Developmental Regulation of Prion Expression in Cattle and Mouse Embryonic Stem Cells

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ABSTRACT

The host encoded cellular prion protein (PrP^C) is an N-linked glycoprotein tethered to the cell membrane by a glycophosphatidylinositol (GPI) anchor. Under certain conditions, PrP^C can undergo conversion into a conformationally-altered isoform (PrP^{Sc}) widely believed to be the pathogenic agent of transmissible spongiform encephalopathies (TSEs). Thus, tissues expressing PrP^C are potential sites for conversion of PrP^{Sc} during TSE pathogenesis. Although much is known about the role of PrP^{Sc} in prion diseases, the normal function of PrP^C is poorly understood. Lines of mice and cattle in which PrP^C has been ablated by gene knockout show no major phenotypical alterations other than resistance to TSE infection. However, recent reports using Prnp-null mouse models have suggested the participation of PrP^C in neural stem/progenitor cell proliferation and differentiation. The first objective in our study was to map the expression of PrP^C in twenty six somatic and reproductive tissues in ruminants. Our second objective was to characterize the ontogeny of PrP^C expression during bovine embryonic and early fetal development. Finally, we used a mouse embryonic stem cell (mESC) model to study the potential role of PrP^C during neurogenesis. In adult tissues, intense expression of PrP^C was detected in the central nervous system (CNS), thymus and testes, whereas the liver, striated muscle and female reproductive tissues showed the lowest expression. We observed that PrP^C was associated with tissues undergoing cellular differentiation including spermatogenesis, lymphocyte activation and hair follicle regeneration. Analyses in bovine embryos and fetuses indicated peaks in expression of PrP^C at days 4 and 18 post-fertilization, stages associated with the maternal-zygote transition and the maternal recognition of pregnancy and initiation of placental attachment, respectively. Later in development, PrP^C was expressed in the CNS where it was localized in mature neurons of the neuroepithelium and emerging neural trunks. Based on these observations, we hypothesized that PrP^C was involved in neurogenesis. We tested this hypothesis in a murine embryonic stem cell model (mESC). mESC were induced to form embryoid bodies (EBs) by placing them in suspension culture under differentiating conditions and allowed to differentiate *in vitro* for 20 days. We detected increasing levels of PrP^C starting on day 12 (8.21- fold higher vs. day 0; $P < 0.05$) and continuing until day 20 (20.77-fold higher vs. day 0; $P < 0.05$). PrP^C expression was negatively correlated with pluripotency marker Oct-4 ($r = -0.85$) confirming that mESC had indeed differentiated. To

provide a more robust system for assessing the role of PrP^C in neural differentiation, mESC were cultured with or without retinoic acid (RA) to encourage differentiation into neural lineages. Induction of EBs with retinoic acid (RA) resulted in an earlier up-regulation of PrP^C and nestin (day 12 vs. day 16; $P < 0.05$). In addition, immunofluorescence studies indicated co-expression of PrP^C and nestin in the same cells. The results of these experiments suggested a temporal link between PrP^C expression and expression of nestin, a marker of neural progenitor cells. We next tested whether PrP^C was involved in RA-enhanced neural differentiation from mESC using a PrP^C knockdown model. Plasmid vectors designed to express either a PrP-targeted shRNA or scrambled, control shRNA were transfected into mESC. Stable transfectants were selected under G418 and cloned. PrP-targeted and control shRNA clones, as well as wild-type mESC, were differentiated in presence of RA and sampled as above. PrP^C expression was knocked down in PrP-targeted shRNA cultures between days 12 and 20 (62.2 % average reduction vs. scrambled shRNA controls). Nestin expression was reduced at days 16 and 20 in PrP^C knockdown cells (61.3% and 70.7%, respectively vs. scrambled shRNA controls). These results provide evidence that PrP^C plays a role in the neural differentiation at a point up-stream from the stages at which nestin is expressed. In conclusion, the widely distributed expression of PrP^C in ruminant tissues suggests an important biological role for this protein. In the present work we have provided evidence for the participation of PrP^C in the differentiation of mESC along the neurogenic pathway.

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INTRODUCTION

The complex nature of prions has intrigued the scientific community during the last 70 years. Since the first indication of scrapie infectivity in 1937 and the experimental transmission of the scrapie agent in sheep the same year, prions and their associated diseases, have been under constant investigation. Furthermore, the formulation of the prion hypothesis in 1966 and the discoveries by Prusiner in 1982, opened a completely new perspective in the understanding of these infectious proteins. One of the most interesting aspects of the prion hypothesis is the presence of a host-encoded isoform (PrP^{C}) with the autocatalytic or induced capacity to change its secondary configuration into a pathogenic isoform (PrP^{Sc}). Despite intense research undergoing during the last 20 years to probe this hypothesis, the most compelling evidence is yet to be reported. Another enigmatic aspect of the prion biology is the potential physiological function of PrP^{C}, a protein that is widely distributed in mammalian tissues and intensely expressed in the nervous system. PrP^{C} has been associated to several biological roles including cellular adhesion, signaling and protection. Recently, one study reported a positive association between PrP^{C} and differentiation of neural cells. These researchers argued that PrP^{C} was associated in a dose-dependent manner with differentiation of multipotent precursors into mature neurons *in vitro*. Moreover, analyses in mouse brain showed the PrP^{C} played a role in the proliferation of neural precursor cells.

In the present work, we described our efforts to better understand the potential function of PrP^{C}. In chapters two and three we mapped the expression of PrP^{C} in 30 different tissues of the bovine. These analyses characterized PrP^{C} as widely distributed protein expressed in organs with different physiological functions. This wide distribution and intense expression in neurons and lymphoreticular cells suggest that PrP^{C} may have an important physiological function. Chapter four focused on the developmental characterization of PrP^{C} during bovine embryogenesis. The stage-specific expression of PrP^{C} during pre-implantation stage suggests the participation of this protein in the early embryonic development. Thereafter analyses in fetuses showed an intense expression of PrP^{C} during the early development of the nervous system. This data led us to hypothesize a potential contribution of PrP^{C} in neurogenesis. This hypothesis was tested in the last chapter of this work. Analysis of PrP^{C} expression in mouse embryonic stem cell (mESC) showed a positive association between PrP^{C} expression and the marker for neural stem/progenitor cells, nestin. The final experiment demonstrated that the knockdown of PrP^{C} expression results in the significant reduction of nestin levels indicating a direct or indirect association between these proteins. This data represents evidence for the contribution of PrP^{C} in neurogenesis and support the reports of others indicating the participation of PrP^{C} as a neural differentiation factor.

CHAPTER I

PRION BIOLOGY AND BOVINE SPONGIFORM ENCEPHALOPATHY

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative, fatal diseases with no early diagnosis, treatment or cure (Collinge, 2001). Several species are affected by TSEs including human (Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker, Kuru, Fatal familial insomnia), bovine (spongiform encephalopathy), ovine (scrapie), deer (chronic wasting disease) and mink (transmissible encephalopathy). TSEs display a wide spectrum of clinical signs, neuropathology and epidemiology that result in difficult diagnosis and control. Despite their diverse presentations, all TSEs stem from the infectious, spontaneous or hereditary conversion of PrP^{C} into the pathogenic isoform PrP^{Sc} (Soto, 2006). The unpredictable properties of PrP^{Sc} and the potential zoonotic transmission of the bovine spongiform encephalopathy (BSE) have generated intense concern in the international community over animal product biosecurity. One of the most critical aspects of the TSE-infectious agent is the capacity to cross the species barrier from cattle to human through the consumption of beef products. Worldwide, 172 people have fallen victim to the variant CJD (vCJD) during 2008, and epidemiologists predict the presentation of more cases in the coming years due to existing pre-clinical or subclinical infection (Collee et al., 2006; NCJDSU, 2008). The first case of BSE was detected in USA in 2003, resulting in the ban of meat importation from USA to 53 countries with a drastic drop in 83% of the imports. Translated into economic terms, BSE produced a loss of 5 billion dollars which corresponded to 4% of the gross domestic agriculture product (Coffey et al., 2005).

The scientific community has been intrigued with the complex nature of prions for over 70 years. The first indication of prion infectivity was reported in 1937 in Scotland after immunization of sheep against louping ill. The vaccines used were accidentally elaborated from brain extracts obtained from animals infected with scrapie (Gordon, 1946). The fact that eight percent of the immunized animals developed scrapie, with the experimental transmission of the agent performed the same year, the infectious capacity of scrapie among sheep and goats was demonstrated (Cullie and Chele, 1939). In 1959, Hadlow suggested that Kuru, a neurodegenerative disease that affected New Guinea tribes might be similar to scrapie due to similarities in epidemiology, clinical signs and pathological findings. This hypothesis was later confirmed in 1965 by the successful transmission of Kuru to chimpanzees after incubation of 18 to 21 months (Gajdusek et al., 1965). One year later, Alper and colleagues reported that the molecular weight of the scrapie agent was significantly lower compared to a conventional virus (Alper et al., 1966). Moreover, Apler showed that the scrapie agent was able to resist doses of ultraviolet radiation (UV) sufficient to

inactivate nucleic acids. These experiments led to the formulation of the protein-only hypothesis, which described the scrapie agent as a particle conformed by a proteinaceous structure devoid of nucleic acids with the unique capacity to autoreplicate (Griffith, 1967). In the 1980s, Prusiner reported abundant experimental data in support of this hypothesis and proposed for the first time the term "prion" to describe the scrapie agent. Prion was defined as small proteinaceous infectious particle which was resistant to inactivation by most procedures that modify nucleic acids (Prusiner, 1982). This controversial suggestion supported the idea of a scrapie agent consisting only of an infectious protein and discredited the model that included a small nucleic acid in the core of the protein. Furthermore, one of the most intriguing aspects of the prion biology was the discovery of a host-encoded cellular prion protein or PrP^{C} (Oesch et al., 1985). This discovery guided to the formulation of the prion hypothesis that postulates that the agent responsible for prion propagation is originated by autocatalytic conversion of PrP^{C} into the pathogenic isoform. Physical contact between both isoforms induces instability of the PrP^{C} mainly α-helicoidal structure and results in its conversion into a predominantly β-sheet configuration characteristic of PrP^{Sc}.

Although the involvement of PrP^{C} in the infection of TSEs has been well documented, the function of PrP^{C} remains inconclusive. During the last decades, there have been several reports describing roles for PrP^{C} as an antioxidant, cytoprotective, cell adhesion and mitogenic agent (Manson et al., 1992; Martins et al., 2001; Zanata et al., 2002; Chiarini et al., 2002). However, recent studies have demonstrated the capacity of PrP^{C} to bind molecules involved in signal transduction such as the neural cell adhesion molecule (N-CAM) and laminin. Binding of PrP^{C} to these molecules results in neurite outgrowth and neuronal proliferation and migration (Santuccione et al., 2005; Graner et al., 2000). A recent report indicated that PrP^{C}-null mice exhibited an impaired capacity of self-renewal of hematopoietic stem cell populations after serial transplantation in the bone marrow (Zhang et al., 2005). Furthermore, PrP^{C} was positively associated with proliferation of neural cells *in vivo* and differentiation of multipotent neural precursors *in vitro* (Steele et al., 2005). Since PrP^{C} is highly expressed in the nervous tissue, PrP^{C} mediation or activation of cell signaling involved in proliferation or differentiation suggests a role for PrP^{C} in the development of the nervous system; however, further evidence is required to elucidate this hypothesis.

The complex presentation of TSEs and the novel properties of the PrP^{Sc} have opened many questions yet to be answered. During the last years, research in prion biology has mainly focused on determination of the pathogenesis of TSEs and the development of diagnostic and therapeutic methods. However, further research in prion biology will continue to be the foundation for understanding the complex nature of TSEs and how these diseases can be controlled.

PRION BIOLOGY

PrP^{C} structure. The structure of PrP^{C} is highly conserved among species and throughout evolution, suggesting an important biological role (Riek et al., 1996; Gossert et al, 2005). Before post-translational modification, PrP^{C} is composed of a sequence of 253 amino acids with a slight variation between species depending on the number of octapeptide repeats (Prusiner and Scott, 1997). The octapeptide repeat region is an eight-amino acid repetitive motif composed of residues P(H/Q)GGG(-/G)WGQ and located in the N-terminal region of the protein (Moore et al., 2006). During protein maturation, PrP^{C} is exposed to several modifications in the rough endoplasmic reticulum (ER) including replacement of the peptide signal located between amino acids 232-253 with a glycophosphatidylinositol (GPI) anchor. Additionally, two asparagines at amino acids 181-197 are glycosylated and one disulfide bridge is added between two cysteine residues 179-214 (Prusiner, 1998; Harris, 2003). The mature protein is divided in two distinct regions: a flexible and essentially unstructured N-terminal region between amino acids 23-125 and a C-terminal region containing three alpha-helicoidal structures and a short beta-sheet motif between amino acids 126-231 (Abid and Soto, 2006). Asparagine glycosylation will determine variations in the biochemistry of the mature protein resulting in mono-, di- or un-glycosylated forms with molecular weights of ~28, 34 and 25 Kb, respectively (Russelakis-Carneiro et al., 2002; Priola and Vorberg, 2006). PrP^{C} is found as a mixture of these forms with variable proportions depending on the tissue and animal species (Russelakis-Carneiro et al., 2002).

The usual cellular location of PrP^{C} is attached by the GPI anchor to membrane domains rich in cholesterol and sphingolipids known as lipid raft (Martins et al., 2002, DeMarco et al., 2005). However, part of the pool of PrP^{C} can be internalized via clathrin-mediated endocytosis and accumulate inside the Golgi

apparatus. Furthermore, some of the internalized protein is recycled to the cytoplasmatic membrane by kinesin anterograde transport (Harris et al, 1996; Sunyach et al., 2003; Hachiya et al., 2004). It is uncommon for membrane-anchored proteins to be internalized by clathrin-mediated endocytosis since they are devoid of a cytoplasmic domain that usually recruits clathrin-coated pits. However, this process may be facilitated by unknown proteins that interact with PrP^{C}, enabling endocytosis of the molecule (Harris et al., 1996). Although the specific location for PrP^{C} conversion has not yet been determined, it is believed that formation of PrP^{Sc} occurs inside the cell and not in the extracellular suspension. PrP^{C} has been detected inside endosomes and lysosomes which potentially participate as conversion sites (Martins et al., 2002). The low pH maintained inside these organelles may facilitate PrP^{C} conversion as demonstrated by experiments in which acid pH favored aggregation of recombinant PrP^{C} into PrP^{Sc}-like structures (Bocharova et al., 2005). Alternatively, the pool of PrP^{C} that remains attached to the plasma membrane may find a favorable environment for conversion in lipid rafts that promote protein interaction and recruit accessory molecules (Hooper and Taylor, 2006).

Prnp gene structure. The prion gene (Prnp) has homologues in all vertebrates with conserved regions between mammals and birds (Premzl and Gamulin 2007). Prnp is located in chromosome 2 in mouse, 13 in bovine and 20 in human (Sparkes et al, 1986; Ryan and Womack, 1993). The 5' flanking region of bovine Prnp shows an 89% homology with the sheep and only 46-62% homology with the mouse, rat, hamster and human sequences (Inoue et al., 1997). Bovine, sheep, mouse and rat Prnp possess three exons with the protein coding sequence located entirely within the third exon (Fig. 1; Chesebro et al., 1985; Oesch et al., 1985; Inoue et al., 1996). Using chloramphenicol acetyltransferase (CAT) plasmids, the promoter region of the bovine gene was detected in the region between -88 and -30 relative to the transcription start site, similar to the rat promoter region (Inoue et al., 1997). Several regulatory regions including the promoter has been identified in the bovine Prnp with the major region of transcriptional control located upstream of the initiation site. The promoter sequence is rich in G+C features, lacks a TATA box and contains potential binding sites for Sp-1 transcriptional factors (Inoue et al., 1997). Several variables have been reported to influence Prnp expression under *in vitro* conditions including nerve growth factor (NGF), rate of prion infection and epigenetic changes (Graner et al., 2000; Bueler et al., 1993; Martins et al., 2002). However, *in vivo* factors such as the physiological status of the host may influence Prnp expression as well. A recent study reported that Prnp gene expression may be affected by heat shock proteins under stress conditions (Haigh et al., 2007).

Polymorphic variations in the coding sequence of the ovine Prnp gene have been reported to control the susceptibility to scrapie. The major mutations associated with susceptibility or resistance are located at codons 136 (A or V), 154 (R or H) and 171 (R, Q or H) (Clouscard et al., 1995; Hunter et al., 1996). Animals with PrP genotypes V136 R154Q171/VRQ, ARQ/VRQ and ARQ/ARQ are the most susceptible to scrapie, whereas homozygous or heterozygous AHQ and heterozygous ARR animals show only marginal susceptibility. ARR/ARR sheep are considered to be fully resistant (Hunter et al., 1996).

Prion Hypothesis. The prion hypothesis or protein-only hypothesis postulates that the agent responsible for prion propagation is originated by autocatalytic conversion of PrP^{C} into the pathogenic isoform (Griffith, 1967). Conversion into PrP^{Sc} involves a drastic alteration in the protein configuration as well as in the biochemical properties. Crystallography studies indicate that in normal state 47% of the PrP^{C} structure is composed of α-helix and only 3% β-sheet secondary configuration. In the conversion process, the β-sheet configuration is increased to 43-45 % and the α-helix structure is reduced to 17-30% (Pan et al., 1993; DeMarco et al., 2005, Fig.2). Therefore, the newly formed PrP^{Sc} structure is highly planar and stable showing strong resistance to temperature, pH, disinfectants and enzymatic degradation (Taylor, 2000).

Additional supporting evidence of the prion hypothesis has been originated from studies that reported resistance to prion infection in mice lacking the Prnp gene (Bueler et al., 1993). These knockout models not only evidenced the requirement of a host-encoded PrP^{C} protein for the infection process but also allowed a better understanding of the pathogenesis of TSEs. However, the most compelling evidence to probe this theory is yet to be reported. Some researchers claimed that a confirmatory experiment will consist in the *in vitro* conversion of PrP^{C} molecules into a pathogenic isoform with the capacity to induce TSE infection (Chesebro, 1998). Mutations induced to recombinant PrP^{C} have resulted in destabilization of the protein configuration and formation of a PrP^{Sc}-like molecule; however, this mutated agent was unable to induce prion disease (Chiesa et al, 1998; Bocharova et al., 2005). It is possible that additional factors including a transitional form of PrP and host-derived proteins or non-protein compounds (chaperones, glycosaminoglycans or short nucleic acids) are required to sustain *in vitro* generation of PrP^{Sc} (Castilla et al., 2005; Aguzzi et al., 2007). Hamster PrP^{C} was only converted to PrP^{Sc} when cell lysate was added to the reaction (Deleault et al., 2005). Moreover, mice co-expressing both human and mice PrP were resistant to prion replication as consequence of the interaction of mice PrP^{C} with an additional factor (termed protein X) that inhibited human PrP^{C} conversion (Telling et al., 1995). Recent

studies reported the *in vitro* generation of PrP^{Sc} molecules using a protein misfolding cyclic amplification technique (PMCA) that allows the repetitive amplification of the misfolding event (Castilla et al., 2005). Although, the newly formed PrP^{Sc} generated by this technique was able to infect wild-type Syrian hamsters, the use of crude brain homogenates to amplificate these molecules may have also resulted in the addition of different components responsible for the infection.

Two distinct models have been proposed to explain the autocatalytic conversion of PrP^{C}, a process not mediated by nucleic acids that challenge the central dogma of molecular biology. The template-assisted model postulates a thermodynamically stable conversion between both PrP isoforms (Fig. 3). PrP^{C} conversion is induced by PrP^{Sc} and is mediated by an intermediate and heterodimeric unit before the formation of a homodimeric PrP^{Sc}. The process is catalyzed by a yet unidentified protein X that has chaperone-like properties and facilitates aggregation of both isoforms (Cohen and Prusiner, 1998). Protein X promotes PrP^{C} conversion by binding a discontinuous epitope in the globular C terminal region of the protein (Kaneko et al., 1997). Newly formed PrP^{Sc} can eventually agglutinate and precipitate, forming amyloid precursors detected in some TSEs (Prusiner, 1990; Cohen et al., 1999). A second model termed nucleation-polymerization, proposes a similar thermodynamic equilibrium between both isoforms (Fig. 3). However, after PrP^{C} conversion, the model describes a highly unstable and transient PrP^{Sc} molecule that would be stabilized only by forming ordered aggregates. The stabilized oligomers act as nuclei to recruit monomeric PrP^{Sc} in a process that displaces the thermodynamic equilibrium and accelerates PrP^{Sc} formation (Caughey, 2001; Caughey and Lansburry, 2003).

Despite a bulk of evidence in support of the prion hypothesis, alternative models suggesting the participation of viral particles, virinos and small RNAs have also been proposed. Co-sedimentation of retroviral RNA with PrP^{Sc}, and purification of short RNA fragments from infectious fractions suggest the participation of nucleic acids as part of the infectious particle (Akowitz et al., 1990, 1994). The virino model describes the TSE agent as a proteinaceous structure containing nucleic acids with a virus-like conformation (Chesebro, 1998). The finding that prions have a variety of strains that correlates with a species-specific symptomology and histopathology in TSEs has also been used as evidence to support the virino model (Chesebro, 1998). However, the strain phenomenon can be explained by the variation in PrP^{Sc} protein secondary structure and not necessarily by the existence of viral strains containing nucleic acids (Prusiner, 1998). Furthermore, several biochemical properties displayed by scrapie agents indicate the absence of nucleic acids. The molecular weight (< 50 kDa), diameter (4 to 6 nm) and width (1 nm) of

the scrapie agent predicted a reduced core volume (14.1 m3) with insufficient capacity to store enough nucleic acids to encode a viral protein (Prusiner, 1982). Additionally, the prion agent has shown high resistance to UV radiation (42,000 J/m2) and nuclease digestion.

PrP^C Function. Despite intense investigation during recent years, the function of PrP^C remains enigmatic. Some studies have suggested a cellular protective role of PrP^C against oxidative stress. Experiments have showed that neurons obtained from Prnp knockout mice and cultured *in vitro* display higher susceptibility to oxidative agents such as hydrogen peroxide, xanthine oxidase and copper ions compared to wild-type neurons (Brown et al., 2002). Moreover, brain tissue collected from Prnp knockout mice exhibited biochemical changes including increased levels of protein carbonyls and lipid peroxidation products, which are indicative of oxidative stress (Wong et al., 2001). An extensive body of evidence has accumulated suggesting the binding of Cu+2 ions to the PrP^C octapeptide repeat region. Copper (Cu+2) is an essential element that participates as an enzymatic cofactor in the biochemical pathways of all aerobic organisms. However, Cu +2 can also catalyze the formation of reactive oxygen species such as the hydroxyl radical (Martins et al., 2001). The binding of Cu +2 to PrP^C may limit the capacity to catalyze the formation of such toxic oxidative radicals (Martin et al., 2001; Vassallo and Herms, 2003). Some researchers have questioned this antioxidant property due to the super-physiological concentrations of Cu +2 required to activate this process (Westergard et al., 2007). Alternatively, PrP^C may modulate the activity of the Cu/Zn superoxide dismutase (Cu/Zn SOD) enzyme that showed cellular protective function against oxidative stress (Wechslberger et al., 2002; Brown et al., 2001). Analyses in brain tissue obtained from Prnp knockout mice have shown only 10-50% of the normal Cu +2 loading capacity and Cu/Zn SOD enzymatic activity. In contrast, the enzymatic activity and copper loading of Cu/Zn SOD was increased in mice over-expressing PrP^C (Brown et al., 1997).

Several lines of evidence have proposed a cytoprotective role of PrP^C against internal or environmental stresses that initiate apoptosis. This anti-apoptotic potential is primarily based on the capacity of PrP^C to inhibit the action of the apoptotic protein Bax (Bounhar et al., 2001). PrP^C-induced blocking of Bax may be direct, for example by inhibiting its mitochondrial translocation, conformational change, or oligomerization. Alternatively, PrP^C may act upstream of Bax, affecting the activity of BH3, Bcl-2 or Bcl-X_L, or downstream, suppressing the effects of Bax in the release of cytochrome c or activation of Apaf-1 and caspases (Roucou et al., 2005; Westergard et al, 2007). PrP^C could also affect the calcium Bax-mediated secretory pathway in the ER. Other studies have reported a close similarity between the

homologous domain of the anti-apoptotic protein Bcl-2 and the PrP^C octapeptide region. This analogy in the protein structure may allow PrP^C to mimic Bcl-2 function and induce cell survival (Roucou et al., 2005; Westergard et al., 2007).

In addition to the cytoprotective role, PrP^C has been also implicated as a cell proliferation and differentiation factor. Recently it was reported that PrP^C-null mice exhibited an impaired capacity of self-renewal of hematopoietic stem cell populations after serial transplantation in the bone marrow (Zhang et al., 2005). The potential mitogenic capacity has also been supported by studies showing a decrease in T lymphocyte proliferation in mice devoid of PrP^C (Bainbridge et al., 2005). The role of PrP^C in differentiation was suggested by high levels of expression in cells that ceased proliferation and became differentiated into neurons during early stages of mice embryogenesis (Tremblay et al., 2007). In a recent report, PrP^C displayed a positive effect in the proliferation of neural precursor cells and showed a positive correlation with neuronal differentiation (Steele et al., 2006). In this study, mice over-expressing PrP^C exhibited an increased multipotent neural precursor proliferation in neurogenic regions of the brain. In contrast, ablation of PrP^C resulted in lower neural precursor differentiation compared to wild-type controls.

The capacity of PrP^C to bind to several different molecules has opened the idea that this protein may exert its function in association with a ligand. The location of PrP^C in the extracytoplasmic face of the lipid bilayer restricts the interaction to transmembrane and secreted proteins. Transmembrane variants of PrP^C could potentially interact with cytoplasmic partners; however, these forms are normally present in low amounts in the absence of predisposing mutations in the PrP^C molecule (Stewart et al., 2001). The membrane association and the interaction with ligands suggest the hypothesis that PrP^C may activate transmembrane signaling processes associated to neuronal survival, neurite outgrowth, and neurotoxicity. The stress inducible protein STI-1 has been implicated as a co-chaperone molecule that form part of the heat shock protein (HSP) macromolecular complexes Hsp70 and Hsp 90 (Zanata et al., 2002). The interaction between PrP^C and STI-1 showed high affinity and specificity resulting in neuroprotective functions through the mediators of the cAMP dependent protein kinase (AMPc/PKA) pathway (Zanata et al., 2002; Chiarini et al., 2002). Additionally, interaction with STI-1 induced neuritogenesis through the MAPK pathway as a parallel effect to neuroprotection (Lopes et al., 2005). Neuronal growth has also been observed during PrP^C interaction with the neuron cell adhesion protein N-CAM after its recruitment from lipid rafts and the activation of Fyn kinase (Santuccione et al., 2005). Treatment of cultured neurons

with recombinant PrP^C enhances neurite outgrowth and neuronal survival, concomitant with activation of several kinases, including fyn, PKC, PKA, PI-3 kinase/Akt and ERK (Kanaani et al, 2005; Santuccione et al., 2005).

Laminin is an important glycoprotein of the basal membrane and plays an important role in neuronal proliferation, growth and migration (Westergard et al., 2007). Studies have showed that PrP^C binding to the receptor of laminin is specific and saturable and results in dendritic extension, neuronal migration, axonomic regeneration and suppression of cell death induced by kainic acid injection (Graner et al., 2000, Martin et al., 2001). PrP^C can also interact with the cytoplasmic protein laminin receptor precursor (LRP), which may serve as an endocytic receptor for cellular uptake of both PrP^C and PrP^{Sc} (Gauczynski et al., 2006).

Although, PrP^C has been reported as a cellular anti-apoptotic factor, some experiments have suggested its involvement as neurotoxin effects. Synthetic peptide PrP 106-126 has been used to mimic the effect of PrP^{Sc} on cultured cells (Selvaggini et al., 1993). Incubation with this peptide showed toxic effects on cultured neurons and neuronal cell lines, but only in those that express PrP^C (Forloni et al., 1993; Brown et al., 1994a). These studies suggest that PrP^C may mediate PrP^{Sc} pathogenic action through a signaling pathway.

PrP^C role in TSE pathogenesis. The pathogenesis of TSEs including the mechanism of neuronal degeneration has not been completely elucidated. It is now becoming clear, however, that PrP^{Sc} kills neurons by virtue of its ability to perturb the normal and physiological activities of PrP^C (Westergard et al., 2007). The presence of PrP^{Sc} alone in neuronal cells may result in toxic effects by several mechanisms including blocking axonal transport, interfering with synaptic function, or triggering apoptotic pathways (Westergard et al., 2007). Alternatively, the potential association between PrP^{Sc} and PrP^C during the pathogenic process may result in the loss of PrP^C anti-apoptotic activity, resulting in neuronal death. Evidence that argues against this theory is based on the small phenotypic effect and lack of any features of TSEs observed after ablation of PrP^C either prenatally or postnatally (Bueler et al., 1992; Mallucci et al., 2002). However, it is possible that neurodegeneration may be consequence of both loss and gain in function, by loss of the cytoprotective activity of PrP^C that may become essential in the disease state due to cellular or organism stress.

Another hypothesis for the TSE-pathogenic effect postulates the alteration or subversion of the normal PrP^{C} neuroprotective function. PrP^{C} may act as a membrane-anchored signal transduction that transmits the PrP^{Sc} toxic effect (Chesebro et al., 2005). Neurons obtained from PrP^{C} knockout mice and cultured in vitro were resistant to apoptosis induced by exposure to the synthetic peptide PrP 106-126 (Brown et al., 1994a). This phenomenon may be the consequence of a PrP^{Sc}-induced aggregation of cell surface PrP^{C} that generate a neurotoxic rather than a neuroprotective signal. Cross-linking of PrP^{C} using anti-PrP antibodies resulted in apoptosis of neurons in vivo (Solforosi et al., 2004). Alternatively, amino acid sequences (PrPΔ 105-125) in the PrP^{C} structure have been reported to have receptor activity with cytoprotective functions. These receptor sequences may be blocked by PrP^{Sc} resulting in the delivery of a neurotoxic signal (Li et al., 2007).

BOVINE SPONGIFORM ENCEPHALOPATHY

Epidemiology. Bovine spongiform encephalopathy (BSE) was reported for the first time in the UK in 1986 (Wells et al., 1987). The disease achieved epidemic proportions during the 1990s with more than 182,000 cases worldwide recorded between November of that year and July of 2008. BSE has been detected in 24 countries worldwide showing a decreasing trend in the number of cases since 2003 (2167), 2004 (879), 2005 (561), 2006 (201), 2007 (169) (OIE, 2008).

The origin of BSE has not been clarified but several theories have been formulated. One of these theories postulates the inter-species transmission from scrapie-infected sheep to cattle. Cows became infected after consumption of protein concentrate made from carcasses of sheep contaminated with scrapie. Scrapie has affected sheep for 200 years, is endemic in the UK and is present in a number of countries worldwide (Schwartz, 2003). Although contamination of feed with infected ovine carcasses is possible; experimental transmission of scrapie agent to the bovine has proved difficult, making this hypothesis improbable (Cutlip et al., 1994). A second theory involves the spontaneous destabilization and conversion of PrP^{C} into PrP^{Sc}. Spontaneous or atypical BSE cases have been reported recently, and animals affected by this disease may have served as a source of protein supplements for cattle feed thus spreading the disease in

this manner (Capiobianco et al., 2007). However, one of the most controversial theories to explain the contamination of animal concentrate is the human origin of BSE. Funeral rituals in areas of India involve the cremation of cadavers and the disposal of human remains to rivers. Some of these remains may have been collected by "bonepickers" and used to elaborate bone meal that eventually might have been contaminated with human TSE. By this process, contaminated bone and meat meal exported from the India to Europe and used for animal consumption may have served as a source of BSE transmission (Colchester and Colchester, 2005).

Causal agent. Detection of PrP^{Sc} is highly correlated with the pathology and diagnosis of BSE. However, the sole presence of PrP^{Sc} as the pathogenic agent of TSEs has been debated. Mice infected with scrapie agent developed lesions in areas of the brain that showed low levels of PrP^{Sc} (Parchi and Gambetti, 1995). Moreover, no brain damage was reported in transgenic mice over-expressing PrP^{Sc} (Chiesa and Harris, 2001). These reports suggest that cellular damage is a consequence not exclusively of PrP^{Sc} but also require the participation of additional cofactors including a transitional form of PrP and host-derived proteins or non-protein compounds (glycosaminoglycans or short nucleic acids; Aguzzi et al., 2007). In this scenario, PrP^{C} may play an important role as a mediator in PrP^{Sc} pathogenesis. Mice infected with PrP^{Sc} in which Prnp gene was knocked out from the beginning or during the infectious process showed that PrP^{C} expression is required for cellular damage (Brandner et al., 1996; Mallucci et al., 2003).

Pathogenesis. There are several origins of prion disease pathogenesis that remain to be understood. It is believed that the TSE agent is passed under natural conditions from one animal to the other through oral ingestion. Indeed, the transmission of the PrP^{Sc} agent by the oral pathway has great relevance in some TSEs (e.g. variant Creutzfeldt-Jakob disease, Kuru and BSE). This is not the case in spontaneous forms of this disease in which genetic predisposition seems to be the causative factor (e.g. spontaneous Creutzfeldt-Jakob). Although oral ingestion of the agent is the most commonly known form of contamination, other ways of infection cannot be overlooked, such as injection of contaminated products, skin injuries or iatrogenic ways (Mabbot and MacPherson, 2006). The transport of PrP^{Sc} through the intestinal mucosa is mediated by microfold cells (M cells) located within the epithelium villus and follicle-associated epithelium (FAE) of the Peyer's patches, through the process of transcytosis (Heppner et al., 2001). Under physiological conditions, M cells sample contents of the intestinal lumen and present these antigens to the host immune system for immunomodelation. Some pathogenic microorganisms and potentially PrP^{Sc} can exploit M cells transcytosis to gain entry into mucosal tissues (Neutra et al., 1996).

The transport of TSE agents across the intestinal epithelium; however, might not be entirely mediated by M-cell-transcytosis. PrP^{Sc}-proteon complexes originated from CJD brain homogenate can be endocytosed by intestinal epithelial cells (Caco-2 cells) and transcytosed in vesicular structures by a ferritin-dependent mechanism (Mishra et al., 2004).

After transport through the intestinal mucosa, PrP^{Sc} particles are captured by several types of cells including macrophages, lymphocytes and dendritic cells (DCs) and presented to follicular dendritic cells (FDCs) in the lamina propia. The precise involvement of macrophages in TSE pathogenesis is uncertain but these cells may mediate the transport and also the impairment of PrP^{Sc} accumulation. A recent *in vitro* study showed that macrophage depletion resulted in an earlier increase in PrP^{Sc} accumulation in the lymphoid tissue (Maignien et al, 2005). Lymphocytes are also situated intraepithelial, but are unlikely to be involved in transporting PrP^{Sc} as they do not acquire measurable levels of this agent following intra-intestinal exposure (Huang et al., 2002). DCs are a distinct lineage from stromal derived FDCs that sample antigens in the periphery and deliver them to lymphoid tissues to initiate an immune response (Shortman and Liu, 2002). The location of DCs beneath intraepithelial M cells and their capacity to acquire intestinal antigens by inserting their dendrites between tight junctions (independently from M cells) make these cells good candidates to transport PrP^{Sc} to lymphoid tissues (Beekes and McBride, 2000).

Analysis of the distribution of the PrP^{Sc} within the nervous system of orally inoculated rodents indicates that the agent subsequently spreads from the gut-associated lymphoid tissue (GALT) to the CNS through the enteric nervous system in a process known as neuroinvasion (Beekes and McBride, 2000). The enteric nervous system is an important component of the autonomic nervous system and regulates intestinal motility and secretions through stimuli from sympathetic and parasympathetic nerves. PrP^{Sc} reaches the CNS by spreading in a retrograde direction along efferent fibers of both sympathetic and parasympathetic nerves until they contact the spinal cord. It is not understood how PrP^{Sc} initially spread from the FDCs to the peripheral nervous system. This process may be mediated by mobile intermediate cells or exosomes derived from FDCs, DCs or macrophages that transport PrP^{Sc} particles from FDCs to the peripheral nervous system (Mabbot and MacPherson, 2006). Potential neuroinvasion through the blood-brain barrier has also been suggested; however, the localization of PrP^{Sc} deposits and blood vessels in brains of patients with vCJD does not reflect a haematogenous pattern (Armstrong et al., 2003; Mabbot y MacPherson 2006).

Histopathological changes. In general, histopathological changes observed in TSEs are associated with spongiform degeneration, vacuolization, astrogliosis and PrP^{Sc} deposition (Budka, 1995, MacDonald et al., 1996). However, astrogliosis and neuronal loss are not evident in BSE compared to other TSEs. The presence of amyloid plaques are rare in classic BSE and are mostly found in the thalamus. In contrast, atypical BSE or Bovine Amyloidotic Spongiform Encephalopathy (BASE) has been characterized by the presence of large plaques mainly in the white matter. The molecular signature of BASE also differs from BSE and resembles CJD (Casalone et al., 2004). Several theories have been proposed to explain BASE presentation including spontaneous occurrence of TSE in animals as in sporadic CJD. Classic BSE is also characterized by spongiosis and vacuolization of the neuropil and neuronal bodies through simple or multiple vacuoles in the neuronal perikarya (Wells et al., 1989). Neural tissues most consistently and severely affected are the solitary tract nucleus, the spinal tract nucleus of the trigeminal nerve, and the central gray matter of the midbrain (Wells et al., 1989). Neuropil vacuolization of the target nuclei is considered to be pathognomic in BSE. Intraneuronal vacuolation is also observed in BSE, but this feature alone in the absence of neuropil vacuolation is not confirmatory. Vacuolated neurons particularly in certain locations such as the red nucleus may be an incidental finding in cattle (Gavier-Widen et al., 2001). Immunohistochemical analysis consistently reveals PrP^{Sc} accumulation in the brain, with distribution similar to but often more widespread than neuropil vacuolation. Patterns of PrP^{Sc} deposition in the brain include intraneuronal, perineuronal, linear, fine punctuate and coarse particulate (Wells and Wilesmith, 1995). PrP^{Sc} cannot readily be detected in tissues outside bovine CNS; however, limited involvement of the Peyer's patches has been documented in experimentally induced and naturally acquired cases of BSE (Terry et al., 2003).

Diagnosis. All diagnostic methods currently available require post-mortem confirmation. Despite characteristic clinical signs, diagnosis of BSE cannot rely solely on the clinical course and requires histopathological analysis. Initial histopathological diagnosis is based on vacuolar changes in the brain, observation of florid plaques, astrogliosis and neuronal loss. However, presentation of cases with no evident or ambiguous histopathological changes requires confirmation through more specific and sensitive methods such as immunohistochemistry, western blot and/or ELISA (Gavier-Widen et al., 2005, OIE, 2004). Given the pivotal role played by PrP^{Sc} in TSE pathogenesis, diagnostic methods rely on PrP^{Sc} detection by specific antibodies and partial proteinase K digestion that allows differentiation between both PrP isoforms. In recent years, some antibodies have claimed to differentiate between PrP^{C} and PrP^{Sc};

however, none of these molecules have proved suitable for direct identification of PrP^{Sc} (Korth et al., 1997; Curin et al., 2004; Zou et al., 2004).

Consistent early accumulation of PrP^{Sc} and vacuolar lesions in the medulla oblongata at the level of the obex (brainstem) make this area of the brain an optimal site for the post-mortem diagnosis of BSE. An appropriate sample for BSE diagnosis should include the solitary and trigeminal tract nuclei (Jeffrey and Gonzales, 2004). Brainstem sample may be conveniently obtained by introducing a commercially available, long, spoon-shaped metal or disposable instrument with cutting edges through the foramen magnum to facilitate sampling of the brainstem. Alternatively, the sample could be obtained by dismantling the calvarium (e.g. when retrieving the whole brain for rabies diagnosis). The brainstem sample should be promptly refrigerated (at 4°C not frozen) or fixed in 10% formaldehyde until shipment to the diagnostic laboratory to avoid post-mortem decomposition (Gavier-Widen 2005, USDA, 2006).

Several diagnostic methods based on immunological techniques have been validated and officially accepted for BSE. Currently, the BioRad diagnostic immunoassay is used as a rapid test for BSE detection. Advantages of this method include easy manipulation, fast results (24 h), high sensitivity, and detection of PrPSc infection during preclinical stages (Soto, 2006). This immunoassay is based on the capture of PrP^{Sc} present in the sample by antibodies attached to a microtitulation plate. Incubation of captured PrP^{Sc} by anti-PrP antibodies allows secondary antibody detection and visualization with a colorimetric reaction. However, high presentation of false positive results by this method recommends the use of confirmatory techniques (USDA, 2005). High specificity, visualization of the spongiform lesions and PrP^{Sc}-specific immunostaining make the immunohistochemistry a primary choice for confirmatory diagnosis of BSE. This method requires formalin-fixation and paraffin-embedding of the samples. Tissue is sectioned and mounted in glass slides. Mounted tissue sections are exposed to proteinase K digestion and epitope unmasking through immersion in citric buffer. PrPSc is detected by incubation with anti-PrP antibodies followed by secondary antibodies associated to horseperoxidase staining or fluorescence dyes (OIE, 2004). Samples with moderate levels of decomposition may not be suitable for the immunohistochemistry method but may be used for western blot analysis. This method has similar specificity compared to immunohistochemistry but does not allow histological analysis. Currently, this technique can be performed in a short period of time with high sensitivity. Tissue sample lysates are treated with proteinase K for PrP^{C} digestion. Total protein contained in samples are separated by electrophoresis in 12% SDS polyacrylamide gels and blotted into nitrocellulose or polyvinylidene

fluoride (PVDF) membranes. PrP^{Sc} is specifically detected by incubation with anti-PrP antibodies followed by secondary antibodies.

The requirement of a pre-mortem diagnostic method is desirable considering the inability to apply conventional methods for nucleic acids or antiserum detection such as PCR or ELISA. During the last years, substantial efforts have been applied in the development of PrP^{Sc}-specific antibodies for the potential diagnostic of infectivity in fluids and tissues. Despite the significant differences in secondary structure between both PrP isoforms, the development of such antibodies is still incomplete (Demart et al., 1999, Groschup et al., 1997, Kascsak et al., 1997, Curin et al., 2004). PrP^{Sc} has been detected in the blood of infected animals, which represents a potential strategy for early diagnosis of TSEs (Castilla et al., 2005). However, levels of PrP^{Sc} in blood are very low making even highly sensitive techniques such as immunocapillary electrophoresis unable to detect the agent (Schmerr and Jenny., 1998). One important step in the development of such a diagnostic tool was the recently reported development of a protein misfolding cyclic amplification technique (PMCA). This method was able to simulate PrP^{Sc} replication in a test tube and increase the detection threshold of this protein by 10 million times (Castilla et al., 2005). This new technology enables an efficient, specific and rapid detection of prions offering great promise for developing a noninvasive early diagnosis of TSEs.

Conclusions. The complex nature of the prion biology has intrigued the scientific community for more than 70 years and yet there are many questions to be answered. The establishment of the controversial prion hypothesis opened a new perspective in protein biology that involved the participation of these molecules in diseases as pathogenic and infectious agents. Not only TSEs, but other neurodegenerative diseases such as Alzheimer's have showed to be the consequence of the misfolding and deposition of host-encoded proteins with severe neuropathology. Currently, there are no ways to cure, treat or immunize against these diseases, and the consequences for public health and economic costs have proven to be tremendous. However, important advances during the last years in the evaluation of prion biology have allowed a better understanding of the pathogenesis of this disease and have opened new opportunities for treatment.

One of the most intriguing aspects in prion biology is the still enigmatic physiological function of the PrP^{C}. This mysterious protein has been implicated in several biological processes including cellular

protection against oxidative stress and apoptosis, cell-to-cell adhesion, cellular proliferation and differentiation. Recently, important evidence has been reported supporting the idea that PrP^C is involved in signaling pathways associated with cellular proliferation and differentiation. In the present work, we first describe the analyses performed to characterize the expression of PrP^C in several somatic and reproductive tissues. These analyses suggest the participation of PrP^C in various physiological processes involving cellular differentiation and tissue development. Therefore, in the final two chapters we describe the efforts to elucidate the potential participation of PrP^C in cellular differentiation by the utilization of developmental models including embryos and embryonic stem cells.

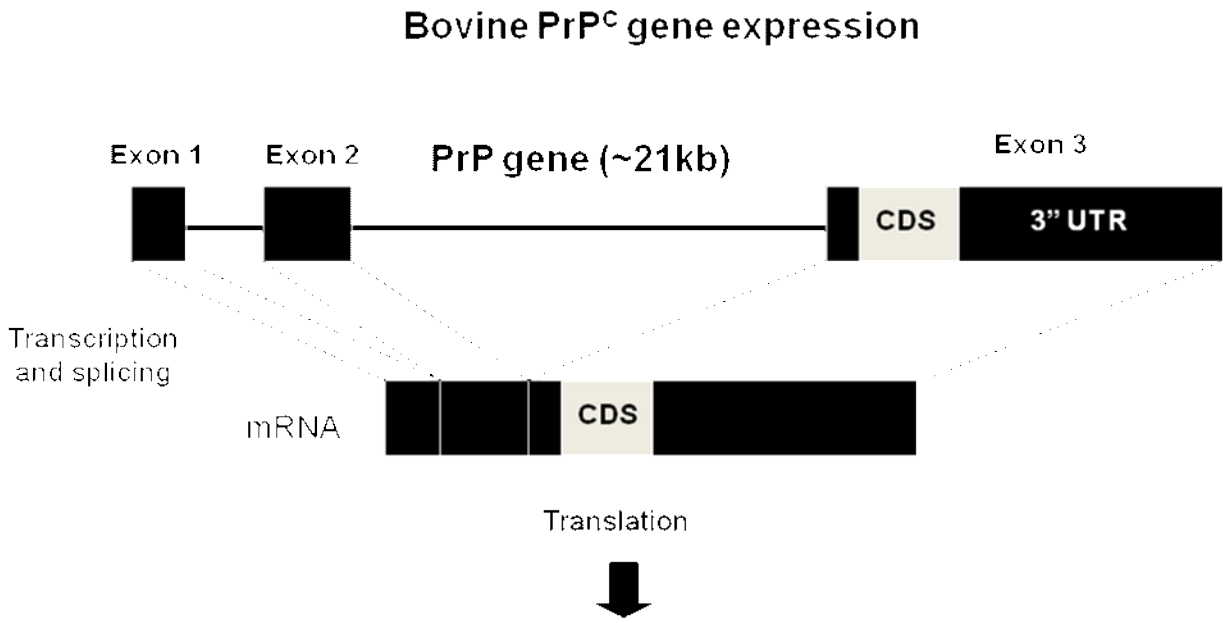

Figure 1. Structure of the Prnp gene and mRNA. The Prnp gene size is aproximate 21 kb. After transcription and splicing, the mRNA molecule is formed by the exons 1, 2 and 3. Exon 3 carries the coding sequence that encodes the PrP^C protein after translation.

PrP isoforms structure

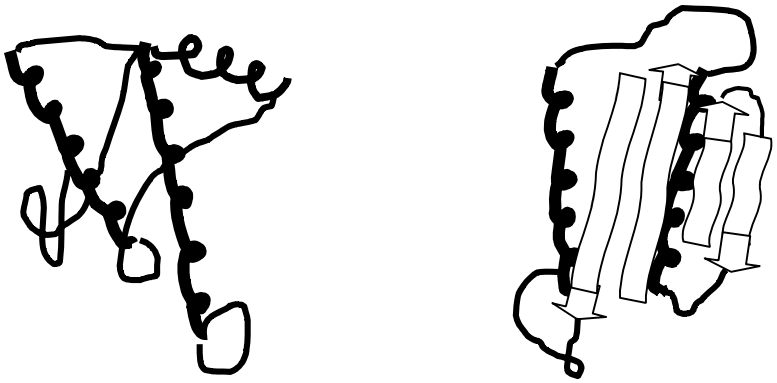

Figure 2. Structure of PrP^{C} and PrP^{Sc} isoforms. PrP^{C} and PrP^{Sc} have important differences in secondary protein configuration. Structure of PrP^{C} is conformed by a high α-helicoidal (47%) and low β-sheet configuration (3%). In contrast, PrP^{Sc} is characterized by high β-sheet (43-45%) and low α-helicoidal (17-30%) proportion.

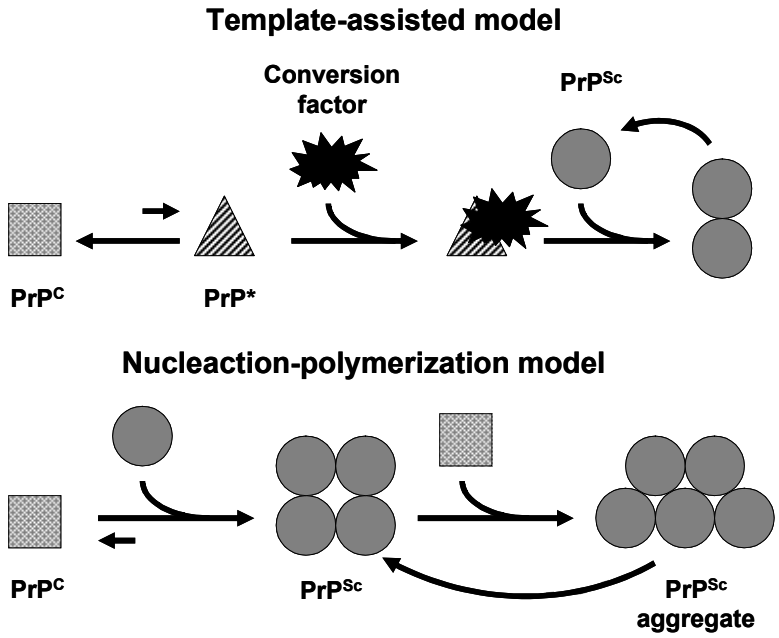

Figure 3. Models for conversion between PrP^{C} and PrP^{Sc}. The template assisted model includes the participation of a conversion factor that mediates PrP conversion. The nucleation-polymerization model describes a PrP^{Sc} heterodimer intermediate complex that induces aggregation and conversion. Both models propose a thermodynamic equilibrium for PrP^{C}-PrP^{Sc} conversion (Soto, 2006).

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CHAPTER II

COMPARATIVE ANALYSIS OF THE EXPRESSION OF THE CELLULAR PRION PROTEIN (PrP^C) IN SOMATIC TISSUES OF THE BOVINE ADULT

INTRODUCTION

The cellular prion protein (PrP^C) is a 250-amino acid glycoprotein commonly found attached by a glycosylphosphotidylinositol (GPI) anchor to lipid rafts in the plasma membrane. Through a poorly understood process, PrP^C is post-transcriptionally converted from a predominantly α-helical structure to a mainly β-sheet isoform (PrP^{Sc}). Substantial evidence indicates that PrP^{Sc} is the principal if not the only component of the agent causing transmissible spongiform encephalopathies (TSEs). This neurodegenerative and infectious group of diseases affects several mammalian species including human (Creutzfeldt-Jakob disease), bovine (spongiform encephalopathy) sheep (scrapie), mink (transmissible encephalopathy) and cervids (chronic wasting disease). Although much is known about the role of PrP^{Sc} in prion diseases, the normal function of PrP^C is poorly understood. One line of investigation proposes that PrP^C is an antioxidant factor that directly or indirectly promotes detoxification of reactive oxygen species (ROS) (Milhavet et al., 2002). Another hypothesis has associated this molecule with cytoprotective activity by blocking the internal or environmental stresses that initiate the apoptotic program (Roucou et al., 2004 and 2005). Furthermore, several authors have recently proposed that PrP^C participate in transmembrane signaling processes associated with cellular survival, replication and differentiation (Mouillet-Richard et al., 2000; Schneider et al., 2003).

Mice devoid of PrP^C have showed resistance to scrapie infection indicating that PrP^C expression is required for the infection process (Bueler et al., 1993). Thus, understanding the tissue-specific expression of PrP^C is crucial considering that cells expressing high levels of PrP^C bear a risk for conversion and accumulation of PrP^{Sc}. Paradoxically, there is a lack of information about the distribution of PrP^C protein in bovine tissues, which are regarded as a source of variant CJD. Tissue-specific analyses in mice and hamsters have demonstrated a ubiquitous presence of PrP^C with intense expression in murine neurons and lymphoreticular cells (Ford et al., 2002a,b; Bailly et al., 2004; Ning et al., 2005). PrP^C has also been localized in human, mouse and bovine digestive cells (Pammer et al., 2000; Marcos et al., 2004, 2005; Amselgruber et al., 2006) and bovine renal cells (Amselgruber et al., 2005). Moreover, reproductive tissues in mice (Ford et al., 2002a) and skin in human (Pammer et al., 1998) have shown cellular-specific immunoreactivity for PrP^C. Lymphoreticular and neural cells actively participate in the pathogenesis of TSEs through the transport, replication and accumulation of PrP^{Sc}. Immune cells are believed to be responsible for the transport of PrP^{Sc} from the intestinal lumen through the enteric wall after oral inoculation. Thereafter, PrP^{Sc} is transported to lymphatic tissues where it replicates and initiates

colonization of the nervous system. After a long period of incubation that ranges from one to five years in cattle, PrP^{Sc} is able to infect the CNS inducing the characteristic spongiform degeneration and neuropathological symptoms.

In the present study, we sought to analyze and compare PrP^{C} expression in fifteen different somatic bovine tissues by western blot and immunohistochemistry. Computerized quantification of western blot bands showed that PrP^{C} was expressed in all tissues analyzed. However, PrP^{C} is differentially expressed, showing intense levels in neural tissues and reduced levels in muscle and liver. Specific antibody staining revealed that PrP^{C} was expressed in cell-specific manner in a wide range of organs including brain, thymus, intestine, lung, and skin. The wide-spread expression of PrP^{C} in bovine tissues suggests that this protein may have important biological functions for this molecule. High levels of PrP^{C} expression in organs not commonly involved in TSE pathogenesis suggest that a higher number of tissues may be at potential risk for PrP^{Sc} infection and transmission.

MATERIAL AND METHODS

Tissue collection

Bovine tissues were obtained from three, 13 month old, healthy Angus steers. Animals were slaughtered at an abattoir located on campus. Samples of the following tissue were collected within 20 min of slaughter: cerebellum, obex, spinal cord (Pars thoracalis), sciatic nerve, mesenteric lymph node, thymus, spleen, liver, pancreas, ileum, kidney, heart, skin and skeletal muscle. Samples for western blot were placed in a glass container and frozen on dry ice. For immunohistochemistry, samples were fixed in 10% formalin.

Western blot

Frozen tissue samples of < 700 mg were thawed and homogenized (10 w/v) in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton-X-100, 1% deoxycholate, 0.1% SDS) using a pestle homogenizer (Fisher Scientific, Hampton, NH, USA). Homogenates were centrifuged at 13,500 rpm for 5 min and the supernatants transferred into a new tube. Total protein concentrations were

determined using a Bicinchoninic acid (BCA) kit (Pierce; Rockford, IL) according to the manufacturer's instructions. For protein denaturation, 50 µl of each homogenized sample was mixed with 50 µl of Laemmli buffer (BioRad Laboratories, Hercules, CA, USA) and heated at 98° C for 5 min. Aliquots containing 20 µg of total protein were added to each lane and separated by SDS-PAGE in 12% gels (BioRad). Electrophoresis was performed at 125V for 60 min. Proteins were then transferred onto PVDF membranes by electroblotting at 100 V for 1h. Membranes were immersed in blocking buffer (LI-COR Corp., Lincoln, NE, USA) for 1 h with shaking. PrP^C was detected by incubation for 1 h in SAF-32 mouse monoclonal anti-PrP (1:400; Cayman Chemical Company, Ann Arbor, MI, USA) directed against amino acid sequence 59-89 located in the N-terminal octapeptide repeat region of the protein. For reference, membranes were co-incubated in rabbit anti-GAPDH (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Both primary antibodies were diluted in 0.1% Tween-20 in blocking buffer. After four washes in 0.1% Tween-20 in phosphate-buffered saline (PBS) for 5 min each, membranes were incubated in secondary IgG fluorescent anti-mouse and anti-rabbit antibodies (1:5000; LI-COR) diluted in 0.1% Tween-20 in blocking buffer for 30 min with shaking. Immunoreactive bands for PrP^C were quantified and added as integrated intensity values using an Odyssey infrared imaging system (LI-COR). Relative expression of PrP^C was corrected by GAPDH expression and standardized to the highest expression value (cerebellum).

Immunohistochemistry

Formalin-fixed tissues were embedded in paraffin and sectioned at 5-7 µm using a microtome (Historange, LKB Bromma, Sweden). Tissue sections were mounted on adhesive coated slides (Newcomer supply; Middleton, Wisconsin) and incubated overnight at 37 °C. Mounted tissues were deparaffinized in xylene and dehydrated in serial alcohol solutions. Slides were subjected to an unmasking protocol that employed unmasking solution (Vector Laboratories., Burlingame, CA, USA) and autoclaving at 120°C for 5 min. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide diluted in 0.1 M PBS (pH 7.4) for 30 min. Tissues were then rinsed two times in 0.1 M PBS and blocked in 2.5% horse serum for 15 min. PrP^C was specifically detected by overnight incubation at room temperature with primary antibody SAF-32 (1:400) diluted in 1.5 % equine serum solution (Vector Laboratories). After two washes in 0.1 M PBS (pH 7.4), bound primary antibody was detected using horse anti-mouse secondary antibody complexed to horseradish-peroxidase for 10 min at room temperature (Vector Lab). Immune

complexes were visualized using 3,3'-diaminobenzidine (DAB) substrate for 5 min or until the signal became visible. Probed sections were then counterstained with hematoxilyn and rehydrated in serial alcohol solutions. Sections were mounted with Permount mounting medium (Fisher Scientific) and coverslides. Neighboring sections processed identically using horse serum instead of primary antibody, served as controls. Digital photos of tissue sections were obtained using bright microscopy (Olympus Vanox-T, Tokyo, Japan).

Data Analysis

Statistical analyses for quantitative western blot were performed using SAS software (version 9.3.1, SAS Institute Inc., Cary, North Carolina, USA). Analyses were repeated at least three times for statistical significance. Analyses of significance ($P < 0.05$) were performed using One-way ANOVA. Expression values for individual tissues were compared to the highest expression (cerebellum) using Dunnet's t-test; whereas, significant differences between tissues were analyzed using Duncan's multiple comparison test.

RESULTS

Western blot

Independent assays comparing the relative levels of PrP^C expression were performed on tissues from three different animals by western blot. PrP^C was detected in all tissue samples in this study. PrP^C showed three distinct migration bands corresponding to molecular weights of 35, 28 and 25 kDa (Fig. 1a). Although we did not specifically analyze bands for glycosylation, each band is likely associated with the di-, mono- and un-glycosylated isoforms of PrP^C (Priola and Vorberg, 2006). The di-glycosylated isoform was predominant form across all tissues; whereas the mono- and un-glycosylated bands showed more variable intensities. Di-glycosylated PrP^C displayed strong bands in CNS tissues and thymus, whereas, immunoreactivity for the same isoform was low in pancreas and liver. In peripheral nerve, intestine, lung and heart the di-glycosylated PrP^C was observed as a doublet. Un-glycosylated PrP^C was undetectable in sciatic nerve and lymphatic tissues. Computerized quantification of PrP^C bands showed the highest ($P<0.05$) PrP^C relative

value in the cerebellum (Fig. 1b). Lowest PrP^C levels were found in liver and pancreas. Expression of PrP^C was higher in neural tissues compared with non-neural tissues with the exception of the thymus, which showed the highest ($P<0.05$) levels of PrP^C among non-neural tissues.

Immunohistochemistry

In order to establish the precise cellular localization of PrP^C within the tissues analyzed, we performed immunohistochemistry using anti-PrP SAF-32 monoclonal antibody. All results are typical for multiple experiments and were in general congruent with the western blot analysis. No staining was observed in the negative control sections incubated with normal horse serum instead of SAF-32 antibody.

Nervous system. Among tissues analyzed, the most intense and wide distribution of PrP^C immunostaining was observed in the nervous system. PrP^C labeling in the cerebellum was confined to the gray matter and appeared homogenous and diffuse on neuron bodies and the neuropil (Fig 2a). At the cellular level, PrP^C immunoreactivity was present in unmyelinated fibers, granule cells, and stellate and basket cells of the molecular layer. Purkinje cells observed in all the extensions of the central layer showed intense PrP^C staining (Fig. 2c). Similarly, PrP^C immunoreactivity was intense in neuronal bodies of the solitary tract nucleus in the obex (Fig. 3a, c). Astrocytes and oligodendrocytes observed around neurons showed moderate levels of PrP^C labeling (Fig. 3c). Immunoreactivity for PrP^C was analyzed in the thoracic portion (Pars thoracalis) of the spinal cord. In this tissue, the pattern of staining was confined to the gray matter (Fig. 4a). PrP^C labeling was also observed in neuronal tracts emerging from the gray matter into the white matter of the spinal cord. Analysis of PrP^C distribution in peripheral nerves was performed in transverse tissue sections obtained from the sciatic nerve. PrP^C labeling was restricted to the neural fibers contained in nerve fascicles (Fig. 5a). No PrP^C reactivity was observed in the connective tissue forming the perineurium or the epineurium.

Lymphoreticular system. Lobules in the cortex of the thymus were intensely labeled for PrP^C (Fig 6a). Observation with higher magnification evidenced a cell-specific staining associated with stromal cells with the appearance of lymphocytes of the T cell lineage (Fig 6c,e). Less intense PrP^C immunoreactivity was detected in epithelial cells located in the medulla. The intense and

wide immunoreactivity observed in the thymus contrasted with a scattered staining detected in the spleen. PrP^C-positive cells with the appearance of myeloid dendritic cells were located in the perilymphoid zones of the red pulp immediately adjacent to nodules of white pulp (Fig. 7a,c,e,f). Mesenteric lymph nodes showed cellular PrP^C staining associated with germinal centers and surrounding areas of secondary lymphoid follicles in the cortex (Fig. 8a). PrP^C-positive cells had the morphology and location of B lymphocytes and follicular dendritic cells (FDCs) (Fig. 8c,e).

Gastrointestinal system. PrP^C immunohistochemical analysis was performed in the ileum section of the intestine. Staining was intense and restricted to enteric neural cells present in the intestinal wall. PrP^C-positive neural cells were observed in the lamina propia within intestinal crypts (Fig. 9a,c). Labeling was also present in neural fibers located in parallel to muscle fibers in the inner circular muscular layer. Between the inner and outer layers of the muscularis, clusters of parasympathetic ganglion cells associated to the myenteric plexus showed intense PrP^C staining throughout the extension of the sections (Fig. 9c,d). In pancreas, PrP^C labeling was restricted to the endocrine tissue conformed by the islets of Langerhans (Fig. 10a,c). No staining was observed in the exocrine pancreatic tissue. Liver tissue showed a weak immunoreactivity PrP^C (Fig 11a).

Skeletal, smooth and cardiac muscle. Skeletal muscle samples were obtained for the gluteus muscle. PrP^C immunoreactivity was not observed in all the extension of the skeletal muscle sections (Fig. 12a). In contrast, neural fibers innervating smooth muscle fibers in the enteric muscularis layers showed strong immunoreaction for anti-PrP antibody (Fig. 9a). Similarly, a homogeneous PrP^C labeling was observed associated with cardiac muscle cells in the myocardium (Figure 13a,c).

Miscellaneous tissues. PrP^C labeling in the lung was mainly associated to the alveolar wall (Fig. 14a). At the cellular level, PrP^C staining appeared to be present in all cells forming the alveolar sacs including pneumocites (Fig. 14c). In the kidney, PrP^C positive staining was observed in cortical convoluted tubules and collective ducts located in the medulla (Fig. 15a). Renal glomeruli showed strong PrP^C labeling associated to extraglomerular mesangial cells, podocytes and endothelial cells (Fig. 15c). The skin tissue sample obtained from the flank area displayed PrP^C labeling in keratinocytes localized in the epidermis and outer sheaths of the hair follicle (Fig. 16a,c). Furthermore, horizontal sections at the level of the dermis allowed the detection of PrP^C immunoreactivity in sebaceous glands (Fig. 16e).

DISCUSSION

Neuropathological findings in animals and humans affected with prions indicate a correlation between PrP^{C} expression and accumulation of PrP^{Sc}. Moreover, the mechanism of the PrP^{C} to PrP^{Sc} conversion does not occur in scrapie-infected *Prnp*$^{0/0}$ mice, indicating the necessity for the infected host cells to express PrP^{C} in order to support conversion or replication (Bueler et al., 1993). Identification of cell types expressing PrP^{C} is necessary to better understand how the agent replicates and spreads from the periphery to the CNS. Furthermore, a spatial localization analysis of PrP^{C} expression may help in the understanding of its cellular function.

The tissue-specific comparative western blot analysis performed in our study is the first reported in the bovine specie. Different PrP^{C} glycosylation patterns were observed among tissues analyzed. Implication for this variability is not completely understood; however, variations in PrP^{C} glycosylation have been shown to affect susceptibility to PrP^{Sc} infection in mouse brain (DeArmond et al., 1999). In our study, the un-glycosylated isoform of PrP^{C} showed undetectable levels in lymphatic tissues. Some authors have proposed that variations in PrP^{C} molecular features could be related to the absence of detectable infectivity in peripheral lymph organs in BSE-affected cattle (Thielen et al., 2001). A significantly higher PrP^{C} expression was observed in neural tissues versus non-neural tissues. We performed a parallel tissue-specific analysis of PrP^{C} mRNA expression using the same tissue samples analyzed by western blot (data not shown). Higher PrP^{C} mRNA expression levels were observed also in neural tissues. However, we found no full correlation when mRNA expression was compared to the protein level. Previous reports of PrP^{C} mRNA using quantitative-PCR analysis in the bovine (Tichopad et al., 2003), hamster (Ning et al., 2005) and sheep (Han et al., 2006) have also shown discordance with western blot analysis. Disparity between PrP^{C} transcription and translation may explain the differences in the efficiency of message and/or protein expression and degradation among tissues.

Computerized quantification of western blot bands showed higher levels of PrP^{C} expression in the cerebellum compared to the spinal cord and peripheral nerves. PrP^{C} expression seems to be more intense in organs with higher proportion of gray matter (e.g. cerebellum) than in those with predominantly white matter structures (e.g. spinal cord). Lower levels of PrP^{C} in peripheral

nerves may be explained by the heterogeneous conformation of this tissue, consisting of a high proportion of collagen and myelin.

Western blot analysis of PrP^C in bovine somatic tissues showed the most intense immunoreactivity in the nervous system. Strong bands associated to PrP^C in lymphoid tissues, especially thymus, indicate that the second major source of PrP^C is located in the lymphoreticular compartment. The remaining organs displayed bands of weak intensity suggesting either low expression of PrP^C in the tissue or cellular-specific immunoreactivity.

PrP^C is mainly found attached to the plasma membrane as a GPI-anchored glycoprotein; however, our immunohistochemical protocol displayed a diffuse PrP^C staining not limited to the plasma membrane but covering the neuronal cytoplasm. Although a proportion of PrPC is located in the cytoplasm, this phenomenon may also be explained by the diffusion of the marker present in the immunoperoxidase method that can create an even distribution of the immunostaining throughout the tissue. Similarly to previous studies, our immunohistochemical analysis detected more intense PrP^C staining in gray matter areas compared to white matter regions of the CNS (Ford et al., 2002b; Diaz-San Segundo et al., 2006). PrP^C signal in the gray matter is mainly associated with the neuropil, defined as the region containing the dendritic tree of neurons, axons terminals and synapses (Taraboulos et al., 1992). Neuropil expression of PrP^C predisposes the accumulation of PrP^{Sc} and correlates with vacuolization observed in histological findings of cattle affected by BSE. Bilaterally symmetric vacuolization of the gray matter neuropil (spongiosis) is considered the most characteristic histological change in BSE (Gavier-Widen et al., 2005). Furthermore, PrP^{Sc} immunoreactivity has revealed that this agent is present in the neuropil but in some cases can spread to neural bodies (Wells and Wilesmith, 1995). Some authors have explained the weak PrP^C immunoreaction in white areas of the CNS by the resistance of myelin-coated pits to protein blotting or penetration by specific PrP antibodies (Taraboulos et al., 1992; Moleres and Velayos, 2007). However, PrP^C labeling has been co-localized with specific markers of astrocytes and oligodendrocytes present in the rat white matter and was detected in glia and axons in the cerebellum of mice (Moleres and Velayos, 2007; Taraboulos et al., 1992). These data may explain the presence of PrP^{Sc} in the white matter of infected brains and support the hypothesis that PrP^{Sc} is axonally transported to the CNS.

Similar to our results, immunoreactivity for PrP^C in mice cerebellum has been described previously in Purkinge, granular cells, and stellate and basket cells of the molecular layer (Ford et

al., 2002b). In Purkinge and granular cells, PrP^C expression has been confirmed by immunogold and GFP-labeling (Lemaire-Vieille et al., 2000; Bailly et al., 2004). Intense PrP^C expression in these cells may explain the specific accumulation of PrP^{Sc} plaques or fine depositions in the molecular and granular layers. These findings have been observed in patients suffering from TSEs that target the cerebellum including Creutzfeldt-Jakob, Gerstmann-Straussler-Scheinker and kuru (Bell and Ironside, 1993).

Consistent early accumulation of PrP^{Sc} in the bovine medulla oblongata at the obex level makes this area of the brain an optimal site for BSE diagnosis. Our analysis in the solitary tract nucleus of the obex detected intense PrP^C immunoreaction in the neuropil, neuroglia and in all neuronal bodies present. In the bovine, areas most consistently and severely affected in the obex are the solitary tract nucleus and spinal tract nucleus of the trigeminal nerve, which vacuolization is considered to be pathognomonic for BSE (Wells et al., 1989). Intense immunoreactivity in neurons of the obex, may explain neuronal degeneration during PrP^{Sc} infection, since neuronal bodies expressing high levels of PrP^C appeared to be particularly sensitive to PrP^{Sc} neurotoxicity (Guentchev et al., 1998; Ford et al., 2002).

Despite the large number of reports, the role of PrP^C in the nervous system has not been completely clarified. Cellular localization of PrP^C along axons and in proximity to presynaptic membrane domains suggest that PrP^C could be involved in synapse formation or transmission (Collinge et al., 1994). Incubation of cultured hippocampal neurons with recombinant PrP^C has been shown to induce rapid elaboration of axons and dendrites, and increase the number of synaptic contacts (Kanaani et al., 2005). Moreover, *Prnp* null mice have been reported to display neurobiological abnormalities related to synapse function such as circadian rhythm (Tobler et al., 1996) and spatial learning (Criado et al., 2005). However, several reports have also described a role for PrP^C against oxidative stress in neurons. Cerebellar granular and neocortical cells cultured from *Prnp* null mice are more susceptible than wild-type counterparts to treatments with agents that induce oxidative stress, including hydrogen peroxide, xanthine oxidase and copper ions (Brown et al., 1997 and 2002). Consistent with these results, brain tissue from *Prnp* null mice exhibits increased levels of protein carbonyls and lipid peroxidation products, which are indications of intense oxidative stress (Wong et al., 2001). Recently PrP^C has also been involved in neurogenesis and differentiation *in vitro* and *in vivo*. Loss- and gain-of-function experiments demonstrate that PrP^C levels correlate with differentiation of multipotent neural precursors into mature neurons *in vitro*. Moreover, PrPC overexpresser mice showed higher cellular proliferation

in the subventricular zone of the nervous system compared to wild-type and knockout mice (Steele et al., 2006).

The thymus showed the highest levels of PrP^C expression among non-neural tissues analyzed. Previous studies have described PrP^C expression in epithelial cells of the medulla and cortex in mice thymus; however, with lower distribution as reported here (Lemaire-Vieille et al., 2000; Ford et al., 2002a). Our analysis showed intense PrP^C labeling in the cortex associated to epithelial cells with the morphology of lymphocytes from the T lineage. Thymus function consists on the development of immunocompetent T lymphocytes derived from the bone marrow (Wheater et al., 1993). PrP^C expression is regulated during lymphocyte development in both thymus and bone marrow (Kubosaki et al., 2001; Liu et al., 2001). Moreover, in mice, PrP^C is believed to be a surface molecule that participates in T lymphocyte activation (Mabbot et al., 1997). All these data suggest that PrP^C participates as a T lymphocyte inducing factor and that this action is initiated at early stages of maturation in the thymus. As part of the lymphoreticular system, the thymus has also been shown to participate in TSEs infection. Studies in mice described a rapid accumulation of PrP^{Sc} in the thymus after inoculation, suggesting that this organ has an active role in replication of the agent (Fraser and Dickinson, 1978; Muramoto et al., 1992).

Despite its known involvement in TSE pathogenesis, the spleen displayed low levels of PrP^C expression in both western blot and immunohistochemical analyses. Most cell types and stromal elements were not reactive to SAF-32 antibody and the staining was restricted to scattered cells concentrated adjacent to the marginal zone of the white pulp. Similar results were reported in the human spleen, where PrP^C was co-localized with MHC class II lineage-defining antigens consistent with the distribution and phenotype of myeloid dendritic cells (DCs) (Burthem et al., 2001). The same study showed that PrP^C was specifically expressed in myeloid DCs and not in follicular dendritic cells (FDCs), B cells and macrophages of the spleen. Myeloid DCs are derived from bone marrow precursor cells or from monocytes and migrate into lymphoid areas after receiving maturation stimulus (Burthem et al., 2001). Migratory myeloid DCs are able to enter the intestinal wall from the bloodstream and sample antigens from the gut lumen providing a potential cellular bridge to lymphoid tissues that serve for PrP^{Sc} replication (Huang et al., 2002). The spleen has an important participation in PrP^{Sc} pathogenesis evidenced by the high titers of infectivity exhibited in mice during early stages of PrP^{Sc} infection. Sheep orally inoculated with BSE agent showed PrP^{Sc} deposition in the same marginal zone of the white pulp where myeloid

DCs PrP^{C}-positive cells were observed in our study (Andreoletti et al., 2006). Nevertheless, some reports have showed that PrP^{Sc} deposition does not occur in the spleen of BSE-infected cattle (Somerville et al., 1997; Buschmann and Groschup, 2005).

Our analysis in the mesenteric lymph node showed PrP^{C}-positive cells in germinal centers and surrounding areas of lymphoid follicles. The location indicates that these cells may be FDCs or/and B lymphocytes (Klein et al., 1998; Thielen et al., 2001). In addition to myeloid DCs, both FDCs and B lymphocytes have direct and indirect participation in PrP^{Sc} pathogenesis. FDCs are critical for PrP^{Sc} replication and accumulation in mice lymphoid tissues and have participation in neuroinvasion (Brown et al., 1999; Mabbot et al., 2000). B lymphocytes have not been directly involved in PrP^{Sc} delivery but are essential for FDCs maturation (Kosco-Vilbois et al., 1997; Chaplin and Fu, 1998). In mice, PrP^{C} is highly express in FDCs, which mediate accumulation of PrP^{Sc} in lymph nodes (Bruce et al., 2000; Schreuder et al., 1998). PrP^{C} is also expressed in bovine FDCs; however, BSE infectivity has been detected only in lymph nodes from experimentally inoculated cows (Wells et al., 1994; Terry et al., 2003) but not from naturally affected cattle (Iwata et al., 2006; Terry et al., 2003). Contrary to our results, Thielen et al, (2001) previously reported undetectable levels of PrP^{C} expression inside the bovine lymphoyd tissue using SAF-32 antibody. Differences in results may be related to the requirement of an epitope unmasking step for the immunoreaction of PrP^{C} in the lymph node. Furthermore, PrP^{C} immunoreactivity may be critically affected by the fixatives used.

Localization of PrP^{C} in the intestine is of considerable importance due to its role as a major route for entry of TSEs agents. Previous reports in hamster, mouse, human, rat, bovine and monkey have described intense PrP^{C} expression in the epithelium of the intestinal wall (Fournier et al., 1998; Ford et al., 2002a; Pammer et al., 2000; Marcos et al., 2004, 2005). In mouse, rat and human, PrP^{C} immunoreactivity was detected in enteroendocrine cells (Ford et al., 2002a; Marcos et al., 2004; Pammer et al., 2000). Other authors have described PrP^{C} immunolabeling in mucous and parietal cells in humans (Fournier et al., 1998; Pammer et al., 2000), in mucous, parietal and goblet cells in hamsters (Fournier et al., 1998, 2000) and in DCs in rat intestine (Miyazawa et al., 2007). In our study, absence of PrP^{C}-specific labeling in the enteric epithelium maybe related to differences in the antibody used. However, we detected intense PrP^{C} staining in neurons located within enteric crypts and inserted in parallel to muscularis fibers. Furthermore, strong PrP^{C} immunoreactivity was observed in the myenteric plexus. Whether enteric neurons participate in PrP^{Sc} transport from intestinal lumen is unknown. Parasympathetic pre-ganglionic neurons in the

vagal trunks derived from the dorsal motor nucleus innervate the mucosa and submucosa of the gastrointestinal tract, via the myenteric plexuses. These motor neurons of the vagus nerves represent a potential route for PrP^{Sc} transport after oral inoculation. This hypothesis is supported by reports of PrP^{Sc} accumulation in the enteric nervous system, especially in the myenteric plexus of BSE-affected cattle and scrapie-affected sheep (Iwata et al., 2006; Terry et al., 2003; van Keulen et al., 1999; Ersdal et al., 2003). However, enteric neurons have also close proximity with other cells types, such as DCs and intraepithelial lymphocytes, which are known to deliver PrP^{Sc} to secondary lymphatic tissue. Therefore, the oral route of transmission seems to be a flexible way to transport PrP^{Sc} to the CNS, either by nervous tissue innervating the intestine or by migratory cells colonizing lymphatic tissue or both.

Previous studies have reported expression of PrP^{C} in mice and bovine pancreatic tissue restricted to a subset of cells in the islets of Langerhans (Ford et al., 2002a; Amselgruber et al., 2006). Mice inoculated with scrapie showed an inflammatory reaction in the endocrine pancreas, which may be associated with PrP^{Sc} accumulation (Ye et al., 1997). PrP^{Sc} deposits have also been described in the pancreas of cervids infected with chronic wasting disease (Sigurdson et al., 2001). Using a panel of antibodies in bovine pancreas, PrP^{C}-specific labeling was co-localized with glucagon producing α-cells (Amselgruber et al., 2006). Nevertheless, a recent study in rat pancreas described co-localization of PrP^{C} inclusions exclusively in insulin producing β-cells (Strom et al., 2007). These authors observed that inclusions of PrP^{C} increased with age and under hyperglycemic conditions suggesting a novel physiological role of PrP^{C} in glucose homeostasis.

As previously described (Ford et al., 2002a), our analysis showed undetectable levels of PrP^{C} in the liver tissue. Low PrP^{C} expression in liver cells may explain the reduced PrP^{Sc} infectivity reported *in vitro* (Bosque et al., 2001). Evidence of PrP^{C} expression in bovine skeletal muscle has important implications for the potential transmission of BSE through the consumption of beef products. Low levels of PrP^{C} expression in bovine skeletal muscle support the idea of a reduced potential for PrP^{Sc} infection in meat. However, PrP^{C} expression in mouse muscle was reported to be 5-10% of that in brain and still sufficient to induce PrP^{Sc} accumulation (Bosque et al., 2001). In our study, we found a similar ratio of PrP^{C} in skeletal muscle in comparison to the brain (4.22%; Fig. 1), which may potentially be sufficient for PrP^{Sc} accumulation. PrP^{Sc} has been detected in skeletal muscle after experimental inoculation of several species including mouse, hamster, goat and sheep (Bosque et al., 2001; Thomzig et al., 2003; Pattison and Millson, 1962; Casalone et al., 2005). Bosque et al, (2001) reported that PrP^{Sc} was accumulated in muscle of

mice after intracerebral inoculation and that muscle and not neural or lymphatic PrP^{C} was the substrate for PrP^{Sc} conversion. These authors also discussed a number of variables that may affect PrP^{Sc} production in muscle including muscle type, prion strain, host species and inoculation route. One or more of these variables may be responsible for the lack of PrP^{Sc} detection in the skeletal muscle from experimentally inoculated cattle (Hamir et al., 2004). Despite the reduced levels of PrP^{C} in skeletal muscle, this protein may play a role in muscle physiology. Studies *in vitro* have implicated PrP^{C} in myocyte differentiation as well as protection against oxidative stress (Brown et al., 1998; Massimino et al., 2006). PrP^{C} expression is up-regulated in regenerating muscle fibers (Sarkozi et al., 1994) and may have a general stress-response effect in various neuromuscular disorders (Kovacs et al., 2004).

Detection of PrP^{C} staining was previously reported in pneumocytes of mice lung tissue (Ford et al., 2002a). Considering the respiratory system as a route for entry in several diseases, the expression of PrP^{C} in the lung may lead to think that there is a potential respiratory transmission route for TSEs. However, this passage of prion pathogenesis has not been clarified. In a previous study in the bovine kidney, PrP^{C} labeling was reported to be restricted to the renal glomeruli (Amselgruber et al., 2005). However, our analysis detected PrP^{C} staining also associated to cortical and convoluted tubules, and collective ducts in the medulla. Disparity in the pattern of staining may be associated to different antibodies used. Expression of PrP^{C} in the kidney can potentially predispose PrP^{Sc} conversion and accumulation in the urinary system. This idea is supported by reports of scrapie-infected hamsters and CJD patients showing PrP^{Sc} urinary excretion (Shaked et al., 2001). Moreover, a study in mice suffering from chronic nephritis showed that urinary inflammation was able to trigger excretion of prion infectivity into urine, which may potentially constitute a vector for horizontal TSEs transmission (Seeger et al., 2005).

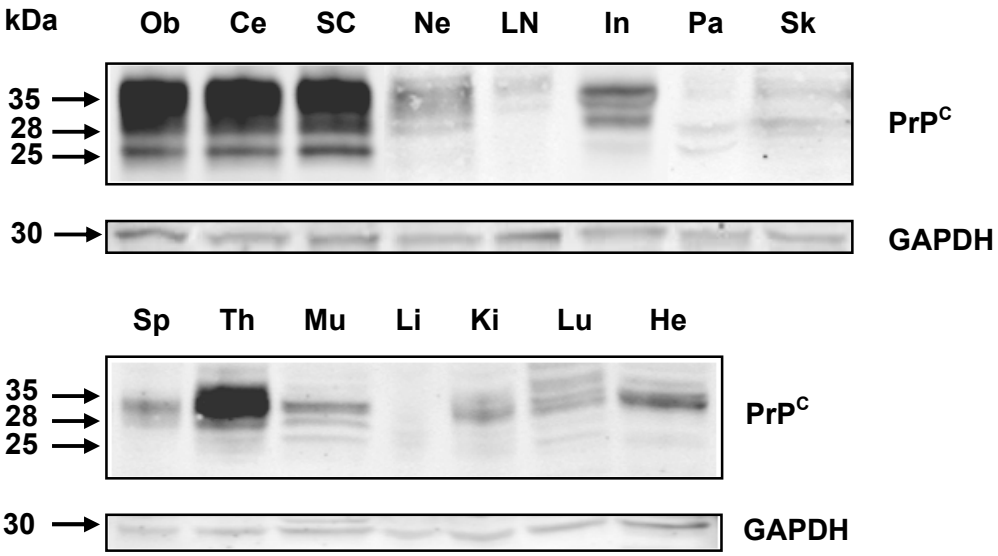
A previous report and our study showed intense PrP^{C} expression in epidermal keratinocytes (Pammer et al., 1998). Additionally, we observed intense PrP^{C} staining in sebaceous glands located in horizontal tissue sections of the skin at the level of the dermis. PrP^{C} expression was also detected in cells surrounding the inner and the outer root sheet of the hair follicle. Interestingly, this group of cells in the hair follicle, including the sebaceous gland, has been described as stem cells responsible for hair follicle regeneration (Levy et al., 2005). This finding supports the idea that PrP^{C} may play a role in induction of cellular differentiation. Intense and specific expression of PrP^{C} in the skin may explain the detection of PrP^{Sc} in the dermis of mice and sheep after oral inoculation (Thomzig et al., 2007). Despite intense expression of PrP^{C} in

keratinocytes and sebaceous cells, deposition of PrP^{Sc} has showed to occur exclusively on peripheral nerve fibers, indicating that PrP^{Sc} is transported by neurons and centrifugally spread to the skin. (Thomzig et al., 2007).

The wide-spread expression and the evolutionary conservation among mammals suggest that PrP^{C} has an important biological function. Several studies have showed roles for PrP^{C} associated with cytoprotection through anti-oxidant and anti-apoptotic effects. These properties have been observed in specialized and mitotically inactive cells such as neurons (Brown et al., 1997 and 2002) and myocytes (Sarkozi et al., 1994; Kovacs et al., 2004). PrP^{C} may be exerting a similar role in other specialized cells where our analysis found intense PrP^{C} expression such as FDCs, podocytes, keratinocytes and pneumocytes. However, in developing cells PrP^{C} expression has been correlated with differentiating and mitogenic processes as showed by studies in neuronal precursors (Steele et al., 2006), myoblasts (Massimino et al., 2006), thymocytes (Kubosaki et al., 2001), lymphocytes (Bainbridge et al., 2005) and hematopoietic cells (Liu et al., 2001). Moreover, several reports have demonstrated that PrP^{C} expression is increased during inflammation in the skin (Pammer et al., 1998), gastric mucosa (Konturek et al., 2005), kidney (Seeger et al., 2005), pancreas and liver (Heikenwalder et al., 2005). All these data suggest that PrP^{C} may have a dual role in damaged tissues that involve protecting cells affected by acute or chronic oxidative stress and also inducing differentiation of immature cells into specialized and functional cells that can serve in tissue regeneration.

Our study presented the first comparative analysis of PrP^{C} expression in bovine tissues. The importance of this study is to lay the foundation for understanding the tissue-specific PrP^{C} expression and to consider the potential participation of more bovine tissues in transmission of BSE infection. Moreover, the wide-spread expression of PrP^{C} in bovine tissues opens the spectrum of possibilities to new cell models for the study of PrP^{C} elusive function.

a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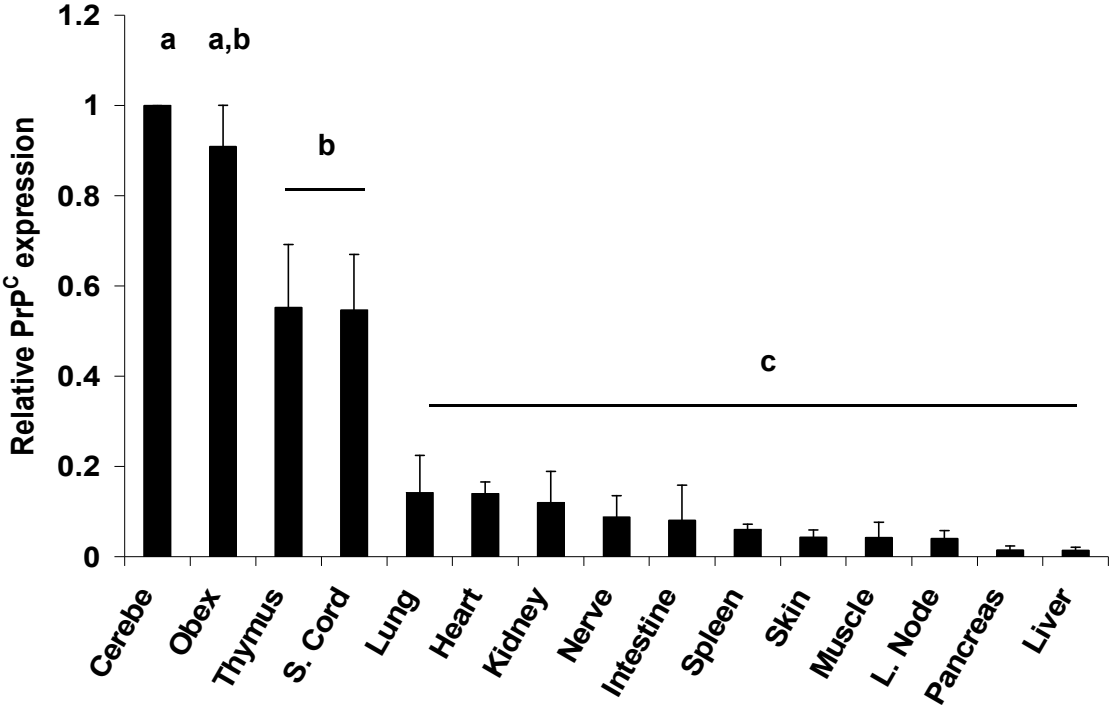

Fig. 1 Western blot analysis of relative PrP^C expression in bovine tissues. (A) PrP^C displayed three distinct migration bands corresponding to molecular weights of 35 kDa (di-), 28 kDa (mono-) and 25 kDa (un-glycosylated). PrP^C was detected in all tissues with higher intensity in neural tissues and thymus. GAPDH was used as control protein (30 kDa). (B) Cerebellum showed the highest ($P<0.05$) levels of PrP^C expression. Among non-neural tissues, the thymus expressed the highest ($P<0.05$) levels of PrPC. Different superscripts indicate significant differences ($P<0.05$). Ob, Obex; Ce, Cerebellum; SC, Spinal Cord; Ne, Nerve; LN, Lymph node; In, Intestine; Pa, Pancreas; Sk, Skin; Sp, Spleen; Th, Thymus; Mu, Muscle; Li, Liver; Ki, Kidney; Lu, Lung; He, Heart.

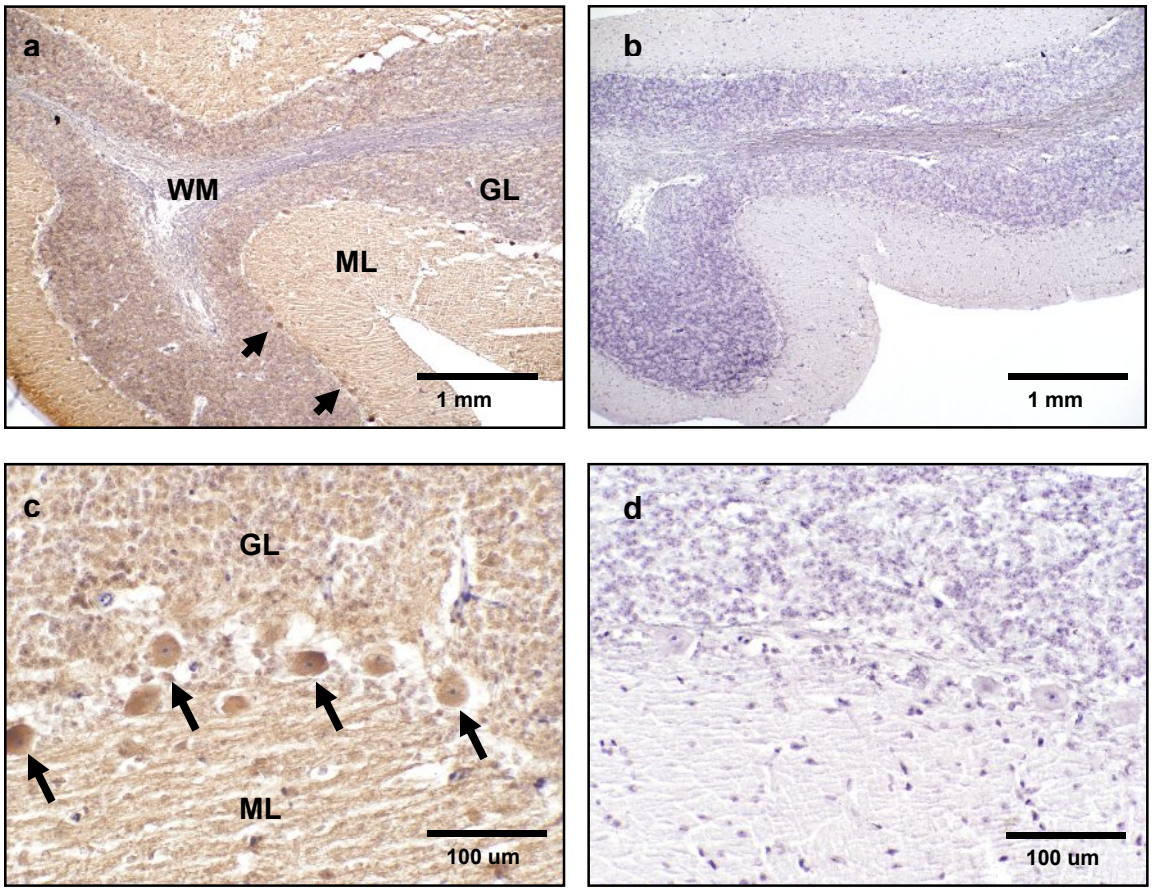

Figure 2. Expression of PrP^C in the bovine cerebellum. (a) Transverse tissue section incubated with SAF-32 antibody and stained using peroxidase. PrP^C staining was confined to the molecular layer (ML), granular layer (GL) and Purkinje cells (*arrows*) located in the gray matter. No staining was observed in the white matter (WM). (c) Higher magnification shows intense PrP^C expression in fibers of the ML, Purkinje cells (*arrows*) and neurons of the GL. (b,d) Serial section incubated with non-immune horse serum instead of SAF-32 antibody shows no staining (negative control).

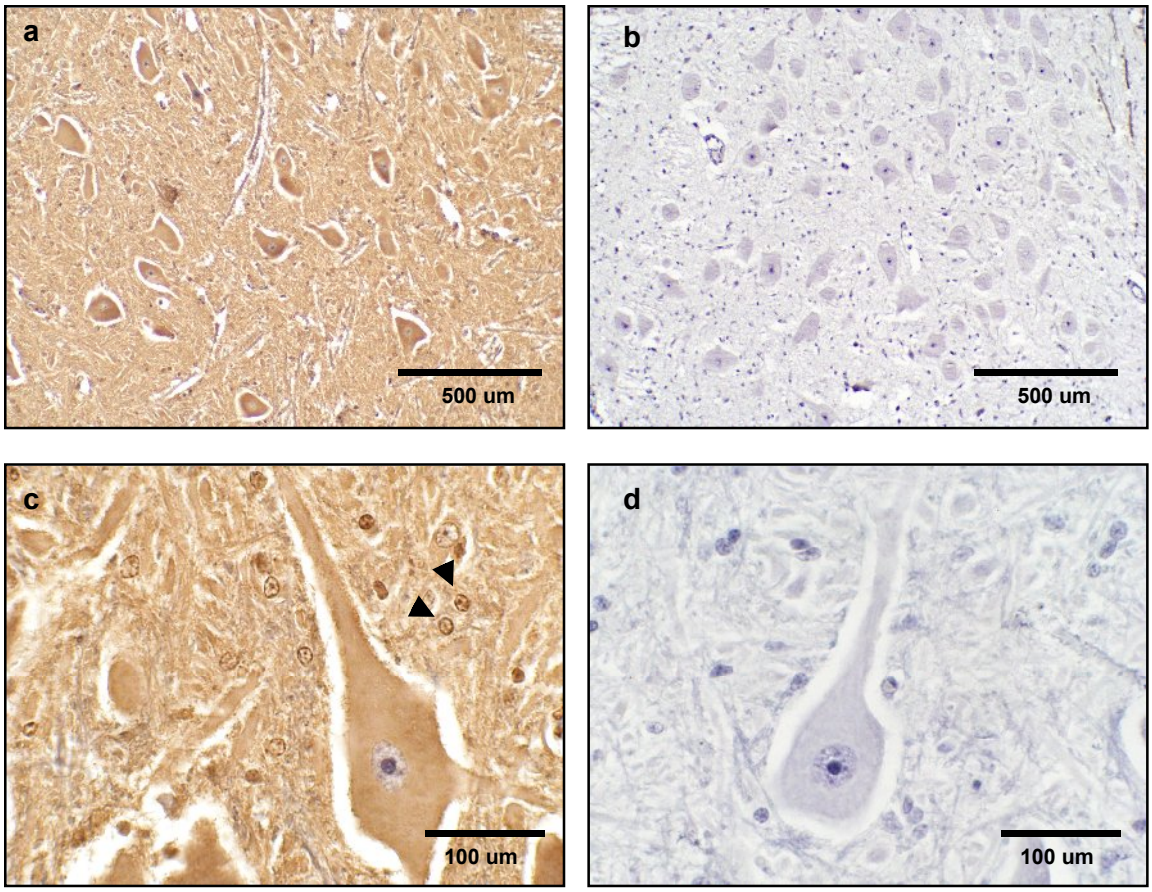

Figure 3. Expression of PrP^C in the bovine obex. (a) PrP^C expression is associated to neuronal bodies, neuropil and neuroglia of the solitary tract nucleus. (c) Higher magnification shows PrP^C labeling in neuronal bodies, appendixes and glial cells (*arrow-heads*). (b,d) Serial section incubated with non-immune horse serum instead of SAF-32 antibody shows no staining (negative control).

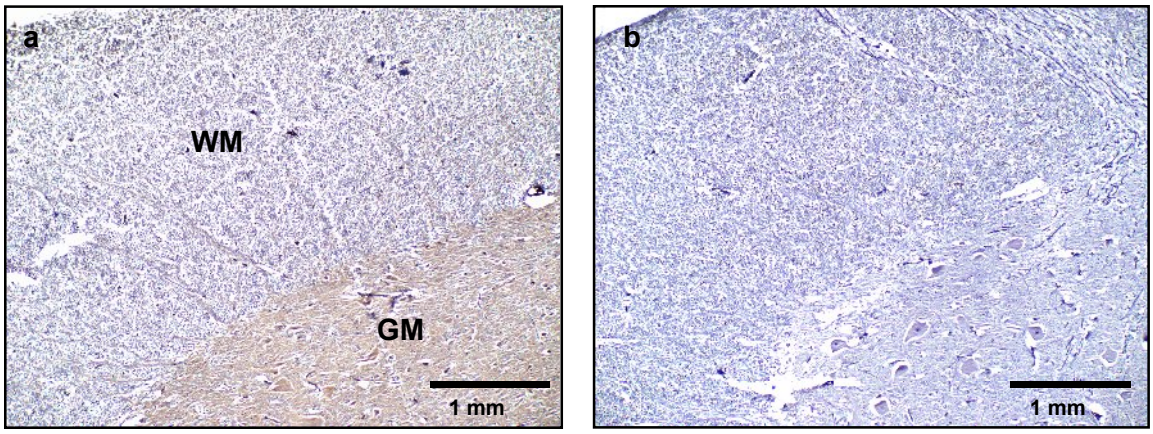

Figure 4. Expression of PrP^C in the bovine spinal cord. (a) Sagittal section showing PrP^C-specific labeling using SAF-32 antibody and peroxidase staining. PrP^C immunoreactivity was restricted to the gray matter. (b) Serial section of the spinal cord incubated with non-immune horse serum instead of SAF-32 (negative control).

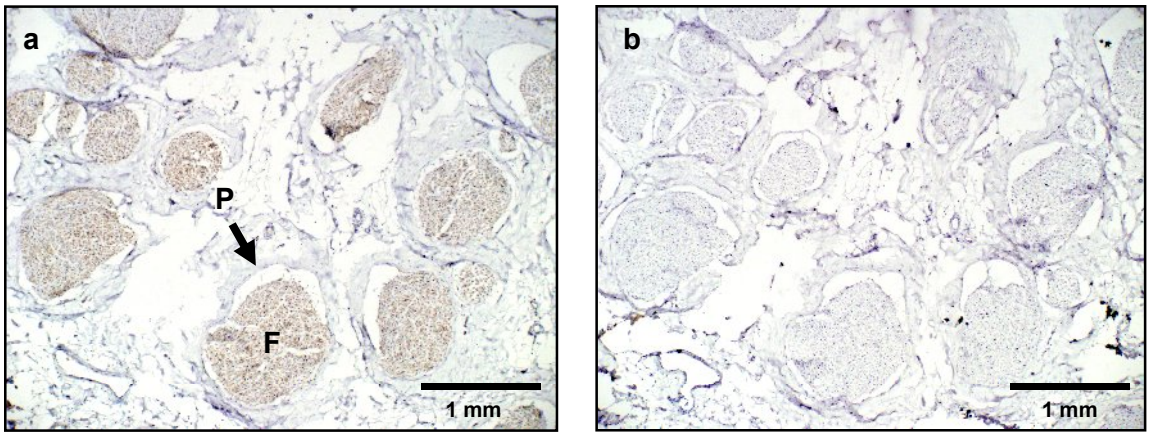

Figure 5. Expression of PrP^C in the bovine syatic nerve. (a) Transverse tissue section incubated with SAF-32 antibody and stained with peroxidase. PrP^C staining is confined to neural fibers associated in fascicles (F). No PrP^C labeling was observed in the perineurum (P). (b) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control).

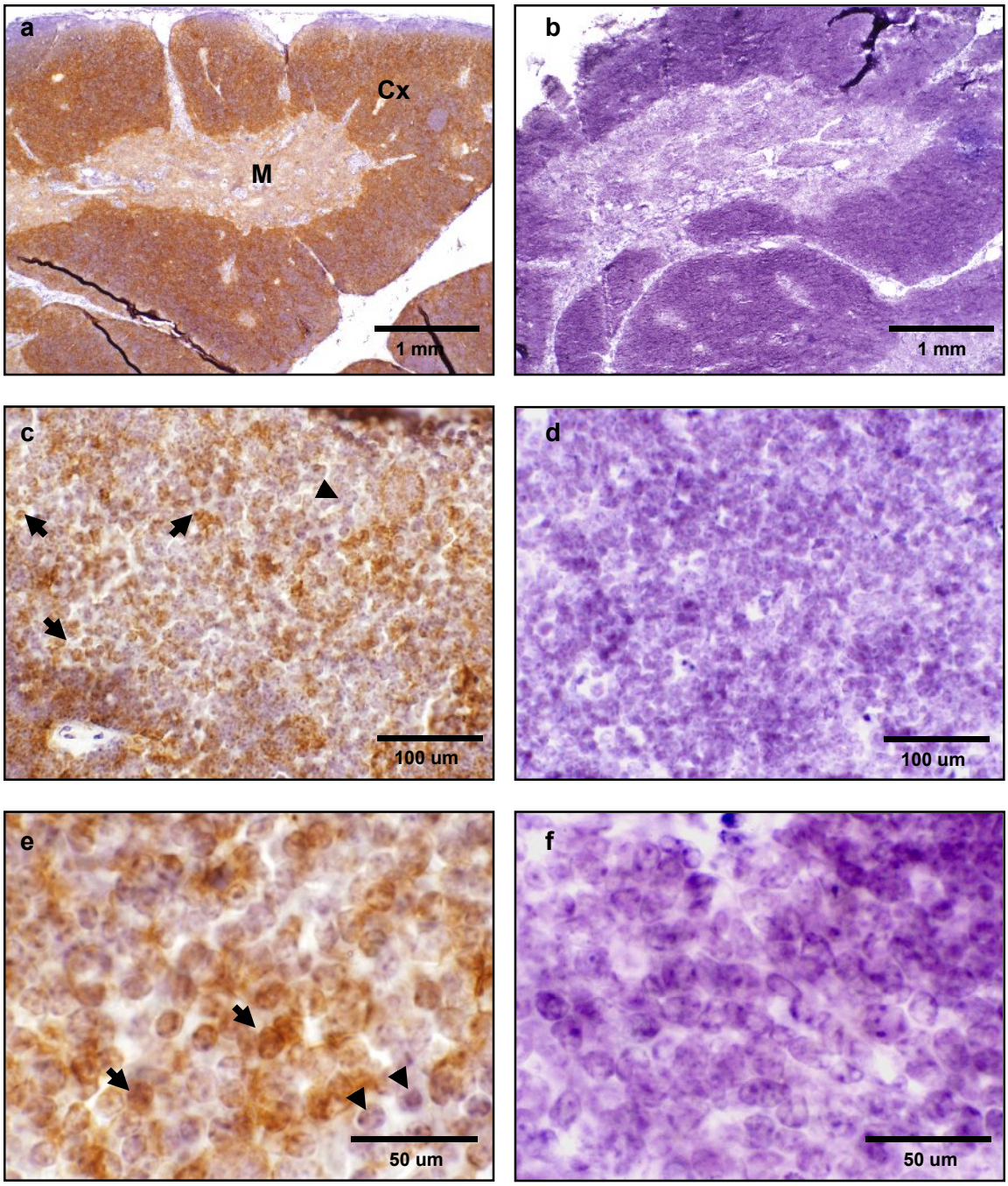

Figure 6. Expression of PrP^C in the bovine thymus. (a). Intense PrP^C-specific labeling is observed in the cortex (Cx) of the thymus. (c,e) Higher magnification in the cortex area shows PrP^C positive (*arrows*) and negative (arrow-heads) stromal cells. (b,d,f) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control).

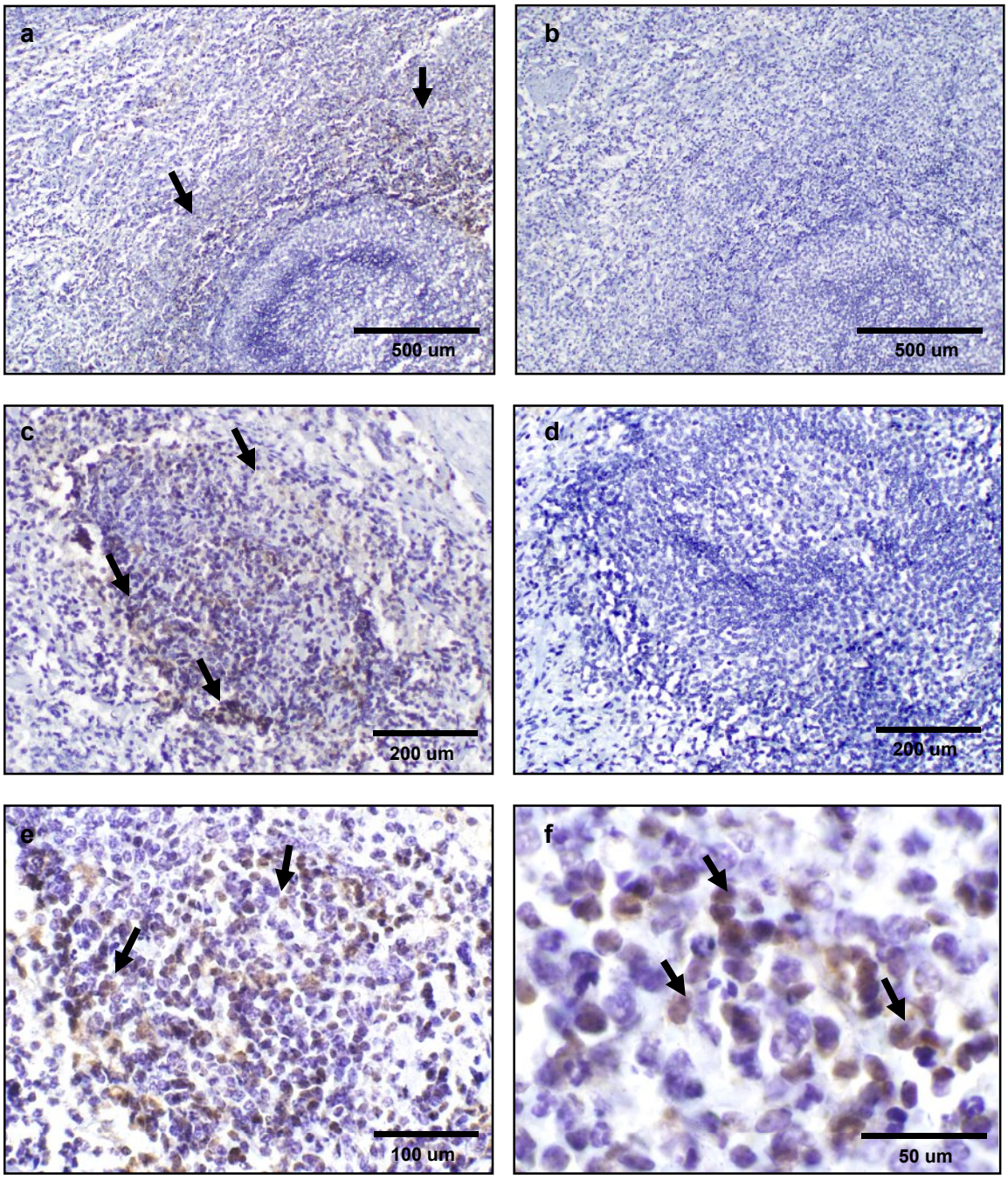

Figure 7. Expression of PrP^C in the bovine spleen. (a,c,e,f) PrP^C staining was associated with cells with the appearance of myeloid dendritic cells (*arrow*) in perilymphoid zones surrounding nodules of white pulp. (b,d) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control).

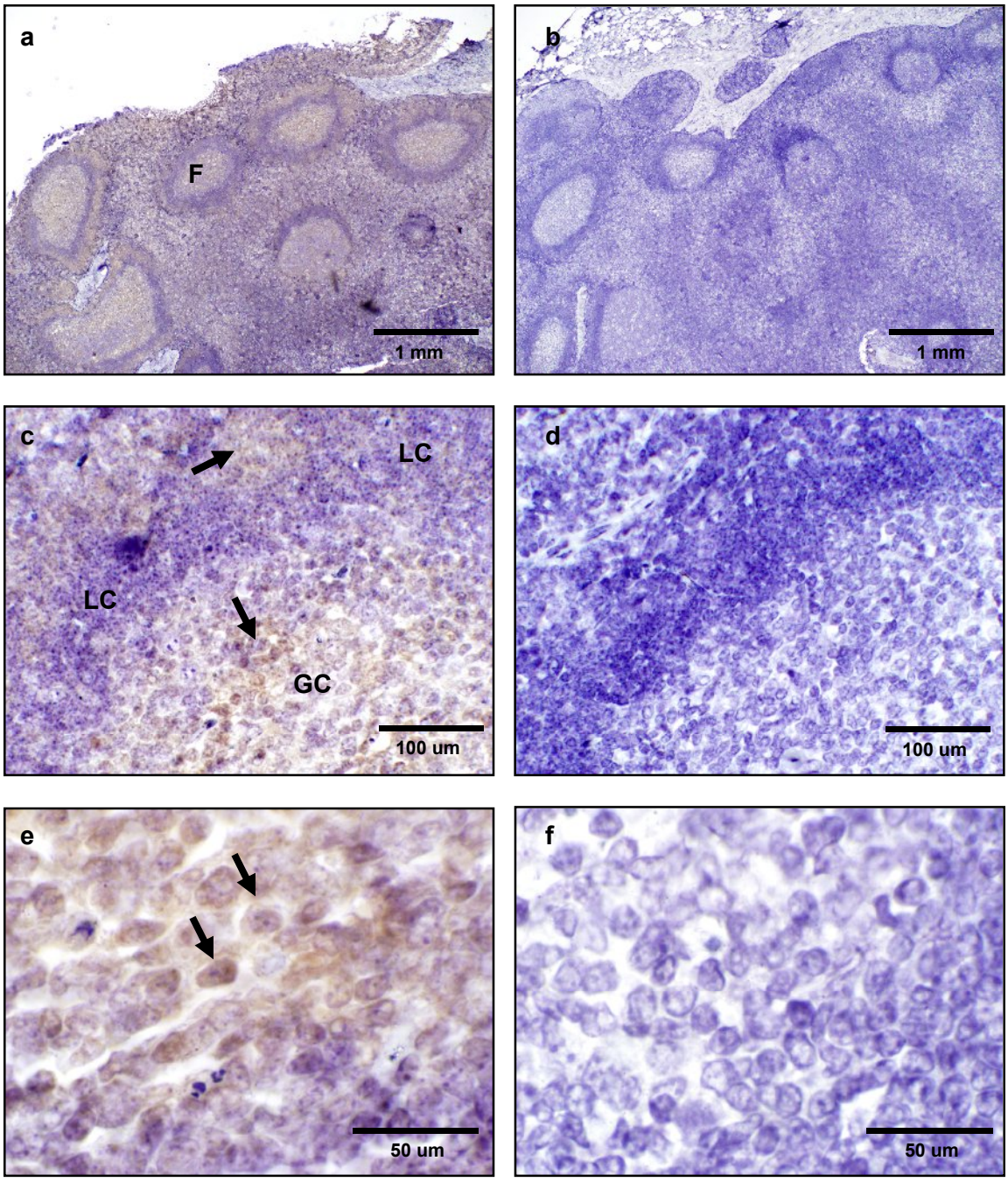

Figure 8. Expression PrP^C in the bovine lymph node. (a) Tissue section shows PrP^C-specific labeling associated with lymphoid follicles in the cortex area. (c,e) Higher magnification evidence of specific PrP^C staining associated with lymphocytes (*arrow*) surrounding the lymphocyte corona (LC) and germinal centers (GC). (b,d,f) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative controls).

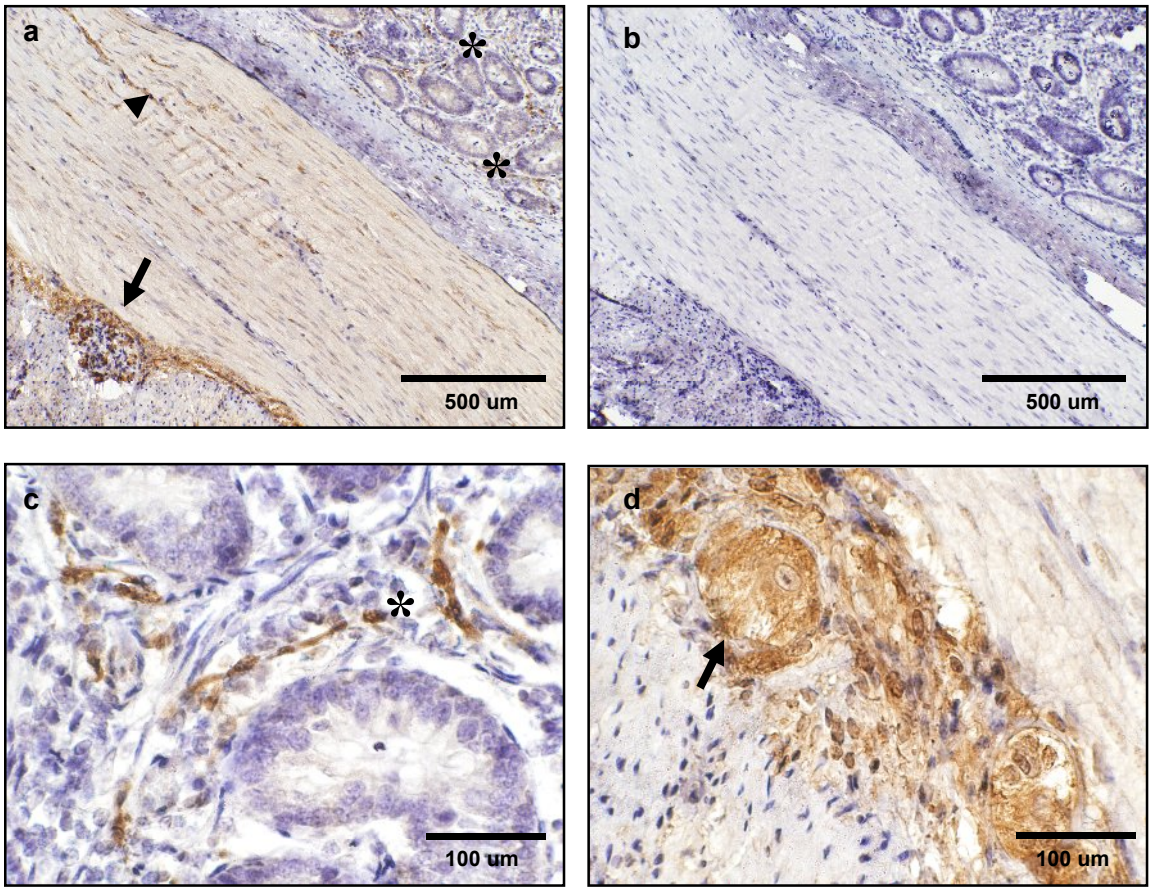

Figure 9. Expression of PrP^C in the bovine ileum. (a) Sagittal tissue section showing PrP^C-specific labeling in enteric mucosa (*), muscularis (*arrowhead*) and myenteric plexus (*arrow*). (c) Higher magnification evidence PrP^C-positive neurons in the lamina propia. (d) PrP^C is highly expressed in parasympathetic ganglion cells forming the myenteric plexus. (b) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control).

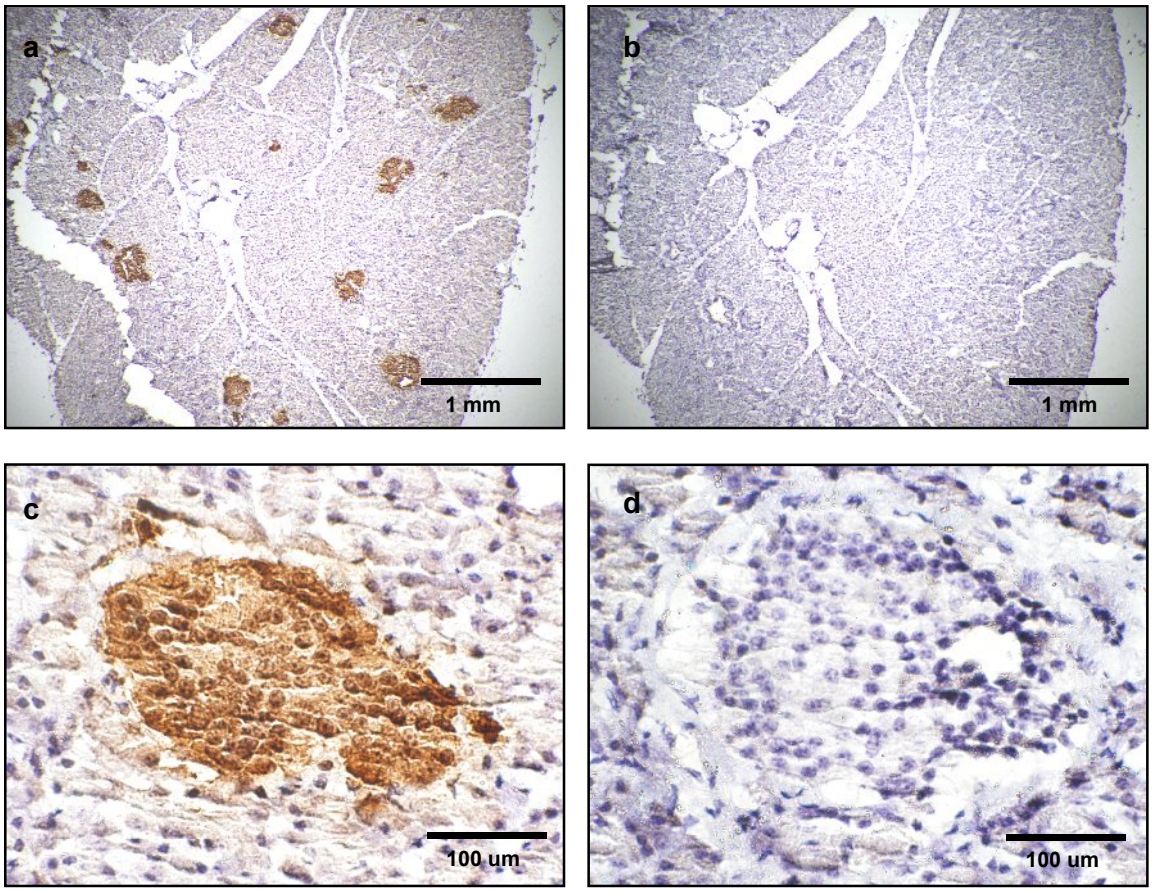

Figure 10. Expression of PrP^C in the bovine pancreas. (a) PrP^C positive staining is restricted to the endocrine pancreas in the islets of langerhans. (c) Higher magnification shows specific PrP^C-positive endocrine cells. (b,d) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control).

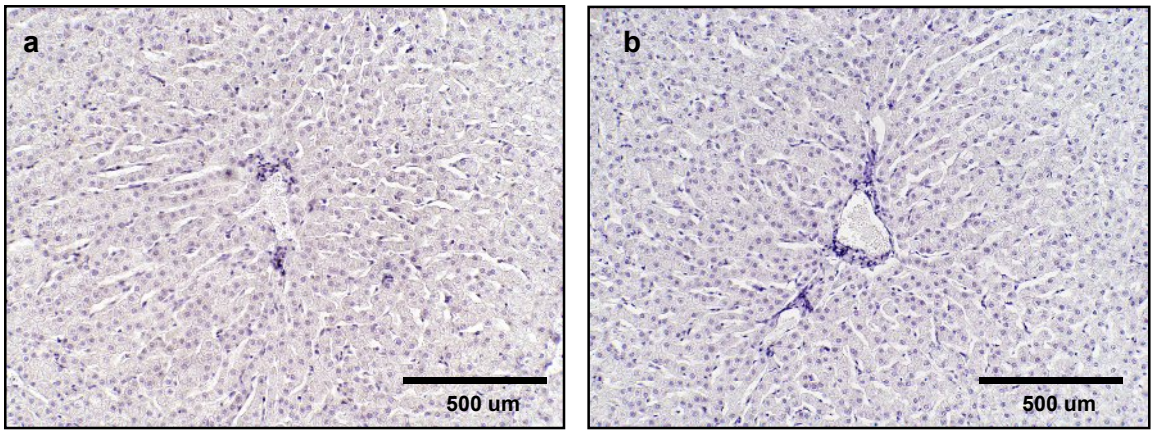

Figure 11. Expression of PrP^C in the bovine liver. (a). No PrP^C staining was observed in the liver tissue after incubation with SAF-32 antibody. (b) Negative control.

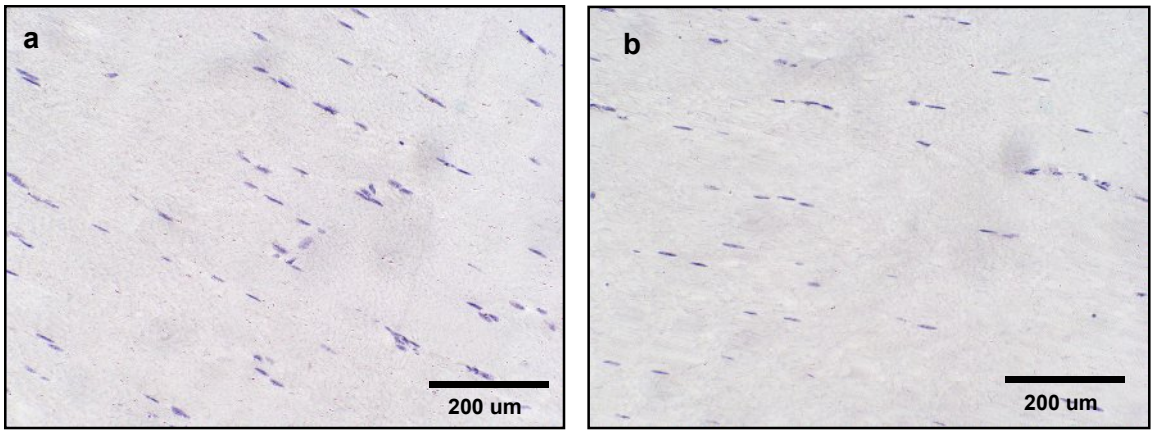

Figure 12. Expression of PrP^C in the bovine skeletal muscle. (a) Tissue section incubated with SAF-32 antibody. No detectable PrP^C expression was observed in the skeletal muscle. (b) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control).

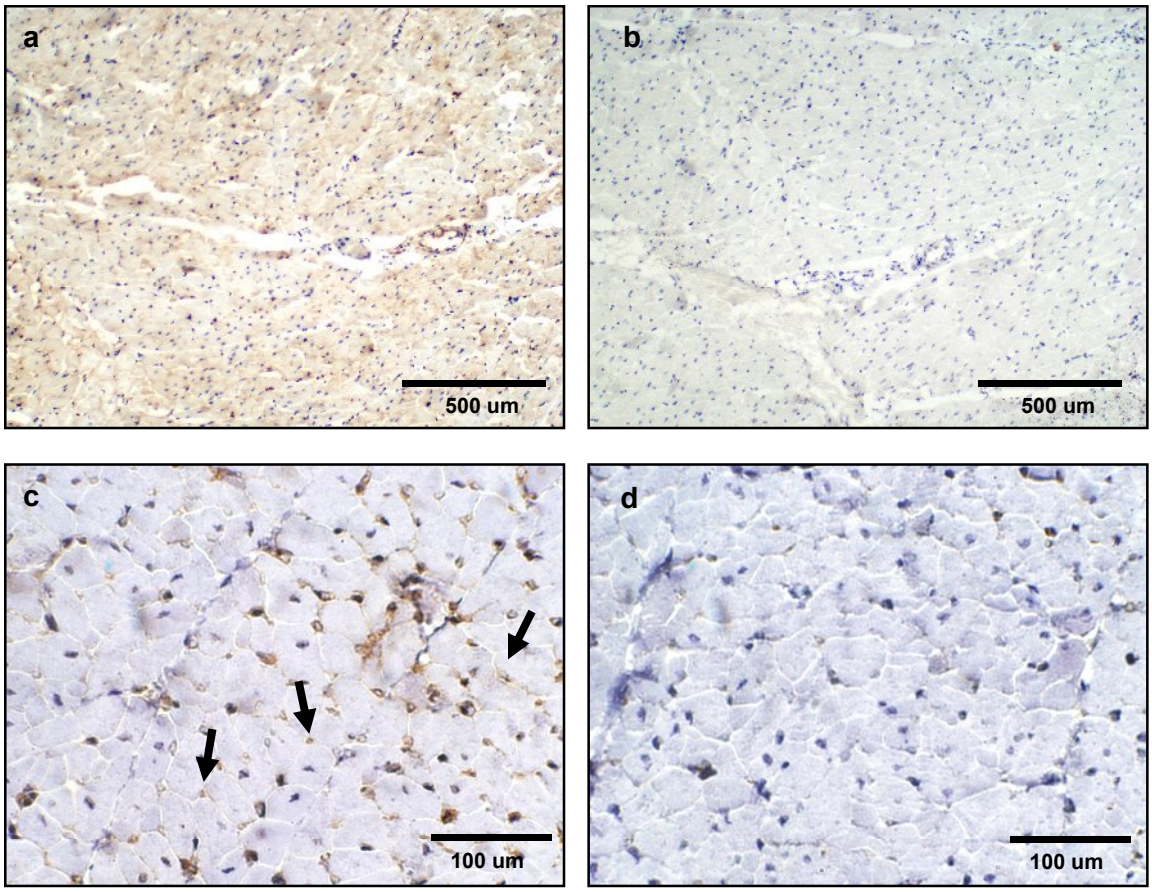

Figure 13. Expression of PrP^C in the bovine cardiac muscle. (a) PrP^C labeling was observed in cardiac muscle cells located in the myocardium. (c) Higher magnification shows PrP^C staining associated with cardiac muscle cells. (b,d) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control).

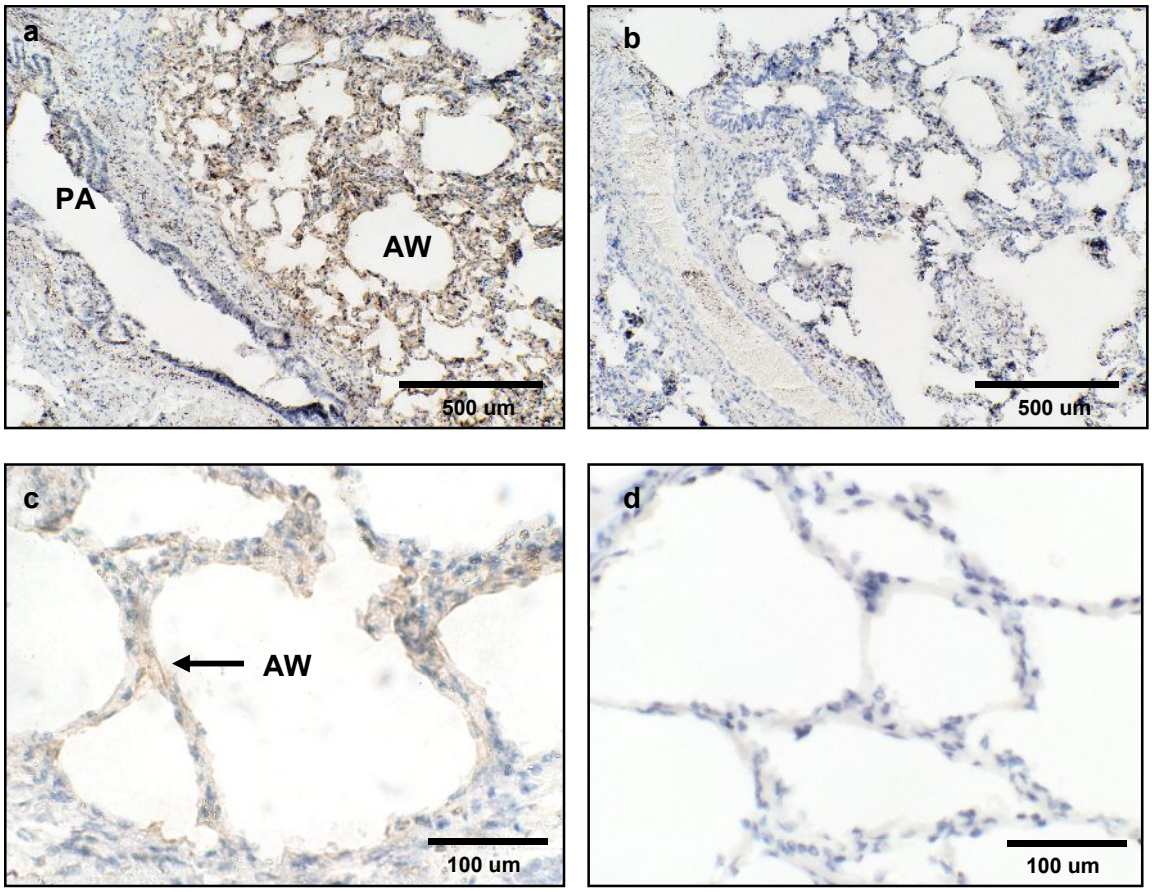

Figure 14. Expression of PrP^C in the bovine lung. (a). PrP^C-specific labeling was observed associated with the alveolar wall (AW). (c) Higher magnification shows PrP^C-specific labeling associated with pneumocites (*arrow*). (b,d) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control). (PA) Pulmonary artery.

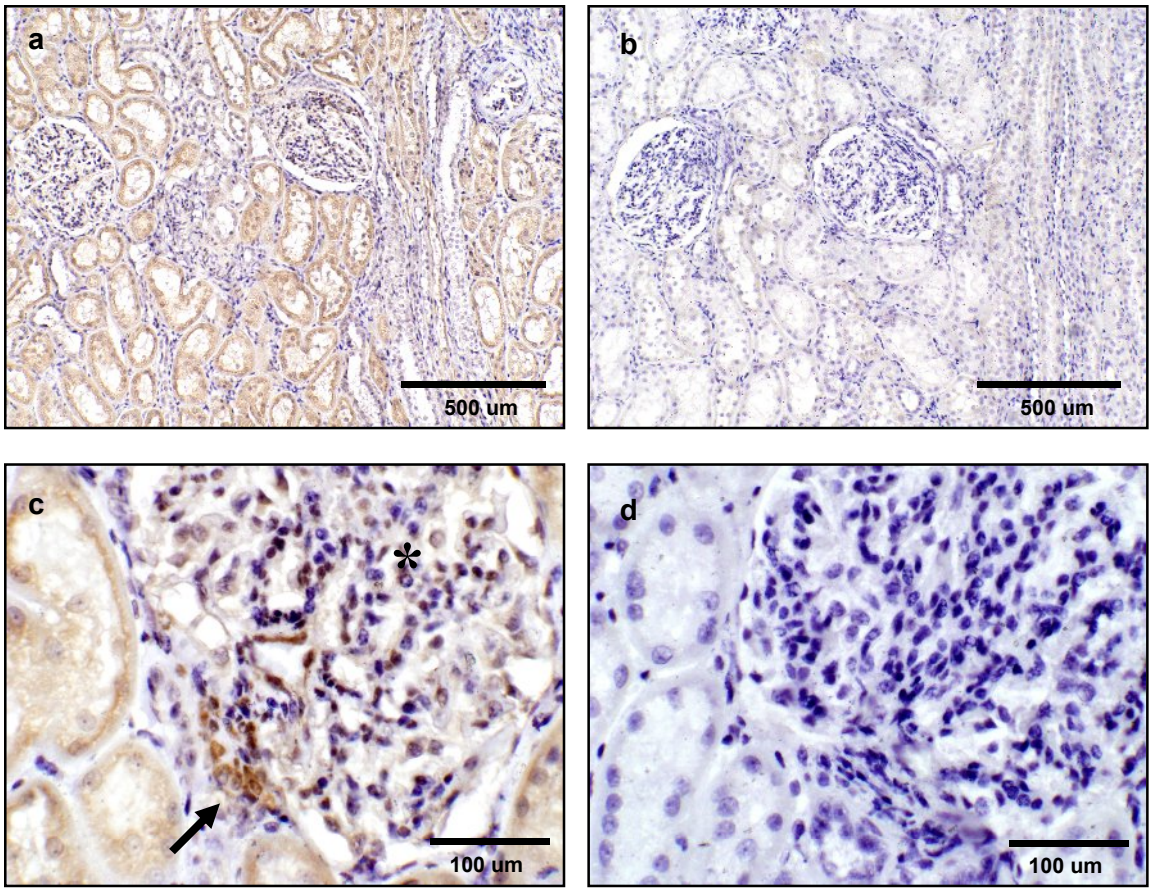

Figure 15. Expression of PrP^C in the bovine kidney. (a) PrP^C expression is associated with cortical convoluted tubules and collective ducts in the medulla. (c) Higher magnification of renal glomerulus shows strong PrP^C staining in extraglomerular mesangial cells (*arrow*). (b,d) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control). Moderate labeling was detected in podocytes and endothelial cells (*).

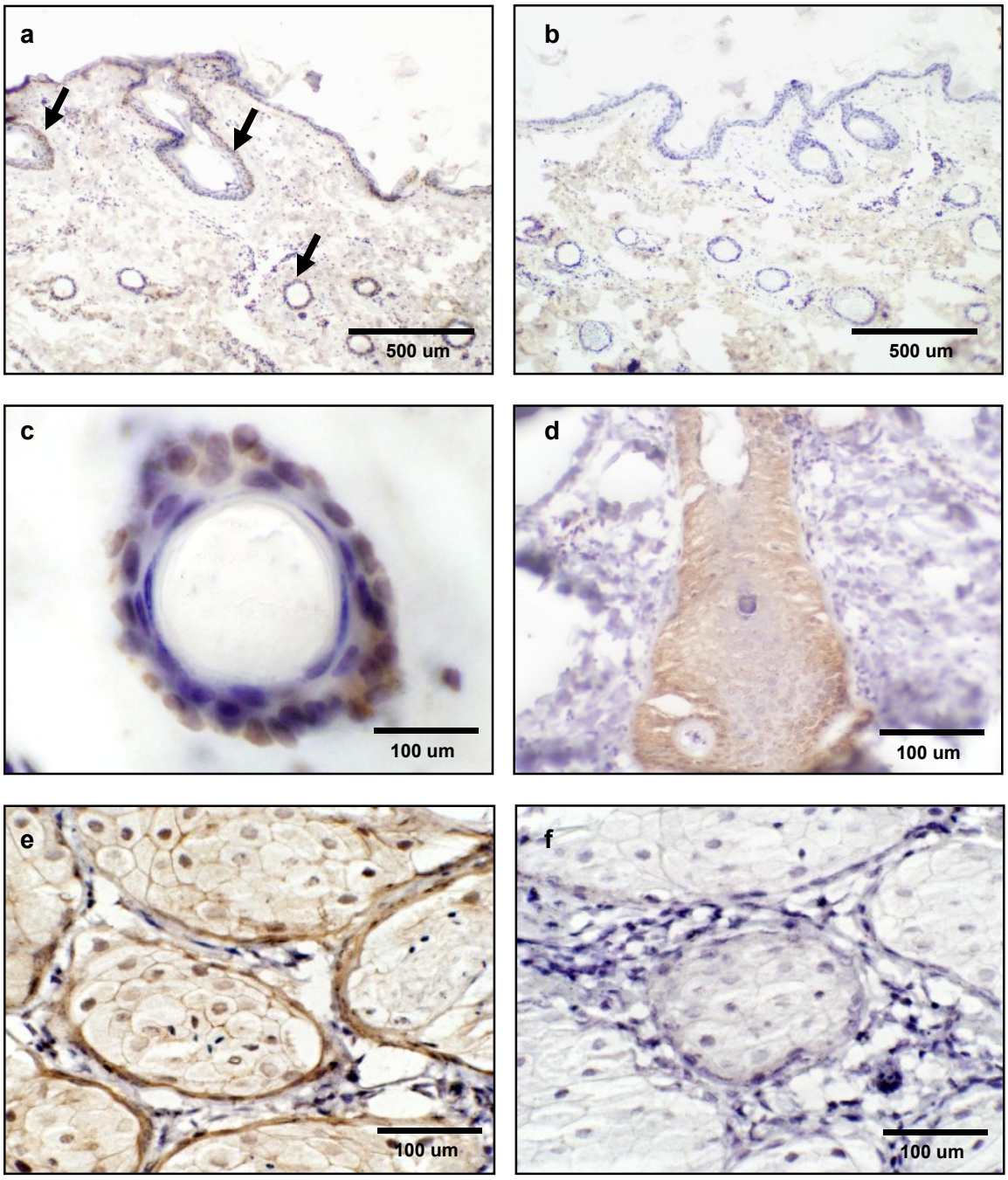

Figure 16. Expression of PrP^C in the bovine skin. (a) PrP^C expression is associated with keratinocytes in the epidermis and hair follicles (*black arrows*). (c) Higher magnification shows that PrP^C is expressed in cells surrounding the hair follicle. (d,e) PrP^C staining was also present in sebaceous glands located in the dermis. (b,f) Serial section incubated with non-immune horse serum instead of SAF-32 antibody (Negative control).

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