Endoplasmic reticulum stress modulates the response of myelinating oligodendrocytes to the immune cytokine interferon-γ

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Interferon-γ (IFN-γ) is believed to contribute to immune-mediated demyelinating disorders by targeting the myelin-producing oligodendrocyte, a cell known to be highly sensitive to the disruption of protein synthesis and to the perturbation of the secretory pathway. We found that apoptosis induced by IFN-γ in cultured rat oligodendrocytes was associated with endoplasmic reticulum (ER) stress. ER stress also accompanied oligodendrocyte apoptosis and hypomyelination in transgenic mice that inappropriately expressed IFN-γ in the central nervous system (CNS). Compared with a wild-type genetic background, the enforced expression of IFN-γ in mice that were heterozygous for a loss of function mutation in pancreatic ER kinase (PERK) dramatically reduced animal survival, promoted CNS hypomyelination, and enhanced oligodendrocyte loss. PERK encodes an ER stress–inducible kinase that phosphorylates eukaryotic translation initiation factor 2α and specifically maintains client protein homeostasis in the stressed ER. Therefore, the hypersensitivity of *PERK+/−* mice to IFN-γ implicates ER stress in demyelinating disorders that are induced by CNS inflammation.

Introduction

The ER is a membranous labyrinthine network that extends throughout the cytoplasm of eukaryotic cells and is contiguous with the nuclear envelope. Approximately one third of all cellular proteins, particularly membrane-spanning and secreted proteins, are translocated into the lumen of the ER, where post-translational modification, folding, and oligomerization occur. The ER is also the site for the biosynthesis of steroids, cholesterol, and other lipids, and it is the major signal-transducing organelle in the cell that continuously responds to environmental cues to release calcium (Kaufman, 1999). A number of cell stress conditions, such as perturbed calcium homeostasis or redox status, elevated secretory protein synthesis rates, altered glycosylation levels, and cholesterol overloading, can interfere with oxidative protein folding. This can subsequently lead to the accumulation of unfolded or misfolded proteins in the ER lumen, which has been referred to ER stress (Ma and Hendershot, 2001; Ron, 2002; Rutkowski and Kaufman, 2004).

Three ER-resident transmembrane proteins have been identified as proximal sensors of ER stress: pancreatic ER kinase (PERK), the kinase encoded by the inositol requiring (IRE) 1 gene, and activating transcription factor (ATF) 6. At the onset of ER stress, the most rapidly activated pathway is translational repression, which is mediated by PERK (Ron and Harding, 2000). PERK couples protein folding in the ER with protein synthesis by phosphorylating the α subunit of the eukaryotic translation initiation (eIF) factor 2, which attenuates the initiation of translation in response to ER stress (Harding et al., 1999; Okada et al., 2002). The cleavage of ATF6 and the activation of IRE1 signaling follow fairly quickly; these processes promote the expression of ER-localized chaperones that facilitate the restoration of proper protein folding within the ER (Yoshida et al., 2001; Calfon et al., 2002; Okada et al., 2002). These protective responses act transiently to maintain homeostasis within the ER, but sustained ER stress ultimately leads to the apoptotic death of the cell (Ma and Hendershot, 2001; Ron, 2002; Rao et al., 2004; Rutkowski and Kaufman, 2004).

Oligodendrocytes produce vast amounts of myelin as an extension of their plasma membrane; a unique, lipid-rich,

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Abbreviations used in this paper: ATF, activating transcription factor; BIP, binding immunoglobulin protein; CGT, ceramide galactosyltransferase; CHOP, CAATT enhancer–binding protein homologous protein; CNP, 2′3′-cyclic nucleotide 3′-phosphodiesterase; CNS, central nervous system; GFAP, glial fibrillary acidic protein; E 14, embryonic day 14; eIF, eukaryotic translation initiation factor; IFN-γ, interferon-γ; iNOS, inducible NO synthase; IRE, inositol requiring; MBP, myelin basic protein; MHC, major histocompatibility complex; MS, multiple sclerosis; NO, nitric oxide; PERK, pancreatic ER kinase; PLP, proteolipid protein; PMD, Pelizaeus-Merzbacher disease; tTA, tetracycline-controlled transactivator; VWM, leukoencephalopathy with vanishing white matter.

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multilamellar sheath that wraps axons in the central nervous system (CNS). On the basis of estimates from morphometric analysis, the mean surface area of myelin membrane per mature oligodendrocyte is 1–20 $\times$ 10^5 μm^2, compared with a perikaryal surface area of only 100–300 μm^2. During the active phase of myelination, each oligodendrocyte in the CNS must produce as much as $\sim$5,000 μm^2 of myelin surface area per day and $\sim 10^5$ myelin protein molecules per minute (Pfeiffer et al., 1993). Perhaps not surprisingly, data from human hypomyelinating diseases and from dysmyelinating animal models suggest that oligodendrocytes rank among the cells that are most sensitive to the disruption of protein translation and protein secretory pathways. Leukoencephalopathy with vanishing white matter (VWM) is caused by mutations in the gene encoding eIF-2B (Leegwater et al., 2001; Fogli et al., 2004). Moreover, the leukodystrophy Pelizaeus-Merzbacher disease (PMD), which results from mutations in the myelin proteolipid protein (PLP) gene, is associated with perturbation of the protein secretory pathway, as are PMD animal models with point mutations in the PLP gene (Bauer et al., 2002; Southwood et al., 2002).

The pleotropic cytokine interferon-γ (IFN-γ), which is secreted by activated T lymphocytes and by natural killer cells, is believed to play a deleterious role in immune-mediated demyelinating disorders such as multiple sclerosis (MS) and experimental allergic encephalomyelitis (Popko et al., 1997; Popko and Baerwald, 1999; Steinman, 2001a). This cytokine, which is normally not present in the CNS, is detectable during the symptomatic phase of these disorders (Panitch, 1992). The administration of IFN-γ to patients with MS leads to a worsening disease course (Panitch et al., 1987), and treatment of such patients with an IFN-γ antibody delays disability progression (Skurkovich et al., 2001). In vitro IFN-γ is capable of promoting apoptosis in purified developing oligodendrocytes (Andrews et al., 1998; Baerwald and Popko, 1998; Feldhaus et al., 2004). Moreover, transgenic mice that ectopically express IFN-γ in the CNS display a tremoring phenotype and myelin abnormalities (Corbin et al., 1996; LaFerla et al., 2000). Nevertheless, the mechanisms by which the presence of IFN-γ leads to oligodendroglial abnormalities and to alterations to the myelin sheath remain poorly understood. We provide evidence for the activation of the ER stress response in oligodendrocytes that are exposed to IFN-γ in culture and in transgenic mice. Moreover, we demonstrate that PERK, a kinase that responds specifically to ER stress, modulates disease severity in animals that ectopically express IFN-γ and serves to protect oligodendrocytes from apoptosis.

Results

IFN-γ–induced apoptosis in rat oligodendrocytes is associated with ER stress

Our initial efforts to characterize the ER stress response in oligodendrocytes exposed to IFN-γ were performed in vitro. Purified oligodendrocyte progenitor cells were allowed to differentiate for 5 d in defined media, at which point $\sim$40% of the cells expressed the myelin protein 2′3′-cyclic nucleotide 3′-phosphodiesterase (CNP) and extended branched processes. These cells did not extend the flat membrane sheets that are characteristic of more mature oligodendrocyte cultures. When treated with 70 U/ml IFN-γ for 48 h, these cells showed abnormal morphological changes, including cell shrinkage and aggregation of cell bodies, followed by detachment from the

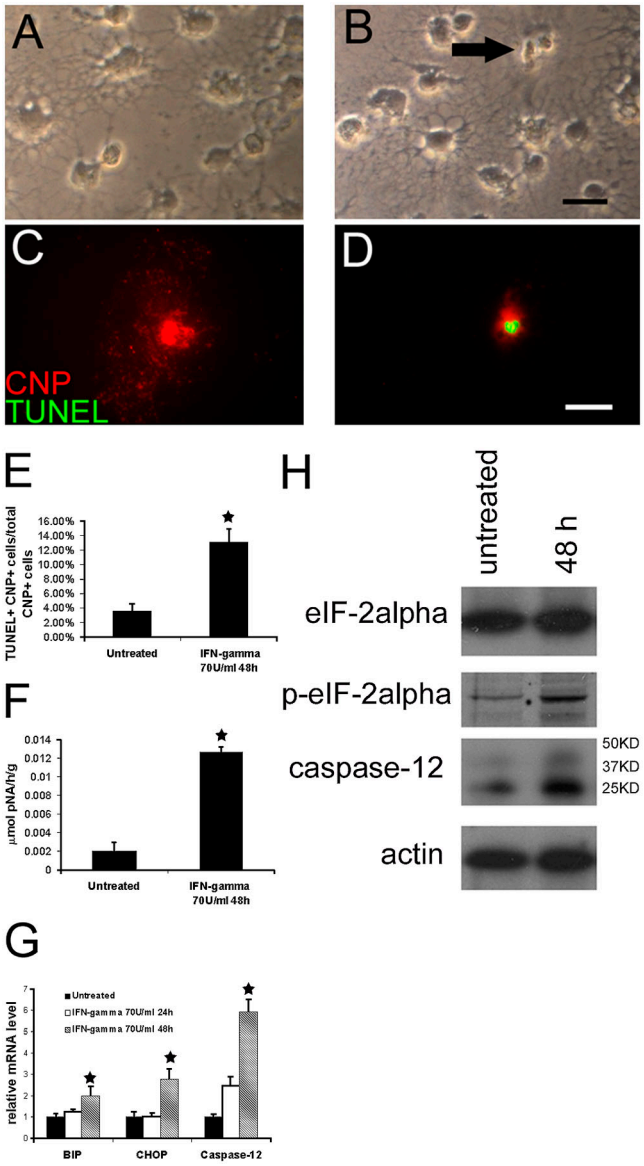

Figure 1. **IFN-γ–induced apoptosis in cultured rat oligodendrocytes is associated with ER stress.** (A) Untreated oligodendrocytes that underwent differentiation for 7 d. (B) Oligodendrocytes that underwent differentiation for 5 d and treatment with 70 U/ml IFN-γ for 48 h, revealing cell shrinkage and aggregation of cell bodies (arrow). (C and D) TUNEL and CNP double labeling for untreated oligodendrocytes that underwent differentiation for 7 d (C) and for oligodendrocytes that underwent differentiation for 5 d and treatment with 70 U/ml IFN-γ for 48 h (D). (E) Quantitation of TUNEL and CNPase double positive cells; *, $P < 0.05$. (F) Caspase-3 activity assay in the oligodendrocyte lysates; *, $P < 0.01$. (G) Real-time PCR analyses of the expression of BIP, CHOP, and caspase-12 in oligodendrocytes treated with 70 U/ml IFN-γ; *, $P < 0.05$. (E–G) Error bars represent standard deviation. (H) Western blot analyses of total eIF-2α, p-eIF-2α, and caspase-12 in oligodendrocytes treated with 70 U/ml IFN-γ. All experiments were repeated at least three times. Bars: (A and B) 30 μM; (C and D) 20 μM.

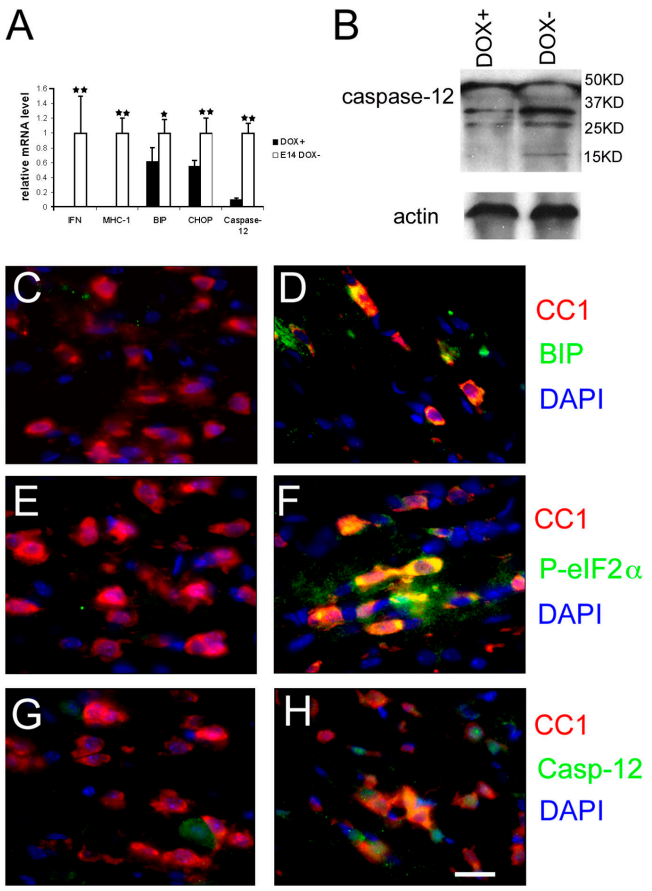

Figure 2. **Hypomyelination induced by ectopically expressed IFN-γ is associated with ER stress.** (A) Real-time PCR analyses for detection of mRNA in the brains of 14-d-old mice ectopically expressing IFN-γ ($n = 3$); *, $P < 0.05$; **, $P < 0.01$. Error bars represent standard deviation. (B) Western blot analyses for caspase-12 in the CNS of 14-d-old double transgenic mice released from doxyclycline at E 14. (C and D) BIP and CC1 double immunostaining in the spinal cord of 14-d-old double transgenic mice that received doxycycline (C) or were released from doxycycline at E 14 (D). (E and F) p-eIF-2α and CC1 double immunostaining in the spinal cord of 14-d-old double transgenic mice that received doxycycline (E) or were released from doxycycline at E 14 (F). (G and H) Caspase-12 and CC1 double immunostaining in the spinal cord of 14-d-old double transgenic mice that received doxycycline (G) or were released from doxycycline at E 14 (H). (C–H) $n = 3$; bar, 30 μM.

culture plate (Fig. 1, A and B). TUNEL and CNP double labeling revealed that IFN-γ induced apoptosis in a significant number of oligodendrocytes (Fig. 1, C–E). Furthermore, the caspase-3 activity in the cell lysates of IFN-γ–treated oligodendrocytes was markedly increased (Fig. 1 F). Thus, 70 U/ml IFN-γ is able to induce apoptosis in oligodendrocytes that are actively synthesizing myelin components.

To determine whether IFN-γ interferes with ER function, we monitored the expression of ER stress markers in cytokine-treated oligodendrocyte cultures. The levels of mRNA that encode the binding immunoglobulin protein (BIP)/78-kD, glucose-regulated protein and the CAATT enhancer binding protein homologous protein (CHOP)/growth and DNA damage protein 153, both of which are associated with the ER stress response, were increased ~2–3 times in oligodendrocytes after exposure to IFN-γ (Fig. 1 G). The phosphorylation of eIF-2α, which inhibits nucleotide exchange on the eIF-2 complex and attenuates most protein synthesis, occurs within minutes after the development of ER stress (Ron, 2002). Western blot analysis revealed that IFN-γ significantly elevated the level of phosphorylated eIF-2α (p-eIF-2α) in oligodendroglial cultures (Fig. 1 H). Caspase-12, an ER-localized caspase, is activated by ER stress and can lead to the cleavage of caspase-3 (Nakagawa et al., 2000; Lamkanfi et al., 2004). The induction of caspase-12 was observed after the treatment of oligodendrocytes with IFN-γ (Fig. 1 G). Moreover, the level of the active fragment of caspase-12 was strongly elevated after 48 h of IFN-γ treatment (Fig. 1 H). These results indicate that IFN-γ–induced apoptosis in cultured oligodendrocytes is associated with the activation of the ER stress pathway.

Hypomyelination induced by ectopic expression of IFN-γ is associated with ER stress

We have generated transgenic mice that allow for the temporally regulated delivery of IFN-γ to the CNS using the tetracycline-controllable system (Lin et al., 2004). To drive tetracycline-controlled transactivator (tTA) expression in astrocytes, we chose the transcriptional regulatory region of the glial fibrillary acidic protein (GFAP) gene, which has been well characterized in transgenic studies (Brenner et al., 1994). *GFAP/tTA* mice were mated with *TRE/IFN*-γ mice to produce animals that were hemizygous for both transgenes. When these mice are maintained on doxycycline, the expression of the IFN-γ transgene is repressed (Fig. 2 A). When double transgenic mice were released from doxycycline at embryonic day 14 (E 14), mRNA for IFN-γ could be detected as early as 10 d after birth (unpublished data). Real-time PCR analysis showed that these mice expressed robust levels of IFN-γ and major histocompatibility complex (MHC) class I, a downstream target of IFN-γ activity, in the CNS at postnatal day 14 (Fig. 2 A). The double transgenic mice that ectopically expressed IFN-γ in the CNS during development are mildly hypomyelinated (see Figs. 4 and 5). This is consistent with observations made on previously generated transgenic mice that expressed IFN-γ constitutively in oligodendrocytes (Corbin et al., 1996). The diminished myelination observed in these mice is correlated with IFN-γ–induced oligodendrocyte apoptosis (see Fig. 7, A and F). IFN-γ up-regulated BIP and CHOP expression ~1.6 and 2 times those of the control levels and strongly enhanced caspase-12 expression in the CNS of these animals (Fig. 2 A). More notable, the level of the active fragment of caspase-12 was also increased in the CNS of these animals (Fig. 2 B). Furthermore, colocalization analysis with the CC1 antibody revealed that oligodendrocytes increased the expression of BIP (Fig. 2, C and D), p-eIF-2α (Fig. 2, E and F), and caspase-12 (Fig. 2, G and H). These data support a link between ER stress and IFN-γ–induced oligodendrocyte apoptosis and hypomyelination during development.

Hypersensitivity of *PERK+/−* mice to the conditional misexpression of IFN-γ

We next pursued a genetic approach to examine the involvement of the ER stress response in the myelin perturbations that

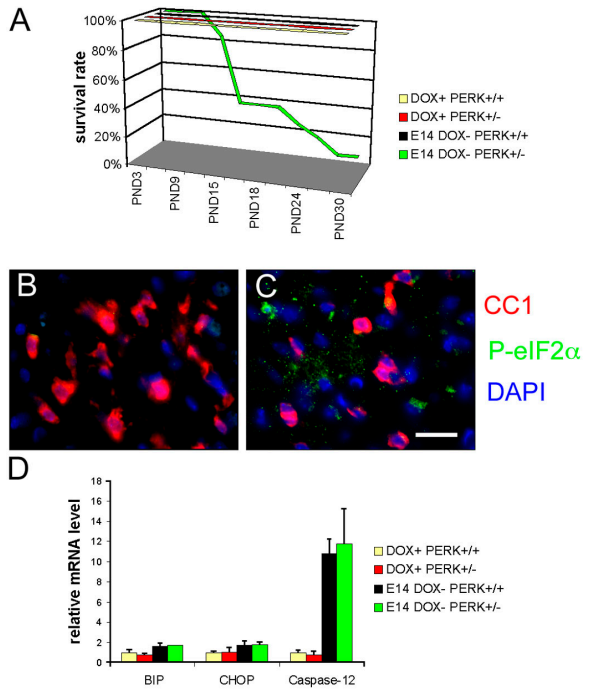

Figure 3. **Hypersensitivity of *PERK+/−* mice to the conditional misexpression of IFN-γ.** (A) Mouse survival curve ($n = 40$ for each group). (B and C) p-eIF-2α and CC1 double labeling in the spinal cord of 14-d-old *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice that received doxycycline (B) or were released from doxycycline at E 14 (C). (B and C) $n = 3$; bar, 30 μM. (D) Real-time PCR analyses of mRNA levels in the brain of 14-d-old mice ($n = 3$). Error bars represent standard deviation.

are elicited by IFN-γ. Three pathways are known to signal ER stress. The IRE1–X-box–binding protein-1 pathway is the oldest and most conserved; however, in mammals, it appears to have been diverted to the control of genes involved in remodeling the ER to high capacity secretion (Ron and Hampton, 2004). Furthermore, both IRE1 and X-box–binding protein-1 mutant mice die at early embryonic stages, making their use to the study of the myelination process ineffective (Ma and Hendershot, 2001). Currently, there are no genetic tools available to explore the ATF6 pathway in mice. The PERK–eIF-2α pathway, on the other hand, contributes to the activation of most ER stress target genes (Harding et al., 2003; Lu et al., 2004). Moreover, although *PERK−/−* mice have a complex phenotype that includes progressive diabetes mellitus, exocrine pancreatic insufficiency, growth retardation, and high mortality (Harding et al., 2001; Zhang et al., 2002), *PERK+/−* mice, although healthy, display evidence of haploid insufficiency (Harding et al., 2000a). Thus, to examine the influence of the ER stress response on myelin and oligodendrocyte abnormalities that are elicited by IFN-γ, we exploited PERK mutant mice (Harding et al., 2001).

GFAP/tTA and *TRE/IFN-γ* mice were crossed with *PERK+/−* mice, and the resulting progeny were intercrossed to obtain double transgenic mice that were homozygous or heterozygous for the *PERK* mutation. As reported, the majority of double transgenic mice with a *PERK−/−* background died within 12 d after birth, regardless of whether they received doxycycline during the entire period or if doxycycline was switched to water at E 14. Double transgenic *GFAP/tTA; TRE/IFN-γ* mice on a *PERK+/+* background, released from doxycycline at E 14, showed the expected minor tremor and ataxia but exhibited good survival. In contrast, the double transgenic mice on a *PERK+/−* background had a much more severe phenotype. These animals were considerably smaller than their IFN-γ–expressing *PERK+/+* littermates or *PERK+/−* animals that did not inherit the combination of *GFAP/tTA* and *TRE/IFN-γ* alleles and that showed severe tremor and ataxia. Approximately two thirds of these mice experienced tonic seizures. Strikingly, >90% of the double transgenic mice that were released from doxycycline at E 14 on a *PERK+/−* background died by postnatal day 27, whereas double transgenic mice on a wild-type background displayed normal survival (Fig. 3 A).

We next investigated the correlation between the severities of the *PERK+/+* and *PERK+/−* animals' phenotype with the animals' capacities to attenuate protein synthesis in response to IFN-γ by examining the level of p-eIF-2α. Highly elevated levels of p-eIF-2α in oligodendrocytes of the CNS were observed in double transgenic mice on a *PERK+/+* background (Fig. 2, E and F). In contrast, only a slight increase in p-eIF-2α immunoreactivity was observed in the CNS of double transgenic mice on a *PERK+/−* background (Fig. 3, B and C). Nevertheless, we did not find that the loss of function mutation in *PERK* significantly affected the RNA levels of BIP, CHOP, and caspase-12 in the CNS of mice misexpressing IFN-γ (Fig. 3 D). Collectively, these data indicate that the reduced capacity to elevate p-eIF-2α levels in response to IFN-γ contributes to the severe phenotype in mice misexpressing IFN-γ on a *PERK+/−* background.

IFN-γ misexpression leads to severe hypomyelination in a *PERK+/−* background

The tremoring phenotype with tonic seizures, which was displayed by *PERK+/−* mice that express IFN-γ in the CNS, is suggestive of myelin perturbations. Immunostaining for myelin basic protein (MBP) was notably reduced in the CNS of 14-d-old

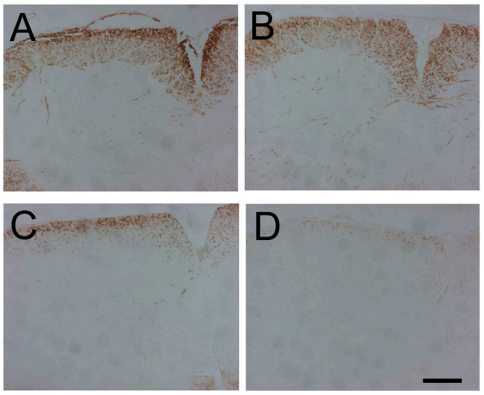

Figure 4. **Double transgenic mice with a *PERK+/−* background develop severe hypomyelination.** (A and C) MBP immunostaining in the spinal cord of 14-d-old double transgenic mice that received doxycycline (A) or were released from doxycycline at E 14 (C). (B and D) MBP immunostaining in the spinal cord of 14-d-old *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice that received doxycycline (B) or were released from doxycycline at E 14 (D). (A–D) $n = 3$; bar, 150 μM.

GFAP/tTA; *TRE/IFN-γ*; *PERK+/−* mice released from doxycycline at E 14, compared with double transgenic mice on a wild-type background (Fig. 4). Moreover, ultrastructural examination revealed that the majority (81% ± 14.9%) of axons in the spinal cord of *PERK+/−* mice that express IFN-γ in the CNS were unmyelinated (Fig. 5). In contrast, double transgenic animals on a wild-type background that were released from doxycycline at E 14 displayed considerably fewer unmyelinated axons (30% ± 12.9%), whereas animals that were maintained continuously on doxycycline to repress IFN-γ expression had even fewer unmyelinated axons (9.8% ± 6.1%). Thus, these data establish a correlation between the severe tremoring phenotype induced by IFN-γ on the *PERK+/−* background and hypomyelination.

Loss of oligodendrocytes after IFN-γ misexpression in *PERK+/−* mice

Next, we examined the status of oligodendrocyte function in these animals to gain insight into the cellular mechanisms that account for the hypomyelination displayed by *PERK+/−* mice expressing IFN-γ in the CNS. We determined the steady-state levels of mRNAs that encode the myelin markers MBP, PLP, and ceramide galactosyltransferase (CGT). Real-time PCR analysis showed that MBP, PLP, and CGT mRNA levels were slightly lower than normal in the brains of 14-d-old double transgenic mice that were on a wild-type background and were released from doxycycline at E 14. These mRNA levels were even lower in the CNS of *GFAP/tTA*; *TRE/IFN-γ*; *PERK+/−* mice released from doxycycline at E 14 (Fig. 6). To determine whether the decreased steady-state levels of myelin protein–encoding mRNAs were caused by reduced numbers of myelinating cells, we determined oligodendroglial numbers in these mice. Compared with control mice, there were slightly fewer oligodendrocytes identified by CC1 immunostaining in the CNS of 14-d-old *GFAP/tTA*; *TRE/IFN-γ* transgenic mice that were on a wild-type background and were released from doxycycline at E 14 (Fig. 7 A). In contrast, very few oligodendrocytes could be detected in the corpus callosum and in the cerebellum of IFN-γ–expressing transgenic mice on a *PERK+/−* background, and oligodendrocyte numbers in the spinal cord of these mice were decreased by >50% (Fig. 7 A).

In addition, the number of oligodendrocytes that were TUNEL positive in the cervical spinal cord of these mice was 2.5 times higher than the number of such cells in double transgenic mice on a wild-type background after release from doxycycline at E 14 (Fig. 7, B–F). Moreover, ultrastructural examination showed that apoptotic oligodendrocytes contained highly condensed chromatin mass, intact membrane, shrunken cytoplasm, and apoptotic body (Fig. 7 G). These data reinforce the hypothesis that the ER stress response is associated with IFN-γ–induced oligodendrocyte apoptosis and indicate that PERK plays a critical role in protecting oligodendrocytes from the detrimental consequences of IFN-γ–induced ER stress.

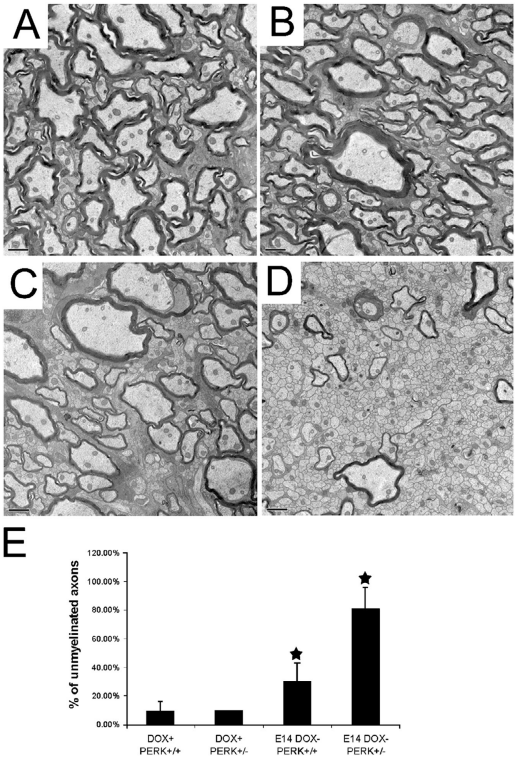

Figure 5. **Double transgenic mice with a *PERK+/−* background develop severe hypomyelination.** (A and B) Ultrastructural examination showing normal myelination in the spinal cord of 14-d-old double transgenic mice (A) and *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice (B) that received doxycycline. (C and D) Ultrastructural examination showing minor hypomyelination in the spinal cord of 14-d-old double transgenic mice (C) and severe hypomyelination in the spinal cord of 14-d-old *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice (D) released from doxycycline at E 14. (A–D) $n = 3$; bars, 1 μM. (E) The percentage of unmyelinated axons in the white matter of the cervical spinal cord was calculated from three mice per time point; *, $P < 0.01$. Error bars represent standard deviation.

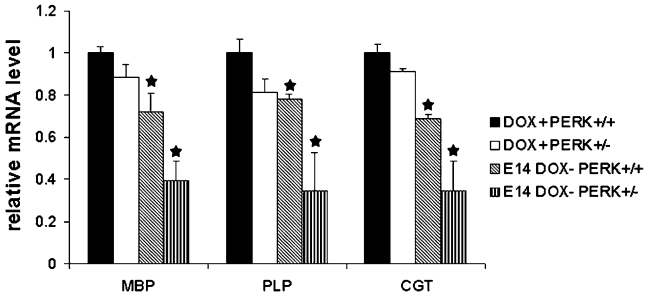

Figure 6. **The levels of MBP, PLP, and CGT mRNA were significantly decreased in the CNS of double transgenic mice with a *PERK+/−* background.** Real-time PCR analyses for myelin gene expression in the brain of 14-d-old mice ($n = 3$); *, $P < 0.05$. Error bars represent standard deviation.

Oligodendrocytes in adult animals are less sensitive to IFN-γ than actively myelinating oligodendrocytes from younger animals

Compared with the actively myelinating oligodendrocytes of young, growing animals, oligodendrocytes in adult mice produce lower levels of membrane proteins and lipids—just enough to maintain homeostasis in the myelin structure (Morell and Quarles, 1999). Thus, the ER of oligodendrocytes in adult animals may have more spare capacity to process an increased

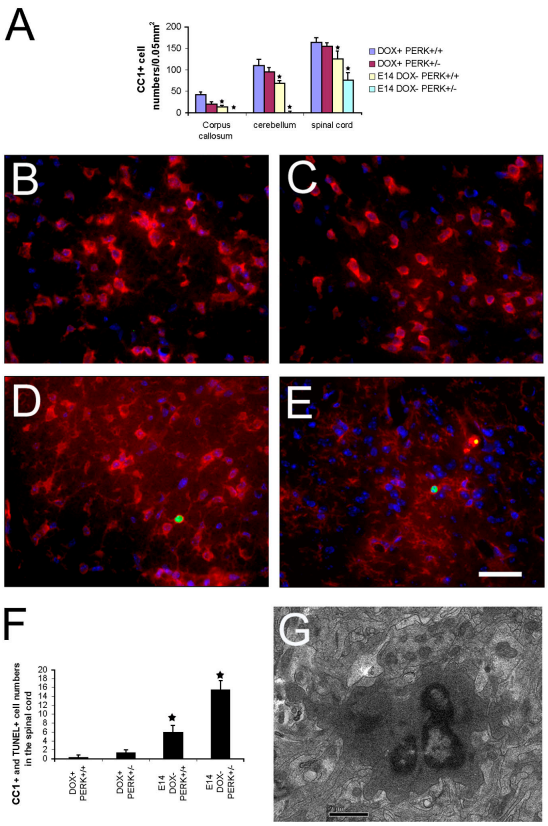

Figure 7. **Double transgenic mice with a *PERK+/−* background lose the majority of oligodendrocytes in the CNS.** (A) Quantitation of CC1-positive cells in the CNS of 14-d-old mice ($n = 3$); *, $P < 0.05$. (B and C) TUNEL and CC1 double labeling in the spinal cord of 14-d-old double transgenic mice (B) and *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice (C) that received doxycycline. (D and E) TUNEL and CC1 double labeling in the spinal cord of 14-d-old double transgenic mice (D) and *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice (E) released from doxycycline at E 14. (B–E) $n = 3$; bar, 60 μM; red fluorescence shows CC1 immunoreactivity; green fluorescence shows TUNEL stain; and blue fluorescence shows DAPI countstain. (F) Quantitation of TUNEL and CC1 double positive cells in the spinal cord of 14-d-old mice ($n = 3$); *, $P < 0.01$. (A and F) Error bars represent standard deviation. (G) Ultrastructural examination showing that apoptotic oligodendrocytes contained highly condensed chromatin mass, intact membrane, shrunken cytoplasm, and apoptosis body; bar, 2 μM.

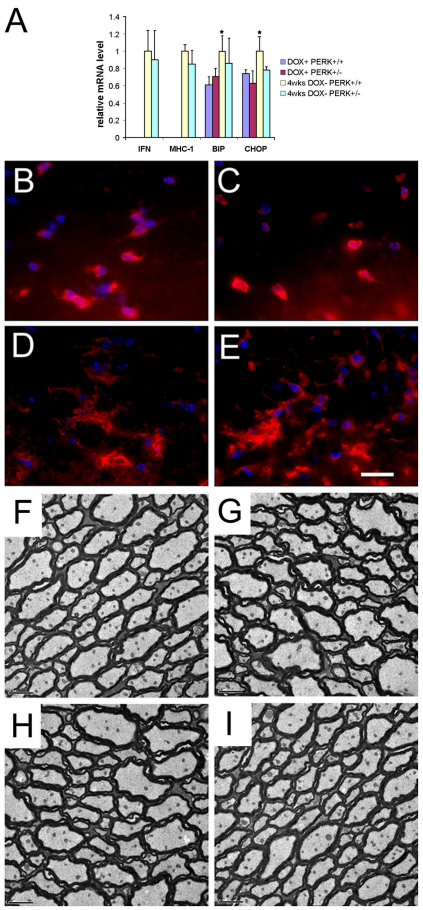

Figure 8. **Oligodendrocytes in adult mice are less sensitive to IFN-γ than actively myelinating oligodendrocytes from younger mice.** (A) Real-time PCR analyses of mRNA levels in the brains of 10-wk-old mice ($n = 3$); *, $P < 0.05$. Error bars represent standard deviation. (B and C) BIP and CC1 double immunostaining in the cerebellum of 10-wk-old double transgenic mice (B) and *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice (C) that received doxycycline. (D and E) BIP and CC1 double immunostaining in the cerebellum of 10-wk-old double transgenic mice (D) and *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice (E) released from doxycycline at 4 wk of age. (B–E) $n = 3$; bar, 60 μM; red fluorescence shows CC1 immunoreactivity; absence of green fluorescence shows that no cells express detectable levels of BIP; and blue fluorescence shows DAPI countstain. (F and G) Ultrastructural examination showing normal myelination in the cerebellum of 10-wk-old double transgenic mice (F) and *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice (G) that received doxycycline. (H and I) Ultrastructural examination showing normal myelination in the cerebellum of 10-wk-old double transgenic mice (H) and *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice (I) released from doxycycline at 4 wk of age. (F–I) $n = 3$; bars, 2 μM.

protein load and, as such, may be less sensitive to disruptions of the protein secretory pathway. To examine this possibility, double transgenic mice were allowed to develop to maturity, at which time IFN-γ expression in the CNS was initiated. Real-time PCR analysis showed that 4-wk-old double transgenic animals released from doxycycline started to express IFN-γ at ∼6 wk of age, and the levels of IFN-γ mRNA and protein in the CNS were comparable with those in developing mice released from doxycycline at E 14 (unpublished data). IFN-γ did not affect oligodendrocyte survival in adult mice, even in mice on a *PERK+/−* background (Fig. 8, B–E). Moreover, ultrastructural examination revealed normal myelin in the CNS of 10-wk-old double transgenic mice with a wild-type or a *PERK+/−* background that were released from doxycycline at 4 wk of age (Fig. 8, F–I). A modest induction of BIP and CHOP by IFN-γ was observed in the cerebellum of 10-wk-old double transgenic mice released from doxycycline at 4 wk of age (Fig. 8 A). Nevertheless, colocalization analysis showed that mature oligodendrocytes did not significantly increase BIP expression (Fig. 8, B–E). Thus, our data indicate that oligodendrocytes from adult animals are less sensitive to the presence of IFN-γ than are actively myelinating oligodendrocytes of growing juvenile mice.

Discussion

The presence of the T cell–derived cytokine IFN-γ within the CNS in immune-mediated demyelinating disorders is believed to contribute to disease pathogenesis (Steinman, 2001b; Wingerchuk et al., 2001). Nevertheless, the mechanism of the cyto-

kine's effect remains unresolved. In this study, we demonstrate that the ability to respond to ER stress modulates the detrimental effects of IFN-γ on the myelination process, suggesting that this cytokine's deleterious effects are mediated, at least in part, by the disruption of the protein secretory pathway in oligodendrocytes. First, our in vitro data indicated that IFN-γ–induced apoptosis in cultured rat oligodendrocytes is correlated with the activation of the ER stress pathway in these cells. Second, we found that hypomyelination and oligodendrocyte apoptosis in mice that ectopically express IFN-γ in the CNS during development were also associated with ER stress. Third, animals with a decreased capacity to respond to ER stress because of an inactive PERK allele exhibit a dramatically increased susceptibility to the presence of IFN-γ in the CNS during development. These mice exhibited a more severe behavioral phenotype and a higher mortality, which correlated with hypomyelination and a striking decrease in oligodendrocyte numbers in the CNS.

Evidence is accumulating that the developmental status of oligodendrocytes influences their susceptibility to IFN-γ. It has previously been demonstrated that cultured, developing oligodendrocytes are significantly more sensitive to the apoptotic-inducing effects of IFN-γ than are mature oligodendrocytes (Andrews et al., 1998; Baerwald and Popko, 1998). We show that this sensitivity correlates with the activation of the ER stress response. Moreover, we have found that cultured immature oligodendrocytes show increased sensitivity to ER stress induction compared with more mature oligodendrocytes, as reflected by a twofold increase in CHOP and BIP mRNA levels after exposure of the cells to tunicamycin (unpublished data). Similarly, we have previously demonstrated that the ectopic presence of IFN-γ selectively promotes hypomyelination in the developing CNS (Corbin et al., 1996). Remarkably, the sensitizing effect of the *PERK* mutation was also restricted to IFN-γ expression in the developing CNS. During development, actively myelinating oligodendrocytes synthesize an enormous amount of myelin membrane, whereas oligodendrocytes in adult animals are responsible for considerably less membrane production, as maintenance of the myelin structure requires only modest new protein synthesis (Morell and Quarles, 1999). We propose that actively myelinating oligodendrocytes may be more sensitive to the adverse effects of IFN-γ because they are synthesizing larger quantities of myelin constituents. This physiological load of ER client proteins likely renders the developing oligodendrocytes closer to a level of physiological ER stress threshold than oligodendrocytes from adult animals.

Our observations may have important implications for immune-mediated demyelinating disorders such as MS, despite the fact that these mostly afflict adults. Reparative remyelination, which follows demyelinating insults, is believed to recapitulate important aspects of developmental myelination and, therefore, might carry with it a susceptibility to ER stress. Moreover, significantly elevated p-eIF-2α immunoreactivity has been observed in oligodendrocytes in the course of experimental allergic encephalomyelitis, an animal model of MS (Chakrabarty et al., 2004). We propose that IFN-γ produced by T cells in MS plaques (Panitch, 1992; Vartanian et al., 1995) promotes ER stress in actively myelinating (remyelinating) oligodendrocytes and may be a contributing factor to the poor remyelination of demyelinated axons that is observed in this disease (Franklin, 2002; Bruck et al., 2003).

The molecular mechanism by which IFN-γ activates the ER stress response in oligodendrocytes is unclear, but it is likely that its presence indicates an increased unfolded protein load on the ER of these cells. It has been observed that IFN-γ stimulates the expression of hundreds of distinct proteins, many of which are membrane spanning (particularly the antigen-presenting MHC molecules of the immune system) in cultured rat oligodendrocytes and in the CNS of mice ectopically expressing IFN-γ (Horwitz et al., 1999; unpublished data). Previous studies have shown that the accumulation of MHC class I heavy chain molecules in the ER of oligodendrocytes results in myelin abnormalities in MBP/MHC class I transgenic mice and that the expression of IFN-γ in the CNS of these mice exacerbates their phenotype (Baerwald et al., 2000). It is possible that IFN-γ stresses the ER of myelinating oligodendrocytes by stimulating MHC class I expression in these cells, which, together with the load of myelin protein, leads to an overload of the capacity of the ER, stress, and cell death.

An alternative, but not mutually exclusive, possibility is that IFN-γ stimulates ER stress in oligodendrocytes through the activation of effector molecules. Oyadomari et al. (2001) demonstrated that nitric oxide (NO) mediates the apoptotic death of pancreatic β cells through the activation of the ER stress pathway. Furthermore, Baud et al. (2004) recently demonstrated that NO is toxic to developing oligodendrocytes, but that mature oligodendrocytes are less sensitive to the presence of NO. IFN-γ has been shown to increase inducible NO synthase (iNOS) expression in certain cell types (Munoz-Fernandez and Fresno, 1998). Nevertheless, we have not observed a significant increase in iNOS expression in our model systems. In purified oligodendrocyte cultures, IFN-γ appears incapable of stimulating increased iNOS expression (unpublished data), which is consistent with previous reports (Bhat et al., 1999; Molina-Holgado et al., 2001). In the double transgenic animals that expressed IFN-γ in the CNS, iNOS mRNA levels increased by $<50\%$ (unpublished data). Thus, the degree to which NO plays a role in the activation of the ER stress pathway in oligodendrocytes in the presence of IFN-γ remains to be determined.

Oligodendrocyte function appears particularly sensitive to disruptions of protein synthesis and to perturbations of the secretory pathway. In addition to the evidence presented in this study for the role of ER stress in immune-mediated demyelinating disorders and for the protective function of PERK, there are genetic myelin disorders that appear to be caused by the disruption of these pathways. VWM is a fatal hypomyelination disease caused by mutations in eIF-2B (Leegwater et al., 2001; Richardson et al., 2004). The disorder affects glial cells of white matter, with oligodendrocytes described as abnormal and "foamy" in appearance (Wong et al., 2000). Although it is unclear whether mutations in eIF-2B directly contribute to the dysfunction of oligodendrocytes, it is clear that the proper regulation of translation initiation is essential for myelination. Moreover, PMD is caused by mutations in the PLP gene that result in the accumulation of improperly folded PLP in the

secretory pathway, which appears to activate the ER stress response in oligodendrocytes (Southwood et al., 2002). Altogether, these disorders suggest that to achieve normal myelin formation, oligodendrocytes must maintain the homeostasis of PERK activity. Too much activity, which correlates with eIF-2B inactivation in VWM patients, results in hypomyelination, whereas the PERK pathway clearly provides a protective role in disorders that result in ER stress in oligodendrocytes.

PERK has a pervasive role in the response to ER stress. In addition to controlling protein synthesis and, thereby, the level of client protein load on the ER (Harding et al., 1999), PERK-mediated eIF-2α phosphorylation also activates a gene expression program with many target genes (Harding et al., 2000b, 2003). Among these is the transcription factor CHOP, whose deletion has been noted to protect cells against death induced by ER stress in various experimental paradigms (Zinszner et al., 1998; McCullough et al., 2001; Oyadomari et al., 2002). Nevertheless, recent scrutiny of CHOP's role in the death of ER-stressed cells suggests that this phenomena reflects a failure of homeostasis rather than the activation of a death-promoting mechanism that is under positive selection. CHOP's target genes include growth and DNA damage protein 34 and ER oxidoreductin 1, which promote recovery from ER stress–mediated translational repression and an oxidizing environment in the ER, respectively. In certain experimental contexts, the higher client protein load and the oxidizing environment of the stressed CHOP+/+ ER is detrimental to cell survival (Marciniak et al., 2004). However, it seems highly plausible that in other contexts, such as the myelinating oligodendrocyte, the role of these CHOP target genes in promoting the recovery of secretory capacity may be beneficial to the organism. We have observed reduced CHOP mRNA induction in adult *PERK+/−* mice that ectopically expressed IFN-γ in the CNS, whereas CHOP mRNA levels in the severely affected developing *PERK+/−* mice were similar to their wild-type littermates. Although the CHOP data from the developing mice need to be considered in light of the severe diminution of these very cells (oligodendrocytes, which probably experience ER stress in these animals), these data raise an interesting possibility that CHOP has a protective role in oligodendrocytes. In support of this possibility, Southwood et al. (2002) presented evidence suggesting that CHOP serves to ameliorate the toxic effects of mutant PLP in oligodendrocytes in a mouse model of PMD.

To summarize, we have demonstrated that the detrimental effects of the immune-cytokine IFN-γ on oligodendrocytes, in vitro and in vivo, are associated with an activation of the ER stress response. Moreover, we have used a genetic approach to show that the ability of oligodendrocytes to respond to ER stress modulates the deleterious actions of IFN-γ on the myelination process. As such, these studies have identified a potentially important clinical target in immune-mediated demyelinating disorders. The presence of this cytokine in demyelinated lesions might significantly contribute to the failed remyelination efforts of demyelinated lesions by activating an ER stress response in newly recruited oligodendrocytes. Therapeutic approaches to alleviate this stress might prove beneficial in enhancing myelin repair in immune-mediated demyelinating disorders.

Materials and methods

Cell culture

Oligodendrocyte progenitors were cultured from neonatal rat brains (Baerwald and Popko, 1998). A mixed glial culture was grown in flasks in medium containing 10% FBS, and when the astrocyte layer became confluent (10–14 d), oligodendrocyte progenitors were separated from astrocytes and microglia by using an orbital shaker. Cells (>95% of which were A2B5 positive, GFAP negative, and CD11b negative) were cultured in 0.5% FBS-containing medium, which also contained 10 ng/ml PDGF and 5 ng/ml FGF (both purchased from R&D Systems). Then, cells were switched to 0.5% FBS medium without PDGF and FGF for differentiation. After 5 d in the differentiating medium, ∼40% of cells were CNP positive. 70 U/ml of recombinant rat IFN-γ (Calbiochem) was added to the cells that had been allowed to differentiate for 5 d. To examine the oligodendroglial response to general ER stress–inducing agents, progenitor cells that had been cultured in differentiation medium for 5 or 7 d were treated with 2 μg/ml tunicamycin (Sigma-Aldrich) for 6 h.

Mice breeding

Line110 *GFAP/tTA* mice and line184 *TRE/IFN-γ* mice (Lin et al., 2004) on the F1 hybrid background (C57BL/6; Swiss Webster) were mated with *PERK+/−* mice (Harding et al., 2001) on the Swiss Webster background to obtain *GFAP/tTA; PERK+/−* and *TRE/IFN-γ; PERK+/−* progeny on an F2 hybrid background. Then, we mated *GFAP/tTA; PERK+/−* mice with *TRE/IFN-γ; PERK+/−* mice to get *GFAP/tTA; TRE/IFN-γ; PERK+/−* mice and *GFAP/tTA; TRE/IFN-γ; PERK+/+* mice. To prevent transcriptional activation of the *TRE/IFN-γ* transgene by tTA, 0.05 mg/ml doxycycline was added to the drinking water and was provided ad libitum. All animal procedures were conducted in complete compliance with the National Institutes of Health's (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Real-time PCR

RNA was isolated from cultured cells and mice brains using TRIzol reagent (Invitrogen) and was treated with DNaseI (Invitrogen) to eliminate genomic DNA. Reverse transcription was performed using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). Real-time PCR was performed with iQ Supermix (Bio-Rad Laboratories) on a real-time PCR detection system (model iQ; Bio-Rad Laboratories). The following primers and probes (Integrated DNA Technologies, Inc.) for real-time PCR were used: mouse glyceraldehyde phosphate dehydrogenase (GAPDH) sense primer (CTCAACTACATGGTCTACATGTTCCA); mouse GAPDH antisense primer (CCATTCTCGGCCTTGACTGT); mouse GAPDH probe (TGACTCCACTCACGGCAAATTCAACG); mouse IFN-γ sense primer (GATATCTCGAGGAACTGGCAAAA); mouse IFN-γ antisense primer (CTTCAAAGAGTCTGAGGTAGAAAGAGATAAT); mouse IFN-γ probe (TGGTGACATGAAAATCCTGCAGAGCCA); mouse MBP sense primer (GCTCCCTGCCCCAGAAGT); mouse MBP antisense primer (TGTCACAATGTTCTTGAAGAAATGG); mouse MBP probe (AGCACGGCCGGACCCAAGATG); mouse PLP sense primer (CACTTACAACTTCGCCGTCCT); mouse PLP antisense primer (GGGAGTTTCTATGGGAGCTCAGA); mouse PLP probe (AACTCATGGGCCGAGGCACCAA); mouse CGT sense primer (TTATCGGAAATTCACAAGGATCAA); mouse CGT antisense primer (TGGCGAAGAATGTAGTCTATCCAATA); mouse CGT probe (CCGGCCACCCTGTCAATCGG); mouse CHOP sense primer (CCACCACACCTGAAAGCAGAA); mouse CHOP antisense primer (AGGTGCCCCCAATTTCATCT); mouse CHOP probe (TGAGTCCCTGCCTTTCACCTTGGAGA); mouse BIP sense primer (ACTCCGGCGTGAGGTAGAAA); mouse BIP antisense primer (AGAGCGGAACAGGTCCATGT); mouse BIP probe (TTCTCAGAGACCCTTACTCGGGCCAAATT); mouse caspase-12 sense primer (ATGCTGACAGCTCCTCATGGA); mouse caspase-12 antisense primer (TGAGAGCCAGACGTGTTCGT); mouse caspase-12 probe (AGTCCAAGATACACTGAAGCTTTGTCCACGTGAT); rat GAPDH sense primer (CCCCAATGTATCCGTTGTGGA); rat GAPDH antisense primer (GCCTGCTTCACCACCTTCTT); rat GAPDH probe (ACATGCCGCCTGGAGAAACCTGCC); rat BIP sense primer (CCTATTCCTGCGTCGGTGTATT); rat BIP antisense primer (GGTTGGACGTGAGTTGGTTCT); rat BIP probe (CCGCATCGCCAATCAGACGCTCCC); rat CHOP sense primer (GAAATCGAGCGCCTGACCAG); rat CHOP antisense primer (GGAGGTGATGCCAACAGTTCA); rat CHOP probe (AGACCACACGGCGGGCTCTGATCG); rat caspase-12 sense primer (AGAATTAATGAAGTTTGCTGGCCG); rat caspase-12 antisense primer (CAGGATGCCGTGGGACATAAA); and rat caspase-12 probe (CCAGAGCACCAGTCCTCCGACAGC).

Western blot analysis

Tissues or cultured cells were rinsed in ice-cold PBS and were immediately homogenized in 5 vol Triton X-100 buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 10 mM PMSF, 15 μg/ml aprotonin, and 6 μg/ml pepstatin A) using a motorized homogenizer. After incubating on ice for 15 min, the extracts were cleared by centrifugation at 14,000 rpm twice for 30 min each. The protein content of each extract was determined by protein assay (Bio-Rad Laboratories). The extracts (40 μg) were separated by SDS-PAGE and were transferred to nitrocellulose. The blots were incubated with primary antibody (see below), and the signal was revealed by chemiluminescence after reacting with HRP-conjugated second antibody. The following primary antibodies were used: anti–eIF-2α (1:500; Santa Cruz Biotechnology, Inc.); anti–p-eIF-2α (1:1,000; Cell Signaling Technology); anti–caspase-12 (1:500; Santa Cruz Biotechnology, Inc.); and antiactin (1:1,000; Sigma-Aldrich).

Caspase-3 activity assay

Activation of caspase-3 was assessed by using the fluorimetric Caspase-3 Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Immunohistochemistry

Anesthetized mice were perfused through the left cardiac ventricle with 4% PFA in PBS. The half saggital brains and transverse cervical spinal cords were removed, postfixed with PFA, cryopreserved in 30% sucrose, embedded in optimal cutting temperature compound, and frozen on dry ice. Frozen sections were cut in a cryostat at a thickness of 10 μm. For immunohistochemistry, frozen sections were treated with −20°C acetone, blocked with PBS containing 10% NGS and 0.1% Triton X-100, and incubated overnight with the primary antibody diluted in blocking solution. Fluorescein, Texas red, or enzyme-labeled secondary antibodies (Vector Laboratories) were used for detection. Immunohistochemistry for CC1 (APC7, 1:50; EMD Biosciences, Inc.), BIP (1:50; Santa Cruz Biotechnology, Inc.), caspase-12 (1:50; Santa Cruz Biotechnology, Inc.), p-eIF-2α (1:50; Cell Signaling Technology), and MBP (1:1,000; Sternberger Monoclonals) was performed. The fluorescent-stained sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories) and were visualized with a fluorescence microscope (model Axioplan; Carl Zeiss MicroImaging, Inc.) using a 40× objective (1.3 oil, model Achrostigmat; Carl Zeiss MicroImaging, Inc.) or a 63× objective (1.4 oil, model Plan-Apochromat; Carl Zeiss MicroImaging, Inc.). Images were captured using a camera (model PXL CCD; Photometrics) connected to a computer (Apple) with the Open Lab software suite. We quantified immunopositive cells by counting positive cells within the white matter of the spinal cord, cerebellum, and corpus callosum. Only those cells with nuclei that were observable by DAPI staining were counted.

TUNEL assay

CNP (1:200; Sternberger Monoclonals) and TUNEL double staining in cultured cells and CC1 and TUNEL double staining in mice tissue were performed using the ApopTag Kit (Serologicals Corp.) following the manufacturer's instructions.

Electron microscopy

Mice were anesthetized and perfused with 4% PFA and 2.5% glutaraldehyde. The cervical spinal cord and white matter of the cerebellum and corpus callosum were processed. Thin sections were cut, stained with uranyl acetate and lead citrate, and analyzed as previously described (Coetzee et al., 1996).

Statistics

Data are expressed as mean ± SEM. Multiple comparisons were statistically evaluated by a one-way analysis of variance between groups test using Sigmastat 3.1 software (Hearne Scientific Software). Differences were considered statistically significant if $P < 0.05$.

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