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Expression of Amino-Terminally Truncated PrP in the Mouse Leading to Ataxia and Specific Cerebellar Lesions

Doron Shmerling,‖ Ivan Hegyi,† Marek Fischer,# Thomas Blättler,† Sebastian Brandner,† Jürgen Götz,* Thomas Rülicke,‡ Eckhard Flechsig,* Antonio Cozzio,* Christian von Mering,* Christoph Hangartner,* Adriano Aguzzi,† and Charles Weissmann*§

*Institut für Molekularbiologie
Abteilung I
Universität Zürich
8093 Zürich
Switzerland

†Institut für Neuropathologie

‡Biologisches Zentrallabor
Universitätsspital Zürich
8091 Zürich
Switzerland

Summary

The physiological role of prion protein (PrP) remains unknown. Mice devoid of PrP develop normally but are resistant to scrapie; introduction of a PrP transgene restores susceptibility to the disease. To identify the regions of PrP necessary for this activity, we prepared PrP knockout mice expressing PrPs with amino-proximal deletions. Surprisingly, PrP lacking residues 32–121 or 32–134, but not with shorter deletions, caused severe ataxia and neuronal death limited to the granular layer of the cerebellum as early as 1–3 months after birth. The defect was completely abolished by introducing one copy of a wild-type PrP gene. We speculate that these truncated PrPs may be nonfunctional and compete with some other molecule with a PrP-like function for a common ligand.

Introduction

PrP^{Sc}, a protein pathognomonic for transmissible spongiform encephalopathies (Prusiner, 1982), is a modified form of PrP^{C} (Oesch et al., 1985). PrP^{C} is expressed in all vertebrates examined, mainly in the brain but also in many other tissues at lower levels (Bendheim et al., 1992). It is encoded by a single exon of a unique gene, synthesized as a precursor protein, and processed into a mature form by removal of its amino terminal signal sequence, glycosylation, and replacement of its carboxy-terminal signal sequence by a GPI residue, which anchors it to the outer cell surface.

Three lines of mice devoid of PrP^{C} have been generated by homologous recombination using different strategies (Büeler et al., 1992; Manson et al., 1994; Sakaguchi et al., 1995). In two of the lines, the mice developed normally and showed no obvious pathological phenotype (Büeler et al., 1992; Manson et al., 1994; Sakaguchi et al., 1995). Electrophysiological abnormalities in the brains of such knockout mice have been reported by one group (Collinge et al., 1994; Whittington et al., 1995; Colling et al., 1996) but not by others (Herms et al., 1995; Lledo et al., 1996) in the same line of mice. An alteration in circadian activity rhythms of these mice has been described (Tobler et al., 1996).

Sakaguchi et al. developed a line of PrP knockout mice in which, in addition to the coding sequence, about 900 bp of the second intron and 450 bp of the 3′ noncoding sequence of the PrP gene were deleted (Sakaguchi et al., 1995). These mice also developed normally, but at about 70 weeks of age showed progressive symptoms of ataxia and an extensive loss of Purkinje cells in the cerebellum (Sakaguchi et al., 1996). Because these symptoms were not observed in the other PrP knockout lines, they were likely not due to deletion of the PrP coding region (Weissmann, 1996).

Common to all PrP knockout mice is resistance to scrapie and inability to propagate the scrapie agent, or prion, as predicted by the "protein only" hypothesis. There is no obvious clue as to the physiological role of PrP other than that it binds copper (Brown et al., 1997) and that it is located at the outer cell surface, suggesting a role as transporter, ligand, or receptor.

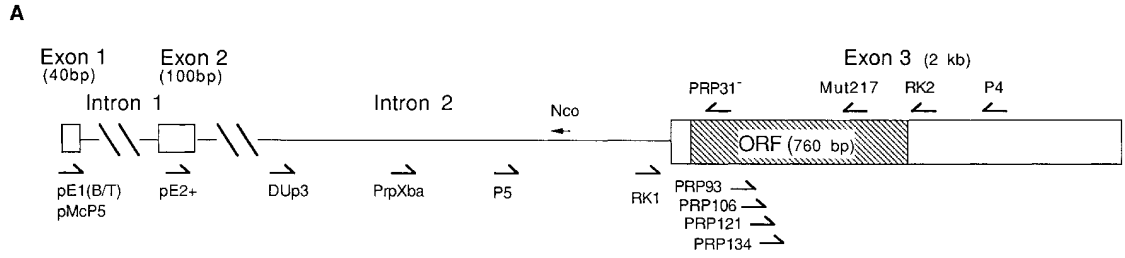
We have undertaken a study of the structure–activity relationship of PrP regarding its capacity to support scrapie pathogenesis and prion propagation. In our first report, we showed that in PrP knockout mice overexpression of recombinant PrP genes with deletions from codons 69 to 84 or from 32 to 80 was able to restore these functions (Fischer et al., 1996). We then prepared a series of deletions extending further toward the carboxyl terminus. Unexpectedly, expression at moderate levels of PrP molecules with deletions from codons 32 to 121 or 134 but not with the shorter deletions 32–80, 32–93, or 32–106 caused ataxia and specific degeneration of the granular layer of the cerebellum within 2–3 months after birth. The defect was completely abrogated if one or more copies of a wild-type murine PrP gene were introduced into mice carrying multiple copies of the truncated gene. We speculate that the truncated PrP may compete with some other molecule with a similar function as PrP for a common ligand.

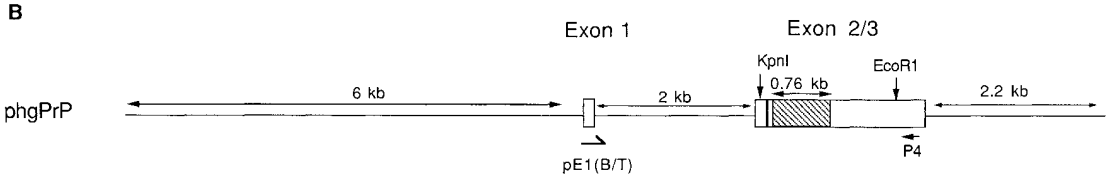
Results

Characterization of Transgenic Mice Expressing Truncated PrP

Fischer et al. developed a PrP-encoding vector based on the murine PrP gene (Figure 1A) from which the large intron had been deleted and that contained 6 kb of 5′ and 2.2 kb of 3′ flanking sequence ("half-genomic construct" or pPrPHG [Figure 1B; Fischer et al., 1996]). This vector, which contains the wild-type PrP sequence, restored susceptibility to scrapie in a dose-dependent manner when introduced into *Prnp*$^{o/o}$ mice. It was subsequently found that even in transgenic mice showing

§To whom correspondence should be addressed.
‖Present address: Core Technologies Department, Novartis Pharma AG, 4002 Basel, Switzerland.
#Present address: Department für Innere Medizin, Abteilung für Infektionskrankheiten, Universitätsspital Zürich, 8091 Zürich, Switzerland.





C

PrP type	Population	Gene copy number*	PrP mRNA*	PrP*	Cerebellar onset (weeks)	Syndrome terminal (months)
Wild		1	1	1		—
"	tga20/tga20	60	5	10		—
A §	e19h/+	150	2	3-4		—
B §	d11/+	30	4	6		—
"	d12/+	40	4	10-12		—&
C	C4	25	2	4		—
"	C15	25	2	3		—
D	D32	15	2.5	3		—
E	E11	70	2	6	6-8	4-9
F	F11	20	1.2	2	5	3-6
"	F35	70	2	2	3-4	2-3

§ From Fischer et al. (1996)
* Relative to wild-type
& Mice homozygous for the transgene also showed no abnormalities

Figure 1. PrP Genes, Transgenes, and Transgenic Mice

(A) Map of the murine PrP gene. The horizontal arrows indicate primers used for various purposes.
(B) Map of the PrP expression vector pPrPHG and the restriction sites and primers used for the construction of the PrP deletion plasmids.
(C) Maps of the exon 2/3 regions of wild-type and truncated PrP expression plasmids and characterization of the PrP transgenic mice. Constructs A and B and the cognate transgenic mice were described earlier (Fischer, 1995) as PrPΔNco and PrPΔN-term, respectively. Gene copy number, PrP mRNA, and PrP levels are given relative to wild type and were determined as described in Experimental Procedures.

several-fold higher expression of PrP in brain than wild-type mice, there was no detectable PrP mRNA in Purkinje cells, suggesting that there might be a Purkinje cell–specific enhancer in the large intron or in a segment of 3′ flanking region absent in the construct (Fischer et al., 1996).

In preparing amino-proximal deletions of PrP, we retained the sequences encoding the amino-terminal signal sequence and the adjoining nine amino acids to ensure transport into the endoplasmic reticulum and proper processing. Because deletion of residues 32–80 did not abolish the capacity of PrP to support scrapie

pathogenesis and prion replication, we prepared a series of deletions extending further towards the carboxyl terminus (Figure 1C). The appropriate constructs were injected into the pronuclei of *Prnp*$^{o/o}$ zygotes, and transgene-carrying animals were identified and bred to *Prnp*$^{o/o}$ mice. In two cases (C15 and D32), injection was into wild-type embryos, and the transgenes were then crossed into *Prnp*$^{o/o}$ mice. The resulting populations, which had a mixed 129/Sv-C57BL/6 background, were analyzed in regard to the gene copy number as well as PrP mRNA and PrP levels (Figure 1C). PrP levels were estimated by quantitation of Western blots of glycosylated as well as deglycosylated brain samples relative to wild type (Figure 2B). Because the PrP molecules had deletions of various lengths, it was necessary to ascertain that the antibody gave a commensurate signal for the different PrP species. This was the case within ± 20% for wild-type PrP and PrP with deletions 32–80 and 32–121 (Figure 2C).

Surprisingly, the mobilities of the major species of PrP molecules with deletions extending to positions 93 (type C), 106 (type D), 121 (type E), and 134 (type F) did not differ noticeably on SDS-polyacrylamide gels (Figure 2B), but they were greater than those of wild-type PrP. There were, however, faster-running minor bands whose mobility showed the expected increase with increasing deletion size (constructs B, C, D, and E). Because these might represent unglycosylated species, we suspected that the aberrant mobilities might be due to the carbohydrate residues and subjected all samples to PNGase digestion. As shown in Figure 2B, this indeed resulted in major PrP bands showing increasing mobility with increasing deletions. In addition, all samples showed a fast-running band with a mobility corresponding to a molecular weight of about 20,000, at varying intensities. Because the band had the same mobility for the different deletion mutants, we conclude that it is a carboxy-terminal PrP fragment resulting from proteolytic digestion. This cleavage likely occurs at least in part in vivo because extraction under different conditions and in the presence of protease inhibitors did not reproducibly reduce the intensity of the band (data not shown) and because such cleavages have been described previously in preparations from brain or from cell culture (Harris et al., 1993; Chen et al., 1995). The origin of this fragment was not further investigated.

Wild-type and truncated Δ32–134 PrP, as expressed in permanently transformed 911 cells, were present at the cell surface, as evidenced by cell surface staining with PrP antibodies and examination by confocal microscopy (data not shown).

The Phenotype of Mice Expressing Truncated PrP

Surprisingly, mice overexpressing PrP with deletions 32–121 (type E) and 32–134 (type F) (Figure 1C) developed pronounced behavioral disorders between 3 and 8 weeks of age. The earliest features were coarse tremor and staggering gait (Figure 3A) followed by paresis of the hind legs; the animals then found it difficult or were unable to right themselves when laid on their sides or

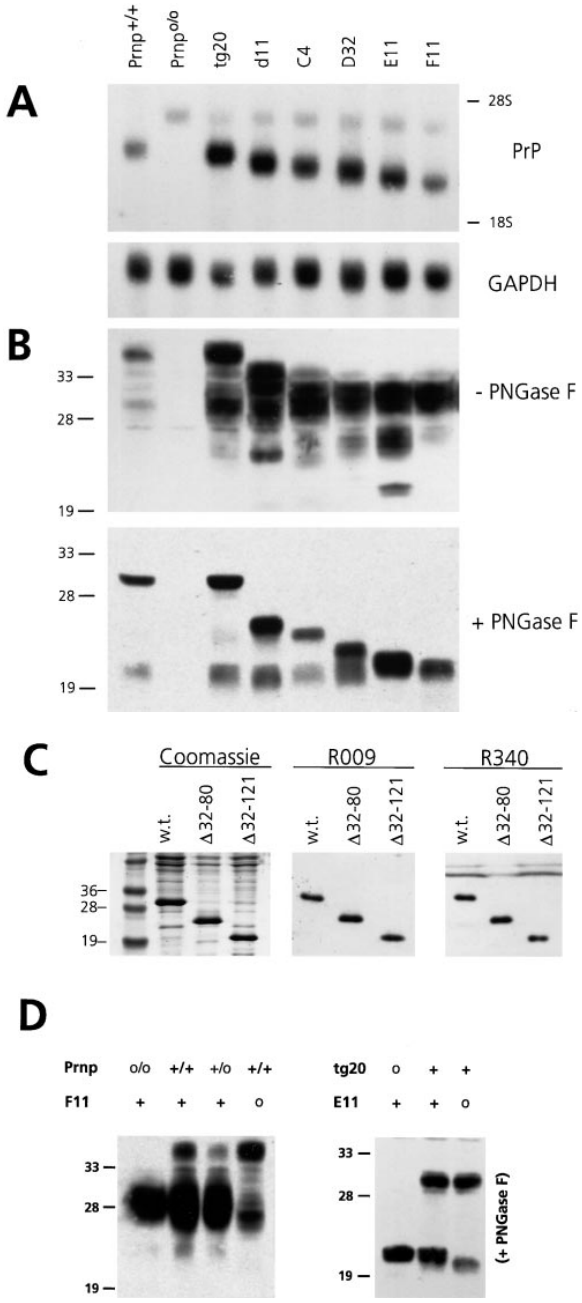


Figure 2. Analysis of Brain Tissue of Transgenic Mice Expressing Truncated PrP and of Wild-Type Mice

(A) Northern analysis of total brain RNA using a PrP mRNA probe (top panel) and, after stripping, a GAPDH probe (bottom panel).

(B) Western analysis using PrP antiserum R340 on untreated (top panel) and deglycosylated (PNGase F-treated; bottom panel) brain protein.

(C) Western analysis of truncated PrP from *E. coli* (320 ng/lane) to show that antibodies R009 (middle panel) and R340 (right panel) interact about equally well with all forms of truncated PrP. The left panel shows a Coomassie blue–stained gel loaded with 4 μg protein per lane.

(D) Coexpression of wild-type and truncated PrP in brains of F1 mice derived from an F11/−, *Prnp*$^{+/o}$ × F11/−, *Prnp*$^{+/o}$ cross (left panel) and an E11/−, *Prnp*$^{o/o}$ × *tga20*/− cross (right panel). Each lane was loaded with 50 μg total brain protein.

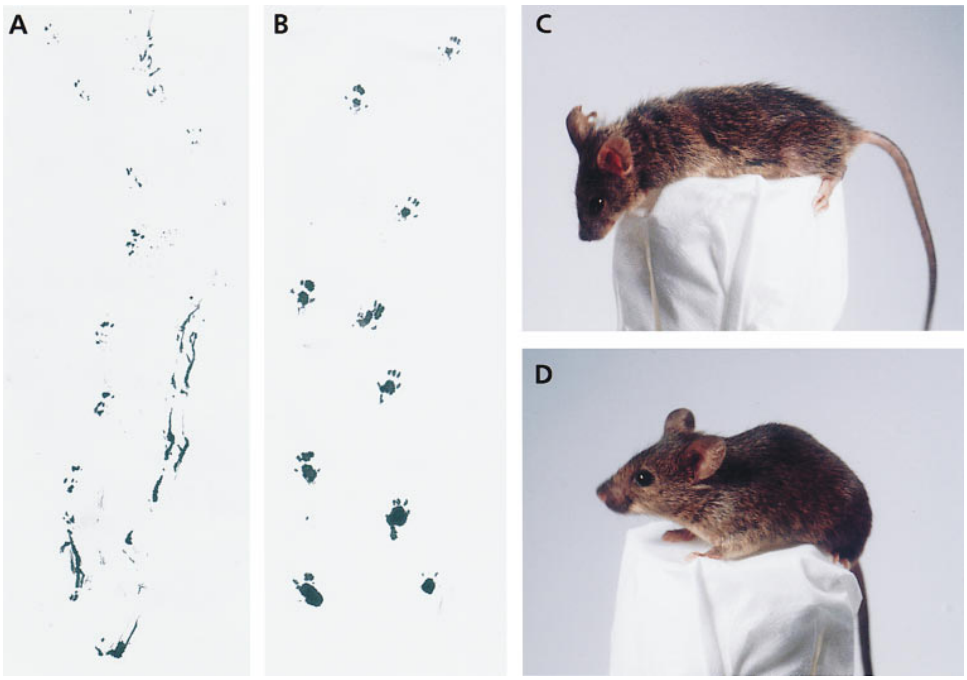


Figure 3. Footprints of PrP Knockout Mice without the *Prnp*Δ32–134 Gene Cluster

The posterior footpads of a 12-week-old F35 mouse and of a *Prnp*$^{o/o}$ littermate were dipped in India ink and the mice were allowed to walk on a sheet of paper. Footprints of the F35 mouse (A) and the control *Prnp*$^{o/o}$ mouse (B). (C) The F35 mouse is droopy due to low tonus and has bedraggled fur, perhaps because of deficient grooming. (D) An alert and happy control mouse. Both mice are 12 weeks old.

backs. The mice were underweight and lost weight as they aged; at 14 weeks wild-type and F11 animals weighed 25.3 ± 1.4 g (n = 6) and 15.8 ± 2.0 g (n = 5), respectively, and moribund animals weighed 12.1 ± 2.1 g (12–25 weeks of age; n = 4). The F11 mice appeared to be weak and more susceptible to parasitic infection, perhaps because of insufficient grooming. Ultimately, the mice were severely handicapped in their movements and had to be sacrificed. F35 mice, which had the higher Δ32–134 transgene copy number (Figure 1C), were the earliest to show symptoms (3–4 weeks after birth) and deteriorated the most rapidly, having to be sacrificed at 2–3 months of age. These mice rarely bred spontaneously and initially were mostly propagated by mating a superovulated F35 female with a normal *Prnp*$^{o/o}$ male and transferring the embryos into healthy fosters or, on occasion, by in vitro fertilization using testicular sperm from a transgenic animal. Currently, F35 mice are being maintained as heterozygotes containing the F35 transgene cluster on a *Prnp*$^{+/o}$ background because, as described below, these mice show no impairment and the pathological phenotype can be recovered by crossing with *Prnp*$^{o/o}$ mice.

Histological examination of the brains of 2–3 month–old animals showed that E11, F11, and F35 but not C4 or D32 mice had massive pathological changes in the cerebellum: a dramatic reduction in the width of the granular cell layer in the hemispheres (Figures 4A–4F) and vermis but not the flocculus (Figures 4G and 4H), intense astrocytosis affecting in particular the Bergmann glia, and irregularly distributed large vacuoles in the white matter of the pons (Figure 4G). Strikingly, the Purkinje cells appeared normal (Figure 4I), and the molecular layer was only modestly reduced in width and still contained a high density of Purkinje cell dendrites (Figures 5E and 5F; MAP2). Luxol-Nissl stainings did not disclose evidence of myelin breakdown (Figures 5G and 5H; LN) despite massive vacuolation of myelinated fiber tracts in the brain stem of some mice. The remainder of the brain showed no obvious changes except for occasional patchy astrocytosis, which was also observed in wild-type litter mates. E11 mice, in addition to these findings, showed severe vacuolation in the brain stem, particularly in the intracerebral portion of the cranial nerves. There was some variation in the severity of the phenotype among individual mice, and one mouse aged 22 weeks displayed only marginal granule cell layer loss despite pronounced cerebellar gliosis. The mice with the shorter truncations of PrP, up to residue 106, show no pathological changes (Figures 4B and 4C).

Expression of PrP in the brain of E11 and F11 mice aged 3–10 weeks (Figures 6A–6F) was studied by immunohistochemistry. Expression was widespread, encompassed both forebrain and cerebellum, and was roughly similar in all mice analyzed as judged by staining intensity. No significant modulation of PrP expression was detected during the time period analyzed. PrP was expressed in all layers of the cerebellum except for the Purkinje cells (Figure 4I). Histoblot analysis failed to reveal protease-resistant PrP and no birefringent deposits could be observed after Congo red staining (data not shown).

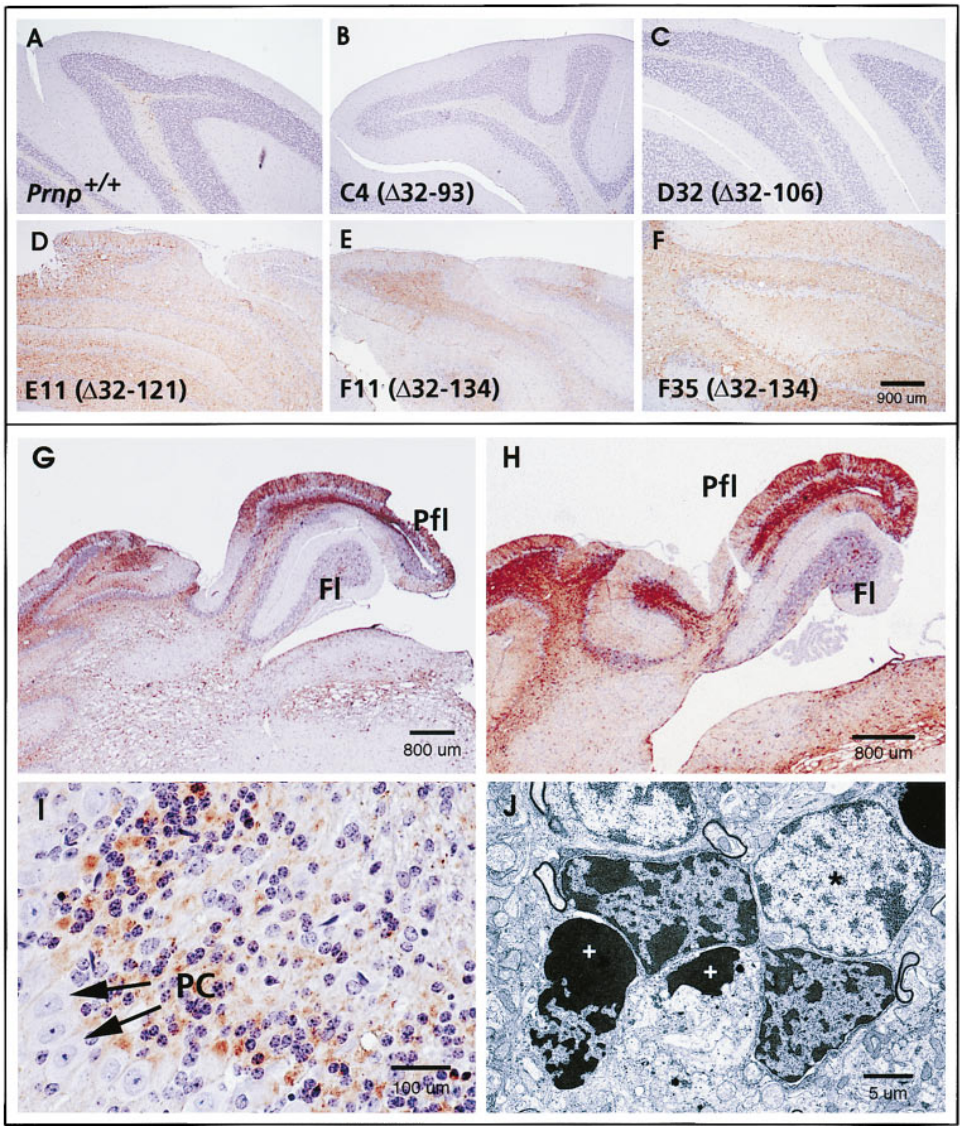


Figure 4. Neuropathology of Transgenic Mice with Amino-Proximal Deletions of Prion Protein

(A–F) Comparison of GFAP immunostains of the cerebellar cortex of various transgenic and wild-type mice. Cerebella of transgenic mice C4 ([B], 8 weeks old) and D32 ([C], 10 weeks old) are histologically normal and indistinguishable from those of wild-type mice (A). Transgenic lines E11 ([D], 8 weeks old), F11 ([E], 25 weeks old), and F35 ([F], 6 weeks old) show a striking reduction of the internal granular layer of the cerebellum and intense astrocytic gliosis.

(G–H) GFAP-stained sections through the cerebellum of E11 ([G], 10 weeks old) and F11 ([H], 12 weeks old) transgenic mice. Prominent astrogliosis and neuronal loss in the internal granular layer, as well as white matter spongiosis, affect the entire cerebellum except for the flocculus (Fl), which is largely unaffected. Pfl, paraflocculus.

(I) At higher magnification, PrP-immunostained sections of the cerebellar cortex of F11 transgenic mice (12 weeks old) reveal strong PrP expression in residual granule cells but no PrP expression in Purkinje cells (PC, arrows). Purkinje cells display no histopathological changes.

(J) Transmission electron microscopy of cerebellar granule cells of F11 transgenic mouse aged 3.5 weeks. Some neuronal cells do not display ultrastructural changes (asterisk), while others are undergoing apoptosis (plus).

TUNEL analysis yielded strong signals in nuclei of degenerating cerebellar granule cells (data not shown) in parallel to detection of nuclear fragmentation and condensation in transmission electron microscopy (Figure 4J). No TUNEL positivity and no morphological evidence of apoptosis was apparent in other regions of the central nervous system.

Furthermore, electron microscopy of the cerebellar granule cell layer (Figure 4J) revealed large numbers of apoptotic bodies in 3-week-old F11 mice and moderate numbers in 10- and 16-week-old F11 mice. No sign of degeneration in neurons of the pyramidal layer and of the dentate gyrus in the hippocampus of the same mice were detected in any transgenic line. Also, we were not

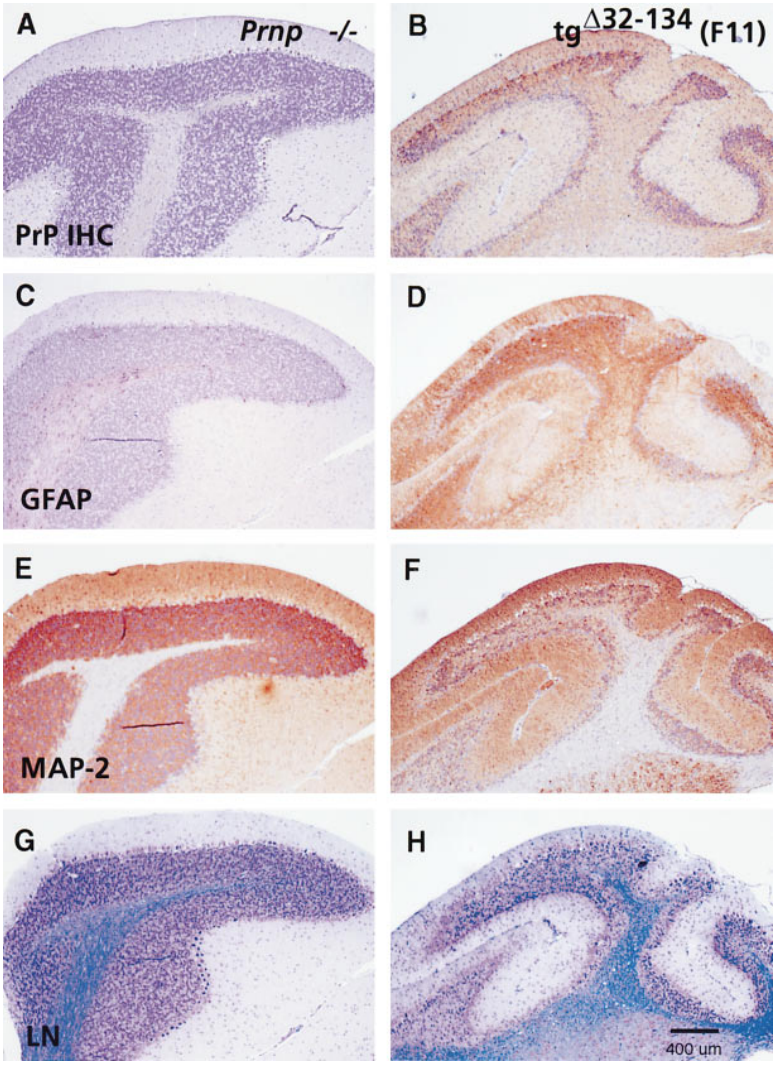


Figure 5. Comparison of Cerebellar Sections from *Prnp*$^{o/o}$ and F11 Mice

Sections were stained for PrP (A and B), GFAP (C and D), microtubule-associated protein (MAP-2, [E and F]), and Luxol-Nissl (LN, [G and H]). Both *Prnp*$^{o/o}$ and F11 mice were 12 weeks old. In contrast to the severely damaged granular cell layer, the molecular layer shows only a modest reduction in width (C and D) and no obvious changes in the density of Purkinje cell dendrites (E and F). The white matter of F11 mice displays no signs of myelin breakdown (G and H).

able to identify intraneuronal inclusion bodies such as those described by Muramoto et al. (1997) in either cerebellar or hippocampal neurons.

The time course of the pathological changes was examined in F11 mice (Figures 7B–7F). Astrocytosis was barely apparent at 3 weeks but pronounced at 4 weeks; individual neurons seemed to be surrounded by astrocytic processes (satellitosis). By 5 weeks pycnotic nuclei dotted the granular layer, which still had the normal width; astrocytosis was intense and extended to the molecular layer. In 1 of 2 5-week-old mice, we observed ongoing synchronous destruction of the granule cell layer leading to large cystic defects. At 6 weeks the granular layer had narrowed; no cystic areas were present, perhaps because resorption of degenerated tissue and subsequent shrinkage had taken place. At 8 weeks there was a dearth of cells in the granular layer, while the Purkinje cell layer appeared unaffected.

In the cerebral hemispheres, neuronal loss was not conspicuous at any time point. However, mild yet significant gliosis was detected in the thalamus and cortex of some mice at the height of cerebellar degeneration. Since gliosis was stationary, we tend to believe that it represents a reaction to toxic metabolites released during the ongoing damage to the cerebellum rather than an intrinsic cortical pathology.

The Cerebellar Defects Are Abrogated by the Introduction of One or More Wild-Type PrP Genes

E11, F11, or F35 mice were crossed with mice carrying wild-type PrP alleles or *tga20* mice carrying a cluster of wild-type PrP-encoding transgenes. All resulting progeny with truncated transgenes *E11* or *F11* and a *tga20* transgene cluster (Table 1, crosses 1, 5, 6, 7, and 8) showed normal behavior and normal brain histology (Figure 7G) as did progeny with the *F11* or the highly pathogenic *F35* gene cluster and with as few as one copy of a normal PrP gene (Table 1, crosses 2, 4, and 9). In a preliminary experiment, it was found that even a cluster of Δ32–93 transgenes (*C4*) abrogated the deleterious effects of the *F11* gene cluster (Table 1, cross 8).

It was possible that expression of wild-type PrP in some way prevented expression of the truncated PrP molecules. Western blots of brain extracts from mice containing the *F11* gene cluster and one or two copies

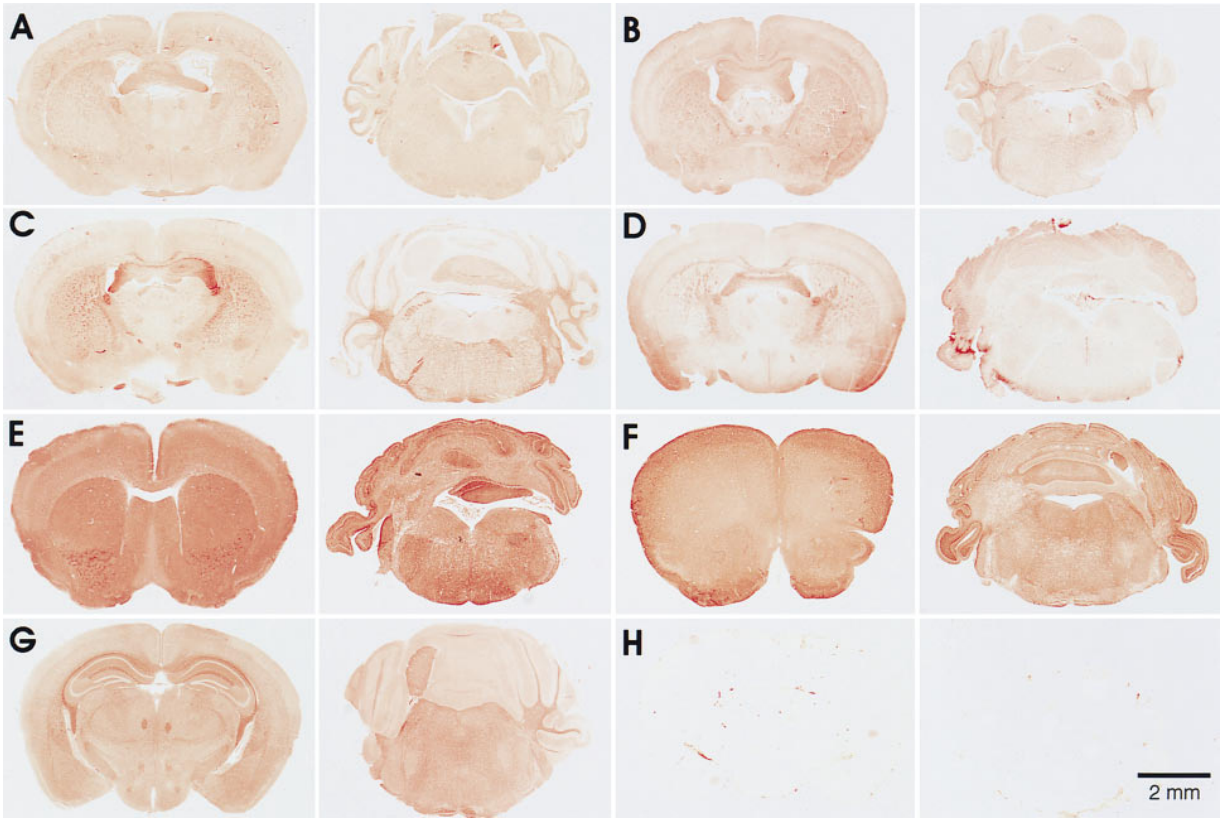


Figure 6. PrP Expression in the Brains of F11 and E11 Transgenic Mice at Various Ages

Coronal sections (left, forebrain; right, cerebellum) of 3-week- (A), 4-week- (B), 6-week- (C), and 12-week-old F11 mice (D); 8-week- (E), and 10-week-old E11 mice (F); 10-week-old wild-type mice (G); and 12-week-old *Prnp*$^{o/o}$ mice (H). While strong PrP immunoreactivity is discernible in brain sections of mice expressing wild-type and mutant PrP proteins, virtually no staining is seen in *Prnp*$^{o/o}$ tissue. Truncated PrP is expressed to similar extents in cerebral and cerebellar cortex in E11 or F11 transgenic mice, but pathological changes are found only in the cerebellum.

of the wild-type *Prnp* allele, however, showed that the level of the truncated PrP was the same as in the absence of wild-type PrP (Figure 2D, left panel). The same was true for the *E11* (Figure 2D, right panel) or *F11* (not shown) gene cluster in the presence of the *tga20* transgenes.

Attempts to Transmit Disease with Brain Extracts from E and F Mice

It has been reported that PrP-null mice overexpressing PrP with a P101L mutation from the cognate transgenes spontaneously develop scrapie-like disease. This disease could be transmitted by inoculation of brain extracts into mice expressing the same type of mutated PrP at a low level, which does not cause spontaneous disease, but the disease could not be transmitted to wild-type mice (Hsiao et al., 1994; Telling et al., 1996).

We inoculated mice overexpressing wild-type PrP (*tga20* mice) with brain extracts from E11 and F11 mice taken at 2.5 months of age. No signs of disease were observed in the indicator mice as late as 60 weeks after inoculation. Further attempts of transmission into mice expressing PrP with 32–121 or 32–134 deletions at low levels are required in view of the reports mentioned above (Hsiao et al., 1994; Telling et al., 1996).

Discussion

We have found that PrP-null mice expressing at a moderate level transgenes encoding PrP with an amino-terminal deletion extending from residue 32 to 121 or 134, but not those with the deletions 32–80, 32–93 or 32–106, exhibit severe neurological symptoms and degeneration of the granular layer of the cerebellum at an early age. It is highly unlikely that this phenotype is due to insertional mutagenesis, not only because it was expressed in mice heterozygous for the insertion but also because it was observed in three independent transgenic populations. We therefore attribute the phenotype to the expression of specific forms of truncated PrPs.

Pathological consequences may result from overexpression of a normal protein in the tissue in which it is normally expressed (Xu et al., 1993; Simson et al., 1994; Sakata et al., 1996) or from ectopic expression or overexpression (McAndrew et al., 1995). Westaway et al. (1994) reported that (uninoculated) transgenic mice harboring high copy numbers of cosmids containing wild-type PrP genes developed truncal ataxia, hindlimb paralysis, and tremors as well as necrotizing myopathy of skeletal muscle, demyelinating polyneuropathy and focal vacuolation of the central nervous system. Disease

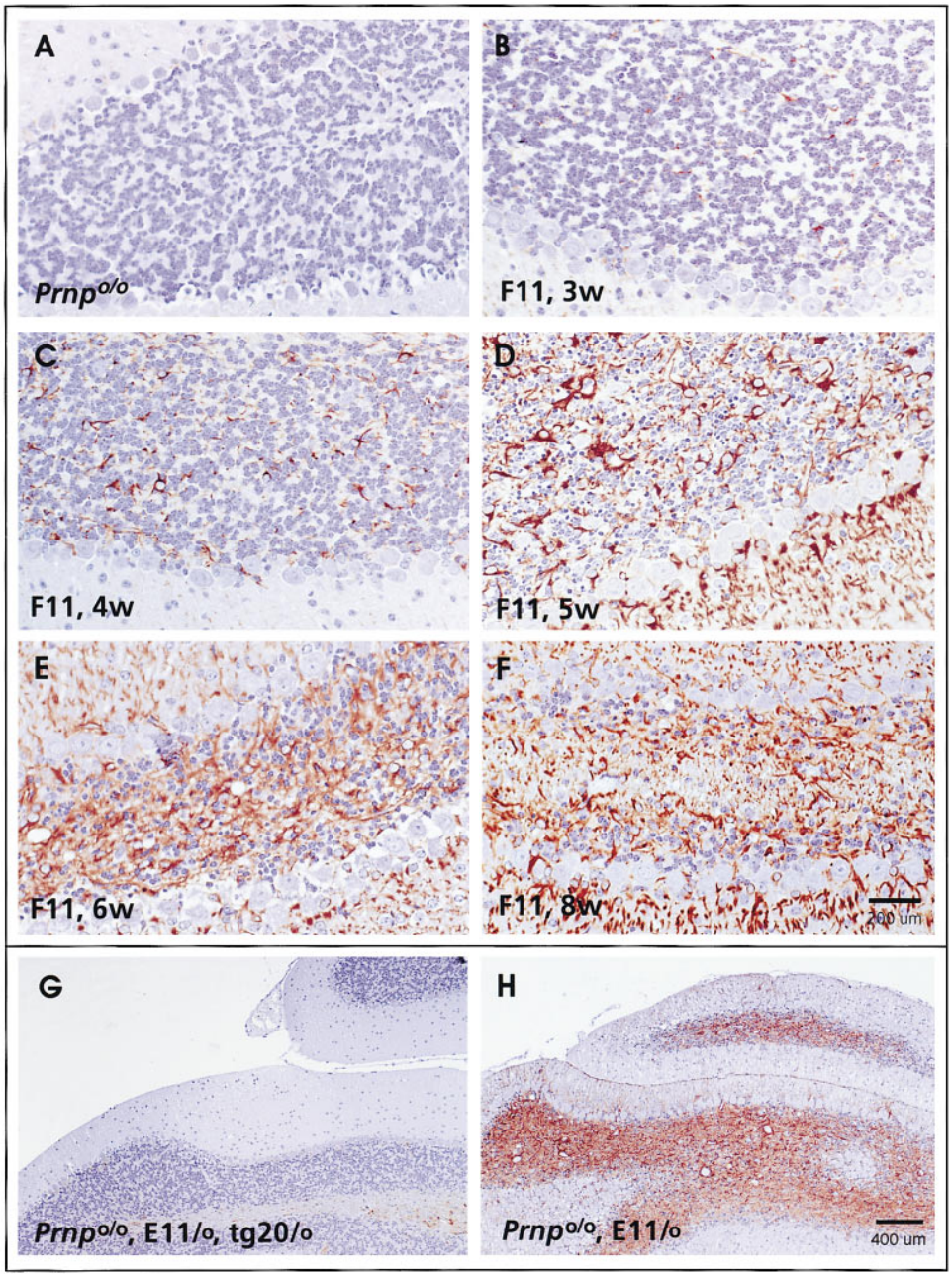


Figure 7. Time Course of Neuropathological Changes in the Internal Cerebellar Granular Layer of F11 Transgenic Mice

Sections were immunostained for GFAP. At 3 weeks (B) and 4 weeks (C) of age, the internal granular layer is still largely intact and similar to the nontransgenic *Prnp*$^{o/o}$ control (A), but incipient astrogliosis is observed. In 5-week-old mice (D), there is prominent diffuse astrogliosis of the whole cerebellar cortex and coarse vacuolation of the granular cell layer. Several apoptotic figures are visible in the granular cell layer. At 6 (E) and 8 (F) weeks, the granular layer has collapsed and shows progressive cell loss as well as extreme gliosis. The Purkinje cells appear intact throughout this process. (G and H) GFAP immunohistochemistry of the cerebellum of mice carrying the E11 transgene cluster on a *Prnp*$^{o/o}$ (H) or on a *tga20* genetic background (G). Introduction of the wild-type transgenes completely abrogated the phenotype seen in E11 transgenic mice, as demonstrated here by the absence of astrogliosis (G).

developed late in life, between 460 and 650 days of age, depending on transgene dosage. Transgenic mice overexpressing PrP from multiple copies of a mouse PrP (B allele) cosmid (obtained from S. Prusiner) were generated in our laboratory and showed the same phenotype; however, *tga20* mice carrying a minitransgene encoding the *Prnp*^{a} allele coding sequence and lacking intron 2 (for an exact description, see Fischer et al., 1996), although expressing at least a similar level of PrP, never showed any disabilities, perhaps because they had a different expression pattern (Fischer et al., 1996) or a different PrP sequence.

A different heritable disorder was observed in wild-type mice overexpressing a mouse–hamster hybrid PrP

Table 1. Wild-Type *Prnp* Alleles Protect against Deleterious Effects of Truncated *Prnp* Alleles[a]

Cross	Parents	Genotype of Offspring	Phenotype
1	*E11*/−, $Prnp^{o/o}$ × ***tga20***/−, $Prnp^{o/o}$	*E11*/−, ***tga20***/−, $Prnp^{o/o}$	1 killed at 11 weeks (**healthy**)[b] 1 killed at 15 weeks (**healthy**)[c] 1 **healthy** at 52 weeks
2	*F11*/−, $Prnp^{o/o}$ × $\boldsymbol{Prnp}^{+/o}$	*F11*/−, $\boldsymbol{Prnp}^{+/o}$	2 **healthy** at 31–32 weeks 1 killed at 12 weeks (**healthy**)[b]
3	*F11*/−, $\boldsymbol{Prnp}^{+/o}$ × $\boldsymbol{Prnp}^{+/o}$	*F11*/−, $\boldsymbol{Prnp}^{+/+}$	1 killed at 11 weeks (**healthy**)[b]
4	*F11*/−, $\boldsymbol{Prnp}^{+/o}$ × $Prnp^{o/o}$	*F11*/−, $\boldsymbol{Prnp}^{+/o}$ *F11*/−, $Prnp^{o/o}$	4 **healthy** at 22–23 weeks 11 **sick** at 5–9 weeks
5	*F11*/−, $Prnp^{o/o}$ × ***tga20/tga20***, $Prnp^{o/o}$	*F11*/−, ***tga20***/−, $Prnp^{o/o}$	2 killed at 14 weeks (**healthy**)[b] 5 **healthy** at 31–32 weeks
6	*F11*/−, ***tga20***/−, $Prnp^{o/o}$ × $Prnp^{o/o}$	*F11*/−, ***tga20***/−, $Prnp^{o/o}$ *F11*/−, $Prnp^{o/o}$	1 **healthy** at 23 weeks 1 **sick** at 6 weeks
7	*F11*/−, ***tga20***/−, $Prnp^{o/o}$ × ***tga20***/−, $Prnp^{o/o}$	*F11*/−, ***tga20***/−, $Prnp^{o/o}$ *F11*/−, $Prnp^{o/o}$	6 **healthy** at 19 weeks 3 **sick** at 6–7 weeks
8	*F11*/−, ***tga20***/−, $Prnp^{o/o}$ × *C4/C4*, $Prnp^{o/o}$	*F11*/−, *C4*/−, $Prnp^{o/o}$ *F11*/−, *C4*/−, ***tga20***/−, $Prnp^{o/o}$	1 **healthy** at 24 weeks 1 **healthy** at 24 weeks
9	*F35*/−, $Prnp^{o/o}$ × $\boldsymbol{Prnp}^{+/+}$	*F35*/−, $\boldsymbol{Prnp}^{o/o}$	5 **healthy** at 29 weeks

[a] *C4*, *E11*, *F11*, and *F35* designate the truncated PrP transgene clusters present in C4, E11, F11, and F35 mice, respectively (see Figure 1C). *tga20* is a gene cluster encoding wild-type PrP. Alleles encoding wild-type PrP are in bold type.
[b] Western blots of brain extracts showed transgene expression levels similar to those of the same transgene on a $Prnp^{o/o}$ background (see Figure 2d).
[c] Histology of the brain was normal.

with a deletion between residues 177 and 200 or 201 and 217, which disrupted the penultimate and last α helix (Riek et al., 1997), respectively (Muramoto et al., 1997). These mice showed cytoplasmic inclusions of PrP-derived deposits in neurons and spontaneously developed fatal CNS illnesses similar to neuronal storage diseases at ages ranging mostly from 90 to 227 days.

The behaviorally challenged transgenic mice described in this paper differ from the mice described by Prusiner and his colleagues in several regards. (1) They exhibit the pathological phenotype even when expressing truncated PrP at only about twice wild-type levels. (2) The phenotype is apparent within a few weeks after birth. (3) The truncated protein is expressed at the cell surface. (4) The histopathology is essentially restricted to the cerebellum with degeneration of the granular layer and coarse vacuolation of the white matter and no obvious pathology in cortex or basal ganglia. (5) There were no PrP-derived deposits.

Strikingly, the pathological phenotype was overcome by introducing as few as a singly copy of the wild-type PrP gene.

How can our findings be explained?

An unspecific toxic effect is unlikely because only granule cells of the cerebellum, with the exception of those in the flocculus that also express PrP, undergo cell death, while other neurons expressing the truncated PrP at similar levels (e.g., in the cortex) do not.

A dominant negative effect, i.e., direct interference of truncated PrP with a function unique to normal PrP, cannot be invoked because pathology develops in mice devoid of PrP. However, a phenotypic transdominant negative effect is conceivable. Assume that in wild-type mice PrP interacts with a presumed ligand, "L_{prp}", to elicit a particular signal and that the same signal is elicited by the interaction of L_{prp} with π, a conjectural protein that has the functional properties of PrP (but is not closely related to it at the DNA sequence level) (Figure 8). This would explain why ablation of PrP has no obvious phenotypic consequences. We postulate that truncated PrP can interact with L_{prp} without giving rise to a signal and that it can compete efficiently with π, thus acting as an inhibitor of the latter. Because the pathogenic effects of truncated PrP are overcome by coexpression of wild-type PrP at comparable levels, the affinity of L_{prp} for truncated PrP would have to be less than for intact PrP but greater than for π. It is interesting to note that the detrimental effects of PrP appear when the truncations extend to or beyond position 121. It has been shown that mature PrP consists of a flexible, disordered tail extending from position 23 to 120, a highly conserved region, while the remainder is folded into a stable, structured globular part (Donne et al., 1997; Riek et al., 1997). It is appealing to imagine that the globular domain binds to a receptor (or ligand), while the tail is responsible for the activation. Several candidate ligands for PrP have been recently suggested (Martins et al., 1997; Rieger et al., 1997; Yehiely et al., 1997).

The hypothesis presented above suggests that there could exist inherited diseases with phenotypes similar to the one we have reported, not only as a consequence of PrP truncations but also of mutations of the presumed ligand L_{prp} or of component(s) of the system transmitting the postulated signal. We are not aware of natural mouse mutations qualifying for this role, but human familial diseases such as congenital cerebellar granule cell hypoplasia and mental retardation (cerebelloparenchymal disorder III) (Norman, 1940; McKusick, 1988), primary degeneration of the granular layer of the cerebellum (Pascual-Castroviejo et al., 1994), or infantile cerebellooptic atrophy (PEHO syndrome) (Haltia and Somer, 1993), all of which present with degeneration of the granule cell layer, are possible candidates.

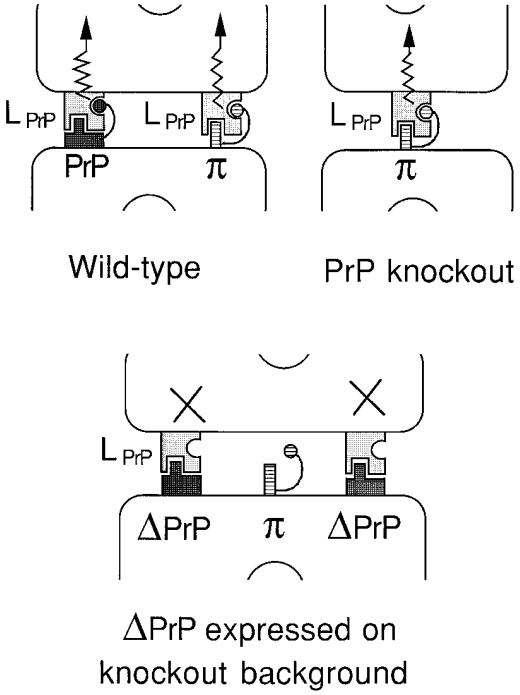


Figure 8. Model to Explain Effects of Truncated PrP

PrP is composed of a globular part (dark-hatched polygon) and a flexible tail (string-and-ball) (Riek et al., 1997) and may interact separately with a presumed ligand, "L_{prp}", via these two domains, thereby eliciting a signal. The same signal might be elicited by the interaction of L_{prp} with π, a conjectural protein that has the functional properties of PrP (top, left), explaining why PrP knockout mice show no phenotype (top, right). The model postulates that PrP lacking the flexible tail can interact with L_{prp} without giving rise to a signal and that it can compete efficiently with π, thus acting as a dominant inhibitor of the latter (bottom). The postulated signal could act through PrP if the deleterious effect is cell autonomous or through L_{prp} if it is not.

Experimental Procedures

PrP Minigenes with Amino-Proximal Deletions

Plasmid PrP12Msc contains a murine PrP cDNA fragment extending from the beginning of the first exon to codon 31 in exon 3 was prepared by PCR, using as upstream primer pE1[B/T] (5′-TGTCGGA TCCAGCAGACCGATTCTGG) and as downstream primer PRP31$^-$ (5′-CCACCCTCCAGGCTTT). This 247 bp fragment was ligated into pBluescript KS$^-$, which had been cleaved with SacI and blunted; the junction between the downstream end of the PCR product (5′-TGG) and the blunted SacI site (5′-CCA) generated an MscI site (5′-TGG′CCA).

PCR products of PrP cDNA starting at codons 94, 107, 122, or 135 were generated using a downstream primer P4 (5′-CCTTGGGAA TGAACAAAGGTTTGCTTTCAAC; third exon) and upstream primers PRP93 (5′-ACCCATAATCAGTGGAACAA), PRP106 (5′-AACCTCAA GCATGTGGCA), PRP121 (5′-GGGGGCCTTGGTGGCTA), and PRP 134 (5′-AGGCCCATGATCCATTT), respectively, and Pfu polymerase (which produces blunt ends). Each PCR product was cleaved with EcoRI in the 3′ noncoding region and ligated into plasmid PrP12Msc that had been cleaved with MscI (to give a blunt end after codon 31) and EcoRI. This yielded plasmids PRP123.C, PRP123.D, PRP123.E, and PRP123.F with deletions of codons 32–93, 32–106, 32–121, and 32–134, respectively.

Finally, the KpnI-EcoRI fragment of pPrPHG (Fischer et al., 1996) was replaced by the KpnI-EcoRI fragment of each of the PRP123 plasmids to yield plasmids pPrPHG.C, pPrPHG.D, pPrPHG.E, and pPrPHG.F. The entire open reading frame was verified by sequencing (Sanger et al., 1977).

Generation, Identification, and Maintenance of Transgenic Mice

The pPrPHG plasmids containing the truncated PrP coding sequences were propagated in *E. coli* XL1 blue, the minigene excised with NotI and SalI, and processed as described (Fischer et al., 1996). Nuclear injections into fertilized oocytes were carried out by conventional methods (Brinster et al., 1985; Wilmut et al., 1991). In all but two cases, zygotes were from *Prnp*$^{o/o}$ mice (with a 129/Sv-C57BL/6 hybrid background); however, C15 mice were from zygotes resulting from a cross between *Prnp*$^{o/o}$ and wild-type C57BL/6 mice and D32 animals from zygotes of a C57BL/6 × DBA/2 cross. Transgenes on a *Prnp*$^{o/o}$ background were identified by PCR using the exon 2 primer pE2$^+$ (5′-CAACCGAGCTGAAGCATTCTGCCT) and the exon 3 primer Mut217 (5′-CCTGGGACTCCTTCTGGTACCGGGTGACGC). For breeding out the *Prnp*$^+$ allele from transgenic mice that had been generated on a wild-type or *Prnp*$^{+/o}$ background, PCR analysis was carried out using primers RK1 (*Prnp* intron 2, 5′-TCAGCCTAAATACTGG GCA), RK2 (*Prnp* exon 3, 5′-GCCTAGACCACGAGAATGC), and RK3 (*neoR* gene, 5′-CAAGGGCCCCTCCTCCTAGAA); RK1 and RK2 gave an 880 bp signal for the *Prnp*$^+$ allele, and RK1 and RK3 gave a 730 bp product for the *Prnp*^{o} allele. Semiquantitative determination of transgene copy number relative to the residue of the PrP knockout gene was carried out with primers P7, PRP134, and Mut217, amplifying by PCR for 21 cycles using ^{32}P-labeled dATP (Fischer et al., 1996). Primers P7 (located in the *neoR* gene within the *PrP*^{o} allele: 5′-TCTATCGCCTTCTTGACGAGTTCTTCTGAG and Mut217 amplify a *Prnp*^{o} amplicon of 210 bp, while PRP134 and Mut217 yield a 265 bp fragment characteristic for the wild-type as well as the truncated alleles.

Northern blot analyses (Sambrook et al., 1989) were performed on RNA extracted by the acid phenol method (Chomczynski and Sacchi, 1987) using as a PrP probe a randomly ^{32}P-labeled (Prime-it, Stratagene) 308 bp amplicon prepared with primers pMcP5 (exon 1, 5′-GTCGGATCAGCAGACCGATTCTGGGCGCT) and PRP31$^-$ on PrP cDNA. This probe hybridizes with all wild-type and truncated PrP mRNAs as well as the "readthrough" RNA from the disrupted *Prnp* locus (Büeler et al., 1992). After stripping, the sample was rehybridized with a GAPDH probe (Fort et al., 1985).

For Western blot analyses, a brain hemisphere was homogenized in 7 vol PBS, 0.5% Nonidet P-40, and 0.5% deoxycholate and the solution was centrifuged 10 min in an Eppendorf centrifuge. For deglycosylation, 50 μg denatured protein were incubated at 37°C for 4 hr with 500 U PNGase F (New England Biolabs) according to the manufacturer's instructions. To inhibit proteolysis, the inhibitors Pefabloc (1 mg/ml), Leupeptin (10 μg/ml), Pepstatin (10 μg/ml), Aprotinin (1 μg/ml) (all from Boehringer, Mannheim), and 0.5 mg/ml EDTA were added. Recombinant PrPs with an N-terminal His tag were expressed in *E. coli* (unpublished data) and purified by Ni-chelate affinity chromatography (Petty, 1996). After electrophoresis of protein samples through 12% or 15% SDS-polyacrylamide gels, samples were transferred to PVDF membranes (Immobilon P, Millipore) and exposed to a rabbit polyclonal anti-PrP antiserum (R340 raised against *E. coli* murine PrP [1:5000; Brandner et al., 1996], or R009 raised against PrP residues 221–234, [1:2000; Barry et al., 1988]), then to peroxidase-labeled anti-rabbit antiserum (1:2500; Amersham) and developed with the ECL detection system (Amersham).

Histopathology

Organs were fixed in 4% paraformaldehyde in PBS (pH 7.5), paraffin-embedded, and cut into 2 μm sections. Brain sections were stained with hematoxylin-eosin, Luxol-Nissl (myelin and neurons), and with commercial antibodies to GFAP (glial fibrillary acidic protein; activated astrocytes), MAP-2 (dendrites), or synaptophysin (synapses). PrP was detected on microwave-treated paraffin sections using monoclonal antibody 8H4 (hybridoma culture supernatant diluted 1:100, a kind gift of Dr. Man-Sun Sy) (Zanusso et al., submitted) and visualized using the peroxidase-anti-peroxidase method.

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