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# Endogenous Progenitors Remyelinate Demyelinated Axons in the Adult CNS

**JoAnn M. Gensert* and James E. Goldman†**
*Department of Physiology and
Cellular Biophysics
†Department of Pathology
and the Center for Neurobiology
and Behavior
Columbia University College of Physicians
and Surgeons
New York, NY 10032

## Summary

**Remyelination occurs in demyelinated CNS regions in diseases such as multiple sclerosis. Identification of the cell type(s) responsible for this remyelination, however, has been elusive. Here, we examine one potential source of remyelinating oligodendrocytes—immature, cycling cells endogenous to adult white matter—and demonstrate that this population responds to demyelination by differentiating into myelinating oligodendrocytes. Dividing cells in subcortical white matter of adult rats were labeled by stereotactic injection of a replication-deficient *lacZ*-encoding retrovirus (BAG). Following a focal demyelination induced with lysolecithin, many of the BAG-labeled cells differentiated into myelinating oligodendrocytes engaging in repair of the lesion. Identification of endogenous cells capable of remyelination provides a target for the study of CNS repair processes in demyelinating diseases.**

## Introduction

Central nervous system (CNS) lesions in demyelinating diseases such as multiple sclerosis (MS) show partial remyelination, an observation also reported in many experimental models of CNS demyelination (Ludwin, 1987; Raine, 1991; Allen and Kirk, 1992; Prineas et al., 1993; Blakemore et al., 1996). The origin and identity of endogenous cells that effect remyelination is an unresolved issue, central to understanding this repair process.

Pathological analyses of demyelinating disorders have suggested two potential sources of remyelinating cells, mature oligodendrocytes and progenitors. Mature oligodendrocytes that have escaped destruction may directly participate in remyelination or may enter a cycling state and reengage a developmental program toward myelination (Raine et al., 1981; Ludwin, 1984; Ludwin and Bakker, 1988; Wood and Bunge, 1991; Wu and Raine, 1992; Duncan et al., 1992; Ludwin and Szuchet, 1993; Raine and Wu, 1993); or, immature cells that are either resident to the lesion or migrate into this area may be responsible for remyelination (Ludwin, 1979; Prineas et al., 1989).

In morphological studies of demyelinated lesions, the cells effecting remyelination are identified after remyelination has already begun, which permits only speculation regarding the source of the remyelinating oligodendrocytes. The origin(s) of these cells, therefore, is not clear. Transplantation of glial populations from neonatal CNS into demyelinated or hypomyelinated regions shows that the adult CNS can support myelination by neonatally derived cells, but still leaves unresolved the question of which endogenous cell type(s) effect remyelination (Vignais et al., 1993; Duncan and Archer, 1994; Blakemore et al., 1996). Finally, although the adult mammalian CNS contains progenitors that can differentiate into oligodendrocytes in culture systems (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989; Armstrong et al., 1992; Noble et al., 1992), their role in remyelination in vivo is not known. Hence, which endogenous cells can engage in remyelination under pathological circumstances has remained unresolved and merits further examination (Compston, 1994; Ludwin, 1994).

In this study, we directly addressed whether cycling cells in the white matter of the adult CNS can differentiate into myelinating oligodendrocytes in response to experimental demyelination produced by lysolecithin. In this model, demyelination is quickly initiated, is detectable ultrastructurally within 30 min of administration, approaches completion by ~96 hr, and is reversible (Hall and Gregson, 1971; Hall, 1972). To identify dividing cells and their progeny in vivo, we labeled cycling cells in the subcortical white matter (cingulum and corpus callosum) of adult rats by stereotactic injection of a replication-deficient retrovirus containing *lacZ* as a heritable marker (BAG; Sanes et al., 1986; Price, 1987; Price et al., 1987). Cells and their progeny were then visualized with Xgal histochemistry or anti-β-galactosidase antibody. Using this approach, we previously identified and characterized a population of cycling cells in the subcortical white matter of adult rats (Gensert and Goldman, 1996). These cells have less complex morphologies than mature glia, do not migrate through the CNS, and do not express markers of either mature astrocytes or mature oligodendrocytes. We observed that very few labeled cells differentiated into myelinating oligodendrocytes and none into astrocytes in the unperturbed white matter. In this report, we show that these cells do differentiate into myelinating oligodendrocytes following demyelination.

## Results

The developmental potential of endogenous cycling cells in subcortical white matter (cingulum and corpus callosum) of the adult rat was examined in the context of experimental demyelination. Cycling cells were first labeled by stereotactic injection of the BAG retrovirus, followed two days later by induction of a focal demyelination by deposition of lysolecithin into the same area. Animals were sacrificed and examined 21 and 28 days post lesion (dpl).

### Lysolecithin Produces a Focal Demyelination

As visualized 28 dpl, the region of lysolecithin injection contains less myelin than the surrounding white matter

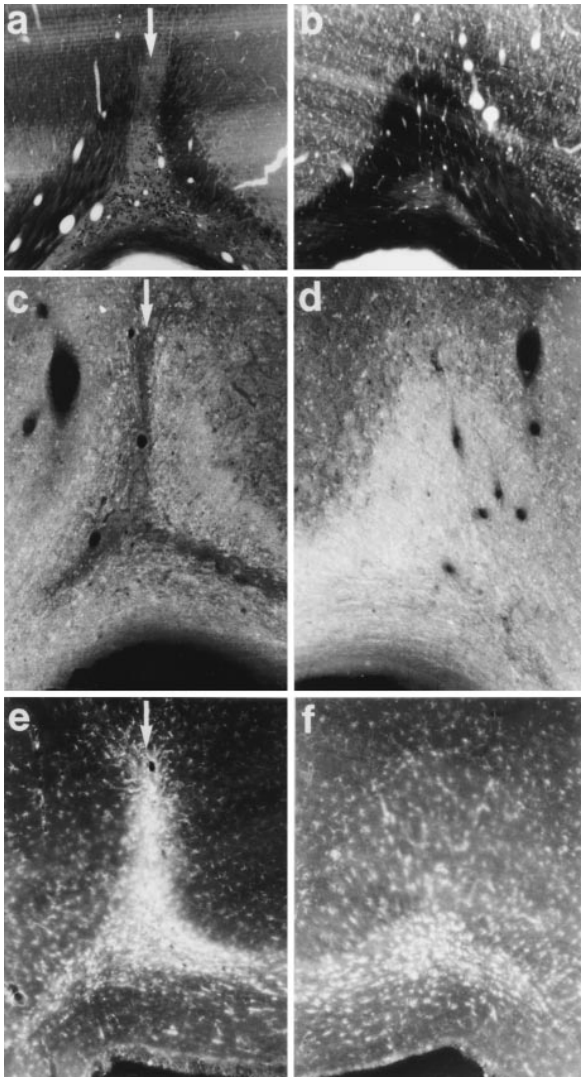


Figure 1. Lysolecithin Produces a Zone of Demyelination

Lysolecithin-induced lesions (A, C, E) and representative sections from the unlesioned contralateral hemisphere (B, D and F) 28 dpl. Demyelinated subcortical white matter (arrow) is delineated by the osmiophilic white matter border (A). There is also a decrease in the immunoreactivity for an oligodendrocyte marker, Rip, in the demyelinated region (arrow in C). A robust astrocyte response is demonstrated in the core of the lesion (arrow) by increased GFAP immunoreactivity (E). Rip is visualized with a fluorescein-conjugated secondary antibody, and GFAP is visualized with an AMCA-conjugated secondary antibody.

and is clearly delineated by a relative paucity of osmiophilic myelin (Figures 1A and 1B) and decreased immunoreactivity for Rip, a marker for cells in the oligodendrocyte lineage (Figures 1C and 1D; Friedman et al., 1989; Daston and Ratner, 1994). Also, the astrogliotic response characteristic of lesions is present, as demonstrated by increased immunoreactivity for glial fibrillary acidic protein (GFAP; Bignami et al., 1972) in the demyelinated area (Figures 1E and 1F).

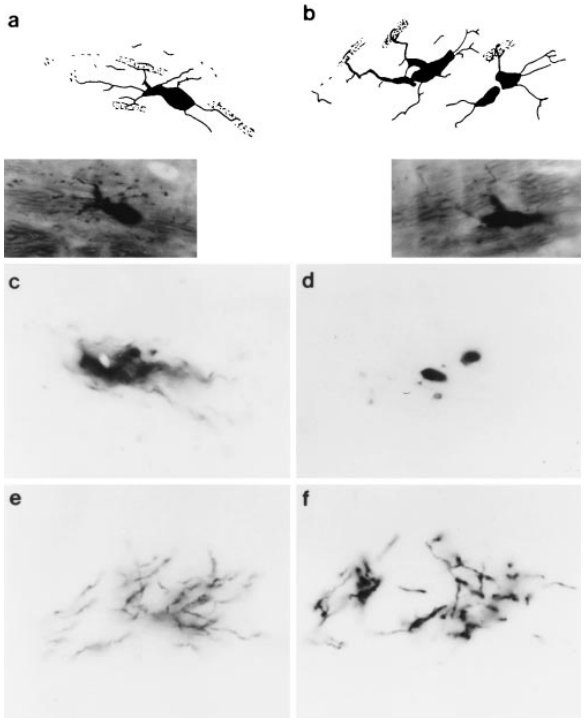


Figure 2. Endogenous Cycling Cells Differentiate into Oligodendrocytes and Remyelinate Experimentally Demyelinated Regions in Adult Rat Brain

Camera lucida drawings of BAG-labeled cells in the lesion 28 dpl were obtained with the aid of a Leitz drawing tube (A and B); axons remyelinated by these BAG-labeled cells are stippled. Corresponding light microscopic photographs are inset; the photograph inset in (B) is of the cell on the left. Light microscopic images of BAG-labeled remyelinating oligodendrocytes in demyelinated subcortical white matter are shown in (C) and (D). Labeled myelin sheaths sectioned longitudinally in the corpus callosum occur in wavy parallel arrrays (C), whereas labeled myelin cut transversely as in (D) resembles a "donut" (see text). Clusters of labeled myelin sheaths course less uniformly through the cingulum, where both the ribbon-like morphology of longitudinally sectioned myelin and labeled "donuts" can also be seen (E and F).

### BAG-Labeled Cells Differentiate along an Oligodendrocyte Pathway and Remyelinate in the Zone of Demyelination

Myelination by BAG-labeled cells was seen 28 dpl. Since the lesioned tissue has suffered serious insult, the axonal and myelin architecture is less organized than in unperturbed white matter. Labeled cells located in the lesion were morphologically complex with multiple fine processes (Figures 2A and 2B). Several elaborated myelin sheaths (Figures 2C and 2D), which, when sectioned longitudinally, appeared linear and ribbon-like (Figures 2C, 2E, and 2F). Labeled myelin sheaths in the corpus callosum occurred in wavy, roughly parallel arrays (Figure 2C), whereas labeled myelin coursed nonuniformly through the cingulum (Figures 2E and 2F). Figure 2D shows a labeled cell with a process that bifurcates, leading to a myelinated axon that is cut transversely;

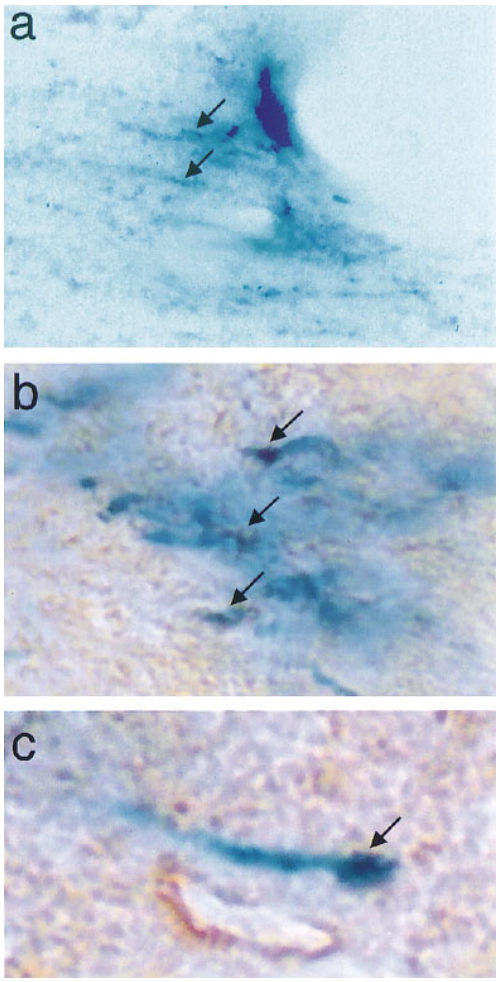


Figure 3. MBP Is Detected in BAG-Labeled Myelin

A BAG-labeled myelinating oligodendrocyte is shown in (A); arrows delineate myelin sheaths. Arrows in (B) and (C) show MBP expression (black/purple) in BAG-labeled myelin (blue).

here the labeled myelin looks like a "donut." These "donuts" can also be seen amidst the labeled myelin in Figures 2E and 2F.

Another example of a BAG-labeled myelinating cell from a different brain is shown in Figure 3A. Myelin basic protein (MBP; Deibler et al., 1972) was detected immunohistochemically in BAG-labeled myelin from the same area (arrows in Figures 3B and 3C). Double labeling of β-galactosidase and MBP is noted at points along the myelin sheaths and at expansions, which likely represent paranodal loops, where the cytoplasm is less densely packed (Szuchet, 1995).

In 100% (4/4) of lesioned brains 28 dpl, extensive myelination by BAG-labeled cells was observed and was confined to the lesion area. BAG-labeled cells outside the lesion area, however, retained the simple morphology seen in control brains (not shown; but see Gensert and Goldman, 1996, for examples). In four rats injected with the BAG virus, but not subsequentially with lysolecithin, we observed only two labeled myelinating oligodendrocytes in slices extensively sampled 28 days post injection (dpi; Gensert and Goldman, 1996). Thus, in the adult rat white matter, oligodendrocyte differentiation to a myelinating stage from a nonmyelinating, cycling state is normally an extremely rare occurrence in the absence of pathology.

### BAG-Labeled Cells Do Not Migrate from the Periphery into the Lesion to Effect Remyelination

Remyelination by BAG-labeled cells was observed when BAG and lysolecithin were introduced into the same area (albeit with a 2 day interval between surgical manipulations, as noted above; Figures 4A and 4B). To determine if BAG-labeled progenitors located outside the lesion would migrate into the lesion to effect remyelination, we first deposited BAG into the cingulum and 2 days later induced a focal demyelination in the subcortical white matter 1.5–2.0 mm lateral to the site of BAG deposition ($n = 2$; Figure 4C). BAG-labeled cells were observed in the cingulum at the injection site. However, no labeled cells were seen in the zone of demyelination (Figure 4D), even though the lesion was fewer than 500 μm from the BAG deposition, in closest proximity. No myelinating oligodendrocytes were found.

### BAG-Labeled Oligodendrocytes Arise from Cells Labeled during the Initial Retroviral Injection

We believe that the β-gal-positive myelinating oligodendrocytes differentiated from the cycling cells that were labeled prior to the lesion, and did not arise from progenitors or mature oligodendrocytes that may have been stimulated to proliferate subsequent to lesioning. For cells to have been labeled at the time of lesioning, infectious BAG retrovirus would have to have been available for 2 days in vivo. To assay how rapidly the BAG titer decreased, we maintained the BAG stock at 37°C, and titered it over the course of 48 hr using NIH 3T3 cells. The titer decayed rapidly: the retroviral stock was unable to infect cells in this assay after even 12 hr (Figure 5). In addition, when retrovirus stock was held at 37°C for 48 hr, then injected into subcortical white matter with a lysolecithin lesion made immediately thereafter, no BAG-labeled cells were observed ($n = 2$; data not shown). Hence, the labeled myelin sheaths are elaborated by cells that had been cycling prior to the lesion.

## Discussion

Our data presented here provide the first clear demonstration that immature, cycling cells endogenous to adult brain respond to demyelination by differentiating into myelinating oligodendrocytes and remyelinating axons. The strategy we employed to address this involves labeling cycling cells with a heritable marker, introduced using stereotactic surgery, and following their response(s) to a focal demyelination induced 2 days later with lysolecithin.

Two technical issues warrant clarification. We assume that the BAG-labeled cells are representative of the population of cycling cells that normally divide in adult brain,

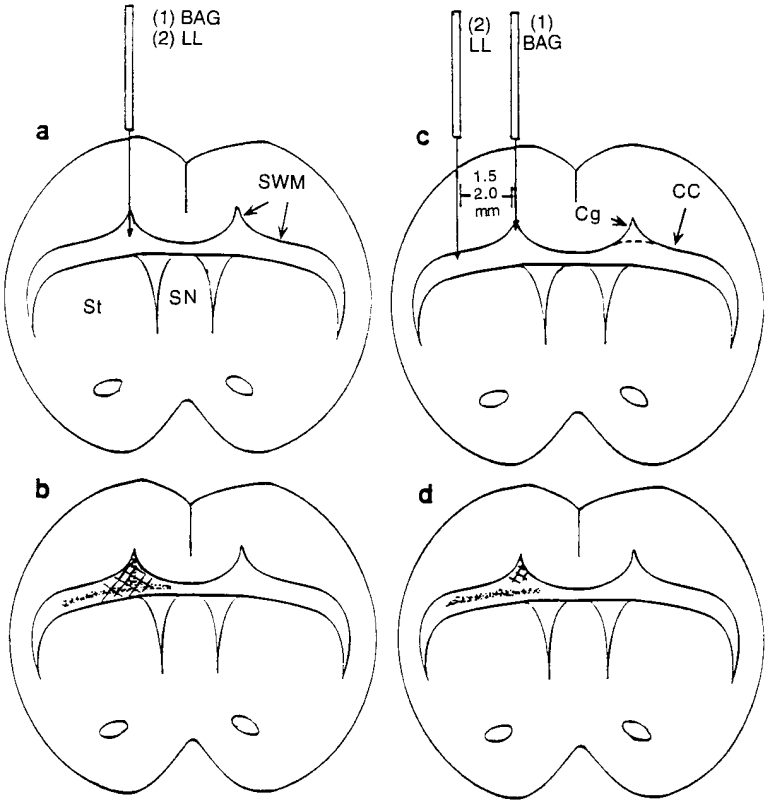


Figure 4. Progenitors Resident to the Lesion Differentiate into Oligodendrocytes

Panels (A) and (C) show the injection experiments. In (A), BAG and lysolecithin (LL) were deposited into the same area with a 2 day interval between surgical interventions; in (C), lysolecithin was deposited 1.5–2.0 mm lateral to the BAG injection. In (B) and (D), the zone of demyelination is represented by the stippled area, and the distribution of the BAG-labeled population by cross-hatches. In (B), remyelination by BAG-labeled cells was seen only in the area where the two zones overlapped. In (D), there was no overlap and no remyelination was observed. The subcortical white matter (SWM) consists of cingulum (Cg) and corpus callosum (CC), as marked. (septal nuclei, SN; striatum, St).

rather than cells induced to divide by the trauma produced by the injection itself. Our previous bromodeoxyuridine (BrdU) incorporation studies demonstrated that there was no statistically significant difference between the numbers of cells that had incorporated BrdU in the area into which BAG had been deposited, through a pulled capillary pipet attached to a Hamilton syringe, and the contralateral, control area (Gensert and Goldman, 1996).

We also assume that the BAG-labeled cells that differentiate into myelinating oligodendrocytes were labeled at the time of BAG deposition, rather than 2 days later as a result of the lysolecithin injection. Our data (above) suggest that residual infectious BAG is not available at the time of lesioning 2 dpi. Hence, any cell that is stimulated to divide by lesioning could not become labeled. Our in vitro titering assay demonstrated that BAG was unable to infect cells after 48 hr incubation at 37°C. Also, no BAG-labeled cells were observed when retrovirus stock, maintained at 37°C for 48 hr, was injected with lysolecithin-induced demyelination immediately following. Furthermore, were infectious retrovirus available after 48 hr at 37°C, a variety of labeled cells would be expected, including astrocytes and microglia, since these proliferate after trauma (Amat et al., 1996). We did not see labeling of these cell types, after the initial BAG injection, after lysolecithin treatment 2 dpi, or after introduction of BAG maintained at 37°C for 48 hr and lysolecithin in rapid succession, further supporting the conclusion that the labeled myelin sheaths are elaborated by cells that had been cycling prior to the lesion.

We targeted the population of cycling cells in subcortical white matter for several reasons. First, white matter is an appropriate place to study issues involving myelin, such as responses to demyelination. Second, although the entire adult brain contains cycling cells, the subcortical white matter contains the greatest concentrations of cycling cells (Hommes and Leblond, 1967; Dalton et al., 1968). This is important because the nature of retroviral infection and integration is such that every cell that is dividing at the time of retrovirus availability is not likely to be infected. Since our studies suggest a low

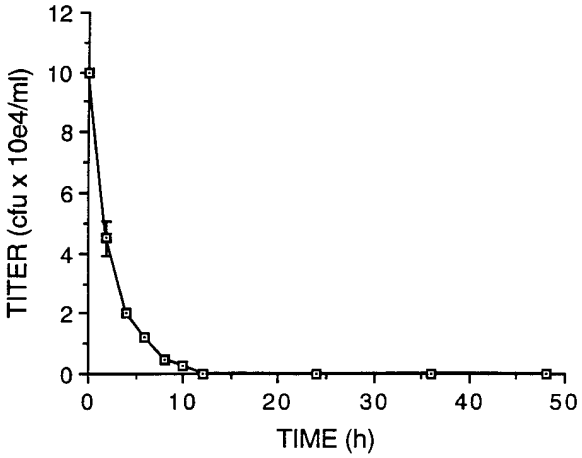


Figure 5. Retroviral Stocks Rapidly Lose Infectious Titer at 37°C

Stocks were titered as described in the text. Graph depicts titer (mean ± SEM) versus time after initial infection of cultures. Standard errors at points other than 2 hr were negligible and do not show clearly in this representation.

labeling efficiency of the BAG retrovirus (data not shown), the size of the targeted population becomes an important factor. Third, the subcortical white matter is a region that is large enough to target repetitively, enabling multiple interventions in the same animal, as well as reliable comparisons among animals.

In considering the degree of remyelination by BAG-labeled cells, one must note that the pool of labeled cells that engages in remyelination belongs to a larger population of cycling cells, many of which were not labeled with BAG, but nonetheless probably participate in this repair process. As noted above, because of the nature of retroviral infection and integration, not all cells that are cycling during the period of retrovirus availability will become labeled; furthermore, immature cells that are not in S-phase when the retrovirus is available, but cycle at some point during the experimental period ($\sim$30 days), would also escape labeling. Presumably these other immature, cycling cells would behave similarly to the labeled ones described here and contribute to the repair process. The degree of remyelination elicited by BAG-labeled cells, therefore, is likely an underestimate of the remyelination capable by the population of cycling cells endogenous to adult brain. In addition, the contribution of other cell types that were not cycling during retrovirus availability—such as myelinating oligodendrocytes and possibly nonmyelinating, but quiescent, oligodendrocyte progenitors—cannot be dismissed. We cannot at present determine quantitatively the possible relative contributions of these various cell types to the repair process.

The exact nature of the cycling cells that participate in this process requires further study. As noted (Gensert and Goldman, 1996), the population of cycling cells labeled by the BAG virus is neuroectodermal and immature, but undoubtedly heterogeneous. Although we cannot draw any conclusions about which specific BAG-labeled cells participate in the repair of these lesions, we have clearly demonstrated that immature cycling cells in this labeled population can be induced to differentiate and effect remyelination. Also, whether the repair is significant enough to restore function of the remyelinated axon is an important issue not easily addressed in this system. Restoration of normal conduction velocity of remyelinated axons through a lesion has been demonstrated, however, in remyelinated spinal cord (Honmou et al., 1996).

Remyelination in the adult brain is often incomplete (Ludwin, 1987; Raine, 1991; Allen and Kirk, 1992; Prineas et al., 1993). In the lysolecithin model, we have observed unmyelinated axons in the lesion area 28 dpl (electron microscopy; data not shown). Perhaps the limitations to remyelination lie partly in the failure of endogenous progenitors to migrate. These cells do not appear to be migratory in the unperturbed CNS (Gensert and Goldman, 1996) or in the lesioned CNS. This failure to migrate appears to be an intrinsic property of the cells, rather than a restriction imposed by adult CNS tissue, since the adult brain permits the migration of neonatal cells transplanted into it (Vignais et al., 1993; Duncan and Archer, 1994; Blakemore et al., 1996).

The lack of migration in response to a lesion can be inferred from two observations. First, morphologically undifferentiated BAG-labeled cells were present at the periphery of the demyelinated area in experiments where the lesion and BAG deposition were to the same area. This observation does not rule out the possibility that some of the cells could have migrated into the lesion, but clearly all do not. Second, when the lesion was produced ventrolaterally to the site of BAG injection (Figure 3C), no myelinating oligodendrocytes were found, and all BAG-labeled cells remained outside of the lesion area. We are unable to dismiss the possibility that the BAG-labeled cells were unable to migrate from the cingulum ventrally to the corpus callosum due to constraints imposed by the different orientation patterns of the axons in each of these structures (Ramon y Cajal, 1995); in transplantation studies, exogenous cells align along axons, rather than against them (Emmett et al., 1991; Andersson et al., 1993). However, we did not observe BAG-labeled cells lining up at the cingulum–corpus callosum border, as if they had migrated part of the way ventrally. Hence, we believe it is the progenitors resident to the lesion that effect remyelination, rather than those at the periphery.

A demyelinating insult that destroys all the resident progenitors, or repeated assaults to the same area with progressive depletion of a progenitor pool, therefore, would diminish the remyelinative capacity in that area. It would be useful to be able to induce migration of the cycling cells at the periphery of a lesion into a demyelinated zone that has suffered depletion of this population. Also, because the population of cycling cells endogenous to adult brain is small, expansion of their numbers prior to remyelination would also be of value.

We do not know as yet what specific stimuli induce these progenitors to differentiate into oligodendrocytes, although damage to the brain per se does not induce oligodendrocyte differentiation, since a stab wound will not produce such development (Gensert and Goldman, unpublished data). Determining specific mechanisms involved in proliferation and differentiation of this population of cells would provide important insights into repair processes in demyelinating diseases and possibly useful information for optimizing repair.

## Experimental Procedures

### Injection of Retrovirus and Lysolethicin-Induced Demyelination

To address whether immature cycling cells in adult subcortical white matter can be induced to differentiate along an oligodendrocyte pathway, a strategy combining stereotactic surgery with retrovirus technology was employed. Cycling cells were labeled with retrovirus (BAG, $10^2$ cfu) introduced stereotactically into female Sprague-Dawley rats (Harlan, Indianapolis, IN; $\sim$200–230 g). According to Harlan, female Sprague-Dawley rats in this body weight category are aged at 10–15 weeks, i.e., P70–P105 (Harlan International Rat Toxicology Models Profiles and Parameters, Volume II, p. 2; Harlan Sprague-Dawley Product Guide, July 1, 1995, p. 7). Retrovirus was introduced into the subcortical white matter (cingulum and corpus callosum) at the coordinates 1.1 mm posterior to Bregma, 1.0 mm lateral to Bregma, and 2.8 mm deep, through small holes drilled in the skull. These holes were filled with absorbable gelatin foam (Gelfoam; Upjohn, Kalamazoo, MI) before suturing. At 2 dpi, the animals were anesthetized, the sutures and Gelfoam were removed, and lesions were induced at the stereotactically defined locus with lysolecithin (1 $\mu$l of a 2% solution in Hanks' Balanced Salt Solution; Calbiochem, San Diego, CA and Gibco, Grand Island, NY, respectively). Following the lesion, the burr holes were again filled with Gelfoam, the wound

was sutured, and the animal was kept warm until recovery and subsequently returned to the animal facility. Animals were sacrificed 21 (n = 2) and 28 (n = 4) dpl.

To minimize any damage resulting from the injections, we used separate Hamilton syringes, each fitted with a pulled capillary pipet (Narishige Scientific Instrument Laboratory, Tokyo, Japan), for the deposition of BAG and of lysolecithin into the brain. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

### Immunohistochemistry and Xgal Histochemistry

Cycling cells that had incorporated into their genome the *lacZ* provided by the retrovirus were visualized using immunohistochemistry with anti-β-galactosidase antibodies or Xgal histochemistry. These were performed as previously described, with minor changes (Gensert and Goldman, 1996). The fixative for perfusion was 3% paraformaldehyde, 0.5% glutaraldehyde, and 2 mM $MgCl_2$ in 0.1 M PIPES (paraformaldehyde from Electron Microscopy Sciences, Fort Washington, PA; others from Sigma, St Louis, MO); for experiments involving osmication, the same fixative with 1% glutaraldehyde was used. The tissue was sectioned coronally at 25 μm using a Vibratome 1000. BAG-labeled cells were identified either with Xgal histochemistry (1 mg/ml, overnight, at 28°C; Sigma Chemical Company) or with anti-β-galactosidase antibodies (1/100, overnight, at 4°C; Promega), secondary antibodies directly conjugated to horseradish peroxidase (1/200, 2 hr, room temperature; Sigma Chemical Company), and subsequent use of diaminobenzidine (DAB, Sigma Chemical Company) for detection. MBP was detected immunohistochemically in the myelin of BAG-labeled cells with an anti-MBP antibody (1/100, overnight, at 4°C; DAKO Corporation, Carpintiria, CA) and the appropriate secondary antibody directly conjugated to horseradish peroxidase, as described above. Immunohistochemistry involving GFAP or Rip were performed as previously described (Gensert and Goldman, 1996). Osmication was performed in 1% osmium tetroxide (Electron Microscopy Sciences) for 15–30 min. Sections were viewed using an Olympus BX-60 microscope with epifluorescence optics.

### Migration of BAG-Labeled Cells toward a Lesion

To address whether immature cycling cells migrate into a lesion to effect remyelination, BAG was deposited at the coordinates noted above, except that it was deposited more superficially at a depth of 2.6 mm (rather than at 2.8 mm), and the lysolecithin-induced lesion, performed 2 dpi, was produced in the subcortical white matter ~1.5–2.0 mm lateral to the site of the BAG injection (see Figure 4). The lesion extended from this site along the corpus callosum, just ventral to the site of BAG deposition. Brains were processed as noted above.

### Titering BAG Retrovirus In Vitro

An in vitro assay was performed to determine the decay of BAG titer over time. NIH 3T3 cells were plated at a density of $3 \times 10^3$ cells/$cm^2$. At ~15 hr after plating, half the media of several wells (4–8 wells per time point) was replaced by media containing BAG retrovirus. The BAG stock ($10^5$ cfu/ml) was maintained at 37°C for the duration of each experiment. Aliquots of BAG were drawn from this stock to infect cells initially (time = 0), every 2 hr through the 12 hr time point, and every 12 hr thereafter through the 48 hr time point. Cultures were fixed and processed for Xgal histochemistry after 3 days, and clusters of labeled cells were counted. To examine whether the BAG titer at 48 hr was sufficient to label cells stimulated to divide subsequent to lesion, retrovirus stock was maintained at 37°C for 48 hr before stereotactic injection, and lysolecithin was introduced immediately following BAG deposition. Brains were analyzed 2–4 dpi.

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