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Identification of a Novel Gene Encoding a PrP-Like Protein Expressed as Chimeric Transcripts Fused to PrP Exon 1/2 in Ataxic Mouse Line with a Disrupted PrP Gene

Aimin Li,[1] Suehiro Sakaguchi,[1,4] Ryuichiro Atarashi,[1] Bhabesh C. Roy,[1] Ryota Nakaoke,[1] Kazuhiko Arima,[1] Nobuhiko Okimura,[1] Juraj Kopacek,[1,3] and Kazuto Shigematsu[2]

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SUMMARY

1. Mouse lines lacking prion protein (PrP^C) have a puzzling phenotypic discrepancy. Some, but not all, developed late-onset ataxia due to Purkinje cell degeneration.

2. Here, we identified aberrant mRNA species in the brain of Ngsk *Prnp*$^{0/0}$ ataxic, but not in nonataxic Zrch *Prnp*$^{0/0}$ mouse line. These mRNAs were chimeric between the noncoding exons 1 and 2 of the PrP gene (*Prnp*) and the novel sequence encoding PrP-like protein (PrPLP), a putative membrane glycoprotein with 23% identity to PrP^C in the primary amino acid structure. The chimeric mRNAs were generated from the disrupted *Prnp* locus of Ngsk *Prnp*$^{0/0}$ mice lacking a part of the *Prnp* intron 2 and its splice acceptor signal.

3. In the brain of wild-type and Zrch *Prnp*$^{0/0}$ mice, PrPLP mRNA was barely detectable. In contrast, in the brain of Ngsk *Prnp*$^{0/0}$ mice, PrP/PrPLP chimeric mRNAs were expressed in neurons, at a particularly high level in hippocampus pyramidal cells and Purkinje cells under the control of the *Prnp* promoter.

4. In addition to the functional loss of PrP^C, ectopic PrPLP expression from the chimeric mRNAs could also be involved in the Purkinje cell degeneration in Ngsk *Prnp*$^{0/0}$ mice.

KEY WORDS: ataxia; knockout mice; prion protein; prion protein-like protein; Purkinje cell

[1] Department of Bacteriology, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
[2] Department of Pathology II, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
[3] Department of Molecular Biology, Institute of Virology, Slovak Academy of Sciences, Dubravskacesta 9, 842, 46, Brastislava, Slovakia.
[4] To whom correspondence should be addressed. e-mail: suehiros-ngs@umin.u-tokyo.ac.jp

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INTRODUCTION

Cellular prion protein (PrP^C) is a membrane glycoprotein expressed constitutively on the neuronal cell surface and to a lesser extent on various other tissues including lymphoreticular cells (Prusiner, 1998). Posttranslational conversion of PrP^C into its proteinase K-resistant isoform (PrP^{Sc}) is implicated in the pathogenesis of a series of transmissible spongiform encephalopathies—i.e., prion diseases including Creutzfeldt–Jakob disease of man and scrapie in animals and in the replication of their etiological agents, prions (Prusiner, 1998). Although PrP^C has been presumed to play a role in cell adhesion, nutrient uptake, or signal transduction on the neuronal cell surface, the physiological function of PrP^C remains to be elucidated.

All 4 so far reported mouse lines homozygous for the disrupted *Prnp* gene (*Prnp*$^{0/0}$) are resistant to prion infection (Büeler *et al.,* 1993; Manson *et al.,* 1994b; Prusiner *et al.,* 1993; Sakaguchi *et al.,* 1995), indicating their loss of PrP^C function, but they have shown curiously discrepant phenotypes (Büeler *et al.,* 1992; Manson *et al.,* 1994a; Moore, 1997; Sakaguchi *et al.,* 1996). Two of them, Ngsk and Rcm0 *Prnp*$^{0/0}$ mice, revealed progressive ataxia due to the degeneration of cerebellar Purkinje cells during old age, in contrast to Zrch and Edbg *Prnp*$^{0/0}$ mice, which have never displayed ataxia. Successful rescue of Ngsk *Prnp*$^{0/0}$ mice from the Purkinje cell degeneration by a transgene encoding the normal mouse PrP^C has indicated that the functional loss of PrP^C is essential for this phenotype (Nishida *et al.,* 1999). However, molecular mechanisms underlying the puzzling discrepancy of phenotypes among the *Prnp*$^{0/0}$ mouse lines have not been well understood.

It is most likely that differences in the structures of targeting constructs could be attributed to this discrepancy by generating alterations in the genomic integrity of *Prnp* loci. The mouse *Prnp* locus is located on chromosome 2, and consists of three exons and two introns, where exon 3 includes the entire open reading frame (ORF) encoding PrP^C and a poly(A) signal (Prusiner, 1991). In the disrupted *Prnp* of Zrch *Prnp*$^{0/0}$ mice, a part of the ORF was replaced with a neomycin-resistant gene (*neo*) (Büeler *et al.,* 1992) while in Edbg *Prnp*$^{0/0}$ mice, *neo* was inserted into a *Kpn* I site of the ORF (Manson *et al.,* 1994a). The alleles of both nonataxic *Prnp*$^{0/0}$ mouse lines thus preserve the whole intron 2, its splicing acceptor (SA), and a part of or the entire ORF sequences. On the other hand, *neo* replaced a part of intron 2, the SA, and the entire ORF in the *Prnp* alleles of ataxic Ngsk and Rcm0 *Prnp*$^{0/0}$ mice (Moore, 1997; Sakaguchi *et al.,* 1995).

One possible mechanism causing the phenotypic discrepancy is that a truncated or fused PrP generated by the disrupted *Prnp* alleles of nonataxic Zrch and Edbg *Prnp*$^{0/0}$ mice might compensate for some aspects of PrP^C function, because a part of or the entire ORF sequences are preserved in these alleles. However, such a protein product has not been identified so far. Alternatively, in addition to the PrP ORF, another unknown genetic element might also be disrupted in the alleles of ataxic *Prnp*$^{0/0}$ mice. For instance, disruption of the proposed Purkinje cell-specific *cis* element located within intron 2 might affect the expression of neighboring genes essential for the longevity of Purkinje cells (Shmerling *et al.,* 1998). However, rescue of Ngsk *Prnp*$^{0/0}$ mice from the Purkinje cell degeneration by the mouse *Prnp* transgene introduced in *trans* has argued against this idea (Nishida *et al.,* 1999).

The remaining possibility is that aberrant transcripts expressed by the disrupted *Prnp* alleles of ataxic $Prnp^{0/0}$ mice might contribute to expression of the phenotype. The intron sequence located adjacent to the SA of the last exon of a β-globin gene was demonstrated to be important for the recognition and cleavage of the pre-mRNA by cleavage/polyadenylation machinery (Antoniou *et al.,* 1998). Because the *Prnp* alleles of the Ngsk and Rcm0 $Prnp^{0/0}$ mice lack the intron 2 sequence including the SA, such a mechanism could possibly cause aberrant gene expression and contribute to the phenotype in Ngsk and Rcm0 $Prnp^{0/0}$ mice.

In the present study, we detected aberrant mRNAs hybridized to a probe consisting of *Prnp* exon 1 and 2 cDNA, but not to the exon 3 probe in the brain of Ngsk $Prnp^{0/0}$ mice. Sequence analysis of their cDNAs revealed that they were chimeric between *Prnp* exon 1/2 and a novel gene encoding the PrP-like protein (PrPLP). The expression of PrPLP mRNA was barely detectable in the brain of adult wild-type and nonataxic Zrch $Prnp^{0/0}$ mice, whereas its ectopic expression under the control of the *Prnp* promoter was detected in neurons including Purkinje cells in the brain of Ngsk $Prnp^{0/0}$ mice. These results suggested that, in addition to the functional loss of PrP^{C}, ectopic expression of a prion protein-like protein (PrPLP) in the brain of Ngsk $Prnp^{0/0}$ mice could be associated with the Purkinje cell degeneration.

MATERIALS AND METHODS

Mice

Ngsk $Prnp^{0/+}$ mice were mated to generate the three different genotypes of Ngsk $Prnp^{0/0}$, $Prnp^{0/+}$, and $Prnp^{+/+}$ (wild-type) mice. Genotype determination of those mice was performed by polymerase chain reaction (PCR) as described previously (Nishida *et al.,* 1999). Zrch $Prnp^{0/0}$ mice were kindly provided by Prof. Weissmann (Neurogenetics Unit, Imperial College School of Medicine, UK). Ngsk/Zrch $Prnp^{0/0}$ mice were obtained by mating between Ngsk and Zrch $Prnp^{0/0}$ mice.

Total RNA Extraction and mRNA Purification

Total RNA was extracted from various mouse tissues by using TRIZOL Reagent (Gibco BRL Life Technologies, Inc., Gaithersburg, USA) and mRNA was purified from them using the mRNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) as recommended in the manufacturer's manual.

Northern Blot Analysis

Total RNA or mRNA was electrophoresed on a formaldehyde-denatured agarose gel and blotted onto a Hybond N membrane (Amersham Pharmacia Biotech) with 10× sodium chloride/sodium citrate. The RNA was fixed onto the membrane by ultraviolet light of 70,000 microjoules per cm^2 for 2 min (UVP, Ltd., Upland, U.S.A.). The membrane was subjected to a prehybridization procedure for 4 hr at

45°C in the buffer (5× sodium chloride/sodium phosphate/EDTA/0.5% SDS/50% formamide/5× Denhardt's solution/10% dextran sulphate/100 μg/ml salmon sperm DNA). A hybridization procedure was performed using an appropriate ^{32}P-labeled DNA probe (BcaBEST Labelling Kit, TaKaRa, Tokyo, Japan) overnight at 45°C in the same buffer. The membrane was washed twice in 2× SSC/0.1% SDS at room temperature for 10 min, once in 1× SSC/0.1% SDS, and twice in 0.1× SSC/0.1% SDS at 65°C for 15 min. Signals were detected by BAS 2000 (Fuji, Tokyo, Japan) or autoradiography on Konica X-ray film.

In situ Hybridization

The brain tissues were sliced to 5 μm thickness after fixing for 16 hr in 4% buffered paraformaldehyde (pH 7.4) at 4°C and embedding in paraffin. The tissues were deparaffinized, digested with 8 mg/ml pepsin for 10 min at 37°C, and soaked for 10 min in 0.25% acetic anhydride/0.1 m*M* triethanolamine hydrochloride (pH 8.0)/0.9% NaCl. The cRNA probes were labeled with digoxigenin (DIG)-UTP (Roche Diagnostics, Mannheim, Germany) using T7 or T3 polymerase (Gibco BRL Life Technologies, Inc.). The tissue sections were subjected to hybridization with the labeled cRNA probes in buffer (50% formamide/10m*M* Tris-HCl [pH 7.5]/ 1m*M* EDTA/0.6*M* NaCl/0.5 mg/ml yeast tRNA/0.25 mg/ml salmon sperm DNA/ 1% skim milk/0.25% SDS/5× Denhart's solution) at 50°C for 16 hr. The sections were washed several times in 4× SSC, and immersed in 50% formamide/2× SSC at 50°C for 30 min. They were digested by 20 μg/ml RNase A at 37°C for 30 min and washed in 0.2× SSC at 60°C for 20 min. Signals were detected by an enzyme-linked immunosorbent assay using alkaline phosphatase conjugated anti-DIG Fab fragments (1:500, Roche Diagnostics, Mannheim, Germany) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP).

Probes

The *Prnp* exon1/2 probe is a full-length cDNA (145 base pairs [bp]) of *Prnp* exon 1 and 2, and the *Prnp* exon 3 probe is a fragment (937 bp) of *Kpn* I-*EcoR* I of *Prnp* exon 3. The PrPLP probe is a fragment (1110 bp) of *Hpa* I–*Hind* III derived from the chimeric 2.2 kilobase (kb) cDNA containing the PrPLP ORF.

3′ Rapid Amplification of cDNA Ends (RACE) and 5′ RACE

In a 3′ RACE system (Gibco BRL Life Technologies, Inc.), 4 μg of total RNA were primed by an adapter primer (5′GGCCACGCGTCGACTAG-TACTTTTTTTTTTTTTTTTTTT-3′, AP) and the first strand of corresponding cDNAs were synthesized by SuperScript II Reverse Transcriptase according to the manual. The cDNAs were then amplified using an Abridged Universal Amplification Primer (5′-GGCCACGCGTCGACTAGTAC-3′, AUAP) and a *Prnp* exon 1 sense primer 501 (5′-GTCGGATCAGCAGACCGATTCT-3′) by Advantage cDNA Polymerase Mix containing TagStart Antibody (Clontech, Heidelberg, Germany) in GeneAmp PCR System 9700 (Perkin-Elmer Co., Foster City, USA). The

RNA–DNA hybrids were denatured at 95°C for 1 min, and then amplified by 25 cycles of the reaction (95°C for 15 sec, 57°C for 30 sec, 68°C for 3 min) with final elongation at 68°C for 7 min.

In a 5′ RACE system (Gibco BRL Life Technologies, Inc.), 1 μg of total RNA was used for synthesis of the first strand of corresponding cDNAs using a PrPLP gene specific antisense primer 322 (5′-GCCACAGGACTCGCTGGTGGAGCTTGCT-3′) according to the manual. The cDNAs were purified by GlassMax DNA Isolation Spin Cartridge (Gibco BRL Life Technologies, Inc.), and then tailed with dCTP by terminal deoxynucleotidyl transferase. The dC-tailed cDNAs were amplified in the GeneAmp PCR System 9700 by nested-PCR using the same conditions as used for the 3′ RACE system. To explain briefly: the dC-tailed cDNAs were amplified using the outer primer pair of an Abridged Anchor Primer (5′-GGCCACGCGTC-GACTAGTACGGGGGGGGGG-3′, AAP) and a PrPLP gene-specific antisense primer 333 (5′-AGCATCTCCTTGGTCACGTTGGC-3′), and then further amplified using the inner primer pair of an AUPA and a PrPLP gene-specific antisense primer 344 (5′-GAGCAGCCTTCGTAGTAGATCC-3′).

Reverse Transcription (RT)-PCR

Four micrograms of total RNA were primed by an oligo-dT primer and the first strand of corresponding cDNAs were synthesized by SuperScript II RNase H^- reverse transcriptase (Gibco BRL Life Technologies, Inc.) as recommended by the manufacturer. The cDNAs were amplified by a *Prnp* exon 1 sense primer 501 and a PrPLP antisense primer 311 (5′-ACAATCTGAAGATCTGTCAACAAG-GAAGTT-3′) using Advantage cDNA Polymerase Mix (Clontech). To explain briefly: the RNA–DNA hybrids were denatured at 95°C for 1 min and followed by 25 cycles of the reaction (95°C for 15 sec, 68°C for 3 min) with final elongation at 68°C for 7 min in the Thermocycler (GeneAmp PCR System 9700).

DNA Sequencing

The amplified DNA fragments were cloned into a PCR 2.1 TOPO (Invitrogen, NV leek, the Netherlands) or into a pBluescript II SK-vector (Stratagene, La Jolla, CA, USA). They were sequenced by a dideoxy chain termination method (Thermo Sequenase premixed cycle sequence kit, Amersham Pharmacia Biotech) using 5′ Texas Red-labelled T3, T7 and reverse M13 primers (SQ 5500; HITACHI Co, Tokyo, Japan).

Database Searches and Computational Analysis

DNA homology search and alignment of amino acids were carried out by the BLAST sequence similarity searching network service at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). A protein analysis for the deduced amino acid sequence was performed using the National Institute of Agronomic Research (INRA) network service (http://w3.toulouse.inra.fr/lgc/multalin/multalin.html) and the ExPASy Molecular Biology Server (http://www.expasy.ch/).

GenBank Accession Numbers

Chimeric PrP/PrPLP 3.4kb cDNA, AF192382; chimeric PrP/PrPLP 2.2kb cDNA, AF192383; PrPLP 3.2 kb cDNA, AF192384; PrPLP 2.0 kb cDNA, AF192385.

RESULTS

The Disrupted *Prnp* Locus of Ataxic Ngsk *Prnp*$^{0/0}$ Mice Encodes Aberrant mRNAs Containing the *Prnp* Exon 1/2 Sequence

In order to investigate whether the disrupted *Prnp* locus of Ngsk *Prnp*$^{0/0}$ mice encodes aberrant mRNA species, total RNAs from the brains of Ngsk *Prnp*$^{+/+}$ (wild-type) and *Prnp*$^{0/0}$ mice were subjected to Northern blotting using the two cDNA probes each corresponding to *Prnp* exon $\frac{1}{2}$ and exon 3, respectively (Fig. 1A). Both probes hybridized with the 2.2 kb authentic PrP mRNA abundantly

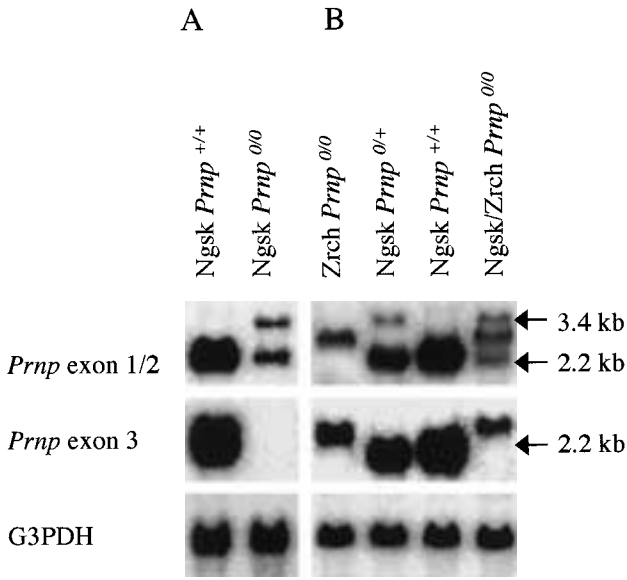

Fig. 1. Aberrant mRNAs were generated in the brain of NgsK *Prnp*$^{0/0}$ mice from the disrupted *Prnp* locus. Ten micrograms of total RNA from brains of 9-week-old mice with the indicated *Prnp* genotypes were analyzed by Northern blot using *Prnp* exon 1/2 and exon 3 probes. (A) Ngsk *Prnp*$^{+/+}$ (wild-type) mice expressed 2.2 kb authentic PrP mRNA, which hybridized to both probes, and Ngsk *Prnp*$^{0/0}$ mice expressed aberrant 2.2 and 3.4 kb mRNAs, which hybridized to *Prnp* exon 1/2 not to exon 3 probe. (B) Zrch *Prnp*$^{0/0}$ mice expressed the 2.4 kb PrP-neo chimeic mRNA, which hybridized to both probes. Ngsk *Prnp*$^{0/+}$ and Ngsk/Zrch *Prnp*$^{0/0}$ mice expressed the aberrant mRNAs in addition to the authentic PrP mRNA in Ngsk *Prnp*$^{0/+}$ and the PrP-neo chimeric mRNA in Ngsk/Zrch *Prnp*$^{0/0}$ mice, respectively. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

expressed in the brain of wild-type mice. Surprisingly, the exon 1/2 probe recognized 2.2 and 3.4 kb mRNAs in the brain of Ngsk *Prnp*$^{0/0}$ mice. The exon 3 probe failed to hybridize to the 2.2 and 3.4 kb mRNAs, indicating that these two mRNAs were distinct from the authentic PrP mRNA. In contrast, the brain of nonataxic Zrch *Prnp*$^{0/0}$ mice did not express such aberrant mRNA species except for the 2.4 kb PrP-neo chimeric transcript (Fig. 1B) as reported previously (Büeler *et al.,* 1992). Moreover, we extended the analysis of the aberrant mRNA expression in the brains of Ngsk *Prnp*$^{0/+}$ and Ngsk/Zrch *Prnp*$^{0/0}$ mice heterozygous for the disrupted Ngsk *Prnp* allele. The exon 1/2 probe hybridized to the aberrant 2.2 and 3.4 kb mRNAs in the brains of both mice in addition to the authentic PrP mRNA and 2.4 kb PrP-neo chimeric mRNA, respectively (Fig. 1B). These findings indicated that the disrupted *Prnp* allele of Ngsk *Prnp*$^{0/0}$ mice, but not that of Zrch *Prnp*$^{0/0}$ mice, encodes the aberrant 2.2 and 3.4 kb mRNAs containing *Prnp* exon 1/2 but lacking exon 3 sequence.

The Aberrant mRNAs Are Chimeric Between *Prnp* Exon 1/2 and the Novel Sequence Encoding a PrP-Like Protein

3′ RACE was carried out to obtain the cDNAs of the aberrant mRNAs from the brain of Ngsk *Prnp*$^{0/0}$ mice. The primer pair, the *Prnp* exon 1-derived primer 501 and AUAP, gave a discrete 2.2 kb product on an agarose gel (data not shown), but failed to amplify the 3.4 kb cDNA presumably due to inefficient priming by AUAP. Therefore, the 3′ end of the amplified 2.2 kb cDNA was sequenced and an antisense oligonucleotide (primer 311) corresponding to the sequence 60 bp upstream from the 3′ end of the cDNA was synthesized. RT-PCR using the primers 501 and 311 successfully produced 2.1 and 3.3 kb cDNAs (data not shown) derived from the 2.2 and 3.4 kb aberrant mRNAs, respectively. The RT-PCR products were inserted into a plasmid vector and nucleotide sequences of several plasmid clones were determined. Combined with the 3′ end sequences of the 3′ RACE product, the two cDNAs were estimated to be 2149 and 3386 bp in length, respectively (Fig. 2A). Both were started by 145 bp sequences identical to the *Prnp* exon 1 and 2, which were followed by novel sequences distinct from the *Prnp* exon 3. The difference between the two cDNAs was the presence or absence of the 1,237 bp insert underlined in Fig. 2A. The highly conserved polyadenylation signal, AATAAA, was observed 20 bp upstream of the poly(A) start site.

Next, we performed 5′ RACE using total RNA extracted from the brain of Ngsk *Prnp*$^{0/0}$ mice in order to confirm the start site of the aberrant mRNAs. It was interesting that 5′ RACE produced four discrete bands including one major band on the agarose gel (data not shown). We successfully cloned three out of the four bands and determined their sequences. Sequence analysis revealed that all three 5′ RACE products started from the *Prnp* exon 1/2 sequences, suggesting that expression of the aberrant mRNAs in Ngsk *Prnp*$^{0/0}$ mice is driven by the *Prnp* promoter. However, the 5′ RACE products showed different DNA inserts just following the *Prnp* exon 1/2 sequences (Fig. 2B). These are most likely to be alternatively spliced products. The sequence of major band was completely identical

A

GTCGGATCAGCAGACCGATTCTGGGCGCTGCGTCGCATCGGTGGCAGGACTCCTGAGTATATTTCAGAACTGAACCATTTCAACCGAGCT 90
*GAAGCATTCTGCCTTCCTAGTGGTACCAGTCCAATTTAGGAGAGCCAAGCAGACT*ACTAAAAGTTAACTCAGAATCAAGCGAAGGCTTTT 180
CTGGAGGTCGAGTTCTGGATCATGATGGAGTGGAGGTCGCTTCGAGTGGAGGTCGCTTCGCGCACCGGATTCACC**ATG**AAGAACCGGCTG 270
M K N R L
GGTACATGGTGGGTGGCCATCCTCTGCATGCTGCTTGCCAGCCACCTCTCCACGGTCAAGGCAAGGGGCATAAAGCACAGGTTCAAGTGG 360
G T W W V A I L C M L L A S H L S T V K A R G I K H R F K W
AACCGGAAGGTCCTGCCCAGCAGCGGCGGCCAGATCACCGAAGCTCGGGTAGCTGAGAACCGCCCAGGAGCCTTCATCAAGCAAGGCCGG 450
N R K V L P S S G G Q I T E A R V A E N R P G A F I K Q G R
AAGCTGGACATCGACTTTGGAGCAGAGGGCAACAGGTACTACGCGGCTAACTATTGGCAGTTCCCTGATGGGATCTACTACGAAGGCTGC 540
K L D I D F G A E G N R Y Y A A N Y W Q F P D G I Y Y E G C
TCTGAAGCCAACGTGACCAAGGAGATGCTGGTGACCAGCTGCGTCAACGCCACCCAGGCGGCCAACCAGGCTGAGTTCTCCCGGGAGAAG 630
S E A N V T K E M L V T S C V N A T Q A A N Q A E F S R E K
CAGGATAGCAAGCTCCACGAGTCCTGTGGCGGCTGATCAAAGAGATCTGCTCCGCCAAGCACTGCGATTTCTGGCTGGAAAGGGGA 720
Q D S K L H Q R V L W R L I K E I C S A K H C D F W L E R G
GCTGCGCTTCGGGTCGCCGTGGACCAACCGGCGATGGTCTGCCTGCTGGGTTTCGTTTGGTTCATTGTGAAGTAAAAATCAATGAAGCTG 810
A A L R V A V D Q P A M V C L L G F V W F I V K *
GCAGCCACAGAGGTGAGCTGGTGGGCAAAGGTAGACAGAGGTAGCCCAGTTCTCTCTATCTAGCCCCCGAGTGTTCTGAAAGTACAACGT 900
GTAGCGTTTCAGGGCATTTCAAAAGTCCCTCCCAAGTACTCCCCCTACTCCACGTGTTTGATAATGTGTTTCAGTGCCCCTATGCTCCAC 990
CCCTGTGAGACCTGGCCTGTTCCTGCCTTTGCAGCTACACTAGGTGAGAAACCAGCCAAAGGATACAAGAATTGTCCTGTGCACTCCACC 1080
TGATTTTCCAACTCCAGGAAACTGAGGCTCACATTGCAGGAGTCCCTGTCCAGCACAGTTAGAAAACAGAAGAGCTGGGTTCCCCTGCTC 1170
TGTCTTCCCTCAGGTTTTCTGCTCTGTGTAGCCTAGAGCTGCCTCCTGATCCCTCCAAGACTCAGGTCCAAGTGCAGTCCCGAATGTGGA 1260
CTGAAATGTGGAAGTCACACCCTGAACTAAGTGGGAAGCCCTGAGCGACAACCGTGCACCTTGACACCTTACGTGAGACAATATAACAAA 1350
AATTAAGCTAAAGTAGACATGACACTAGACAACGGCTCAGAAATCAGATTGTAGGGAAATTTGCTATGCACCTCCCGGAGAGCAGCTTCA 1440
GGGCAGGATGTGTTTGTGGTGTCTAGATGCGTCTTCACAAGGACTGGTGTTTGCTGGTTGGGCCCCTGCAATGTCTGCAAAGTTAAGAAA 1530
ACCTTTTTAAGTGACACATTGTGCGTGCGTGTGCATGCCTGTGTGTGTGTGTGTGTGTGTGTGTTTATTTGTTTTCTAGATAGA 1620
AATTTTTCTATGTAGCCCAGGTTGACCCCATAATCATGATCTTCTGGCCTCTGCCTCCTGAATGCTGGGATTGCAGACATGCCACCAAGC 1710
CCAATATGTGTTTACTTCTGATGTCCCCTGGCCCTATGTGCATGTGTGTTCTTAGGCATCTTAAGATGCTCACTGCGCACAGAGCTCTCT 1800
GCAAACACTGTCTGTTTTTAAAACCCACTTTGGTAAAAGCTAATCTCCGTGAATCATTTAACACTCCCAAAATATAATTATTAGCCAAAA 2890
AGCCTAAGAGAAATGTCATCTTTAGGAAGACCCAATAAAGAAGTGGTGTCTACCATCATAAGAACTGATATGCCACTTTGTGAACTTAAA 1980
TATATATTAAAAATATGAATGTTACCTAAATGTTTAAAAATCTTTTAAGATTCTTCCACAGGTTTTCCTCACCTTTCAGGACTCCAGGAG 2070
TTGCTGAGCGGGATCTGGAAATAACTGCTACTGGGTGTAGTCTCTTGTAAACGGAGTATCTGCCAGGGGGGTCGGGTATTGTCAAAGGAT 2160
TCATCTGCAACATTCCAGAACACGAGTGCTGATAGCAGTACTTCGAGTGCTGAGTTCCCAGTGCAATCGGTTCTTCTCAAGGACACCTCT 2250
GGCAGGTGTCACATGGTAAAGGGACATGAAGCAGGGCGGAACCTTTACCACTGGACAGACAGCCCAGAGAAACCAGCCTGCTGCAGTACG 2340
CCACTGCTGCCAAGACACACCTCCTCAGTCTACTGATCCTAAACCAGAGAGCAGGCCCAGATCACACAGTTTACCCCTTGCACGCCATTT 2430
TAAATATCAGACAATAAAGAAGGAATAGTTAGTTCTTAGATGGTGATGTTGGTGTTTCAGCTCCAAGCTTAAGGCTTAGTTACCCGGGTG 2520
CACAAAGCTGTAGACAACCAACTTCCTTCCAGCGTGAAAATGTGTAGGCTGGACAGAGTCCGTGCTCCACGATCGCTGAGTTCTTTCAGA 2610
CCATTGGTTGTCACACCCACAAGCCTAACTGTGACAGCCAATGAGTGTCCATGTGATTTTGACTGCAAATTTCGCTTATTTGATTTTTAG 2700
CGGAGACGTCCTTGCTCTTACTTGTGGATCAGGAGAGGTTTTCTTAGCCCTTCTAAGTGTTCCCAACCCGTGCGCTGTAGAACAGATTGC 2790
GCAAACTGACAGCAGAGTAAAAGGGGGCGGGGCTTCACTTCCGGCATCCCACCAGTCCACGCCCGCCGTTTTCACCTCTCCAGACTCGCC 2880
ACTCCTTTATTCATTTGCAACTGCAAATGTAGCAATTTGCAATCCTAATACAGGGCCAGGGCGGTGGATACCTTAAAGTTTTATTTGACT 2970
TCATTTAGTGCTTTCTGTCCATTTTTAATGACTTTTGTTTTTTTTGTTTTTGTTTCTCCAGCCCCATCTCCTCCCTCTGCCCTAGTATGAA 3060
ACTTGTACCCCTTTACTGTGTTTAAATATAAAGAAGAGGGGTTTTGTTTTGTTACATTACAACCCTTAAAATACAACGATCACTTGTATA 3150
ATATTTGTTTCTCTGCGGTTTGTATTCCTGATTTAGTGGAATGCCATTAAACTCTCTGGGTCAGTATCTAACTAGTTAACATCTAACTAG 3240
TCCTTTTAGATGACCATGAAGTATTCCACAGTACAGATGTGCTGCAAGTGACTTGATAACTTCCTTGTTGACAGATCTTCAGATTGTTCT 3330
TATCTTGCAAAC**AATAAA**CACCCATACATATACAGACAAAAAAAAAAAAAAAAAA

B

266 bp insert

···*TTTAGGAGAGCCAAGCAGACT*GTACCAAGGATGCAGGAAATTCCTGTCCAAAGACCAGGCCTCTTCCGCCTCTTATCTGTCTGCTTTG
TCCTGGATGGACTTCACCTCGTGAAGATTTGACTCTGTGTCCTACAGATAGCCAAAGTTTGGCTGTGAGGGACAAAGGGACTCAGAGAAA
GCTTAGACTAAAAGTTAACTCAGAATCAAGCGAAGGCTTTTCTGGAGGTCGAGTTCTGGATCATGATGGAGTGGAGGTCGCTTCGAGTGG
AGGTCGCTTCGCGCACCGGATTCACC**ATG**AAGAACCGGCTGGGTACATGGTGGGTGGCCATCCTCTGCATG···

103 bp insert

···*TTTAGGAGAGCCAAGCAGACT*ACTAAAAGTTAACTCAGAATCAAGCGAAGGCTTTTCTGGAGGTCGAGTTCTGGATCATGATGGAGTG
GAGGTCGCTTCGAGTGGAGGTCGCTTCGCGCACCGGATTCACC**ATG**AAGAACCGGCTGGGTACATGGTGGGTGGCCATCCTCTGCATG···

No insert

···*TTTAGGAGAGCCAAGCAGACT*ATTCACC**ATG**AAGAACCGGCTGGGTACATGGTGGGTGGCCATCCTCTGCATG···

C

TTCCGGAGAAGTGAGGGCTCCAAGCTTCAGAGGCCACAGTAGCAGAGAACCGAGATTCACC**ATG**AAGAACCGGCTGGGTACATGGTGGGT
GGCCATCCTCTGCATG···

to the 5′ end sequences of the two cDNAs of 2.2 and 3.4 kb mRNAs, indicating that they represent major transcripts in the brain of Ngsk *Prnp*$^{0/0}$ mice.

In the novel sequence following the *Prnp* exon 1/2 sequences, we identified an ORF encoding the predicted protein product of 179 amino acids with a molecular weight of 20,441 dalton (Fig. 2A). Its N-terminal domain comprising highly hydrophobic 23 amino acids is likely to represent a signal peptide, and its C-terminal region is also hydrophobic, which may be compatible with a transmembrane domain. Although the predicted protein lacks the consensus C-terminal serine residue, the site of glycosylphosophatidylinositol (GPI) anchor in PrP^{C} (Stahl and Prusiner, 1991), it is also possible that this C-terminal region may be cleaved out during the attachment of the GPI anchor. Moreover, conserved Asn-linked glycosylation sites are found at codons 99 and 111. These features are consistent with the characteristics of membrane glycoproteins, like PrP^{C}, and the two proteins interestingly revealed 23% identity in the amino acid sequence on a homology search as shown in Fig. 3 (Thompson *et al.,* 1994). We designated this putative protein as a PrPLP. It lacks a copper-binding octapeptide repeat region present in the N-terminal half of PrP^{C} but has a high homology with PrP^{C} at C-terminal half region, suggesting that PrPLP might make a similar globular structure to PrP^{C} (Riek *et al.,* 1996).

Ectopic Expression of Chimeric PrP/PrPLP mRNAs Under the Control of the *Prnp* Promoter in the Brain of Ngsk *Prnp*$^{0/0}$ Mice

We detected the PrPLP expression in the brain of neonate wild-type mice and performed 5′ RACE on the PrPLP mRNA from wild-type mice, which gave a single band on an agarose gel (data not shown). Sequence analysis revealed that its 5′ sequences (61 bp) preceding the PrPLP ORF were distinct from those of the PrP/PrPLP chimeric mRNAs in Ngsk *Prnp*$^{0/0}$ mice (Fig. 2C), indicating an unique start site of the normal PrPLP mRNA. This strongly indicates that the PrPLP sequence is derived from a novel gene distinct from *Prnp,* and the PrP/PrPLP chimeric mRNAs in Ngsk *Prnp*$^{0/0}$ mice are intergenic.

Fig. 2. cDNA sequences of the chimeric PrP/PrPLP and normal PrPLP mRNAs. (A) A complete cDNA sequence (3,386 bp) of the 3.4 kb chimeric PrP/PrPLP mRNA is shown. The cDNA for the shorter 2.2 kb transcript lacks the underlined 1,237 bp sequences. The cDNAs share the 145bp *Prnp* exon 1/2 sequence (italicized capitals) at their 5′ termini. A conserved poly(A) signal (bold capitals) is found 20 bp upstream of the poly(A) tail. A single ORF starts from the methionine codon (bold capitals) at nucleotide position 256 and terminates by the stop codon (*) at position 793. The ORF would specify a protein consisting of 179 amino acids with molecular sizes of 20,441 daltons. Predicted amino acid structures of the protein product are shown by the single-letter designation. Arrows indicate the junctions of the variable 5′ untranslated region which is preceded by the 5′ terminal *Prnp* exon 1/2 sequence and followed by the conserved sequence starting from 7 bp upstream of the initiator codon of ORF. (B) The cDNAs of the variable 5′ untranslated regions containing different inserts derived from the chimeric PrP/PrPLP mRNAs are shown. The inserts locate in the 5′ untranslated sequence between the 5′ terminal *Prnp* exon 1/2 sequence (italicized capitals) and conserved sequence starting from 7 bp upstream of the intiator codon of ORF. The 103 bp insert is the major insert as seen in A. The other two are minor ones, one is a 266 bp insert including the 103 bp while the other contains no insert. (C) The cDNA for the normal PrPLP mRNA contains the unique sequences at the 5′ terminus followed by the conserved sequence starting from 7 bp upstream of the intiator codon of ORF.

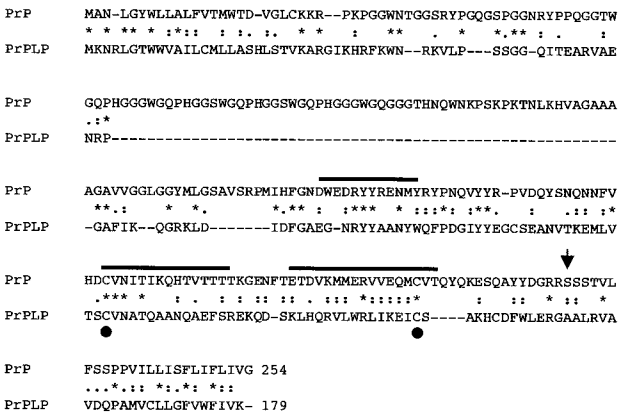

Fig. 3. Primary amino acid sequence similarities between PrP^C and PrPLP. The mouse PrP^C and PrPLP are aligned using a CLUSTAL W program (Thompson *et al.,* 1994). Asterisks (*) indicate identical amino acids, single dots (·) weakly similar amino acids, and double dots (:) strongly similar amino acids, respectively. Three lines indicate three α-helix regions of PrP^C and closed circles indicate sites of conserved cysteine residues of PrP^C (Riek *et al.,* 1996). An arrow indicates the serine residue of a GPI anchor attachment site of PrP^C.

Next, we performed *in situ* hybridization for the chimeric PrP/PrPLP mRNA on the brain sections of 20-week-old wild-type and Ngsk $Prnp^{0/0}$ mice. Consistent with the Northern blot analysis, no signal was detectable in the brain of wild-type mice (Fig. 4B and F). On the other hand, in Ngsk $Prnp^{0/0}$ mice, the chimeric PrP/PrPLP mRNA was expressed abundantly in neurons and non-neuronal cells throughout the brain, with the strongest signal detected in pyramidal cells of the hippocampus and Purkinje cells of the cerebellum (Fig. 4A and E). This expression pattern is the same as that for the PrP mRNA in the brain of wild-type mice (Fig. 4D and H). These results showed that the disrupted *Prnp* allele of Ngsk $Prnp^{0/0}$ mice caused the ectopic expression of PrP/PrPLP mRNA in the brain under the control of the *Prnp* promoter.

DISCUSSION

In the present study, we identified a novel sequence with an ORF encoding a putative membrane glycoprotein, PrPLP, with a significant homology to PrP. This sequence was highly expressed as chimeric mRNAs containing the *Prnp* exon 1/2 sequences at its 5′ end in the brain of ataxic Ngsk $Prnp^{0/0}$ mice, but not in nonataxic Zrch $Prnp^{0/0}$ mice. The 5′ sequence of the normal PrPLP mRNA is unique and distinct from the *Prnp* exon 1/2 sequences, strongly indicating that the novel sequence is derived from an independent PrPLP gene presumably located downstream of *Prnp*. Very recently, Moore *et al.* have identified the same ORF using a different experimental approach (Moore *et al.,* 1999). By genomic walking and large-scale

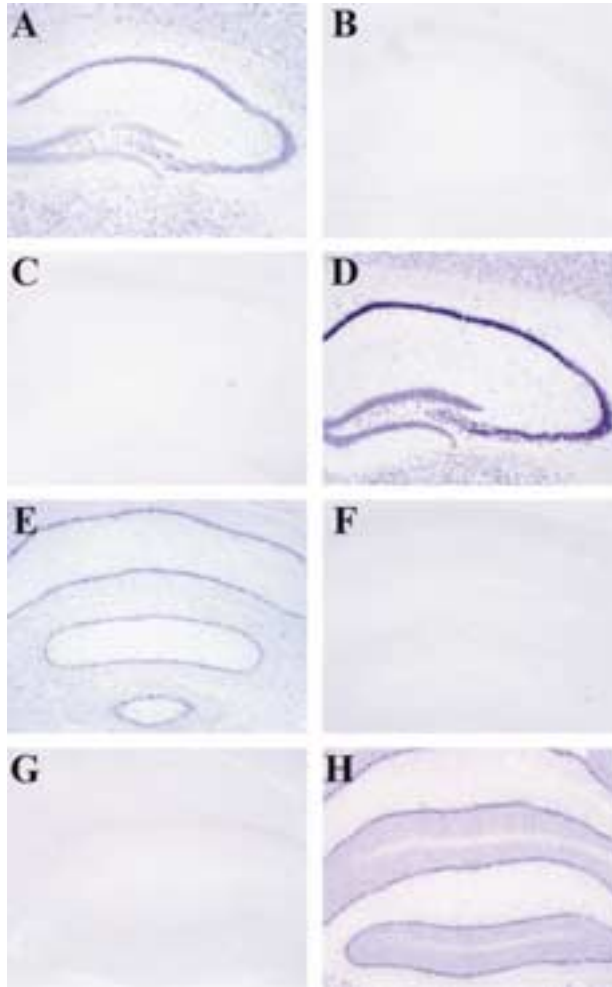

Fig. 4. Ectopic expression of the chimeric PrP/PrPLP mRNA expression in hippocampal and cerebellar regions of Ngsk *Prnp*$^{0/0}$ mice. *In situ* hybridization analysis of the brain tissues (hippocampus, A–D; cerebellum, E–H) of 20-week-old Ngsk *Prnp*$^{0/0}$ (A, C, E, G) and wild-type (B, D, F, H) mice was carried out using PrPLP (A, B, E, F) and PrP (C, D, G, H) antisense cRNA probes, independently. The chimeric PrP/PrPLP mRNA is expressed in neurons and non-neuronal cells of Ngsk *Prnp*$^{0/0}$ mice, in particular in pyramidal neurons of the hippocampus (A) and in Purkinje cells (E). There is no expression of PrPLP mRNA in the brain of wild-type mice (B, F). Cell distribution of the chimeric PrP/PrPLP mRNA in Ngsk *Prnp*$^{0/0}$ mice is nearly identical to that of PrP mRNA in wild-type mice. Original magnification: $\times$ 10.

sequencing, they mapped the ORF about 16 kb downstream of *Prnp* and designated the novel gene encoding the ORF as *Prnd* and its protein product as doppel (Dpl). Together, the chimeric mRNAs are likely to be generated as a consequence of intergenic splicing between *Prnp* and *Prnd* in Ngsk *Prnp*$^{0/0}$ mice.

The PrPLP/Dpl is likely to be a membrane glycoprotein because it has a signal peptide-like stretch at the N-terminal region and a hydrophobic domain at the C-terminus, which is compatible with a signal for a GPI anchor or a transmembrane domain. PrP^{C}, a GPI-anchored membrane glycoprotein, is reported to have a potential to associate physically with 37-kDa laminin receptor precursor, which binds the matrix protein laminin and mediates attachment, differentiation, movement, and growth of the cells (Rieger *et al.,* 1997). It would be conceivable that the PrPLP/Dpl might function as a cell adhesion molecule.

We detected that the ectopic expression of the chimeric mRNA encoding PrPLP/Dpl was regulated by the *Prnp* promoter in the brain of Ngsk *Prnp*$^{0/0}$ mice due to the intergenic splicing. The chimeric mRNAs were generated from the disrupted *Prnp* allele of Ngsk *Prnp*$^{0/0}$ but not from that of Zrch *Prnp*$^{0/0}$ mice. Moore *et al.* reported another ataxic mouse line, Rcm0 *Prnp*$^{0/0}$ mice, which also expressed the chimeric mRNAs (Moore *et al.,* 1999). The *Prnp* alleles of both Ngsk *Prnp*$^{0/0}$ and Rcm0 *Prnp*$^{0/0}$ mice lack a part of intron 2 including the SA site which are preserved in the allele of Zrch *Prnp*$^{0/0}$ and Edbg *Prnp*$^{0/0}$ mice. Antoniou *et al.* demonstrated that a particular sequence set within the last intron of the β-globin gene was necessary for the efficient 3′-end formation of the mRNA including the cleavage and polyadenylation of pre-mRNA (Antoniou *et al.,* 1998). It is conceivable that the structure of the disrupted *Prnp* alleles of ataxic *Prnp*$^{0/0}$ mice lacking a part of intron 2 is likely to be involved in the unusual intergenic splicing. The pre-mRNA synthesis started from the *Prnp* exon 1 proceeds to the 16 kb downstream *Prnd* due to inefficient cleavage at the poly(A) site of the *Prnp* exon 3, and then the intergenic splicing occurs, skipping the *Prnp* exon 3 due to the lack of the *Prnp* intron 2 (Fig. 5). Recent reports have demonstrated that the C-terminal region of RNA polymerase II is physically associated with multiple factors involved in pre-mRNA elongation, splicing, cleavage at the poly(A) site, and polyadenylation (Steinmetz, 1997). Moreover, the splicing factor U1snRNP is reported to regulate the polyadenylation process (Gunderson *et al.,* 1994). These emerging findings suggest a critical interaction of the *Prnp* intron 2 sequence with a sophisticated complex of transcription factors to make a mature PrP mRNA. Elucidation of this interaction would provide an important insight into the understanding of the transcription machinery.

We detected the ectopic PrPLP/Dpl expression in Purkinje cells of the ataxic Ngsk *Prnp*$^{0/0}$ mice, not in nonataxic Zrch *Prnp*$^{0/0}$ mice, suggesting that the ectopic expression of PrPLP/Dpl is likely to be involved in the Purkinje cell degeneration of Ngsk *Prnp*$^{0/0}$ and Rcm0 *Prnp*$^{0/0}$ mice. We have also recently demonstrated that the Purkinje cell degeneration could be rescued by expressing the normal mouse PrP^{C} in Ngsk *Prnp*$^{0/0}$ mice as a transgene (Nishida *et al.,* 1999). These results suggest that absence of PrP^{C} and overexpression of PrPLP would be required for the Purkinje cell degeneration. Interestingly, similar observations were recently reported by Shmerling *et al.,* that expression of the truncated PrPs lacking residues

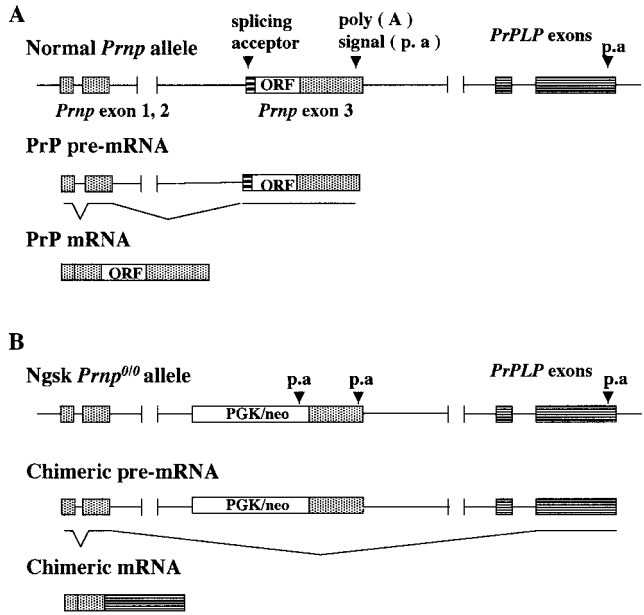

Fig. 5. Model for ectopic PrPLP mRNA expression in the brain of Ngsk *Prnp*$^{0/0}$ mice. (A) On the normal *Prnp* allele, PrP pre-mRNA starts from the *Prnp* exon 1 and is cleaved and polyadenylated at the appropriate termination site of *Prnp* exon 3. The intervening sequences, intron 1 and 2, are spliced out and mature PrP mRNA is generated from the pre-mRNA. (B) On the Ngsk *Prnp*$^{0/0}$ allele, pre-mRNA started from *Prnp* exon 1 proceeds to the termination site of the 16 kb downstream *Prnd* gene due to the lack of a part of the *Prnp* intron 2 sequences. Intergenic splicing takes place between *Prnp* and *Prnd* genes to generate chimeric mRNA encoding PrPLP whose expression is under the control of *Prnp* promoter.

32–121 or 32–134 in the Zrch *Prnp*$^{0/0}$ background caused ataxia due to granular cell death in the cerebellum and that this abnormal phenotype could be rescued by introducing a wild-type PrP gene (Shmerling *et al.,* 1998). They proposed a hypothesis to explain this phenomenon. In brief, a protein they assumed to be in wild-type mice can bind to an unidentified PrP^{C} ligand with a lesser affinity compared to PrP^{C} and elicit a particular signal. In Zrch *Prnp*$^{0/0}$ mice expressing the truncated PrP, the truncated PrP compete with the ligand for the assumed protein, which disturbs the signal eliciting from the assumed protein and cause the granular cell death. According to the hypothesis, PrPLP/Dpl might function like the truncated PrPs and prevent a signal essential for Purkinje cell survival in ataxic Ngsk *Prnp*$^{0/0}$ mice, because the primary amino acid structure of the truncated PrPs has a structural homology to PrPLP/Dpl. However, the exact relationship between the ectopic PrPLP/Dpl expression and the Pukinje cell degeneration is still enigmatic. Generation of transgenic mice harbouring the transgene encoding PrPLP/Dpl in the Zrch *Prnp*$^{0/0}$ background is urgently needed to elucidate mechanisms involved in the neurodegeneration in Ngsk *Prnp*$^{0/0}$ mice. The discovery of PrPLP/Dpl will

provide a new clue in prion research as well as in understanding other neurodegenerative processes.

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