TECHNOLOGY REPORT

Inducible Site-Specific Recombination in Myelinating Cells

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Summary: To explore the function of genes expressed by myelinating cells we have developed a model system that allows for the inducible ablation of predetermined genes in oligodendrocytes and Schwann cells. The Cre/loxP recombination system provides the opportunity to generate tissue-specific somatic mutations in mice. We have used a fusion protein between the Cre recombinase and a mutated ligand-binding domain of the human estrogen receptor (CreERT) to obtain inducible, site-specific recombination. CreERT expression was placed under the transcriptional control of the regulatory sequences of the myelin proteolipid protein (PLP) gene, which is abundantly expressed in oligodendrocytes and to a lesser extent in Schwann cells. The CreERT fusion protein translocated to the nucleus and mediated the recombination of a LacZ reporter transgene in myelinating cells of PLP/CreERT mice injected with the synthetic steroid tamoxifen. In untreated animals CreERT remained cytoplasmic, and there was no evidence of recombination. The PLP/CreERT animals should be very useful in elucidating and distinguishing a particular gene’s function in the formation and maintenance of the myelin sheath and in analyzing mature oligodendrocyte function in pathological conditions.

Key words: myelinating cells; CreERT; recombination

INTRODUCTION

The myelin sheath is the multilayered membrane structure that surrounds most nerve axons and facilitates rapid nerve conduction velocities by promoting salutary conduction. Oligodendrocytes are responsible for myelinating the central nervous system (CNS), and Schwann cells myelinate the peripheral nervous system (PNS). Myelinating cells express a number of molecules critical to their differentiated function (Morell and Quarles, 1999; Baumann et al., 2001; Garbay et al., 2000). A better understanding of the molecular mechanisms by which these cells form and maintain the myelin sheath will likely advance our understanding of this critical structure, as well as facilitate the development of strategies for the repair of the injured sheath in demyelinating diseases such as multiple sclerosis and chronic inflammatory polyneuropathy (Scherer, 1997; Duncan et al., 1997).

The examination of mouse mutants, spontaneous and experimentally engineered, that are specifically deficient in a particular myelin component has been an invaluable tool in the analysis of the myelination process (Werner et al., 1998; Suter et al., 1999; Martini, 2000; Yool et al., 2000). To date, these mutations have been present in all cells at all stages of development in the animals examined. Such models are satisfactory for molecules that are “myelin specific,” but to determine the function of molecules more widely expressed, the analysis of myelinating cell-specific mutants is preferable.

Fortunately, new tools have been developed that allow for the generation of conditional genome alterations by controlling the tissue or cellular specificity of the introduced mutations (Gao et al., 1999). Conditional somatic mutations can be obtained using a recombination system based on the bacteriophage P1 site-specific Cre recombinase (Kuhn et al., 1995; Nagy, 2000) under the control of either a tissue- or cell-specific promoter. The Cre recombinase efficiently catalyzes molecular recombination at specific 34-bp sequences termed loxP sites. Any DNA segment flanked by loxP sites, in the same orientation, will be deleted in cells expressing the Cre recombinase but will remain intact in nonexpressing cells. Numerous studies have demonstrated that Cre-mediated recombination can occur in a variety of cell types (Sauer et al., 1989; Kuhn et al., 1995; Kellendonk et al., 1999; Feltri et al., 1999; Nagy et al., 2000). Furthermore, several recent studies have described mice expressing Cre recombinase under the control of the

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myelin basic protein (MBP) promoter that allow for the introduction of oligodendroglial-specific alterations (Niwa-Kawakita et al., 2000; Hisahara et al., 2000; Sato et al., 2001).

In addition to spatial specificity, the control of temporal specificity is also a desirable feature of an in vivo recombination system. For example, specific molecules may play distinct functions in the formation versus the maintenance of the myelin sheath. Steroid hormone receptors have been exploited to confer temporal control to the Cre recombinase (Metzger et al., 1995; Kellen-donk et al., 1996). For example, Feil et al. (1996) developed a system for regulating the temporal specificity of recombination by generating a Cre recombinase fused to a mutated ligand binding domain of the human estrogen receptor (ER). The mutant ER (ERT) is activated by the synthetic steroid tamoxifen but not by endogenous estradiol (Feil et al., 1996). The CreERT fusion protein translocates from the cytoplasm of the cell, where it is functionally inactive, to the nucleus when bound to tamoxifen, thus allowing temporally (tamoxifen) controlled somatic mutagenesis of floxed target genes.

We set out to generate a mouse model system that would allow for the spatial and temporal control of gene inactivation in myelinating cells. Toward this goal, transgenic mice were generated in which CreERT expression was driven to myelinating cells using the regulatory sequences of the murine proteolipid protein (PLP) gene (Wight et al., 1993; Griffiths et al., 1998). The expression of transgenes driven by this PLP transcriptional control region is cell-type specific, primarily restricted to myelinating oligodendrocytes in the CNS (Wight et al., 1993; Fuss et al., 2000, 2001) and Schwann cells in the PNS (Mallon et al., 2002). Furthermore, PLP is expressed earlier than MBP during development, beginning during embryonic life and at very early developmental stages of the oligodendroglia lineage (Wight et al., 1993; Belachew et al., 2001; Mallon et al., 2002). PLP-promoter-driven CreERT transgenic mice should allow for the molecular assessment of myelinating cell development and function in vivo from the progenitor stage to mature myelinating cells.

Previous studies have shown that the first half of the mouse PLP gene, containing 2.4 kb of 5’ flanking DNA, exon 1 and intron 1, is sufficient to direct both spatial and temporal transgene transcription that accurately reflects the expression of the endogenous PLP gene (Wight et al., 1993; Fuss et al., 2000, 2001). Three independent lines were generated carrying the PLP/Cre-ERT transgene (Fig. 1A). Mice of all PLP/CreERT lines were fertile, transmitted the transgene in a Mendelian fashion, and showed no signs of disease. RT-PCR data, generated using a primer set that amplifies a region that spans the intron in the transgene, demonstrates the presence of transgene-derived mRNA primarily in CNS (brain and spinal cord), but also weakly in heart and testis in animals of each line (Fig. 1B). PLP/DM20 mRNA expression has previously been shown to be present in heart at levels at about 0.1% of that present in the brain (Campagnoni et al., 1992), and PLP-driven mRNA was detected in the testis of transgenic animals (Fuss et al., 2000).

To examine the expression of the CreERT protein in the three transgenic lines, we performed Western blot analyses using polyclonal antibody against the Cre protein (Kellendonk et al., 1999) (Fig. 1C). In brain samples from transgenic mice of the three different lines, a protein with an apparent molecular weight of 67 kDa was detected. This corresponds to the expected size of recombinant CreERT fusion protein. No expression of CreERT protein was detectable in wild-type animal (Fig. 1C).

We further characterized the expression of the CreERT protein by immunohistochemistry in adult sections of brain, optic, and sciatic nerves using the same antisera as used for immunoblotting. Expression of the CreERT protein is localized predominantly in white matter areas, with a scattered distribution within corpus callosum, anterior commissure, cerebellum, spinal cord, optic, and sciatic nerves (Fig. 2), and is consistent with the endogenous expression of the PLP gene (Wight et al., 1993). The expression profile is essentially the same for all three PLP/CreERT transgenic lines. At the same time, no expression of CreERT protein is detectable in wild-type animals (data not shown).

To assess the identity of the CreERT-positive cells in the CNS we performed several double-labeling immunohistochemistry experiments on frozen brain sagittal sections and examined areas with relatively simple morphology, such as cerebellum (Fig. 3A–C). Analysis of CreERT labeling using confocal imaging techniques revealed CreERT-positive cell bodies in all white matter tracts (red labeling, Fig. 3A, D). For a more accurate localization of oligodendrocytes and their myelin sheath, monoclonal antibodies specific for MBP and PLP were used. As expected, cells positive for CreERT could be identified in areas that were positive for MBP or PLP (green labeling, Fig. 3B and 3C, respectively). CreERT-derived fluorescence was confined to cell bodies. Nevertheless, a precise colocalization of cell bodies double positive for CreERT and MBP or PLP in myelin-rich CNS areas was not easily obtained since MBP and PLP are myelin-associated and do not accumulate in the cell bodies. Occasionally single cells could be distinguished at the edges of the white matter tracts where the overall MBP or PLP stain was less, with clear CreERT-positive cell bodies and MBP- or PLP-positive cytoplasm and processes (Fig. 3A–C). To assess the identity of CreERT-positive cells more accurately, we used antibodies that specifically label oligodendrocyte cell bodies (clone CC1 of adenosomatous polyposis coli protein and gluthathione-s-transferase Pi [GST-Pi]), processes and somata of astrocytes (anti-glial fibrillary acidic protein [GFAP]), and neuronal cell bodies and dendrites (clone SM132 of neurofilament H) for double-labeling experiments (Fig. 3D–I). As representative CNS regions, we analyzed corpus callosum, spinal cord and cerebellum. These studies clearly demonstrated that neither astrocytes nor neurons were labeled by CreERT (Fig. 3H, I). In general, a major-
ity of the CreERT-positive cells were also CC1 (Fig. 3D, E) or GST-Pi (Fig. 3F, G) positive. The above data confirmed a restricted expression pattern of CreERT in myelinating oligodendrocytes in the CNS of PLP/CreERT mice.

To examine the steroid response of CreERT-expressing oligodendrocytes, we compared the intracellular localization of the chimeric protein upon treatment of transgenic mice with tamoxifen or oil carrier alone. Immunohistochemistry analyses were performed on frozen spinal cord sections using polyclonal anti-Cre antibody and DAPI to stain cell nuclei. In the absence of tamoxifen treatment, all CreERT protein was excluded from the nucleus (Fig. 4A, B). Three days of 1 mg/day tamoxifen treatment was sufficient to induce nuclear translocation of CreERT (Fig. 4C, D).

The specificity of Cre-mediated recombination was tested functionally by crossing the PLP/CreERT-expressing mice to animals harboring a floxed β-galactosidase (LacZ) reporter transgene (Araki et al., 1995). In the reporter mice, LacZ expression is only activated in cells that express functional Cre recombinase, which removes a stop signal from the floxed transgene. Double-transgenic animals were treated for 8 consecutive days with a single daily injection of oil carrier or 1 mg/day of tamoxifen. The recombination event was analyzed by PCR in various tissues of the PLP/CreERT:floxed LacZ reporter mice. Recombination of the floxed LacZ transgene was scored by amplification of a 384-bp PCR product using primers described by Niwa-Kawakita (2000), AG10 and Z6 (Fig. 5). These primers also allowed amplification of a 1972-bp product, corresponding to the nonrecombined transgene, which was observed in all samples (Fig. 5). In tamoxifen-treated floxed LacZ reporter mice carrying the PLP/CreERT transgene, the

FIG. 1. Transgene construct and PLP/CreERT expression in transgenic mice. (A) Structure of the PLP/CreERT transgene and strategy to the detection of CreERT mRNA by RT-PCR. To generate transgenic PLP/CreERT mice we used the PLP cassette containing 2.4 kb of the 5'-flanking DNA, exon 1 and intron 1 of the PLP gene (black-and-white diagonally striped boxes), which have been shown to drive abundant oligodendrocyte expression (Fuss et al., 2000). For transcription termination, a simian virus (SV) 40 poly(A) signal sequence (horizontally striped box) is present 3' of the cDNA sequence encoding CreERT (CreERT box). White boxes represent vector sequences. (B) RT-PCR analysis of CreERT mRNA expression. RNA was isolated from brain (B), spinal cord (SC), liver (L), spleen (Sp), kidney (K), heart (H), lung (Lu), and testis (T) of 3-week-old PLP/CreERT mice. RT-PCR products corresponding to PLP/CreERT mRNA (fusion between PLP exon 1 and CreERT coding sequence) and GAPDH mRNA used as an internal control are indicated. (C) Western blot analysis of CreERT expression. Twenty μg of total protein of brain from 3-week-old PLP/CreERT and littermate control mice were analyzed. Membrane was incubated with polyclonal anti-Cre recombinase and monoclonal anti-actin antibodies. All three expressing transgenic lines and wild-type (wt) sample are showed. The expected specific band sizes are 67 kDa and 42 kDa for recombinant CreERT fusion protein and actin, respectively.
384-bp fragment specific for the deleted transgene was amplified principally in the CNS samples analyzed, thus confirming the spatial and temporal specificity of the PLP transgene for the CNS. Nevertheless, consistent with the PLP/CreERT expression pattern described above, recombination was also weakly observed in heart and testis. At the same time, no deleted transgene was amplified in oil carrier treated animals (Fig. 5).

To determine the cellular localization of Cre-mediated recombination in the CNS and PNS, various tissue samples from PLP/CreERT:floxed LacZ reporter mice, treated by oil carrier or tamoxifen, were analyzed for expression of β-galactosidase by X-gal staining (Fig. 6). In reporter mice carrying the PLP/CreERT transgene, specific staining was observed in various regions of the CNS, only after tamoxifen treatment (Fig. 6A, B). Blue-stained cells were found primarily in white matter tracts of brain (Fig. 6C–F) and optic nerve (Fig. 6G). To a far lesser extent X-gal conversion was also detected in the sciatic nerves (Fig. 6H), confirming that the PLP promoter cassette used includes elements that stimulate transcription in Schwann cells (Puckett et al., 1987; Mellon et al., 2002). Immunofluorescence staining for both LacZ and Cre recombinase protein detected LacZ reporter expression in a significant proportion of Cre positive cells (Fig. 6A).

Finally, dual label immunocytochemistry for both LacZ and CC1 primarily, although not exclusively, detected recombination in oligodendroglial cells, as assessed by LacZ reporter expression, after Cre-mediated recombination (Fig. 7B and C). It is unclear whether the lack of uniform labeling of LacZ-positive cells by the oligodendroglial marker reflects a population of oligodendrocytes that do not express CC1, as the work of Fuss et al. (2000) would suggest, or whether the PLP/CreERT transgene is expressed in nonoligodendroglial cells, or some combination of these two possibilities.

The combination of the PLP promoter and the induction of the CreERT recombinase by tamoxifen yields a highly selective conditional mutagenesis system for myelinating cells. The PLP/CreERT transgene is expressed primarily in white matter tracts of the CNS, with less expression detected in Schwann cells, and even less in the heart and testis. In the CNS of these mice, cells labeled by their CreERT expression could be clearly identified as mature oligodendrocytes by double immunocytochemical studies with oligodendrocyte-specific markers.

Expression of the PLP gene was initially thought to occur exclusively in the CNS, but PLP is clearly also expressed in Schwann cells (Puckett et al., 1987). The
PLP expression cassette used in these studies contains transcriptional control elements that drive expression also in the PNS (Wight et al., 1993; Jiang et al., 2000; Mallon et al., 2002), where it is expressed in both myelinating and nonmyelinating Schwann cells (Jiang et al., 2000). The PLP promoter remains active after sciatic nerve transection (Jiang et al., 2000), which suggests that the inducible system described here could be used to induce recombination in experimental models of peripheral nerve injury.

The availability of the PLP/CreERT² mice should allow for the detailed characterization of the function of proteins expressed by oligodendrocytes. The targeted expression of the Cre recombinase to these cells provides

**FIG. 3.** Oligodendroglial CreERT² expression examined by confocal imaging. To assess the identity of the Cre-positive cells (red) in brain we performed double labeling (green) on 5-week-old frozen brain sagittal sections with antibodies specific for myelin sheath: MBP (A, B) and PLP (C); oligodendrocyte cell bodies such a CC1 (D, E) and GST-Pi (F, G); neurons: neurofilament H (H); and astrocytes: GFAP (I). Panels A and B show sections from line 3, panels C, D, E, F, G, and I display sections from line 46, and panel H is from line 16. Pictures show double labeling, with single (A/D) or double laser channel, localized in cerebellum (A/B/C), corpus callosum (D/E), or cerebellum (F/G/H/I). Scale bars = 10 μm, except for D and E, which equal 25 μm.
the means to tease out the oligodendroglial role played by genes expressed in cell types in addition to oligodendroglia. The inducible feature of the CreERT fusion protein provides the opportunity to characterize the function of oligodendroglial proteins during the myelination process, as well as in mature animals. Furthermore, as demonstrated by the floxed LacZ reporter gene, the PLP/CreERT animals can be used to temporally activate gene expression specifically in oligodendrocytes. A major problem with transgenic studies using a developmentally regulated promoter has been the expression of transgenes throughout development as well as in mature cells. Thus, investigating the impact of a protein in the mature cell cannot be separated from its potential to alter the development of the cell. The ability to activate cre-mediated recombination selectively in oligodendrocytes provides the opportunity to investigate the function of proteins in mature oligodendrocytes that have developed normally. These animals offer another tool in our efforts to comprehensively characterize the myelination process and at the same time investigate the function of mature oligodendrocytes in normal and pathological environments.

MATERIALS AND METHODS

Generation of the PLP/CreERT Transgenic Line

For cell type-specific expression of the Cre recombinase, a 2-kb CreER T fragment, containing the Cre open reading frame fused to a mutated form of the estrogen receptor ligand binding domain, was first isolated from pCreERT plasmid (Feil, 1996), which was kindly provided by Dr. Pierre Chambon. This T4 DNA polymerase-treated EcoRI DNA fragment was inserted into the SmaI site of a modified version of the vector pNEB193 (Fuss et al., 2000). The pPLP/CreERT plasmid was constructed by cloning the 2.1-kb AscI-PacI restriction fragment containing the CreERT sequence into the 14.5-kb PLP promoter cassette containing 2.4 kb of the 5’ H11032 flanking DNA, exon 1, intron 1 of the mouse PLP gene and a SV40 poly(A) signal sequence (Fuss et al., 2000). The final 16.5-kb pPLP/CreERT construct was cut with ApaI and SacII to release a 13-kb PLP/CreERT fragment containing the transgene. For detection of the transgene by PCR analysis the CreERT primers sense 5’-GAT GTA GCC AGC AGC ATG TC-3’
and reverse 5′-ACT ATA TCC GTA ACC TGG AT-3′ were used to amplify a 532-bp PCR product.

**Detection of CreERT mRNA Synthesis in PLP/CreERT Mice**

CreERT mRNA was detected by reverse transcriptase (RT)-PCR. Total RNA was extracted from brain, spinal cord, liver, kidney, heart, lung, and testis tissues of 3-week-old PLP/CreERT mice with the TRIzol Reagent method (Life Technologies). cDNA was synthesized for 10 min at 37°C using 200 units of Moloney murine leukemia virus RT and 2 μg of RNA and was amplified by 30 cycles of PCR at 55°C using primers sense (5′-TCA GAG TCG CAA ACC TCG AG-3′) and reverse (5′-GAT GTA GCC AGC AGC ATG TC-3′) that amplify a 1.5-kb fragment of the PLP/CreERT cDNA. As an internal control, a 400-bp cDNA fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified in the same condition using the sense and reverse primers 5′-CCA CTC ACG GCA AAT TCA ACG GCA-3′ and 5′-TCC AGG CGG CAC GTC AGA TCC ACG-3′, respectively.
Western Blot Analysis of CreERT Expression

Total protein extracts were prepared from brain of 3-week-old transgenic PLP/CreERT mice. Tissues were homogenized in 1% sodium dodecyl-sulfate (SDS), PBS (pH 7.3) for 30 s using a manual potter, and boiled for 10 min. Insoluble material was removed by centrifugation at 5000 × g for 10 min. Protein concentrations were determined using a modified Lowry assay (Bio-Rad, Inc.). Twenty micrograms of each protein sample was resolved on 10% Bis-Tris gel (Novex), transferred to PVDF membranes (Millipore Corp.) and probed overnight at 4°C with polyclonal anti-Cre antibody (Covance BabCo) or 1 h room temperature with monoclonal anti-actin (clone AC-40; Sigma) at dilution of 1:5000 and 1:500, respectively. Antibodies were detected by enhanced chemiluminescence detection system following the instructions of manufacturers (ECL Plus; Amersham).

Immunohistochemical Analysis

For immunohistochemistry, 5-week-old transgenic PLP/CreERT mice and nontransgenic littermate negative controls were used. Anaesthetized mice were perfused intracardially with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.5. Isolated tissues were postfixed for 1 h room temperature with monoclonal anti-actin (clone AC-40; Sigma) at dilution of 1:5000 and 1:500, respectively. Antibodies were detected by enhanced chemiluminescence detection system following the instructions of manufacturers (ECL Plus; Amersham).

Tamoxifen-Induced Nuclear Translocation of CreERT Recombination

To address the CreERT-mediated recombination, the PLP/CreERT mice and nontransgenic littermate negative controls were used. Anaesthetized mice were perfused intracardially with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.5. Isolated tissues were postfixed for 1 h in the same fixative, cryopreserved in 30% sucrose in PB for 48 h at 4°C, embedded in OCT and frozen before cryostat sectioning (10–30 μm). Sections were permeabilized in −20°C acetone for 10 min, washed in PB, and blocked for 2 h at room temperature (RT) in PB containing 10% (v/v) normal goat serum and 0.1% (v/v) Triton X100 and incubated overnight at 4°C in the primary polyclonal antibody (1:5000 dilution) generated against the Cre protein (Covance/BabCo) (Kellendonk et al., 1999), diluted in the same blocking solution. After sections were rinsed in PB containing 0.1% (v/v) Triton X100, bound primary antibodies were detected by incubation with an anti-rabbit Cy3-coupled for 2 h RT. Sections were mounted in Vectashield mounting medium (Vector Laboratories Inc.). For immunoperoxidase staining Cre immunoreactivity was also detected, following the primary antibody, by the avidin-biotin complex method (Vectastain ABC-Elite kit; Vector Laboratories) and visualized with diaminobenzidine (DAB) chromogen (Fig. 2). For double labeling, monoclonal primary antibodies used with polyclonal Cre antibody were directed against MBP (SMI99 clone; Sternberger Monoclonals Inc.), PLP (clone plpc1; Serotec), Adenomatous polyposis coli protein (APC) (clone CC1; Calbiochem-Novabiochem Corp.), glutathione-S-transferase Pi form (GSTPi) (Biotrin Int Ltd.), neurofilament H (SMI32 clone; Sternberger Monoclonals Inc.), and glial fibrillary acidic protein (GFAP) (SMI22 clone; Sternberger Monoclonals Inc.) The above procedure was repeated using anti-rabbit Cy3-coupled and anti-mouse conjugated to biotin secondary antibodies followed by streptavidin fluorescein (Jackson Immuno Research) to detect Cre polyclonal and mouse monoclonal antibodies, respectively. Images were generated on a Leica TCS-NT laser scanning microscope.

FIG. 7. Analyses of β-galactosidase expression pattern by confocal imaging. To assess the localization of β-galactosidase-expressing cells after Cre-mediated recombination, we performed double immunofluorescence labeling of β-galactosidase (red) and Cre recombinase (green) (A) or β-galactosidase (green) and CC1 (red) (B, C), on frozen tissue sections from 5-week-old line 3 PLP/CreERT :floxLacZ mice treated for 8 consecutive days with tamoxifen. Panel shows staining of spinal cord (A), anterior commissure (B), and cerebellum (C). Yellow cells correspond to colocalization of both labeling. Scale bars = 10 μm.
tonically with 100 μl of suspension (1 mg tamoxifen) or with the sunflower oil carrier alone. Immunofluorescence labeling against Cre was performed on spinal cord cryosections as previously described and nuclei were visualized with DAPI staining by adding 0.01% DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Sigma) in Vectashield mounting medium.

Recombination Analysis

PCR was also used to analyze CreER<sup>T</sup>-mediated excision of the floxed CAT cassette from the floxLacZ target allele in various tissues of PLP/CreER<sup>T</sup>-floxLacZ double heterozygotes after 8 days of treatment with tamoxifen or oil carrier. To explore the junction between the β-actin promoter and LacZ, the primers AG10 and Z6 described in Niwa-Kawakita (2000) were used. Recombination was scored by amplification of a 384-bp PCR product. The same primer set also allowed amplification of a 1972-bp fragment corresponding to the nonrecombined transgene.

Beta-Galactosidase Histochemistry

Recombination of the floxed LacZ transgene was analyzed by identification of β-galactosidase-expressing cells after staining with 5-bromo-4-chloro-3-indolylβ-D-galactopyranoside (X-gal) as described by Wight et al. (1993). Briefly, anaesthetized mice were perfused intracardially with PBS followed by 1% paraformaldehyde, 0.5% glutaraldehyde in PBS, pH 7.3, and finally in the same fixative containing 10% sucrose. Tissues were immersed in the fixative solution containing 10% sucrose. Half-brain (hand-cut sagittally along the interhemispheric fissure), sciatic, and optic nerves were directly whole stained. Upon dropping, other half-brain sank in the same solution containing 25% sucrose (usually overnight at 4°C), and was embedded in OCT and frozen before cryostat sectioning (30 μm). The X-gal stain consisted of incubation at 37°C overnight in 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl<sub>2</sub>, 0.02% NP-40, and 0.01% sodium deoxylcholate in PBS. Whole organs or sections were rinsed twice with 3% DMSO in PBS followed by 1% paraformaldehyde, 0.2% glutaraldehyde. Whole organs were photographed and embedded in OCT before cryostat sectioning (30 μm). Then sections were dehydrated in ethanol (70, 80, 95, 100%), cleared in xylene, and mounted with Permount (Fisher Scientific). Recombination of the floxLacZ transgene was also analyzed by immunocytochemical detection on cryostat sections of β-galactosidase, with a polyclonal antibody (Cappel Research Reagents; ICN), and double labeling with the monoclonal antibody against APS protein, as described previously, or the polyclonal anti-Cre antibody using the tyramide signal amplification system (APS, NEN Life Science Products) according to the manufacturer's instructions. Streptavidin-Alexa Fluor 488 conjugate (Molecular Probes) was used to visualized biotin-labeled tyramide. The second antigen (β-galactosidase) was detected by a Cy3-conjugated goat anti-rabbit IgG serum.

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