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

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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 (Bueller et al., 1993; Manson et al., 1994a). During the course of disease, PrP is converted from the normal cellular protease-sensitive form (PrPSc), attached to the cell membrane by a glycoprophosphatidylinositol (GPI) anchor, to the disease-associated form (PrPSc) 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 (Bueller 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 (Nsrgk, Rcm0 and ZrchII) 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Prnd expression was detected in the CNS of the ZrchI (Moore et al., 1999) or NPU null mice (N. L. Tuzi & J. C. Manson, unpublished observation).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 ~ 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
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; Peoč’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<sup>[108F189V]</sup> 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 (B0<sup>RCM</sup> and B0<sup>NPU</sup>) 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.

1. **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<sup>[108F189V]</sup>) (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 B0<sup>NPU</sup> and B0<sup>RCM</sup> respectively.

#### 301V challenge of mice.

**301V challenge of mice.** B0<sup>NPU</sup> and B0<sup>RCM</sup> 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 B0<sup>RCM</sup> and B0<sup>NPU</sup> 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.

**Lesion profiles.** Mice were killed by cervical dislocation and their brains removed and fixed in 10% formal saline. Haematoxylin and eosi-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.

**PrP immunocytochemistry.** Following fixation in formal saline the brains were treated with formic acid (98%) for 90 min followed by washing 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 e4H (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.

**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% agarose–formaldehyde denaturing gel and transferred to Nytran (Dupont) by capillary transfer overnight. RNA was fixed to the membrane by baking at 80 °C for 2 h before probing for a 35P-labelled 540 bp PCR fragment generated from the Dpl ORF (Moore et al., 1999). Membranes were hybridized overnight using ULTRAbib (Ambion) and washed according to the manufacturer’s instructions. A 936 bp KmⅡ/EcoRI 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 EcoRI and NotI).

### 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

![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.](image)

Table 1. 301V incubation times

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>No. of $Prnp$ alleles</th>
<th>Expression of $Prnd$*</th>
<th>Incubation time (days)†</th>
<th>SEM</th>
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<tr>
<td>$BO^{NPU}$</td>
<td>1</td>
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<td>195</td>
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</tr>
<tr>
<td>$BO^{RCM}$</td>
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<td>yes</td>
<td>191</td>
<td>1·6</td>
<td>17</td>
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<tr>
<td>BB‡</td>
<td>2</td>
<td>no</td>
<td>134</td>
<td>1·0</td>
<td>15</td>
</tr>
</tbody>
</table>

* 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 $Prnp^{(HPF/BV)}$ 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. 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 profiling 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.

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 homogenerate. 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...
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 BORCM and BONPU 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 BONPU mice succumbed to disease in 195 days ± 0.9 SEM and the BORCM 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 BORCM and BONPU 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 BONPU and BORCM 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 BORCM and BONPU (Fig. 2). A high degree of vacuolation was also observed in the cochlear nucleus (Fig. 3) and in the CA1 region of the hippocampus (Fig. 4). Vacuolar change in the cochlear nucleus of 301V terminal brains from BONPU (a) and BORCM (b) mice. Coronal sections stained with haematoxylin and eosin. Magnification bar, 100 µm.

**Fig. 3.** Vacular change in the cochlear nucleus. Vacular change in the cochlear nucleus of 301V terminal brains from BONPU (a) and BORCM (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 BONPU (a) and BORCM (b) mice or normal brain challenged BONPU (c) and BORCM (d). Coronal sections stained with haematoxylin and eosin. Magnification bar, 50 µm.
Effect of Prnd expression in CNS on TSE

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 BONPU; (b, e) 301V BORCM; (d) normal brain BONPU; (f) normal brain BORCM. (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 BORCM and BONPU groups of mice (Fig. 2). The pattern of vacuolation observed for the BB mice was similar to the BORCM and BONPU 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 BORCM and BONPU 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 BORCM and BONPU 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 BORCM and BONPU 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\textsuperscript{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\textsuperscript{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\textsuperscript{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 Pgrp\textsuperscript{-/-} 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 Zrch1 Pgrp\textsuperscript{-/-} 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\textsuperscript{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.

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**References**


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