Journal of General Virology (2002), **83**, 705–711. Printed in Great Britain

Expression of doppel in the CNS of mice does not modulate transmissible spongiform encephalopathy disease

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Late onset ataxia reported in three independently derived PrP null lines of mice has been attributed to the overexpression of the doppel protein in the CNS of these mice rather than to the loss of PrP. The central role of PrP in the transmissible spongiform encephalopathies (TSEs), the proximity of the gene which encodes doppel (*Prnd*) to the PrP gene (*Prnp*) and the structural similarity shared by PrP and doppel have led to the proposition that ataxia which develops during TSE disease could, in part, be due to doppel. In order to address this hypothesis, we have crossed our two inbred lines of PrP null mice, which either express (RCM) or do not express (NPU) the *Prnd* gene in the CNS, with mice expressing two *Prnp*$^{a[108F189V]}$ alleles of the PrP gene. We have found that the TSE infection does not influence the level of expression of *Prnd* in the CNS at the terminal stages of disease. Moreover, we have demonstrated that the level of expression of *Prnd* in the CNS has no influence on the incubation period, vacuolar pathology nor amount or distribution of PrP^{Sc} deposition in the brains of the TSE-infected mice. Doppel has therefore no apparent influence on the outcome of TSE disease in transgenic mice, suggesting it is unlikely to be involved in the naturally occurring TSE diseases in other species.

Introduction

The transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases that affect animals and humans. The PrP protein plays a central role in these diseases as demonstrated by the resistance of PrP null mice to TSE disease (Bueler *et al.*, 1993; Manson *et al.*, 1994a). During the course of disease, PrP is converted from the normal cellular protease-sensitive form (PrP^{C}), attached to the cell membrane by a glycophosphatidylinositol (GPI) anchor, to the disease-associated form (PrP^{Sc}) which is resistant to proteases and accumulates in and around cells of the CNS.

To elucidate the role of PrP in TSE disease and the normal function of PrP, a number of lines of PrP null ($PrP^{-/-}$) mice have been generated. The first two lines of $PrP^{-/-}$ mice produced (ZrchI and NPU) developed and reproduced normally (Bueler *et al.*, 1992; Manson *et al.*, 1994a). However, more subtle phenotypic alterations such as altered circadian rhythm (Tobler *et al.*, 1996), electrophysiological defects (Collinge *et al.*, 1994) and alterations in copper binding and superoxide dismutase activity in the CNS (Brown *et al.*, 1997) have subsequently been attributed to the loss of PrP in these mice. Three further lines of PrP null mice (Nsgk, Rcm0 and Zrch II) have since been reported. These lines all present with an ataxic phenotype and Purkinje cell loss from approximately 50 to 70 weeks of age (Moore *et al.*, 1999; Rossi *et al.*, 2001; Sakaguchi *et al.*, 1996). A recent study investigating the cause of ataxia in these mice mapped a gene 16 kb downstream of the murine *Prnp* gene (PrP), termed *Prnd*. This gene encodes a PrP-like protein named doppel (Dpl). This gene was found to be expressed in the CNS of all PrP null mice that went on to develop the ataxic phenotype, but no *Prnd* expression was detected in the CNS of the ZrchI (Moore *et al.*, 1999) or NPU null mice (N. L. Tuzi & J. C. Manson, unpublished observation). It has been suggested that overexpression of Dpl in $Prnp^{-/-}$ mice causes Purkinje cell loss and ataxia and that this ataxia can be prevented by expressing wild-type PrP (Nishida *et al.*, 1999; Rossi *et al.*, 2001). Furthermore, a recent study reported that by reducing the amount of *Prnd* mRNA, the onset of ataxia was delayed by 6 months (Rossi *et al.*, 2001).

Dpl and PrP have $\sim 25\%$ amino acid sequence identity (Moore *et al.*, 1999) and Dpl has been shown to be anchored to the cell surface via a GPI anchor (Silverman *et al.*, 2000), as is

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PrP (Stahl *et al*., 1990). Detailed analysis of recombinant Dpl has shown that the protein is very similar to PrP both in structure and in topology (Mo *et al*., 2001). However, Dpl lacks the octapeptide repeats and conserved amino acid region 106–126 found in the N terminus of PrP, and thus resembles a truncated form of PrP. Indeed, transgenic mice expressing truncated versions of PrP that lack the octapeptide repeats and amino acid region 106–126 (d32–121 or d32–134 PrP), and hence resemble Dpl, have been reported to develop spontaneous behavioural disorders at a young age, including ataxia (Shmerling *et al*., 1998). It has been suggested that the structural similarity between PrP and Dpl may result in the two proteins competing for binding to the same ligand (PrPL) (Weissmann & Aguzzi, 1999). It has also been proposed that binding of PrP to PrPL generates a survival signal; however, binding of Dpl or truncated forms of PrP to PrPL would not result in a survival signal being generated, resulting in Purkinje cell death and ataxia.

Since PrP has a central role in TSE disease, the *Prnp* and *Prnd* genes are in close proximity and the proteins share a structural similarity, it has been proposed that Dpl might also play a role in TSE disease. A number of studies have investigated whether there was any evidence to involve Dpl with altered susceptibility to TSE diseases in humans. The human Dpl gene, *PRND*, has been sequenced from control individuals and patients with CJD. Although four polymorphisms within the coding region of *PRND* have been found in two studies, there is no apparent association between these polymorphisms and human TSE diseases (Mead *et al*., 2000; Peoc'h *et al*., 2000).

In a further study, grafts of Dpl-deficient cells were implanted into the brains of adult PrP null mice. When the grafts were inoculated with the RML isolate of TSE agent, classical signs of TSE pathology were observed in the grafts, demonstrating that the absence of Dpl in the grafts did not lead to absence of TSE disease pathology (Behrens *et al*., 2001).

However, rather than being an absolute requirement for TSE disease, Dpl may be capable of modulating the outcome of TSEs, resulting in alterations in incubation time or in the targeting or intensity of pathological lesions in the brain. In order, therefore, to investigate this hypothesis, we crossed our two inbred lines of PrP null mice which either express (Rcm0) or do not express (NPU) the *Prnd* gene in the CNS, with mice expressing two copies of the $Prnp^{a[108F189V]}$ allele (Moore *et al*., 1998) of the PrP gene (BB mice). This PrP targeted line of transgenic mice was chosen because of the relatively short incubation period observed when BB mice are challenged with the TSE agent 301V. Critically, these three lines of mice all share an identical genetic background, 129/Ola, thereby removing any effects due to non-specific genetic differences. We have inoculated these lines of mice (BO^{RCM} and BO^{NPU}) with the mouse-passaged 301V strain of BSE. We have compared the level of *Prnd* expression in the infected and uninfected mice of each line, to establish whether *Prnd* expression is altered in the CNS during TSE infection. We have also investigated whether *Prnd* expression in the CNS can modulate TSE disease by comparing the incubation times and pathological lesions in the CNS of the two lines of TSE infected mice.

Methods

■ **Generation of mouse lines.** The gene-targeted transgenic mice, termed BB, in which the polymorphisms at 108 and 189 were altered from L108T189 to F108V189 ($Prnp^{a[108F189V]}$) (Moore *et al*., 1998), were crossed with two lines of PrP null mice, either NPU (Manson *et al*., 1994a) or Rcm0 (Moore *et al*., 1995). The F1 mice generated were termed BO^{NPU} and BO^{RCM} respectively.

■ **301V challenge of mice.** BO^{NPU} and BO^{RCM} mice were inoculated under halothane anaesthesia intracerebrally (i.c.) with 20 μl of a 1% brain homogenate (in PBS) prepared from brains of VM mice terminally infected with the 301V strain of TSE. A group of BB mice was also inoculated as controls for the targeting of CNS pathology with 301V. Signs of TSE illness were scored as previously described (Fraser & Dickinson, 1968). Incubation times were calculated as the time interval between inoculation and terminal illness. A control group of BO^{RCM} and BO^{NPU} mice were inoculated by the i.c. route with 20 μl of a 1% brain homogenate prepared from a normal, non-TSE challenged mouse brain.

■ **Lesion profiles.** Mice were killed by cervical dislocation and their brains removed and fixed in 10% formal saline. Haematoxylin and eosin-stained coronal sections (6 μm) were scored for vacuolation on a scale of 0 to 5 in nine standard grey matter areas and three white-matter areas, as described previously (Fraser & Dickinson, 1967, 1968).

■ **PrP immunocytochemistry.** Following fixation in formal saline the brains were treated with formic acid (98%) for 90 min before being placed in fresh formal saline for a minimum of 24 h. The brains were trimmed, dehydrated in alcohol and impregnated with wax in a cycle lasting approx. 7 h. Sections (6 μm) were mounted on Superfrost plus glass slides, air dried at room temperature overnight then for 2 days at 37 °C. Sections were immunostained using mouse monoclonal antibody 6H4 (Prionics AG) as described. Briefly, sections were de-waxed and rehydrated prior to autoclaving at 121 °C for 15 min followed by treatment with 98% formic acid for 5 min. Endogenous peroxidases were inhibited using methanol and 1% hydrogen peroxide. Sections were incubated with 5% normal rabbit serum prior to the addition of the primary antibody. To each test slide a 1:1000 dilution of primary antibody was added and left to incubate overnight at room temperature. The biotinylated rabbit anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, USA) was added at a 1:400 dilution and bound antibody was visualized using the ABC kit (Elite) and diaminobenzidine tetrahydrochloride. Sections were counterstained lightly with haematoxylin. Normal mouse serum in place of primary antibody was used as a control. All washes were done using buffer consisting of PBS–0·1% BSA.

■ **Northern blotting.** Total RNA was isolated from terminal brains using RNAzol B, based on the guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi, 1987). Total RNA (50 μg) was separated on a 1·0% agarose–formaldehyde denaturing gel and transferred to Hybond-N (Amersham Pharmacia) by capillary transfer overnight. RNA was fixed to the membrane by baking at 80 °C for 2 h before probing for a *Prnd* transcript using a ^{32}P-labelled 540 bp PCR fragment generated from the Dpl ORF (Moore *et al*., 1999). Membranes were hybridized overnight using ULTRAhyb (Ambion) and washed according to the manufacturer's instructions. A 936 bp *Kpn*I–*Eco*RI fragment from *Prnp* exon 3 was used to generate the PrP probe.

Prior to probing the membrane to correct for loading, membranes were stripped by adding to 0·1% SDS heated to 100 °C and shaken until cool. The stripped membrane was hybridized as before and probed with a ^{32}P-labelled murine B-actin probe (GenBank acc. no. BE689156, isolated with restriction enzymes *Eco*RI and *Not*I).

Results

Northern blot analysis of BO^{RCM} and BO^{NPU} mice

The levels of *Prnd* and *Prnp* mRNA were determined by Northern blot analysis on total RNA prepared from three 301V i.c. challenged terminal brains of BO^{RCM} and BO^{NPU} mice and one each from normal brain challenged controls. This revealed two RNA species of approximately 3·5 and 2·0 kb in both the 301V infected and the normal brain challenged BO^{RCM} mice (Fig. 1a). No difference in the levels of mRNA were detected between the TSE infected and uninfected BO^{RCM} mice. Prnd mRNA was not detected in the brains of BO^{NPU} mice which had been infected with 301V or challenged with normal brain homogenate. When the Northern blot was hybridized with a *Prnp* probe, one mRNA species of approximately 2·1 kb was detected in brains from all animals (Fig. 1b). The amount of mRNA loaded was visualized by hybridizing the membrane with a murine *β*-actin probe (Fig. 1c). These results confirm that *Prnd* mRNA is expressed in the presence of *Prnp* transcript and that the level of *Prnp* mRNA is not affected by the expression of *Prnd* mRNA. Thus there is no evidence

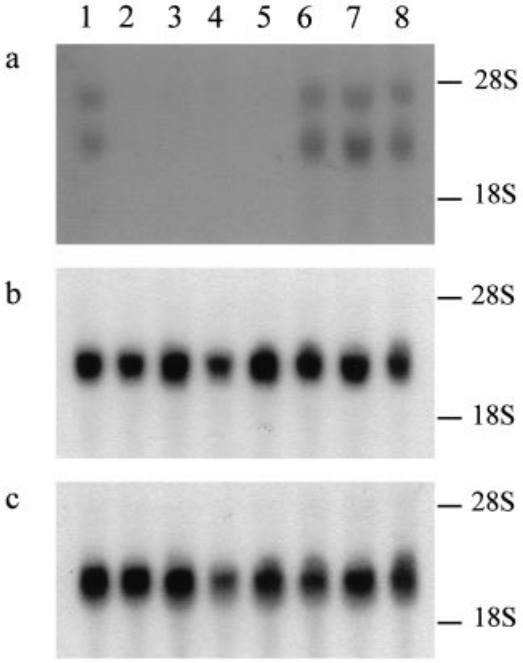

Fig. 1. Doppel and PrP mRNA expression in 301V challenged BO^{NPU} and BO^{RCM} mouse brains. Northern blot analysis on 50 μg of total RNA prepared from terminal brains of 301V challenged, BO^{NPU} (lanes 3–5) and BO^{RCM} (lanes 6–8), or normal brain challenged BO^{NPU} (lane 2) and BO^{RCM} (lane 1) mice. (a) A radiolabelled probe from the ORF of Dpl was used to detect the presence of *Prnd* mRNA. (b) A radiolabelled probe from murine *Prnp* exon 3 was used to detect *Prnp* mRNA and (c) the stripped membrane probed for *β*-actin mRNA.

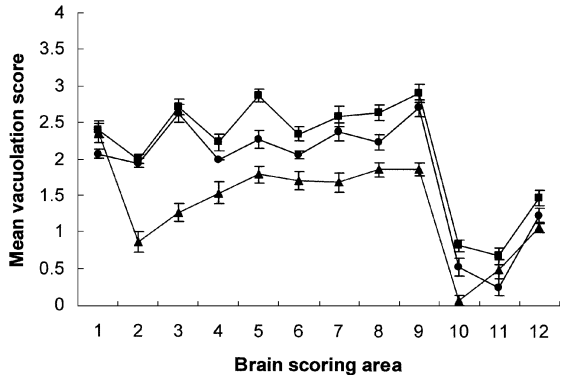

Fig. 2. Analysis of brain vacuolation by lesion profiling. BO^{RCM} (●), BO^{NPU} (■) or BB (▲) transgenic mice were challenged with the TSE agent 301V via the i.c. route. Animals were culled and brains removed at the terminal stages of disease. Nine grey matter (1–9) and three white matter areas (10–12) of terminal brains were scored semi-quantitatively for vacuolation. Lesion profiles were constructed by scoring 14 to 23 brains from each group on a scale of 0 to 5. The mean scores for each brain area are shown (error bars ± SEM). Lesion profile scoring areas: 1, dorsal medulla; 2, cerebellar cortex; 3, superior colliculus; 4, hypothalamus; 5, medial thalamus; 6, hippocampus; 7, septum; 8, thalamic cortex; 9, forebrain cortex; 10, cerebellar white matter; 11, mesencephalic tegmentum; 12, pyramidal tract.

Table 1. 301V incubation times

SEM, standard error of the mean; *n*, number of animals in the group.

Mouse strain	No. of *Prnp* alleles	Expression of *Prnd**	Incubation time (days)†	SEM	*n*
BO^{NPU}	1	no	195	0·9	23
BO^{RCM}	1	yes	191	1·6	17
BB‡	2	no	134	1·0	15

* Determined from Northern blot analysis of RNA from 301V challenged terminal brains.
† Mean incubation taken as time from TSE inoculation until death.
‡ BB transgenic mice express *Prnp*$^{a[108F189V]}$ alleles (Moore *et al.*, 1998). BO^{NPU} are NPU *Prnp*$^{-/-}$ × BB mice; BO^{RCM} are Rcm0 *Prnp*$^{-/-}$ × BB mice (Rcm0 *Prnp*$^{-/-}$ overexpress doppel in the CNS).

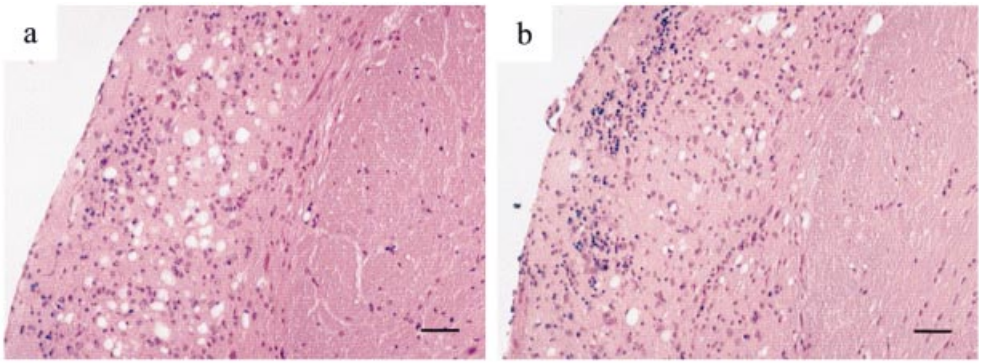

Fig. 3. Vacuolar change in the cochlear nucleus. Vacuolar change in the cochlear nucleus of 301V terminal brains from BO^{NPU} (a) and BO^{RCM} (b) mice. Coronal sections stained with haematoxylin and eosin. Magnification bar, 100 μm.

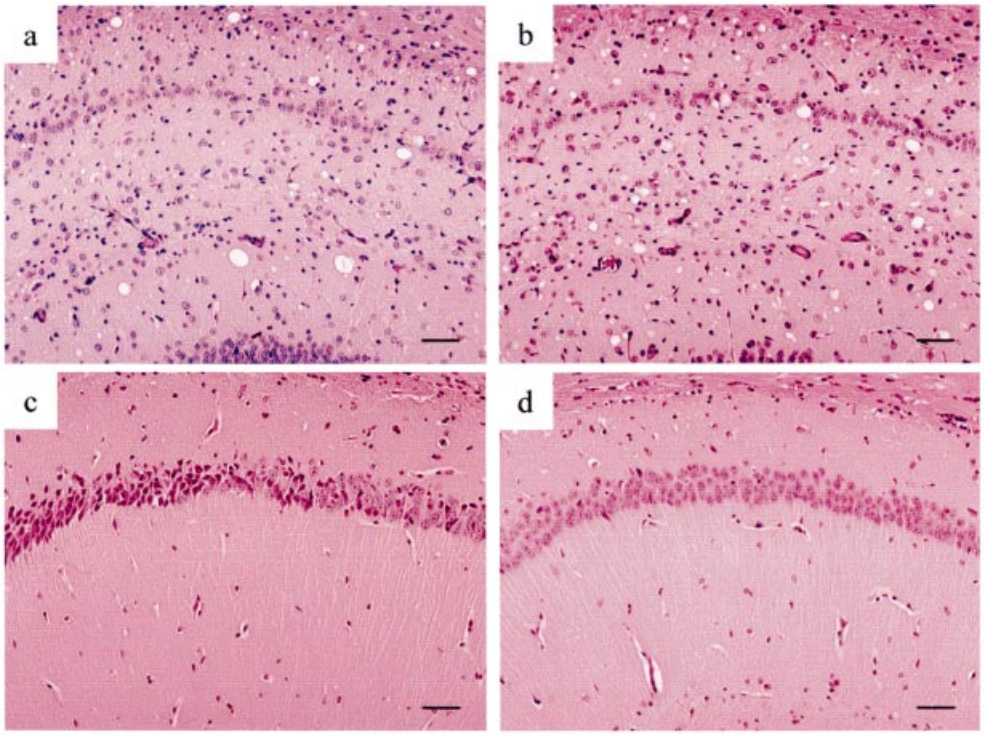

Fig. 4. Neuronal loss and vacuolar change in the CA1 region of the hippocampus. Neuronal loss and vacuolar change in the CA1 region of the hippocampus of 301V terminal brains from BO^{NPU} (a) and BO^{RCM} (b) mice or normal brain challenged BO^{NPU} (c) and BO^{RCM} (d). Coronal sections stained with haematoxylin and eosin. Magnification bar, 50 μm.

from these experiments that the expression of the *Prnd* gene is altered in the CNS of animals infected with TSE disease.

Prnd expression does not influence incubation time

Despite major differences in the expression of *Prnd* in the brains of the BO^{RCM} and BO^{NPU} mice there is essentially no difference in the incubation time of disease following i.c. challenge with the 301V strain of TSE (Table 1). The BO^{NPU} mice succumbed to disease in 195 days ± 0·9 SEM and the BO^{RCM} mice in 191 days ± 1·6 SEM. Thus *Prnd* expression in the CNS does not apparently influence the incubation time of TSE disease in these mice. The incubation times in both the BO^{RCM} and BO^{NPU} mice were longer than in homozygous BB mice, reflecting the effect of gene dosage of *Prnp* on incubation time (Manson *et al.*, 1994b), since BB mice possess two functional PrP alleles whereas BO^{NPU} and BO^{RCM} mice possess only one.

Pathological lesions in the CNS are not affected by expression of *Prnd*

Lesion profile analysis revealed extensive vacuolation in the superior colliculus, thalamus and cortex of the forebrain in the BO^{RCM} and BO^{NPU} (Fig. 2). A high degree of vacuolation was also observed in the cochlear nucleus (Fig. 3) and in the

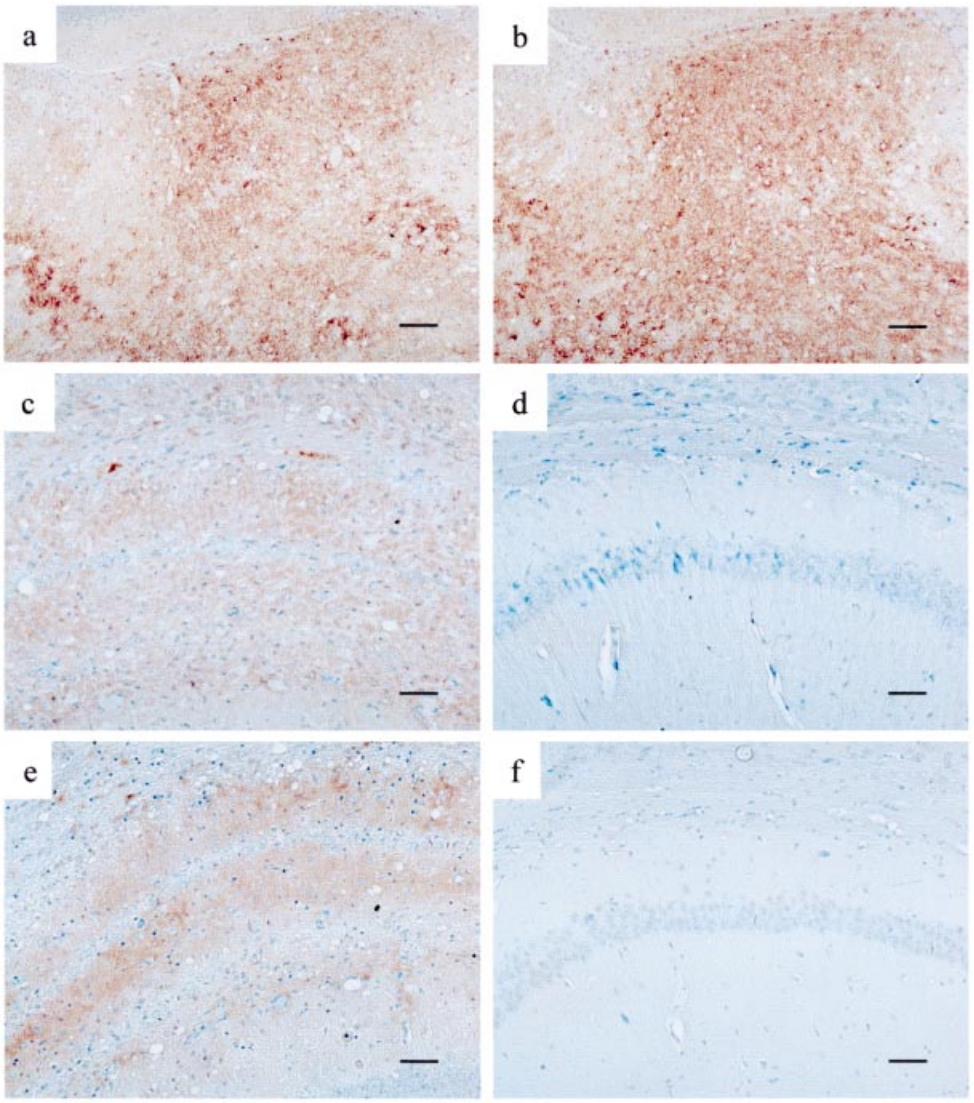

Fig. 5. PrP deposition in the hippocampus and thalamus. PrP staining of 301V terminal brains or normal brain challenge controls. PrP was detected on coronal sections using the monoclonal antibody 6H4, developed by an immunoperoxidase method and lightly counterstained with haematoxylin. (a, c) 301V BO^{NPU}; (b, e) 301V BO^{RCM}; (d) normal brain BO^{NPU}; (f) normal brain BO^{RCM}. (a, b) Dorsal lateral geniculate nucleus of the thalamus; magnification bar, 100 μm. (c–f) CA1 of the hippocampus; magnification bar, 50 μm.

cortex and CA1 region of the hippocampus (Fig. 4) in both lines of mice. Although there was some variation in the amount of vacuolation observed in different brains within the same group of animals, there was no difference in the overall pattern or extent of vacuolation observed between the BO^{RCM} and BO^{NPU} groups of mice (Fig. 2). The pattern of vacuolation observed for the BB mice was similar to the BO^{RCM} and BO^{NPU} mice but the degree of vacuolation was lower in most areas of the brain. This could be due to the difference in expression levels of PrP since BB mice possess two $PrP^{[108F189V]}$ alleles whereas BO^{RCM} and BO^{NPU} mice possess only one. Thus expression of *Prnd* in the CNS does not influence either the targeting or amount of vacuolar pathology detected in the CNS at the terminal stage of TSE disease.

Expression of *Prnd* does not effect PrP deposition or neuronal loss

Severe neuronal loss was observed in the hippocampus, in particular the pyramidal cells of the CA1 region. The degree of neuronal loss was variable between animals of the same genotype, but no major differences between the different lines of mice were observed (Fig. 4). The extent of PrP deposition was determined in the terminal brains of BO^{RCM} and BO^{NPU} mice with PrP deposition being most evident in the CA1 region of the hippocampus, the dorsal lateral geniculate nucleus and thalamus (Fig. 5). Although variation was again observed within each genotype group, no major differences between the BO^{RCM} and BO^{NPU} mice were observed and patterns of PrP

deposition seen in these two lines of mice were comparable with that of BB mice (data not shown). Immunocytochemistry was also performed on brains from animals inoculated with a normal mouse brain homogenate. These showed no neuronal loss or PrP deposition (Fig. 5). Furthermore, no cross-reaction of the anti-PrP antibody with Dpl was evident since no signal was detected in brain sections of normal brain challenged BO^{RCM} mice that overexpress Dpl in the CNS (Fig. 5) or unchallenged RCM PrP null mice (data not shown).

Discussion

PrP is central to the TSE diseases and mutations and polymorphisms in the coding region of PrP can influence the outcome of TSE disease. However, it is also clear that factors other than the coding region of PrP influence TSE phenotype. The close proximity of the *Prnd* gene to the *Prnp* gene, the structural similarity of Dpl to PrP and its apparent ability to induce ataxia in mice when overexpressed in the CNS suggested Dpl as an ideal candidate for influencing TSE disease. We have, however, found no evidence that expression of the *Prnd* gene is altered in the CNS in mice terminally infected with TSE. The brains of uninfected and 301V infected BO^{RCM} mice have similar levels of *Prnd* mRNA transcripts, whereas there is no evidence for *Prnd* mRNA in the CNS of either infected or uninfected BO^{NPU} mice. This suggests that expression of Dpl mRNA is not induced in the CNS in the terminal stages of TSE disease. This is further supported by our finding that *Prnd* mRNA could not be detected by Northern blot analysis of terminal brains from SV and VM mice challenged i.c. with another TSE agent, ME7 (data not shown). Moreover, we have found no evidence that different expression levels of *Prnd* in the CNS of transgenic mice alter the course of TSE disease following i.c. challenge with 301V, since there was no difference in incubation times and terminal pathology of mice with or without *Prnd* expression in the CNS.

Dpl is not normally expressed in the CNS of adult mice but is expressed in the periphery. Dpl mRNA was shown to be present in the gut and endothelial cells of the brain and spleen of 6-day-old mice with the highest levels being detected in testis and heart, with spleen and skeletal muscle showing lower levels and brain, kidney, liver and lung having barely detectable levels in 9-week-old mice (Li *et al.*, 2000). Therefore, Dpl may have a function primarily in the periphery rather than the CNS of adult mice. It has also been reported that Ngsk $Prnp^{-/-}$ mice showed evidence of demyelination and axon loss in the PNS. However, whether this is due to Dpl overexpression or the absence of PrP is unclear as Zrch I $Prnp^{-/-}$ mice (which do not overexpress Dpl) were also reported to show demyelination of the sciatic nerve (Nishida *et al.*, 1999). Thus if Dpl were to influence the outcome of TSE disease it may achieve this through events in the periphery rather than in the CNS. With TSE diseases the most natural route of infection is via the periphery where events such as uptake and replication of infectivity and its transport to the CNS are likely to be controlled by a number of different factors. Experiments are currently under way to address the effect of Dpl expression in the CNS on TSE disease when mice are challenged via the peripheral route but, at present, there is no evidence for alterations in Dpl expression leading to different outcomes of TSE disease with peripheral routes of infection. However, since *Prnd* expression is altered in the CNS of BO^{RCM} mice, this may not result in alterations in the peripheral events of TSE disease in this line of mice. In order to address this issue we have produced a line of mice in which the *Prnd* gene has been ablated resulting in an absence of *Prnd* expression throughout the mouse. Inoculation of this line of mice with TSE strains by i.c. and peripheral routes will establish if expression of *Prnd* influences any aspect of TSE disease in mice. This work is currently in progress.

In summary, we have found no evidence that the expression of *Prnd* is altered in the terminal stages of TSE infection or that *Prnd* expression in the CNS influences the incubation time of TSE disease. Moreover, the expression of *Prnd* in the CNS does not appear to alter either the targeting or the intensity of pathological lesions in the brain of animals terminally infected with TSE disease. Thus we have demonstrated that Dpl has no apparent role to play in the TSE diseases in i.c. inoculated mice. However, experiments are currently under way to determine whether expression of Dpl can affect naturally occurring TSE diseases.

We are grateful to I. McConnell and her staff, especially J. Foster, V. Thomson and J. Beaton, for care and clinical scoring of the mice and D. Drummond for inoculation. We would also like to thank S. Mack for tissue processing and section cutting, A. Boyle for the lesion profiles and Dr L. Jamieson and A. Suttie for advice on the ICC analysis.

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Received 12 September 2001; Accepted 23 November 2001