Mature Astrocytes Transform into Transitional Radial Glia within Adult Mouse Neocortex That Supports Directed Migration of Transplanted Immature Neurons

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Neuronal migration is an essential step in normal mammalian neocortical development, and the expression of defined cellular and molecular signals within the developing cortical microenvironment is likely crucial to this process. Therapy via transplanted or manipulated endogenous precursors for diseases which involve neuronal loss may depend critically on whether newly incorporated cells can actively migrate to repopulate areas of neuronal loss within the adult brain. Previous studies demonstrated that embryonic neurons and multipotent precursors transplanted into the neocortex of adult mice undergoing targeted apoptosis of pyramidal neurons migrate long distances into neuron-deficient regions, undergo directed differentiation, accept afferent synaptic input, and make appropriate long-distance projections. The experiments presented here: (1) use time-lapse digital confocal imaging of neuronal migration in living slice cultures to assess cellular mechanisms utilized by immature neurons during such long distance migration, and (2) identify changes within the host cortical astroglial population that may contribute to this migration. Prelabeled embryonic day 17 mouse neocortical neurons were transplanted into adult mouse primary somatosensory cortex undergoing targeted apoptotic degeneration of callosal projection neurons. Four to 7 days following transplantation, living slice cultures containing the region of transplanted cells were prepared and observed. Sequential time-lapse images were recorded using a video-based digital confocal microscope. Transplanted cells displayed bipolar morphologies characteristic of migrating neuroblasts and moved in a saltatory manner with mean rates of up to 14 μm/h. To investigate whether a permissive glial phenotype may provide a potential substrate for this directed form of neuronal migration, slice cultures were immunostained with the RC2 monoclonal antibody, which identifies radial glia that act as a substrate for neuronal migration during corticogenesis. RC2 does not label mature stellate astrocytes, which express glial fibrillary acidic protein (GFAP). RC2 expression was observed in glial cells closely apposed to migrating donor neurons within the slice cultures. The timing and specificity of RC2 expression was examined immunocytochemically at various times following transplantation. RC2 immunostaining within regions of neuronal degeneration was transient, with peak staining between 3 and 7 days following transplantation. Strongly RC2-immunoreactive cells that did not express GFAP were found within these regions, but not in distant cortical regions or within control brains. RC2-positive cells were identified in recipient transgenic mice which express β-galactosidase under a glial specific promoter. Coexpression of RC2 and β-galactosidase identified these cells as host astroglia. These results demonstrate that adult cortical astrocytes retain the capacity to reexpress an earlier developmental phenotype that may partially underlie the observed active migration of transplanted neurons and neural precursors. Further understanding of these processes could allow directed migration of transplanted or endogenous precursors toward therapeutic cellular repopulation and complex circuit reconstruction in neocortex and other CNS regions.

Key Words: apoptosis; astrocyte; migration; neocortex; neuronal degeneration; radial glia; RC2; transplantation.

INTRODUCTION

Transplantation of immature neurons, neural precursors, or manipulation of endogenous neural precursors to repopulate and reconstruct complex circuitry within the adult central nervous system (CNS) offers potential restoration of the normally irreversible loss of function observed following damage or disease that leads to neuronal cell death (23, 42, 43, 51, 76). The transplantation of embryonic or genetically engineered cells into some models of subcortical CNS damage has been shown to ameliorate symptoms associated with injury, but the restoration of damaged cellular circuitry does not appear to be necessary for such recovery of function.
(21, 27, 44, 66). The successful recovery of function following neural transplantation in these models is believed to be primarily achieved by the local release of neurotransmitter or trophic factors from donor cells transplanted close to the injured area, or in some cases limited local innervation (1, 8). In other areas of the adult CNS, such as the neocortex, it is likely that functional recovery will require reconstruction of the complex cellular circuitry and architecture that underlie higher brain functions (33, 34, 43, 48, 72).

Precise cytoarchitecture and specific cellular connections subserve the complex motor, sensory, and cognitive functions of the neocortex. Neocortical neurons are organized into distinct laminae and have complex axonal projections often over great distances. Following damage to the neocortex, significant functional recovery may depend critically on whether neurons can migrate into appropriate laminar locations, integrate into the host cytoarchitecture, differentiate into the appropriate type of neuron, and then reconstruct the specific disrupted local and distant projection patterns of the damaged host neurons (6, 12, 13, 68, 70, 77, 78).

We have examined local microenvironmental control over neuronal and neural precursor migration, differentiation, integration, and connectivity following transplantation using an approach of targeted apoptotic neuronal degeneration in a variety of specific neuronal populations in vivo. For example, neuronal degeneration of the population of lamina II/III neocortical pyramidal neurons with callosal projections in developing or adult cortex is initiated by photodeactivation of a retrogradely targeted chromophore, using very deeply penetrating long-wavelength light. This results in highly specific neuronal cell death via apoptosis that occurs in the targeted population of neurons over the following 2–3 weeks (48, 49, 71, 72). This relatively synchronous initiation of apoptosis mimics developmental programmed cell death, and our prior experiments demonstrate that these events induce signals activated during normal development and organizational refinement in the nervous system (89). Intermixed neurons of other types, glia, nonneural cells, and vasculature remain intact, allowing neighboring cell populations to alter cellular and molecular signaling in response to and in compensation for this perturbation of the neuronal environment and circuitry.

Following transplantation into adult neocortex undergoing targeted neuronal degeneration, embryonic neurons and neural precursors migrate long distances from the site of transplantation (48, 72), integrate into appropriate laminar locations within the cortex (48, 72, 76), differentiate into mature neurons (34, 48, 72, 76), and make long-distance axonal projections specific for their cellular phenotype (34). Heterotopic control transplants of cerebellar neurons do not undergo these directed cellular events (48), and the migration and differentiation responses of transplanted neurons are limited to the 3- to 4-week period of active neuronal degeneration (72, 89). These findings suggest that the microenvironment of the adult neocortex undergoing this targeted cell death provides spatially and temporally appropriate cellular and molecular signals that permit and/or instruct neuronal development long after both neurogenesis and corticogenesis are normally completed. Understanding the mechanisms of and potential controls over newly incorporated cell migration and integration within injured adult brain will contribute to therapeutic repopulation and reconstruction of complex CNS circuitry.

Neuronal migration is a critical step in the normal development of the cerebral cortex (31, 32, 64, 65). Postmitotic neurons migrate outward from deep within proliferative zones to form cortical laminae in a temporally regulated manner, with the oldest neurons forming deeper layers and the youngest forming more superficial layers in an “inside-out” manner (3). The migrating cortical neuroblasts reach their final laminar location by a distinctive mode of saltatory cellular locomotion (31, 32, 85). Radial glia provide a precise scaffolding as the primary pathway for neuronal migration in the developing neocortex (62, 63, 64, 65, 79), although tangential migration and migration along other substrates such as axons has also been demonstrated (29, 50, 57, 58, 59, 60). Radial glia are an exclusively developmental cell type in mammals with a distinctive bipolar morphology and antigenic phenotype. A monoclonal antibody, RC2, has been developed as an immunocytochemical marker that selectively distinguishes radial glia from other cells in the central nervous system (19, 56). When the period of neuronal migration is complete, the glial scaffolding that supported neuronal migration during cortical development becomes disassembled, and the radial glia transform into mature stellate astrocytes (14, 16, 19, 41, 55, 61, 69, 88).

The present experiments examine: (1) the cellular morphology and the mechanisms of migration of donor neurons transplanted into adult host primary somatosensory (S1) cortex undergoing selective degeneration of callosal projection neurons, utilizing a living-slice culture technique and time-lapse video microscopy; (2) the reexpression of a developmentally regulated radial glial phenotype by host astroglia as a potential substrate for this migration, using immunocytochemical methods. Mature stellate astrocytes, oligodendroglia, and extracellular matrix within the adult CNS are generally thought to be inhibitory to neuronal migration (22, 26, 30, 32, 36, 46, 72, 83, 85). The migration of transplanted neurons in regions of selective apoptotic degeneration of callosal neurons within the neocortex of adult mice suggests the hypothesis that a permissive and/or instructive microenvironment exists within the
host cortex. Reexpression of a permissive radial glial phenotype by host astroglia within neuron-deficient regions of adult neocortex may play a part in the observed migration.

We found that embryonic neurons transplanted into regions of adult primary somatosensory cortex undergoing targeted neuronal degeneration actively migrated into regions of ongoing neuronal apoptosis with a bipolar morphology, rate, and saltatory mode of migration similar to that of migrating neuroblasts during normal corticogenesis. In addition, host astrocytes within these regions of neuronal degeneration dedifferentiated toward a radial glial phenotype, including an elongated morphology and expression of the immunohistochemical marker RC2.

MATERIALS AND METHODS

This study is based on data from 69 C57B/6J and Tg[X;GFAP,lac2]Mes3 transgenic (kindly provided by A. Messing) adult mice (living slices n = 17; histology n = 52). Callosal projection neurons in lamina II/III of adult mouse S1 cortex were targeted for photolytic cell death, and dissociated embryonic day 17 (E17) neurons or immortalized multipotent neural precursor cells (C17.2; kindly provided by E. Snyder) were transplanted into spatially defined regions undergoing neuronal degeneration. Four to 7 days after neuronal transplantation, the migration of transplanted donor neurons in living cortical slices was observed in vitro. Some of the living slices used in these migration studies were fixed following the period of observation and processed for RC2 immunocytochemistry. A separate group of control and experimental brains was processed specifically for RC2 and GFAP immunocytochemistry at three different times following transplantation. The brains of transgenic mice and the brains of wild-type mice transplanted with C17.2 precursor cells were processed for combined RC2 immunocytochemistry and X-gal histochemistry to identify β-gal activity within transgenic host astroglial cells and transplanted C17.2 cells respectively.

Surgical procedures. Detailed chlorin e₆ injection and laser exposure methods for inducing specific neuronal degeneration have been described previously (48, 72). Briefly, 2- to 4-week-old mice were deeply anesthetized with Avertin, and a segment of the skull overlying S1 cortex was removed. Glass micropipettes with tip diameters of 30–60 µm were used to microinject a total volume of approximately 500 nl of fluorescein latex nanospheres conjugated with chlorin e₆, divided among 5 to 10 injection sites evenly spaced throughout S1. Following surgery, the bone was replaced, and pups were returned to their dams. Two weeks later, degeneration of approximately 65–90% of lamina II/III pyramidal neurons (48, 72) was induced within a spatially defined region of the contralateral S1 cortex by exposure through intact dura to a continuous-wave long wavelength (674 nm) laser with custom optics (Candela Lasers, MA). Slow, progressive, apoptotic degeneration of callosal projection neurons labeled with chlorin e₆ occurred over the following 3 to 4 weeks in agreement with prior results (48, 72). Apoptotic neuronal degeneration has been previously shown to be limited to the callosal projection neurons of laminae II/III within spatially defined regions of neocortex (48, 71, 72). Three-dimensional cytoarchitecture of the cortex was maintained unperturbed, leaving other neurons, non-neuronal cell types, and vasculature completely intact.

Two weeks after laser exposure, at 6 to 8 weeks of age, suspensions of E17 cells or C17.2 precursors were labeled with a second type of latex nanosphere containing rhodamine. These nanospheres preferentially label neurons in vitro (49), persist within neurons indefinitely, and do not secondarily label host cortical neurons (48, 72). Suspensions of cells were also labeled with the lipophilic dyes DiI or PKH, which label cellular membranes and outline cell somata and processes of both neurons and glia (4, 37, 38). Injections of prelabeled donor cell suspensions were placed through the center of degenerating neocortical area S1 and spanned laminae I through VI, occasionally reaching the corpus callosum. Embryonic neurons or precursors were transplanted into somatosensory cortex of 55 6- to 8-week-old mice undergoing targeted neuronal apoptosis and into the intact somatosensory cortex of 6 age-matched controls. Other controls included transplantation of nonviable embryonic cells (after several freeze/thaw cycles) into three adult mice undergoing targeted neuronal apoptosis and seven adult mice undergoing targeted neuronal apoptosis which had sham injections with removal of the skull, but no transplantation of cells.

Neocortical dissection techniques. Isolation and double fluorescence labeling of E17 neocortical neurons pooled from the entire cortex followed methods previously reported (34, 48, 72). Prior to transplantation, donor cell suspensions were assessed by exclusion of trypan blue to ensure cellular viability of greater than 75%. Suspensions were adjusted to a final concentration of 4 × 10⁴ viable cells per microliter, for a total of 2–4 × 10⁵ cells per injection site and 2 × 10⁴ cells per animal. Following transplantation, all remaining cells were cultured; somatic morphology, neurite extension, and granular compartmentalization were further monitored in vitro to ensure the health of transplanted neurons. In a set of control transplantations, E17 neurons rendered nonviable by four successive freeze/thaw cycles (viability by trypan blue exclusion less than 1%) were transplanted into S1 cortex undergoing targeted neuronal apoptosis to confirm that the results were specific to viable cell transplants.
Cell culture. RC2 hybridoma cells were maintained by standard techniques (39). Media from confluent flasks was spun at 2000 rpm for 10 min, and the supernatant was removed and diluted 1:4 in 0.1 M PBS with 0.1% Triton X-100 for use in RC2 immunocytochemistry. C17.2 cells were maintained as previously described (73, 76) until the day of transplantation, when a nearly confluent plate of cells was trypsinized, gently triturated, washed, and resuspended in sterile medium.

Preparation of living slices. Cortical slices were prepared by a modification of standard methods (9). Four to 7 days following transplantation, brains were quickly removed and placed in ice-cold Hank's balanced salt solution (HBSS; Gibco). Then 150-µm-thick slices of living cortex were cut in a chilled sterile HBSS bath on a vibrating microtome (Vibratome) and collected in sterile ice-cold HBSS. Slices were examined quickly using a Zeiss inverted microscope equipped with epifluorescence. Regions of living cortical slices containing donor neurons labeled with fluorescent nanospheres and Dil or PKH were trimmed to a final size approximately 3 by 4 mm, spanning the full depth of the neocortex. Using a spatula, slices were transferred onto a 20-µl bed of chicken plasma (Sigma) on a glass coverslip (Fisher) sealing a hole in the bottom of a 35-mm culture dish (Falcon). Approximately 10 µl of thrombin (Sigma) was added to the plasma, and the plasma/thrombin solution was allowed to set for 5-10 min in an incubator. Oxygenated, warmed HBSS was added to cover the surface of the slice, and the culture dish was placed onto a warmed stage (36-38°C). Oxygenated HBSS was replaced at least once per hour during the 6- to 16-h observation period.

Analysis of living slices. Living cortical slices were quickly scanned using a Zeiss inverted microscope with a 25× objective to locate the position of prelabeled transplanted neurons. A field, 625 µm in diameter, containing cortical laminae II/III in the region of selective neuronal degeneration was chosen, and migrating donor neurons in this field were continuously observed for up to 16 h using a 40× oil immersion objective. Images of migrating neurons were visualized using a silicon-intensified video camera (Dage-MTI) and a digital confocal imaging software package (Vaytek). Out-of-focus haze was removed, and images were collected at 10- to 30-min intervals. Serial images were recorded and analyzed for morphological changes and rate of migration with a video-based image analysis program (Java, Jandel Scientific). The positions of individual migrating neurons over time were digitized, and the total distance traveled by each migrating neuron was calculated. The mean rate for each migrating neuron was computed.

Tissue preparation for immunocytochemical analysis. Living cortical slices from five experiments in which migration was observed were fixed in 4% paraformaldehyde overnight following the observation period and processed for RC2 immunocytochemistry. In addition, the brains of 55 mice were processed specifically for RC2 and GFAP immunocytochemistry at three different times: 4 days (n = 8), 1 week (n = 33), or 2 weeks (n = 14) following donor neuron transplantation into areas of selective neuronal degeneration. The mice were deeply anesthetized with Avertin and then transcardially perfused with heparinized saline followed by 2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) with 75 mM L-Lysine and 10 mM Napier (11). The brains were postfixed in the same fixative and 40-µm-thick serial sections cut through the region of cellular transplantation on a vibrating microtome (Vibratome). Sections were processed for fluorescence immunocytochemistry and observed with a Zeiss microscope equipped with epifluorescence and Nomarski DIC optics. Additional sections of neocortex from 1-day-old mouse pups and 6-week-old adult mice were immunostained for RC2 and GFAP both as controls to verify that the RC2 antibody is specific for radial glia (19, 39, 40, 56) and to identify and visualize radial glia and mature astrocytes respectively in vivo at the appropriate developmental ages. GFAP and X-gal histochemistry was also performed in several 6-week-old Tg(X;GFAP,lacZ)Mes3 mice to identify lacZ transgene expression in mature astrocytes of adult transgenic animals (11).

Immunocytochemistry. Immunocytochemical staining for the developmentally regulated glial markers RC2 (antibody, gift of Dr. M. Edwards, Eunice Kennedy Shriver Center; and hybridoma cells, gift of Dr. K. Hunter, Rockefeller University), and glial fibrillary acidic protein (GFAP, Zymed) was performed on fixed living slices and on Vibratome sections of mouse brain. Samples were rinsed in 0.1 M PBS with 0.3% Tween 20, incubated in blocking solution (0.1% PBS with 0.3% Tween 20, 3% whole goat serum, and 5% bovine serum albumin) for 1 h, and placed into the primary antibody solutions (1:4 RC2; 1:200 GFAP) for 24 h at 4°C. The tissue was washed several times in blocking solution and then incubated in goat anti-mouse IgM conjugated to FITC (Sigma; diluted 1:50 in PBS) or goat anti-mouse IgG conjugated to CY3 (Amersham; diluted 1:500 in PBS) for 1 h at room temperature. To identify β-galactosidase-expressing cells, sections from selected brains were then processed for X-gal histochemistry by standard methods (73). Following further washes with 0.1 M PBS, sections were dry mounted on gelatin-coated slides, dehydrated in successive ethyl alcohol washes, and mounted in Fluoromount (Gurr).
RESULTS

Active migration of transplanted neurons within living slice cultures. From four to seven days following transplantation spanning all cortical laminae (and see 34, 48, 72) through regions of primary somatosensory (S1) cortex of adult mice undergoing neuronal degeneration in S1, embryonic day 17 neurons actively migrated from positions including laminae VI, V, and IV to regions of selective cell death within lamina II/III of living slice cultures. Transplanted neurons were observed up to several hundred micrometers from the injection site, consistent with previously reported findings of migration of up to 1 mm or more obliquely across normal intervening laminae V and IV (72) over a 2-week period (34, 48). Serial time-lapse video micrographs revealed that transplanted neurons in living slice cultures exhibited a characteristic migratory behavior similar to that seen during corticogenesis. More than 50% of identified neurons (n = 57 examined continuously for 6–16 h) migrated at mean rates between 4–14 µm/h (Fig. 1b). Neurons moved through the adult host cortex in a developmentally typical saltatory fashion: the neuronal cell body remained stationary for extended periods of up to 2 h, then moved rapidly in the direction of the leading process for a distance, and then paused again. During a given recording session for any individual neuron, this process was repeated many times, and many cells moved total distances greater than 150 µm. During the active phase of saltatory migration, 43% of migrating donor neurons traveled for short periods at consistent speeds of 10–25 µm/h, and a few (n = 4) migrated at speeds up to 25–30 µm/h.

In contrast, E17 neurons did not move significant distances within control slice cultures following transplantation into lamina II/III of the intact neocortex of control mice. Fifty-three percent of observed neurons (n = 32) transplanted into control cortex moved at the mean rate of less than 1 µm/h, and the remaining neurons moved at mean rates of less than 2 µm/h (Fig. 1a). Over the total period of observation (up to 16 h) all of these neurons stayed within 12 µm of their initial location: 71% stayed within 10 µm of their initial localization, while the remaining 29% were found 10–12 µm away from the point where they were first observed.

We examined the morphology of transplanted E17 neurons in cortex undergoing neuronal degeneration using digital confocal video microscopy. Donor neurons were identified by Dil, PKH, or fluorescent nanosphere labeling. Identified donor neurons displayed bipolar morphologies with elongated cell bodies, thick leading processes, and thinner trailing processes (Fig. 1c). The leading processes tended to be more complex and branched than the trailing processes. These morphologic features are characteristic of migrating embryonic neurons during normal mammalian corticogenesis (31, 32, 53, 57, 62, 63, 64). Embryonic neurons transplanted into the intact somatosensory cortex of control animals were rounded in appearance, approximately 15–20 µm in diameter, lacked processes, and remained localized within the injection site (not shown).

Reexpression of the radial glial cell marker RC2 within living slices. Living slices from the in vitro migration studies were fixed following the period of observation, and processed for RC2 immunocytochemistry, to evaluate the possible contribution of radial glia as substrates for this migration. Within adult host cortex undergoing neuronal degeneration, RC2-positive glia were identified closely apposed to migrating donor neurons (Fig. 1d). The RC2-expressing cells were typically bipolar with elongated rather than stellate processes. These cells resembled transitional astroglial forms described during the transformation of radial glia to stellate astrocytes during late corticogenesis (14, 16, 19, 41, 55, 61, 69, 88). RC2-positive cells were observed in a variety of orientations within the cortical slices and were not preferentially oriented in a radial direction from pia to ventricle (see Discussion). Immunocytochemical staining with the RC2 monoclonal antibody permitted identification of glial cells within living slice cultures in which migrating donor neurons had been observed with time-lapse video microscopy. We performed further specific immunocytochemical studies to examine the specificity and time-course of reexpression of this glial antigen normally expressed only transiently during early cortical development.

Developmental expression of glial markers. We examined intact cortex from both immature (1-day-old) and adult (6-week-old) mice with the immunocytochemical markers RC2 and GFAP to verify the developmental expression of both of these markers. The antigen recognized by the RC2 antibody has been previously described to follow a specific developmental timetable, with high levels of expression by radial glia during embryonic brain development, lower levels in early postnatal transitional glia, and none in astrocytes after the second postnatal week (14, 19, 41, 55), when mature and activated astroglia express GFAP. Within the developing neocortex of immature mouse brain, RC2 staining revealed palisades of radially oriented bipolar cells, which extended processes toward the pial surface (Fig. 2a). We also observed a variety of RC2-positive multipolar cells (Fig. 2b) with limited expression of GFAP, consistent with transitional forms intermediate in the transformation of radial glia to astrocytes previously described at this developmental stage (14, 16, 19, 41, 55, 61, 69, 88). No RC2-immunoreactive cells were observed in cortex from intact adult mouse cortex (data not shown), consistent with the previously described loss of this antigen by the second postnatal week of development in the mouse (56). Many stellate
FIG. 1. Developmental rates of migration, neuronal migratory morphology, and reexpression of radial glial phenotype. Active neuronal migration by transplanted immature neurons within living slice cultures coincides with reexpression of a radial glial phenotype and the marker RC2 within regions of adult mouse neocortex undergoing targeted apoptotic neuronal degeneration. Immature neurons transplanted into intact control neocortex fail to migrate and the radial glial phenotype is not observed. (a) Mean rates of migration for E17 neurons transplanted into intact primary somatosensory cortex of control brains; slice cultures were prepared 4 to 7 days following transplantation (n = 32 cells, from 5 separate slice experiments). (b) Mean rates of migration for E17 neurons transplanted into somatosensory cortex of experimental brains undergoing targeted neuronal death (n = 57 cells, from 9 separate slice experiments). (c) Migrating donor neuron prelabeled with fluorescent nanospheres and PKH, visualized by digital confocal microscopy deep within a living slice culture of a region of adult mouse primary somatosensory cortex undergoing targeted pyramidal neuron degeneration. A complex leading process (long arrow) extends in the direction of movement. The elongated cell body (short arrow) underwent saltatory migration between the simpler trailing process (arrowhead) and the direction of migration. Scale bar, 20 µm. (d) Reexpression of the developmentally regulated radial glial cell marker RC2 within a living slice culture supporting neuronal migration. RC2-expressing cells (arrows) were visualized by digital confocal microscopy deep within the slice culture, surrounding transplanted immature neurons that underwent active migration. Scale bar is 40 µm.
GFAP-positive cells were seen in the intact adult mouse cortex (Fig. 2c), representing mature astrocytes (7, 20).

Glial markers following transplantation of embryonic neurons into adult cortex. RC2-immunoreactive cells were found in adult neocortex undergoing neuronal degeneration 7 days after transplantation of E17 neurons (Fig. 2d). These cells had a variety of forms, and were generally elongated and bipolar rather than stellate in shape. The RC2-positive cellular processes of these cells extended distances up 100 µm through the surrounding neuropil and were similar to transitional glial morphologies (Fig. 2b). No cellular RC2 staining was observed in cortex undergoing neuronal degeneration without transplanted cells or following transplantation of nonviable neurons, and the appearance of these control cases was identical to that of negative controls in which we omitted the primary antibody (data not shown). The differential pattern of expression of RC2 in conditions in which neuronal migration of transplanted neurons is observed compared with conditions without migration suggests that reexpression of this glial phenotype in adult cortex may be permissive and/or instructive for neuronal migration.

Two days following transplantation of E17 cells into regions of neuronal degeneration in adult somatosensory cortex, RC2-immunoreactive glia were seen closely apposed to donor neurons outside of the injection track. Only very rare RC2-immunoreactive glia were observed at this early time-point surrounding transplanted donor neurons in intact cortex. Similar results were found one week following neuronal transplantation (Figs. 3a and 3b), and following transplantation of C17.2 cells (not shown). RC2-positive glia were not observed in age-matched animals that had not received transplants into either intact or degenerating S1 (data not shown). Fourteen days after transplantation, no RC2-expressing cells were observed in intact S1 and fewer RC2-positive glial cells overall were seen in degenerating S1, relative to earlier times.

Two conditions may contribute to the observed reexpression of a radial glial phenotype by astrocytes within adult cortex: the presence of signals provided by embryonic neurons, as well as the relative absence of local mature neurons or signaling by them. The transplantation of embryonic neurons led to a transient reexpression of the radial glial marker RC2 in adult neocortex which was substantially more pronounced and prolonged in regions of targeted neuronal degeneration compared with intact brain, and which temporally overlapped with the period of maximal cellular migration following transplantation (72). Interestingly, high levels of RC2 expression were seen in the corpus callosum, a brain region normally devoid of adult neurons, when occasional E17 neurons or C17.2 cells were transplanted through the deepest lamina VI of cortex and into the corpus callosum (data not shown). Taken together, these results suggest the hypothesis that glial phenotype might be regulated by signals provided by neurons in the surrounding cortical environment.

Double-labeling experiments that combined immunocytochemistry for RC2 and GFAP in the same section revealed that 7 days following transplantation of embryonic neurons into cortex undergoing targeted neuronal degeneration, most of the RC2-positive glial cells (Fig. 3a) were not immunostained with GFAP (Fig. 3b), indicating that these cells underwent significant phenotypic change. In contrast, the few RC2-positive glial cells that could be found in intact S1 (Fig. 3c) were all GFAP-positive as well (Fig. 3d). In all conditions, other astrocytes that were GFAP-positive were not RC2-positive (Fig. 3b).

RC2-immunoreactive cells in the adult cortex are host astrocytes. RC2-immunoreactive cells were found in adult neocortex undergoing neuronal degeneration after transplantation of E17 neurons (Fig. 2d). RC2-expressing cells were not labeled with the fluorescent markers used to identify donor cells. The lack of donor cell markers (PKH/DiI) within RC2-expressing cells strongly suggested that these cells were of host origin. RC2-positive cells in cortex transplanted with C17.2 precursor cells did not express the donor cell marker β-galactosidase by X-gal staining (data not shown), further supporting the conclusion that the RC2-expressing cells were of host origin.

To definitively determine the origin of the RC2-positive cells, we repeated our transplantation experiments using as recipients Tg(X;GFAP, lacZ)Mes3 transgenic mice in which the Lac-Z transgene is driven by the GFAP promoter (11). GFAP-expressing mature astrocytes were directly identified within host cortex of these mice by dense nuclear staining with the X-gal reaction (Figs. 4a and 4b). Combined RC2 immunocytochemistry and X-gal histochemistry showed that RC2-positive glial cells surrounding migrating E17 neurons within cortical regions of neuronal cell death cortex expressed the host-specific transgene (Figs. 4c and 4d). Taken together, these results definitively demonstrate that adult cortical astroglia are capable of transiently dedifferentiating and assuming elements of a radial glial phenotype which may be permissive and/or instructive for neuronal migration in the adult cortex and excludes the possibility that the observed RC2-positive cells were exclusively of donor origin.

**Discussion**

The two central findings of these experiments are that: (1) embryonic neurons transplanted into regions of adult primary somatosensory cortex undergoing targeted neuronal degeneration actively migrate into regions of ongoing neuronal death with a morphology,
FIG. 2. (Top) Expression of glial markers and cellular morphology in developing and experimental mouse primary somatosensory cortex. (a) Palisades of radially oriented radial glia (arrow) labeled by RC2 staining of postnatal day 1 (P1) mouse cortex. (b) Bipolar and multipolar (arrow) cellular forms of transitional radial glia during the transformation of bipolar radial glia into stellate astrocytes identified by RC2 staining of an adjacent area of P1 mouse cortex. (c) No RC2-positive cells are seen in intact adult mouse cortex, but a subset of stellate astrocytes (arrowhead) are identified by staining for GFAP. No RC2-positive cells were seen in negative controls in which the primary antibody
rate, and mode of migration similar to normal corticogenesis; and (2) host astrocytes within these regions de-differentiate toward a developmentally regulated radial glial phenotype, including an elongated morphology and expression of the immunochemical marker RC2.

Transplanted neurons observed to migrate within slice cultures of adult neocortex display bipolar morphologies characteristic of migrating neuroblasts during corticogenesis and move in a saltatory manner at rates similar to those seen during development. Glial

was omitted, or following transplantation of non-viable neurons into either intact adult cortex or adult cortex undergoing targeted neuronal apoptosis. (d) Many elongated RC2-immunoreactive cells (arrow) were present in adult mouse neocortex undergoing neuronal degeneration seven days following transplantation of viable E17 neurons. Scale bar for a–d, 50 µm.

FIG. 3. The radial glial marker RC2 is reexpressed by a subset of astroglia that acquire elongated transitional radial glial morphology surrounding actively migrating transplanted embryonic neurons. Double immunofluorescence labeling with RC2 and GFAP following transplantation of E17 neurons into adult mouse cortex undergoing targeted pyramidal neuronal degeneration (a,b) or intact control cortex (c,d). (a) Astroglia in cortex undergoing neuronal degeneration elongate and express RC2 (long arrows) but typically not GFAP following transplantation of E17 neurons. Some intermediate form glia express both (arrowhead). (b) The same field as in “a” imaged for GFAP labeling. The RC2-positive cell indicated by an arrowhead is both RC2- and GFAP-labeled. The two elongated RC2-positive cells indicated in panel “a” (long arrows) are not stained for GFAP in panel “b.” Other astroglia were labeled for GFAP alone in all conditions (short arrow). (c) Only very rare cells in intact cortex express RC2 (arrow) following transplantation of embryonic neurons. None express it in the absence of transplantation. (d) The same field as in “c” imaged for GFAP labeling. The rare RC2-positive cells always coexpressed GFAP, and typically retained stellate astrocyte morphology (arrow). Scale bar for a–d, 50 µm.

FIG. 4. (Bottom) Astroglia that reexpress a transitional radial glial phenotype in adult mouse cortex undergoing neuronal degeneration are of host origin. Embryonic wild-type neurons were transplanted into adult cortex of Tg(X;GFAP;lacZ)Mes3 transgenic mice in which the lac-Z transgene is driven by the GFAP promoter, allowing endogenous astrocytes to be directly identified by their dense nuclear X-gal labeling. RC2-expressing glia were not labeled by physical fluorescent prelabels or β-galactosidase used to identify donor cells in other experiments. (a) Endogenous host astrocytes in intact somatosensory cortex of Tg(X;GFAP;lacZ)Mes3 transgenic mice labeled with GFAP antibody (arrow). (b) The same field as “a” viewed under Nomarski DIC optics shows dense X-gal reaction product in the nucleus of the same astrocyte (arrow). Another GFAP expressing cell is out of the plane of focus. (c) RC2-labeled transitional radial glial cell (arrow) adjacent to migrating immature neurons in a cortical region undergoing targeted neuronal cell death. (d) The same field as “c” viewed under phase contrast optics identifies the RC2 expressing cell as being of host astrocytic origin by the presence of dense X-gal product in the nucleus (arrow). Scale bar for a–d, 20 µm.
cells within neuron-deficient adult murine cortex adjacent to migrating donor neurons can express the developmentally regulated radial glial cell marker RC2, without expressing the mature astrocytic marker GFAP. The reexpression of RC2 by glial cells within adult cortex is transient and both temporally and spatially correlated with the active migration of transplanted neurons, consistent with the hypothesis that radial glial cells may provide a substrate for at least some of the observed neuronal migration. The absence of fluorescent and genetic donor cell identifying labels, and glial cell coexpression of RC2 and the host-specific Lac-Z transgene following neuronal transplantation within neuron-deficient cortical regions of recipient mice expressing Lac-Z under control of a GFAP promoter, both demonstrate that astrocytic cells of the host adult neocortex reexpress a radial glial phenotype in vivo. Therefore, under the appropriate conditions, adult neocortex is capable of providing a microenvironment that supports neuronal migration long after both neurogenesis and corticogenesis are normally completed. During development, the neocortex is formed by neurogenesis and successive migration of neurons from the germinal matrix outward through the intermediate zone to eventually form the layers of cortex in an inside-out manner (3). Developing cortex is the site of both long-distance neuronal migration and apoptotic neuronal cell death (10, 32, 35, 64). Neurogenesis and neuronal migration in the mammalian neocortex are limited to the embryonic and early postnatal periods and cease as normal development proceeds. The absence of sustained neurogenesis, the disappearance of developmentally regulated neuronal, glial, and extracellular signals, and the expression of inhibitory signals during brain maturation are thought to underlie the relative lack of cellular plasticity observed in the adult neocortex (5, 22, 36, 83). Transplantation of neural precursors or embryonic neurons into nonneurogenic areas of the adult brain, combined with manipulation of the signals controlling neuronal migration and differentiation, may overcome the lack of plasticity in the adult mammalian neocortex and increase the capacity for repair following neuronal damage.

A number of studies have demonstrated that transplantation of embryonic neurons or neural precursors during early brain development and corticogenesis can result in significant migration, integration, and differentiation of the transplanted cells in a manner appropriate to the site of engraftment and developmental stage of the recipient (5, 21, 42, 51, 75). This approach has utility for the study of basic developmental processes (52, 54) and for the potential replacement of defective gene products (45, 51, 74). Embryonic neurons transplanted into the ventricular zone of neonatal ferrets are found in appropriate laminar locations within the overlying host neocortex a few days later (52, 53), as are isochronic transplants of E17 neurons into mouse neocortex (48). In contrast, embryonic neurons or neural precursors transplanted into intact adult neocortex or into neocortex lesioned with injections of kainic acid fail to differentiate or to migrate away from the site of injection (48, 72, 76), suggesting that the intact adult neocortical microenvironment is not normally instructive and/or supportive of neuronal migration.

The present results confirm and extend at the cellular level prior findings obtained by serial histologic analysis that embryonic neurons and neural precursors transplanted into regions of neocortex undergoing targeted apoptotic neuronal degeneration undergo active and directed migration to repopulate neuron-deficient areas. Previous work has shown that these transplanted neurons receive synaptic contacts with host tissue, express neuronal antigens, assume pyramidal morphologies, and make appropriate long-distance and local projections (34, 43, 48, 72, 76). Host interneurons within these cortical regions have been shown to upregulate neurotrophins in response to the targeted apoptotic degeneration of neighboring pyramidal neurons (89). The migration, integration, and differentiation of transplanted neurons in these prior experiments occurred long after the ages at which these events normally take place during neocortical development. The current data extend these findings to the cellular level and examine the individual morphology, rate, and mode of migration of transplanted neurons. Taken together, these results suggested the hypothesis that adult neocortex undergoing targeted neuronal degeneration can reexpress developmental signals to recreate a microenvironment which can guide the migration and differentiation of transplanted precursors.

In specific cases, long-distance migration of neuronal cells has been observed following transplantation into adult brain regions. Transplants of SVZ cells into the rostral migratory stream have been shown to migrate to the olfactory bulb in adult rodents (46), but unlike neocortex, the SVZ/olfactory bulb system is one of the few regions of active neurogenesis in the adult brain. Embryonic Purkinje neurons transplanted into the cerebellum of adult PCD mutant mice devoid of Purkinje cells specifically migrate to and differentiate within appropriate positions in the cerebellar architecture (81, 82, 87), yet similar transplants into intact wild-type adult cerebella or into PCD mutants at a stage when Purkinje cells have not yet disappeared fail to differentiate or migrate. Similarly, embryonic Purkinje neurons transplanted into the cerebellum of Lurcher mutant mice also migrate long distances within the host brains to correct locations (18). These studies provide further evidence that a relatively neuron-deficient but otherwise intact adult brain can support the migration of transplanted embryonic neurons.

Embryonic neurons transplanted into an appropriate
adult neocortical environment appear to migrate via mechanisms similar to those used by migrating neuroblasts during corticogenesis. The morphological features of transplanted embryonic neurons we observed migrating within areas of adult cortex undergoing neuronal degeneration resemble cells seen in static images of migrating neuroblasts in developing cortex (62, 63, 64). Migrating transplanted neurons were bipolar cells with thick, complicated leading processes and thin, simple trailing processes. In contrast, nonmigrating cells such as those seen following transplantation into intact adult cortex tended to remain as spherical cells without processes.

The morphology of migrating donor neurons argues for a form of active cellular locomotion similar to that identified during normal neurogenesis and neuronal migration in the developing cortex (31, 32). Transplanted neurons shown by time-lapse video microscopy migrated using a saltatory mode of locomotion similar to that observed both in normal developing ferret cortex (57, 58) and in vitro models of neuronal migration (24, 25, 85). The neuronal soma followed the trajectory of the leading process to advance through the surrounding cortical environment, then the soma remained motionless for an extended period of time before moving forward after the leading process again. The rates at which the transplanted neurons migrate (10–14 µm/h) were also similar to those reported in the above studies. In contrast, the rates suggested for nonradial or tangential migration of neocortical cells during development (57) or of SVZ cells within the rostral migratory stream (47) are much more rapid, approximately 30 µm/h. Both of these forms of neuronal migration are thought to be independent of radial glial guidance. The morphology of migrating embryonic neurons also differs from the morphology of the symmetric spindle-shaped migrating SVZ cells within the rostral migratory stream, which do not migrate on radial glia, but rather undergo a distinct “chain migration” (47). These results support the hypothesis that transplanted neurons utilize ontogenically conserved mechanisms of locomotion that at least partially recapitulate the radial glial dependent migration of neuroblasts during normal corticogenesis.

Mature stellate astrocytes, oligodendroglia, and extracellular matrix are generally thought to be inhibitory to neuronal migration (14, 15, 16, 19, 22, 41, 55, 61, 69, 88), but recent evidence suggests that in the appropriate setting the mature central nervous system can reexpress embryonic markers and signals that support both neuronal migration (80, 82, 86) and axonal outgrowth (67). In the cerebellum of the PCD mutant mouse, a population of glial cells in the host adult brain reexpresses developmentally regulated markers for Bergmann glia which are thought to serve as a substrate for migrating transplanted Purkinje neurons (80, 82). Radial glial cells are the primary substrate for neural migration in the developing neocortex (64) and if reexpressed in mature cortex might support neuronal migration. Radial glial cells support neuronal migration in the adult songbird brain (2). Taken together with these prior results, the current findings suggest that reexpression of a permissive radial glial substrate within neuron-deficient regions could partially underlie the observed migration of transplanted neurons within the adult brain. In the present study, cells surrounding migrating donor neurons within regions of cortex undergoing apoptotic neuronal degeneration expressed the radial glial cell marker RC2, but not a marker of stellate astrocytes GFAP. The reexpression of a radial glial phenotype observed following transplantation of embryonic neurons into neuron-deficient regions of cortex is transient, but the temporal expression overlaps with the period of maximal neuronal migration (72).

The cells that reexpressed RC2 within regions of adult neocortex supporting neuronal migration are mature astroglia of the host neocortex. We confirmed this by several lines of evidence to exclude the possibility that the RC2-expressing cells seen within host neuron-deficient cortical regions could be of donor origin. Radial glial elements have been demonstrated within transplants of embryonic neural tissue (26, 90), and transplanted glia have been shown to migrate widely throughout adult cortex (91). However, none of the RC2-positive glial cells within our experimental slice cultures contained the physical fluorescent or genetic lac-Z markers used to prelabel donor cells. Together these results strongly suggest that the RC2-expressing cells were of host origin, but the theoretical possibilities existed that they both divided sufficiently to dilute the physical prelabels in some experiments, and stopped expressing the transgene in the other experiments. To definitively exclude the possibility that the RC2-positive glial cells were of donor origin, we demonstrated coexpression of RC2 and a host-specific lac-Z transgene following transplantation of wild-type embryonic neurons into transgenic mice in which lac-Z was driven by a glial specific promoter.

Expression of RC2 but not GFAP by glial cells surrounding transplanted neurons undergoing active migration is in contrast to what is often seen in reactive gliosis following trauma to the cortex. Up regulation of GFAP by reactive astrocytes at the site of cortical trauma is a hallmark of reactive gliosis, and has long been considered to lead to the formation of gliotic scars and to be inhibitory to neuronal regeneration (17, 22, 67, 83). Inhibition of the gliotic response to cortical injury has recently been demonstrated following infusion of RF60, a diffusible factor from embryonic neurons that is thought to control glial differentiation (40). Cells of the astroglial lineage may exist in a spectrum
from radial glia to mature stellate astrocyte to reactive astrocyte with corresponding limited support of plasticity within the brain. Control over glial phenotype may have important implications in the future success of transplantation therapy in the central nervous system, as will a better understanding of the interactions between neurons and glia that regulate the differentiation state of glia within damaged brain.

The state of differentiation of neurons within cortex appears to influence the developmental state of astrocytic cells, and the glial differentiative pathway is likely bidirectional (39). In cell culture experiments, exposure of adult astrocytes to RF60 in the absence of mature neurons allows reexpression of the radial glial phenotype by astrocytic cells (39, 41). This result is supported by other in vitro experiments in early developmental rat cortical slice cultures in which neurons survive but do not mature into adult phenotypes, and radial glial cells were not observed to differentiate into mature stellate astrocytes (28). In contrast, in slice cultures from later in development in which neurons do mature into adult phenotypes, radial glial cells disappear and are replaced by mature stellate astrocytes. These results support the possibility that glial cells within adult neocortex can be switched from mature stellate forms which express GFAP and are not supportive of neuronal migration, to earlier developmental bipolar forms which express RC2 and support neuronal migration.

In the present experiments, long-distance neuronal migration of transplanted embryonic neurons and significant up regulation of RC2 by host glial cells was only seen in experimental conditions in which there was both a depletion of mature neurons and the presence of viable immature neurons or precursors. The relative increase in proportion of embryonic to mature neurons may be responsible for the observed radial dedifferentiation of host astrocytic cells. We also observed a striking expression of RC2 by callosal astrocytes following transplantation of viable embryonic neurons into or adjacent to the corpus callosum. The corpus callosum is essentially devoid of mature neuronal cell bodies. In the work of others, embryonic striatal neurons did not migrate following transplantation into intact striatum containing normal numbers of mature neurons, and RC2 expression in these studies was limited to transplanted cells, without host cell expression (26). These results support the idea that mature neurons may supply signals that act to maintain the mature astrocytic phenotype, and embryonic neurons may supply signals that act to maintain an earlier radial glial form.

Several observations suggest that while some transplanted neurons in the present study may have migrated along RC2-positive glia, others traveled on other substrates such as axons. Not all migrating neurons lay close to RC2-positive glial cells, and transplanted neurons did not always migrate along the axis at which RC2-positive glial cells were aligned. It is known that neurons can migrate along axonal processes and other substrates (29, 50, 57, 58, 59, 60, 65). It is possible that transplanted donor neurons in the present study migrated along a variety of substrates, and that reexpression of RC2 may be only one of several elements that contribute to making the cortical microenvironment permissive or instructive for neuronal migration in the adult brain.

In summary, the results of the present study indicate that embryonic neurons transplanted into adult mouse neocortex (1) actively migrate into regions undergoing targeted apoptotic neuronal degeneration, at rates and with bipolar morphologies typical of migratory neuroblasts seen during normal murine corticogenesis, and (2) a population of adult host glial cells in cortical areas adjacent to migrating donor neurons transiently dedifferentiate to a developmentally regulated transitional radial glial phenotype. The transplanted embryonic neurons move in a saltatory fashion and at rates comparable to those observed during normal development, suggesting that the transplanted embryonic neurons use a form of locomotion similar to that used in the developing brain. Glia transform from stellate astrocytic morphology to elongated forms, and they reexpress the radial glial surface marker RC2 but not GFAP, during the period that the transplanted neurons are migrating. Taken together, these results suggest that transplanted immature neurons and neural precursors at least partially use the processes of nearby RC2-positive glia as a substrate for directed migration and utilize mechanisms of locomotion similar to those used by migrating neuroblasts during normal corticogenesis. Such directed and developmentally relevant migration to cellular locations under appropriate conditions could facilitate cellular repopulation and circuit reconstruction of neuron-deficient neocortex or other regions of the CNS. This supports the idea that even in the adult brain, the reconstruction of complex neocortical or other CNS circuitry may be possible via transplantation of neuronal precursors or manipulation and control of recently identified endogenous multipotent neural precursors. Further understanding of mechanisms and controls for potentially facilitating and guiding the directed migration and differentiation of transplanted or endogenous neural precursors could contribute new approaches for repopulation of regions of neuronal loss within the neocortex and more broadly within the CNS toward the therapeutic reconstruction of complex circuitry.

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