

Prion-like protein Doppel expression is not modulated in scrapie infected cells and in the brains of patients with Creutzfeldt–Jakob disease

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Abstract Doppel protein has been discovered in *prnp* knock-out mouse lines, with overproduction of this protein in the brain causing ataxia and neurodegeneration. We investigated whether Doppel expression (i) affected or was affected by the course of prion propagation in neuroblastoma cells, or (ii) modulated Creutzfeldt–Jakob disease pathogenesis. No change in Doppel production was detected in N2a cells, before or after infection. Transient murine Doppel gene expression had no effect on N2a viability or PrP^{Sc} production. A sensitive immunometric assay revealed low levels of Doppel in human brain, reflecting weak transcription of the corresponding gene. No difference in brain Doppel levels was observed between Creutzfeldt–Jakob disease patients and controls, adding further evidence that Doppel is unlikely to be involved in prion disease pathogenesis.

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Key words: Prion; Doppel; Creutzfeldt–Jakob disease; Brain; mRNA; Immunoassay

1. Introduction

Prions cause a group of fatal neurodegenerative diseases, including Creutzfeldt–Jakob disease (CJD) in humans [1]. The main event in the pathogenesis of these diseases is the conversion of the cellular prion protein (PrP^c), a normal ubiquitous protein encoded by the gene *PRNP*, to an abnormal isoform, scrapie prion protein (PrP^{Sc}) [1]. PrP^c is a glycoprotein, about 210 amino acids long when mature; it is present in large amounts in neurons [2], binds copper with high affinity [3], and may be involved in cell signalling [4].

Prion infections are post-translational disorders, as PrP^{Sc} accumulates in the brains of infected animals with no change in PrP mRNA levels [5]. However, prion infections may modulate the transcription of various genes [6–10].

A new mouse gene, *prnd*, encoding the PrP-like protein Doppel (Dpl, for *downstream prion protein-like*) was recently discovered 16 kb downstream of *prnp* [11,12]. The unexpected overexpression of *prnd* in two specific lines of *prnp* knock-out mice (*Prnp*^{0/0}, *Ngsk*, and *RcmO* lines), led to late-onset ataxia associated with Purkinje cell degeneration [11,12]. The role of this protein in conferring this phenotype was confirmed in transgenic PrP^{0/0} mice overproducing Dpl [13,14]. Furthermore, the ataxic phenotype was corrected by introduction of a wild-type *prnp* transgene [15]. This suggests that Dpl and PrP interact, possibly on the basis of the similarities between the two proteins. Both proteins are glycosylphosphatidylinositol-anchored, and share 25% C-terminal sequence homology, resulting in a α -helix-rich folding pattern [11,16,17]. *Prnd* is expressed at low levels in the brains of wild-type adult mice, and transcription levels are highest in the testis in mice, sheep and cattle [11,18]. In humans, Dpl is expressed in large amounts in the Sertoli cells, mature spermatozoa and seminal fluid [19].

Recent studies have investigated the possible modulation of prion diseases by ectopically produced Dpl in experimentally infected animals [20]. The influence of Dpl in cultured infected cells and in humans with prion diseases is unknown. We therefore investigated whether Dpl production affected, or was affected by, the course of prion propagation in the chronically infected neuronal cell model, N2a. We then investigated whether human Dpl (HuDpl) production was modified in the brains of patients who died from CJD.

2. Materials and methods

2.1. Antibodies

Monoclonal antibodies (mAbs) were raised against recombinant human Dpl (rHuDpl 28–152) (rHuDpl); they do not recognize the mouse protein. Anti-Dpl polyclonal antibody (pAb) DDC39, anti-PrP mAb 3F4, pAb P45–66 and SAF mixtures have been described elsewhere [19,21].

2.2. Construction of a mouse Dpl-encoding plasmid

The open reading frame of the mouse Dpl (MoDpl) gene was amplified by polymerase chain reaction (PCR) from mouse genomic

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Abbreviations: CJD, Creutzfeldt–Jakob disease; Dpl, Doppel protein; mAb, monoclonal antibody; OD, optical density; pAb, polyclonal antibody; PK, proteinase K; PrP^c, cellular prion protein; PrP^{Sc}, scrapie prion protein

DNA (gift of Dr. Andrew Goldsborough, Montpellier, France) using the following primers: sense 5'-GACCAGGAATTCATGAAGAACCGGCTGGGTACA-3' and antisense 5'-GACCAGGAATTCTTACTTCACAATGAACCAAAC-3'. The purified PCR product was digested with *EcoRI* (New England Biolabs) and ligated into pcDNA3 (Invitrogen). Constructs were verified by sequencing.

2.3. Cell culture transfection

The N2a#58 clone was chronically infected with the 22L prion strain as previously described [21]. Where indicated, cells were transiently transfected with Lipofectamine (Invitrogen), according to the manufacturer's instructions.

2.4. RT-PCR analysis

Total RNA (5 µg) extracted from adult 129 SV mouse brain and testis was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen). Equivalent amounts of cDNA were used for *prnd* study, using the primers described by Moore et al. ([11]): DW 189 and DW 96. Human cDNAs from brain and testis (Clontech, Palo Alto, CA, USA) were amplified using primers Dplex1 (forward) 5'-GAGGCTCCAGAGGCACACT-3' and Dplex2 (reverse) 5'-CTCGGCTCCAAGTCAATGT-3' and β -actin (*actb/ACTB*) primers *ACTB* ex 1 (forward) 5'-CCAGAGCAAGAGAGGCATCC-3' and *ACTB* ex 2 (reverse) 5'-GCTGGGGTGTGAAGGTCTC-3' under standard conditions, with an annealing temperature of 58°C.

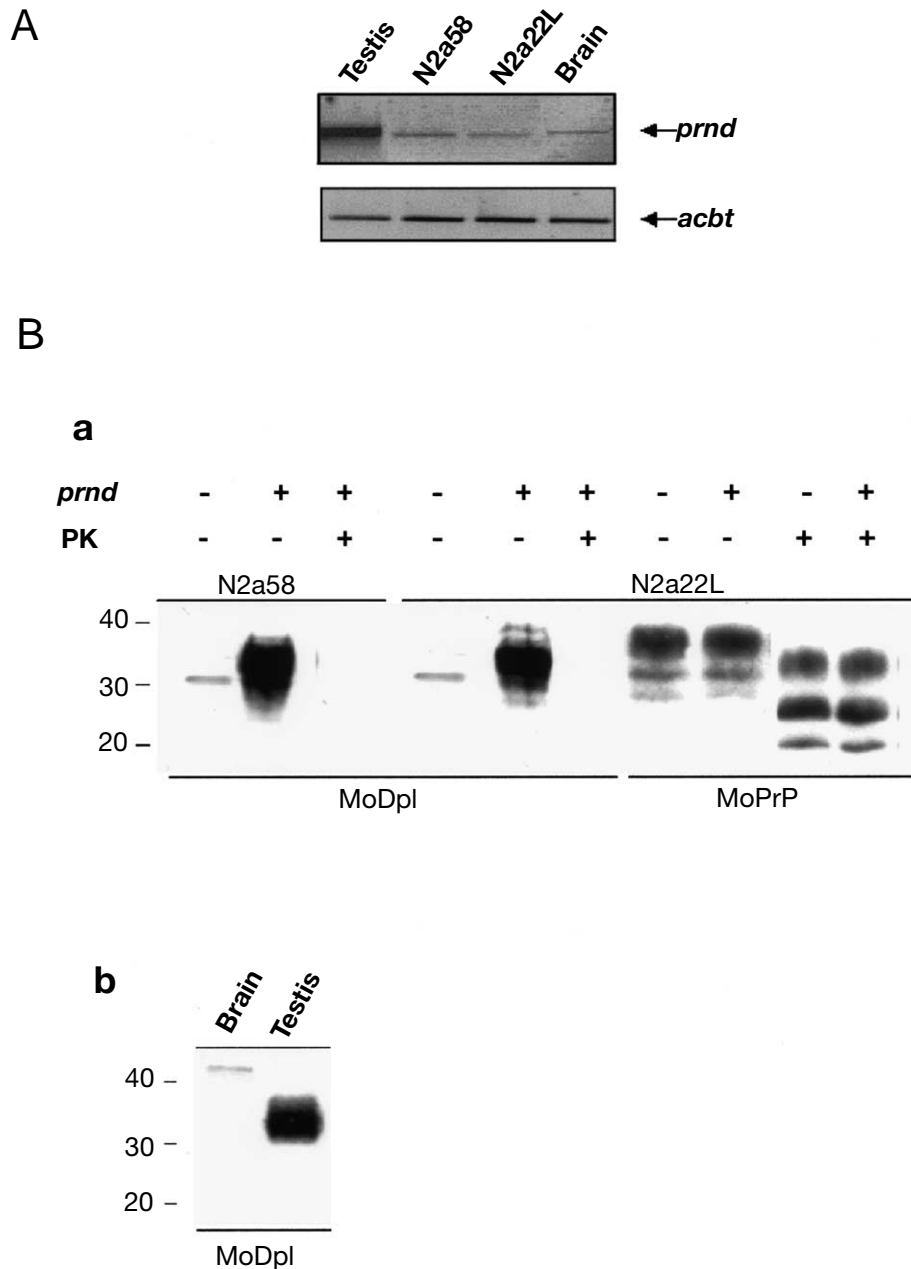


Fig. 1. A: Levels of *prnd* mRNA in control and infected N2a#58 cells and in mouse brain. RT-PCR was performed with the mouse cDNA, using *prnd*-specific primers. The results obtained are compared with those obtained with *actb* primers. B: a: Transient expression of MoDpl in N2a cells and effect on PrP^c and PrP^{Sc} production. Dpl and PrP were detected by Western blotting, directly or after digestion by PK, with pAb DDC39 (Dpl), P45–66 (PrP, PK–) or SAF mixture (PrP, PK+). Where indicated, cells were transiently transfected with a plasmid encoding the murine *prnd* gene, and analyzed 72 h after transfection. The two bands in the first and fourth lanes were not specific. Molecular weight is indicated in kDa. b: Dpl is not detected in the mouse brain by Western blotting. Dpl levels in the brain (90 µg total protein loaded) and testis (30 µg) were investigated by Western blotting with pAb DDC39 (1/10000). The band observed in the brain lane was not specific. Molecular weight is indicated in kDa.

2.5. Cell and tissue extract preparations and Western blotting

Cells were lysed in lysis buffer (150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris-HCl pH 7.5), centrifuged and mixed directly with Laemmli buffer or treated with proteinase K (PK; 16 µg/mg total protein; Roche Diagnostic) for 30 min at 37°C. Digestion was stopped by incubation with Pefabloc (1 mM; Roche Diagnostic) for 5 min on ice. The suspension was centrifuged (20 000 × g, 45 min, 4°C), and pellets were suspended in Laemmli buffer.

Brain tissues were obtained from autopsies of 10 sporadic CJD patients of both sexes, CJD diagnosis was confirmed by neuropathological examination and PrP^{Sc} detection in frontal cortex homogenates (either type 1 or 2). No mutations were detected in *PRNP*. Thirteen non-CJD patients of both sexes (10 patients with amyotrophic lateral sclerosis, one with stroke, one with Alzheimer's disease, and one with metabolic encephalopathy) were analyzed for comparison. Testes were obtained at autopsy from non-CJD patients; mouse brain and testes were obtained from 129 SV mice. Tissues were frozen and stored at -80°C until assay. Tissues were homogenized at 4°C in lysis buffer (10% w/v), centrifuged and protein concentrations were determined by the BCA technique (Bio-Rad).

For Western blotting, samples were run in 12% polyacrylamide gels containing sodium dodecyl sulfate and glycine and proteins were detected as previously described [19,21], using the antibodies indicated in the text.

2.6. Enzymatic immunoassay for HuDpl

The tetrameric form (G4) of acetylcholinesterase (AChE) was covalently coupled to mAb anti-Dpl as previously described [22]. Immunometric assays were performed with brain homogenates (8 mg/ml total protein) or rHuDpl (28–152), as previously described [23], with Dop79 as capture antibody and Dop28-AChE as tracer [23].

2.7. Affinity chromatography

BrCN Sepharose 4B was incubated overnight with 3–5 mg Dop28 in 0.1 M carbonate buffer, 0.5 M NaCl, pH 8.5. The gel was washed in carbonate buffer, incubated for 2 h in 0.1 M Tris-HCl, pH 8, washed and resuspended in 0.01% NaN₃ in 0.1 M phosphate buffer pH 7.4. We incubated 250 µl of brain homogenate (8 mg protein/ml) for 2 h at 4°C with 50 µl of affinity gel. The mixture was centrifuged for 10 min at 15 000 × g and 100 µl of supernatant was used for immunometric assay.

3. Results and discussion

We first investigated the presence of *prnd* transcripts and MoDpl in control and chronically infected N2a cells. Prion propagation in the infected cells was assessed by detecting PrP^{Sc} by Western blotting. *Prnd* transcripts were faintly detected by RT-PCR in both cases, with no evidence of marked up- or down-regulation following infection (Fig. 1A). The level of *prnd* transcription in the N2a cell line was similar to that in mouse brain, and considerably lower than that in the testis (Fig. 1A) [11].

We used wild-type and infected N2a lysates for Western blotting, to determine whether *prnd* mRNA was translated into detectable amounts of MoDpl (Fig. 1B,a). MoDpl was detected in neither normal nor infected cells, but was readily detected in the testis, as previously reported (Fig. 1B,b) [16]. No MoDpl was detected in mouse brain. As the detection limit for Western blotting for rHuDpl was estimated to be about 300 ng (data not shown), the method may not have been sensitive enough to detect small amounts of the protein in these cells. However, no significant change in *prnd* expression was observed during infection of the N2a cell line with the 22L scrapie strain. These ex vivo results are similar to the results obtained in vivo in mice injected intracerebrally with scrapie, in which prion infection does not affect *prnd* expression levels in the central nervous system [20].

We then investigated whether the ectopic production of

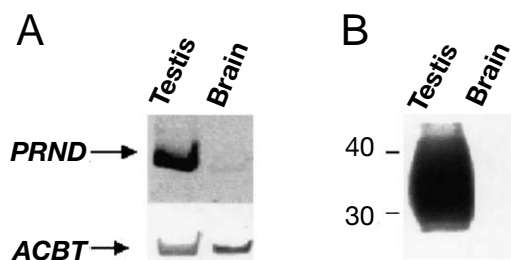


Fig. 2. A: *PRND* transcripts are detected in human brain. RT-PCR on cDNA from human organs was carried out using *ACTB*- and *PRND*-specific primers. B: Dpl is not detected in the human brain by Western blotting. HuDpl levels in the brain (90 µg total protein loaded) and testis (8 µg) were investigated by Western blotting with pAb DDC39 (1/10 000). Molecular weight is indicated in kDa.

MoDpl affected the course of prion propagation in N2a cells. We carried out transient transfection experiments with the murine *prnd* gene (Fig. 1B,a). No change in cell morphology or growth rate was observed following MoDpl production. MoDpl synthesis did not affect level of PrP^c or PrP^{Sc} production. These data are consistent with a recent report [14] indicating that the level of *prnd* expression in the brain does not affect the incubation period, vacuolar pathology or the amount or distribution of PrP^{Sc} deposition in the brains of mice intracerebrally inoculated with prions. Finally, no PK-resistant form of MoDpl was generated, indicating that, despite the structural similarity of the two proteins, PrP^{Sc} did not impose its conformational imprinting on Dpl.

Similar conclusions concerning the role of Dpl in prion pathogenesis were drawn from experiments involving infected neuroblastoma cells and experimentally infected mice [14]. We nonetheless extended the study to a spontaneous human prion disease, sporadic CJD. Genetic variations in the coding sequence of *PRND* are not associated with susceptibility to or the expression of sporadic CJD [24]. However, this lack of genetic association did not rule out the possibility of changes in HuDpl production in the brains of patients with the disease.

We found that *PRND* was transcribed less strongly in the brains of non-CJD individuals than in the testes (Fig. 2A). HuDpl was not detected by Western blotting in the control (Fig. 2B) or in CJD brains, even after immunoprecipitation (data not shown). We therefore designed a specific immunoassay, to increase the sensitivity of detection. The two-site immunometric assay was developed with a combination of two mAbs – Dop79 and Dop28 – which provided the most sensitive detection (Fig. 3A), using rHuDpl as the standard. The detection limit of the assay was below 10 pg/ml.

Brain homogenates from five CJD and five non-CJD patients were assayed for HuDpl before and after affinity chromatography with mAb Dop28. The absorbance obtained with untreated homogenates was strongly decreased by this chromatography step, narrowing the blank value (Fig. 3A), indicating that HuDpl was specifically detected by the immunometric test. The amount of HuDpl in brain homogenates was estimated to be around 15–50 ng/g total protein, using rHuDpl as a standard. This is about one thousandth the amount present in the testis (35–50 µg/g) or seminal plasma (7–15 µg/g) [19]. We therefore determined HuDpl levels directly in brain homogenates from controls and sporadic

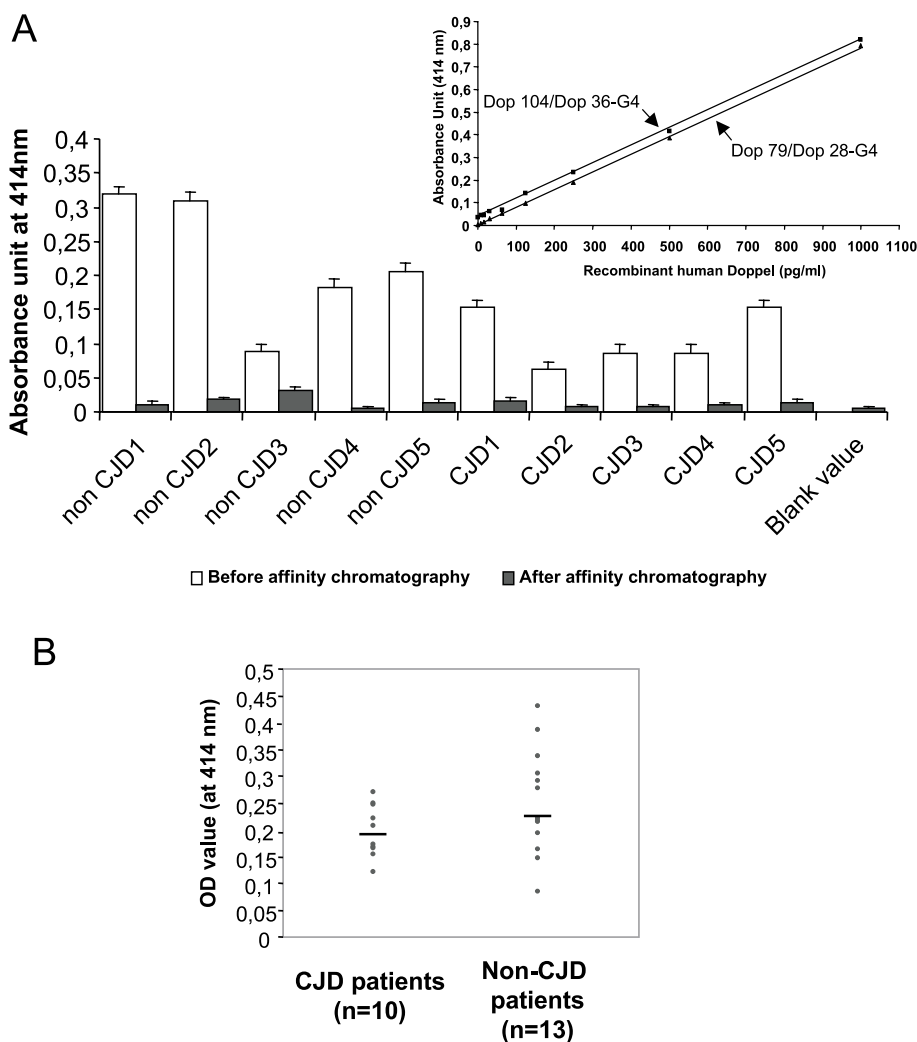


Fig. 3. A: Detection of Dpl in human brain homogenates, using a two-site immunoassay before and after depletion by affinity chromatography. Standard curves were established and the Dop79–Dop28 combination was selected for the sample assay. We used 100 μ l of elution product, or of homogenate centrifuged for 10 min, for the immunometric assay. B: Analysis of brain homogenates from 10 CJD and 13 non-CJD patients. 100 μ l of brain homogenates were assayed as described. Absorbance values, with the median, are represented on the graph for both series.

CJD patients (Fig. 3B). No significant difference was observed between CJD patients (median optical density (OD): 0.190; range: 0.120–0.269) and non-CJD patients (median OD: 0.221; range: 0.083–0.430; Kolmogorov–Smirnov test: $P=0.27$). *PRNP* codon 129 genotypes and PrP^{Sc} types did not seem to affect HuDpl levels in sporadic CJD, but this finding is based on limited observation. Our results thus suggested that HuDpl production was unaffected in the brain of sporadic CJD patients. For comparison, PrP^c is present at a concentration of about 1–10 μ g/g total protein in humans (J. Grassi, personal communication) and sheep [25]. Therefore, the abundance of PrP^c in the brain is more than 1000 times greater than that of Dpl, precluding toxic effects of the protein in the brain, as observed in PrP^{0/0} mice overproducing Dpl [13,14].

In conclusion, weak transcription of the *PRND* gene in human brain led to very low levels of HuDpl, detected only with a highly sensitive immunometric test. Neither mRNA nor Dpl levels appeared to be modified by prion infection in chronically infected N2a cells or in sporadic CJD. These data, and those obtained in experimentally infected animals [20], indicate that Dpl is probably not involved in prion dis-

ease pathogenesis, in the central nervous system at least. However, the putative interaction between Dpl and PrP^c may provide clues as to the enigmatic function of PrP^c. Dpl is thought to be involved in male fertility [19,26] and the new immunoassay presented here opens up possibilities for the sensitive detection of HuDpl in human fluids and tissues.

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