SUPPLEMENTARY METHODS

Cell culture

Primary Schwann cell cultures derived from sciatic nerves of adult CD11b-TK\textsuperscript{int-30} mice and their wild-type littermates were prepared according to established protocols (Morrissey et al., 1991; Weidner et al., 1999). Sciatic nerves were dissected out, stripped free of epineurium, cut into multiple segments of approximately 1 mm, and placed as explants into tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (Pen/Strep). When the cell outgrowth around the explants had reached near confluence, explants were lifted and gently transferred into new dishes. Cells forming the first outgrowth were trypsinized, transferred into new dishes, and cultured separately in DMEM + 10% FBS + 1% Pen/Strep (fibroblasts are the first cells to grow out from primary nerve explants). Over 6 weeks, explants were transferred 4 times and then dissociated following enzymatic digestion and plated onto dishes coated with mouse laminin (BD Biosciences; Mississauga, ON, Canada), as described before by Weidner et al. (Weidner et al., 1999). After one cycle of cytosine arabinoside (10 µM) to eliminate proliferating fibroblasts, the culture medium was switched to fresh DMEM + 10% FBS + 1% Pen/Strep to which 2 µM of forskolin and 20 µg/ml of bovine pituitary extracts were added to stimulate Schwann cell proliferation. Unless specified otherwise, all reagents for cell culture were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Prior to experimentations, we established that Schwann cell and fibroblast cultures were 99% and 98-99% pure, respectively, based on multiple immunofluorescence labeling using the following antibodies: (1) anti-p75 neurotrophin receptor (p75\textsuperscript{NTR}; a marker of Schwann
cells; Dr. Moses Chao), (2) anti-S100β (a marker of Schwann cells), (3) anti-fibronectin (a marker of fibroblasts), and (4) anti-CD45 (a marker of leukocytes). For more details about these primary antibodies and their dilution, please refer to Supplementary Table 1.

To confirm that the HSV-1 TK<sup>mt-30</sup> transgene was expressed specifically by cells of myeloid origin, and not by other cell types in the sciatic nerve, cultures of Schwann cells and fibroblasts obtained from CD11b-TK<sup>mt-30</sup> transgenic mice or their wild-type littermates were treated or not with GCV. For this experiment, Schwann cells and fibroblasts were seeded at 2.5 x 10<sup>4</sup> cells/well and positive control cells (i.e. GL261-CMV-TK) at 1 x 10<sup>5</sup> cells/well in BD Falcon<sup>TM</sup> CultureSlides (BD Biosciences) in their respective cell culture medium. Three days later, the medium was changed, GCV at 1 µM or PBS added, and cells allowed to grow for another 3 days. Cells were then fixed with 4% PFA in PBS (pH 7.4) for 20 min and immediately processed for immunofluorescence following our previously described protocol (Pineau and Lacroix, 2007), with the following modifications: 1) blocking in Tris-buffered saline (TBS) + 0.2% Triton X-100 + 5% normal serum for 15 min, 2) incubation for 1 hr at room temperature in the primary antibodies listed above, 3) incubation for 1 hr in secondary antibodies conjugated with fluorophores Alexa 488 or Alexa 568 (1:200 dilution; Invitrogen Canada Inc., Burlington, ON, Canada), and 4) counterstaining in DAPI (1:5,000 dilution; Invitrogen Canada Inc.) for 2 min. Following immunofluorescence, chambers were gently removed from the slides using the Safety Removal Fixture provided with CultureSlides and slides coverslipped with polyvinyl alcohol/2.5% diazabicyclooctane (Sigma-Aldrich Canada Ltd.).
For quantification of Schwann cells (p75\textsuperscript{NTR}/S100\beta\textsuperscript{+}) and fibroblasts (fibronectin\textsuperscript{+}/S100\beta\textsuperscript{-}), the number of labeled cells was estimated by the optical fractionator method using the Bioquant Nova Prime software (Bioquant Image Analysis Corporation, Nashville, TN). First, the outline of each well was traced at 4X magnification and then sampled at 100X. The counting parameters were as follows: sampling grid size, 225 x 225 \(\mu\)m; counting frame size, 50 x 50 \(\mu\)m. Cells were counted only if their nuclei laid within the dissector area and did not intersect forbidden lines.

**Tissue processing**

For the experiments involving *in situ* hybridization (ISH) or ISH combined to immunohistochemistry, mice were overdosed with a mixture of ketamine and xylazine and transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA), pH 9.5, in borax buffer. After perfusion with the fixative, brains, sciatic nerves, dorsal root ganglia (DRGs), and spinal cords were dissected out, post-fixed for 2 days, and placed overnight in a 4% PFA-borax/10% sucrose solution until tissue processing. For the purpose of histological and immunohistochemical labeling, animals were perfused instead with cold PBS followed by 4% PFA, pH 7.4, in PBS. Sciatic nerves were post-fixed for 1 hr only and then placed in a PBS/20% sucrose solution until sectioning.

Frozen brains were mounted on a microtome and cut into 30-\(\mu\)m coronal sections from the olfactory bulb to the end of the medulla. Tissue sections were collected in a cold cryoprotectant solution and stored at -20 °C. Sciatic nerves and DRGs were cut longitudinally or coronally using a cryostat (model CM3050S; Leica Microsystems, Richmond Hill, Ontario, Canada) set at a thickness of 14 \(\mu\)m. Sciatic nerve and DRG
sections were collected directly onto slides that have a permanent positive charged surface (Surgipath Canada Inc., Winnipeg, Manitoba, Canada), separated into three (DRGs) or four (sciatic nerves) different series of adjacent sections, and then stored at -20 °C. Spinal cord segments L2-L5 were cut coronally at a thickness of 30 µm and collected directly onto slides into seven different series of adjacent sections, as previously described (Pineau and Lacroix, 2007).

For the sciatic-sciatic nerve graft experiments in which the number of YFP⁺ axonal profiles was counted proximal to the surgical repair site and at various distances into the grafts, the entire sciatic nerves, including the grafts, were placed on microscope slides and immediately coverslipped with polyvinyl alcohol/2.5% diazabicyclooctane (Sigma-Aldrich Canada Ltd.). Images of optical sections through these nerves were obtained at 2.5-µm intervals, at 10X magnification, using a confocal laser scanning microscope (Fluoroview, Olympus, Center Valley, PA) and following protocols published by English and colleagues (English et al., 2005; Groves et al., 2005). Stacks of optical sections of adjacent microscope fields were stitched together using Adobe Photoshop (v7.0).

**Quantification of ISH signal and histological and immunohistochemical labeling**

ISH signal was quantified by two different approaches. When it was possible to assign hybridization signal to individual cells (e.g. for neurotrophins), cells were counted individually. Otherwise, the average density of hybridization signal was measured (for all other mRNAs). ISH signal was quantified using nuclear emulsion-dipped slides (for neurotrophins, Tie2, and p75NTR) or on Bio-Max MR x-ray films (for growth-associated genes). For experiments involving sciatic nerve longitudinal sections, hybridization
signal was quantified within predefined 1-mm sciatic nerve segments, starting from the site of injury (identified by the 10-0 suture node) up to 4 mm distal to the lesion. Thus, a total of 4 sciatic nerve segments were analyzed per animal (n = 2-3 sections per mouse).

For experiments involving DRGs (i.e. quantification of mRNAs coding for growth-associated genes), hybridization signal was quantified within two randomly selected sections of ipsilateral L5 DRGs from each animal. Finally, for experiments involving L2-L5 spinal cord coronal sections, ISH signal was measured bilaterally within a circular frame of predetermined diameter centered over the motoneuronal group of Rexed's lamina IX (n = 3-4 sections per lumbar segment per mouse). For each section analyzed, the outline of the predefined segment/area was first traced or centered manually under bright-field illumination at 10X magnification. The number of cells expressing positive mRNA signal was then counted, using our previously published method (Pineau and Lacroix, 2007), and results expressed as an average number of positive cells per mm$^3$.

Optical density measurements were performed as described elsewhere (Boivin et al., 2007; Turrin et al., 2007). All data collection was performed blind with respect to the identity of the animals.

For the quantification of macrophages (CD68), granulocytes/inflammatory monocytes (Gr-1), T cells (CD3), B cells (CD45R/B220), and macrophages that ingested myelin debris (CD68/ORO), the number of labeled cells was estimated by the optical fractionator method using the Bioquant Nova Prime software. First, the outline of the longitudinal segments were traced, as described above, and then sampled at 100X magnification. The counting parameters were as follows: sampling grid size, 225 x 225 µm; counting frame size, 50 x 50 µm; dissector height, 14 µm. Cells were counted only if
their nuclei laid within the dissector area, did not intersect forbidden lines, and came into focus as the optical plane moved through the height of the dissector.

For the quantification of myelin staining (LFB staining) and immunolabeling for CD31 and Galectin-3, the proportional area of tissue occupied by labeling within a region of interest (ROI) was measured, as previously described (Boivin et al., 2007). This method of quantification was chosen because cells immunostained with the Galectin-3 antibody could not be counted individually. Briefly, the outline of the longitudinal sections (n = 2 sections per mouse) were traced manually at 4X magnification. The area of tissue occupied by labeling in this sampling area was then measured using the Bioquant Nova Prime software on video images of tissue sections transmitted by a high-resolution Retiga QICAM fast color 1394 camera (1392 x 1040 pixels; QImaging, Burnaby, British Columbia, Canada) installed on a Nikon (Tokyo, Japan) Eclipse 80i microscope. Thresholding values in Bioquant Image were chosen such that only labeled product resulted in measurable pixels on the digitized image. Contrast between positive signal and background was maximized and held constant between all images.

For quantification of the number of axonal profiles in ipsilateral L5 dorsal and ventral roots, the outline of the cross-section was traced at 20X magnification, a grid of 50 µm x 50 µm positioned over the root, and all axons counted at 100X magnification. The Toluidine Blue-stained sections chosen for this analysis were taken at the same distance from the DRG.

Axon growth into PN grafts and the number of blood vessels per sciatic nerve cross-section were quantified by counting the total number of YFP-labeled axons and CD31-immunoreactive blood vessels, respectively, at pre-determined distances from the
host-graft interface/lesion site. In the case of intact nerves (i.e., unlesioned), the center of the peripheral nerve segment was used for quantification of CD31⁺ blood vessels. Blood vessels associated with the epineurium were excluded from this analysis. For the experiment in which regeneration of SCI axons was quantified within PN grafts, the pre-determined distance was set at 200 µm.

**Flow cytometry analysis of sciatic nerve Schwann cells**

To address the question of whether depletion of CD11b⁺ myeloid cells may have affected Schwann cell function, rather than Schwann cell proliferation, the expression of Galectin-3 by Schwann cells was examined *in vivo* using flow cytometry. This was done on Schwann cells purified from sciatic nerve distal stump biopsies obtained from mice generated from crossing S100β-EGFP with CD11b-TKmt⁻³⁰ mice. In this experimental setting, Schwann cells were gated based on their expression of S100β (i.e., GFP) and p75NTR. Surface and intracellular expression of Galectin-3 was then examined in Schwann cells purified from S100β-EGFP/CD11b-TKmt⁻³⁰ transgenic mice or their wild-type littermates treated with either saline or GCV.

Schwann cell purification was performed according to a protocol adapted from Salomon *et al.* (Salomon et al., 2001). Briefly, animals were anesthetized and their sciatic nerves dissected out, cut into multiple small segments, and immediately placed into 1.5-ml microtubes (1 mouse sciatic nerve per tube) containing DMEM, 10 mM HEPES, and bovine serum albumin (BSA; 5mg/ml). Sciatic nerve segments were then quickly centrifuged and incubated at 37°C for 30 min in fresh culture medium containing DMEM + HEPES + BSA to which 1.6 mg/ml of collagenase (type IV; Sigma-Aldrich Canada Ltd.) and 200 µg/ml of DNase I (Sigma-Aldrich Canada Ltd.) were added. Cells were
gently dissociated by repeated pipetting through a 1,000-µl pipet, reincubated at 37°C for 30 min, and dissociated again to a single-cell suspension using the same pipet. Cells were centrifuged at 6,000 rpm for 5 min and washed twice with PBS. Cell concentration and percentage viability were estimated using a hemocytometer and the Trypan blue exclusion method.

For intracellular staining of Schwann cells, cells were fixed with 2% PFA for 30 min, washed with PBS, and permeabilized with 0.1% saponin (Sigma-Aldrich Canada Ltd.) in PBS for an additional 30 min at room temperature. After blocking for 30 min with 10% normal goat serum in PBS, cells were incubated with a polyclonal anti-p75NTR antibody (Chemicon) and a monoclonal anti-Galectin-3 antibody for 30 min at room temperature. For negative controls, primary antibodies were omitted. The complete description of the primary antibodies and the dilution used for flow cytometry are given in Supplementary Table 1. Cells were then washed with PBS and incubated for 30 min with PE-conjugated goat anti-rabbit (1:1,000 dilution; Jackson ImmunoResearch, West Grove, PA) and Cy5-conjugated goat anti-rat (1:1,000 dilution; Invitrogen Canada Inc.) secondary antibodies. Finally, cells were washed again and resuspended in PBS. A total of 20,000 events were analyzed in duplicate for each sciatic nerve sample using CellQuest Pro software on a FACSCalibur (BD Biosciences).
References for Supplementary Methods


Turrin NP, Plante MM, Lessard M, Rivest S (2007) Irradiation does not compromise or exacerbate the innate immune response in the brains of mice that were transplanted with bone marrow stem cells. Stem Cells 25:3165-3172.

and remyelinate central nervous system axons in a phenotypically appropriate manner that correlates with expression of L1. J Comp Neurol 413:495-506.
SUPPLEMENTARY DISCUSSION

Correlation of our in situ hybridization findings on neurotrophin expression with mRNA measurements obtained in previous studies (continued from the main text)

Our results differ, however, from the Funakoshi study with regard to the expression of NT-3, which we have found to be regulated exactly like the other three neurotrophins. Using RNase protection assays, Funakoshi et al. have previously reported that NT-3 mRNA is strongly expressed in the intact sciatic nerve from adult rats. After sciatic nerve transection, NT-3 mRNA expression was found to decrease to almost undetectable levels in the nerve distal stump at 6-12 hrs post-lesion and then to progressively increase up to baseline levels after 2 weeks (Funakoshi et al., 1993). One way to explain why NT-3 was found to be constitutively expressed in intact nerves in the Funakoshi study and not in ours is the different animal species used (rats vs. mice). In humans, Sobue et al. have reported that NGF, BDNF, and NT-3 mRNAs are barely detectable in peripheral nerves from healthy subjects, whereas mRNA levels for all neurotrophins are elevated in nerve segments obtained from patients suffering from various peripheral neuropathies (Sobue et al., 1998). Although we cannot comment on the specificity of the results published by Funakoshi et al., our study has clearly established that the riboprobes used in our ISH analyses gave specific labeling in the adult mouse nervous system.

Mechanisms by which CD11b+ myeloid cells could regulate blood vessel formation/stabilization

Although it is not perfectly clear at present how myeloid cells could regulate angiogenesis, some studies have suggested that these cells could secrete proangiogenic factors and produce proteases that may facilitate sprouting angiogenesis or release
angiogenic factors that are sequestered in the matrix (De Palma et al., 2005). Another possible mechanism by which myeloid cells may influence angiogenesis is through blood vessel stabilization. One of the molecular pathways regulating this process involves the angiogenic factors angiopoietin (Angpt)1 and Angpt2 and their Tie2 receptor. It has been proposed that one function of Ang1 is to stabilize vessels and make them leak-resistant by facilitating communication between endothelial cells and surrounding support cells presumably derived from the bone marrow (Yancopoulos et al., 2000; Jain, 2003). However, whether these cells express the CD11b^Tie2^ phenotype has yet to be demonstrated. Together, these results suggest that CD11b^ myeloid cells or a subset of these cells regulate angiogenesis after neural injury, although further studies will be needed to clarify the mechanisms involved in this process and how this process affects nerve regeneration and repair.

In recent years, it has become increasingly evident that axonal growth and guidance in the nervous system and angiogenesis are regulated by common signaling systems, including neurotrophins/Trk receptors (or p75NTR), ephrins/Ephs, semaphorins/neuropilins/plexins, VEGF/neuropilins/VEGF receptors, netrins/DCC/UNC5, Slits/Robos, and neuroregulins/erbB receptors (for review, see (Carmeliet and Tessier-Lavigne, 2005; Klagsbrun and Eichmann, 2005; Kermani and Hempstead, 2007)). From our transplant experiments, we were able to show that regenerating axons, in several occasions, were perfectly aligned with blood vessels, or vice-versa. Even more fascinating are examples of misrouted axons following the trajectory of blood vessels, or vice-versa. What is unclear is whether the pattern of branching of regenerating axons was self-organized or determined by blood vessel
branching. Thus, although we did not directly assess whether the formation of new vessels was required for axon regeneration, the results presented here show that recovery of sciatic nerve functions was compromised in animals with deficient angiogenesis. These results suggest that regenerating axons and blood vessels respond to common cues provided by myeloid cells after peripheral nerve injury and that a close interaction between them is probably needed to reach target tissues and allow for functional recovery.
References for Supplementary Discussion


