Two-photon imaging of remyelination of spinal cord axons by engrafted neural precursor cells in a viral model of multiple sclerosis

Authors
Greenberg, M. L
Weinger, J. G
Matheu, M. P
et al.

Publication Date
2014-05-19

DOI
10.1073/pnas.1406658111

License
CC BY 4.0

Peer reviewed
Two-photon imaging of remyelination of spinal cord axons by engrafted neural precursor cells in a viral model of multiple sclerosis

Milton L. Greenberg,a,b,1 Jason G. Weinger,c,d,1 Melanie P. Mathieu,a,b,2 Kevin S. Carbajal,c,d,3 Ian Parke,e,a,b, Wendy B. Macklin,1 Thomas E. Lane,b,c,d,4,5, and Michael D. Cahalan8,a,b,5

a Department of Physiology and Biophysics, b Institute for Immunology, c Department of Molecular Biology and Biochemistry, d Sue and Bill Gross Stem Cell Center, e Department of Neurobiology and Behavior, and f Multiple Sclerosis Research Center, University of California, Irvine, CA 92697; and g Department of Cell and Developmental Biology, University of Colorado School of Medicine, Aurora, CO 80045

Contributed by Michael D. Cahalan, April 18, 2014 (sent for review February 18, 2014)

Neural precursor cells (NPCs) offer a promising approach for treating demyelinating diseases. However, the cellular dynamics that underlie transplanted NPC-mediated remyelination have not been described. Using two-photon imaging of a newly developed ventral spinal cord preparation and a viral model of demyelination, we describe the motility and intercellular interactions of transplanted mouse NPCs expressing green fluorescent protein (GFP) with damaged axons expressing yellow fluorescent protein (YFP). Our findings reveal focal axonal degeneration that occurs in the ventral side of the spinal cord within 1 wk following intracranial instillation with the neurotropic JHM strain of mouse hepatitis virus (JHMV). Axonal damage precedes extensive demyelination and is characterized by swelling along the length of the axon, loss of YFP signal, and transected appearance. NPCs engrafted into spinal cords of JHMV-infected mice exhibited diminished migration velocities and increased proliferation compared with transplanted cells in noninfected mice. NPCs preferentially accumulated within areas of axonal damage, initiated direct contact with axons, and subsequently expressed the myelin proteolipid protein gene, initiating remyelination. These findings indicate that NPCs transplanted into an inflammatory demyelinating microenvironment participate directly in therapeutic outcome through the wrapping of myelin around damaged neurons.

Significance

Stem cell transplantation has emerged as a promising cell-based therapy for the treatment of demyelinating diseases such as multiple sclerosis (MS). This study provides the first real-time imaging of transplanted stem cell-mediated remyelination in a mouse model of MS. Whereas current treatments solely delay disease progression, transplanted stem cells actively reverse clinical disease in animal models. Using two-photon microscopy and viral-induced demyelination, we describe a technique to visualize cellular migration and remyelination in the mouse spinal cord. Transplanted neural precursor cells physically wrap damaged axons with newly formed myelin, preserving axonal health.

www.pnas.org/cgi/doi/10.1073/pnas.1406658111

PNAS Published online May 19, 2014 E2349–E2355
dorsal spinal cord during demyelinating disease progression (19–23). However, because engrafted NPCs preferentially migrate to regions deep within the ventral spinal cord (24), standard dorsal-side 2P in vivo imaging techniques are not suitable for visualization deep in the ventral side. Using a ventral-side imaging preparation, we now demonstrate that NPCs transplanted into the spinal cords of JHMV-infected mice under pathologic conditions migrate directionally, take up residence in regions of axonal degradation, colocalize with damaged axons, and facilitate remyelination through direct interactions with axons.

Results

Live-Cell Imaging of Axonal Degeneration in the Ventral Spinal Cord.

To establish a system for ex vivo imaging in the ventral side of the murine spinal cord, we isolated the spinal cord from thoracic vertebra 4 to lumbar vertebra 2. The explanted spinal cord was embedded in a 5% agarose gel to maintain spinal cord integrity during superfusion with oxygenated medium (Fig. 1A) and was subsequently imaged by 2P microscopy. We used transgenic Thy1-YFP mice, which express yellow fluorescent protein (YFP) in a subset of medium- to large-caliber axons (25, 26), to study axonal pathology in the ventral spinal cord during the course of demyelination following JHMV infection. Lesions in the ventral spinal cord contained axons displaying a spectrum of “focal axonal degeneration” (FAD) (an established scale of axonal damage (20)) morphologies (Fig. 1B). Long, continuous axons with no damage are defined as “FAD stage 0”; FAD stage 1 axons contain focal swellings progressing along the length of the axon; and FAD stage 2 axons have gaps separating areas of YFP fluorescence. Analysis of FAD at various time points following JHMV infection revealed a progression of axonal pathology (Fig. 1C), similar to studies examining FAD in the dorsal spinal cord following myelin oligodendrocyte glycoprotein (MOG) immunization (20). Whereas damaged axons were not observed in the noninfected mouse (Fig. 1D), rapid FAD progression was readily observed 7 d following JHMV infection, confirming that FAD was a result of JHMV infection (Fig. 1E and Movie S1).

Whereas FAD has been shown to be associated with mitochondrial and myelin damage (20), it is unknown whether FAD stage 2 axes are completely transected. To determine the extent of axonal transection in axons that had lost YFP signal, JHMV-infected spinal cord sections were examined for axonal damage by immunofluorescence microscopy of downstream SMI-32 staining, which detects a nonphosphorylated epitope in neurofilament H (27). In areas where the axonal YFP signal was markedly diminished and fractured, SMI-32 staining appeared punctate or absent, indicating severely damaged axons (Fig. 2A). We observed various stages of axonal damage along the length of a single axon (Fig. 2B), including areas of intact YFP signal without SMI-32 (intact healthy axon), YFP signal concomitant with SMI-32 (intact but damaged axon), no YFP or SMI-32 signal (transected area of axon without continuous neurofilament), and SMI-32 without YFP signal (intact but damaged axon). The latter case indicates that although a loss of YFP fluorescence correlates with varying degrees of axonal damage, it does not necessarily indicate complete axonal transection. We conclude that loss of YFP signal correlates with varying degrees of axonal damage, but not necessarily an irrevocably transected axon. Whereas both FAD 1 and FAD 2 axons had areas with axonal damage, axons exhibiting loss of both YFP and SMI32 at multiple loci were more common in FAD 2 axons (Fig. 2C). To verify that axonal regions devoid of both YFP and SMI-32 signal are completely transected, we performed immunohistochemical staining for SMI-31, an accepted marker for undamaged axonal regions (phosphorylated neurofilament H) (28). We were unable to detect SMI-31 without YFP signal along YFP-expressing axons (Fig. 2D). These findings support the conclusion that YFP SMI-32+ axonal regions are transected.

Live-Cell Imaging of NPC Dynamics in the Ventral Spinal Cord.

To observe engrafted NPC behavior, GFP-NPCs were transplant- ed intraspinally at thoracic vertebra 10 (5, 24, 29) and were monitored ex vivo under 2P excitation. We first confirmed that GFP-fluorescent cells were indeed NPCs and that the fluorescence did not arise from other cells that may have phagocytosed GFP-NPCs. Spinal cord slices were stained for the ionized calcium-binding adaptor molecule 1 (Iba-1), a marker of activated macrophages and microglia (30). Despite a high number of activated macrophages and microglia in the spinal cord 3 wk following transplant, GFP fluorescence did not overlap with Iba-1 staining (Fig. 3A), demonstrating that the GFP fluorescence observed in the spinal cord was not due to engulfed NPCs and this was consistent with earlier studies (5).

At day 1 following GFP-NPC transplantation into a noninfected mouse, clusters of cells had limited motility at the site of transplant (Fig. S1A). In control noninfected mice, GFP-NPCs moved with an average velocity of 1.9 ± 0.1 μm·min⁻¹ (n = 5). NPC motility characteristics were altered in the JHMV-infected demyelinated spinal cord, where GFP-NPCs moved with a lower average velocity of 0.9 ± 0.05 μm·min⁻¹ at the transplant site (n = 6) (Fig. S1B). In contrast to the limited motility observed at the injection site, GFP-NPCs located 300 μm or farther from the transplant site were observed to move more rapidly in both infected and noninfected mice. (Fig. 3B and Movie S2). At day 1 posttransplant, GFP-NPCs were observed distal to the transplant site in three of five noninfected and three of six JHMV-infected mice. GFP-NPCs >300 μm from the transplant site had an average velocity of 5.0 ± 0.7 μm·min⁻¹ in the JHMV-infected spinal cord, compared with 9.3 ± 0.6 μm·min⁻¹ in the noninfected spinal cord (Fig. 3C). We applied directional persistence analysis (31) to further characterize NPC migration to describe the direction of cell movement over time; a value of 1 represents migration along a straight line without turning, 0 represents neutral
movement, and −1 represents migration directly toward the track origin (Methods). The directional persistence of transplanted NPCs was significantly lower in the JHMV-infected spinal cord, compared with the noninfected spinal cord (Fig. 3C).

When examined 14 d after transplant, GFP-NPCs were observed in dense clusters in the ventral area of the thoracic region of both the noninfected and the JHMV-infected spinal cord. A subset of GFP-NPCs expressed Ki-67, a nuclear marker of cellular proliferation (32), indicating proliferation occurred 2 wk after transplant (Fig. 4A). Proliferation was significantly increased within the JHMV-infected spinal cord, in which 55.7 ± 6.8% of DAPI+ GFP-NPCs expressed Ki-67, compared with 14.4 ± 2.2% in the noninfected spinal cord (Fig. 4B). Two-photon imaging revealed that GFP-NPCs exhibit dynamic morphologies in the ventral spinal cord. Cells were observed to proliferate, with large (~10 μm) “buds” protruding off clusters of GFP-NPCs (Fig. 4C and Movie S3), and GFP-NPCs actively extended and retracted processes in both the noninfected and the JHMV-infected spinal cord (Fig. S2 and Movie S4).

**GFP-NPCs Preferentially Colocalize with Damaged Axons.** One week following NPC transplantation into the JHMV-infected Thy1-YFP mouse, GFP-NPCs closely associated with stage 1 and stage 2 FAD axons (Fig. 5A and B). Large clusters of GFP-NPCs preferentially established residence in regions with extensive axonal damage as determined by the FAD index (Fig. 5C). Following migration to FAD lesions, GFP-NPCs initiated intercellular interactions with stage 1 and stage 2 FAD axons (Fig. 5D and Movie S5), gathering YFP fluorescent segments of stage 2 axons together (Fig. 5E and Movie S6). Because loss of YFP signal does not necessarily indicate a transected axon, GFP-NPC contact with an axon may alter YFP fluorescence within intact FAD axons. Therefore, we examined GFP-NPCs that transiently contacted Thy1-YFP axons at 2 wk posttransplant. Axonal pathology has been associated with a decrease in YFP fluorescence (20), suggesting that NPC interactions with FAD axons may stabilize axons. In transient interactions, as the colocalization of an NPC and an axon diminished, YFP fluorescence rapidly decreased in the axon, changing the axon from FAD 1 to FAD 2 (Fig. 6A–C and Movie S7). Analysis of multiple GFP-NPCs revealed that the migration of NPCs away from damaged axons was strongly correlated with the loss of axonal YFP fluorescence (Fig. 6D).

**Engrafted GFP-NPCs Remyelinate Axons.** To determine whether transplanted NPCs produce myelin following interactions with damaged axons, we cultured NPC neurospheres isolated from mice that express GFP under the control of the myelin proteolipid protein promoter (PLP-GFP) (33). Two weeks following transfer into a JHMV-infected spinal cord, NPCs formed stable interactions with damaged axons and expressed GFP, indicating that transplanted NPCs differentiated into myelinating cells and expressed myelin genes (Fig. 7A). In these cells, GFP fills the cytoplasm of the cell body, revealing that differentiated NPCs wrapped around damaged axons (Movie S8), with the GFP fluorescence extending around the length of the axon (Fig. 7B). Correspondingly, YFP fluorescence increased (Fig. 7C), indicating increased axonal health based on earlier reports correlating axonal health/integrity with YFP expression (20). Furthermore, we found GFP-NPCs wrapped around YFP+ axons and myelin basic protein (MBP)

---

*Fig. 2. Loss of YFP signal correlates with axonal damage in both intact and transected axonal regions. (A) Spinal cord section from a Thy1-YFP mouse imaged 23 d p.i. by immunofluorescence microscopy, colabeled with α-SMI-32 (red) to show damaged axons. White asterisks indicate lesions with multiple SMI-32+ damaged axons. (B) Loss of YFP signal does not necessarily indicate axonal transection. Fluorescence image shows axonal regions without damage (yellow arrow, YFP+SMI-32+) and damaged axonal regions without YFP signal (red arrow, YFP+SMI-32+). White asterisk represents a lesion with multiple SMI-32+ axons. (C) Example of complete axonal transections (white arrowheads, YFP+SMI-32+) in an axon with intact damaged regions with YFP signal (white arrows, YFP+SMI-32+). (D) Fluorescence image shows YFP+ axonal regions without damage (white arrow, YFP+SMI-31+), damaged axonal regions with YFP signal (yellow arrow, YFP+SMI-31+), and damaged/transected axonal regions without YFP signal (white arrowhead, YFP+SMI-31+). (Scale bars, 20 μm.)

*Fig. 3. GFP-NPC motility in the spinal cord 1 d posttransfer. (A) Immunohistochemistry illustrating localization of mouse GFP-NPCs (green) in the spinal cord of a C57BL/6 mouse 3 wk posttransfer. Activated microglia and macrophages labeled with anti-ionized calcium-binding adapter molecule 1 (Iba-1) are shown in red, overlaid with DAPI-stained nuclei (blue). (B) Time-lapse two-photon image in an explanted noninfected C57BL/6 spinal cord, showing transferred GFP-NPC (green) motility distal from the transplant site 1 d posttransfer. Panels depict cell positions at the times indicated (min), together with superimposed tracks showing movement beginning at 0:00 (Movie S2). (Scale bars, 40 μm.) (C) Distribution of instantaneous GFP-NPC cellular velocities in the spinal cord of a C57BL/6 mouse 1 d posttransfer. Overall mean is indicated in red (P < 0.0001). (D) Scatter plot of GFP-NPC directional persistence (dp) in the spinal cord (noninfected, dp = 0.62 ± 0.03; JHMV-infected, dp = 0.48 ± 0.04; P = 0.027).

*Fig. 5. GFP-NPCs Preferentially Colocalize with Damaged Axons. One week following NPC transplantation into the JHMV-infected Thy1-YFP mouse, GFP-NPCs closely associated with stage 1 and stage 2 FAD axons (Fig. 5A and B). Large clusters of GFP-NPCs preferentially established residence in regions with extensive axonal damage as determined by the FAD index (Fig. 5C). Following migration to FAD lesions, GFP-NPCs initiated intercellular interactions with stage 1 and stage 2 FAD axons (Fig. 5D and Movie S5), gathering YFP fluorescent segments of stage 2 axons together (Fig. 5E and Movie S6). Because loss of YFP signal does not necessarily indicate a transected axon, GFP-NPC contact with an axon may alter YFP fluorescence within intact FAD axons. Therefore, we examined GFP-NPCs that transiently contacted Thy1-YFP axons at 2 wk posttransplant. Axonal pathology has been associated with a decrease in YFP fluorescence (20), suggesting that NPC interactions with FAD axons may stabilize axons. In transient interactions, as the colocalization of an NPC and an axon diminished, YFP fluorescence rapidly decreased in the axon, changing the axon from FAD 1 to FAD 2 (Fig. 6A–C and Movie S7). Analysis of multiple GFP-NPCs revealed that the migration of NPCs away from damaged axons was strongly correlated with the loss of axonal YFP fluorescence (Fig. 6D).

**Engrafted GFP-NPCs Remyelinate Axons.** To determine whether transplanted NPCs produce myelin following interactions with damaged axons, we cultured NPC neurospheres isolated from mice that express GFP under the control of the myelin proteolipid protein promoter (PLP-GFP) (33). Two weeks following transfer into a JHMV-infected spinal cord, NPCs formed stable interactions with damaged axons and expressed GFP, indicating that transplanted NPCs differentiated into myelinating cells and expressed myelin genes (Fig. 7A). In these cells, GFP fills the cytoplasm of the cell body, revealing that differentiated NPCs wrapped around damaged axons (Movie S8), with the GFP fluorescence extending around the length of the axon (Fig. 7B). Correspondingly, YFP fluorescence increased (Fig. 7C), indicating increased axonal health based on earlier reports correlating axonal health/integrity with YFP expression (20). Furthermore, we found GFP-NPCs wrapped around YFP+ axons and myelin basic protein (MBP)
Axonal damage in MS is considered a secondary event that occurs following myelin loss in response to accumulation of myelin-reactive lymphocytes within the CNS and this is supported by experimental autoimmune encephalomyelitis (EAE), an autoimmune model of neuroinflammation and demyelination. More recently, through use of viral models of demyelination as well as EAE, emerging evidence supports the possibility that axonal damage precedes demyelination (40–44). Within 1 wk following infection of Thy1-YFP mice with JHMV, we observed axons with FAD, characterized by discontinuous YFP fluorescence, consistent with earlier reports describing axonopathy in JHMV-infected mice (41, 45). Our findings indicate that axonal damage occurs early following JHMV infection of the CNS before robust immune-mediated demyelination. Previous in vivo imaging studies of the demyelinated spinal cord suggest that loss of fluorescence in axons indicates total axonal transection (20, 23). However, we show that loss of YFP fluorescence can occur in an intact axon and does not necessarily mean axonal transection. Although it is possible a variety of cell types may interact with axonal segments, we show that axonal YFP fluorescence changes reversibly during and subsequent to interaction with transplanted NPCs, judged by colocalization with transplanted NPCs. The observed variability in the amount of axonal YFP fluorescence change may be a result of the extent of axonal damage at the time of interaction or the length of the imaging window. These results extend previous studies from our laboratory that demonstrated axonal sparing following NPC transplantation into JHMV-infected mice (8).

Physical engagement of damaged axons by transplanted NPCs increases axonal YFP fluorescence, and disengagement of NPCs

colocalized with GFP fluorescence, further confirming that transplanted NPCs actively participate in remyelination (Fig. 7D and Fig. S3).

Discussion

Our results indicate that transplanted GFP-NPCs preferentially migrate to regions of axonal damage, proliferate, and actively remyelinate axons. NPCs have been shown to selectively colonize areas of white matter damage and facilitate remyelination in preclinical animal models of neuroinflammatory demyelination (5, 8, 34, 35). However, the migration of transplanted NPCs and their interactions with damaged axons has not previously been visualized. In this study, we established a system for stable ex vivo imaging to observe single NPC behavior in the ventral spinal cords of mice, using a model of viral-induced demyelination. Although the etiology of MS is unknown, numerous factors including both genetic and environmental influences are considered important in initiation and maintenance of disease. Viral infection has long been considered a potential triggering mechanism involved in demyelination, and numerous human viral pathogens have been suggested to be involved in eliciting myelin-reactive lymphocytes and/or antibodies that subsequently infiltrate the CNS and damage the myelin sheath (36–39). As such, viral models of neuroinflammation/demyelination are relevant and have provided important insights into mechanisms associated with disease. Moreover, the molecular mechanisms governing how engrafted NPCs interact with demyelinated axons within an inflammatory environment resulting from demyelination derived from a persistent viral infection have not been defined. Therefore, the present study uses the JHMV model of demyelination to characterize NPC migration kinetics as well as their ability to physically engage demyelinated axons and promote remyelination. To accomplish this, we used transgenic Thy1-YFP mice and 2P microscopy to visualize axonal damage following JHMV infection and imaged real-time interactions of transplanted GFP-NPCs with damaged axons, allowing insight into mechanisms by which transplanted NPCs contribute to amelioration of clinical and histopathological disease.
from axons rapidly diminishes YFP signal intensity. Loss of YFP fluorescence may occur in intact axons due to impaired microtubular transport (46), axonal swelling resulting in retraction of proteins from damaged areas (47), changes in reactive oxygen and nitrogen species that initiate mitochondrial pathology (48), or changes in ion channel expression (49) that do not allow for a favorable environment for YFP fluorescence. However, to account for the rapid changes in YFP fluorescence, we propose that interactions with NPCs may reverse acidification and diminish elevated internal calcium concentrations present in axons in inflammatory lesions through effects on NMDA receptors (23) or acid-sensing ion channels (50). YFP fluorescence is particularly sensitive to acidic pH (51), indicating that restoration of normal physiological pH may underlie rapid increases in axonal YFP fluorescence observed during remyelination. Continued studies examining these possibilities are required to better understand the nature of axonal damage in response to CNS viral infection and the ability of NPCs to protect and repair damaged axons.

We have previously shown that transplanted GFP-NPCs preferentially colonize areas of demyelination and this positional migration is guided, in part, by expression of the chemokine receptor CXCR4 on transplanted NPCs that respond to enriched expression of the chemokine ligand CXCL12 that is enriched within areas of white matter pathology (5). However, the motility of engrafted NPCs was not examined. Our results support and extend those earlier findings by showing that NPCs recognize and directly interact with damaged axons. However, it is possible that reduced GFP-NPC motility is due to inflammatory signals in the JHMV-infected spinal cord. The molecular signals mediating this interaction are to this point undefined, although previous studies have clearly shown that NPCs are capable of expressing adhesion molecules that may engage axons (52).

Engagement of damaged axons by transplanted GFP-NPCs is the first step in events leading to NPC differentiation and remyelination (53, 54). We have previously shown that the majority of transplanted NPCs into JHMV-infected mice differentiate into oligodendroglia, the myelinating cells of the CNS supporting an important role for transplanted cells in participating in remyelination (5, 8). Moreover, transplantation of NPCs lacking the transcription factor Olig1 into JHMV-infected mice results in diminished clinical and histologic recovery that correlates with altered lineage fate commitment as Olig1-deficient NPCs preferentially differentiate into astrocytes (55). Until now, studies have shown that transplanted NPCs directly or indirectly contribute to endogenous remyelination (11, 55), although evidence of direct remyelination...
was not entirely conclusive. Whereas we have previously shown that GFP expression from transplanted NPCs colocalized with MBP (55), our real-time imaging of PLP-expressing NPCs definitively demonstrates that transplanted NPCs directly remyelinate axons and are not merely stimulating endogenous oligodendroglia to remyelinate. We show that NPC-derived oligodendrocytes express PLP, a protein necessary for myelination, and extend and cover axons within ~20 min. Finally, this study also provides a model system to better understand the physical nature of NPC interactions with damaged axons.

**Methods**

**Mice and Virus.** C57BL/6 (National Cancer Institute) and Thy-1-YFP (25) (Jackson Laboratory) mice were isolated and intracranially (i.c.) with 150 plaque-forming units (PFU) of JHMV strain J2-2v-1 (JHMV) in 30 μl sterile Hank’s balanced saline solution (HBSS) (5, 24). Mice were killed by inhalation of halothane (Sigma-Aldrich) at various days postinjection (p.i.), and spinal cords were removed and processed for analysis. For tissues used for immunohistochemistry, mice were fixed by cardiac perfusion. Mice expressing eGFP driven by the mouse myelin PLP gene promoter were used to generate NPCs that express eGFP only, following terminal differentiation into mature myelinating oligodendrocytes (33). All experiments were approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

**Cell Culture, Reagents, and Transplantation.** Enhanced green fluorescent protein-expressing NPCs (GFP-NPCs), derived from C57BL/6 mice, were cultured as previously described (24). In addition, NPCs from PLP-GFP mice were isolated and transplanted as previously described (34, 55). Transplantation culture in DMEM/F12 (Invitrogen) supplemented with 1× B27 (Invitrogen), 1× insulin-transferrin- selenium (Invitrogen), 1× penicillin-streptomycin (Invitrogen), 40 ng/ml T3 (Sigma-T67407), and 20 ng/ml human recombinant EGF (Sigma-E9644) for 5 d, PLP-GFP neurons were dissociated with 1× Tryple (Gibco-12563-011) for 20 min at 37 °C and centrifuged for 5 min at 500 × g, 4 °C. Cells were resuspended and triturated in 1× HBSS followed by three washes with 1× HBSS. GFP-NPCs or PLP-GFP-NPCs were transplanted (2.5 × 10^6 in 2.5 μl HBSS per mouse) at spinal cord T10 at day 14 p.i. into C57BL6 and Thy-1-YFP mice. As previously published, this time point for transplantation was chosen because virus and inflammation have waned whereas demyelination has peaked (5).

**Spinal Cord Preparation, Two-Photon Imaging, and Analysis.** Two-photon imaging was performed using a previously described system (56) with a Chameleon Ultra II Ti:Sapphire laser (Coherent). Spinal cords were isolated from thoracic vertebra 4 to lumbar vertebra 2, embeod in 2.5% agarose gel to maintain the integrity of the spinal cord, oriented with the ventral side facing the dipping objective, and superfused with warm, oxygenated medium as applied for ex vivo imaging of other explanted organs (56). Explanted spinal cords were imaged with a laser excitation of 900 nm. Five hundred twenty- and 560-nm images were acquired with a 20× water immersion objective, and superfused with warmed, oxygenated medium as applied to improve visual separation of eGFP and eYFP. In final videos, pixel intensities over a set threshold at the same position were considered colocalized.

**Immunohistochemistry.** The murine spinal cord was extracted and processed for optimal cutting temperature compound and resin-embedded sections as previously described (56). For immunofluorescence staining, slides were prepared with goat serum and block BSA as goashield in 1× PBS + 0.5% BSA. Preimmune serum was used as a negative control (56). Following suggested protocols (56), primary antibodies included rabbit anti-Iba1 (1:200, DakoCytomation); rabbit anti-MBP (1:200, Chemicon); mouse anti-SMI-31 (phosphorylated neurofilament H; 1:1,000; EMD Millipore), or mouse anti-SMI-32 (nonphosphorylated neurofilament H; 1:1,000; Covance). Anti-SMI-31 and anti-SMI-32 were blocked as described above with the addition of 5% (v/v) bovine serum albumin (BSA) (56). For immunofluorescence staining, slides were permeabilized with 0.1% Triton X-100, and primary antibodies included rabbit anti-Iba1 and mouse anti-SM (1:200, Zymed Laboratories); rabbit anti-MBP (1:200, Microm Immunochemicals); mouse anti-SM (1:1,000; Invitrogen) was used. DAPI Fluoromount-G (Southern Biotech) was used to visualize nuclei. Colocalization of overlapping fluorescence was determined using Imaris 7.3 (Bitplane). Pixel intensities over a set threshold at the same position were considered colocalized.

**Statistical Analysis.** Statistical significance for velocity measurements in Fig. 3C was determined using Student’s t test. The Mann–Whitney U test was used to calculate significance for the nonnormally distributed directional persistence measurements in Fig. 3D. A P value <0.05 was considered significant. Data are presented as mean ± SEM.

**ACKNOWLEDGMENTS.** We acknowledge the California Institute for Regenerative Medicine core facilities at the University of California, Irvine. This work is supported in part by National Institutes of Health (NIH) Grants R01 GM-41514 (to M.D.C.) and R01 NS-074987 (to T.E.L.) and the National Multiple Sclerosis Society (NMSS) Collaborative Center Research Award CA1058-A-8 (to T.E.L. and M.D.C.), NMSS Grant RG4925, NIH Training Grant T32 HD-075673 (to M.L.G.), and NMSS Postdoctoral Fellowship FG 1960-A-1 to J.G.W.


