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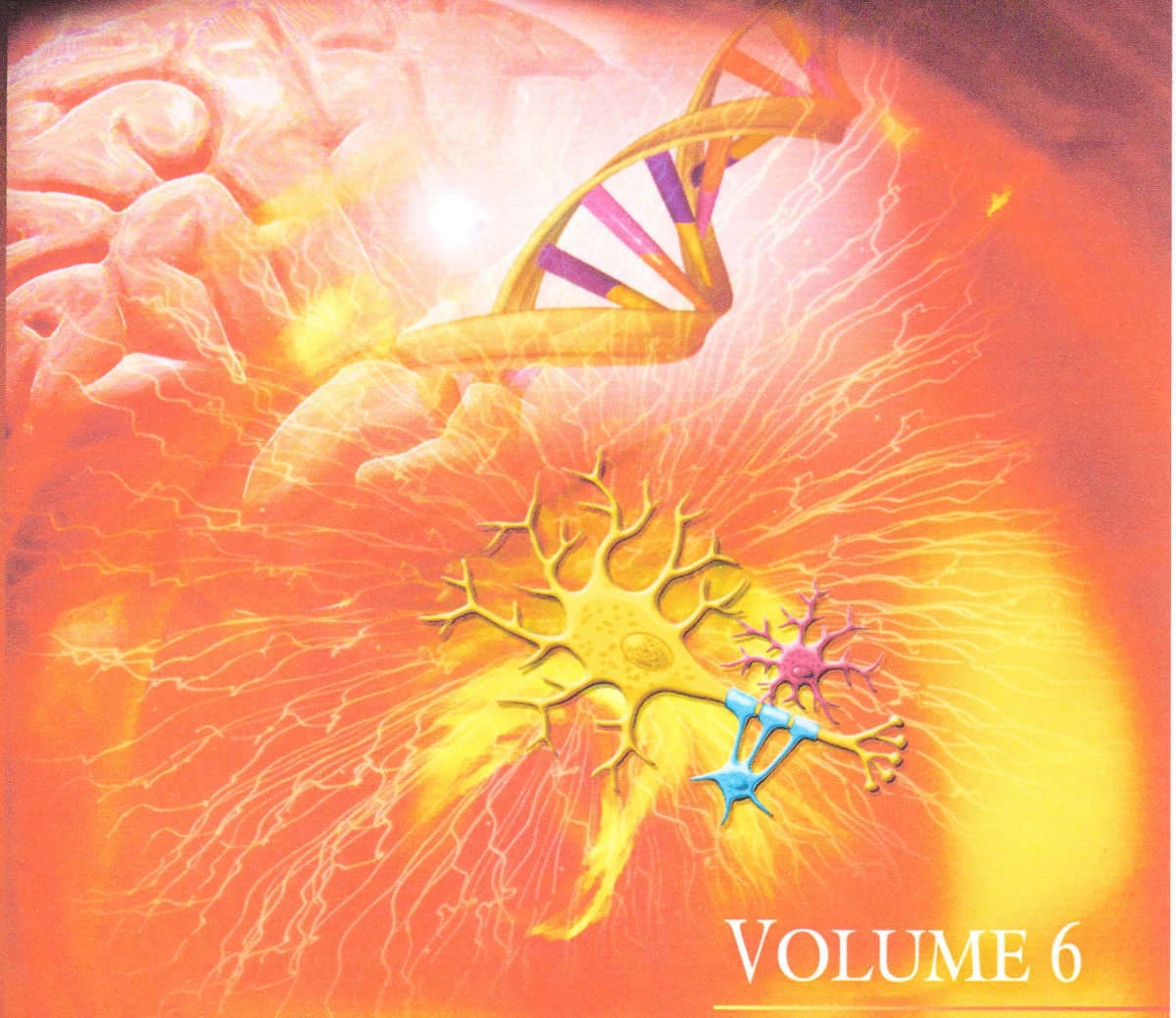

VOLUME 6

HORIZONS IN NEUROSCIENCE RESEARCH

NOVA

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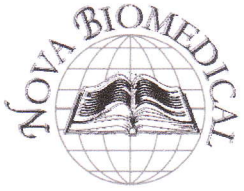

Nova Science Publishers, Inc.
New York

Library of Congress Cataloging-in-Publication Data

ISBN 978-1-62100-080-8 (eBook)
ISSN 2159-113X

Published by Nova Science Publishers, Inc. † New York

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In: Horizons in Neuroscience Research. Volume 6
Editors: A. Costa and E. Villalba
ISBN 978-1-62100-063-1

Chapter 12

THE PRION GENE COMPLEX: FROM PRION DISEASES TO MALE FERTILITY

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ABSTRACT

Prions are infectious pathogens that cause a group of invariably fatal neurodegenerative diseases in both humans and animals. A hallmark of prion diseases or transmissible spongiform encefalopaties (TSE) is the conversion of the cellular prion protein (PrP^{C}), expressed by the prion protein gene (PRNP), into an abnormally folded isoform (PrP^{Sc}), that cause the bovine spongiform encephalopathy and scrapie in sheep. In humans, there are presently five known subtypes: Kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, fatal insomnia and new variant CJD (vCJD). Spontaneous cerebellar neurodegeneration and ataxia syndromes in certain strains of $PRNP^{-/-}$ mice led to the discovery of a novel gene, PRND, which encodes a prion-like protein designated as Doppel. PRND gene is located 16–52 kb (depending on the species) downstream from PRNP. PRND contributes together with PRNP, with the recently discovered PRNT (that encodes the prion protein testis specific - Prt) and with SPRN (shadow of prion protein gene that encodes Shadoo) genes, to the so called ''prion gene complex". The normal physiological function of PrP^{C} remains largely unclear. Although not involved in the etiology of the TSE, Doppel as well as Prt are highly expressed in the testicular tissue, which allied to the sterility of $PRND^{-/-}$ mice suggest an important physiological role on male fertility. More recently, some reports proposed the

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quantification of Doppel as a predictor of tumor malignancy. The biological role and the potential biomedical applications for prion proteins will be further discussed.

1. INTRODUCTION

Prions are the major contributing agents of transmissible spongiform encephalopathies (TSEs) in mammals. Research regarding the identification of the infectious nature of prion diseases and the discovery of the chemical nature of this type of infectious agent have been recognized by two Nobel Prizes in Physiology or Medicine: one in 1976 to Daniel Carleton Gajdusek (for his work on kuru, the first human prion disease demonstrated to be infectious) and the other in 1997 to Stanley B. Prusiner. A hallmark of prion diseases - whether sporadic, dominantly inherited, or acquired by infection - is the conversion of the cellular prion protein (PrP^{C}), expressed by the prion protein gene (PRNP), into an abnormally folded isoform, designated as PrP^{Sc} (prion protein associated with scrapie), which is the major component of infectious prions (Prusiner et al., 1998). This transconformation is produced by an autocatalytic process induced by PrP^{Sc} itself and is the mechanism responsible for TSEs, such as bovine spongiform encephalopathy (BSE), scrapie in sheep, and the human equivalent, CJD. Infection is mediated by passage of PrP^{Sc} (devoid of nucleic acid) from host to host (the protein-only hypothesis), provided they contain a functional PRNP gene (Prusiner 1998, DeArmond and Bouzamondo, 2002). Due to its neuroinvasiveness, PrP^{Sc} reaches the mammalian brain and aggregates leading to neuronal death (Prinz et al., 2003).

Spontaneous cerebellar neurodegeneration and ataxia syndromes in certain strains of $PRNP^{-/-}$ mice led to the discovery of a novel gene, PRND, which encodes a prion-like protein designated as Doppel. PRND gene is located 16–52 kb (depending on the species) downstream from PRNP (Moore et al., 1999). PRND contributes, together with PRNP and with the recently discovered PRNT (that expresses prion protein testis specific - Prt) and SPRN (shadow of prion protein gene that encodes Shadoo) genes, to the so called "prion gene complex". PRNP, and its homologues, SPRN, PRND and PRNT, show similar gene organizations, which encompass two or three exons (Premzl and Gamulin, 2007). Nonetheless these genes present distinct expression patterns, suggesting different biological functions.

Analysis of both adult and fetal human tissues confirmed the ubiquitous but variable expression profile of PRNP, with the highest levels observed in the central nervous system (CNS) and testis. Contrastingly, although human PRND shows a wide tissue expression pattern in fetal tissues, it is expressed exclusively in adult testis, whereas all three PRNT isoforms were detected only in adult testis, implying that PRND is developmentally regulated (Makrinou et al., 2002). Doppel is expressed on both Sertoli and germ cells in mice, rats, swine (Behrens et al., 2002, Serres et al., 2006), humans (Peoc´h et al., 2002), ovine (Espenes et al., 2006) and bovine (Rondena et al., 2005), which allied to the sterility of $PRND^{-/-}$ mice (Behrens et al., 2002) suggest an important physiological role on male fertility. Our results strength this hypothesis as a possible association between PRND gene polymorphisms and ram semen traits/freezability and embryo production was identified (Baptista et al., 2008, Pereira et al., 2009). Moreover the direct involvement of Doppel protein in enhancing spermatozoa fertilizing ability was determined by Pimenta et al. (in press).

The purpose of this review is to appraise key issues in the genetics and biology of the prion protein family crossing from prion diseases to male fertility. The biological role and the potential biomedical applications for prion proteins will be also discussed.

2. PRION GENE COMPLEX

2.1. Genetic Background, Structural and Biochemical Properties

2.1.1. Genetic Background

2.1.1.1. PRNP

Prions are the product of a single gene that is highly conserved in mammals (Comincini et al., 2001). Mammalian PRNP is a housekeeping gene, present in both eutherians and fish (Premzl and Gamulin, 2007) that has been characterized in several species, like hamster (Li and Bolton, 1997), human, sheep, mouse (Lee et al., 1998) and bovine (Hills et al., 2001). These analyses have identified, as conserved features of eutherian (placental mammals) PRNP promoters, their GC richness and a lack of TATA box. However, there are some differences in gene structure and regulation of gene expression among species. PRNP genes contain three exons, with exons 1 and 2 encoding the 5'UTR region of mRNA in mouse, sheep (Lee et al., 1998), rat (Saeki et al., 1996) and bovine (Hills et al., 2001), but only two exons in human, the first of which encodes the 5´UTR region (Lee et al., 1998). Two or three exons are transcribed alternatively in the mRNA encoded by Syrian hamster PRNP (Li and Bolton, 1997). Complete ORF and 3' UTR region are encoded by the 3' terminal PRNP exon. There is a single known transcription start site in all eutherian PRNPs, except for rodent species (mouse, rat, hamster) which have multiple transcription start sites (Premzl et al., 2005). The PRNP is clearly upregulated in the CNS (Central Nervous System) at both fetal and adult stages, and PrP^C is predominantly localized to synaptic membranes (Herms et al., 1999). Interestingly, PRNP mRNA in adult testis is also elevated to levels almost as high as in the brain (Makrinou et al, 2002). In the reproductive tract of the ram, PrP^C is mainly observed in the epididymis and only very weakly in the testis (Ecroyd et al, 2004).

2.1.1.2. PRND

The first discovered prion protein gene PRNP homologue was Doppel gene (PRND), which lies adjacent to PRNP in the genomic sequence (located 20kb (Makrinou et al., 2002), 16 kb (Moore et al., 1999), 16.8 kb (Comincini et al., 2001), and 52 kb (Essalmani et al., 2002) 3´to PRNP, in humans, mouse, cattle and sheep, respectively). It was proposed that PRND and PRNP arose by an early gene duplication event of an ancestral PRN gene (Moore et al, 1999). For instance, the human, sheep and cattle PRND genes map to the same chromosomal location (respectively 20p12-pter, OA13q17/18 and BTA13q17) as the PRNP gene (Comincini et al., 2001).

PRND gene contains either two (as described in sheep, cattle (Comincini et al., 2001; Tranulis et al., 2001) and goat (Uboldi et al., 2005)) or three (as described in mouse and human (Moore et al. 1999)) exons.

The ruminant genes exhibit a similar structure, with a non-coding 5′ exon longer than that found in human and mouse, and with the entire coding sequence found within a larger second exon that also contains the 3′UTR region (Moore et al., 1999; Comincini et al., 2001).

2.1.1.3. SPRN

The third member of the prion gene family, termed SPRN (for Shadow of prion protein gene), encoding the Sho protein (also named Shadoo, the Japanese word for shadow), was annotated in eutherians and fish (Premzl et al., 2003 and 2004; Watts et al., 2007). SPRN is not part of the PRN genomic locus (containing PRNP and PRND), and instead can be found on chromosomes 7, 10 and 22, in mice, humans and sheep, respectively (Lampo et al., 2007; Watts et al., 2007). SPRN comprises two exons, with the open reading frame (ORF) contained within a single exon (as PRNP and PRND), exon 2 (Premzl et al., 2003; Watts et al., 2007).

2.1.1.4. PRNT

The forth member of the prion gene family, termed PRNT (prion protein testis-specific gene), was described closer to PRND than PRNP in the human genomic sequence (situated 3 kb in human (Makrinou et al., 2002), and 6 kb in cattle (Kocer et al., 2007), 3′ downstream of PRND), and emerged possibly due to a duplication event that occurred early during eutherian species divergence (Makrinou et al., 2002; Harrison et al., 2010).

It has a two-exon structure (Makrinou et al., 2002) as described in primates, and predicted in cow, sheep, horse and dog, through the two exon RNA structures that align to 90% of human PRNT (Harrison et al., 2010). While the eutherian PRNP and SPRN promoters incorporate CpG islands, the PRND and PRNT promoters do not include CpG islands (Makrinou et al., 2002; Premzl and Gamulin, 2007) suggesting that these genes are expressed in a tissue-specific manner. However, although human PRND is expressed in a testis-specific manner in adult tissues (unlike its mouse homologue, as described in Moore et al., 1999) but not in human fetal tissues, all three isoforms of human PRNT are exclusively expressed in the adult testis and are not present in any of the fetal tissues (including testis), which implies that PRND, unlike PRNT, is developmentally regulated (Makrinou et al., 2002). The goat and bovine genomes, contain a 100% 227-bp-long PRNT homologous region, both with a premature stop codon, and a second AUG initiation codon located downstream (Kocer et al, 2007).

2.1.2. Gene Expression and Structural and Biochemical Properties of Prion and Prion-Like Proteins

2.1.2.1. PrP^C

Alper et al. in 1966, from inactivation studies using ionising and UV-irradiation, concluded that the target size of the scrapie infectious agent (50–150 kDa) was too small for a virus but more characteristic of a protein. Many experimental results were left unexplained until Prusiner took up the inactivation studies and performed systematic analysis using not only chemical and physical, but also enzymatic procedures (Prusiner et al., 1982).

PrP^{Sc} is derived from the benign, cellular isoform of the prion protein (PrP^C) by a posttranslational process involving a profound conformational change (Kaneko et al., 1995).

Although PrP^C is mainly thought of as a glycoprotein attached to the cell surface by a GPI anchor, PrP^C is actually synthesized as a family of four members: the secretory membrane anchored glycoprotein (^{Sec}PrP), two (N- and C-) transmembrane forms with opposite topologies (^{Ntm}PrP and ^{Ctm}PrP), and a cytosolic soluble form (^{Cyt}PrP) of PrP (Hegde and Rane, 2003). PrP^C presents a folded domain at the C terminus that consists of a three-helix structure with a single disulfide bond and a pair of short B-strands (Mo et al., 2001).

The function of PrP^C is still largely unknown, although there is some evidence that the octarepeat sequence (octapeptide PHGGGWGQ repeated four times) in the N-terminal unstructured region of the protein binds Cu(II) ions, which indicate that PrP^C can exist in a Cu-metalloprotein form in vivo (Brown et al., 1997). PrP^C may have a protective effect against neuronal insults. In particular, PrP^C is upregulated following ischemic brain damage, in both humans and mice (McLennan et al, 2004; Weise et al., 2004) and has a neuroprotective activity against apoptosis in vivo (Nishida et al., 1999; Moore et al., 2001; Rossi et al., 2001, Westergard et al., 2007). Among other alternatives, PrP^C could also be a signal transduction protein, as its activation *in vitro* triggers a signalling pathway for which the terminal targets in both neuronal and non-neuronal cells are the MAP kinases ERK1/2 (Schneider et al., 2003).

PRNP is expressed in a broad range of vertebrate tissues such as spleen, lymph nodes, lung, heart, kidney, muscle and mammary glands. It is also found in the gonads (spermatogenic cells and ovaries) but most abundantly occurs in the CNS (Miranda et al., 2011).

2.1.2.2. Shadoo

Shadoo (or Sho) is a hypothetical GPI-anchored protein encoded by the SPRN gene ('shadow of the prion protein'), exhibiting homology and domain organization similar to the N-terminus of PrP. Sho expression engenders a PrP^C-like neuroprotective activity and overlaps PrP^C , but is low in cerebellar granular neurons (CGNs) containing PrP^C and high in PrP^C-deficient dendritic processes (Watts et al., 2007).

Shadoo (Japanese shadow), demonstrates a number of biochemical and cell biological properties also exhibited by PrP^C. Sho is predicted to be extracellular and GPI-anchored, includes N-glycosylation at one or two sites and a cleavage event likely positioned N-terminal to the hydrophobic (composed of aliphatic amino acids) tract (Premzl et al., 2003; Lampo et al., 2007; Watts et al., 2007). In PrP^C, the hydrophobic region also plays a role in the conversion into PrP^{Sc} (Norstrom et al., 2005). The center of both PrP^C and Sho comprises a functionally conserved and ancient activity domain contributing to neuroprotection, and observed loss of Sho protein during prion infections might be related to interference by parenchymal PrP^{Sc} with the physiological protective activity of Sho (Watts et al., 2007).

In sheep, a highly relevant species in prion matters, SPRN shows a high level of homology with the bovine, and to a lesser extent with the human SPRN sequence, and Sho expression presented high levels in cerebrum and cerebellum, and low levels in testis, lymph node, jejunum, ileum, colon and rectum (Lampo et al., 2007).

2.1.2.3. Doppel

In sheep, TSE susceptibility is influenced by polymorphisms of the PRNP gene, with the alleles coding for alanine, arginine and arginine at positions 136, 154 and 171 of the prion protein associated with a high resistance to classical scrapie and BSE (Hunter, 2007).

Nevertheless, this resistance is not absolute, leading to a growing interest in other genes and proteins which could have an influence on TSE susceptibility in sheep. One of these genes is PRND (which encodes a prion-like protein designated Doppel or Dpl), a PRNP homologue found near the PRNP gene and having structural and biochemical similarities with PRNP (Lampo et al., 2007). However, some structural differences and the lack of Dpl overexpression in patients with PrP-induced diseases suggest that Dpl is not involved in prion pathology (Behrens et al., 2001).

Like PrP^C, Dpl is a GPI anchored glycoprotein (structured by three α-helices and two β-sheets), although it has only a 25% amino acid similarity to PrP^C and lacks the distinctive PrP^C repeats and the hydrophobic domain (Silverman et al., 2000). In ovine testis, Dpl protein appears to carry two N-glycans, but apparently lacks O-glycans (Espenes et al., 2006). After being synthesized in the endoplasmic reticulum, the Dpl polypeptide is processed at its C- and N- terminus and is then exposed to the cell membrane (Uelhoff et al., 2005).

PRND, is expressed in the testis of mice, humans, sheep, goats and cattle (Moore et al., 1999; Tranulis et al., 2001; Peoc'h et al., 2002; Rondena et al., 2005; Kocer et al., 2007), which together with sterility detected in PRND-knockout mouse lines (sperm from Dpl deficient mice appear to be unable to undergo the normal acrosome reaction and fertilize the oocyte) strongly suggest that Dpl may play a major role in male fertility (Behrens et al. 2002; Paisley et al., 2004).

Interestingly, haploid spermatozoa lacking the Dpl gene ($PRND^-$) are perfectly fertile when generated in the context of a heterozygous ($PRND^{+/-}$) mouse. This may be related to the fact that sperms spend much of the maturation time in the form of syncytia, with maturating cells connected to each other by cytoplasmic bridges which may allow sufficient amounts of Doppel protein to be transferred from $PRND^+$ to $PRND^-$ spermatids, and rescue fertility (Behrens et al., 2002). Furthermore Dpl is also required for sperm to contribute to embryonic development beyond the morula stage and the elevated levels of DNA damage observed in sperm from Dpl knockout mice ($PRND^{-/-}$), indicate that it is involved in protection from oxidative stress (Paisley et al., 2004).

Previous work from our group, identified a possible association between Dpl gene polymorphisms (synonymous 78G>A substitution in codon 26, coding for Alanine) and ram semen traits/freezability and embryo production. In fact, a classification function was estimated, using data from post-swim-up semen motility and concentration and Day 6 embryo production rate, allowing the identification of the Dpl homozygous GG genotype with 86.7% of accuracy. On the other hand, we found a positive influence of Dpl GA genotype on fresh (higher spermatozoa viability) and thawed (lower total and head abnormalities) ovine semen (Baptista et al., 2008; Pereira et al., 2009).

Although PRND is permanently expressed in the Sertoli cells, its expression in the testicular germ cells varies according to species, especially in the ejaculated spermatozoa where its detection was not always possible. Ovine Dpl protein was detected in the seminiferous epithelium in the final stages of spermatogenesis associated with maturation phase spermatids and subsequently within Sertoli cells, but was not detected on ovine ejaculated spermatozoa. Contradictorily Dpl expression was detected in bovine and human ejaculated spermatozoa and in boar epididymal epithelial cells, suggesting also a possible epididymal origin of Dpl (Espenes et al., 2005; Rondena et al, 2005; Serres et al, 2006; Kocer et al, 2007). In goat, PRND was found to be expressed in testes and ovaries (with higher levels in testes) at various development stages, which differs from the expression pattern

described in sheep in which no expression was observed in prepubertal testis (Espenes et al., 2006; Kocer et al., 2007), suggesting that at least in goat, Doppel might be involved in testis differentiation (Kocer et al., 2007).

The involvement of Dpl in the motility of human spermatozoa was suggested by Peoc'h et al. (2002) after finding this protein on the flagella of mature ejaculated spermatozoa. Latter Pimenta et al. (in press) showed that ram sperm supplementation with 190 $ngmL^{-1}$ of recombinant Dpl (rDpl) during in vitro capacitation significantly improves spermatozoa motility, vigour, viability and fertilization rate. This observation suggests an important function for Dpl during ovine sperm capacitation and also in the consequent fertilization process. Furthermore, during the capacitation process, ovine spermatozoa were supplemented with different concentrations (40, 80 and 190 $ngmL^{-1}$) of Dpl protein and the enhanced spermatozoa viability was achieved regardless of dosage. This may argue in favour of putative physiological functions of soluble forms of Dpl and could be of fundamental importance for the potential biomedical applications of this prion like protein.

2.1.2.4. Prt

Homology between human PrP^C and Prt is lower (44% similarity and 30% identity) than between ORFs (open reading frames) from PRND and PRNT (50% similarity and 42% identity). In bovine, PRNT encodes for an N-terminally truncated protein of 55 aa in length, homologous (55% identity) to its human counterpart (Kocer et al., 2007). In the goat, PRNT is weakly and stochastically expressed in both testes and ovaries at various development stages (Kocer et al., 2007). No signal peptides were predicted for Prts, which suggests that Prts are intracellular proteins (Premzl and Gamulin, 2007). The function of this prion protein remains unknown.

3. The Central Nervous System Invasion

A widely discussed model predicts that prion neuro-invasion consists of two distinct phases: lympho-invasion and neuro-invasion proper (Aguzzi, 2001). The second phase has long been suspected to involve peripheral nerves, and may depend on expression of PrP^C by nerves (Glatzel et al., 2000).

3.1. Transmission Routes

Prions, the agents causing TSEs, colonize the brain of hosts after oral, parenteral, intralingual, or even transdermal uptake, and can be experimentally or naturally transmitted via prion-contaminated food, blood, milk, saliva, feces, nasal secretions, urine and placenta (Gough et al., 2010; Haybaeck et al., 2011). Upon oral challenge, an early rise in prion infectivity can be observed in the distal ileum of infected organisms. There, Peyer's patches acquire strong immunopositivity for the prion protein. PrP^{Sc} accumulates and is amplified in follicular dendritic cells (FDCs) within Peyer's patches and other isolated lymphoid follicles possibly by an interaction with dendritic cells or macrophages. Following accumulation in gut-associated lymphoid tissues, PrP^{Sc} is thought to move to the enteric nervous systems

(ENS) by an interaction with FDCs or dendritic cells. As a result of neuroinvasion into the ENS, PrP^{Sc} spreads to the CNS (Wells, 1994; Vankeulen, 1996; Ano et al., 2009). Scarification of the most superficial layers of the skin, and subsequent administration of prions, has been known for a long time to be a highly efficacious method of inducing prion disease (Taylor et al., 1996). Following inoculation by skin scarification, TSE agent accumulation upon FDCs in the draining lymph node is critical for the subsequent transmission of disease to the brain (Glaysher et al., 2007). Recently, Haybaeck et al. (2011) demonstrated that prions can also be transmitted through aerosols. Aerogenic exposure to prions is very efficacious and can lead to direct invasion of neural pathways without an obligatory replicative phase in lymphoid organs (without the need of a functionally intact immune system). Airborne prions follow a pathway of direct prion neuroinvasion along with olfactory neurons which extend to the surface of the olfactory epithelium. This previously unappreciated risk for airborne prion transmission may warrant re-thinking on current prion-related biosafety guidelines and health standards in diagnostic and scientific laboratories being potentially confronted with prion infected materials.

Several different cell types have been proposed to have a role in prion spreading. Of these, some of the most important players are described below.

3.1.1. Dendritic Cells (DCs)

DCs are hemopoietic (bone marrow derived) specialized antigen-presenting cells and essential mediators of immunity and tolerance. DC and monocyte lineages originate from a common progenitor, the monocyte and dendritic cell progenitor (MDP) (Liu et al., 2010). DCs were implicated as potential vectors of prions in oral and haematogenous spread of the agent (Huang et al., 2002; Aucouturier et al., 2011). Using co-cultures of prion-loaded BMDCs (bone-marrow-derived-dendritic-cells) and cerebellar neurons, Langevin et al. (2010) characterized the transfer triggered by direct cell-cell contact of the prion protein and the resulting infection of the neuronal cultures, suggesting an active transport of prion aggregates, in accordance with a role of TNTs (tunnelling nanotubes). Gousset et al. (2009) demonstrated that TNTs are in fact involved in the spreading of PrP^{Sc} from the peripheral site of entry to the PNS by neuroimmune interactions with dendritic cells, and within neurons in the CNS. Also, DCs have the ability to migrate to the CNS during prion diseases. DCs were in fact detected in the cerebral cortex, subcortical white matter, thalamus and medulla oblongata of C57BL/6 mice intraperitoneally infected with the mouse-adapted KFu strain of Gerstmann-Sträussler-Scheinker syndrome, a human genetic prion disorder (Roscicarelli et al., 2005).

3.1.2. Follicular Dendritic Cells (FDC)

FDCs are mesenchymal (nonhemopoietic) cells, located in lymphoid follicles within the microenvironment of germinal centers. These cells retain native Ags in the form of immune complexes on their membrane for months, and present these Ags to B cells during the secondary response. FDC rescue bound B cells from apoptosis, and induce the differentiation of B cells into long-term memory B cell clones. Furthermore, FDC have also been implicated in the pathogenesis of TSEs (Muñoz-Fernández et al., 2006). TSE infection adversely affects the maturation and regression cycle of FDCs, supporting the hypothesis that TSEs cause an abnormality in immune function (McGovern et al., 2009). FDCs, especially those expressing

PrP^C, could also serve as a prion "reservoir" during the latency phase, thus playing a key role during the scrapie lymphoinvasion (Toppets et al., 2011). In fact, temporary dedifferentiation of FDCs by treatment with an inhibitor of the lymphotoxin-beta receptor signalling pathway (LTbetaR-Ig), blocked the early accumulation of PrP^{Sc} and TSE infectivity within the draining lymph node, in C57BL/Dk mice inoculated (by skin scarification) with the ME7 strain of scrapie, indicating that FDCs are essential for the accumulation of PrP^{Sc} and infectivity within lymphoid tissues and subsequent neuroinvasion (Mohan et al., 2005).

3.1.3. Membranous Epithelial Cells (M-Cells)

Intestinal epithelial cells are bound to each other by tight junctions. This close-packed structure forms a highly selective barrier for macromolecules and limits the access of pathogenic bacteria to the underlying host tissues (Turner, 2006). Gut epithelia are composed of two different epithelial types. One is the villous epithelium, and the other is the follicle-associated epithelium (FAE), which overlies gut-associated lymphoid tissues (GALTs) such as Peyer's patches. The FAE is considerably different from the surrounding villous epithelium, in that it contains membranous (M) cells, which are specialized epithelial cells. Because M cells have a high capacity for the transcytosis of a wide range of macromolecules, they act as an antigen sampling system from the gut lumen to mucosal immune cells. Recently, Miyazawa et al. (2010), demonstrated that bovine M cells are also able to deliver agents of TSE. Moreover, M-cell differentiation (dependent on signals transmitted by intra-epithelial B-cells) is necessary (and sufficient) for active transepithelial prion transport in vitro (Aguzzi et al., 2003).

3.1.4. B-Cell Lymphocytes

B cells not only play a pivotal role in humoral immunity, but also are involved in a broad spectrum of immune responses, including antigen presentation and T-cell function regulation (Matesanz-Isabel et al., 2011). Using a panel of immune-deficient mice inoculated with prions intraperitoneally, Klein et al. (1997) found that defects affecting only T lymphocytes had no apparent effect, but that all mutations that disrupted the differentiation and response of B lymphocytes prevented the development of clinical scrapie. Also, scrapie developed after peripheral inoculation in mice which had differentiated B cells but no functional follicular dendritic cells, which led to the conclusion that differentiated B cells are crucial for neuroinvasion by scrapie. Moreover, a subpopulation of B cells was recently identified that may contribute to the trafficking of prions to the spleen during early pathogenesis of the disease (Edwards et al., 2010).

3.2. Brain Invasion and Damage

In nature, prion diseases are usually transmitted by extracerebral prion infection, but clinical disease results only after invasion of the CNS (Klingeborn et al., 2011). Sympathectomy appears to delay the transport of prions from lymphatic organs to the thoracic spinal cord, which is the entry site of sympathetic nerves to the CNS. However, denervated (sympathetic fibers) mice eventually develop scrapie, probably due to an alternative (as the

possible access through the vagal nerve), low-efficient route of entry (Baldauf et al., 1997; McBride et al., 2001; Aguzzi, 2003; Aguzzi et al., 2003). The brain, as the primary site of prion replication, can reversely provide infectious prions to peripheral tissues, becoming a source of intestinal prion accumulation. In fact, following intracerebral inoculation with prions, PrP^{Sc} was detected in the ileum (Peyer's patches) of infected mice (Lawson et al., 2010).

The mechanism of neurodegeneration in prion diseases is still poorly understood, and not much is known about the mechanism by which prions actually impair neuronal function and cause cell death. Interestingly, there is increasing evidence that the infectious and neurotoxic properties of PrP conformers are not necessarily coupled, as transgenic mouse models revealed that some PrP mutants interfere with neuronal function in the absence of infectious prions. On the other hand, propagation of prions can occur without causing neurotoxicity. Consequently, it appears plausible that two partially independent pathways exist, one pathway leading to the propagation of infectious prions and another one that mediates neurotoxic signalling (Resenberger et al., in press). There is now a growing body of evidence that PrP^{C}, in addition to serving as a precursor of PrP^{Sc}, acts as a signal transducer or mediator of the neurotoxic effects of PrP^{Sc} (Harris and True, 2006). When mice were produced that did not express PrP, they appeared developmentally and behaviourally normal. These mice do not propagate prions or develop pathology when inoculated with mouse-adapted prion strains, confirming the requirement of PrP for animals to contract prion diseases, and suggesting that PrP^{Sc} is not directly neurotoxic to non-prion protein expressing cells (Bueler et al., 1992 and 1993; Brandner et al., 1996). *Cre* recombinase-induced PrP knockout (PrP transgene construct flanked by loxP sites, allowing its conditional excision) after prion inoculation, reversed spongiosis and prevented hippocampal neuronal cell loss indicating that targeting neuronal PrP during established prion infection can prevent clinical onset and that early neurological damage can be reversed (Mallucci et al., 2007). In control mice with GPI-anchored PrP, intracerebral or sciatic nerve inoculation resulted in rapid CNS neuroinvasion and clinical disease. In contrast, in anchorless PrP mice, these routes resulted in slow and infrequent CNS neuroinvasion, showing that anchored PrP was an essential component for the rapid neural spread and CNS neuroinvasion of prion infection (Klingeborn et al., 2011). Historically, prion diseases have been characterised neuropathologically by neuronal vacuolation (spongiosis), brisk reactive proliferation of astrocytes and microglia, and by the deposition of amyloid plaques (Brandner, 2003). Several hypotheses have been put forward to explain the neurotoxicity that leads to apoptosis, among them, oxidative stress (Milhavet et al., 2000), microgliamediated damage (Betmouni et al., 1996), and even the involvement of copper (Wong et al., 2001) leading to increased levels of caspase 3, Fas activation, and up-regulation of the transcription factor c-jun (Fraser et al., 2002). However the mechanism of prion-induced cell death still remains obscure (Brandner, 2003).

For PrP^{C}, a variety of physiological functions have been proposed, including roles in metal ion trafficking, cell adhesion, and signal transduction, protection against apoptotic and oxidative stress, and formation and maintenance of synapses (Pauly and Harris, 1998; Mouillet-Richard et al., 2000; Mange et al., 2002; Westergard *et al.*, 2007). However, the evidence in favor of each of these hypothesized functions is not definitive (Solomon et al., 2010)

3.3. Therapeutics

Most current approaches to treatment of prion diseases are based on inhibiting accumulation of PrP^{Sc} (Trevitt and Collinge, 2006). Identification of the cellular pathways activated by neurotoxic forms of PrP would allow development of an entirely new class of anti-prion therapeutics based on blocking these pathways (Solomon et al., 2010). Candidate therapeutic targets for prion diseases include the following four factors: the PrP^{C}; the PrP^{Sc}; the cellular factors associated with the conversion of PrP^{C} into PrP^{Sc}; and the cellular factors responsible for the neurodegenerative process (Sakasegawa et al., 2007). Aspirin, an anti-inflammatory drug, is a known ERK 1/2 (mitogen-activated protein) inhibitor and prevents neurodegenerative disorders. PrP (106-126) induces neurotoxicity by the overexpression of PrP^{C} and activation of ERK1/2. Recent data (Jeong et al., 2011) suggests that ERK1/2 is a key modulator of the protective effect of aspirin on PrP-106-126-mediated cellular prion protein overexpression and neurotoxicity and also suggest that aspirin may prevent neuron cell damages caused by the prion peptide. The calcium-dependent phosphatase Calcineurin (CaN) is hyperactivated both in vitro and in vivo as a result of PrP^{Sc} formation. CaN activation mediates prion-induced neurodegeneration, suggesting that inhibition of this phosphatase could be a target for therapy. To test this hypothesis, prion infected wild type mice were treated intra-peritoneally with the CaN inhibitor FK506 at the clinical phase of the disease. Treated animals exhibited lower degree of neurodegeneration (higher number of neurons and a lower quantity of degenerating nerve cells), reduced severity of the clinical abnormalities and increased survival time. Taken together, these findings may provide a novel strategy for therapy at the clinical phase of the disease (Mukherjee et al., 2010).

4. Prions in Diseases

4.1. Spongiform Encephalopathies

The neurodegenerative disorders included among the human prion diseases are CJD, Gerstmann–Straussler–Scheinker disease (GSS), fatal insomnia and kuru. The animal diseases include scrapie of sheep and goats, transmissible mink encephalopathy, chronic wasting disease of mule deer and elk, feline spongiform encephalopathy and BSE. These disorders have been collectively classified as the 'transmissible spongiform encephalopathies' because they can be transmitted to humans and to animals and because vacuolar degeneration of the gray matter neuropil are the most characteristic neuropathological features (DeArmond and Bouzamondo, 2002; Marsh et al., 2005). The prion diseases are characterized by a triple aetiology being sporadic, most cases of CJD; inherited, familial CJD and most cases of GSS disease; or infectious, this last category includes the vast majority of cases namely BSE, scrapie and kuru (Prusiner et al., 1998; Fornai et al., 2006). As referred, a wealth of data supports the contention that scrapie prions are devoid of nucleic acid and seem to be composed exclusively of a modified isoform of PrP, the PrP^{Sc}. The normal cellular PrP^{C} is converted into PrP^{Sc} through a process whereby a portion of its α-helical and coil structure is refolded into β-sheet (Prusiner et al., 1998). Several factors can switch on this mechanism, by interfering at various levels with the synthesis and/or metabolism of PrP^{C}. This might occur

following mutation of PRNP gene, that leads to synthesis of misfolded prion proteins, or exogenous PrP^{Sc} "contamination", that enhances deposition of cytosolic forms of PrP^{C}, or finally as a consequence of an excess of PrP^{C} synthesis and/or an inefficient degradation by the ubiquitin–proteasome system. According to Fornai et al. (2006) these conditions might actually occur in inherited, "infectious", and sporadic prion disorders, respectively. In different TSE phenotypes, PrP^{Sc} exhibits disease-specific properties, including distinctive cleavage sites after proteolytic treatment, ratio of glycoforms, and deposition patterns, all features useful in providing a means of strain identification (Bessen and Marsh, 1992, Hill et al., 1997 and 1998).

The prototypical TSE, scrapie, has been observed in European sheep for >200 years, whereas BSE in domestic cattle (Bos taurus and Bos indicus; hereafter, cattle) dates to 1986, presumably resulting from scrapie- and/or BSE-infected cattle feed. Thus, BSE seems to be a more recent phenomenon associated with modern agricultural practices. Unfortunately a new variant of CJD, designated vCJD, was transmitted to humans by ingestion of BSE-contaminated food products in Great Britain and to a lesser extent in the rest of Europe (Hill et al., 1997, Scott et al., 1999, Casalone et al., 2004). The unprecedented biological properties of the BSE agent, which circumvents the so-called "species barrier" between cattle and humans and adapts to different mammalian species, has raised considerable concern for human health (Hill et al., 1997, Casalone et al., 2004).

Given the hypothesis that the novel prion strain causing BSE was originated from exposure of cattle to sheep scrapie, the European Community decided to engage in a selection program based on the known genotypes of the PRNP that have been associated with different grades of susceptibility to scrapie. Hence a genetic basis for this susceptibility was identified in sheep and 4 different single nucleotide mutations in the coding region of PRNP gene, were linked to different resistance to scrapie disease (Hunter, 1998, 2007), a "table of susceptibility" with 5 groups of different grades of resistance (R1-R5) was elaborated, being the genotypes ARR/ARR (R1) and VRQ/VQR (R5) respectively the less and the most susceptible to this disease. In 2003, the European Community decided (Decision n°100/2003) to implement this selection program intending to eradicate scrapie from its member states and several countries have established breeding programmes to create disease-resistant national flocks, with the ram as a major selection target. This allows the introduction of Scrapie-resistant genes into sheep populations within a short period eliminating the most susceptible. However, specially in small populations and in breeds with unfavourable ARR allele frequencies, such strategy increases the risk that valuable genetic diversity may be lost due to selective breeding for disease-resistant genotypes (Ehling et al., 2006, Gama et al., 2006). Above all doubts have been raised as to whether the intense selection for the classical PRNP genotype alone might have undesirable consequences, namely a blind selection for other genes, especially those related with reproduction and/or linked to the PRNP gene, like PRND and PRNT. Moreover a preliminary evidence for a correlation between Doppel polymorphism and PRNP genotypes, as well as with scrapie susceptibility was established by our team (Mesquita et al., 2010). In the portuguese sheep breeds, an association between the heterozygotia in the codon 26 of PRND gene and ARQ/AHQ PRNP genotype and the absence of PRND polymorphic variants in the rams belonging to the more resistant scrapie susceptibility group (characterized by the ARR/ARR PRNP genotype) was observed. As it is now well established that Dpl is related with male fertility (Pereira et al., 2009, Pimenta et al., in press) and that PRND gene polymorphisms may be associated with ovine semen

traits/freezability and fertility (Baptista et al., 2008), the putative undesirable consequences of the widespread selection for the PRNP genotype on genetic diversity and reproduction traits must be considered.

On the other hand, large variations in the prevalence rates of the classical and also atypical forms of scrapie as well as in the surveillance plans among the European countries have been reported (Del Rio Vilas and Pfeiffer, 2010, Orge et al., 2010, Rodrigues Martinez et al., 2010). Therefore it seems likely that the European Community breeding program for sheep must be revised although maintaining the intense surveillance of scrapie and BSE for public health safety.

4.2. Prions and Cancer

Doppel, a membrane-bound glycoprotein mainly expressed in the testis of adult healthy people, is generally absent in the CNS. In mice, Dpl is expressed in adult testis and heart, while in the brain it is detectable only during embryogenesis (Li et al., 2000). In humans, in addition to testis, immunometric assays revealed low levels of Dpl in healthy brains (Peoc'h et al., 2003). When overexpressed in neurologic tissue, Dpl is neurotoxic and causes a neurodegenerative disease (Moore et al., 1999). Recently, ectopic expression of Dpl was found in two different tumor types, specifically in glial and haematological cancers (Comincini et al., 2004, 2007). Dpl is ectopically expressed in astrocytoma specimens which are human brain tumors that arise from astrocytes, the most abundant type of glial cells of the CNS. Astrocytomas are graded I-IV according to their histological features. Low-grade astrocytomas typically display a long clinical history with relatively benign prognosis, whereas the prognosis of high-grade astrocytomas is devastating. However, low-grade astrocytomas have an inherent tendency to progress toward more malignant forms. Real-time PRND gene expression profiling, revealed large differences between each grade of malignancy, suggesting that PRND mRNA quantitation might be useful to distinguish astrocytoma subtypes, in disease stratification and in the assessment of specific treatment strategies (Comincini et al., 2007). Most low-grade astrocytomas (83%) show a membrane-bound Dpl, like human healthy testis tissue, whereas the majority of high-grade astrocytomas (75%) display a cytosolic Dpl. Dpl, at the onset of the disease, is likely to be anchored to the outer leaflet of the plasma membrane, through its GPI moiety. Afterwards, as the tumor progresses, Dpl could fail to anchor to the membrane and localize within the cytoplasm. Taken together, these findings show that in astrocytomas, Dpl might constitute an additional tool to characterize the glial tumor progression (Rognoni et al., in press).

Travaglino et al. (2005) investigate Dpl expression and distribution in normal bone marrow cells, in leukaemic cell lines and in bone marrow cells from patients with acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS), in order to evaluate its possible ectopic expression in such disorders. The differential expression of Dpl observed in AML and MDS compared with healthy subjects makes it a possible leukaemia-associated antigen with important diagnostic and therapeutic applications. As for other tumour-associated antigens, Dpl may elicit an immune response that could be the basis of a therapeutic approach. Moreover, the quantification of Dpl levels might aid in the assessment of minimal residual disease after treatment in acute leukaemia. Dpl expressing HL-60 (human premyeloid) and K562 (human myelogenous leukaemia) cell lines may provide a useful in

vitro model to study the protein function as well as the development of cancer immunotherapy responses.

Conclusions and Future Perspectives

Prions are pathological agents causing sporadic, genetic and infectious neurodegenerative disorders. Although the molecular mechanisms of these diseases are not well understood, the "protein-only" hypothesis suggests a mysfolded PrP (PrP^{Sc}) as the etiologic agent. Although prion diseases are usually transmitted by extracerebral prion infection, clinical disease results only after invasion of the CNS. Several different cell types have been implicated in prion lympho and neuro-invasion, such as dendritic cells, follicular dendritic cells, membranous epithelial cells and B-cell lymphocytes, among others. However, recently described (Haybaeck et al., 2011) airborne pathway of direct prion neuroinvasion without an obligatory replicative phase in lymphoid organs should warrant re-thinking on current prion-related biosafety guidelines. The mechanism of neurodegeneration in prion diseases is still poorly understood, and not much is known about the mechanism by which prions actually impair neuronal function and cause cell death, although it appears plausible that two partially independent pathways exist, one leading to the propagation of infectious prions and another one that mediates neurotoxic signalling. Historically, prion diseases have been characterised neuropathologically by neuronal vacuolation (spongiosis), brisk reactive proliferation of astrocytes and microglia, and by the deposition of amyloid plaques (Brandner, 2003). Several different prion linked neurodegenerative disorders have been described in humans and animals and collectively classified as 'transmissible spongiform encephalopathies'. Selection programs intending to eradicate certain prion diseases, as scrapie in sheep, based on different genetic grades of resistance and susceptibility, should take into consideration that such strategy increases the risk that valuable genetic diversity may be lost along the process. Therapeutical approaches describing promising anti-prion agents that are effective in animal models and in cell culture experiments, have yet to prove its efficacy in humans and production animals. Identification of the cellular pathways involved in the physiological function of PrP^{C} would allow development of an entirely new class of anti-prion therapeutics based on blocking these pathways. PRNP is no longer alone, and new key players, like Shadow, PRNT and PRND need to be taken into consideration, as a family of prion genes that interact among them. Some, like PRND and PRNT seem to play a major role in reproduction field and further studies could elucidate possible causes of male infertility. Moreover, the quantification of PRND expression levels and Dpl ectopic expression in glial and haematological cancers might constitute an additional tool to characterize tumor progression. Despite the numerous scientific data reported so far, several aspects concerning toxic and physiological function of these unusual infectious particles, termed prions, remain obscure and need to be further enlightened.

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