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Short Communication

Physiological Expression of the Gene for PrP-Like Protein, PrPLP/Dpl, by Brain Endothelial Cells and its Ectopic Expression in Neurons of PrP-Deficient Mice Ataxic Due to Purkinje Cell Degeneration

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Recently, a novel gene encoding a prion protein (PrP)-like glycoprotein, PrPLP/Dpl, was identified as being expressed ectopically by neurons of the ataxic PrP-deficient (*Prnp*$^{-/-}$) mouse lines exhibiting Purkinje cell degeneration. In adult wild-type mice, PrPLP/Dpl mRNA was physiologically expressed at a high level by testis and heart, but was barely detectable in brain. However, transient expression of PrPLP/Dpl mRNA was detectable by Northern blotting in the brain of neonatal wild-type mice, showing maximal expression around 1 week after birth. *In situ* hybridization paired with immunohistochemistry using anti-factor VIII serum identified brain endothelial cells as expressing the transcripts. Moreover, in the neonatal wild-type mice, the PrPLP/Dpl mRNA colocalized with factor VIII immunoreactivities in spleen and was detectable on capillaries in lamina propria mucosa of gut. These findings suggested a role of PrPLP/Dpl in angiogenesis, in particular blood-brain barrier maturation in the central nervous system. Even in the ataxic Ngsk *Prnp*$^{-/-}$ mice, the physiological regulation of PrPLP/Dpl mRNA expression in brain endothelial cells was still preserved. This strongly supports the argument that the ectopic expression of PrPLP/Dpl in neurons, but not deregulation of its physiological expression in endothelial cells, is involved in the neuronal degeneration in ataxic *Prnp*$^{-/-}$ mice. *(Am J Pathol 2000, 157:1447–1452)*

The cellular prion protein (PrP^C), a membrane glycoprotein anchored by a glycosyl-phosphatidylinositol moiety, is highly expressed in the central nervous system, in particular by neurons, and to a lesser extent in lymphoreticular tissues such as lymph nodes and spleen.[1] Its protease-resistant isoform, PrP^{Sc}, is implicated as the etiological agent and in pathogenesis of transmissible spongiform encephalopathies or prion diseases, but the physiological function of PrP^C has not been well elucidated.[1] To address the function of PrP^C, several mouse lines deficient for PrP^C (*Prnp*$^{-/-}$) were independently generated by different groups using a conventional homologous recombination technique.[2–5] However, they exhibited a puzzling discrepancy in their phenotypes; two lines of *Prnp*$^{-/-}$ mice, Ngsk *Prnp*$^{-/-}$ and Rcm0 *Prnp*$^{-/-}$ mice, developed ataxia in old age due to cerebellar Purkinje cell degeneration, whereas the others, Zrch *Prnp*$^{-/-}$ and Edbg *Prnp*$^{-/-}$ mice, have never shown such an abnormal phenotype. Ngsk *Prnp*$^{-/-}$ mice were rescued from the neuronal degeneration by introduction of a transgene encoding the normal mouse PrP^C, indicating an essential role for the functional loss of PrP^C in the degeneration.

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We recently identified a novel gene that encodes a putative glycoprotein, namely PrP-like protein (PrPLP), consisting of 179 amino acids with 23% identity to PrP^C in the primary amino acid structure.[6] Moore et al, who independently discovered the same gene, designated it as *Prnd* and its product as doppel (Dpl).[5] In the brain of ataxic but not non-ataxic $Prnp^{-/-}$ mice, unusual intergenic splicing between the PrP gene (*Prnp*) and the downstream *Prnd* occurred, probably due to the deletion of the *Prnp* intron 2 sequence including its splicing acceptor.[5,6] The intergenic splicing rendered the PrPLP/Dpl expression under the control of the *Prnp* promoter, which caused its overexpression in neurons including Purkinje cells of ataxic $Prnp^{-/-}$ mice.[5,6] Taken together, these results suggest that in addition to the functional loss of PrP^C, the ectopic PrPLP/Dpl expression could also be involved in the neuronal degeneration in ataxic $Prnp^{-/-}$ mice.

In the present study, we examined physiological expression of PrPLP/Dpl mRNA in various tissues and found its expression in the endothelial cells of brain, spleen, and gut of neonatal wild-type mice. Moreover, we demonstrated that the physiological regulation of PrPLP/Dpl mRNA expression in brain endothelial cells was still preserved even in ataxic Ngsk $Prnp^{-/-}$ mice exhibiting ectopic PrPLP/Dpl mRNA in nearly all neurons including Purkinje cells.

Materials and Methods

Ngsk $Prnp^{-/+}$ mice were intercrossed to generate Ngsk $Prnp^{-/-}$ and $Prnp^{+/+}$ (wild-type) mice. Genotypes of the mice were determined by polymerase chain reaction (PCR) as described previously.[7] Zrch $Prnp^{-/-}$ mice were kindly provided by Prof. C. Weissmann (Neurogenetics Unit, Imperial College School of Medicine, London, UK).

Total RNA isolated from various tissues of mice by Trizol reagent (Gibco BRL Life Technologies, Inc., Rockville, MD) according to recommendation in the manufacturer's manual, was electrophoresed on a formaldehyde-denatured agarose gel and blotted onto a Hybond N membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) with 10× SSC. After the fixation of RNA by ultraviolet light (UVP, Ltd., Upland, CA), the membrane was subjected to a prehybridization procedure for 4 hours at 45°C in buffer containing 5× SSPE, 0.5% sodium dodecyl sulfate (SDS), 50% formamide, 5× Denhardt's solution, 10% dextran sulfate, and 100 μg/ml salmon sperm DNA. A hybridization procedure was performed using an appropriate ^{32}P-labeled DNA probe (BcaBEST Labeling 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 minutes, once in 1× SSC/0.1% SDS, and twice in 0.1× SSC/0.1% SDS at 65°C for 15 minutes. Signals were detected by BAS 2000 (Fuji, Tokyo, Japan) or autoradiography on Konica X-ray film.

In situ hybridization was performed as described elsewhere.[6] Briefly, the brain tissues were fixed for 16 hours in 4% buffered paraformaldehyde (pH 7.4), embedded in paraffin, and sliced at 5 μm thickness. The spleen and ileum tissues, immediately frozen by cold isopentane, were sliced into 10 μm thickness by a cryostat. The tissue sections were hybridized with digoxigenin (DIG)-UTP-labeled cRNA probes (Roche Diagnostics, Mannheim, Germany), which were made by using T7 or T3 polymerase (Gibco BRL Life Technologies). The sections were washed several times in 4× SSC and immersed in 50% formamide/2× SSC at 50°C for 30 minutes. They were then treated with RNase A at 37°C for 30 minutes and washed in 0.2× SSC at 60°C for 20 minutes. Signals were detected by an enzyme-linked immunosorbent assay for brain or GenePoint System (Dako, Copenhagen, Denmark) for spleen and ileum. After *in situ* hybridization, the tissues were incubated in 0.3% H_2O_2 solution for 30 minutes at room temperature to prevent endogenous peroxidase activity. The tissues were incubated with rabbit anti-factor VIII (1:400) serum (Dako) overnight at 4°C after blocking with normal goat serum. The signals were detected by incubation with biotinylated goat anti-rabbit Ig (1:500), avidin-conjugated horseradish peroxidase (1:500; Dako), and 3-amino-9-ethyl carbazole (Dako).

The PrPLP/Dpl, *Prnp* exon1/2, and *Prnp* exon 3 probes were prepared as described elsewhere.[6] The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe is a fragment comprising positions 395 to 1005 of the cDNA (NM 008084.1).

Results and Discussion

To elucidate the physiological expression profiles of the PrPLP/Dpl mRNA, total RNA samples from various tissues of 9-week-old wild-type mice were subjected to Northern blotting with the probe covering the PrPLP/Dpl coding region. Two discrete signals of 2.0 and 3.2 kb were detectable at a high level in testis and heart, to a lesser extent in spleen and skeletal muscle, but barely detectable in brain, kidney, liver, and lung of the wild-type mice (Figure 1A). The distribution of PrPLP/Dpl mRNA among the tissues was distinct from that of PrP mRNA (2.2 kb), which was expressed most abundantly in brain and ubiquitously in the remaining tissues including kidney, liver, spleen, skeletal muscle, lung, heart, and testis (Figure 1A). This indicated that the PrPLP/Dpl expression is regulated independently from the PrP mRNA expression under physiological conditions.

In contrast, in 9-week-old Ngsk $Prnp^{-/-}$ mice, the PrPLP/Dpl mRNAs (2.2 and 3.4 kb) were up-regulated in the brain and kidney (Figure 1B). The *Prnp* exon 1/2 but not exon 3 probe detected the same mRNA species (Figure 1B), indicating that they were intergenic spliced products generated from the *Prnp* allele of Ngsk $Prnp^{-/-}$ mice and their expression was under the control of PrP promoter as described previously.[6] Lack of a part of *Prnp* intron 2, including its splicing acceptor in the disrupted *Prnp* allele of Ngsk $Prnp^{-/-}$ mice, is likely to be involved in the unusual intergenic splicing in the brain and kidney. 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

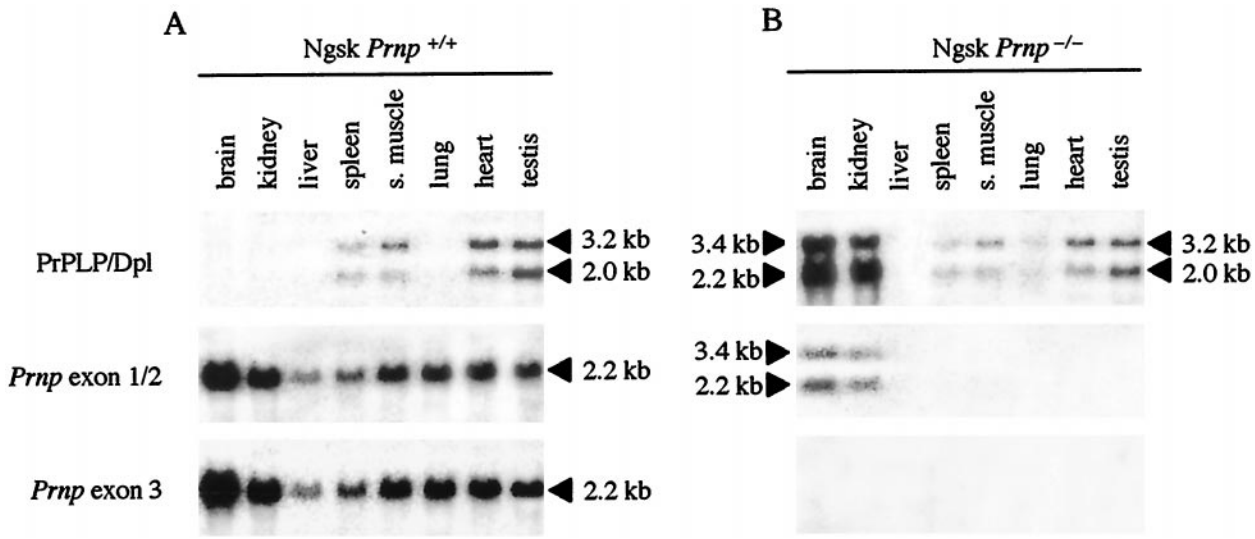

Figure 1. Northern blotting for PrPLP/Dpl and PrP mRNAs in various tissues of 9-week-old wild-type (Ngsk $Prnp^{+/+}$, **A**) and NgsK $Prnp^{-/-}$ mice (**B**) using the indicated probes. s. muscle, skeletal muscle.

the intergenic splicing occurs, skipping the *Prnp* exon 3 due to the lack of the splicing acceptor. However, expression profiles of the PrPLP/Dpl mRNA in the tissues other than brain and kidney were indistinguishable between Ngsk $Prnp^{-/-}$ and wild-type mice, and the PrPLP/Dpl mRNA expressed in spleen, skeletal muscle, heart, and testis of Ngsk $Prnp^{-/-}$ mice did not hybridize with the *Prnp* exon 1/2 probe (Figure 1B). Thus, despite the PrP promoter being active in these tissues (Figure 1A), intergenic splicing between *Prnp* and *Prnd* was unlikely to occur in these tissues of Ngsk $Prnp^{-/-}$ mice. Since a significant level of the PrPLP/Dpl mRNA was physiologically expressed in these tissues but not in brain and kidney of wild-type mice (Figure 1A), it is conceivable that the intergenic splicing is inhibited in the cells where the *Prnd* promoter is active. Alternatively, a tissue-specific factor(s) might be involved in some aspects of the intergenic splicing process in the brain and kidney.

To make an inquiry into the role of PrPLP/Dpl, we extended Northern blot analysis to the brain of younger wild-type mice, which revealed a transient expression of the PrPLP/Dpl mRNA in the brain soon after birth. As shown in Figure 2A, a detectable level of the PrPLP/Dpl mRNA was already expressed in the brain at birth, reached a maximal level at 6 days, and decreased thereafter, reaching an undetectable level by 8 weeks. *In situ* hybridization and immunohistochemistry were carried out to identify the cell type expressing the PrPLP/Dpl mRNA in the brain of 6-day-old wild-type mice. *In situ* hybridization gave linearly aligned or patchy signals of PrPLP/Dpl mRNA distributed throughout the brain (Figure 2B). The control sense probe did not give any signal (data not shown). This morphological expression strongly suggested that the cells with PrPLP/Dpl mRNA signals were other than neurons and glial cells. Immunohistochemical staining with the rabbit anti-serum against factor VIII-related peptide,[8] a specific marker of endothelial cells, gave signals that colocalized with those for PrPLP/Dpl mRNA in the brain of 6-day-old wild-type mice (Figure 2C).

We next examined the PrPLP/Dpl mRNA expression in spleen and gut of 6-day-old wild-type mice by *in situ* hybridization. In the spleen, signals for PrPLP/Dpl mRNA were distinctly detectable at the marginal region of white pulps (Figure 3A) and appeared to colocalize with the immunoreactivities of factor VIII on the consecutive section (Figure 3B). In the gut, the signals corresponding to PrPLP/Dpl mRNAs were detectable on the linearly extended structures in villi and other regions, the majority of which were easily recognized as capillaries by their morphological characteristics (Figure 3D). However, the PrPLP/Dpl mRNA expression was below the level of detection by *in situ* hybridization in these tissues of adult mice (data not shown). These results indicated that PrPLP/Dpl was preferentially expressed by endothelial cells in various tissues of neonatal mice in a developmentally regulated fashion.

Brain endothelial cells are major constituents of the blood-brain barrier (BBB). At about 1 week after birth, when the PrPLP/Dpl mRNA shows its maximal expression (Figure 2A), brain endothelial cells show maximal proliferation activity[9] and BBB maturation is completed.[10] Cell adhesion molecules such as intercellular adhesion molecule-1 and platelet endothelial cell adhesion molecule-1 CD31 are highly expressed on endothelial cells and promote angiogenesis or BBB maturation via cell-cell interactions in the same period.[11,12] The PrPLP/Dpl is a putative membrane glycoprotein with 23% identity to PrP^C in the primary amino acid structure.[5,6] PrP^C is reported to physically associate with 37-kd laminin receptor precursor, which binds to the matrix protein laminin and mediates attachment, differentiation, movement, and growth of the cells.[13] It would be conceivable that PrPLP/Dpl might also play a role in the endothelial cell adhesion mediating angiogenesis, in particular BBB maturation in the brain, soon after birth.

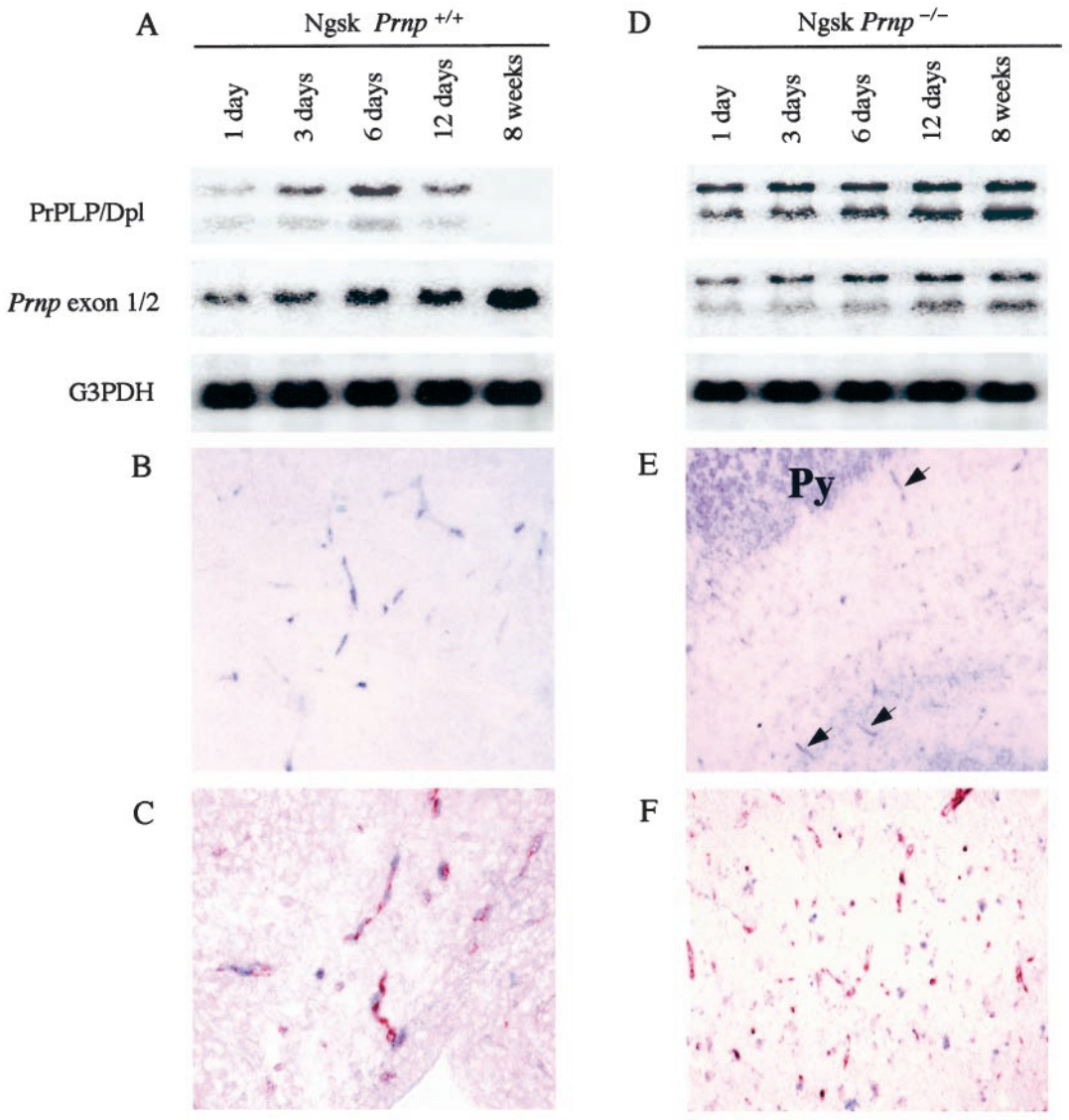

Figure 2. Northern blotting for PrPLP/Dpl and PrP mRNAs in the brain of wild-type (Ngsk $Prnp^{+/+}$) mice (**A**) and of NgsK $Prnp^{-/-}$ mice (**D**) at ages of 1, 3, 6, and 12 days and 8 weeks after birth. PrPLP/Dpl mRNA was transiently expressed in the former soon after birth, but the expression was deregulated in the latter due to intergenic splicing. *In situ* hybridization for PrPLP/Dpl mRNA with (**C** and **F**) or without (**B** and **E**) co-immunostaining of factor VIII, a marker of endothelial cells, on the brain sections of Ngsk $Prnp^{+/+}$ (**B** and **C**) and $Prnp^{-/-}$ (**E** and **F**) mice. The blue and red stains represent PrPLP/Dpl mRNA and factor VIII immunoreactivity, respectively. Linear or patchy hybridization signals colocalized with factor VIII immunoreactivities are observed throughout the brain of 6-day-old Ngsk $Prnp^{+/+}$ mouse (**B** and **C**), whereas the 6-day-old Ngsk $Prnp^{-/-}$ mouse brain shows the PrPLP/Dpl mRNA expression in neurons in addition to endothelial cells (**arrows** in **E**). In the brain of 8-week-old Ngsk $Prnp^{-/-}$ mice (**F**), there is no PrPLP/Dpl mRNA signal colocalized with the factor VIII immunoreactivity despite its ectopic expression in neurons. Py, pyramidal cells in the hippocampus. Original magnifications, ×100 (**B**, **C**, and **E**) and ×80 (**F**).

In contrast to the wild-type mice, Northern blotting revealed the constitutive expression of PrPLP/Dpl mRNAs at an abundant level in the brain of Ngsk $Prnp^{-/-}$ mice even at 8 weeks, which was mostly, if not entirely, hybridized to the *Prnp* exon 1/2 probe (Figure 2D). According to our previous study, this is likely to be mainly derived from the ectopic PrPLP/Dpl mRNA in neurons. However, it may be possible that the PrPLP/Dpl expression in brain endothelial cells was also deregulated in the ataxic Ngsk $Prnp^{-/-}$ mice because of the intergenic splicing. Furthermore, this deregulated PrPLP/Dpl might affect the function of the cells, resulting in neuronal degeneration in Ngsk $Prnp^{-/-}$ mice. To examine this possibility, we carried out immunohistochemistry together with *in situ* hybridization on the brain sections of 6-day- and 8-week-old Ngsk $Prnp^{-/-}$ mice. In the 6-day-old brain sections, the PrPLP/Dpl mRNAs were abundantly expressed by neurons throughout the brain, with the strongest signal in pyramidal cells of the hippocampus (Figure 2E) and Purkinje cells of the cerebellum (data not shown). The signals were also readily detectable in endothelial cells of the 6-day-old brain (Figure 2E, arrows). On the other hand, in the brain of 8-week-old Ngsk $Prnp^{-/-}$ mice there was no PrPLP/Dpl mRNA signal colocalized with immunoreactivities of the factor VIII-related peptide, although abundant PrPLP/Dpl transcripts were still present in neu-

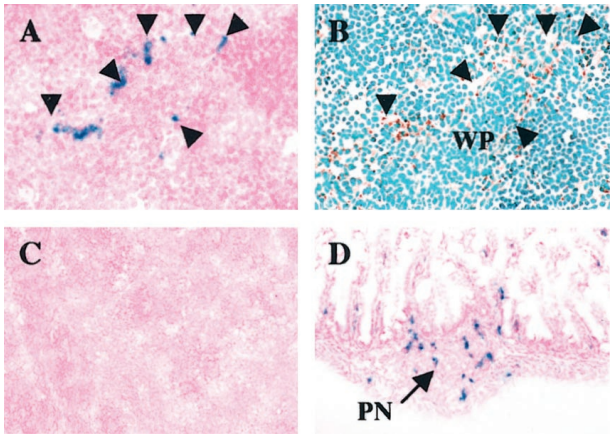

Figure 3. *In situ* hybridization for PrPLP/Dpl mRNA (**A**) and immunohistochemical staining with rabbit antiserum against factor VIII-related peptide (**B**) on consecutive sections of the spleen of 6-day-old neonatal wild-type mice. **Arrowheads** indicate the same sites on each section. WP, white pulp. **C:** Negative control of *in situ* hybridization using the sense cRNA probe of PrPLP/Dpl. **D:** *In situ* hybridization for PrPLP/Dpl mRNA on a section of gut from 6-day-old neonatal wild-type mice. **Arrow** indicates Peyer's node (PN). Original magnifications, ×66 (**A–C**) and ×55 (**D**).

rons (Figure 2F). These results indicated that the physiological regulation of PrPLP/Dpl expression in brain endothelial cells was preserved even in Ngsk *Prnp*$^{-/-}$ mice. This argues against the impaired function of brain endothelial cells due to deregulated expression of PrPLP/Dpl. So it is most likely that the ectopic expression of PrPLP/Dpl in neurons is directly associated with the neuronal degeneration in the ataxic *Prnp*$^{-/-}$ mice.

We previously demonstrated that the ataxic Ngsk *Prnp*$^{-/-}$ mice were rescued from the Purkinje cell degeneration by introducing a normal mouse PrP gene, suggested that two molecular events, ie, the functional loss of PrP^{C} and the ectopic PrPLP/Dpl expression, might be required for the neurodegeneration in ataxic *Prnp*$^{-/-}$ mice.[7] Schmerling et al reported that a transgene encoding N-terminal truncated PrP (PrPΔ32–135) rendered the non-ataxic Zrch *Prnp*$^{-/-}$ mice ataxic by inducing massive granule cell or Purkinje cell death, which could be prevented by introduction of the normal mouse PrP gene.[14] Since the conformational structure of the truncated PrP resembles that of PrP/Dpl, the same mechanisms might be involved in the neurodegeneration in Ngsk *Prnp*$^{-/-}$ and PrPΔ32–135 transgenic mice. Weissmann and Aguzzi recently proposed a hypothesis for molecular mechanisms underlying the neuronal degeneration.[15] They hypothesized two putative molecules; one is a ligand of PrP^{C} (PrP$_{L}$) eliciting a signal necessary for the survival of neurons, and the other is protein π that binds to PrP$_{L}$ with lower affinity but also capable of generating the survival signal. According to the hypothesis, in the non-ataxic *Prnp*$^{-/-}$ mice, protein π compensates for the function of PrP^{C}. However, in the ataxic mice, the ectopically expressed PrPLP/Dpl or truncated PrP preferentially interact with PrP$_{L}$, displacing protein π but without eliciting the survival signal. Identification of these putative molecules is urgently needed to evaluate the validity of this hypothesis. However, there could be an alternative possibility: that the ectopically expressed PrPLP/Dpl itself may elicit a signal for apoptosis in neurons and PrP^{C} functions as an anti-apoptotic factor by competing with PrPLP/Dpl. This idea is consistent with a previous finding that neuronal cell lines derived from ataxic *Prnp*$^{-/-}$ mice rapidly died by apoptosis in serum-free medium, but the apoptosis was prevented by overexpression of PrP^{C} or Bcl-2 protein.[16] Moreover, it was reported that the primary cultured cerebellar neurons derived from the non-ataxic *Prnp*$^{-/-}$ mice were more susceptible to the apoptosis-inducing oxidative stress than wild-type neurons, suggesting the anti-apoptotic function of PrP^{C}.[17]

However, further studies are necessary to understand the exact relationship between the ectopic expression of PrPLP/Dpl and the Purkinje cell degeneration. The transgenic mice expressing PrPLP/Dpl in Purkinje cells with the non-ataxic *Prnp*$^{-/-}$ mouse background would provide definitive evidence for the role of ectopic PrPLP/Dpl in the neuronal degeneration.

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