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OLIGODENDROCYTE DEGENERATION AND RECOVERY AFTER FOCAL CEREBRAL ISCHEMIA

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Abstract

The vulnerability of oligodendrocytes to ischemic injury may contribute to functional loss in diseases of central white matter. Immunocytochemical methods to identify oligodendrocyte injury in experimental models rely on epitope availability, and fail to discriminate structural changes in oligodendrocyte morphology. We previously described the use of a lentiviral vector (LV) carrying eGFP under the myelin basic protein (MBP) promoter for selective visualization of oligodendrocyte cell bodies and processes. In this study, we used LV-MBP-eGFP to label oligodendrocytes in rat cerebral white matter prior to transient focal cerebral ischemia, and examined oligodendrocyte injury 24 hours, 48 hours and one week post-reperfusion by quantifying cell survival and assaying the integrity of myelin processes. There was progressive loss of GFP+ oligodendrocytes in ischemic white matter at 24 and 48 hrs. Surviving GFP+ cells had non-pyknotic nuclear morphology and were TUNEL-negative, but there was marked fragmentation of myelin processes as early as 24 hours after stroke. One week after stroke, we observed a restoration of GFP+ oligodendrocytes in ischemic white matter, reflected both by cell counts and by structural integrity of myelin processes. Proliferating cells were not the main source of GFP+ oligodendrocytes, as revealed by BrdU incorporation. These observations identify novel transient structural changes in oligodendrocyte cell bodies and myelinating processes, which may have consequences for white matter function after stroke.

Keywords

lentivirus transduction; white matter; oligodendrocyte progenitor; ischemia

Oligodendrocyte survival and preservation of myelin integrity is critical for normal axon function following white matter injury. There is growing evidence in disease models and in transgenic knock-out mice lacking expression of myelin proteins that primary dysfunction in oligodendrocytes can lead to secondary axonal injury (for review, see Kassmann and Nave,

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2008). The neurological impairments resulting from injury to these white matter components are pronounced in conditions as diverse as perinatal brain injury, multiple sclerosis, traumatic spinal cord injury, and stroke.

The vulnerability of oligodendrocytes to ischemic injury has been demonstrated in numerous *in vitro* models and is mediated by both oxidative and excitotoxic mechanisms (Matute et al., 2002, 2007; McDonald et al., 1998; Park et al., 2004; Stys, 2004). In ischemic acute brain and spinal cord slices, injury has been demonstrated by a loss of immunoreactivity to oligodendrocyte-specific markers and the appearance of pyknotic nuclei (Li and Stys, 2000; Tekkok and Goldberg, 2001). *In vitro* studies using white matter preparations from transgenic mice with oligodendrocyte-specific expression of fluorescent markers showed ischemic injury marked by decreased fluorescence intensity in myelin (Salter and Fern, 2005).

Evidence of ischemic white matter injury *in vivo* was demonstrated at early time points during focal ischemia, when ultrastructural changes included oligodendrocyte swelling, separation of myelin sheaths from axons, and axon swelling (Pantoni et al., 1996). In permanent and transient focal ischemia models, histopathology in ischemic white matter was indicated by a relative loss of immunoreactivity for oligodendrocyte and myelin proteins beginning as early as 24 hours after stroke (Irving et al., 2001; Valeriani et al., 2000).

In vitro ischemia models are amenable to pharmacological manipulations and are valuable for examining acute responses of oligodendrocytes to injury and protection, but may be an incomplete reflection of what happens *in vivo*, where vasculature and inflammatory responses are additional factors. *In vivo* studies have been limited by the availability of epitopes in injured tissue, and provide little information regarding structural changes that may occur differentially in the oligodendrocyte cell body versus the myelin processes. Structural changes in oligodendrocytes occurring in response to ischemia have not been well-defined in long-term survival studies.

Oligodendrocyte replacement and remyelination contribute to functional recovery in demyelinating diseases (Keirstead and Blakemore, 1999) and may be involved in initial recovery of function in a model of spinal cord injury (Rabchevsky et al., 2007). Hypoxic-ischemic injury in the neonatal brain stimulates generation of new oligodendrocytes in the infarct core and white matter (Zaidi et al., 2004). However, the potential for oligodendrocyte replacement in ischemic adult white matter has not been well-established. Determining the fate of white matter oligodendrocytes after focal ischemia in long-term survival studies may provide insight into putative endogenous repair mechanisms, such as those described in traumatic spinal cord injury (SCI) models (Rabchevsky et al., 2007; Tripathi and McTigue, 2007) and demyelination models (Gensert and Goldman, 1997).

We previously described a method for visualization of white matter oligodendrocytes using a lentiviral vector to selectively target GFP expression to oligodendrocytes via myelin basic protein (MBP) promoter-driven expression of GFP (McIver et al., 2005). In this study, we used LV-MBP-eGFP to label rat white matter oligodendrocytes prior to focal cerebral ischemia. With enhanced visualization of oligodendrocyte morphology via cytosolic GFP expression, we were able to more closely examine survival of and morphological changes in white matter oligodendrocytes occurring 24 hours, 48 hours and one week reperfusion after ischemia.

Experimental procedures

Lentiviral transduction in vivo

HIV-derived recombinant lentivirus carrying a 1.9 kb fragment of the myelin basic protein promoter (MBP; generously shared by Alex Gow) driving expression of cytosolic eGFP was constructed as previously described (McIver et al., 2005). Briefly, recombinant lentivirus was prepared by transient co-transfection of HEK 293T cells with the MBP-eGFP transfer vector and lentiviral helper plasmids (pMDLgpRRE, RSV-Rev and pMDG for VSV-G pseudotyping) using the calcium phosphate co-precipitation method (Dull et al., 1998; Zufferey et al., 1998; Lee et al., 2004). Medium was exchanged 24 hours later, and at 48 hours post-transfection lentivirus was harvested, filtered through a 0.45 µm filter and concentrated by ultracentrifugation (90 minutes at 25,000 rpm), and resuspended in Ringers solution. Infection of HEK 293 cells with the virus was used for titering by flow cytometry at 24 hours post-infection. Titers for preparations used here were 4.5×10^7 infectious units/ml. Virus suspensions were aliquotted and stored at -80°C until used.

Adult Sprague Dawley male rats (Charles Rivers Laboratories, Wilmington, Massachusetts) weighing 200–250 g were used for this study. Animal surgery and care were carried out in accordance with the Institute of Laboratory Animal Research (ILAR) Guide for the Care and Use of Laboratory Animals; all procedures were approved by the Animal Studies Committee at Washington University.

Briefly, rats were anesthetized with isoflurane (3% induction, 1.75% maintenance) while securely positioned in a stereotaxic frame (Stoelting, Wood Dale, Illinois). A midline scalp incision was made, and a Hamilton syringe was lowered into the left lateral corpus callosum through a small burr hole (+2.2 mm lateral; +0.5 mm posterior; -2.4 mm ventral to Bregma). LV-MBP-eGFP (4.5 µl) was delivered at a rate of 0.45 µl/minute via a nanoinjector pump (Stoelting), after which the needle was left in place for 5 minutes to ensure complete diffusion of the virus.

Surgical Procedures for Focal Cerebral Ischemia (MCAo)

Six days post-injection, rats underwent either sham surgery or transient focal ischemia via 60 minute middle cerebral artery occlusion (MCAo) with an intraluminal nylon suture, as previously described (Plesnila et al. 2001). Briefly, the left external carotid artery (ECA) was exposed and loosely ligated. A 25 mm 3-0 surgical nylon suture with a coated blunt end was introduced into the ECA stump and advanced 19–20 mm from the bifurcation of the common carotid artery (CCA) into the circle of Willis to occlude the MCA. The suture ligating the proximal ECA was tightened to prevent bleeding and to immobilize the intraluminal suture inserted into the MCA. Incisions were sutured and the animals were allowed to recover from anesthesia during the occlusion. After 60 minutes of MCAo, rats were re-anesthetized and ischemia was terminated by removing the intraluminal suture. A laser-Doppler probe was used to measure cerebral blood flow (CBF) at several time points to confirm ischemic conditions: CBF was measured directly above the MCA territory at the onset of occlusion and prior to reperfusion to confirm sustained reduced blood flow (<20% baseline CBF), and at the onset of reperfusion to confirm sufficient restoration of blood flow (>60% CBF). Animals not meeting these criteria were omitted from the study. Sham control animals were subjected to the same procedures, without the occlusion of the MCA with intraluminal suture.

Animals were assigned to the following groups, prior to MCAo: 24 hours reperfusion (n = 11 stroke, 7 sham); 48 hours reperfusion (n = 11 stroke, 7 sham); 1 week reperfusion (n = 12 stroke, sham). Mortality, typically from delayed incomplete reperfusion and/or hemorrhage, was equal among groups (n = 2–3 per stroke group).

To track the fate of proliferating cells, a separate set of animals (n = 4 stroke; 1 sham) received daily intraperitoneal injections of 50 mg/kg BrdU (Sigma-Aldrich; St. Louis, Missouri) daily following stroke, and animals were sacrificed one week post-reperfusion.

Tissue processing

Rats were sacrificed at each designated time point by intracardiac perfusion with PBS. Brains were extracted and coronally sectioned at 3 mm using a brain matrix. Sections were incubated in triphenyl tetrazolium chloride (TTC; Sigma-Aldrich) for 3 minutes at 37°C, fixed in 4% paraformaldehyde for 24 hours, and cryoprotected through sucrose gradients (10–30%) for at least 24 hours each. TTC-stained sections were scanned for documentation of infarct size, and 20 µm serial cryosections were collected (every 4th section) on 3 sets of slides. One set was used for Luxol Fast Blue (LFB) staining as follows: slides were dehydrated in 70, 80, and 95% ethanol (EtOH, 5 min each), incubated in 1% LFB (in 95% EtOH + 10% acetic acid) overnight at 60°C in a sealed container, rinsed in 95% EtOH, water (2 × 1 min), 0.05% lithium carbonate (1 min), 70% EtOH, and water. The last three steps were repeated until optimal contrast between white and gray matter regions was obtained.

The second set was used for nuclear counterstaining (Hoechst 33342; Invitrogen, Carlsbad, California) and quantification and morphological assessment of GFP⁺ oligodendrocytes. The third set was used for immunohistochemical detection of oligodendrocyte progenitor cells (OPCs; NG2) or for colocalization with cell proliferation (BrdU) or death (TUNEL) markers. Briefly, sections were rinsed with PBS (3 × 5 min) and blocked for 30 minutes using 2% normal goat serum (Sigma) and 0.1% Triton-X (Sigma) in PBS. Tissue was incubated overnight at 4°C in anti-rabbit NG2 (Millipore, Billerica, Massachusetts; 1:100) or anti-rabbit BrdU (1:1500; Megabase, New York) in blocking serum, rinsed with PBS (3 × 5 min), and incubated for 1–2 hours at room temperature in goat anti-rabbit Alexa-594 secondary antibody (Invitrogen; 1:1000). Detection of BrdU also required antigen retrieval with 10 minutes incubation in 2 N HCl at 37°C followed by 20 minutes incubation in 0.2 M boric acid solution, prior to the blocking step, a process which dampened GFP expression. For detection of cell death, *in situ* cell death TUNEL assay (Millipore) was used according to protocol.

All sections were treated with Hoechst 33342 nuclear stain (Invitrogen), coverslipped in Vectashield mounting media (Vector Labs, Burlingame, California), and visualized under laser scanning confocal (Zeiss, Maple Grove, Minnesota) microscopy. Digital color micrographs were further processed using Adobe Photoshop.

Data acquisition

Prior to data collection, an observer blind to experimental conditions identified the virus injection site for each animal based on increased cellularity and autofluorescence along the needle track, as previously reported (McIver et al., 2005). To assess adequacy of the virus infusion, GFP⁺ cells were examined in the periventricular white matter near the midline (an area not affected by stroke after MCAo). Animals with fewer than 100 GFP cells in the examined area were excluded from further data analysis (sham controls n=1, MCAo 24 hrs, n=2; 48 hrs, n=3; 1 week, n=2). Infarct volume determined by TTC was similar in included and excluded animals. The final number of animals included data analysis were: sham controls n=5 (24hrs); n=4 (48hrs); n=5 (1wk); and MCAo n=7 (24hrs); n=5 (48hrs); n=7 (1wk).

For each animal, 60 µm anterior and 120 µm and 180 µm posterior to the injection site, images of a pre-defined region of the ipsilateral white matter (marked ~3.5 mm from the

midline; delineated by the box in Figure 1) were collected with a 10x objective using confocal imaging software (Zeiss). These three sections were selected for analysis to ensure that all data were collected from white matter regions within the area targeted by the virus injection. Image files were opened with Metamorph Image Analysis (Molecular Devices) and GFP+ oligodendrocytes were counted manually and averaged. To account for the temporally regulated changes in transduction levels, the average counts of GFP+ oligodendrocytes in experimental animals were normalized as percent of sham controls (matched by survival time after virus injection). In this method of normalization, the number of GFP+ oligodendrocytes in sham controls is considered to be a representative sample of white matter oligodendrocytes.

Within the same pre-defined region of the lateral corpus callosum, but spanning 6 sections (2 anterior, 4 posterior to injection site), GFP+ cells were visualized at higher magnification for morphological analysis. GFP+ oligodendrocytes with cell bodies that resided completely within the z-plane were selected, and a 15.3 μm z-stack was collected with a 60x objective ($n = 10\text{--}14$ cells per animal). The stack was then flattened into a maximum intensity projection, exported into Adobe Photoshop, and processed to enhance the intensity and contrast of myelin processes (Figure 1B). The modified images were imported into Metamorph and the threshold was adjusted to reduce background pixilation while retaining a well-defined cell body. Two circle regions, with 20 μm and 35 μm radii, were positioned around the cell body. The processes were defined as intact if they maintained contiguous with cell bodies and extended to the proximal (20 μm) and distal (35 μm) circles. The number of cells analyzed were: 24 hrs: sham $n = 62$, MCAo $n = 89$; 48hrs: sham $n = 53$, MCAo $n = 69$; 1 week: sham $n = 66$ sham, MCAo $n = 89$. All cell and process counts were normalized as a percent of sham controls. Pairwise comparisons were performed using Two-way ANOVA and t-tests with Bonferroni correction to determine statistical significance.

Results

Examination of oligodendrocytes in rat white matter using LV-MBP-eGFP

We previously showed that injection of LV-MBP-eGFP in the corpus callosum of adult mice resulted in selective GFP expression in white matter oligodendrocytes (McIver et al, 2005). In this study, we used LV-MBP-eGFP to visualize oligodendrocytes in rat white matter. In uninjured, sham control brains, stereotaxic injections of LV-MBP-eGFP directed to the lateral corpus callosum resulted in widespread expression of GFP in oligodendrocytes throughout the ipsilateral white matter (Figure 1A), spreading approximately 1 mm anterior and 2 mm posterior to injection site (data not shown). Because a subset of oligodendrocytes express GFP, it was possible to visualize individual cell bodies and myelin processes using confocal imaging to capture the entire cell in 3-dimensions (Figure 1B). By tracing individual processes back to the cell body, we were able to quantify the number of intact proximal and distal processes (20 μm and 35 μm radii, respectively) for each oligodendrocyte (Figure 1C).

Focal ischemia induced by 60 minutes MCAo causes white matter injury

2,3,5-triphenyltetrazolium chloride (TTC), an indicator of mitochondrial viability, is commonly used for visualization of the infarct area, which is demarcated by a lack of staining (Hatfield, et al., 1991). In animals subjected to 60 minute MCAo, a substantial portion of the ipsilateral corpus callosum and external capsule was surrounded by infarcted gray matter at 24 and 48 hours reperfusion after ischemia (Figure 2). After one week reperfusion the infarct border was ill-defined, likely due to positive TTC staining of infiltrating cells that accumulate in ischemic tissue (Danton and Dietrich, 2003; Garcia et al., 1994). White matter remained unstained even in non-pathological tissue (see Figure 2,

contralateral hemisphere). To confirm ischemic white matter injury, we used Luxol Fast Blue (LFB) myelin staining in the same brain sections, since decreased LFB staining has been shown to be a sensitive indicator of myelin injury after ischemia (Irving et al., 2001). Relative to the contralateral side, there was decreased LFB staining in the ipsilateral hemisphere, which corresponded topographically to the TTC-defined infarct. Loss of LFB staining was detected in ipsilateral striatum and cortex, and in the lateral corpus callosum as early as 24 hours reperfusion. By 48 hours reperfusion, LFB staining in myelin was further disrupted, and by one week reperfusion LFB was substantially reduced (Figure 2). For each time point examined, direct comparison of TTC with LFB confirmed this model produced white matter infarcts. Furthermore, these regions of ischemic white matter partially overlapped with the distribution of GFP+ oligodendrocytes produced by stereotaxic injections of LV-MBP-eGFP, specifically in the lateral corpus callosum and external capsule (Figure 1A). This approach allowed for verification that the white matter analyzed fell within the ischemic territory, and that the area of analysis for quantification of oligodendrocyte injury fell near, yet outside, the ischemic core, where viability is more likely. Since epitope availability in injured tissue is a concern, and because the available oligodendrocyte-specific antibodies do not completely label both the cell bodies and the processes, we did not use immunostaining of oligodendrocytes as a secondary indicator. Instead, we assessed cell survival by normalizing the number of GFP+ oligodendrocytes in injured white matter to the number of GFP+ cells in time-matched sham controls, as a representative indicator of oligodendrocyte survival.

Ischemic injury in oligodendrocytes transduced with LV-MBP-eGFP

Oligodendrocyte loss and myelin degeneration occurs by 24–48 hours post-reperfusion—We used two independent indicators of cell death to measure ischemic white matter injury: the presence of TUNEL+ nuclei, and pyknotic nuclear morphology visualized as small round nuclei with intense Hoechst staining. By 24 hours after stroke, there was extensive cell death in the white matter as evidenced by the presence of TUNEL+ and pyknotic nuclei (Figure 3A). TUNEL+ cells were found in white matter near the TTC-indicated ischemic boundary (Figure 3Ai), and were more numerous in the lateral corpus callosum and external capsule (Figure 3Aii–iii), and within the infarct core. Throughout each of these regions, we found GFP+ oligodendrocytes with intact cell bodies and TUNEL-negative, non-pyknotic nuclei (Figure 3B–D), suggesting that some surviving GFP+ oligodendrocytes persisted in regions of significant cell death.

To determine time-dependent changes in oligodendrocyte viability in ischemic white matter, we counted surviving GFP+ oligodendrocytes within a region of the lateral corpus callosum (see Figure 1A). Within the same region, we assayed morphological injury in surviving GFP+ oligodendrocytes by quantifying intact processes that were contiguous with the cell body (refer to Figure 1B, C). The loss of GFP+ oligodendrocytes was not statistically significant at 24 hours after stroke, relative to sham controls (Figure 4A; $68.7 \pm 17\%$; $p = 0.24$; $n=5$ sham, 7 MCAo animals). High resolution examination of surviving oligodendrocyte morphology indicated a significant loss of intact processes proximal ($66.0 \pm 6.5\%$; $p < 0.001$) and distal ($59.2 \pm 7.6\%$; $p < 0.001$) to the cell body (Figure 4B; $n=62$ cells sham; $n=89$ cells MCAo). By 48 hours reperfusion after stroke, there were significantly fewer GFP+ oligodendrocytes compared to controls (Figure 4C; $49.7 \pm 8.0\%$; $p < 0.005$; $n=4$ sham, $n=5$ MCAo animals), as well as a significant loss of intact processes (Figure 4D; proximal $46.3 \pm 5.5\%$; and distal $33.8 \pm 6.5\%$; $p < 0.001\%$; $n=53$ cells sham; $n=69$ cells MCAo). Every GFP+ oligodendrocyte sampled in ischemic white matter at these time points ($n = 158$) had intact, non-pyknotic nuclei, indicating that these cells were viable (data not shown). In most cells sampled, remnants of GFP+ myelin processes were detected, as evidenced by GFP fragments scattered near the perimeter of the cell body (Figure 4B and

D). These results indicate that surviving GFP+ oligodendrocytes begin to show marked fragmentation of myelin processes both proximal and distal to the cell body as early as 24 hours post-reperfusion, and that significant oligodendrocyte loss occurs in ischemic white matter by 48 hours after ischemia.

Recovery of white matter oligodendrocytes occurs by one week post-reperfusion—We examined cell survival in white matter one week after reperfusion and observed numerous GFP+ oligodendrocytes throughout the corpus callosum and external capsule. In the region sampled for quantitative studies, GFP+ cell counts were not significantly different from sham controls ($79.7 \pm 12\%$, $p = 0.275$; $n=5$ sham, $n=7$ MCAo animals; Figure 5A). Furthermore, these oligodendrocytes exhibited intact proximal and distal processes, similar in number to those in sham white matter (Figure 5B; proximal $87.9 \pm 7.7\%$; distal $79.1 \pm 9.9\%$, $p = 0.1$; $n=66$ cells sham; $n=89$ cells MCAo). Many TUNEL+ nuclei were still present in regions of ipsilateral white matter, but they did not colocalize with GFP+ oligodendrocytes (data not shown). Furthermore, from 48 hours to one week post-reperfusion, there was a significant increase in the number of intact proximal (<0.05) and distal ($p<0.05$) processes of oligodendrocytes measured in ischemic white matter. Collectively, these data provide evidence for endogenous repair mechanisms of oligodendrocyte lineage cells after focal cerebral ischemia.

Response of NG2+ white matter oligodendrocyte progenitor cells after focal ischemia

Oligodendrocyte progenitor cells (OPCs) persist in adult CNS and are capable of differentiating into mature myelinating oligodendrocytes (Berry et al., 2002). Adult OPCs are marked by immunoreactivity to NG2 (Levine and Card, 1987; Nishiyama et al., 1996), which has been used to assess the response of OPCs to various types of white matter injury (for review, see Levine et al., 2001). To explore the response of adult OPCs relative to oligodendrocyte loss and restoration, we examined time-dependent changes in NG2 immunoreactivity in ischemic white matter. As previously reported (Levine et al., 2001), NG2 was expressed in OPCs throughout the cortex and striatum, in ependymal cells lining the lateral ventricle, and on the cell membranes of white matter OPC bodies and processes in the uninjured brain (Figure 6A). Relative to sham controls, by 48 hours after reperfusion NG2 immunoreactivity was substantially increased throughout the ipsilateral hemisphere, especially in the white matter (Figure 6B). These NG2+ cells were hypertrophic, with large cell bodies and retracted processes. By one week reperfusion, increased NG2 immunoreactivity persisted in ischemic white matter, where NG2+ cells remained hypertrophic but began to resume normal morphology, showing numerous ramified processes (Figure 6C). Confocal images of white matter NG2+ cells with GFP+ oligodendrocytes demonstrate that these were non-overlapping populations of cells (Figure 6, far right column).

To examine the possibility that reactive microglia or macrophages acquired immunoreactivity for NG2, as has been previously reported (Matsumoto, et al., 2008), we assessed colocalization of the monocyte lineage marker, Iba1, with NG2. Colocalization of Iba1 and NG2 was prominent in ischemic gray matter, but accounted for less than 20% of NG2+ cells residing in the white matter (data not shown). Collectively, these results highlight a distinct response of white matter OPCs to ischemic injury which may contribute to oligodendrocyte restoration following ischemia.

Increased immunoreactivity may be associated with either up-regulation of NG2, an increase in the number of OPCs, or both. Up-regulation of NG2 expression likely enhances detection of OPCs and may contribute to an apparent increase in the number of OPCs in ischemic

white matter. Nonetheless, these results suggest that focal ischemia induces increased NG2 immunoreactivity in white matter cells, and may also stimulate proliferation of NG2+ OPCs.

Proliferating cells are not the main source of GFP+ oligodendrocytes in post-ischemic white matter

In sham controls and in uninjured contralateral hemisphere, detection of BrdU incorporation was primarily limited to cells proliferating in the subventricular zone, as has been reported (data not shown; Jin and Glavan, 2007; Garcia-Verdugo et al., 1998). One week after ischemia, BrdU incorporated into numerous nuclei within ipsilateral white matter. Examination of the lateral corpus callosum, within the same region analyzed for cell survival, revealed that the majority of GFP+ oligodendrocytes were BrdU-negative. In ischemic white matter sampled from four animals, only 4.1 $\pm$ 2.7% of GFP+ cells were BrdU-positive (data not shown). Likewise, the vast majority of BrdU-positive cells were not labeled with GFP (Figure 7). Figure 7 shows a representative sample demonstrating numerous GFP+ oligodendrocytes among BrdU+ nuclei, with only one instance of colocalization. These results indicate that the restoration of GFP+ oligodendrocytes in white matter occurring between 48 hours and one week reperfusion is not completely attributable to differentiation of proliferating cells.

Discussion

Understanding mechanisms of ischemic injury in *in vivo* models of stroke is a requisite step towards designing therapeutic interventions. In human focal ischemia, infarcts often involve the white matter (Ransom et al, 2004), and thorough examination of cellular injury in white matter is critical in animal stroke models. Previous studies investigating ischemic white matter injury *in vivo* assessed injury based on relative changes in expression of oligodendrocyte- and myelin-specific markers (see Ness et al., 2005 for review), but these epitopes may be transiently lost or masked under pathological conditions. Even in healthy tissue, visualization with these antibodies is typically limited either to the cell body (e.g. CC-1) or to myelin (e.g. MBP, PLP), and may be insensitive to changes in oligodendrocyte morphology that occur in response to injury.

Transgenic mice that express oligodendrocyte-lineage-specific fluorescent reporters allow complete visualization of oligodendrocyte cell bodies and processes (Mallon et al., 2002; Murtie et al., 2007; Belachew et al., 2001), and studies using acute preparations from these mice have provided insight into ischemic oligodendrocyte injury (Salter and Fern, 2005). However, in the lines generated thus far, fluorescent reporters are not completely restricted to mature, myelinating oligodendrocytes, but can also be expressed in progenitor cells (Mallon et al., 2002; Yuan et al., 2002). Moreover, high expression levels in white matter tracts make it difficult to delineate individual myelin processes, and therefore limit measurements of injury to assays that are insensitive to subtle changes in morphology. Electron microscopy is highly sensitive to detection of cellular injury and has been used to describe ultrastructural changes in oligodendrocytes occurring early after ischemia (Pantoni et al., 1996). EM provides the morphological detail required for assessing subtle differences in cell viability, but ultrastructural quantification of injury requires repeated sampling over selective, small regions, which may obscure the relative proximity to the lesion. Moreover, most of these studies have been limited to measuring acute changes in ischemic white matter. Little is known regarding the time-dependent degenerative changes or the regenerative capacity of myelinating oligodendrocytes in *in vivo* ischemia models.

In this study, we used a lentiviral vector to spatially target GFP expression specifically to mature myelinating oligodendrocytes in rat white matter. By directing LV-MBP-eGFP injections to the lateral corpus callosum, we showed GFP expression in a subset of

oligodendrocytes, and used confocal microscopy to obtain 3-dimensional representations of myelin processes and respective cell bodies. We applied this technique to more closely examine the time course of white matter oligodendrocyte injury induced by transient MCAo, a commonly used *in vivo* stroke model. Using TTC and LFB staining, we confirmed that this model produces infarcts involving the white matter, as previously reported (Irving et al., 2001). TTC stained tissue was used to document the total infarct area, and then subsequently processed for visualization of white matter injury on the cellular level. This is important because MCAo can produce variable infarct sizes even when cerebral blood flow measurements are consistently reduced during the occlusion (Durukan and Tatlisumak et al., and observations). Here, the use of TTC allows us to confirm post-hoc whether the white matter region we sampled for quantification fell within ischemic boundaries.

We found a decreased number of GFP+ oligodendrocytes in the lateral corpus callosum beginning as early as 24–48 hours reperfusion after stroke, consistent with the time course of oligodendrocyte injury reported in other studies (Irving et al., 2001; Valeriani et al., 2000). Relative to the distribution of GFP+ oligodendrocytes observed in sham white matter, we also observed a time-dependent decrease in the number of surviving GFP+ oligodendrocytes along the medial-lateral extent of the corpus callosum, with almost complete absence in the external capsule by 48 hours after ischemia (data not shown; McIver et al., 2006). It is unlikely that decreased GFP expression was due to inactivity of the MBP promoter, as MBP transcription increases following focal ischemia (Gregersen et al., 2001). As indicated in Figure 3, some surviving GFP+ oligodendrocytes were detected in regions of ischemic cell death, residing near but not colocalizing with TUNEL+ nuclei, and in most cases GFP expression persisted in the remaining, albeit fragmented, processes. Given these findings, it is unlikely that the decrease in GFP+ cells quantified in this study results from down-regulated activity of the MBP promoter, or similarly, decreased transcription. Widespread necrosis in the striatum and cortex is a defining feature of the infarct core in this stroke model (Belayev et al., 1996), so it is likely that GFP+ oligodendrocytes undergo cell death, resulting in loss or degradation of GFP. Tissue within the infarct core undergoing pan-necrosis is not the main target for neuroprotection or subsequent repair. Here, we aimed to examine oligodendrocytes closer to the infarct boundary, where there may be greater potential for recovery. Because variability in infarct size is an intrinsic feature of this experimental model, we cannot rule out the possibility that some cells assayed in this study fell within the infarct core. Use of experimental stroke models that produce well-defined infarcts limited to white matter may be useful for future studies characterizing injury and recovery in peri-infarct regions (e.g. Sozmen et al., 2009).

Surviving GFP+ oligodendrocytes exhibited significant fragmentation of myelin processes at 24 and 48 hours post-reperfusion, but retained viable cell bodies, as evidenced by TUNEL-negative, non-pyknotic nuclei (Figure 3, data not shown). These results indicate that degeneration of myelin processes can precede oligodendrocyte cell death. This is a compelling prospect in light of evidence showing differential localization of unique glutamate receptor subunits on myelin processes (Karadottir et al., 2005; Micu et al., 2006; Salter and Fern, 2005). Myelinating processes are integrally associated with underlying axons and therefore may receive signals released both extracellularly and from axon fibers, so it would not be surprising if molecular pathways mediating myelin injury differ from those in the cell body (Stys, 2004).

Preservation of the cell body and nucleus during myelin degeneration presents the intriguing possibility that remyelination occurs by structural recovery of previously injured oligodendrocytes. By one week reperfusion after focal ischemia, we observed recovery in both the number of intact processes and in the number of GFP+ oligodendrocytes in a region of ischemic white matter. Unlike the structural fragmentation of processes observed 24–48

hours after ischemia, by one week the white matter was populated by GFP+ oligodendrocytes with intact process numbers similar to sham controls, and significantly greater than that measured 48 hours after ischemia.

In addition to restoration of oligodendrocyte structure there was also recovery of GFP+ cell numbers in ischemic white matter. The repopulation of GFP+ oligodendrocytes visualized in this study was most likely derived from cells already resident in white matter, rather than cells migrating in from gray matter areas. Using a non-cell-selective promoter, we previously demonstrated a tropism of this lentiviral vector for white matter glial cells when injected into the corpus callosum (McIver et al., 2005).

Dividing cells are not the only source of GFP+ oligodendrocytes in post-ischemic white matter

Using BrdU incorporation, we tracked the response of cells proliferating during one week post-reperfusion to determine whether they differentiated into GFP+ oligodendrocytes. Here, we found numerous BrdU+ cells in the same region of ischemic white matter where increased GFP+ oligodendrocytes were found. Of many GFP+ oligodendrocytes, less than 5% were BrdU-positive. Furthermore, colocalization with the oligodendrocyte cell marker, CC1, revealed that only 11% of oligodendrocytes residing in ischemic white matter were labeled with BrdU (data not shown). Given these findings, it is unlikely that dividing cells account for the full extent of oligodendrocyte recovery described in this study. The bioavailability of BrdU is limited (Kriss and Revesz, 1961), so it is possible that our data underestimate the number of GFP+ oligodendrocytes generated from proliferating cells. However, BrdU+/CC1+ oligodendrocytes were detected within one week after spinal cord injury using a similar dosing regimen (Tripathi and McTigue, 2007). Even a single pulse of BrdU, given 24 hours after spinal cord demyelination, was sufficient to track newly generated oligodendrocytes one week later (Watanabe et al., 2002).

OPCs are a likely source of newly generated GFP+ oligodendrocytes

It is now widely accepted that adult OPCs are responsible for remyelination after injury. In spinal cord injury and demyelination models, it has been well documented that OPCs respond to oligodendrocyte loss by proliferating and differentiating into myelinating oligodendrocytes (for review, see Levine, 2001). For example, in spinal contusion models oligodendrocyte loss was concomitant with increased NG2 expression and cell proliferation early after injury, and was followed by an increase in oligodendrocytes within weeks post-injury (Horky et al., 2006; Lytle and Wrathall, 2007; Rabchevsky et al., 2007; Tripathi and McTigue, 2007; Zai and Wrathall, 2005). In these studies, oligodendrocyte replacement was prominent in gray and white matter regions bordering the lesion, and was associated with functional recovery. Similarly, in a demyelination model, retroviral labeling of endogenously proliferating cells demonstrated that resident NG2+ progenitors contribute to remyelination (Gensert and Goldman, 1997).

Little attention has been given to the response of NG2+ progenitor cells and oligodendrocyte replacement after ischemic injury in adult brain, and existing studies have been primarily limited to examination of cellular changes in the gray matter (Tanaka et al., 2001, 2003; Lee et al., 2003; Mandai et al., 1997), where myelination is minimal compared to white matter. Nonetheless, these studies showed a time-dependent increase in NG2 immunoreactivity, coincident with restoration of oligodendrocytes and myelin in peri-infarct cortex by 1–2 weeks reperfusion (Tanaka et al, 2001 and 2003). Within the infarct core, however, there was sustained loss of NG2+ OPCs, oligodendrocytes and myelin. These results are in contrast to our findings of NG2 expression in ischemic white matter and may be associated with reported differences in the physiological properties of gray versus white matter OPCs

(Dimou et al., 2008). We found an increase in NG2 immunoreactivity throughout ischemic white matter, which was sustained by 1 week reperfusion. At 48 hours post-reperfusion, NG2+ cells appeared hypertrophic with retracted processes, which may reflect mitotic morphology. By 1 week post-reperfusion, NG2 cells resumed a normal morphology with ramified processes, and co-resided with GFP+ oligodendrocytes. The marked increase in NG2 immunoreactivity observed here, especially near the infarct core, could reflect the known contribution of these cells to the formation of the glial scar, as has been previously suggested (Badan et al., 2003; Popa-Wagner et al., 2007). Proliferating NG2+ cells did not account for a substantial proportion of BrdU+ cells, as only 8% of BrdU+ cells were NG2+ one week after ischemia (not shown). Given these findings, it is likely that the majority of restored GFP+ cells examined in our study were generated from non-dividing OPCs that were infected with LV-MBP-eGFP (since LV alone is not cell-specific), and latently expressed GFP via differentiation-dependent utilization of the MBP promoter.

Potential sources for BrdU+ cells in post-ischemic white matter

In models of spinal cord injury and demyelination, sources of cell replacement and factors mediating proliferation and differentiation may differ from those in cerebral white matter. For example, in the brain the subventricular zone (SVZ) is a local source of actively dividing cells. Neural stem cells in the SVZ respond to ischemic injury by increasing in proliferation rate and decreasing cell cycle length (Zhang et al., 2007). Similarly, we found increased numbers of BrdU+ cells in the SVZ in stroke animals, compared to sham (data not shown). Since there is evidence that SVZ cells contribute to neurogenesis in peri-infarct cortical areas via migration through the corpus callosum (Ohab et al., 2006), it is possible that some of the BrdU+ cells we found in ischemic white matter were neurogenic. Microglia residing in the SVZ and striatum also proliferate after stroke and may play a supportive role in neurogenesis (Thored et al., 2009). Though we did not closely examine the proliferation of microglia in ischemic white matter, we did observe an increase in immunoreactivity for the microglia marker, Iba1 (data not shown). The capacity of SVZ-derived cells to generate oligodendrocytes has been demonstrated in demyelinating lesions in the corpus callosum (Menn et al., 2006; Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002), but has not yet been reported after ischemia.

Conclusion

Recovery of function commonly occurs in cases of human stroke (Jorgensen et al., 1999), and in animal models of stroke it is associated with axon sprouting (Carmichael, 2006). It will be valuable to further elucidate endogenous pathways leading to oligodendrocyte repair after ischemic demyelination, since these mechanisms may also contribute to functional recovery of white matter after stroke.

In our examination of oligodendrocyte survival and morphology after ischemia, we provide evidence supporting the regenerative capacity of white matter oligodendrocyte lineage cells, reflected by restoration of cell numbers and intact myelin processes. Lentiviral gene delivery not only enhances visualization of morphological changes of oligodendrocytes in response to injury, but also provides an effective method to assess potential protective effects of novel transgene expression. This may be a valuable approach for the many diseases in which remyelination after injury is a therapeutic goal.

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Abbreviations

BrdU	Bromodeoxyuridine
CBF	cerebral blood flow
CNS	central nervous system
eGFP	enhanced green fluorescent protein
LFB	Luxol Fast Blue
LV	lentiviral vector
MBP	myelin basic protein
MCAo	Middle Cerebral Artery occlusion
OPC	oligodendrocyte progenitor cells
SVZ	subventricular zone
TTC	triphenyl tetrazolium chloride
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Figure 1. Visualizing white matter oligodendrocyte morphology with lentiviral vector LV-MBP-eGFP

One week after stereotaxic microinjection in rat lateral corpus callosum, fluorescence image (A) of the ipsilateral hemisphere shows GFP-expressing oligodendrocytes distributed throughout the corpus callosum and external capsule. The region for cell counts in lateral corpus callosum is indicated by the white box. Dashed line shows lateral ventricle. Morphology of an individual GFP+ oligodendrocyte, demonstrated in maximum-intensity image projection of 15 μm stack (B). Method of quantification of proximal and distal myelinating processes after threshold selection (C). Circles show 20 and 35 μm distances from cell body. Scale bars: 400 μm in A; 20 μm in B and C.

Figure 2. Time course of white matter injury following 60 min transient middle cerebral artery occlusion (tMCAo)

Brightfield images show infarct delineation by absence of TTC and Luxol Fast Blue (LFB) staining at 24 hrs, 48 hrs, and 1 week after stroke. The TTC-identified infarct region is less distinct at 1 week, likely due to infiltration of inflammatory cells. Right columns show LFB histochemistry at 10x from contralateral or ipsilateral regions of lateral corpus callosum.

Figure 3. Oligodendrocyte injury 24 hrs after tMCAo

TTC-stained brain section shows infarct area and white matter regions for distribution of GFP+ (green) and TUNEL+ (red) cells (A). TUNEL+ nuclei reside near GFP+ oligodendrocytes and are increasingly distributed along the medial (i) and lateral (ii) corpus callosum and external capsule (iii). GFP+ oligodendrocytes are not TUNEL+. Higher magnification (B) of external capsule (from dashed box in iii) shows surviving GFP+, TUNEL- oligodendrocytes with fragmented and swollen myelinating processes. Images of GFP only (C) show fragmented oligodendrocyte processes (arrowhead), and nuclear stain (D; Hoechst 33342) show non-pyknotic nuclei of GFP+ cells (arrows). Scale bars: 20 μ m.

Figure 4. Loss of GFP+ oligodendrocytes and myelin processes after focal ischemia

Confocal fluorescence images (A–D) show GFP+ cells in lateral corpus callosum from sham controls (left) and time-matched ischemic rats (right) at 24 hrs (A, B) and 48 hrs (C, D) after MCAo. A and C are 10x images used for cell counts (24hrs: sham n=5, MCAo n=7 animals; 48hrs: sham n=4, MCAo n=5 animals; counts are averaged across three sections, and expressed as a percent of the sham control). B and D are 60x images used for oligodendrocyte process counts (24hrs: sham n=62 cells, MCAo n=89 cells; 48hrs: sham n=53 cells, MCAo n=69 cells). TTC insets show regions selected for quantitative analysis, shown in bar graphs at right. Compared to sham controls, loss of GFP+ oligodendrocytes in ischemic white matter was observed at 24 (A) and 48 hours (C) after ischemia ($p < 0.001$, at

48 hrs). Loss of intact myelin processes occurred as early as 24 hours after ischemia (B), with only ~50% remaining intact by 48 hours, relative to sham controls (D; $p < 0.001$; asterisk, statistically significant difference based on t-test with Bonferroni correction,).

Figure 5. Restoration of GFP+ oligodendrocytes in white matter one week after ischemia
 Confocal fluorescence images (A 10x, B 60x) show GFP+ cells in lateral corpus callosum from sham controls (left; n=5 animals; n=66 cells) and time-matched ischemic rats (right; n=7 animals; n= 89 cells) at 1 week after MCAo. Statistical analysis using t-test with Bonferroni correction shows no significant difference between sham and stroke groups for counts of GFP+ cells (A; averaged counts across three sections, and expressed as a percent of the sham control) or oligodendrocyte processes (B).

Figure 6. Increased NG2 immunoreactivity in ischemic white matter

Confocal images show changes in NG2 immunoreactivity relative to sham controls (A) at 48 hrs (B) and 1 week (C) after stroke, in the ipsilateral hemisphere (A, B, C; 10x montages; scale bar = 1mm), lateral corpus callosum and external capsule (i; scale bar = 500µm). Maximum intensity projections from a 15µm z-stack show NG2 expression (ii, iii; red) and GFP+ cells (iii; green) in the lateral corpus callosum (scale bar = 50µm). Increased gain was required for detection of hemispheric NG2 immunoreactivity at low magnification in sham controls (A), which revealed weak NG2 expression in the white matter (A-i), where NG2 was expressed on OPC processes (A-ii, iii) near GFP+ cells (A-iii). At 48 hrs after ischemia there was an overall increase in NG2 immunoreactivity (B), most strikingly in the white matter (B-i), where NG2+ cells had retracted processes and large cell bodies (B-ii), but remained distinct from the few surviving GFP+ oligodendrocytes (B-iii). One week after ischemia (C), increased NG2 immunoreactivity persisted in the white matter (C-i), but NG2+ cells begin to exhibit typical morphology with numerous ramified processes (C-ii), in areas with repopulated GFP+ oligodendrocytes (C-iii).

Figure 7. BrdU-incorporation and colocalization with GFP+ oligodendrocytes one week after ischemia

Confocal imaging of the lateral corpus callosum (A) revealed numerous BrdU+ nuclei (red) and GFP+ oligodendrocytes (green), with only one instance of colocalization (arrow indicates BrdU+ nuclei (B) colocalized to GFP+ cell (C)). Scale bars = 50 μ m.