Brief Communications

Diminishing Apoptosis by Deletion of Bax or Overexpression of Bcl-2 Does Not Protect against Infectious Prion Toxicity *In Vivo*

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B-cell lymphoma protein 2 (Bcl-2) and Bcl-2-associated X protein (Bax), key antiapoptotic and proapoptotic proteins, respectively, have important roles in acute and chronic models of neurologic disease. Several studies have implicated Bax and Bcl-2 in mediating neurotoxicity in prion diseases. To determine whether diminishing apoptotic cell death is protective in an infectious prion disease model we inoculated mice that either were null for proapoptotic Bax or overexpressed antiapoptotic Bcl-2. Interestingly, genetic manipulation of apoptosis did not lessen the clinical severity of disease. Moreover, some disease parameters, such as behavioral alterations and death, occurred slightly earlier in mice that are null for Bax or overexpress Bcl-2. These results suggest that Bax and Bcl-2 mediated apoptotic pathways are not the major contributing factor to the clinical or pathological features of infectious prion disease.

Key words: **PrP; home cage; amyloid; cell death; necrosis; transmissible**

Introduction

The pathological events observed in neurodegenerative disease culminate in a dramatic loss of neurons in the brain but the importance of cell death pathways in this process is controversial (Yuan et al., 2003; Shacka and Roth, 2006; Steele and Yi, 2006). Genetic manipulations of proapoptotic or antiapoptotic proteins have, in many cases, ameliorated pathological or clinical features in mouse models of neurodegenerative disease, including amyotrophic lateral sclerosis, ischemia, and axotomy (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Kostic et al., 1997; Gould et al., 2006; Tsai et al., 2006; Li et al., 2007). However, in other studies modification of apoptosis has not altered the symptoms of neurodegeneration (Sagot et al., 1995; Selimi et al., 2000; Kang et al., 2003; Chiesa et al., 2005; Coulpier et al., 2006). Thus, apoptotic pathways have significant disease-modifying effects in a subset of neurodegenerative diseases.

Prion diseases (PrD) are a unique class of neurodegenerative disorders manifesting in genetic, sporadic, and infectious forms, affecting humans as well as wild and domesticated animals (Prusiner, 1998; Aguzzi and Heikenwalder, 2006; Caughey and Baron, 2006). In infectious forms of the disease, the normal cellular isoform of the prion protein, PrP (termed PrP^{C}), is structurally converted into PrP^{Sc}, a self-perpetuating and aggregation-prone conformation of the protein (Prusiner, 1998). The ongoing conversion of PrP^{C} to PrP^{Sc} is required for prion toxicity; however, beyond this observation little is known about the mechanism by which this process triggers neurotoxicity (Aguzzi and Heikenwalder, 2006).

Prion infections cause characteristic lesions in the brain: spongiform vacuolation, aggregates of PrP, activation of microglia, proliferation of astrocytes, and ultimately loss of neurons (Aguzzi and Heikenwalder, 2006). The mechanism(s) of neuronal loss is unknown, but various forms of neuronal death, including apoptosis, have been documented in diverse hosts with PrD (Liberski et al., 2004). Apoptosis has been described in the brains of patients affected by Creutzfeldt-Jakob disease and fatal familial insomnia and in prion-infected hamsters, mice, and sheep (Liberski et al., 2004). Several studies (discussed below) have suggested important roles for B-cell lymphoma protein 2 (Bcl-2)-associated X protein (Bax) and Bcl-2 in the neurotoxic pathway caused by PrP mutants (Chiesa et al., 2005; Li et al., 2007; Nicolas et al., 2007).

Bax and Bcl-2 are at the core of the apoptotic pathway located at the mitochondria where multiple cell death signals converge to trigger apoptosis (Yuan et al., 2003). Downstream of apoptotic stimuli, Bax induces cell death by intramembranous homo-oligomerization and resultant permeabilization of the mitochondrial outer membrane, leading to release of cytochrome *c* and activation of caspases (Danial and Korsmeyer, 2004). The Bcl-2

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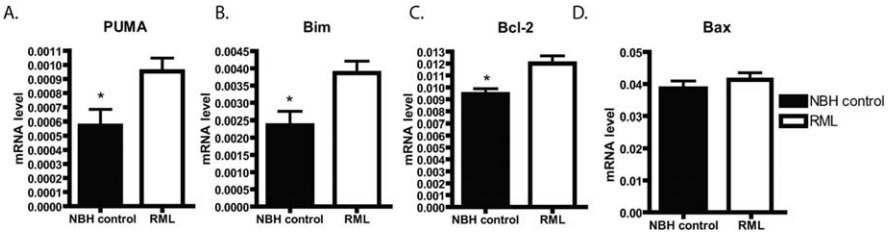

Figure 1. Alterations in the expression of proapoptotic and antiapoptotic genes in prion disease. RT-PCR was performed on trizol extracted brain samples from CD1 mice between 4 and 4.5 months after infection with prions or normal brain homogenate as a control. ***A–D***, Levels of PUMA (***A***), Bim (***B***), *Bcl-2* (***C***), and *Bax* (***D***) were examined. *$p < 0.05$, Student's *t* test.

family is comprised of proapoptotic and antiapoptotic members, and is defined by the presence of up to four conserved domains within their primary structure (Reed, 2006). Proapoptotic members can be further subdivided into more fully conserved, "multidomain" members containing Bax homology (BH) 1–3 or "BH3-only" members, such as Bim and Puma, which activate Bax.

To address directly the role of apoptosis in infectious PrD, we inoculated *Bax* knock-out (KO) and *Bcl-2* neuronal overexpression transgenic (Tg) mice with the Rocky Mountain Laboratory (RML) strain of murine prions. To our surprise, we observed that *Bax* deficient and *Bcl-2* overexpression Tg mice were not protected against prion toxicity. In fact, some features of PrD, such as behavioral alterations and survival, were made worse, whereas accumulation of proteinase-K resistant PrP and pathological changes were unaltered by diminishing apoptosis.

Materials and Methods

RNA extraction and quantitative RT-PCR. Total RNA was prepared from brain homogenates using trizol (Invitrogen, Carlsbad, CA) and cDNA was synthesized with SuperScript III (Invitrogen) using random primers. Quantitative real-time (RT)-PCRs using SYBR green fluorescent reagent were performed in an ABI PRISM 7700 (Applied Biosystems, Foster City, CA). Relative amounts of mRNAs were calculated from the values of comparative threshold cycle by using β actin as a control. Primer sequences were designed by Primer Express software (Applied Biosystems) and are identical to those reported in (Hetz et al., 2007).

Mouse strains, prion inoculations, and behavioral analysis. All mouse experiments were approved by the Massachusetts Institute of Technology Committee on Animal Care. Food and water were provided *ad libitum* and mice were group housed ($n = 2$–5 per cage) while being maintained on a 12 h light/dark cycle. *Bax* KO mice (Knudson et al., 1995) were obtained from The Jackson Laboratory (Bar Harbor, ME) at N8 generations of backcross to C57BL/6J, and were backcrossed one additional time. All mice used in our studies were generated by intercrossing *Bax*+/− by *Bax*+/− mice to generate littermate controls. *Bcl-2* Tg mice (Dubois-Dauphin et al., 1994) were kindly provided by Nancy Forger (University of Massachusetts, Amherst, MA). This line was maintained on a mixed background composed of C57BL/6 and DBA/2 and was crossed once to C57BL/6 for ovarian transfer rederivation. For breeding we crossed *Bcl-2* Tg+/− males to Tg−/− females. For prion injections, mice were injected intracranially with 30 μl of 0.1% brain homogenate containing ~5.5 log LD_{50}/30 μl infectious units of RML murine prions. Behavioral analysis was performed essentially as described by Steele et al. (2007). Briefly, mice were placed in new cages containing minimal bedding for a 24 h video recording using dim red lighting during the dark cycle. Behavioral analysis of videos was performed using HomecageScan 2.0 with definitions as previously defined with the exception that "remain low" and "sleep" were merged under the title "rest" because the steel coat color in some of the *Bax* KO mice made it difficult to distinguish these two classes of inactivity.

Proteinase-K treatment and Western blotting. Ten percent homogenates (weight/volume) of whole brain were made in PBS from tissue frozen at 4.5 and 5.0 months post inoculation (mpi). Tissue was homogenized in a glass dounce homogenizer and sonicated, and then large debris were pelleted by low speed centrifugation (~500 g for 5 min). Homogenates were diluted to 1% in lysis buffer consisting of PBS and 1% Triton X-100 and 1% Tween 20 and treated with 50 μg/ml proteinase-K (PK) for 1 h at 37C, followed by boiling in NuPAGE lithium dodecyl sulfate running buffer (Invitrogen) and electrophoresed in NuPAGE Novex (Wadworth, OH) Bis-Tris midi gels (Invitrogen). Proteins were transferred to nitrocellulose membranes which were blocked using 5% milk and probed with SAF83 (Cayman Chemical, Ann Arbor, MI) to detect PrP and an antibody against β-tubulin was used for a loading control. A Licor Odyssey was used to detect infrared conjugated secondary antibodies (Rockland, Gilbertsville, PA).

Neuorpathological analysis. Brains were immersion fixed in formalin, paraffin embedded, and sectioned coronally. Five micron sections were stained with hematoxylin and eosin. For glial fibrillary acidic protein (GFAP) immunostaining, five micron thick paraffin sections were deparaffinized and then incubated in 98.6 degree waterbath at pH 6.0 for 40 min, after which the sections were stained using an automated stainer, using an rabbit anti-cow GFAP antibody at 1:400 dilution (Dako, High Wycombe, UK). A blinded analysis of vacuolation and gliosis in the hippocampus, thalamus, striatum, cortex, and cerebellum was conducted. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining was performed according to the manufacturer's protocol (Roche, Welwyn Garden City, UK).

Statistical analysis. Statistical analysis was performed using GraphPad Prism. Student's *t* test was used for analysis of RT-PCR data, log rank test was used for survival data, and Wilcoxon rank sum test (nonparametric) was used for behavioral analysis.

Results

Expression levels of Bcl-2 family members

Alterations in the expression levels of proapoptotic and antiapoptotic proteins can be indicative of activation of particular cell death pathways. Therefore, we examined the expression levels of several key proapoptotic and antiapoptotic genes in the brains of RML infected CD1 mice that were killed between 4 and 4.5 months after infection, which is ~3–4 weeks before the mice become moribund. By quantitative RT-PCR, we observed a significant increase in the RNA levels of Puma and Bim, two potent activators of Bax-dependent apoptosis, when compared with control mice injected with normal brain homogenate ($p < 0.05$, Student's *t* test, $n = 4$ mice per group; Fig. 1*A*,*B*). In addition, a small but reproducible increase in the mRNA levels of Bcl-2 was observed in prion infected mice, possibly reflecting a compensatory effect (Fig. 1*C*). Similar changes in expression of Bcl-2 were observed in mice infected with another strain of prions, 139A (data not shown). We did not observe significant changes in the mRNA levels of Bax (Fig. 1*D*) or Bcl-X_L (data not shown) in RML infected mice. Because similar changes in the expression of these upstream regulators, Bim and Puma, were observed in a mouse model of amyotrophic lateral sclerosis (Hetz et al., 2007) where Bax deletion significantly delayed cell loss and behavioral onset (Gould et al., 2006), our RT-PCR results suggested that prion infection may activate the main proapoptotic machinery.

Prion disease in Bax knock-out mice

Because Bax is required for neuronal apoptosis in a variety of disease models (Chiesa et al., 2005; Gould et al., 2006; Heitz et al., 2007; Li et al., 2007) and its deletion delays disease symptoms in several models of neurodegeneration (Gould et al., 2006; Tsai et al., 2006; Li et al., 2007), we hypothesized that *Bax* KO mice would be protected against prion toxicity. To test this, we inoculated C57BL/6 *Bax* KO and wild-type (WT) littermate controls with 5.5 $\log LD_{50}$/30 μl of RML prions intracranially (i.c.). We monitored their home cage behaviors using a high resolution automated system which presented robust diagnosis of PrD in mice in a previous study (Steele et al., 2007). After disease onset, RML infected mice show a striking increase in physical activity as manifested by increases in behaviors such as walking and jumping; other behaviors, such as grooming and hanging decrease during later stages of PrD. We video recorded and analyzed prion-infected *Bax* KOs (n = 13–16) and WT littermates (n = 14–18) twice monthly beginning at 3 months postinoculation (mpi), which is just before onset of symptoms in RML-infected mice (Steele et al., 2007).

The overall profile of behavioral changes was similar in prion-infected *Bax* KO and control mice. However, several behavioral changes previously shown to be altered in RML PrD in C57BL/6 mice were observed in the *Bax* KO mice before their WT littermate controls, suggesting that some PrD symptoms have an earlier onset in *Bax* KO mice. The prion-inoculated *Bax* KOs had a subtle increase in "walking" over controls at 3.0 and 3.5 mpi (Fig. 2*A*), a subtle decrease in "resting" at 3.0 and 3.5 mpi (Fig. 2*B*), no change in grooming (Fig. 2*C*), and a small decrease in "hang vertical" at 4.0 mpi (Fig. 2*D*). Interestingly, later in disease progression two behaviors, "walk" and "rest," were also significantly altered between *Bax* KO and control mice. Importantly, the behavior of uninoculated *Bax* KO mice was indistinguishable from uninoculated WT controls with the exception of "eating," which was slightly elevated in *Bax* KO mice (data not shown).

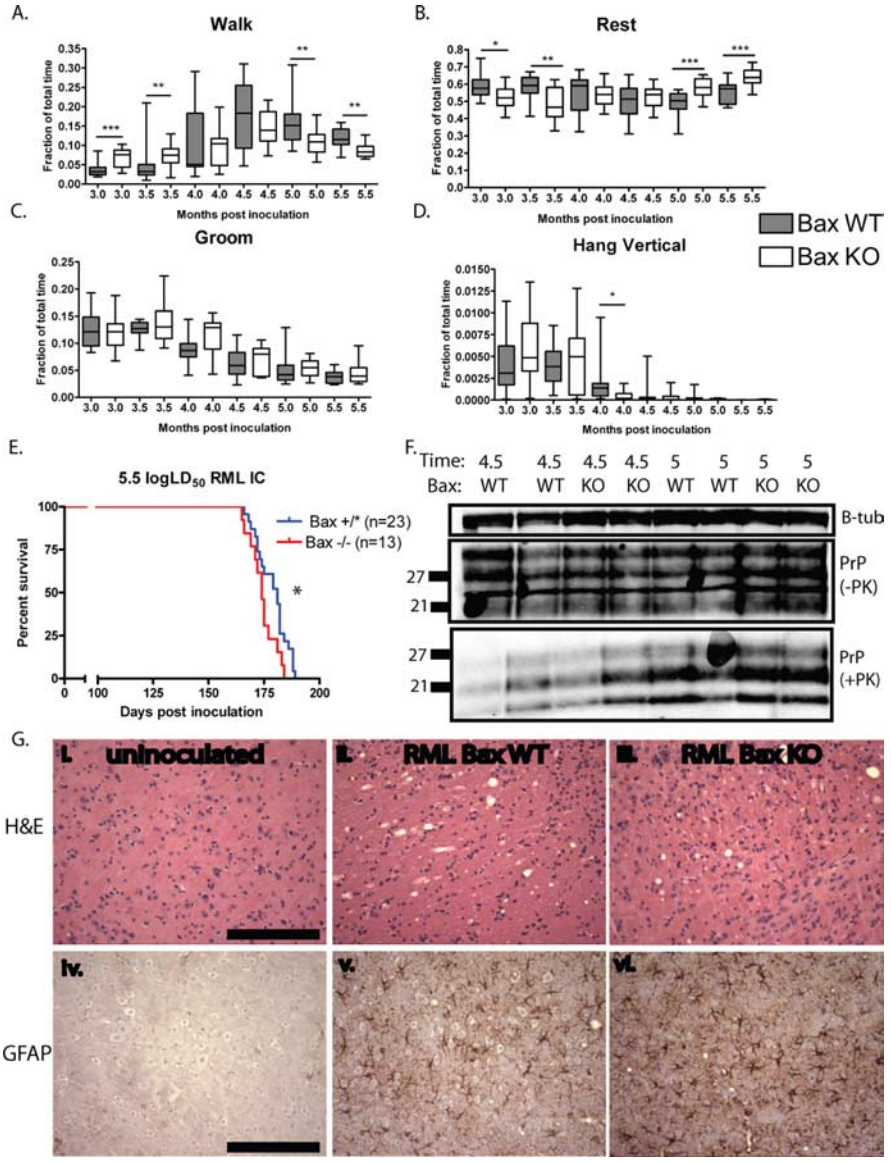

Figure 2. Deletion of *Bax* does not protect against prion disease. ***A–D***, Box-and-whisker plots for behaviors known to be altered in prion disease such as walk (***A***), rest (***B***), groom (***C***), and hang vertical (***D***) are shown as for *Bax* knock-out (white bars) and controls (gray bars). Sample sizes were n = 13–16 at each time point for *Bax* KOs and n = 14–18 for WT controls. Statistically significant results are denoted with asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) and were computed using a Wilcoxon rank sum test. The top and bottom whiskers represent the maximum and minimum values for each time point whereas the box represents the 25th to 75th percentiles, and the line represents the median value. ***E***, *Bax* KO mice die faster from prion infection (p = 0.041, log rank test). ***F***, Accumulation of proteinase-K-resistant PrP was measured at 4.5 and 5.0 mpi by Western blotting n = 2 mice per genotype per time point. ***G***, The neuropathological changes were similar in prion-infected *Bax* KO and control mice, shown at 5 mpi, in terms of vacuolation in hematoxylin- and eosin-stained sections of uninoculated WT (***i***), prion-inoculated *Bax* WT (***ii***), and *Bax* KO (***iii***). Gliosis measured by staining for GFAP was performed on sections from uninoculated WT mice, which did not show any staining (***iv***), and on prion-inoculated *Bax* WT (***v***) and *Bax* KO (***vi***) samples. Scale bar, 100 μm.

Given that the behavioral onset in prion-infected *Bax* KOs was subtly enhanced, we next measured the survival and pathology of these mice after prion inoculation. We observed a 7 d enhancement of disease in *Bax* KO mice in terms of median survival (median survival for *Bax* WT, n = 23, was 181 dpi, and for *Bax* KO, n = 13, was 174 dpi; p = 0.041) (Fig. 2*E*). This result suggests that deletion of Bax causes a very subtle enhancement of disease. Next, we inspected brains from prion-infected *Bax* KO and control mice at 4.5 and 5 mpi, ~1–1.5 months before morbidity. Mock-inoculated controls of both genotypes did not show any spongiform changes, nor was there any gliosis or evidence for neuronal loss (Fig. 2*Gi*,*iv*). Brains from prion-inoculated *Bax* KO and WT controls showed dramatic vacuolation visualized using a hematoxylin and eosin stain (Fig. 2*Gii*,*iii*). Widespread gliosis, a hallmark of neurodegeneration, was visualized with a stain for GFAP in the brains from prion-infected *Bax* KO and WT mice (Fig. 2*Gv*,*vi*). There were no obvious differences in pathology between *Bax* KO and controls in the hippocampus, thalamus, striatum, cortex, and cerebellum. To examine cell death in

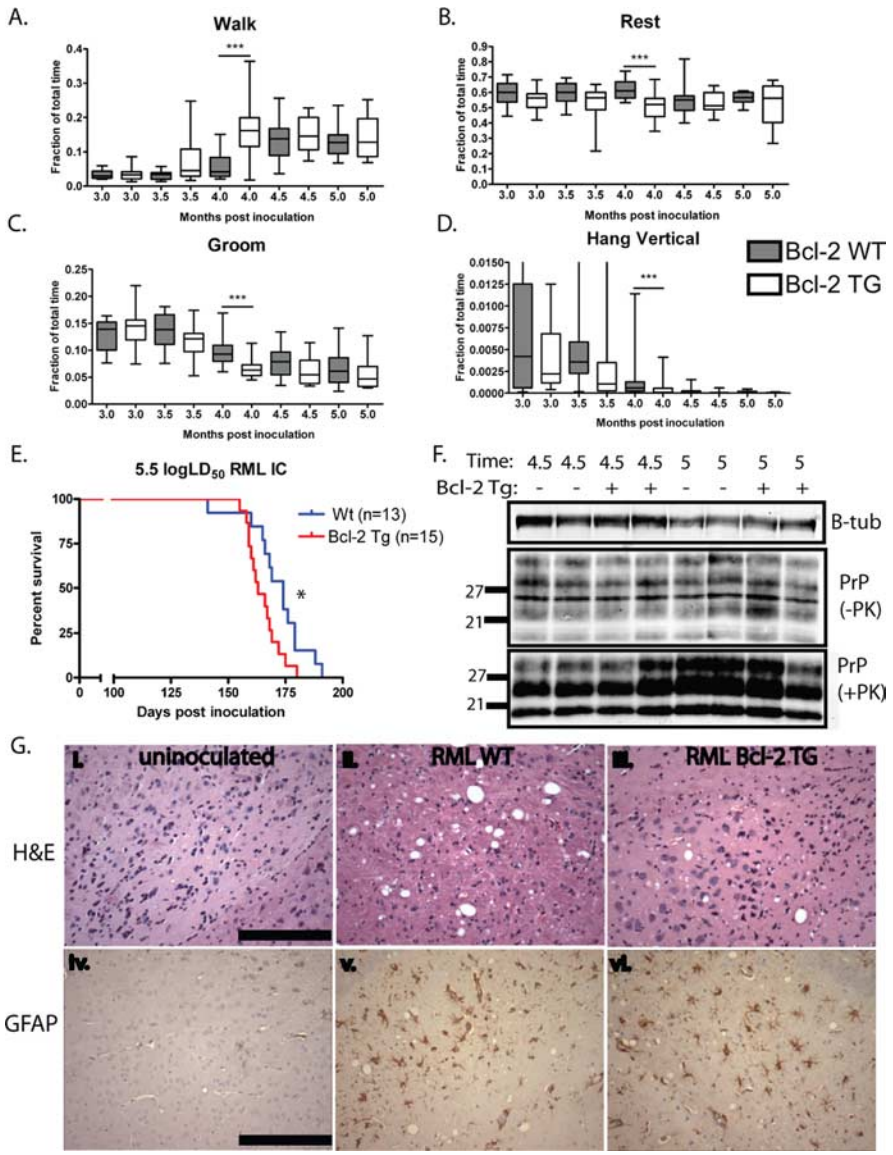

Figure 3. Overexpression of *Bcl-2* in neurons does not protect against prion disease. ***A–D***, Box-and-whisker plots for several behaviors known to be altered in prion disease such as walk (***A***), rest (***B***), groom (***C***), and hang vertical (***D***) are shown as for *Bcl-2* TG (gray bars) and controls (white bars). Statistically significant results are denoted with asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) and were computed using a Wilcoxon rank sum test. ***E***, *Bcl-2* Tg mice die faster from prion infection ($p = 0.03$, log rank test. ***F***, Accumulation of proteinase-K resistant PrP was measured at 4.5 and 5.0 mpi by Western blotting $n = 2$ mice per genotype per time point. ***G***, The neuropathological changes were similar in prion-infected *Bcl-2* Tg and control mice, shown at 5 mpi, in terms of vacuolation in hematoxylin- and eosin-stained sections of uninoculated WT (***i***), prion-inoculated WT (***ii***) and *Bcl-2* Tg (***iii***). Gliosis measured by staining for GFAP was performed on sections from uninoculated WT mice which did not show any staining (***iv***) and on prion-inoculated WT (***v***) and *Bcl-2* Tg (***vi***) samples. Scale bar, 100 μm.

PrD brains, we performed TUNEL staining on samples taken at 4.5 and 5.0 mpi. The brains of *Bax* WT and *Bax* KO were almost completely devoid of TUNEL-positive cells with the exception of the granular layer of the cerebellum, which showed an equivalent amount of TUNEL staining between both genotypes (and no staining in mock inoculated controls) (data not shown).

Finally, we assayed the formation of PK-resistant PrP, the classic surrogate marker for PrD, by treating brain homogenates from *Bax* KO and control mice ($n = 2$ per group) at 4.5 and 5.0 mpi with PK. PK-resistant PrP is the classic surrogate marker for the buildup of prion infectivity and the amount of aggregated PrP in the brain. Digestion with PK suggested that *Bax* KO ($n = 2$) and WT controls ($n = 2$) had similar amounts of prion deposition at the time points examined (Fig. 2*F*).

Prion disease in Bcl-2 neuronal overexpression transgenic mice

Because deleting Bax had a surprising and subtle enhancing effect on prion disease, we tested another mouse model with diminished apoptosis. Bcl-2 has a broad antiapoptotic function and its overexpression protects neurons from damage and death in a variety of models (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Chen et al., 1997; Kostic et al., 1997; Nicolas et al., 2007). We obtained mice (C57BL/6, DBA/2 hybrids) that specifically overexpress human Bcl-2 in neurons under the neuronal enolase promote (Martinou et al., 1994). We inoculated *Bcl-2* Tgs and WT littermate controls with 5.5 $\log LD_{50}$/30 μl RML prions (i.c.) and analyzed their behavior twice monthly beginning at 3 mpi. Similar to what we observed in the *Bax* KOs infected with prions, several known PrD-related behavioral alterations occurred earlier in the *Bcl-2* Tg mice compared with controls. For example, an increase in "walk" occurred earlier in *Bcl-2* Tgs at 4.0 mpi (Fig. 3*A*), and "rest" (Fig. 3*B*), "groom" (Fig. 3*C*), and "hang vertical" (Fig. 3*D*) all showed decreases first in *Bcl-2* Tgs at 4.0 mpi.

The *Bcl-2* Tgs also succumbed to PrD slightly faster than the WT controls; the median survival of *Bcl-2* Tgs was 11 d shorter than that of controls (the median survival for WT controls was 174 dpi, whereas for *Bcl-2* Tgs the median survival was 163 dpi; $p = 0.030$, log rank test) (Fig. 3*E*). Thus, in this model of PrD, too, there is a subtle enhancement of incubation time, suggesting that Bcl-2 over-expression does not protect against prion toxicity and may even cause a very subtle enhancement of infectious PrD.

At the neuropathological level, brains from prion-infected *Bcl-2* Tgs and control mice at 4.5 and 5 mpi showed similar dramatic vacuolation (Fig. 3*Gii*,*iii*) and gliosis in all regions examined (Fig. 3*Gv*,*vi*). There were no obvious differences in pathology in the hippocampus, thalamus, striatum, cortex, and cerebellum. We also assayed the amount of proteinase-K resistant PrP by treating brain homogenates from *Bcl-2* Tgs and controls at 4.5 and 5 mpi with 50 μg/ml PK; no differences were noted ($n = 2$ mice per group per time point) (Fig. 3*F*).

Discussion

By RT-PCR we observed small alterations in the expression of proapoptotic and antiapoptotic genes in prion-inoculated mice before overt illness, consistent with other reports (Park

et al., 2000; Lyahyai et al., 2006). These results suggested that apoptotic pathways could be involved in mediating prion-induced neurodegeneration. Accordingly, we challenged two well characterized mouse lines with demonstrated resistance to apoptosis with RML prions. Surprisingly both deletion of Bax and overexpression of Bcl-2 had subtle enhancing effects on PrD.

The role of apoptosis in genetic forms of PrD has been studied in several systems, both *in vitro* and *in vivo*. Apoptotic cell death has been observed in primary cultured neurons infected with prions (Cronier et al., 2004), treated with proteasome inhibitors (Ma et al., 2002), or both (Kristiansen et al., 2005). In a Tg mouse expressing a repeat expansion of PrP associated with disease in humans, deletion of Bax did not affect the behavioral phenotype, but rescued the cerebellar granular neurons from death (Chiesa et al., 2005). The complexity of prion-induced death is underscored in a series of previous studies examining the roles of Bax and Bcl-2 in mediating mutant PrP toxicity *in vivo*. For example, Li and colleagues deleted Bax from two additional mutant PrP Tg models and observed that Bax deletion delayed cell loss in one deletion mutant line (PrPΔ32-134) although not affecting cell loss in another deletion mutant line (PrPΔ105-125) (Li et al., 2007). A previous study demonstrated a protective role of Bcl-2 overexpression in a mutant PrP Tg (PrPΔ32-134), as Bcl-2 overexpression delays neuronal loss and extends survival in these Tg mice (Nicolas et al., 2007). Finally, one group determined that the toxicity induced by ectopic expression of the PrP ortholog, doppel (Dpl), in the brain of mice lacking PrP is diminished by deletion of Bax (Heitz et al., 2007) whereas another group failed to detect a rescue after deletion of Bax in a different model of Dpl toxicity (Dong et al., 2007). Thus, Bax-dependent and -independent pathways may mediate the toxicity of ectopic Dpl expression in the CNS.

One study of infectious bovine prions in *Bax* KO mice did not detect any differences between *Bax* KOs and controls in terms of survival or pathological changes (Coulpier et al., 2006). Our results are overall quite complementary to this study with the slight discrepancy that we see a small enhancement of disease in terms of the survival of *Bax* KOs inoculated with RML prions. This could be the result of using different prion strains or simply that the effect that we observed in the *Bax* KOs is subtle. Because we did not observe protection against cell death in prion-infected *Bax* KOs and neuronal *Bcl-2* overexpression Tgs, the mechanism by which infectious PrP mediates toxicity must differ from that of mutant PrP and/or Dpl related toxicity, cases in which deletion of Bax and overexpression of Bcl-2 confer protection. The mechanism of cell death in infectious PrD may occur through Bax- and Bcl-2-independent pathways, nonapoptotic pathways or another possibility is that if apoptotic pathways are diminished (by deletion of Bax or overexpression of Bcl-2) then alternate pathways are rapidly induced. The fact that we observed subtle enhancements of some PrD symptoms may arise from altered brain structure/function in *Bax* KO and *Bcl-2* mice, which both possess more neurons because of reduced developmental cell death or because some low level of neuronal apoptosis may be beneficial in prion-inoculated mice perhaps by reducing the production of infectious prions. Our results suggest that therapeutic targeting of Bax- and Bcl-2-dependent apoptotic pathways will not be a viable strategy for combating infectious PrD.

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