

Primary demyelination in transgenic mice expressing interferon- γ

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Ever since the use of interferon- γ to treat patients with multiple sclerosis resulted in enhanced disease, the role of IFN- γ in demyelination has been under question. To address this issue directly, transgenic mice were generated that expressed the cDNA of murine IFN- γ in the central nervous system by using an oligodendrocyte-specific promoter. Expression of the transgene occurred after 8 weeks of age, at which time the murine immune and central nervous systems are both fully developed. Directly associated with transgene expression, primary demyelination occurred and was accompanied by clinical abnormalities consistent with CNS disorders. Additionally, multiple hallmarks of immune-mediated CNS disease were observed including upregulation of MHC molecules, gliosis and lymphocytic infiltration. These results demonstrate a direct role for IFN- γ as an inducer of CNS demyelination leading to disease and provide new opportunities for dissecting the mechanism of demyelination.

Primary demyelination is characterized by the active destruction of the myelin sheath that insulates and protects the axons of neurons. It is the most pronounced pathologic feature of multiple sclerosis (MS) and is responsible for much of the pathophysiology of disease. Evidence from epidemiological and biological studies of MS, as well as animal models of demyelinating diseases created to mimic MS, indicates that demyelination is multifactorial and complex with both genetic and environmental influences implicated. Consistent in MS and these models is an immune response in the CNS including an increase in major histocompatibility complex (MHC) expression, macrophage and microglial activation, an increase in expression of adhesion molecules by endothelial cells, enhanced permeability of the blood-brain barrier, and upregulation of the T helper 1 (Th1) cytokine network¹⁻¹⁰. The Th1 inflammatory cytokine, IFN- γ , is expressed in immune-mediated CNS disorders like multiple sclerosis (MS) and animal models of demyelinating disease, as well as in response to infections of the CNS by pathogens like viruses^{2,3,5-7}. IFN- γ is further implicated in demyelinating diseases as it is found in both plaques and the cerebral spinal fluid from MS patients^{5,7}.

Interferon- γ , through direct or indirect mechanisms, can upregulate the expression of a number of immune regulatory molecules including MHC class I and II and inflammatory cytokines¹¹. However, intracerebral injection of IFN- γ into rodents did not result in demyelination¹², and the use of anti-IFN- γ an-

tibodies in the treatment of Theiler's virus-induced demyelination and in experimental allergic encephalitis (EAE) enhanced both diseases^{13,14}. Because IFN- γ also influences viral replication¹⁵, as well as T-cell proliferation and activation¹⁶, the precise role it plays in the demyelination process is unclear. To better understand the role of IFN- γ in the CNS and in demyelination, IFN- γ was exclusively expressed in the CNS using transgenic mouse technology. This enabled us to examine the direct consequences of IFN- γ expression without the complications of the use of viruses^{3,6}, the breakage of the blood-brain barrier by intracranial injection¹², the use of adjuvants and pertussis toxin employed with EAE (ref. 2, 17), or the adoptive transfer of autoreactive T cells¹⁸.

The cDNA of the mouse IFN- γ gene was cloned behind the myelin basic protein (MBP) promoter to direct expression to CNS oligodendrocytes (Fig. 1a) and injected into BALB/cByJ \times C57BL/6 fertilized eggs. The founder line, BG10, expressed the MBP-IFN- γ construct, and progeny were subsequently bred onto the BALB/cByJ (H-2^d) background to the F₄ level. Tissue-specific expression of the spliced transgene mRNA was demonstrated by polymerase chain reaction coupled with reverse transcription (RT-PCR) (Fig. 1b) by using primers flanking an intron within the construct. The correctly sized and spliced RT-PCR product was restricted to nervous system tissue and was not found in liver, kidney, spleen, pancreas, lung, heart or thymus. Transgene expression at the RT-PCR level was found only after 8 weeks of age and was detected in both male and female mice. Expression of IFN- γ protein was identified in the white matter of brains by immunofluorescence with an IFN- γ -specific monoclonal antibody. Uniformly, male MBP-IFN- γ transgenic mice showed symptoms of shaking, hind-limb weakness, hunched posture, ruffled appearance, swollen eyes and a wasting syndrome beginning as early as 8 weeks of age with increasing signs over time leading to death in 10 of 10 mice by 8 months of age. Disease was less frequent (50%) in female mice and clinical signs did not appear until about 6 months of age (5 of 10 mice). The founder mouse was female and did not develop clinical disease yet showed pathology in the CNS similar to that of other transgenic mice. Similar disease linkage to a particular sex has been observed in other murine models of immune-mediated disorders^{19,20}. It is interesting that transgenic mice expressing IFN- γ within the pancreas also showed increased disease in males over females¹⁹, and this suggests an association of hormone responses with IFN- γ -induced immune

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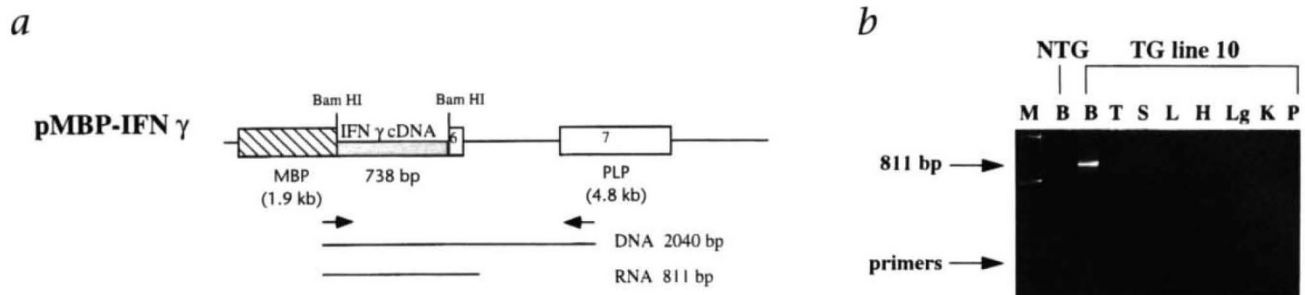


Fig. 1 MBP-IFN- γ transgenic mice: Transgene construct and expression. *a*, pMBP001 contains 1.9 kb of the *MBP* gene promoter/enhancer sequences, and polyadenylation and splice signals from the *PLP* gene. The murine IFN- γ cDNA was cloned into the *Bam*HI site of pMBP001 to give pMBP-IFN- γ . Expression of spliced mRNA in transgenic mice was determined by RT-PCR using primers that hybridized within the transgene and the *PLP* exon 7 as indicated by arrows, with the resulting RT-PCR product of 811 bp. *b*, Brain-specific expression of the transgene was demonstrated by RT-PCR analysis of total RNA extracts from the brain (B), thymus (T), spleen (S), liver (L), heart (H), lung (Lg), kidney (K), and pancreas (P) of a 6-month-old MBP-IFN- γ transgenic (TG) mouse and the brain from a 6-month-old nontransgenic (NTG) littermate. M, molecular weight markers (100-bp marker from Gibco-BRL, Gaithersburg, MD, upper band 1500 bp, lower band 600 bp).

responses. Because expression of IFN- γ did not occur until after 8 weeks of age, this allowed the CNS of the mice to develop normally before IFN- γ exposure. This was advantageous because it provided a sudden, continuous source of IFN- γ

throughout the CNS without the limitations in distribution found with mechanical injection or the influences of other factors upregulated during pathogen infection. The delay in expression is not consistent with prior work that demonstrated

Fig. 2 Pathologic changes in the CNS of MBP-IFN- γ transgenic mice. *a*, Colocalization of MBP antigen within F4/80-staining macrophages/microglia in a 12-week-old MBP-IFN- γ transgenic mouse by confocal microscopic analysis. Sagittal sections were double-stained for immunofluorescence and analyzed by laser scanning confocal microscopy (scale bar, 50 μ m). The panels labeled 1–3 show a region of the cortex stained with antibodies to F4/80 (1, green), MBP (3, red), and the merged view with both labels (2, areas of colocalization of MBP and F4/80 are presented in yellow). Arrows denote macrophages/microglia with colocalization of MBP staining. The single panel (4) shows a similar region of the cortex stained with antibodies to CD31 (green, endothelial cells) and MBP (red). No colocalization of signal is observed in the CD31/MBP stained section. *b*, Increased expression of MHC class I in the brains of MBP-IFN- γ transgenic mice as detected by immunohistochemical analysis of sagittal sections with monoclonal antibody to MHC class I. Section 1 is from an MBP-IFN- γ transgenic 6-month-old male mouse, section 2 is from a transgenic 12-week-old male, section 3 is from a transgenic 12-week-old female, section 4 is from a transgenic 4-week-old male, and section 5 is from a 12-week-old nontransgenic male. *c*, Increased expression of MHC and F4/80 in the brain of a female 12-week-old MBP-IFN- γ transgenic mouse. Sagittal brain sections from transgenic and nontransgenic mice were stained with antibodies to MHC class I, MHC class II and F4/80 as indicated. The panels are consecutive sections of low-power views of the hippocampus, fimbria and corpus callosum (scale bar, 400 μ m; original magnification \times 40). Similar results were found in multiple sections from five transgenic and five nontransgenic mice.

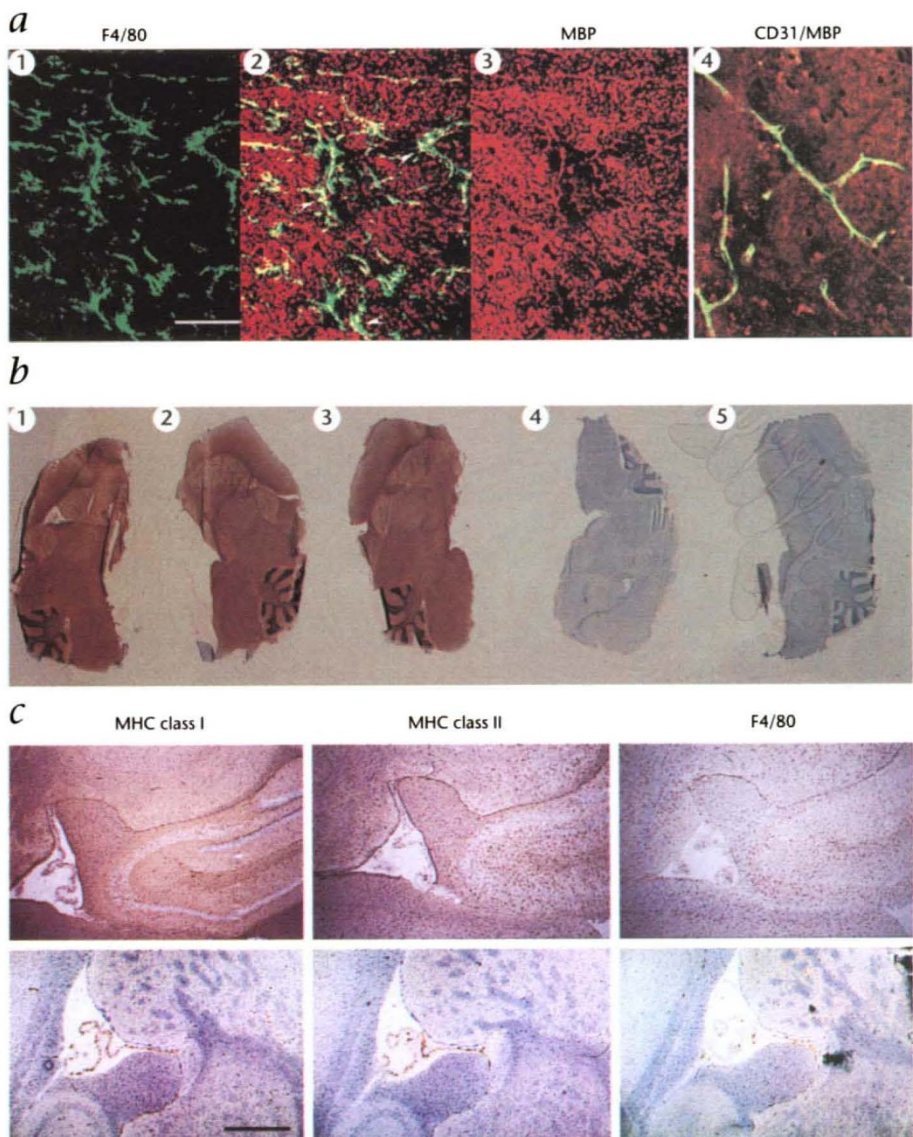
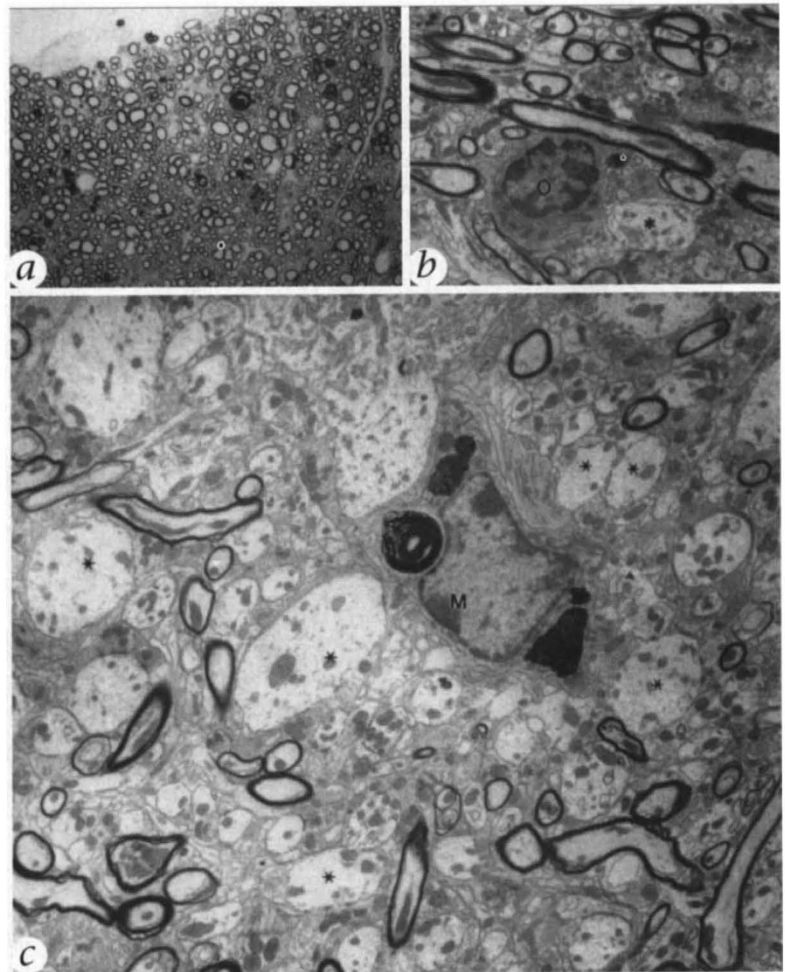


Fig. 3 Primary demyelination detected by electron microscopy of the spinal cord of a 6-month-old female MBP-IFN- γ transgenic mouse. *a*, Sections of the spinal cord (1 μ m thick and embedded in plastic) show small focal areas of demyelination in association with degenerating myelin whorls (original magnification, $\times 1100$). *b*, Normal appearing oligodendrocytes (O) in association with demyelinated axons (*) (original magnification, $\times 6250$). *c*, Multiple demyelinated axons (stars) in association with a macrophage/microglial cell (M) with engulfed myelin debris (original magnification $\times 7800$).



that this promoter expresses transgenes early in life and continuously through adulthood, consistent with endogenous MBP expression^{21,22}, and is most likely due to insertion site influences within the genome on the transgene. The observed changes are thus specifically attributable to the effect of IFN- γ on a mature adult CNS and not owing to any developmental defect caused by transgene expression²³.

Degradation of the myelin sheath and primary demyelination occurred in MBP-IFN- γ -expressing transgenic mice as seen by both confocal and electron microscopy. Double-immunofluorescent labeling with antibodies to MBP and F4/80, a macrophage/microglia marker, revealed both the presence of a large number of activated macrophages/microglia throughout the brain of transgenic male and female mice as early as 8 weeks of age, and the colocalization of MBP within these activated macrophages/microglia (Fig. 2*a*). The presence of myelin degradation products within phagocytic cells of the CNS suggested an active demyelination process. By contrast, similarly treated brain sections from nontransgenic and transgenic mice expressing a viral transgene, lymphocytic choriomeningitis virus nucleoprotein (LCMV-NP)²², under the same MBP promoter, revealed that microglia expressing F4/80 throughout the CNS did not colocalize with MBP signal (data not shown). Further, the analysis of 6-month-old MBP-IFN- γ transgenic mice by electron microscopy demonstrated primary demyelination (Fig. 3). Demyelinated axons, as well as axons with degenerating myelin sheaths, were found throughout the brain and adjacent to macrophage/microglia that were engulfing myelin debris. Although macrophage/microglia were commonly detected at the sites of demyelination, infiltrating T cells were only rarely observed (data not shown). Despite the presence of demyelinated axons, oligodendrocyte cell bodies were morphologically normal. The presence of morphologically normal oligodendrocytes suggests that expression of the transgene itself was not detrimental to these cells as had been previously reported in other transgenic systems²⁴.

Expression of IFN- γ in the CNS resulted in increased expression of MHC class I and II molecules (Fig. 2, *b* and *c*). The level of MHC expression was equivalent in male and female mice and remained high over the lifetime of the animal. Immunohistochemical (Fig. 2, *b* and *c*) and double-label immunofluorescent analyses by confocal microscopy showed that the increase in MHC class I and II expression was widespread in both white and gray matter, and localized to endothelial cells, microglia and oligodendrocytes, but not to neurons or astrocytes (M.S.H., C.F.E., F.G. Klier and M.B.A.O., manuscript in preparation). Although the lack of MHC expression on neurons *in vivo* was anticipated from earlier publications^{25,26}, the absence

on astrocytes *in vivo* was unexpected⁴⁵. Expression of MHC on astrocytes may require an additional factor or alternate pathway of induction, and this is currently under investigation. Previous work had established the ability of oligodendrocytes to express MHC class I and II under certain tissue culture conditions including in the presence of IFN- γ (ref. 27, 28) as well as during viral infection^{4,29}. The presence of activated microglia and astrocytes was demonstrated by immunohistochemical staining with antibodies to F4/80 (Fig. 2*c*) and glial fibrillary acidic protein (GFAP) (data not shown), respectively. By contrast, in transgenic mice before their 8th week of life, MHC class I and II expression was not detectable on oligodendrocytes and limited to a few endothelial cells and to cells within the choroid plexus. Microglia and astrocytes at this time and in nontransgenic mice showed limited expression of F4/80 (Fig. 2*c*) and GFAP (data not shown). Infiltrating lymphocytes were predominantly CD8-positive (90%) and were found only in transgenic mice. These cells were scattered diffusely throughout the parenchyma of the brain and spinal cord (data not shown). Their limited numbers (200 T cells per sagittal brain section) prohibited recovery from the CNS and analysis of their immune specificity. The T-cell infiltration correlated with an increase in endothelial cell expression of ICAM-1, an adhesion molecule involved in lymphocyte homing and migration (data not shown), and the presence of serum proteins, albumin and IgG, on the parenchymal side of CNS endothelial vessels (data not shown) suggesting leakage of the blood-brain barrier.

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Neither ICAM-1 elevation nor leakage in the blood-brain barrier was noted in nontransgenic mice, or in MBP-IFN- γ transgenic mice before their 8th week of life. Specificity for the action of IFN- γ was further ensured when other transgenic mice that utilized the same MBP promoter expressing β -galactosidase²¹ or a LCMV-NP (ref. 22) as transgenes did not show elevations of MHC class I or class II, activated microglia or astrocytes, or T-cell infiltration over a 1-year period of observation.

Constitutive expression of IFN- γ in the CNS upregulated a number of cytokines. RNase protection assays detected significant levels of IFN- γ in 8-week-old or older transgenic mice, as well as increased message levels of interleukin-12p40 (IL-12p40), tumor necrosis factor- α (TNF- α), lymphotoxin β , IL-1 α , and IL-1 β when compared with nontransgenic mice (data not shown). No changes in the levels of mRNAs for IL-6, IL-10, IL-12p35, IL-1 receptor, TNF- β , IFN- β , transforming growth factors TGF- β 1 and TGF- β 2, or migration inhibition factor (MIF) were detected in transgenic and control mouse brains. Thus, constitutive expression of IFN- γ in the CNS directly activated the expression of mRNAs for IL-12p40, TNF- α , lymphotoxin β , IL-1 α , and IL-1 β ; of these, TNF- α has been implicated in oligodendrocyte or myelin injury¹¹.

In a similar study²³, transgenic mice were generated with oligodendrocyte expression of IFN- γ . However, these mice expressed IFN- γ early in life and exhibited profound pathologic defects to the myelin sheath including severe hypomyelination, inability to propagate, and premature death due to this early IFN- γ expression. In addition, these mice shared some of the features found in the mice presented here including demyelination and upregulation of immune molecules. Although both models give evidence of demyelination following IFN- γ expression in the CNS and of support for an active role for IFN- γ in immune-mediated demyelination, the mouse model presented here is not complicated by the developmental defects due to IFN- γ overexpression during CNS development.

This transgenic model of constitutive IFN- γ expression in the adult brain offers a unique opportunity to dissect the molecular mechanisms involved in demyelinating disease. In these mice, IFN- γ may be directly deleterious to oligodendrocytes as has been shown *in vitro*²⁰. It may also act indirectly by activating cells and factors that subsequently injure myelin. Macrophages/microglia are activated in these mice as a result of IFN- γ expression, and these cells release potentially toxic factors like TNF- α and nitric oxide that can alter oligodendrocyte function^{11,31}. Similarly, IFN- γ may activate myelin-specific autoreactive lymphocytes, as has been reported for transgenic mice that developed lymphocytes specifically against islets as a result of pancreatic β -cell-specific IFN- γ expression¹⁹. Future experiments breeding these mice with cytokine or MHC gene knockout mice will further identify factors that play roles in demyelination and will likely provide unique pharmacologic strategies for the treatment of demyelinating diseases like MS.

Methods

The generation and screening of transgenic mice. PCR amplification of the cDNA was done to facilitate cloning by adding restriction enzyme sites using primers that hybridize to the 5' and 3' ends of the cDNA and amplify a region including the entire coding region of the gene: Primer 1, which hybridizes to base pairs (bp) 1–32, 5'–GGATCCGAATTC-

GAGCTCGGTACCCAATTCTG–3'; and primer 2, which hybridizes to bp 743–710, 5'–GGATCCGAATTCCTTATCACATATAAGTTG–3'. The 743-base pair product was cloned into the TA vector system (Invitrogen, San Diego, CA), and then digested with *Bam*HI for cloning. The cDNA was cloned into the *Bam*HI site of a plasmid, pMBP001, containing sequences from –1907 to +36 of the MBP promoter, followed by a polylinker region linked to a portion of the proteolipid protein (PLP) gene as described²¹. The PLP sequences provided splice and polyadenylation signals and included PLP exon 6, intron 6 and exon 7. A linear DNA fragment for microinjection was obtained by *Not*I digestion and injected by the Scripps Research Institute Transgenic Facility into BALB/cByJ \times C57BL/6J fertilized eggs. Transgenic founders were identified by PCR amplification of the novel IFN- γ construct from samples of DNA extracted from tail biopsies. Total RNA was prepared by a standard guanidium isothiocyanate procedure.

Immune staining of CNS tissue. Mice were anesthetized and perfused with 40 ml saline. Brains and spinal cord were removed, snap-frozen and stored at –20 °C. Immunohistochemistry was performed on 10- to 12- μ m cryostat sections as described²². Sections were stained with the following primary antibodies: anti-CD8 (anti-Ly-2 and Ly-3, PharMingen, La Jolla, CA), anti-CD4 (anti L3T4, PharMingen), anti-H–2 monotypic antigen (MHC class I), anti-Ia antigen (MHC class II) (Boehringer Mannheim, Indianapolis, IN), anti-GFAP (Dako, Carpinteria, CA) and anti-F4/80 (Serotec, Washington, DC). The second antibody was biotinylated and was used in conjunction with the Vectastain Elite ABC (peroxidase) kit (Vector Laboratories, Burlingame, CA). Staining was detected using diaminobenzidine as a chromogen. Sections were counterstained in Mayer's hematoxylin (Sigma Chemical Co.). Immunofluorescence staining for confocal microscopy was performed on similarly prepared, cut and fixed cryostat brain sections. Sections were blocked with Superblock (ScyTek, Logan, Utah) and stained with primary antibodies; rat anti-mouse F4/80, rat anti-mouse CD31 (PharMingen) and mouse anti-rat myelin basic protein (Boehringer Mannheim). Secondary fluorescent antibodies used were Texas red-labeled horse anti-mouse IgG and fluorescein isothiocyanate (FITC)-labeled rabbit anti-rat IgG (Vector Laboratories), and the slides were mounted in Vectashield (Vector Laboratories). Double-immunolabeled sections were examined with a Bio-Rad (MRC 600; Hercules, CA) laser scanning confocal microscope equipped with an argon/krypton mixed gas laser.

Electron microscopy. Six-month-old mice were perfused with Trump's fixative as previously described¹⁴. Brains and spinal cords were extracted and placed in fixative for 24–48 hours at which time they were embedded into Araldite plastic. Sections 1 μ m thick were cut and stained with 1% toluidine blue. The brain stem, thalamus, hippocampus and corpus callosum were examined. Selected areas were trimmed for electron microscopy focusing primarily on white matter tracks.

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