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Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain

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PrP knockout mice in which only the open reading frame was disrupted ('Zürich I') remained healthy. However, more extensive deletions resulted in ataxia, Purkinje cell loss and ectopic expression in brain of Doppel (Dpl), encoded by the downstream gene, *Prnd*. A new PrP knockout line, 'Zürich II', with a 2.9 kb *Prnp* deletion, developed this phenotype at ~10 months (50% morbidity). A single *Prnp* allele abolished the syndrome. Compound Zürich I/Zürich II heterozygotes had half the Dpl of Zürich II mice and developed symptoms 6 months later. Zürich II mice transgenic for a *Prnd*-containing cosmid expressed Dpl at twice the level and became ataxic ~5 months earlier. Thus, Dpl levels in brain and onset of the ataxic syndrome are inversely correlated.

Keywords: cerebellar syndrome/doppel protein/gene deletion/granule cells/prion protein

Introduction

PrP, the prion protein, plays a central role in the pathogenesis of transmissible spongiform encephalopathies such as scrapie or BSE. The normal form of PrP, designated PrP^C, is encoded by a single-copy gene (Basler *et al*., 1986) and is expressed in the brain of healthy and prion-infected organisms to about the same extent (Chesebro *et al*., 1985; Oesch *et al*., 1985).

Several PrP knockout lines have been generated that differ in the design of the gene disruption (Figure 1). The first two lines described, hereafter called $Prnp^{0/0}$ Zürich I and $Prnp^{-/-}$ Edinburgh, developed and reproduced normally (Büeler *et al*., 1992; Manson *et al*., 1994). In $Prnp^{0/0}$ Zürich I mice, PrP codons 4–187 (72% of altogether 254 codons) were replaced by a neomycin (*neo*) cassette. Mice homozygous for the disrupted gene expressed transcripts containing the *neo* and the residual *Prnp* sequence in the brain, but no PrP^C-containing protein was detected. In the $Prnp^{-/-}$ Edinburgh mice the PrP gene was disrupted by the insertion of a *neo* cassette into a unique *Kpn*I site following residue 93 of the PrP open reading frame (ORF). No PrP mRNA or PrP-related protein was detected in the brain of homozygous $Prnp^{-/-}$ Edinburgh animals.

In a third PrP knockout line, here designated $Prnp^{-/-}$ Nagasaki, a 2.1 kb genomic DNA segment comprising 0.9 kb of intron 2, 10 bp of 5′ non-coding region, the entire PrP ORF and 0.45 kb of the 3′ non-coding region, was replaced with a *neo* cassette (Sakaguchi *et al*., 1996). These mice developed normally but exhibited severe ataxia and Purkinje cell loss in later life (Sakaguchi *et al*., 1996) as well as demyelination of peripheral nerves (Nishida *et al*., 1999). Because this phenotype was abolished by introduction of a PrP transgene (Nishida *et al*., 1999) it was concluded that both ataxia and peripheral nerve degeneration were due to the absence of PrP. A fourth line, Rcm0, was cited in Moore *et al*. (1999) and Silverman *et al*. (2000) as resembling the Nagasaki line with respect to the extensive PrP gene deletion and the ataxic syndrome.

Recently a gene, *Prnd*, was found 16 kb downstream of the murine PrP gene, which comprises an ORF encoding a 179-residue protein (Moore *et al*., 1999). The predicted protein, which was named Doppel (Dpl), has ~25% identity with the C-terminal two-thirds of PrP. *Prnd*-derived mRNA is expressed from a promoter upstream of exon 1 of *Prnd* at relatively high levels in testis and heart but at very low levels in brain of wild-type mice. However, in Nagasaki and Rcm0 mice, but not in Zürich I mice, *Prnd*-specific RNAs were present at relatively high levels in brain. These transcripts originate at the *Prnp* promoter, run beyond the *Prnd* ORF and are processed by one or more splicing events that link the 3′ end of the second PrP exon directly or indirectly to the Dpl-encoding exon (Moore *et al*., 1999; Li *et al*., 2000). Dpl has been identified as an N-glycosylated, GPI-linked protein in testes of normal, and in brain of Rcm0 PrP knockout, mice carrying the extensive PrP gene deletion (Silverman *et al*., 2000). The cerebellar syndrome was therefore attributed to the ectopic expression of Dpl in the brain (Moore *et al*., 1999; Li *et al*., 2000).

Here, we describe the generation of a further PrP knockout line, hereafter called $Prnp^{-/-}$ Zürich II, in which the PrP-encoding exon and its flanking regions were replaced by a *loxP* site. The homozygous mice, like their $Prnp^{-/-}$ Nagasaki counterparts, developed progressive ataxia and age-dependent Purkinje cell loss starting at 5–6 months, with half the mice affected by ~10 months, and showed ectopic expression of two *Prnd*-derived mRNAs and Dpl in brain. A single wild-type PrP allele fully corrected the deleterious phenotype in agreement with previous reports (Sakaguchi *et al*., 1996; Nishida *et al*., 1999). The F1 offspring of a cross between the Zürich I and Zürich II lines showed partial complementa-

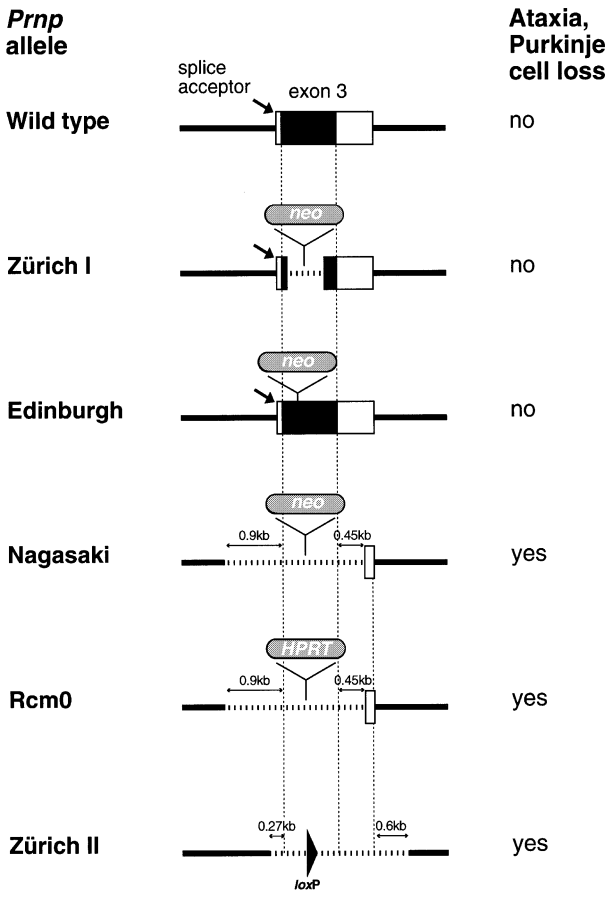

Fig. 1. Strategies used to create various PrP knockout lines. The black boxes represent PrP ORF; white boxes, non-coding *Prnp* regions; grey boxes, sequences inserted into the gene; dotted line, deleted regions of *Prnp*. *neo*, neomycin phosphotransferase; HPRT, hypoxanthine phosphoribosyltransferase; *loxP* (black arrowhead), a 34 bp recombination site from phage P1.

tion, in that they remained healthy 6 months longer than the Zürich II mice, despite the absence of a PrP ORF. The level of *Prnd*-derived mRNAs and Dpl in brain was half that in Zürich II mice.

Introduction into Zürich II mice of a cosmid comprising both the *Prnd* gene and a *Prnp* locus lacking the PrP ORF resulted in accelerated development of the ataxic phenotype and was associated with increased levels of *Prnd*-derived transcripts and Dpl in brain. These results suggest that the allele with deletions extending beyond the PrP ORF is pathogenic in a dose-dependent fashion and that this pathogenicity is due to ectopic expression in brain of Dpl (Moore *et al*., 1999; Li *et al*., 2000) rather than to the absence of PrP. N-proximally truncated PrP, which resembles Dpl, also causes ataxia that, as in the case of ectopic Dpl expression, is counteracted by full-length PrP (Shmerling *et al*., 1998).

Results

Mice carrying a deletion of the PrP ORF and its flanking sequences develop a cerebellar syndrome

We generated a PrP-deficient mouse line by gene targeting in embryonic stem cells, as described in Materials and methods and in Figure 2A. In this mouse the wild-type PrP allele was replaced by the *Prnp lox1* allele (to be referred to hereafter as $Prnp^{-}$ Zürich II allele), in which 0.26 kb of intron 2, 10 bp of the 5′ flanking sequence, the entire ORF, the 3′ non-coding region of exon 3 and 0.6 kb of 3′ adjacent sequence were replaced by a 34 bp *loxP* sequence.

Mice carrying the $Prnp^{-}$ Zürich II allele were bred to homozygosity; hemizygous Zürich II ($Prnp^{-/+}$) and homozygous Zürich II ($Prnp^{-/-}$) mice were born in the ratio expected from Mendelian inheritance. In brains of $Prnp^{-/-}$ Zürich II mice no PrP-specific mRNA was found (Figure 2D), nor could PrP be detected by immunoblotting (Figure 2E) or immunohistochemistry (Figure 4B).

$Prnp^{-/-}$ Zürich II mice were normal until ~6 months of age, when they began to develop intention tremor and trembling gait (Figure 3A); this phenotype, which was taken as criterion for diagnosis of the cerebellar syndrome, was present in 50% of the mice by 10 months, and in 100% by 13 months ($n = 63$) (Figure 3B). Progression of the disease led to hypotony of the hind limbs and impaired equilibrium on a moving surface (Crawley and Paylor, 1997) as compared with wild-type animals (P.Valenti and A.Cozzio, data not shown). The hindlegs seemed more affected than the forelegs. Histological analysis revealed a severe age-dependent loss of Purkinje cells in $Prnp^{-/-}$ Zürich II mice (Figure 4A), which was most prominent in the vermis regions I–VIII (Figure 5), the simple lobule, crus I and II ansiform lobules, paramedian lobule and copula pyramis, while vermis regions IX and X were less affected (data not shown). The average cell loss in vermis I–VIII was ~40 and 80% at 30 and 63 weeks, respectively. Concomitant with the gaps in the monolayer of Purkinje cells, the thickness of the molecular layer in these areas was reduced, probably due to the loss of the dendritic trees of the Purkinje cells. Importantly, the granular cell layer as the major afferent source of the Purkinje cells showed no alterations that could be held responsible for secondary damage of their target cells.

$Prnp^{0/0}$ Zürich I mice showed no ataxia or Purkinje cell loss up to at least 80 weeks (Figures 4 and 5).

Mice homozygous for the *Prnp lox2* allele (in which the PrP reading frame and surrounding regions were flanked by *loxP* sequences) were derived from the same founder as the $Prnp^{-/-}$ Zürich II mice, as explained in Materials and methods. These animals also remained healthy for at least 57 weeks and showed no Purkinje cell loss (data not shown).

Northern analysis of 5 μg of poly(A)$^{+}$ mRNA from Zürich II mouse brains showed two *Prnd*-specific RNAs of ~4.3 and 2.7 kb, with about the same intensity as the bands from the same amount of poly(A)$^{+}$ mRNA from testis, which were slightly smaller, 3.9 and 2.2 kb (Figure 6A). The molecular weights are similar to those determined by Li *et al*. (2000), 3.4 and 2.2 kb, but considerably higher than those reported by Moore *et al*. (1999), 2.7 kb and 1.7 kb for both brain and testis, perhaps because of discrepancies in the molecular weight determinations. No *Prnd*-specific bands were detected in wild-type brain. $Prnp^{0/0}$ Zürich I brain showed a single weak band at

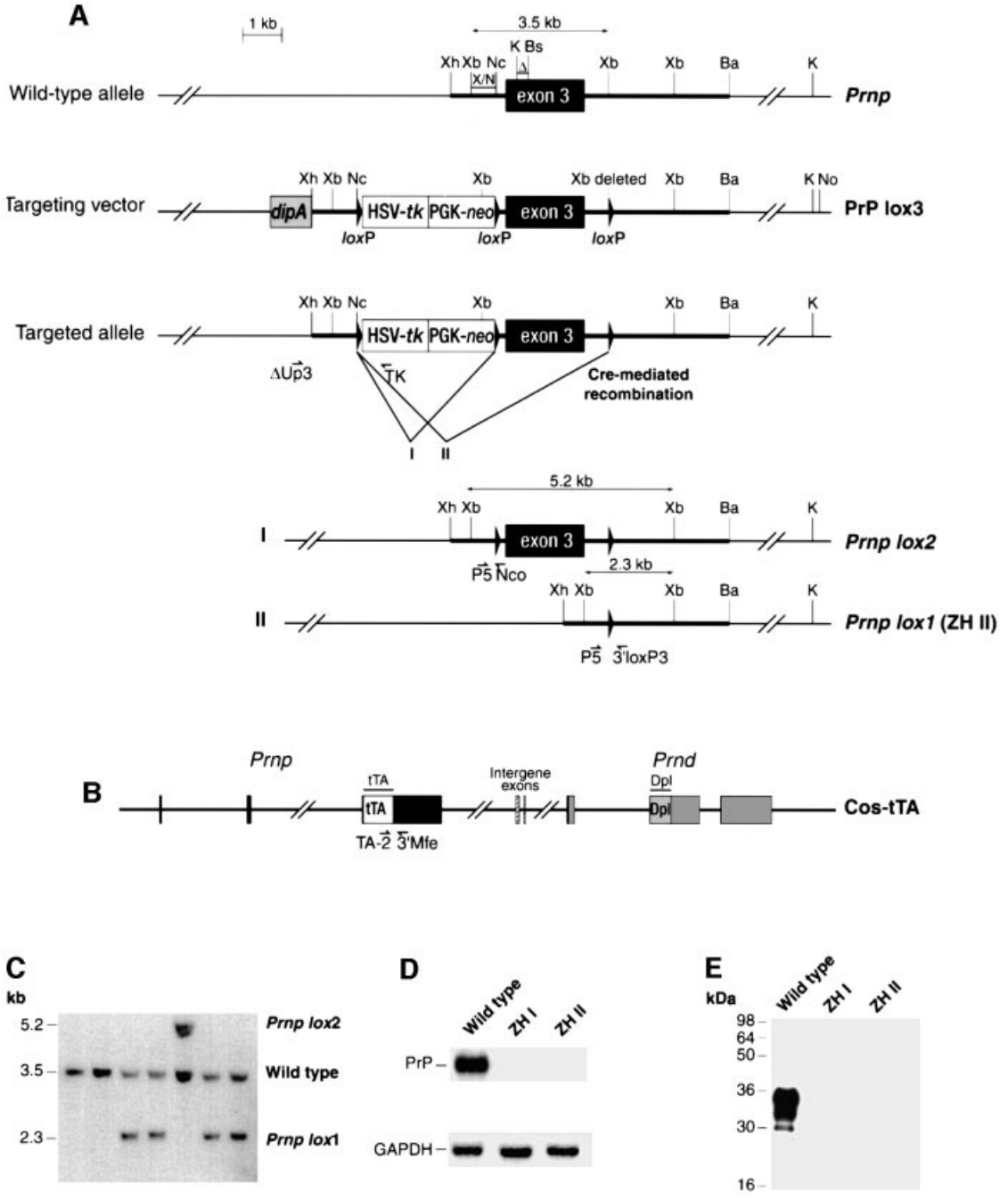

Fig. 2. Strategy for production of *Prnp*$^{-/-}$ Zürich II knockout mice and Cos-tTA transgenic mice. (**A**) Deletion of PrP exon 3 and its flanking regions. The targeting vector PrP lox3, containing the *loxP*-flanked positive–negative selection cassette HSV-*tk*/PGK-*neo* and a *loxP* site downstream of exon 3, was introduced by homologous recombination. Cre-mediated recombination yielded either allele I, in which only the selection cassette was deleted, or II, where both the selection cassette and exon 3 and its flanking regions were removed. Probes (X/N; Δ) and PCR primers (ΔUp3; TK; P5; Nco; 3′loxP3) used for detection of homologous recombinants, Cre-deleted alleles and for northern blot hybridizations are indicated. Xh, *Xho*I; Xb, *Xba*I; Nc, *Nco*I; Bs, *Bst*EII; Ba, *Bam*HI; K, *Kpn*I; No, *Not*I. (**B**) Cos-tTA was derived from cos6.I/LnJ-4, which contains the *Prnp* and *Prnd* loci, by replacing the PrP ORF in exon 3 by the tTA ORF. Black boxes, *Prnp* exons; white box, tTA ORF; hatched boxes, intergene exons; dark grey boxes, *Prnd* non-coding regions; light grey box, Dpl ORF. tTA and Dpl are probes used for Southern and northern analyses; TA-2 and 3′Mfe are primers used for PCR analysis of tail DNA. (**C**) Southern blot of tail DNA derived from the F1 progeny of a chimeric founder that had *Prnp lox2* (see AI), *Prnp lox1* (see AII) and wild-type alleles in the germline. Ten micrograms of DNA were digested with *Xba*I and hybridized with probe X/N. (**D**) Northern blot of total brain RNA (10 μg) from wild-type, Zürich I (ZH I) and Zürich II (ZH II) knockout mice, hybridized with probe Δ (Büeler *et al*., 1992) and, after stripping, with GAPDH probe, as described in Materials and methods. (**E**) Western blot of total brain homogenate (60 μg total protein) of wild-type, ZH I and ZH II mice. PrP was detected with monoclonal antibody 6H4.

~3.4 kb, with ~1/20th the intensity of the sum of the two bands in Zürich II brain. Its origin was not investigated further, but the transcripts may reflect synthesis from the *Prnd* promoter (Moore *et al*., 1999).

Western analysis using a polyclonal antibody against recombinant Dpl (Figure 6B) reveals a strong Dpl band in brain of Zürich II mice, almost as intense as in testes of wild-type mice, but no detectable Dpl in

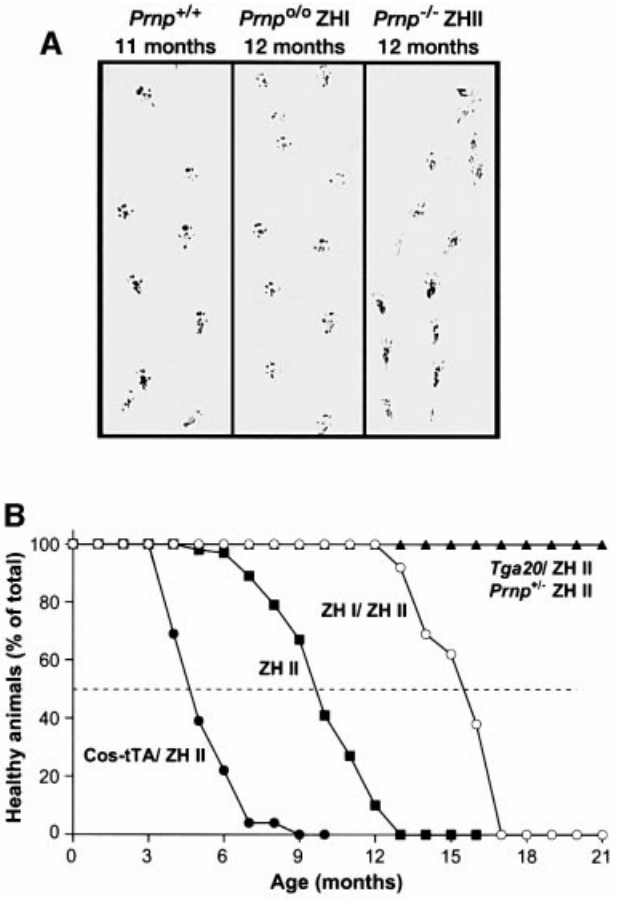

Fig. 3. Development of cerebellar syndrome in ageing $Prnp^{-/-}$ Zürich II mice. (**A**) Footprints of wild-type ($Prnp^{+/+}$), Zürich I ($Prnp^{0/0}$ ZHI) and Zürich II ($Prnp^{-/-}$ ZHII) mice at 11–12 months of age. The hind feet were dipped in ink and the animals prompted to walk on filter paper. Zürich II mice are unable to walk straight and show a generally reduced step length compared with Zürich I and wild-type animals. (**B**) Time-course of the cerebellar symptoms (intention tremor and trembling gait) in Zürich II (ZH II) mice (n = 63), F1 of Zürich I × Zürich II (ZH I/ZH II) mice (n = 13), Zürich II mice transgenic for cosmid Cos-tTA (Cos-tTA/ZH II) (n = 23) or a *Tga20* allele (*Tga20*/ZH II) (n = 17) and Zürich II mice containing a single wild-type allele ($Prnp^{+/-}$ ZH II) (n = 3 for observation over 20 months; n = 19 for observation over 7–8 months).

Zürich I or wild-type brain (less than ~5% of that in Zürich II brain).

F1 offspring of a Zürich I × Zürich II cross have a mitigated ataxic phenotype

Five breeding pairs ($Prnp^{0/0}$ Zürich I × $Prnp^{-/-}$ Zürich II) yielded 33 F1 offspring with a compound heterozygote $Prnp^{0/-}$ Zürich I/II genotype; 13 of these were evaluated over the long term (Figure 3B). They remained healthy until 12 months of age and only then began exhibiting the same cerebellar symptoms as those seen in $Prnp^{-/-}$ Zürich II mice at 5–6 months. After 17 months, all F1 mice showed symptoms (Figure 3B). Histological analysis revealed that at 6 weeks both homozygous $Prnp^{-/-}$ Zürich II and compound heterozygous $Prnp^{0/-}$ Zürich I/II mice showed a normal laminar arrangement (Figure 5). At 30 weeks of age, the compound heterozygotes showed no statistically significant cell loss (average of vermis I–VIII, –15%), and at 63 weeks, when $Prnp^{-/-}$ Zürich II mice had lost 80% of their Purkinje cells, the heterozygotes showed an average loss of only 30%. At 95 weeks, ~70% of the Purkinje cells were lost (Figures 4A and 5), with the exception of vermis IX–X where the loss was only ~25% (data not shown). Offspring of intercrosses of compound heterozygous $Prnp^{0/-}$ Zürich I/II mice showed co-segregation of the cerebellar phenotype with the Zürich II allele. Among 28 offspring, six mice were homozygous $Prnp^{0/0}$ Zürich I, nine were homozygous $Prnp^{-/-}$ Zürich II and 13 animals were compound heterozygous Zürich I/II. At 30 weeks of age, all seven homozygous Zürich II mice observed showed the cerebellar phenotype; two mice were killed for histological examination and showed ~40% Purkinje cell loss (data not shown). The six Zürich I mice remained healthy for up to at least 48 weeks.

As expected, the two *Prnd*-specific RNA bands (Figure 6A) as well as the Dpl protein bands (Figure 6B) in Zürich I/Zürich II brains had about half the intensity of those in Zürich II brain.

A cosmid expressing Prnd-derived mRNAs but not PrP mRNA in brain of Zürich II mice causes accelerated appearance of the ataxic syndrome

The DNA segment contained in $Prnp^{b}$ cosmid cos6.I/LnJ-4 extends from 19 kb upstream to 19 kb downstream of the PrP ORF and comprises the *Prnd* locus (Westaway *et al*., 1994; Moore *et al*., 1999). In connection with a different project, the PrP ORF was replaced with the 900 bp *tet*-dependent transactivator ORF (Gossen and Bujard, 1992; Furth *et al*., 1994; Schultze *et al*., 1996). Nuclear injection yielded founders transgenic for this modified cosmid, Cos-tTA, (A.Cozzio, D.Rossi and C.Weissmann, unpublished data). In order to determine whether an allele containing the flanking regions of the third PrP exon but not the PrP ORF could abrogate the ataxic syndrome, the cosmid transgene was bred into Zürich II mice. Unexpectedly, mice homozygous for the Zürich II ($Prnp^{-}$) allele and containing Cos-tTA showed a much accelerated course of the disease: onset of ataxia was at ~19 weeks (50% morbidity) (Figure 3B) and Purkinje cell loss in vermis I–VIII was 40 and 70% at 6 and 30 weeks, respectively (Figure 5). Expression of tTA at a 4-fold higher level in mice transgenic for a similar vector [mouse line *Prnp*-tTA/F959 (Tremblay *et al*., 1998)], which, however, does not contain the *Prnd* locus, has no deleterious effects up to at least 10 months of age (A.Servadio and D.Rossi, unpublished results). For reasons that are not immediately clear, brains of Zürich II mice containing the *Prnd* cosmid showed about twice the level of chimeric *Prnd*-derived mRNAs (Figure 6A) and Dpl protein (Figure 6B) compared with Zürich II brain. Maybe the replacement of the PrP ORF by a prokaryotic sequence impairs splicing to the acceptor site of the third exon (Lavigueur *et al*., 1993; Zandberg *et al*., 1995; Chiara *et al*., 1996; Dominski and Kole, 1996). Thus, there is again an inverse correlation between the time to appearance of ataxic symptoms and the level of *Prnd*-derived mRNAs and Dpl.

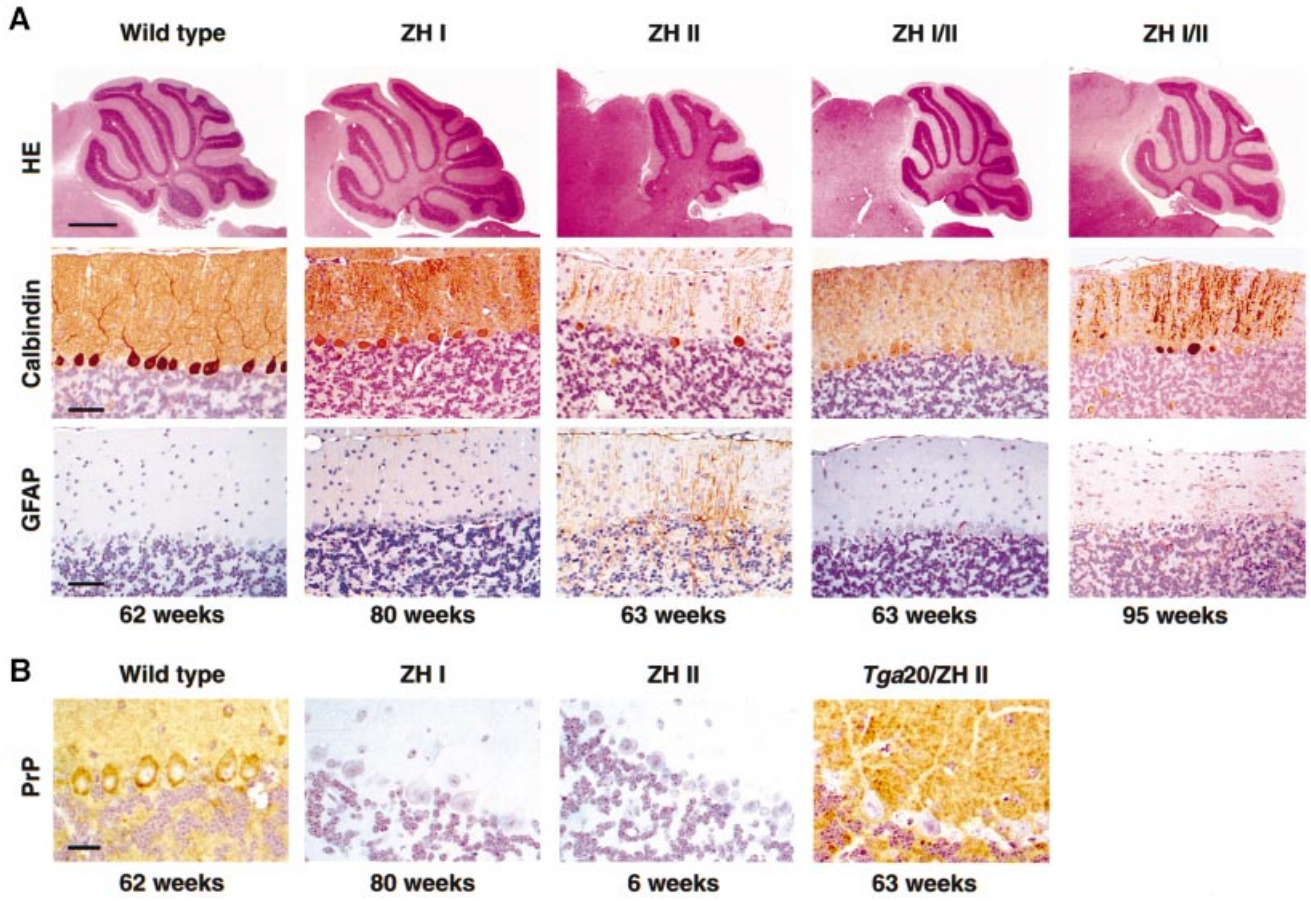

Fig. 4. Histological and histochemical analysis of cerebellar cortex of wild-type and PrP knockout mice at different ages. (**A**) The top row shows parasagittal sections of wild-type, Zürich I (ZH I), Zürich II (ZH II) and F1 progeny of Zürich I × Zürich II (ZH I/II) mice stained with HE. Scale bar, 1 mm. The middle row shows cerebellar cortical sections stained for calbindin (specific for Purkinje cells). Wild-type and ZH I mice show an intact Purkinje cell layer at 62 and 80 weeks, whereas ZH II mice present extensive cell loss at 63 weeks. ZH I/II mice show progressive Purkinje cell loss from 63 to 95 weeks. Scale bar, 70 μm. The sections were stained on different occasions, resulting in different intensities. The bottom row shows GFAP staining of the cerebellar cortex with slight astrocytosis in ZH II and ZH I/II mice at the latest time points. Scale bar, 70 μm. (**B**) Immunohistochemical staining for PrP reveals high PrP levels in Purkinje cells and lower levels in the granule cell and molecular layers of wild-type mice. No PrP is detected in ZH I and ZH II tissue. *Tga20*/ZH II mice show high PrP levels in the molecular and granule cell layers, but not in Purkinje cells and their dendritic trees. Scale bar, 25 μm.

Abrogation of the effects of the Zürich II allele by a single Prnp wild-type allele or a PrP-encoding transgene cluster

Hemizygous $Prnp^{-/+}$ Zürich II mice exhibited no cerebellar symptoms up to at least 100 weeks (Figure 3B). Because, as described above, a single Zürich II allele causes disease at ~68 weeks (50% morbidity), we conclude that a single $Prnp^{+}$ allele can abrogate the deleterious effects of the $Prnp^{-}$ Zürich II allele for practically the lifespan of the mouse.

In a further experiment, $Prnp^{-/-}$ Zürich II mice were crossed with *Tga20* mice. These mice carry multiple PrP transgenes devoid of the large intron on a Zürich I PrP knockout background and express PrP in most areas of the brain with the distinct exception of Purkinje cells (Fischer *et al*., 1996). The F1 progeny were back-crossed with Zürich II mice to eliminate the Zürich I allele. None of the resulting offspring presented clinical symptoms by 90 weeks of age (Figure 3B). There was no loss of Purkinje cells at 63 weeks and 40% loss at 90 weeks (Figure 5), showing that expression of PrP strongly mitigated the cerebellar phenotype even when it was not expressed in Purkinje cells at a discernible level (Fischer *et al*., 1996) (Figure 4B).

Discussion

Five independent PrP knockout mouse lines have been reported. Three of these show cerebellar symptoms and loss of Purkinje cells on ageing, namely the $Prnp^{-/-}$ Nagasaki mice (Sakaguchi *et al*., 1996), the Rcm0 mice of Moore *et al*. (Moore *et al*., 1999; Silverman *et al*., 2000) and the $Prnp^{-/-}$ Zürich II mice (this paper), while the $Prnp^{-/-}$ Edinburgh (Manson *et al*., 1994) and the $Prnp^{0/0}$ Zürich I mice (Büeler *et al*., 1992) do not. The strategies used to abolish PrP differed in an important respect: in the lines remaining healthy, PrP expression was abrogated either by placing an insert within the PrP coding region (the Edinburgh mice) or by replacing the coding region between codons 3 and 188 by a *neo* cassette. In contrast, the Nagasaki, Rcm0 and Zürich II lines were generated by deleting not only the ORF, but also 5′ flanking sequences extending into the second intron and 3′ non-coding sequences. As shown in Figure 1, the three lines have in

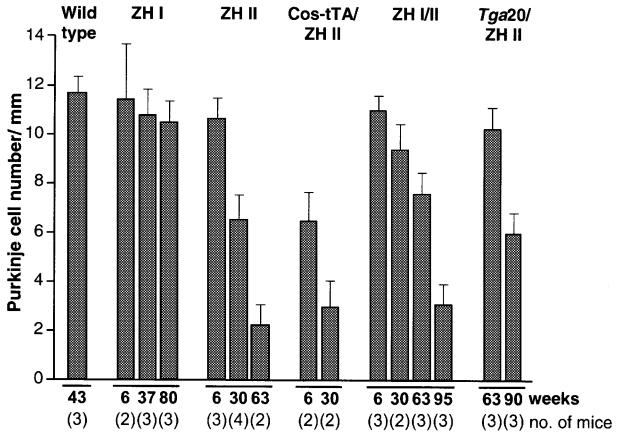

Fig. 5. Purkinje cell counts in PrP null and wild-type mice. Purkinje cells were counted in parasagittal sections stained with calbindin. Each bar represents the average cell count per mm in vermis I–VIII. The cell numbers in Zürich II (ZH II) mice were reduced as compared with Zürich I (ZH I) mice and wild-type mice at 30 weeks ($P < 0.1$) and strongly reduced at 63 weeks ($P < 0.05$). In ZH II mice transgenic for the tTA cosmid (Cos-tTA/ZH II), reduction was already apparent at 6 weeks ($P < 0.1$). In F1 offspring of a Zürich I × Zürich II cross (ZH I/II) and in Zürich II mice transgenic for a *Tga20* allele (*Tga20*/ZH II), reduction in cell numbers, as compared with wild type, only became significant after 90–95 weeks ($P < 0.05$). The statistical significance of Purkinje cell loss was analysed separately for each genotype by non-parametric statistics (Kruskal–Wallis test, followed by the Mann–Whitney U-test). The age at which mice were killed and the number of mice examined are indicated at the bottom of the figure.

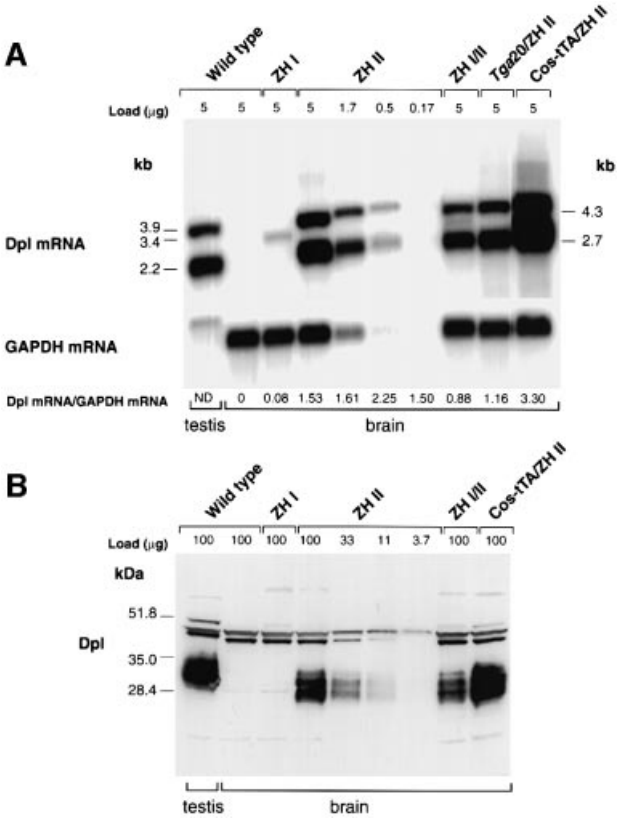

Fig. 6. Analysis of Dpl mRNA and protein levels in testis of wild-type and in brain of wild-type and PrP knockout mice. (**A**) Northern blot analysis was performed on poly(A)$^+$ mRNA using a labelled Dpl ORF probe. After stripping, the membrane was re-hybridized with a GAPDH probe. For quantification in brain (bottom panel), the value resulting from the sum of both Dpl transcripts was normalized relative to GAPDH. (**B**) Western blot analysis was performed on tissue homogenates as described in Materials and methods. Strain designations are as in Figure 5.

common the loss of 270 bp upstream of the PrP reading frame and of 450 bp downstream. Whilst the deleted sequences in the Nagasaki mice were replaced by a *neo* cassette, which, at least in some cases, causes an abnormal phenotype (Fiering *et al*., 1995), those in the Zürich II mice were replaced by a 34 bp *loxP* sequence, which is not known to cause deleterious effects.

Although the cerebellar phenotype resulting from extended deletions in the PrP gene can be rescued by a wild-type PrP allele, it is clearly not caused by the absence of PrP, because the Zürich I and the Edinburgh mice remain healthy despite their lack of PrP (Weissmann, 1996). In the Edinburgh mice no PrP-specific mRNA was detected, so that significant levels of any fusion protein containing PrP sequences are unlikely. In the Zürich I mice the *neo* cassette was inserted between the third and the 188th codon of the PrP sequence; although its coding and 3′ non-coding sequence were in-frame with the 67 residual PrP codons, there were two termination codons in between, which would preclude read-through. The relevant DNA segment from the Zürich I mice currently in use was resequenced and the presence of the termination codons confirmed (data not shown). Therefore, the presence of a PrP fragment or a fusion product is not responsible for maintaining the normal phenotype in either of the two lines.

The fact that the knockout lines showing the cerebellar phenotype lack sequences flanking the PrP ORF suggested that critical information was partly or entirely located in these regions. The pathological phenotype could come about either by loss of function, if these flanking regions controlled the formation or encoded part or all of some essential protein or RNA, or by gain of function, if the extended deletion resulted in the production of a deleterious product. The finding that introduction of a wild-type *Prnp* allele, either by breeding or as a transgene, abrogated the ataxic phenotype could be accommodated by either explanation: because the *Prnp* allele contains the flanking sequences, it could supply the missing function conjectured by the loss-of-function hypothesis. Alternatively, within the framework of the gain-of-function hypothesis, PrP might overcome the pathogenic effect of a postulated deleterious product.

The discovery of *Prnd*, the gene encoding Dpl, and its expression in the brains of Zürich II, Nagasaki and Rcm0 mice, suggested that the PrP knockout alleles in these animals give rise to a deleterious product. Analysis of brain-derived cDNAs indicated that in wild-type mice Dpl mRNA is very weakly expressed, mainly from a promoter upstream of exon 1 of *Prnd*, whilst the strong expression in Nagasaki and Rcm0 mice is due to chimeric RNAs that originate at the *Prnp* promoter, run all the way across and past the *Prnd* ORF and are processed by one or more splicing events that link the 3′ end of the second PrP exon directly or indirectly to the Dpl-encoding exon (Moore *et al*., 1999; Li *et al*., 2000). This intergenic splicing, which was also detected by PCR at very low levels in wild-type mice, is greatly enhanced in the ataxic mice because

the splice acceptor site upstream of the PrP-encoding exon (Figure 1) is deleted, thus diverting the splice to a downstream acceptor site. *Prnd*-specific mRNA was expressed undiminished in brain of Nagasaki mice 'cured' by the introduction of a PrP-expressing transgene (Nishida *et al*., 1999).

The hypothesis that expression of Dpl in brain is responsible for the ataxic syndrome is supported not only by the fact that in Zürich I knockout mice containing a single Zürich II allele onset of ataxia and Purkinje cell degeneration is retarded, but also by the finding that Dpl expression in the brain at twice the level of that in Zürich II mice, found in mice transgenic for a cosmid devoid of the PrP ORF but containing *Prnd*, accelerates appearance of the symptoms.

Why should overexpression of Dpl cause ataxia and concurrent overexpression of PrP restore normal function? Shmerling *et al*. (1998) found that introduction into Zürich I $Prnp^{0/0}$ mice of an amino-proximally truncated transgene encoding PrP devoid of the octa repeats and the conserved 112–126 region (PrPΔ32–134) leads to ataxia and degeneration of the cerebellar granule cell layer within weeks of birth. Moreover, introduction of a single wild-type PrP allele prevented the disease. They proposed that PrP interacts with a ligand to elicit an essential signal and that a conjectured PrP-like molecule with lower binding affinity can fulfil the same function in the absence of PrP. According to this hypothesis, in PrP knockout mice the truncated PrP could interact with the ligand, displacing the PrP-like molecule, without, however, eliciting the survival signal. If PrP has the higher affinity for the ligand, it would displace its truncated counterpart and restore function. Because Dpl resembles the truncated PrP, it might cause disease by the same mechanism (Moore *et al*., 1999; Silverman *et al*., 2000). However, because the promoter used to express the truncated PrP is active in granule cells but not in Purkinje cells, while the wild-type PrP promoter directing the ectopic expression of Dpl is active in Purkinje cells (Li *et al*., 2000), the cellular targets may be different. Indeed, targeting the truncated PrP to Purkinje cells causes ataxia and degeneration of Purkinje cells (E.Flechsig, R.Leimeroth and C.Weissmann, unpublished data).

The wild-type *Prnp* allele or a *Prnp*-containing cosmid gives rise to PrP expression in Purkinje cells that may counteract the conjectured deleterious effect of Dpl. Interestingly, the *Tga20* transgene also has this beneficial effect, although there is no detectable expression in Purkinje cells. Perhaps PrP expressed on the synapses of neighbouring cells can supply the protective effect or can be physically transferred, as shown for other GPI-linked proteins (Kooyman *et al*., 1995; Anderson *et al*., 1996; Brunschwig *et al*., 1999; McHugh *et al*., 1999). Interestingly also, the ataxia provoked by expression of truncated PrP in Purkinje cells can be abrogated by the *Tga20* allele, perhaps by the same mechanism (E.Flechsig, R.Leimeroth, I.Hegyi and C.Weissmann, unpublished data).

It would thus seem that chronic ablation of PrP *per se* has only quite modest effects such as alterations in circadian activity rhythms and sleep patterns (Tobler *et al*., 1996) and demyelination in the peripheral nervous system with old age (Nishida *et al*., 1999). The hypothesis that expression of Dpl in Purkinje cells causes cell death and ataxia and that the deleterious effects can be counteracted by concomitant expression of PrP is attractive and strongly supported by our results.

Finally, it is worth re-emphasizing the pitfalls that may beset the interpretation of knockout experiments. In the instances described, deletions of the PrP-encoding exon gave rise to a severe phenotype that could be reversed by introduction of an intact PrP-expressing transgene, the classical experiment correlating a phenotype with ablation of a gene. Nonetheless, in this case the conclusion was misleading, because the ataxic phenotype did not result from the deletion of the PrP ORF but from the incidental up-regulation of a deleterious gene product whose pathogenicity was offset by wild-type PrP.

Materials and methods

Generation of PrP knockout mice by homologous recombination and Cre-mediated site-directed recombination

For the deletion of the PrP ORF-containing exon and its flanking regions, a targeting vector PrP lox3 (Figure 2A) was constructed that contained a 7.1 kb genomic segment extending from the *Xho*I cleavage site 1.4 kb upstream of *Prnp* exon 3 to the *Bam*HI site 3.7 kb downstream of exon 3. For the selection of homologous recombinants, a *loxP*-flanked cassette that contained the herpes simplex virus type I thymidine kinase (HSV-*tk*) gene (Mansour *et al*., 1988) and the neomycin resistance gene (*neo*) controlled by the phosphoglycerol kinase promoter (PGK) (Soriano *et al*., 1991) was inserted at the *Nco*I site 0.26 kb upstream of exon 3. A further *loxP* site was introduced at the *Xba*I site 600 bp downstream of exon 3. A diphtheria toxin chain A (*dipA*) cassette for selection against non-homologous recombinants was placed at the 5′ end (Palmiter *et al*., 1987). The targeting construct was cloned in pBS-KS(–).

E14.1 ES cells, a subline of E14TG2a cells (Hooper *et al*., 1987) derived from 129/OlaHsd mice (Harlan UK Ltd, UK) were obtained from K.Rajewsky and cultured on irradiated mouse embryonic fibroblasts in ES medium (Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 100 U penicillin/ml, 100 μg streptomycin/ml, 0.5 μg leukaemia inhibitory factor (LIF)/ml).

PrP lox3 (10 μg) was linearized with *Not*I and electroporated into ~3×10^7 ES cells in 800 μl of phosphate-buffered saline (PBS) using a Bio-Rad gene pulser (240 V, 500 μF); G418 (0.4 mg/ml) was added after 24 h. ES cell clones were screened in pools of eight by PCR using primers ΔUp3 (5′-CAAGGGCCCCTCCTCCTAGAA) and TK (5′-ACGCGC-AGCCTGGTCGAA). Positive clones were confirmed by Southern blot analysis. A homologous recombination event identified by PCR from within the selection marker located in the 5′ region was detected in 82 of 1046 (7.8%) G418-resistant clones. However, concurrent insertion of the third *loxP* site (3′ of exon 3) was a rare event found in only five of the 82 clones (6%). To eliminate the *loxP*-flanked selection cassette, 10 μg of Cre-expressing pBS185 (Sauer and Henderson, 1990) were electroporated into 10^7 ES cells in 800 μl of PBS as above and the cells were plated on to T75 feeder-cell-coated flasks. After 72 h the cells were replated at different densities (10^3–10^6 cells per plate) on to 90-mm feeder plates and immediately exposed to 0.2 μM 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (Bristol-Myers Squibb, Stamford, CT). Of the surviving 1–4% of the ES cell colonies, half had retained the *loxP*-flanked PrP exon 3 (*Prnp lox2* genome configuration) and half had undergone recombination between the first and third *loxP* site, giving rise to the *Prnp lox1* knockout allele (Figure 2A). The *Prnp lox2* allele was screened for by PCR using primers P5 (5′-GCTTTCTTC-AAGTCCTTGCTCCTGCTGTAG) and Nco (5′-TATTGCATGGTT-GTTACGCC), and the *Prnp lox1* allele with primers P5 and 3′loxP3 (5′-GGGCAGGAGAAACAGTTTGG). Deletions were confirmed by Southern analysis. Positive ES cell clones with normal karyotypes were injected into C57BL/6 blastocysts.

One of the *Prnp lox2*-containing clones gave rise to a highly chimeric animal that produced an F1 generation carrying either the *Prnp lox1* or the *Prnp lox2* allele in addition to the wild-type allele (Figure 2C), indicating that the founder was triple chimeric for the two mutated and the wild-type

allele in its germline. Both lines were bred to homozygosity; both mutant alleles showed Mendelian inheritance.

Mice transgenic for the transactivator cosmid Cos-tTA

Cosmid cos6.I/LnJ-4 (Westaway *et al*., 1994) comprises the *Prnp* and the *Prnd* locus. The Cos-tTA vector is derived from it by replacing the PrP ORF with the tetracycline-controlled transactivator (tTA) ORF (Gossen and Bujard, 1992). A 0.9 kb PCR product of the tTA ORF was prepared using pUD-Combi (kindly provided by H.Bluethmann) as template and the primers ATG (5′-CATG*CCATGGC*TAGATTAGATAAAAGTA) (*Nco*I site italicized) and MfeI (5′-GCG*CAATTG*TTACTTGTCATC-GTCGTCC) (*Mfe*I site italicized). A fragment extending from the *Xba*I site within PrP intron 2 to the initiator ATG of the PrP ORF was prepared by PCR with the template NZWBamHI (Büeler *et al*., 1992) and the primers XbaI (5′-GTGAGCCTGCCAAAAC*TCTAGA*TGTTTCCTG) (*Xba*I site italicized) and AflIII (5′-TTCG*ACATGT*TGACTGATCTGC) (*Afl*III site italicized). The amplicon harbouring the tTA ORF was joined by its 5′-*Nco*I end to the *Nco*I-compatible 3′-*Afl*III end of the PrP amplicon and cloned into *Xba*I- and *Sma*I-restricted pTM1 (Moss *et al*., 1990) in a three-way ligation. The *Nco*I–*Mfe*I fragment of this intermediate plasmid, comprising 260 bp of intron 2, 10 bp of 5′ non-coding region and the entire tTA ORF, was introduced into *Nco*I–*Mfe*I-cleaved NZWBamHI, replacing the PrP ORF and 80 nucleotides of 3′ non-coding region by the tTA ORF, to yield NZW-tTA. The 3.7 kb *Bam*HI–*Mfe*I fragment of NZW-tTA was inserted in a three-way ligation into an intermediate construct containing the 18 kb *Pme*I–*Sal*I fragment from the PrP cosmid cos6.I/LnJ-4. The tTA-containing *Pme*I–*Sfi*I fragment from this construct was ligated in a two-way ligation to the *Pme*I–*Sfi*I backbone fragment of the original cosmid to yield the final vector Cos-tTA (Figure 2B). Cos-tTA was digested by *Not*I and electrophoresed through a 0.5% agarose gel (Seakem GTG; FMC Bioproducts, Denmark). The tTA/*Prnp* insert was electro-eluted using BIOTRAP (Schleicher & Schuell), ethanol precipitated, resuspended in injection buffer (10 mM Tris–HCl pH 7.5, 5 mM NaCl, 0.1 mM EDTA) and filtered through a Micropore filter (Millipore Ultrafree MC 0.45 μm).

Injections into pronuclei of fertilized oocytes from $Prnp^{0/0}$ Zürich I mice were performed by conventional methods. Founders were identified by Southern blot analysis of tail DNA and crossed with $Prnp^{-/-}$ Zürich II animals. Offspring positive for the cosmid were identified by PCR using primers TA-2 (5′-GCACCATACTCACTTTTGCCCTTTAGA) and 3′Mfe (5′-CAGGGGTATTAGCCTATGGGGGACACAG) (Figure 2B) and mated with $Prnp^{-/-}$ Zürich II mice to eliminate the $Prnp^{0/0}$ Zürich I allele.

Southern blot analysis

About 10 μg of genomic DNA were cleaved with appropriate restriction enzymes, electrophoresed through 1% agarose gels, depurinated and transferred to Hybond N^+ membranes. After prehybridization, hybridization and washing (Church and Gilbert, 1984), membranes were exposed to X-ray film or scanned with a PhosphorImager (Molecular Dynamics, USA).

The various *Prnp* alleles were detected with an α-^{32}P-labelled 650 bp *Xba*I–*Nco*I fragment (X/N probe, Figure 2A) covering a segment of intron 2 upstream of the *loxP* site. *Xba*I digestion produces fragments of 3.5 kb for $Prnp^+$, 4.0 kb for $Prnp^0$ (=$Prnp^0$ Zürich I), 2.3 kb for *Prnp lox1* (=$Prnp^-$ Zürich II) and 5.2 kb for *Prnp lox2*. The Cos-tTA transgene was detected in *Xba*I-digested genomic DNA, using as probe the tTA ORF (cleaved out of pUD-Combi, a gift from H.Bluethmann, with *Nco*I and *Bam*HI) or a 0.9 kb PCR product of the tTA ORF used for the cloning of Cos-tTA (Figure 2B).

Northern blot analysis

Total RNA was prepared using the RNeasy kit (Qiagen) following the manufacturer's instructions. Poly(A)$^+$ mRNA was purified using the Oligotex mRNA kit (Qiagen). RNA was electrophoresed through a 1% agarose gel and transferred to Hybond-N^+ (Amersham). Hybridizations were carried out according to standard techniques (Sambrook *et al*., 1989) using an α-^{32}P-labelled probe (1–2 $\times$ 10^6 c.p.m./ml). Probe Δ was used for PrP mRNA (Büeler *et al*., 1992) (Figure 2A), cloned Dpl ORF for Dpl mRNA (Figure 2B) and the tTA ORF fragment described above for tTA transcripts. Filters were stripped by boiling in 0.5% SDS, 20 mM Tris–HCl pH 7.5 for 10 min, tested for residual signal and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Fort *et al*., 1985).

Western blot analysis

Brain homogenates (10% w/v) were prepared in PBS containing 0.5% Nonidet P40, 0.5% sodium deoxycholate by passing brains successively through 18- and 22-gauge needles. Homogenates were centrifuged at 1500 *g* for 10 min and supernatants were adjusted to 8 mg/ml total protein. Samples (60 μg for PrP and 100 μg or as indicated for Dpl) were electrophoresed through 16% SDS–polyacrylamide gels (NOVEX™, San Diego, CA) and transferred to PVDF membranes (Immobilon-P, Millipore, USA). PrP was detected with monoclonal antibody 6H4, which recognizes residues 143–151 of PrP (Korth *et al*., 1997) (1:10.000, Prionics AG, Switzerland). Dpl was probed with polyclonal anti-Dpl rabbit antiserum 2234 (1:5000; raised against recombinant Dpl), pre-adsorbed for 1 h at 37°C with 10% wild-type brain homogenate. Blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG1 antibodies (1:5000, Zymed, San Francisco, CA) for PrP or with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (1:5000, Dako, Glostrup, Denmark) for Dpl. Blots were developed using the enhanced chemiluminescence kit SuperSignal West Pico (Pierce, Rockford, IL) and exposed to BIOMAX MR-1 film (Kodak, Rochester, MN). Appropriate film exposures were scanned with a laser densitometer (Molecular Dynamics).

Immunohistochemistry

For immunohistochemical staining of PrP, sections were heated three times for 5 min in a microwave oven in 10 mM sodium citrate pH 6, and incubated with polyclonal anti-PrP rabbit antiserum R340 (1:50; Brandner *et al*., 1996). Visualization was with the Tyramide Signal Amplification kit (NEN, Life Science, Boston, MA)

Histopathology and morphometric analysis

Mice were deeply anaesthetized and preterminally perfused with 1 ml of 1 mM EDTA in PBS and subsequently with 3% paraformaldehyde/1% glutaraldehyde in PBS. Brains were paraffin-embedded and cut into 2 μm sections. Sections were stained with haematoxylin–eosin (HE) and for glial fibrillary acidic protein (GFAP; polyclonal antibody, 1:300 from Dako). Biotinylated secondary antibodies (goat anti-rabbit; Dako) were used at a dilution of 1:200 and visualization was with avidin–biotin complex (Dako) according to the manufacturer's instructions. For quantification of Purkinje cells in the cerebellum, sections were stained with an anti-calbindin monoclonal antibody (1:5000; Sigma Chemical Company, St Louis, MO) and visualized by a biotinylated rabbit anti-mouse antibody (1:200; Dako) and avidin–biotin complex. Purkinje cells were counted at 200× magnification using a counting grid with square side length 100 μm. Ten adjoining squares were counted in each area indicated.

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