively, and immobilized in a stereotaxic head holder. Using sterile technique, a midline incision exposed the saggital suture and lambda, and a burr hole was made 3 mm to the right and 3 mm anterior to the lambda. A Hamilton syringe (Hamilton, Reno, NE) was inserted through the hole to a depth of 3 mm below the dural surface, and 10  $\mu\text{l}$  of Ham's-F12 medium were injected slowly containing a combination of  $3 \times 10^5$  C6-R cells and  $5 \times 10^4$  neural progenitors or  $3 \times 10^5$  fibroblasts and  $5 \times 10^4$  neural progenitors or  $5 \times 10^4$  neural progenitors alone. After withdrawing the syringe, the craniotomy defect was filled with bone wax and the incision closed with silk suture (Ethicon). In other adult rats, a lesion was produced by ibotenic acid injection (39). Fifteen micrograms was divided into three fractions that were delivered at 2, 3, and 4 mm from the dural surface, at the defined coordinates, 1 week prior to the implantation of neurons alone or in combination with C6-R cells.

Animals were sacrificed 11 days postimplantation. They received an ip overdose of pentobarbital (120 mg/kg), followed by transcardial perfusion through the left ventricle with 4% paraformaldehyde in PBS after vascular washout with PBS. The brain was removed and postfixed O/N in 4% paraformaldehyde/PBS. They were placed in PBS equilibrated with 15% sucrose overnight, embedded in Optimal Cutting Temperature Compound (Miles, Elkhart, IN), and frozen, and 40- $\mu\text{m}$  cryostat sections were analyzed for PKH-26 and GFP fluorescence. Alternatively, tissue was embedded in 3% agar in PBS and 100- $\mu\text{m}$  Vibratome sections were obtained.

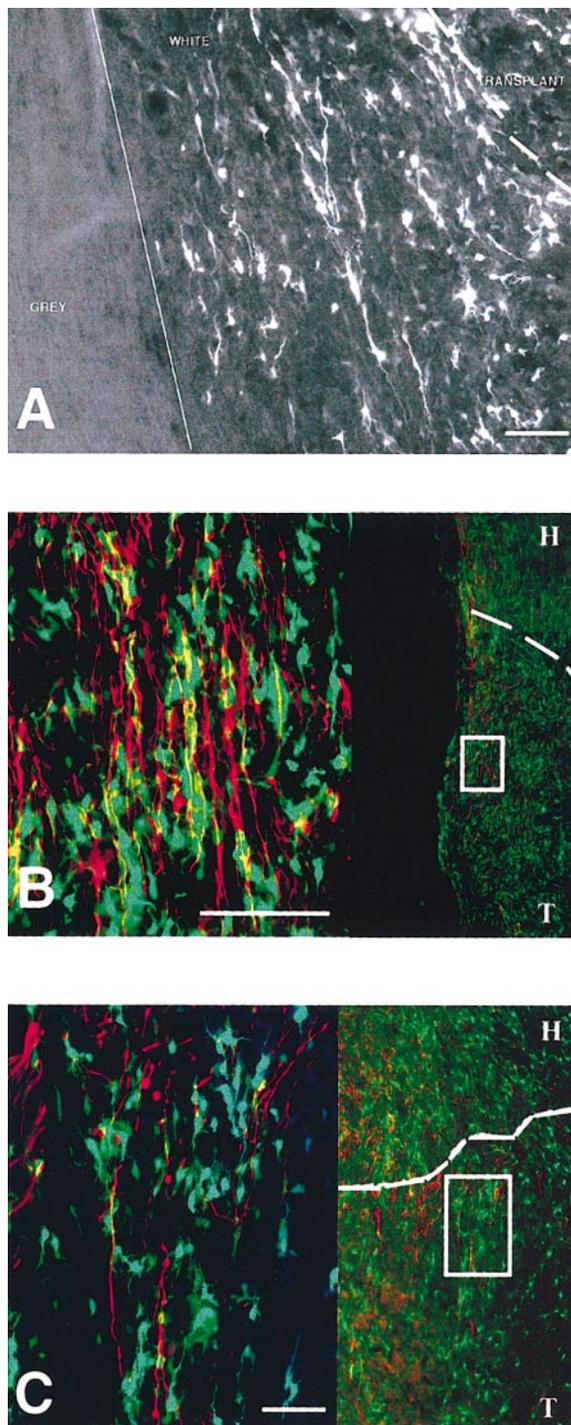
The cross-sectional area of the dispersion of C6-R cells and neurons was measured in every fifth cryostat section as digitized images obtained using NIH Image software and Scion Image, coupled to a Cohu CCD video camera. The volume covered by the dispersed cells was obtained by multiplying the mean of cross-sectional areas by the sum of all 40- $\mu\text{m}$  sections. In the first experiment, only the linear extent of the medial–lateral and dorsal–ventral dispersions was measured in all sections and the mean was calculated.

### *Implantation of Cells in the Spinal Cord*

For lesion implantation studies, six female Sprague–Dawley rats were anesthetized with an intraperitoneal



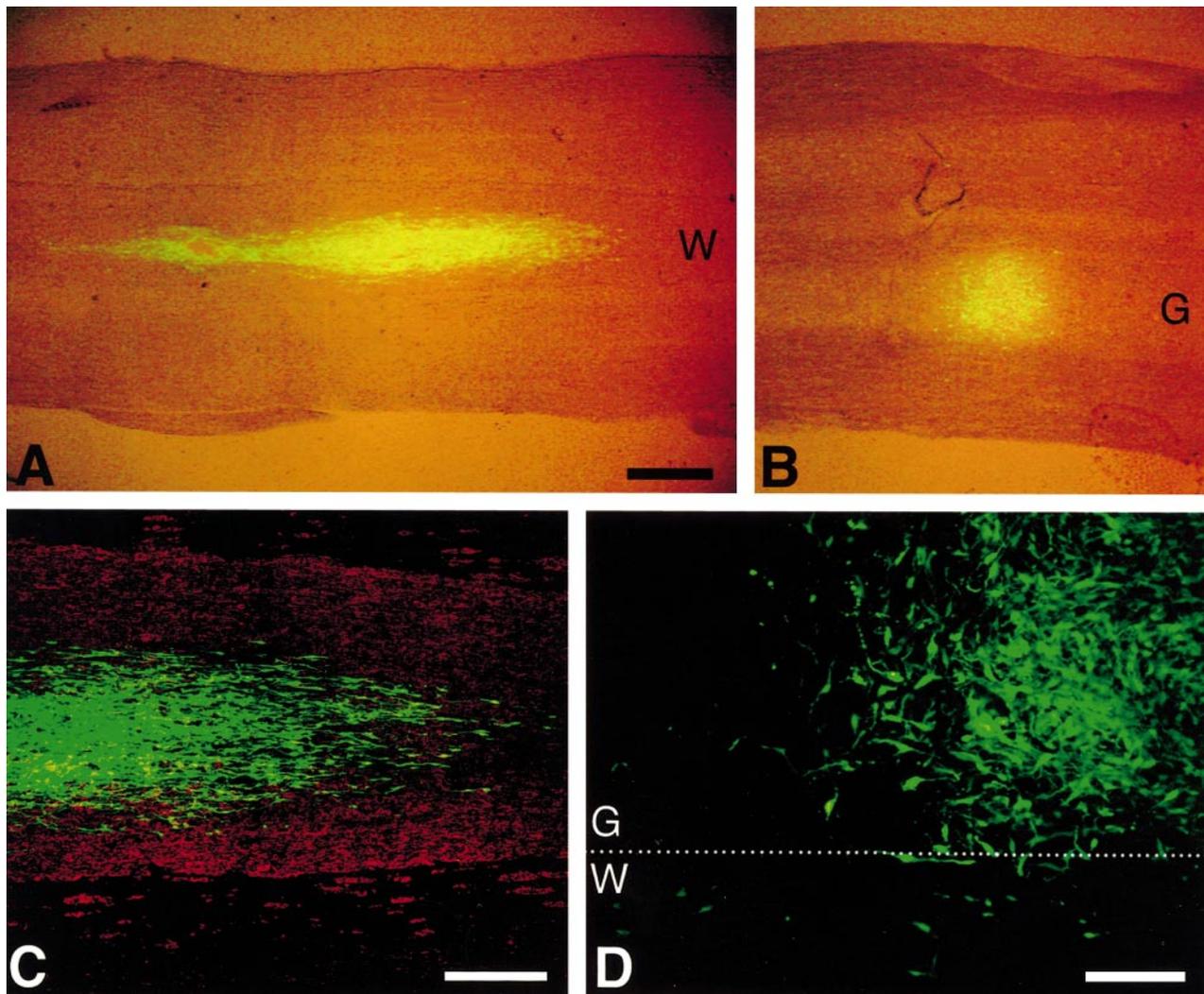
**FIG. 1.** C6-R cells 5 days after implantation in adult brain. (A) GFP-labeled C6-R cells distributed along the corpus callosum (CC). The insert shows the corresponding anatomical structures with the target for implantation (\*). (B) A higher power view from panel A shows that many C6-R cells are highly polarized and align along the corpus callosum (Bars = 50  $\mu\text{m}$ ). (C) Codistribution of C6-R cells into the corpus callosum and the hippocampus (H) probably in the CA1 region. The presence of cells in the hippocampus is likely the result of a deeper penetration of the injection needle in certain cases combined with selective migration of the C6-R cells as seen consistently in the corpus callosum.



**FIG. 2.** Implantation of C6-R cells into a spinal cord cavity. Immediately following a partial hemisection of the spinal cord, C6-R cells were transplanted along with gelfoam into the cavity. C6-R cells express GFP and appear white in panel A. The straight line represents the interface between the grey matter and the white matter and the broken line in the upper right corner represents the interface between the transplant and the host white matter. Note the preference of the C6-R cells for the white matter. Panels B and C each contain low-power fluorescent micrographs on the right and the boxes represent the location of higher magnification micrographs

injection of acepromazine maleate (0.7 mg/kg; Fermenta Animal Health Co., Kansas City, MO), ketamine (95 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA), and xylazine (10 mg/kg, Bayer Co., Shawnee Mission, KS) and underwent laminectomy at the C3–4 level to expose one spinal cord segment. A microscalpel was used to open the dura and pia matter and to make a shallow incision in the right dorsal spinal cord. A fine-tipped glass-pulled microaspiration device was then used to extend the lesion laterally and ventrally. This lesion completely disrupted the lateral funiculus and partially lesioned the ipsilateral ventral funiculus and gray matter but left the dorsal columns intact. The rostrocaudal extent of the lesion cavity was 2–3 mm. A piece of Gelfoam soaked with C6-R cells in growth medium was implanted into the cavity, and then another 10  $\mu$ l of cells suspended in growth medium was slowly injected onto the Gelfoam with a 10- $\mu$ l Hamilton syringe. The dura was closed with three interrupted 10-O silk sutures, and the muscle and skin were closed in layers. After the surgery, animals were kept on heating pads, closely observed until awake, and then returned to their home cages. Cyclosporin A (Sandoz Pharmaceuticals, East Hanover, NJ) was administered subcutaneously at a dose of 1 mg/100 g body weight starting 2 days before the transplantation procedures and was continued for 4 days after the operation. For immunocytochemistry, sections were washed three times with PBS for 5 min, incubated for 5 min in 0.2% Triton/PBS, and rinsed again three times in PBS for 5 min. Nonspecific binding was prevented by incubating the sections in 10% normal goat serum (NGS) in PBS for 1 h at room temperature. The sections were then incubated at room temperature overnight with mouse monoclonal antibody RT97, which recognizes the 200-kDa neurofilament subunit in its phosphorylated form (Boehringer Mannheim, Germany, at 1:100 dilution in 2% NGS/PBS) or with a mouse monoclonal antibody TuJ-1, which recognizes beta III-tubulin (BabCO, Richmond, CA, at 1:100 dilution in 2% NGS/PBS). The slides were rinsed three times for 5 min with PBS and incubated with rhodamine-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:200 in 2% NGS/PBS for 2 h. Sections were then rinsed three times for 5 min in PBS, coverslipped using Vectashield, and examined under fluorescent illumination.

shown on the left. In the low magnification micrographs, the broken lines represent the interface between host (H) and transplant (T). C6-R cells are visualized by green fluorescence in both panels. In panel B, TuJ-1-positive axons are red. In panel C, RT-97-positive axons are red. Note the growth of axons into the transplants with C6-R. All panels are sagittal sections at 12 days postimplantation; top is rostral and bottom is caudal (all bars = 0.1 mm).



**FIG. 3.** Migration of C6-R in adult rat spinal cord. One week following implantation, C6-R cells (yellow) were observed to migrate extensively along the rostrocaudal axis in the white matter (A) but fairly symmetrically in the grey matter (B); panels A and B are combined fluorescence and bright-field images to show grey (G) and white (W) matter regions. The identification of the white and grey matter regions was confirmed by immunofluorescence using monoclonal antibodies against myelin basic protein shown in red in panel C with C6-R cells in green. A higher power fluorescence view of C6-R cells in grey matter bordering white matter is shown in panel D, and the dotted line represents the border between grey and white matter (MBP stain is not shown here to allow better visualization of C6-R cells at the G/W border). Note increased longitudinal orientation of the C6-R cells in the white matter vs the grey matter. Bars = 500  $\mu\text{m}$  in A and B, 200  $\mu\text{m}$  in C, and 100  $\mu\text{m}$  in D.

Animals were euthanized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg; Abbott Laboratories, North Chicago, IL) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, and spinal cords were dissected, immersed in 0.1 M PB at 4°C overnight, and cryoprotected in 30% sucrose (in 0.1 M PB containing 0.5 mM thimerosal) for 3–5 days. Spinal cord specimens were embedded in OCT compound (Fisher Scientific, Pittsburgh, PA) and kept at  $-20^{\circ}\text{C}$  before being cut into 20- $\mu\text{m}$  sections on a cryostat and mounted onto microscope plus/slides (Fisher Scientific, Pittsburgh, PA).

For transplantation into uninjured spinal cord, female Long-Evans hooded rats (190–210 g) were anesthetized with intraperitoneal injection of pentobarbital (45 mg/kg) and a laminectomy was performed to expose thoracic segments T9–10. A sterile glass needle was connected to a sterile 2.5- $\mu\text{l}$  Hamilton syringe and 1  $\mu\text{l}$  of a suspension containing  $5 \times 10^5$  C6-R cells was injected slowly during the course of 10 min into one site or into each of two sites separated by 4–6 mm along the rostral and caudal axis. The surgical wound was closed in layers. After 7 days of survival, experimental and control animals were sacrificed and spinal cords

were sectioned as described. For immunocytochemistry staining of spinal cord tissue, sections were washed three times with 50 mM Tris-buffered solution, pH 7.6, containing 1.5% sodium chloride (1.5 T) and incubated in cold methanol for 30 min. They were then rinsed three times for 5 min with 1.5 T. Nonspecific binding was prevented by incubating the sections in 10% NGS in 1.5 T for 2 h at room temperature. The sections were incubated at 4°C overnight with a mouse monoclonal antibody against myelin basic protein (Sternberger Monoclonals Incorporated, Lutherville, MD) at 1:1000 dilution in 1% NGS in 1.5 T. The slides were rinsed three times for 10 min with 1.5 T and incubated with Alexa Fluor 568-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) at 1:400 dilution in 1% NGS in 1.5 T for 45 min at room temperature. Sections were then rinsed three times for 10 min with 1.5 T, mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL), and examined under bright-field and fluorescent illumination.

## RESULTS

### *Migration Pattern of C6-R Cells in the Brain*

To investigate the behavior of C6-R cells in the adult brain, GFP-labeled cells were implanted using a stereotaxic frame into the rat brain above the splenium of the corpus callosum. At 5 days postimplantation, most of the C6-R cells were found to be aligned along the corpus callosum (Fig. 1A). In contrast, transplants of the parental cell line C6 tended to form aggregates near the site of implantation (17). Observation of C6-R cells at higher magnification showed that many of these cells had a highly polarized morphology and were aligned preferentially along the corpus callosum within the coronal plane of sectioning (Fig. 1B), while C6 cells had polygonal shapes as they did in culture. In some brains implanted with C6-R, cells were observed in the hippocampus (see below, Fig. 7) and occasionally they were found in both areas in the same brain (Fig. 1C).

### *Migration Pattern of C6-R Cells in the Spinal Cord*

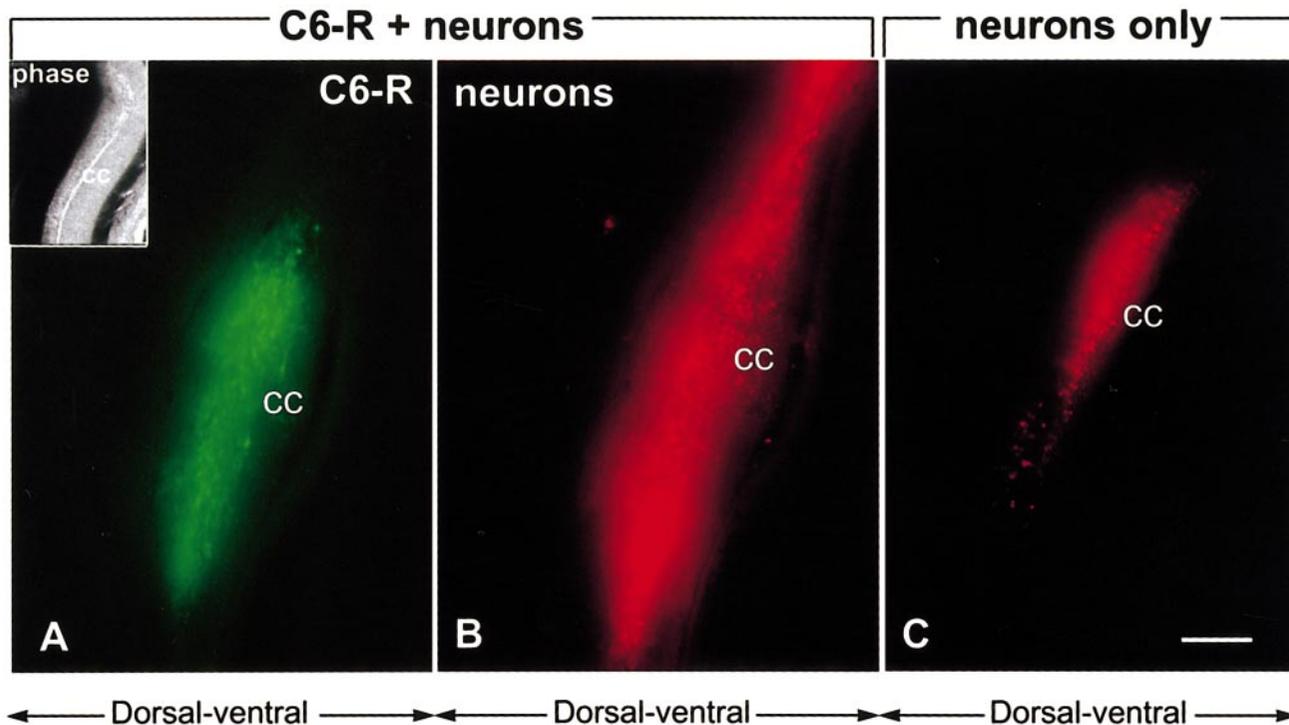
Given the migration and alignment of C6-R cells along the corpus callosum, it was of interest to determine whether these cells showed a similar preference for other white matter tracts, for example, in the spinal cord. In a first set of experiments, C6-R cells with Gelfoam were introduced into lesions that completely disrupted the lateral funiculus and partially lesioned the ipsilateral ventral funiculus and gray matter but left the dorsal columns intact. After 6 or 12 days, C6-R cells were found to have migrated into the host tissue. In all animals the patterns of migration of the C6-R

cells were similar and consisted of preferential longitudinal migration along the white matter (Fig. 2A). There was little or no infiltration in grey matter. We observed staining for TuJ1 and RT-97 antibodies on long fibers traversing the transplants suggesting axonal outgrowth from the host into the transplant (Figs. 2B and 2C). This axonal growth into the C6-R transplant is remarkable as it was not observed in previous experiments using fibroblast transplants as controls (27).

To determine whether C6-R cells exhibited a similar pattern of integration and migration in uninjured tissue, we microinjected 1  $\mu$ l of C6-R cells into uninjured rat spinal cords. Observations 1 week later revealed dramatic migration along the rostrocaudal axis of the spinal cord in white matter. C6-R cells were widely distributed along the rostrocaudal axis of white matter extending for as long as 2 mm, but their lateral distributions were limited primarily within the white matter regions (Figs. 3A and 3C). In contrast, it appeared that transplants into the grey matter (Figs. 3B and 3D) distributed much more uniformly, showing little preference along the rostrocaudal axis and extending much less than in white matter. Quantitation of the ratio of the maximal linear extents of the C6-R cells along the rostrocaudal axis (R) and in the horizontal plane (H) indicated that grey matter distributions were nearly symmetrical (with the ratio of R/H = 1.2) while white matter profiles were highly polarized (R/H = 4). This confirms a strong preference of C6-R for migration in white matter. Examination at high magnification revealed that the processes of the C6-R cells were aligned preferentially along the rostrocaudal axis of the spinal cord in white matter regions but were much more randomly distributed in grey matter regions.

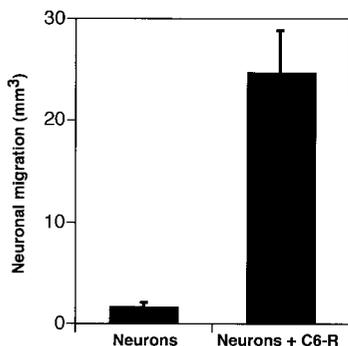
### *Coinplantation of C6-R Cells and Neurons*

Given that C6-R cells distributed extensively in a restricted pattern in particular along white matter tracts, we tested whether C6-R cells were able to support grafting of neurons into the adult rat brain. In the first experiment, GFP-labeled C6-R cells and PKH-26-labeled embryonic rat neurons were implanted using the same stereotaxic coordinates (31) in Sprague-Dawley rats. Neurons only were implanted contralaterally in the same rats, using complementary coordinates. Eleven days after implantation, the pattern of neuronal migration on the right side, which received C6-R cells together with neurons, was compared with that on the left side, which received exclusively neurons. Both GFP-labeled C6-R cells and PKH-26-labeled neurons were distributed along the corpus callosum (Fig. 4B vs Fig. 4C). A more extensive migration of neurons was seen for those neurons that were coinjected with C6-R cells (Fig. 4B), while the neurons injected alone (Fig.



**FIG. 4.** Coimplantation of C6-R cells with neurons. PKH-26 LGE neurons, dissociated into single cell suspension, were injected stereotaxically either alone (C) or with GFP-labeled C6-R cells (B). C6-R cells (A) migrated along the corpus callosum. The insert in panel A is a corresponding phase image. All fluorescence pictures represent the same magnification. Bar = 100  $\mu\text{m}$ .

4C) had a more limited dispersion. The average migration distance of the neurons coinjected with C6-R was 388  $\mu\text{m}$  and only 216  $\mu\text{m}$  for neurons implanted alone ( $n = 2$ ). When we considered the maximal dispersion in the orthogonal direction, the neurons coimplanted with C6-R dispersed through a distance of 68.4  $\mu\text{m}$  while the neurons implanted alone dispersed through a distance of 26.4  $\mu\text{m}$  only ( $n = 2$ ). Comparison between the two sides implanted showed greater dispersion of neurons on the side coinjected with C6-R cells, suggest-

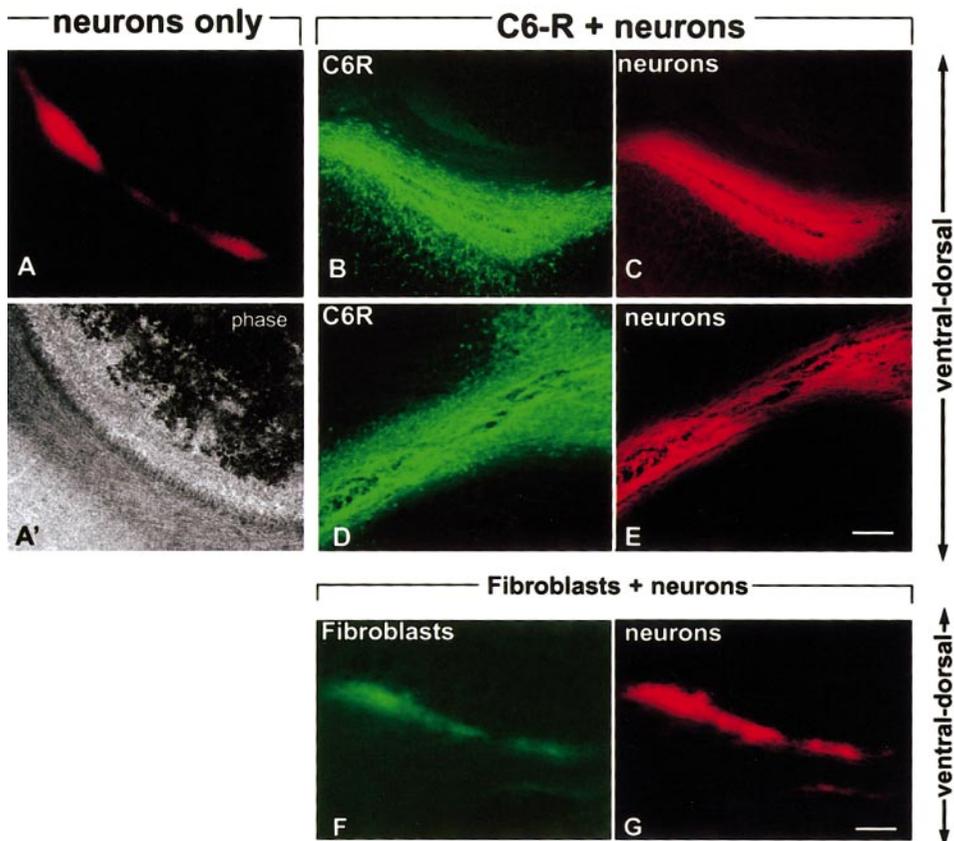


**FIG. 5.** Migration of PKH-26-labeled rat LGE neurons. Mean volume of neurons at 11 days, following coimplantation of C6-R cells, was 24.71  $\pm$  4.00  $\text{mm}^3$  and for implantation of neurons alone was 1.65  $\pm$  0.40  $\text{mm}^3$ . Volumes were calculated from measurements of a series of cross-sectional areas covering the entire implantation site.

ing that C6-R cells may serve as a scaffold and/or substrate for neuronal migration.

In a second type of experiment, we injected different animals with either neurons alone or neurons and C6-R cells. This is in contrast to the first experiment, where animals received injections on both sides of the brain. Qualitatively, similar results were obtained in the second experimental design with C6-R again promoting migration of neurons. For a more accurate quantification of cell migration, volumetric analysis was used, instead of two-dimensional measurements. Eleven days after implantation, the average volume of neurons coimplanted with C6-R cells was 24.71  $\pm$  4.00  $\text{mm}^3$  ( $n = 2$ ), about 14 times the average volume of neurons alone, which was 1.65  $\pm$  0.40  $\text{mm}^3$  ( $n = 3$ ; Fig. 5). A similar effect on neuronal dispersion was observed (volume = 24.28  $\text{mm}^3$ ) when 1/6 the number of C6-R cells (i.e.,  $5 \times 10^4$ ) was used. Neurons implanted alone appear as a narrow well-demarcated band (Figs. 6A and 7B), while neurons coimplanted with C6-R cells comigrated with them, suggesting that C6-R cells can promote neuronal dispersion and migration in both the corpus callosum and the hippocampus (Figs. 6C, 6E, and 7D).

A similar pattern of migration was obtained when embryonic mouse neurons were implanted alone in the adult rat brain. The average volume of neurons im-



**FIG. 6.** Migration of PKH-26 rat LGE neurons along the corpus callosum. (A) Eleven days after implantation of neurons alone, the pattern of the labeled neurons was limited to a thin band. (A') Phase image corresponding to panel A. Panels B and C and panels D and E are corresponding views of sections from brain showing GFP-labeled C6-R cells following coimplantation with neurons. C6-R cells are green (B and D), and PKH-26 labeled neurons are shown in red (C and E). Notice the dramatic increase in the width of the neuronal migration (in the ventral–dorsal direction) when they were coimplanted with C6-R cells in comparison with the more restricted pattern observed when neurons alone were implanted (A). When neurons (G) were coimplanted with fibroblasts (F) the pattern of neuronal migration resembled that of neurons alone (panel A). All pictures represent the same magnification. Bars = 100  $\mu\text{m}$ .

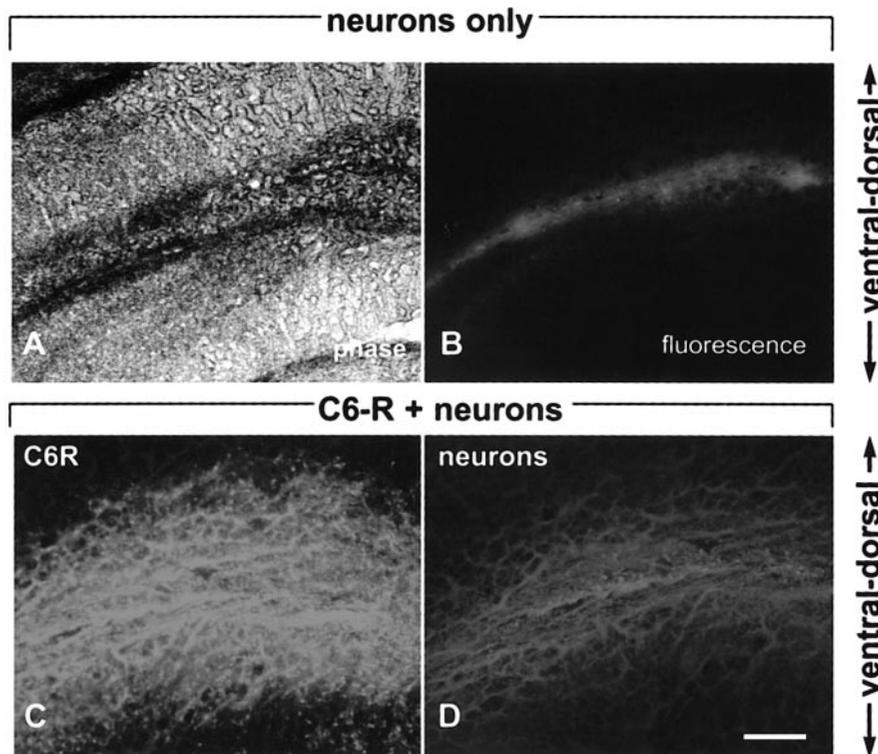
planted alone was  $4.28 \pm 0.46 \text{ mm}^3$  ( $n = 3$ ) and for neurons coimplanted with C6-R cells it was  $13.6 \text{ mm}^3$  ( $n = 1$ ). In a control group in which neurons were coimplanted with fibroblasts ( $n = 2$ ), the average volume dispersion of neurons was  $6.87 \pm 0.14 \text{ mm}^3$ , significantly lower than the value obtained for neurons coimplanted with C6-R cells, but only marginally greater than that obtained with neurons alone. The pattern of migration of neurons coimplanted with fibroblasts was very similar to the pattern of migration of neurons implanted alone, without the ventral–dorsal expansion observed when neurons were coimplanted with C6-R cells (Fig. 6).

#### *Coimplantation of C6-R Cells and Neurons in Ibotenic Acid-Lesioned Brain*

The usefulness of potential transplantation protocols may be significantly limited if they are ineffective in neural sites where lesions have occurred. Therefore, we

were interested in testing the behavior of C6-R cells in sites that had previously been injured. For this purpose we chose ibotenic acid, an excitatory toxin that produces neuronal degeneration while sparing axons (20) that is commonly used experimentally to produce a local lesion in the brain. We implanted C6-R cells 1 week after ibotenic acid injections into the lesion site and compared the results with C6-R in nonlesioned brain. GFP-labeled C6-R cells migrated extensively through the lesion in the cortex, corpus callosum, and hippocampus, in contrast to the much more restricted distribution of C6-R cells observed in the intact brain (Figs. 8A vs Fig. 8B). This suggests that C6-R cells are able to integrate into a degenerative lesion even more extensively than in intact brain.

To determine whether neurons could also migrate along C6-R cells in lesions, we coimplanted LGE neurons along with C6-R cells. When animals received single implantations of PKH-26-labeled E14.5 rat em-



**FIG. 7.** Migration of PKH-26 rat LGE neurons in the hippocampus. Phase contrast (A) and fluorescence views of the same field of hippocampus 11 days after implantation with neurons alone (B). Note the thin band of neurons. Panels C and D show corresponding views in the hippocampus of a brain section following coimplantation of GFP-labeled C6-R cells (C) with PKH-26-labeled neurons (D). Note that the profile of neurons alone (B) is much more restricted than when neurons (D) were coimplanted with C6-R cells (C). All pictures represent the same magnification. Bar = 100  $\mu$ m.

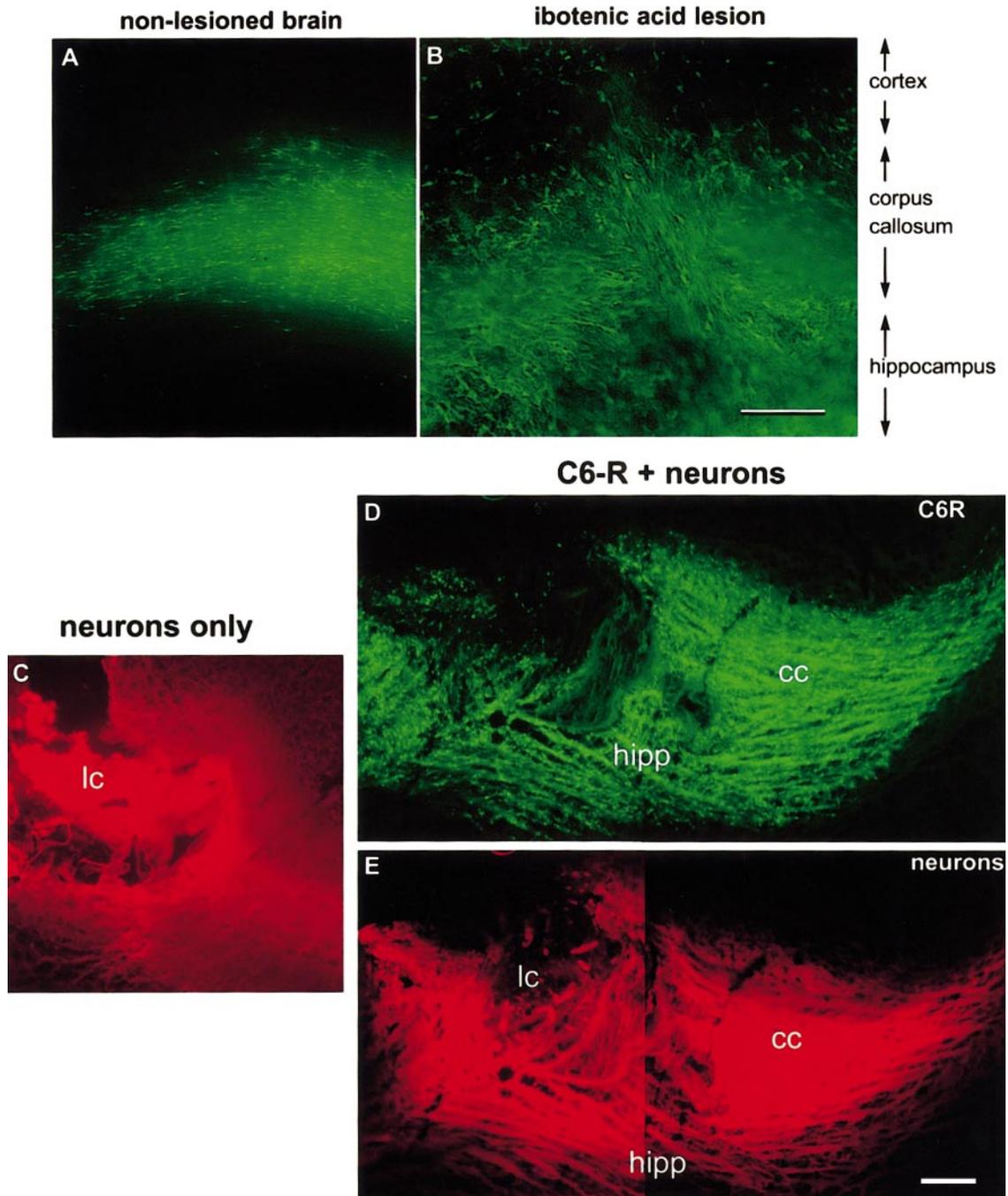
bryonic neurons into ibotenic acid lesions, the neurons remained primarily in the lesion cavity (Fig. 8C). However, when neurons were coimplanted with C6-R cells, they followed the pattern of migration of GFP-labeled C6-R cells (Fig. 8D), in both the corpus callosum and the hippocampus, and extended into the surrounding parenchyma (Fig. 8E).

## DISCUSSION

We studied the behavior of GFP-labeled C6-R cells in the CNS *in vivo* and their ability to promote migration and integration of embryonic neurons in the adult rat brain. Following implantation in the brain, C6-R cells assumed a radial morphology and aligned along the corpus callosum, with occasional dispersion into the hippocampus. In the spinal cord, C6-R cells exhibited a strong preference for the white matter where they migrated dramatically along the rostrocaudal axis and were aligned along this longitudinal axis. In contrast these cells infiltrated fairly uniformly but less extensively in grey matter regions. C6-R cells were also able to infiltrate into injured neural tissue where they dispersed widely.

The preference of C6-R for white matter parallels the invasiveness of gliomas as described by Scherer (38) and the preference of embryonic (13) and neonatal rat astrocytes (7, 16) for white matter when they are homografted into adult rat brain. When C6-R cells were found in grey matter regions in the spinal cord and occasionally in the brain (e.g., the basal ganglia) they were disorganized and did not appear to migrate as robustly as in white matter but they did infiltrate locally. The behavior of C6-R cells was quite different in the developing brain, where they exhibited a radial-like distribution that was in register with the native radial glia in the forebrain (10). The different types of alignment of C6-R cells observed in the developing vs the mature brain suggest that C6-R cells can respond, at least to a certain degree, to environmental factors in the embryonic brain that are distinct in the adult.

During development, many neurons migrate along radial glial cells (15, 33). In the postnatal period (except in cerebellum, where it is delayed further), it is believed that factors responsible for radial glia and their maintenance are lost, leading to differentiation of radial glia into astrocytes. Such factors could include neuregulin/GGF, which plays a critical role in neuronal



**FIG. 8.** Implantation of GFP-labeled C6-R cells with or without neurons in ibotenic acid-lesioned brain. In normal adult brain (A), C6-R exhibited a restricted distribution along the corpus callosum. This is in contrast to the more widespread migration of C6-R cells that was observed in lesioned brain (B). Panels A and B represent the same magnification. Panels C–E show coimplantation of rat LGE neurons and C6-R cells in ibotenic acid-lesioned brain. When implanted alone (C), PKH-26-labeled neurons were observed mostly in the lesion cavity (lc). When C6-R cells were coimplanted with neurons, PKH-26-labeled neurons (E) followed migration of GFP-labeled C6-R cells (D) in the corpus callosum (cc) and the hippocampus (hipp) that are beyond the margins of the cavity created by the lesion. Panels D and E represent the same fields and C–E are the same magnification. All bars = 100  $\mu$ m.

migration during development (1, 37). In addition, Cajal–Retzius cells release diffusible factors that can induce transient rejuvenation of Bergmann radial glia in the adult cerebellum (42). Interestingly, like radial glia, C6-R cells maintain the ability to promote neuronal migration, and they may also stimulate process outgrowth in the adult CNS as astrocytes do during development.

While C6-R is a stable cell line that appears to retain a radial phenotype *in vivo* for much longer periods than do native radial glia, this phenotype is not completely stable *in vivo*. For example, 15 days after injection of GFP-labeled C6-R cells into the embryonic brain, we observed GFP-positive radial cells as well as limited numbers of stellate cells resembling more differentiated glia (10). At 3 weeks after implantation of cells in the adult brain, the majority of brains had only small numbers of GFP-positive C6-R cells but  $\sim 1/3$  had small tumors; in contrast, C6 cells formed large tumors in every brain (17). Although long-term survival of C6-R cells in the spinal cord needs to be investigated, we have observed large number of these cells both 1 (Fig. 3) and 2 weeks following implantation in the spinal cord *in vivo* (K.H. and M.G., unpublished observations). The extensive migration of C6-R cells in adult white matter suggest that they can provide a unique migratory scaffold for an extended period of time and may promote regrowth of host neurons as well as integration of embryonic neurons following cotransplantation.

Migration of grafted neurons is more restricted than that of astrocytes (26) and they exhibit regional specificity (30, 44). The success of grafting donor neurons diminishes with increasing maturity of the host (30) and may be improved by cotransplantation with other cells such as radial glia. Furthermore, pathways of axonal migration can differ from those of astrocyte migration as seen with transplants in neonatal rat brain (47). These observations underscore the dramatic dispersion of neurons that we observed when they were coimplanted with C6-R cells both in the intact brain and in ibotenic acid-lesioned brain. Moreover, the neurons closely paralleled the dispersion of C6-R cells in anterior–posterior, ventral–dorsal, and medial–lateral directions, suggesting they migrate along the C6-R cells *in vivo*. The large volumes of neurons observed upon cotransplantation with C6-R suggest that neuronal survival may also be enhanced by the C6-R cells.

Stem cells that are present in the adult CNS of rodents, primates, and humans (4, 8, 12, 19) hold great potential for regeneration. However, these cells and other candidates for grafting may benefit from interactions with glia that can promote migration, survival, and differentiation of neurons during development (18). Therefore, it is interesting that when neurospheres prepared from embryonic spinal cord stem cells were cocultured with C6-R

cells they appeared to migrate along the processes of C6-R cells and expressed neuronal markers (J.M.N. and I.F., unpublished observations). The ability of C6-R cells to migrate from the graft site and become oriented along the rostral–caudal axis in white matter in the injured spinal cord (Fig. 2) raises the possibility that radial glia may facilitate expansion and differentiation of host neuronal stem cells or grafted stem cells in the mature CNS.

Recent studies have underscored the potential utility of olfactory ensheathing glia cells in promoting axonal regeneration and functional recovery following injury to the CNS (24, 25, 34–36). These cells derived from the olfactory bulb where regeneration occurs throughout the lifetime of the organism have remarkable ability to migrate long distances through mature grey and white matter as well as through injured tissue (36) and promote nerve regeneration (24, 25). It is interesting to note that the olfactory ensheathing glia can migrate through grey matter while most other glia including C6-R migrate preferentially in white matter, although C6-R can also infiltrate somewhat in grey matter. We showed here that C6-R also migrated extensively into brain lesions and facilitated the movement of neurons.

The present studies on the behavior of C6-R cells in the CNS and their ability to promote neuronal migration in the brain demonstrate that transplanted radial glia such as C6-R can alter the milieu *in vivo* to make it more favorable for neuronal migration and possibly for regeneration. These radial cells are particularly interesting because they promote both neuronal migration and process outgrowth in culture (10) and *in vivo*. Further studies are needed to determine whether enhanced neuronal migration and axonal growth result in synapse formation and functional connectivity. Given that genetically modified fibroblasts producing growth factors have been found to facilitate neuronal regrowth following injuries to the spinal cord (14, 29) and brain (6, 45), it may be advantageous to introduce such genes into C6-R cells. Such approaches will show whether cotransplantation of neurons with radial-like glia cells can improve functional recovery in experimental animal models of degenerative and developmental disorders and injury to the nervous system. If models for radial glia prove to be useful in promoting integration of transplanted neurons or regeneration from host neurons or stem cells, then it will be important to identify factors that extend the persistence of radial glia *in vivo*. In this regard, it is interesting to note a recent report that mature astrocytes can transform into transitional radial glia in the brain and support directed migration of immature neurons (23). While the factors responsible for maintaining radial glia remain unclear, the C6-R cells may serve as a good model to test the utility of radial glia in promoting nerve growth in the adult CNS.

## ACKNOWLEDGMENTS

We thank Drs. David Zagzag and Wise Young for advice on transplantation in the brain and spinal cord, respectively. Supported by NIH Grant NS38112.

## REFERENCES

- Anton, E. S., M. A. Marchionni, K.-F. Lee, and P. Rakic. 1997. Role of GGF/neuregulin signalling between migrating neurons and radial glia in the developing cerebral cortex. *Development* **124**: 3501–3510.
- Brustle, O. 1999. Building brains: Neural chimeras in the study of nervous system development and repair. *Brain Pathol.* **9**: 527–545.
- Brustle, O., and R. D. McKay. 1996. Neuronal progenitors as tools for cell replacement in the nervous system. *Current Opinion in Neurobiology* **6**: 688–695.
- Cameron, H. A., and R. McKay. 1998. Stem cells and neurogenesis in the adult brain. *Curr. Opin. Neurobiol.* **8**: 677–680.
- Deacon, T., J. Schumacher, J. Dinsmore, C. Thomas, P. Palmer, S. Kott, A. Edge, D. Penney, S. Kassissieh, P. Dempsey, and O. Isacson. 1997. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. *Nat. Med.* **3**: 350–353.
- Dickinson-Anson, H., I. Aubert, F. H. Gage, and L. J. Fisher. 1998. Hippocampal grafts of acetylcholine-producing cells are sufficient to improve behavioural performance following a unilateral fimbria-fornix lesion. *Neuroscience* **84**: 771–781.
- Emmett, C. J., J. M. Lawrence, G. Raisman, and P. J. Seeley. 1991. Cultured epithelioid astrocytes migrate after transplantation into the adult rat brain. *J. Compar. Neurol.* **310**: 330–341.
- Eriksson, P. S., E. Perfilieva, T. Björk-Eriksson, A.-M. Alborn, C. Nordborg, D. A. Peterson, and F. H. Gage. 1998. Neurogenesis in the adult human hippocampus. *Nat. Med.* **11**: 1313–1317.
- Folkerth, R. D., and R. Durso. 1996. Survival and proliferation of nonneural tissues, with obstruction of cerebral ventricles, in a parkinsonian patient treated with fetal allografts. *Neurology* **46**: 1219–1225. [see comments]
- Friedlander, D. R., P. A. Brittis, T. Sakurai, B. Shif, W. Wirschansky, G. Fishell, and M. Grumet. 1998. Generation of a radial-like glial cell line. *J. Neurobiol.* **37**: 291–304.
- Gage, F. H. 2000. Mammalian neural stem cells. *Science* **287**: 1433–1438.
- Gage, F. H., G. Kempermann, T. D. Palmer, D. A. Peterson, and R. Jasodhara. 1998. Multipotent progenitor cells in the adult dentate gyrus. *J. Neurobiol.* **36**: 249–266.
- Goldberg, W. J., and J. J. Bernstein. 1988. Fetal cortical astrocytes migrate from cortical homografts throughout the host brain and over the glia limitans. *J. Neurosci. Res.* **20**: 38–45.
- Grill, R., K. Murai, A. Blesch, F. H. Gage, and M. H. Tuszynski. 1997. Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. *J. Neurosci.* **17**: 5560–5572.
- Hatten, M. E. 1999. Central nervous system neuronal migration. *Annu. Rev. Neurosci.* **22**: 511–539.
- Hatton, J. D., M. H. Nguyen, and U. H. Sang. 1993. Differential migration of astrocytes grafted into the developing rat brain. *Glia* **9**: 113–119.
- Hormigo, A., D. R. Friedlander, P. A. Brittis, D. Zagzag, and M. Grumet. 2001. Reduced tumorigenicity in the brain of rat glioma cells mediated by hygromycin phosphotransferase. *J. Neurosurg.* **94**: 598–604.
- Jacobson, M. J. 1991. *Developmental Neurobiology*, 3 ed. Plenum Press, New York.
- Kempermann, G., and F. H. Gage. 1998. Closer to neurogenesis in adult humans. *Nat. Med.* **4**: 555–557.
- Kohler, C., and R. Schwarcz. 1983. Comparison of ibotenate and kainate neurotoxicity in rat brain: A histological study. *Neuroscience* **8**: 819–835.
- Kordower, J. H., T. B. Freeman, R. A. Bakay, C. G. Goetz, and C. W. Olanow. 1997. Treatment with fetal allografts. *Neurology* **48**: 1737–1738. [letter; comment]
- Kordower, J. H., T. B. Freeman, B. J. Snow, F. J. Vingerhoets, E. J. Mufson, P. R. Sanberg, R. A. Hauser, D. A. Smith, G. M. Nauert, D. P. Perl, and C. W. Olanow. 1995. Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. *N. Engl. J. Med.* **332**: 1118–1124. [see comments]
- Leavitt, B. R., C. S. Hernit-Grant, and J. D. Macklis. 1999. Mature astrocytes transform into transitional radial glia within adult mouse neocortex that supports directed migration of transplanted immature neurons. *Exp. Neurol.* **157**: 43–57.
- Li, Y., P. M. Field, and G. Raisman. 1997. Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells. *Science* **277**: 2000–2002.
- Li, Y., P. M. Field, and G. Raisman. 1998. Regeneration of adult rat corticospinal axons induced by transplanted olfactory ensheathing cells. *J. Neurosci.* **18**: 10514–10524.
- Lindsay, R. M., and G. Raisman. 1984. An autoradiography study of neuronal development, vascularization and glial cell migration from hippocampal transplants labelled in intermediate explant culture. *Neuroscience* **12**: 513–530.
- Liu, Y., D. Kim, B. T. Himes, S. Y. Chow, T. Schallert, M. Murray, A. Tessler, and I. Fischer. 1999. Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. *J. Neurosci.* **19**: 4370–4387.
- Martinez-Serrano, A., and A. Björklund. 1997. Immortalized neural progenitor cells for CNS gene transfer and repair. *Trends Neurosci.* **20**: 530–538.
- McTigue, D. M., P. J. Horner, B. T. Stokes, and F. H. Gage. 1998. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. *J. Neurosci.* **18**: 5354–5365.
- Olsson, M., C. Bentlage, K. Victorin, K. Campbell, and A. Björklund. 1997. Extensive migration and target innervation by striatal precursors after grafting into the neonatal striatum. *Neuroscience* **79**: 57–78.
- Paxinos, G., and C. Watson. 1997. *The Rat Brain*. Academic Press, San Diego.
- Prasad, K. N., E. D. Clarkson, F. G. La Rosa, J. Edwards-Prasad, and C. R. Freed. 1998. Efficacy of grafted immortalized dopamine neurons in an animal model of parkinsonism: A review. *Mol. Genet. Metab.* **65**: 1–9.
- Rakic, P. 1990. Principles of neural cell migration. *Experientia* **46**: 882–891.
- Ramon-Cueto, A., and J. Avila. 1998. Olfactory ensheathing glia: Properties and function. *Brain Res. Bull.* **46**: 175–187.
- Ramon-Cueto, A., M. I. Cordero, F. F. Santos-Benito, and J. Avila. 2000. Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. *Neuron* **25**: 425–435.
- Ramon-Cueto, A., G. W. Plant, J. Avila, and M. B. Bunge. 1998. Long-distance axonal regeneration in the transected adult rat

- spinal cord is promoted by olfactory ensheathing glia transplants. *J. Neurosci.* **18**: 3803–3815.
37. Rio, C., H. I. Rieff, P. Qi, and G. Corfas. 1997. Neuregulin and erbB receptors play a critical role in neuronal migration. *Neuron* **19**: 39–50.
38. Scherer, H. J. 1940. The forms of growth in gliomas and their practical significance. *Brain* **63**: 1–35.
39. Shetty, A. K., and D. A. Turner. 1996. Development of fetal hippocampal grafts in intact and lesioned hippocampus. *Prog. Neurobiol.* **50**: 597–653.
40. Shihabuddin, L. S., J. Ray, and F. H. Gage. 1999. Stem cell technology for basic science and clinical applications. *Arch. Neurol.* **56**: 29–32.
41. Smith, G. M., U. Rutishauser, J. Silver, and R. H. Miller. 1990. Maturation of astrocytes in vitro alters the extent and molecular basis of neurite outgrowth. *Dev. Biol.* **138**: 377–390.
42. Soriano, E., M. Alvarado-Mallart, N. Dumesnil, J. A. Del Rio, and C. Sotelo. 1997. Cajal-Retzius cells regulate the radial glia phenotype in the adult and developing cerebellum and alter granule cells migration. *Neuron* **18**: 563–577.
43. Wictorin, K., C. F. Lagenaur, R. D. Lund, and A. Bjorklund. 1991. Efferent projections to the host brain from intrastriatal striatal mouse-to-rat grafts: Time course and tissue-type specificity as revealed by a mouse specific neuronal marker. *Eur. J. Neurosci.* **3**: 86–101.
44. Wictorin, K., C. C. Ouimet, and A. Bjorklund. 1989. Intrinsic organization and connectivity of intrastriatal transplants in rats as revealed by DARPP-32 immunohistochemistry: Specificity of connections with the lesioned host brain. *Eur. J. Neurosci.* **1**: 690–701.
45. Winkler, J., S. T. Suhr, F. H. Gage, L. J. Thal, and L. J. Fisher. 1995. Essential role of neocortical acetylcholine in spatial memory. *Nature* **375**: 484–487.
46. Zhang, W., W.-H. Lee, and L. C. Triarhou. 1996. Grafted cerebellar cells in a mouse model of hereditary ataxia express IGF-I system genes and partially restore behavioral function. *Nat. Med.* **2**: 65–71.
47. Zhou, H.-F., and R. D. Lund. 1992. Migration of astrocytes transplanted to the midbrain of neonatal rats. *J. Compar. Neurol.* **317**: 145–155.