Normal neurogenesis and scrapie pathogenesis in neural grafts lacking the prion protein homologue Doppel

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The agent that causes prion diseases is thought to be identical to PrPSc, a conformation of the normal prion protein PrPC. Recently a novel protein, termed Doppel (Dpl), was identified that shares significant biochemical and structural homology with PrPC. To investigate the function of Dpl in neurogenesis and in prion pathology, we generated embryonic stem (ES) cells harbouring a homozygous disruption of the Prnd gene that encodes Dpl. After in vitro differentiation and grafting into adult brains of PrPc-deficient Pnp0/0 mice, Dpl-deficient ES cell-derived grafts contained all neural lineages analyzed, including neurons and astrocytes. When Prnd-deficient neural tissue was inoculated with scrapie prions, typical features of prion pathology including spongiosis, gliosis and PrP Sc accumulation, were observed. Therefore, Dpl is unlikely to exert a cell-autonomous function during neural differentiation and, in contrast to its homologue PrPC, is dispensable for prion disease progression and for generation of PrPSc.

INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders which occur naturally in man and in a variety of animals (Aguzzi et al., 2001). One of the hallmarks of TSEs is the accumulation in the brain of a protease-resistant protein, PrPSc, which is a conformation of a normal host-encoded protease-sensitive isoform, designated PrPc (Prusiner, 1982). PrPc is attached by a glycolipid anchor to the cell surface and is expressed by many cell types, including neurons and astrocytes. When Prnd-deficient neural tissue was inoculated with scrapie prions, typical features of prion pathology including spongiosis, gliosis and PrPSc accumulation, were observed. Therefore, Dpl is unlikely to exert a cell-autonomous function during neural differentiation and, in contrast to its homologue PrPC, is dispensable for prion disease progression and for generation of PrPSc.

Prnp0/0 and Edbg Pnp+/− show only minor electrophysiological and circadian rhythm defects (Büeler et al., 1992; Collinge et al., 1994; Tobler et al., 1996); whereas Ngsk Pnp−/− mice develop ataxia with advancing age, due to cerebellar Purkinje cell degeneration (Sakaguchi et al., 1996; Moore et al., 1999). Two additional Pnp knockout lines, Zürich II and Rcm0, confirmed the observations on the Nagasaki line (Moore et al., 1999; Rossi et al., 2001).

Sequence analysis of a cosmid containing Pnp revealed a novel gene encoding a protein of 179 residues, christened Dpl ('downstream of the Pnp locus' or 'doppel', german for 'double'), 16 kb downstream of the murine Pnp gene. The predicted protein showed ~25% identity with the carboxy-proximal two thirds of PrPC (Moore et al., 1999). Dpl may contain three alpha helices (as does PrPC) and two disulphide bridges between the 2nd and 3rd helix, (Lu et al., 2000; Silverman et al., 2000). Dpl mRNA is expressed at high levels in testis, less in other peripheral organs and at very low levels in brain of adult wild-type mice. However, significant Pnd mRNA transcripts were detected during embryogenesis and in the brains of newborn mice, arguing for a possible function of Dpl in brain development (Li et al., 2000).

In the brain of ataxic Ngsk but not of healthy Zrch and Edbg Pnp−/− mice, an intergenic splicing event places the Dpl locus under the control of the Pnp promoter, probably due to the deletion of the Pnp intron 2 sequence including its splicing acceptor. This causes overexpression of Dpl in neurons and possibly Purkinje cell degeneration. Taken together, these results show that ectopic Dpl expression rather than functional loss of PrPC may be responsible for neuronal degeneration in ataxic Pnp−/− mice. However, Ngsk Pnp−/− mice were rescued from the

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neuronal degeneration by introduction of a Prnp transgene. This suggests that Dpl toxicity may be dose-dependent and can be antagonized by PrP C (Nishida, 1999). Interestingly, overexpression of an amino-proximally truncated transgene encoding PrP C-devoid of the octa repeats and the conserved 106–126 region (PrPA32–135) into Zürich 1 Prnp ΔKO mice caused ataxia and degeneration of the cerebellar granule cell layer within weeks of birth, and introduction of a single intact PrP C allele prevented the disease (Shmerling et al., 1998). Therefore PrP C and truncated PrP C interact genetically. Because Dpl resembles truncated PrP C (Moore et al., 1999), it might cause disease by the same mechanism.

In this study we have investigated the role of Dpl in neurogenesis and in prion pathogenesis. We induced neural differentiation of embryonic stem (ES) cells carrying a homozygous null mutation of the Prnd locus, but a normal Prnp locus. Dpl-deficient ES cells were found to undergo normal neurogenic differentiation and were capable of giving rise to all neural cell lineages when transplanted into host brains. After inoculation with scrapie prions, Dpl-deficient neural grafts showed spongiosis, gliosis and unimpaired accumulation of PrP SC and infectivity similar to wild-type neuroectodermal grafts. We conclude that Dpl is not required for neural differentiation and that Prnd-deficiency does not prevent prion pathogenesis in neural grafts.

RESULTS AND DISCUSSION

To inactivate Prnd in ES cells, the Prnd genomic locus was cloned and used to generate a targeting construct (termed PrndKO), in which the Dpl open reading frame (ORF) was replaced with a neomycin resistance gene flanked by loxP sites. A diphtheria toxin α (DTα) chain gene was added for selection against random integrants (Figure 1A). After electroporation of PrndKO into E14.1 ES cells and G418 selection, 1/382 ES cell clones had undergone homologous recombination at the Prnd locus as assessed by PCR and Southern analysis, yielding the Prndneo allele (Figure 1C and D). In order to generate ES cell clones deficient for Dpl production, the second Prnd allele was inactivated in Prndneo heterozygous cells by selection with high concentrations of G418 (Figure 1B). At 4 mg/ml G418, 22 ES cell clones out of 106 cells plated survived selection, two of which (clone PrndH1B and PrndH1Bb) were homozygous for Prndneo (Figure 1C and D). Undifferentiated Prndneo/neo ES cell clones were similar in morphology to Prndneo heterozygous and wild-type control cells (data not shown).

To investigate the role of Dpl in neural development, Prndneo/neo, PrndΔneo and wild-type ES cells were differentiated into neuroectoderm in vitro following a procedure that was established previously (Benninger et al., 2000) and transplanted into the brains of adult mice PrndΔneo ES cell clones that had also undergone high G418 selection were used as controls to ensure that gene targeting, high G418 selection and prolonged in vitro passaging had no adverse effect on neural differentiation efficiency. Twenty-nine days after transplantation, grafts deficient for Prnd (PrndΔneo/neo and control grafts (PrndΔneo/Δneo) consisted of neural tissue with neurons, astrocytes and oligodendrocytes as identified by immunostaining for MAP2, NeuN, synaptophysin, GAP43, GFAP and S-100 protein (Figure 2 and data not shown). These results indicate that Dpl is not required for the differentiation of ES cells into neural cells, and argue against a cell-autonomous function for Dpl during neurogenesis. To detect a possible influence of the genotype on the development of glial or neuronal cells, we have assessed the cellular density and the contribution of astrocytes, oligodendrocytes and neurons to the graft tissue and found no difference between PrndΔneo/neo and PrndΔneo/Δneo control grafts (Figure 3).

Next, we investigated whether Dpl is required for the development of spongiform pathology and for accumulation of PrP SC and infectivity. Prnd-deficient and control ES cells were differentiated in vitro and transplanted into the caudoputamen of Prnp ΔKO mice. Inoculation with RML prions was carried out 35–45 days after transplantation, and mice were kept for up to 211 days after
Function of Doppel in prion disease

Inoculation. In previous experiments we had shown that this time period is sufficient to allow the formation of PrPSc in grafts (Brandner et al., 1998).

One Prmdneo/neo and two Prmd+/neo mice were killed 52 days after inoculation and were analyzed for the presence of disease-associated PrPSc. In these grafts, we found very small amounts of proteinase K-resistant prion protein by histoblotting, as was expected from the short incubation time and infectivity, as determined by transmission into tga20 indicator mice. Instead, prolonged incubation for 195–203 days in two additional Prmdneo/neo and two Prmd+/neo mice led to significant accumulation of PrPSc in all transplants irrespective of their genotype (Figure 4), and transmission into indicator mice confirmed the presence of infectivity (Table I). Analysis of paraffin sections of long-term infected grafts confirmed typical histopathological hallmarks of scrapie, i.e. vacuolation and astrogliosis (Figure 2M and N) that were strictly confined to the grafts as described earlier (Brandner et al., 1996a, 1998). Therefore, the absence of Prmd in neural tissue does not affect disease progression of experimental prion pathogenesis, at least in the neurografting paradigm.

In this study we have investigated the role of the PrPc homologue Dpl in neural differentiation and prion pathogenesis. Both Prnp and Prnd are expressed during embryogenesis and in the brains of newborn mice, suggesting a role for these proteins in neurogenesis. However, neuronal development proceeds normally in mice lacking Prnp. Likewise, in the absence of Dpl, the differentiation of ES cells into neurons and astrocytes is not impaired. Several similarities between both proteins may indicate similar biological functions. The subcellular localization of PrPc and Dpl is similar; both are GPI-anchored membrane proteins. In addition, the primary amino acid sequence is 23% identical between both proteins, and the structure of Dpl is predicted to share significant homology with PrPc (Mo et al., 2001). Thus a possible role of PrPc and Dpl during neuronal development may

Fig. 2. Histological characterization of non-infected and Scrapie-infected Prmdneo/neo neuroectodermal grafts. Histological analysis of non-infected Prmd+/+ (A–D), Prmdneo/neo (E–H) grafts and Prmd+/neo grafts (I–L). Intraventricularly or intraparenchymally placed ES cell-derived grafts of all genotypes showed no difference in their differentiation pattern, as they contained astrocytes (GFAP immunostain, B, F, J) and showed regular immunoreactivity for synaptophysin (C, G, K) and MAP-2 (D, H, L). Inoculation with mouse prions (185 days) resulted in typical histopathological hallmarks of scrapie, such as vacuolation (M) and astrogliosis (N) but not yet significant loss of neuropil, as indicated by normal staining pattern for synaptophysin and MAP-2. Scale bar: 500 µm (A, E, I, M) and 200 µm (all other panels).
be masked by functional redundancy. To address this question it will be necessary to generate ES cells and mice lacking Prnp as well as Prnd and to study whether the lack of both PrP\(^{C}\) and Dpl will result in impaired neurogenesis.

The ability of Dpl-deficient ES cells to give rise to normal neural grafts enabled us to investigate a potential role of Dpl in prion disease. The typical histological features of scrapie were observed, including spongiosis and gliosis. Also, typical PrP\(^{Sc}\) depositions developed in neuroectodermal grafts devoid of Prnd. The Dpl protein resembles an N-terminally truncated PrP\(^{C}\) protein lacking the octamer repeats, a version of PrP\(^{C}\) that is capable of PrP\(^{Sc}\) propagation (Flechsig et al., 2000). Prnd transcription was not detected in adult neurons, although Prnd mRNA was reported to be present in endothelial cells of the brain (Li et al., 2000). Likewise, we have not detected Dpl protein in the brains of terminally ill scrapie mice (data not shown).

Recently, two groups have searched for possible linkage disequilibrium of Prnd alleles in human prion diseases. Four polymorphisms in Prnd were detected, but no strong association was found between any of these polymorphisms and human prion diseases (Mead et al., 2000; Peoc'h et al., 2000). Taken together, these findings further argue against an important function of Dpl in neurons during prion disease. On the other hand, the experimental approach described here does not rule

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Fig. 3. Quantitation of cell populations in Prnd\(^{+/+}\), Prnd\(^{+/neo}\) and Prnd\(^{neo/neo}\) grafts. Neurons, astrocytes and oligodendrocytes were counted on an area of 400 \(\times\) 400 \(\mu\m\) (as described in Benninger, 2000). There was no apparent difference in distribution, ratio and number of these cell types in grafts of either genotype.

52 days

<table>
<thead>
<tr>
<th>Graft Prnd(^{neo/neo})</th>
<th>Graft Prnd(^{+/neo})</th>
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<tr>
<td>A</td>
<td>B</td>
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195-203 days

<table>
<thead>
<tr>
<th>Graft Prnd(^{neo/neo})</th>
<th>Graft Prnd(^{+/neo})</th>
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<td>C</td>
<td>D</td>
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Fig. 4. Detection of disease-associated PrP\(^{C}\) in intraparenchymal grafts derived from Prnd\(^{neo/neo}\) and Prnd\(^{neo/neo}\) ES cells. (A–D) Frozen sections with intraparenchymally located grafts (outlined with asterisks). Histoblots of these grafts without (E–H) and with (I–L) proteinase K treatment. After short incubation time (52 days), minute amounts of proteinase resistant PrP were detectable in both, Prnd\(^{neo/neo}\) and Prnd\(^{+/neo}\) grafts, while prolonged incubation time (195–203 days) led to strong accumulation of proteinase resistant PrP\(^{C}\). The central irregular shaped region in the Prnd\(^{neo/neo}\) graft (H, L) results from regressive changes and calcifications which are occasionally observed in ES cell-derived neuroectodermal grafts of all genotypes.
Function of Doppel in prion disease

Table I. Determination of prion infectivity by transmission of graft tissue into tg20 indicator mice

<table>
<thead>
<tr>
<th>Genotype of graft</th>
<th>Prion inoculum</th>
<th>incubation time (days)</th>
<th>Latency of scrapie in tg20 indicator mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prnd\textsuperscript{neo/neo}</td>
<td>$3 \times 10^6$ LD\textsubscript{50}</td>
<td>195</td>
<td>63\textsuperscript{a}, 68\textsuperscript{a}, 78\textsuperscript{a}, 86\textsuperscript{a}</td>
</tr>
<tr>
<td>Prnd\textsuperscript{+/neo}</td>
<td>$3 \times 10^6$ LD\textsubscript{50}</td>
<td>203</td>
<td>65\textsuperscript{b}, 65\textsuperscript{c}, 67\textsuperscript{b}, 68\textsuperscript{c}</td>
</tr>
<tr>
<td>Prnd\textsuperscript{+/neo}</td>
<td>$3 \times 10^6$ LD\textsubscript{50}</td>
<td>52</td>
<td>86\textsuperscript{b}, 86\textsuperscript{b}, 86\textsuperscript{b}, 86\textsuperscript{b}</td>
</tr>
<tr>
<td>Prnd\textsuperscript{+/neo}</td>
<td>$3 \times 10^6$ LD\textsubscript{50}</td>
<td>52</td>
<td>81\textsuperscript{b}, 84\textsuperscript{b}, 86\textsuperscript{b}</td>
</tr>
<tr>
<td>Prnd\textsuperscript{+/neo}</td>
<td>$3 \times 10^6$ LD\textsubscript{50}</td>
<td>52</td>
<td>74\textsuperscript{b}, 86\textsuperscript{b}, 86\textsuperscript{b}, 86\textsuperscript{b}</td>
</tr>
<tr>
<td>Prnd\textsuperscript{+/neo}</td>
<td>mock</td>
<td>188</td>
<td>&gt;86\textsuperscript{b}, &gt;86\textsuperscript{b}, &gt;86\textsuperscript{b}, &gt;86\textsuperscript{b}</td>
</tr>
<tr>
<td>Prnd\textsuperscript{+/neo}</td>
<td>mock</td>
<td>201</td>
<td>&gt;86\textsuperscript{b}, &gt;86\textsuperscript{b}, &gt;86\textsuperscript{b}</td>
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Long-term infection of Prnd\textsuperscript{+/-/neo} and Prnd\textsuperscript{neo/neo} grafts (195–203 days) leads to accumulation of high titres of infectivity, as inferred from short incubation times of indicator mice. Short-term infection of Prnd\textsuperscript{+/-/neo} and Prnd\textsuperscript{neo/neo} grafts (52 days) led also to accumulation of infectivity, but at lower levels than in long-term infected grafts, resulting in longer incubation times of indicator mice. Transmission of mock-infected graft tissue (188–201 days after mock-infection) did not result in scrapie.

\textsuperscript{1}Indicator mouse with terminal scrapie disease.

\textsuperscript{2}Indicator mouse with incipient scrapie disease at the time of writing.

\textsuperscript{3}Indicator mouse with no sign of scrapie disease.

out a role for Dpl in peripheral prion pathogenesis and PrP\textsubscript{Sc} transport to the brain. The latter possibility is intriguing, because Prnd is expressed in the spleen, a major peripheral reservoir of PrP\textsubscript{Sc} and prion infectivity (Li et al., 2000). Mice lacking Prnd derived from the ES cells described here will doubtlessly help in addressing this question.

METHODS

Cells and tissue culture conditions. E14.1 ES cells (derived from 129 Ola/Ola mice) were cultured on γ-irradiated (30 Gy) mouse embryo fibroblast feeder layers. Culture medium was Glasgow minimal essential medium (Gibco 21710-025) supplemented with non-essential amino acids (Gibco 11140-025), 1 mM pyruvate (Gibco 11160-070), 0.1 mM β-mercaptoethanol (Sigma), 15% fetal calf serum (FCS; Sigma) and leukemia inhibitory factor (LIF 10\textsuperscript{3} U/ml; Gibco 13275).

Induction of differentiation. Subconfluent ES cell cultures from 75 cm\textsuperscript{2} culture area were trypsinized, spun and transferred into Petri dishes with 12 ml differentiation medium, where they spontaneously formed embryoid bodies (EB) which did not attach to the dishes. The medium-free medium formulation Neurobasal (Gibco 21710-025) supplemented with B27 (Gibco 17504) and G5 supplement (Gibco 17503) containing: insulin, transferrin, selenite, biotin, hydrocortisone, basic fibroblast growth factor epidermal growth factor and glutamine (73.5 mg/l). Medium was replaced on days 1, 2, 3, 4, 6 and 8 by entirely removing EB from Petri dishes and transferring them into a 15 ml Falcon tube where they sedimented by gravity. Transplantation was carried out after 8 days of in vitro differentiation.

Generation of Dpl-deficient ES cells. The genomic Prnd locus was cloned from genomic DNA of E14.1 ES cells by PCR. In the targeting vector, the Prnd ORF was replaced with a floxed neomycin resistance selection cassette. A DTrα gene was inserted for selection against random integrants.

Subconfluent ES cells (20 cm\textsuperscript{2}) were trypsinized, spun and resuspended in 200 μl PBS containing linearized targeting vector (20–40 μg). Electroporation conditions were 0.8 kV, 3 μF, in 0.4 cm Bio-Rad electroporation cuvettes with Bio-Rad GenePulser. Cells were then transferred to a gelatin-coated tissue culture dish with culture medium containing LIF. G418 was added after 24 h at 0.3 mg/ml. After 8 days, surviving colonies were transferred to 96-well plates and expanded. Southern blot analysis was carried out to confirm homologous recombination of the construct into the Prnd locus. Genomic DNA of ES cells was digested with Apal and an external probe radioactively labeled with \textsuperscript{12}P was used to confirm integration by homologous recombination. The frequency of homologous recombination was 1/384 clones. High G418 selection of the Prnd\textsuperscript{neo/neo} ES cell clone was performed with 4.0 mg/ml G418.

Animals and transplantation procedure. Adult male or female Prnp\textsuperscript{0/0} mice (genetic background C57BL/6 × 129 SV) were used as graft recipients. For transplantation, mice were anaesthetized according to published protocols and placed in a stereotaxic frame (Narishige SR 6N). The coronal suture was identified after a longitudinal incision of the scalp. The syringe containing the EB was positioned at 2.5 mm paramedian and then advanced 3 mm through the coronal suture into the caudoputamen region and EB were injected (Coordinates in relation to bregma: anterior 0 mm, lateral 2 mm, ventral 2.5 mm). Injection of embryoid bodies was done as follows: a 10-μl Hamilton syringe (model 701RN) with electropolished 26G pointstyle needle was back-filled with intact EB. After insertion of the plunger, 0.5 ml EB suspension was injected. Passage of the EB through the narrow needle resulted in dispersion upon injection into the brain. Twenty-three mice received Prnd\textsuperscript{+/-/neo} and 16 mice received Prnd\textsuperscript{neo/neo} grafts as a control.

Preparation of the infectious inoculum and inoculation procedure. Inoculation with mouse prions derived from the Chandler strain was performed according to published protocols (Brandner et al., 1996a). The RML5 (Rocky Mountains Laboratory) inoculum was prepared from brains of terminally ill CD-1 mice.

We inoculated 17 grafted Prnp\textsuperscript{+/-/0} mice intracerebrally with 30 μl of the scrapie prion inoculum 6 weeks after the grafting procedure. For control experiments, four grafted mice did not receive any inoculum. For determination of infectivity in grafts,
we collected six frozen sections (10 µm thickness) at the level where sections for histoblots were obtained, and processed them as described earlier (Brandner et al., 1996a), and inoculated them into groups of four Ipa20 mice intracerebrally.

**Histological analysis.** Histological analysis of mature grafts was carried out 29–25 days after transplantation of embryoid bodies. For analysis of growth and differentiation, grafts were analyzed between 29 and 45 days after transplantation, while analysis of scrapie- and mock-infected grafts was carried out up to 211 days after inoculation.

For routine sections, brains were removed, immersion-fixed in 4% buffered paraformaldehyde for at least 4 h, cut in coronal slices of ∼2 mm thickness and dehydrated through graded alcohols. After paraaffin embedding, sections of 3 µm nominal thickness were cut and mounted on coated slides (Super Frost). Hematoxylin & Eosin (H&E), and Luxol-H&E stains as well as immunocytochemical stains were performed. Immunostains were carried out as described (Benninger et al., 2000).

**Histoblots.** Histoblots were carried out according to published protocols (Taraboulos et al., 1992; Brandner et al., 1996b). Frozen sections of 8 µm thickness were mounted on uncoated glass slides, immediately pressed on a nitrocellulose membrane wetted in lysis buffer and air-dried for at least 24 h. For detection, they were rehydrated in Tris buffered saline, and limited proteolysis was performed using proteinase K concentrations of 20, 50 and 100 µg/ml at 37°C for 4 h. The sections were then denatured in 3 M guanidinium thiocyanate for 10 min and blocked for 1 h in 5% non-fat milk serum. The primary antibody (anti-PrP, 6H4, monoclonal, Prionics Zürich) was used at a dilution of 1:5000 in 1% non-fat milk overnight at 4°C. Visualization was achieved with 1% non-fat milk serum. The primary antibody (anti-PrP, 6H4, monoclonal, Prionics Zürich) was used at a dilution of 1:2000. Visualization was achieved with 1% non-fat milk overnight at 4°C. For detection, they were rehydrated in Tris buffered saline, and limited proteolysis was performed using proteinase K concentrations of 20, 50 and 100 µg/ml at 37°C for 4 h. The sections were then denatured in 3 M guanidinium thiocyanate for 10 min and blocked for 1 h in 5% non-fat milk serum. The primary antibody (anti-PrP, 6H4, monoclonal, Prionics Zürich) was used at a dilution of 1:5000 in 1% non-fat milk overnight at 4°C. Detection was accomplished with an alkaline phosphatase-conjugated goat anti-mouse antibody at a dilution of 1:2000. Visualization was achieved with nitroblue tetrazolium using 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride (Boehringer, Mannheim) according to the manufacturer’s protocols.

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