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# Prion proteins and infertility: insight from mouse models

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**Abstract.** A wealth of evidence points to an abnormal form of the prion protein called PrP<sup>Sc</sup> as the transmissible agent responsible for prion diseases. However, the physiological function of its normal conformer, the cellular prion protein (PrP<sup>C</sup>), is still unknown. Recently, a homologue of PrP<sup>C</sup> was discovered and denoted Doppel (Dpl). In contrast to PrP, mice deficient

for Dpl suffer from an important pathological phenotype: male sterility. This phenotype shifts the attention from the brain, where most of the investigations on Dpl have been performed, to testis, raising the hope to resolve the long lasting search of PrP<sup>C</sup> function.

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## Prion diseases

Prion diseases are lethal encephalopathies affecting many animal species. Although prion diseases may present with certain morphological and pathophysiological parallels to other progressive encephalopathies, such as Alzheimer's and Parkinson's disease, they are unique in that they are transmissible. In the last decade, the public interest in prions has risen because strong circumstantial evidence (Aguzzi, 1996; Aguzzi and Weissmann, 1996; Collinge et al., 1996; Bruce et al., 1997; Hill et al., 1997) suggests that bovine spongiform encephalopathy (BSE) is the cause of variant Creutzfeldt-Jakob disease (vCJD).

The nature of the infectious agent causing transmissible spongiform encephalopathies (TSEs) is still not completely clear, but may consist essentially of PrP<sup>Sc</sup>, an abnormally folded, protease-resistant, beta-sheet rich isoform of a normal

cellular protein termed PrP<sup>C</sup> (Prusiner, 1982). Conversion of PrP<sup>C</sup>, which is expressed abundantly in the central nervous system, to PrP<sup>Sc</sup>, is a central event in prion pathogenesis (Prusiner, 1991). However, the molecular mechanisms of conversion remain unclear. PrP<sup>C</sup> is essential for the development of prion disease, since its ablation confers resistance to scrapie (Büeler et al., 1993).

After peripheral inoculation, prions colonize the lymphoreticular system where replication of PrP<sup>Sc</sup> is abundant. Functional follicular dendritic cells are required for prion replication (Montrasio et al., 2000). In the progress of the disease, prions find access to the central nervous system probably through elements of the autonomic nervous system (Glatzel et al., 2001). After reaching the brain, prions cause neuronal loss, astrocytes activation and vacuolation, which are hallmarks of TSEs. However, the mechanisms leading to damage in the brain remain largely unknown.

## The discovery of Doppel and its implication in prion disease

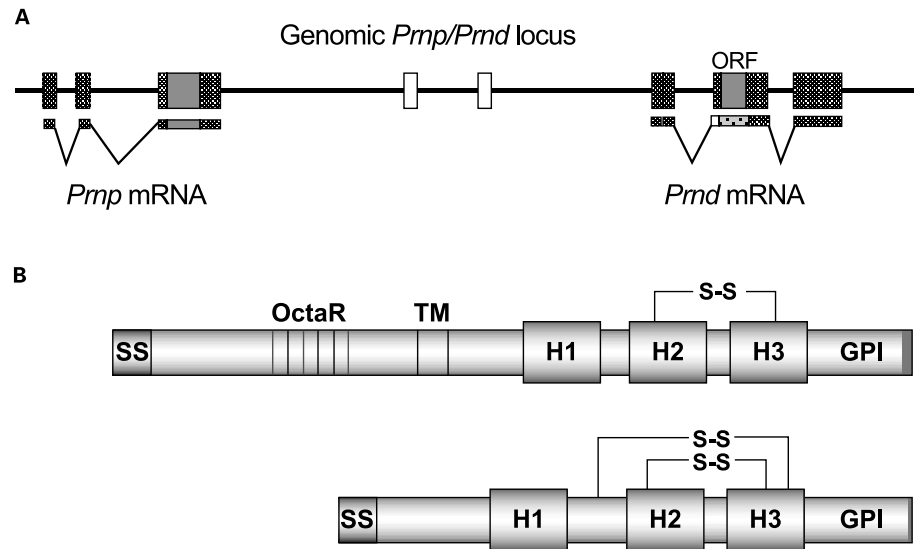
The recent discovery of Dpl created a stir in the prion field. Dpl and PrP<sup>C</sup>, while sharing only 25% identity in primary sequence, have remarkable similarities in the three dimensional structures (Luhrs et al., 2003). Dpl resembles N-terminally truncated PrP: it is GPI anchored, and it has three  $\alpha$ -helices. However, it has two (rather than one) disulphide bridges between the second and the third helices (Fig. 1). The *Prnd* gene, which encodes Dpl, is evolutionarily conserved, suggesting an essential biological function (Lu et al., 2000; Silverman et al., 2000; Mo et al., 2001).

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**Fig. 1. (a)** Genomic *Prnp/Prnd* locus. Genomic exons are represented by rectangles, open reading frames (ORF) by black rectangles and introns by lines. mRNA transcripts generated from the loci are also shown. **(b)** Structural comparison of PrP<sup>C</sup> and Doppel. S-S = disulphide bridges; TM = transmembrane domain; OctaR = octameric repeats. H1, H2 and H3 rectangles represent alpha-helix 1, 2 and 3 respectively.

If Dpl is structurally similar to PrP<sup>C</sup>, is it involved in prion pathogenesis and can it be converted into a Dpl<sup>Sc</sup> form? We addressed this question with a neural grafting paradigm. Neural stem cells homozygous for the disruption of the *Prnd* locus were grafted in the brains of *Prnp* knockout mice. The *Prnd* null grafts were found to undergo normal neural differentiation, ruling out an important function of Dpl during brain development. After inoculation with scrapie, grafts lacking *Prnd* showed unimpaired prion pathogenesis and an infectivity level similar to wild-type grafts (Behrens et al., 2001). Similarly, overexpression of Dpl in the brain of transgenic mice does not modulate prion pathogenesis (Tuzi et al., 2002). Several groups have searched for possible linkage disequilibria of *Prnd* alleles in human prion disease but no polymorphisms were found to be associated with them (Mead et al., 2000; Peoc'h et al., 2000). Interestingly, a relation between a cluster of familial CJD mutations and the structure of Dpl has been described. These F-CJD alleles may cause mutant PrP to become a weak mimetic of Dpl structure or function (Mastrangelo et al., 2002).

Dpl resembles an N-terminally truncated PrP<sup>C</sup> protein lacking the octamer repeats (Fig. 1b). The latter version of PrP<sup>C</sup> is actually capable of supporting PrP<sup>Sc</sup> propagation, suggesting that Dpl protein could be susceptible to conversion into "Dpl<sup>Sc</sup>" (Flechsig et al., 2000). However, presently no evidence suggests any conversion of Dpl into a misfolded beta-strand-rich, protease-resistant conformation and any implication in prion pathogenesis.

#### The physiological function of PrP<sup>C</sup>

The physiological role of PrP<sup>C</sup> is still unclear. So far, the functions attributed to PrP<sup>C</sup> are disparate and quite confusing: they include a membrane receptor, an effector in signal transduction, a regulator of apoptosis, a copper binding protein, and a regulator of synaptic transmission, among others.

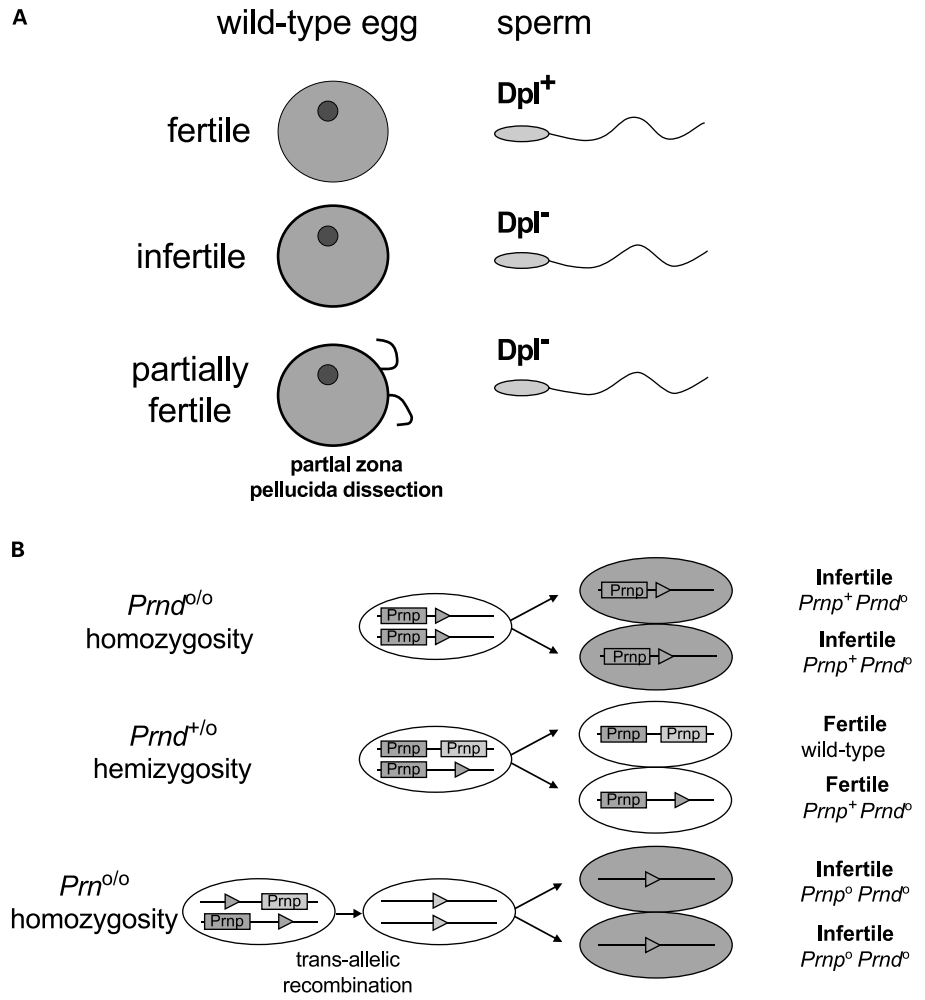
Postnatal ablation of PrP does not result in neurodegeneration (Mallucci et al., 2002); hence prion pathology is unlikely to be caused by simple loss of PrP<sup>C</sup>. However, the unknown func-

tion of PrP<sup>C</sup>, upon conversion to PrP<sup>Sc</sup>, could be altered or rendered unspecific and this may be one cause of neurodegeneration. Therefore, elucidation of the physiological function of PrP is of highest importance and may be crucial for understanding prion diseases (Aguzzi and Heikenwalder, 2003).

To achieve this goal, several efforts have been dedicated to the identification of prion binding proteins. In fact, many candidates have been found, but none of them has revealed any *in vivo* function in which PrP<sup>C</sup> would be involved. Also, the PrP binding partners are disparate and, as a whole, rather confusing. For example, PrP was reported to bind to transmembrane proteins (N-CAM, laminin receptor, dystroglycan complex) (Keshet et al., 2000; Gauczynski et al., 2001; Schmitt-Ulms et al., 2001) and to apoptotic molecules (Bax, Bcl-2) (Kurschner and Morgan, 1995; Bounhar et al., 2001). None of these binding partners have been shown to be implicated in prion pathogenesis. Despite considerable efforts aimed at connecting PrP<sup>C</sup> with partners with known functions, a clear-cut understanding of PrP<sup>C</sup> function is still lacking.

#### PrP<sup>C</sup> and testis

Research on PrP<sup>C</sup> has been largely focused to the brain, where PrP<sup>C</sup> is expressed at high levels. However, PrP<sup>C</sup> is expressed in many peripheral tissues, and investigation of its role in specific peripheral tissues might help in assign it a specific function. In mouse testis, PrP mRNA is detected in Sertoli cells, primary and secondary spermatocytes, and spermatids in the seminiferous tubules. Protein expression is highest in the cytoplasm of the head of maturing spermatozoa, in the ductus deferens and epididymis, and in the cytoplasmic droplet excluded from the spermatozoan head during maturation (Ford et al., 2002). PrP<sup>C</sup> is present in mature sperms and, in contrast to processing in other cells, PrP<sup>C</sup> is truncated in sperm cells in its carboxy terminus and lack a glycosyl phosphatidyl inositol (GPI) anchor. Therefore, during sperm maturation, PrP<sup>C</sup> changes from its regular GPI anchored form to a carboxy terminally truncated peptide no longer associated with membrane



**Fig. 2.** A possible model for the function of Dpl in fertilization. **(a)** Male sterility in mice that lack Doppel may be due to the incapability of Doppel deficient sperm to fertilize eggs. The process that appears to be disturbed relates to the acrosomal reaction and to the penetration of the zona pellucida. In fact, mechanical dissection of the zona pellucida restores, at least in part, fertility. **(b)** Interestingly, haploid spermatozoa lacking the Doppel gene ( $Prnd^o$ ) are perfectly fertile when generated in the context of a heterozygous  $Prnd^{+/o}$  mouse. Instead,  $Prnd^o$  sperm are infertile when generated in a  $Prnd^{o/o}$  mouse.  $Prn^o$  sperm, which lack PrPC and Dpl, are also infertile.

rafts. However, mutant mice lacking PrP are fertile and give birth with a normal litter size. PrPC was also reported to protect sperm cells during specific stress situations (Shaked et al., 1999). This would be consistent with the fact that the absence of PrPC in PrP deficient mice does not cause any major apparent damage and does not result in male sterility (Büeler et al., 1992).

*Physiological function of Dpl in testes*

Dpl is expressed strongly in testis and heart, at lower levels in spleen and skeletal muscle and is absent in brain of wild-type mice. However, significant levels of *Prnd* mRNA have been detected during embryogenesis and in the brain of newborn mice (Li et al., 2000).

In mouse testis, Dpl expression is detected in stage specific manner in spermatids (Behrens et al., 2002). In humans, Dpl is present in Sertoli cells, on the flagella of spermatozoa and seminal plasma (Peoc'h et al., 2002).

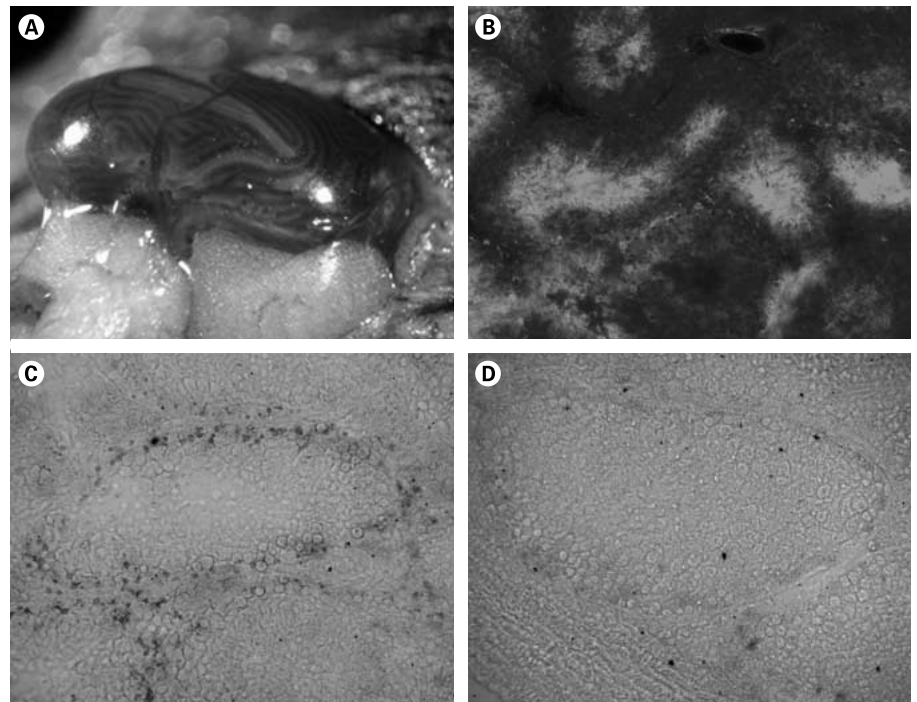
To investigate the physiological function of Dpl, we generated mutant mice lacking Dpl. The mutants develop normally and have a normal behavior, yet knockout males are infertile. The overall histological appearance of Dpl deficient testes was normal: we found normal distribution and counts of spermato-

gonia, spermatocytes and Sertoli cells. However, mature spermatozoa from mutant mice were reduced in numbers, less mobile, mostly malformed and unable to fertilize oocytes in vitro. Many  $Prnd^{neo/neo}$  sperm head were lacking a discernible acrosome. Only after partial disruption of the zona pellucida was *in vitro* fertilization of  $Prnd^{neo/neo}$  partially restored, therefore implicating acrosome malformation as a crucial reason for sterility (Fig. 2) (Behrens et al., 2002).

The molecular mechanism of Dpl action on the acrosome is unclear. Dpl may be present at the acrosome surface through its GPI anchor and may be directly involved in sperm-egg interaction. If this appears to be true, anti-Dpl antibodies might block *in vitro* fertilization of wild-type sperm. Alternatively, Dpl might be required for normal morphogenesis of acrosome during development or to trigger acrosomal reaction. Sperm malformation could result from a failure in acrosome biogenesis. A better characterization of Dpl function is required and identification of Dpl partners could help; in contrast to PrP, so far none have been identified for Dpl.

*Do PrP and Dpl accomplish redundant functions?*

The similarities between PrPC and Dpl in primary amino acid sequence, structure and subcellular localization suggest



**Fig. 3.** Injection of lentivirus into the testis. **(a)** Lentivirus was injected into the right testis. Trypan blue was added to the solution in order to visualize the injected material. Most seminiferous tubules can be reached by this technique, as indicated by the translucent blue stain. **(b)** Under fluorescence, trypan blue (which emits red light) can be detected within the seminiferous tubuli of testicular frozen sections. **(c-d)**  $\beta$ -Galactosidase staining on mouse testis cryosections injected with a lentiviral vector that encodes *lacZ* **(c)** and mock-inoculated control testis **(d)**. Both mice were sacrificed one month after injection.

related biological functions (Mo et al., 2001; Behrens and Aguzzi, 2002). Therefore, a possible role of PrPC and Dpl during development may be masked by functional redundancy. The presence of Dpl could also mask a minor function of PrPC in testis or more strongly affect spermatogenesis.

Therefore, we generated *Prn<sup>0/0</sup>* mutant mice lacking both PrPC and Dpl. We took advantage of trans-allelic meiotic recombination (Herault et al., 1998), showing that this strategy is not restricted to specific loci and can be widely used as a powerful genetic tool for complex genomic applications.

Surprisingly, the double mutant *Prn<sup>0/0</sup>* mice do not show any obvious abnormalities apart from male infertility, and morphological analyses of sperm do not provide evidence for a difference in phenotype compared to the single mutant *Prnd<sup>neo/neo</sup>* mice. It appears therefore that disruption of PrP in Dpl deficient mice does not induce any obvious histological abnormalities, and fails to accentuate the sperm phenotype. Thus, developmental expression of Dpl does not compensate and suppress any pathological brain phenotype that might have been brought about by the absence of PrPC. We conclude that the mild phenotypes that are described in mice lacking PrPC are unlikely due to functional compensation by Dpl.

The lack of redundancy is surprising in the light of the high structural similarity between PrPC and Dpl (Fig. 1b) and the evolutionary conservation of both proteins. Therefore, it is conceivable that there are additional as yet unidentified proteins belonging to the PrPC/Dpl protein family that have retained a structure similar to PrPC and Dpl.

#### *Reverse genetics using lentiviral vectors*

Significant progress has been made recently in the realm of *in vivo* gene delivery to the testis. The newly developed testis

cell transplantation (Brinster, 2002) and viral-vector mediated gene transfer provide powerful tools to examine spermatogenic defects, to generate transgenic animals, and even to correct male infertility. Recently, restoration of spermatogenesis by lentiviral gene transfer has been achieved in infertile mice (Ikawa et al., 2002). We are using a similar approach in our laboratory. Testes transduced with a replication-defective lentiviral vector encoding *lacZ* were positive for  $\beta$ -gal staining (Fig. 3). We intend to take advantage of this technique to study the reverse genetics of Dpl. We are now transducing testes from *Prnd<sup>neo/neo</sup>* or *Prn<sup>0/0</sup>* mice with lentiviruses encoding full-length Dpl or different truncated versions of Dpl. Subsequently, we are studying the possible restoration of fertility by mating transduced males, and/or by performing *in vitro* fertilization. This technique has an excellent potential for yielding crucial information about the function of Dpl while obviating for the expensive and time-consuming need to generate new transgenic mouse lines.

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