Progress on low susceptibility mechanisms of transmissible spongiform encephalopathies

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Abstract: Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a group of fatal neurodegenerative diseases detected in a wide range of mammalian species. The “protein-only” hypothesis of TSE suggests that prions are transmissible particles devoid of nucleic acid and the primary pathogenic event is thought to be the conversion of cellular prion protein (PrPC) into the disease-associated isoform (PrPSc). According to susceptibility to TSEs, animals can be classified into susceptible species and low susceptibility species. In this review we focus on several species with low susceptibility to TSEs: dogs, rabbits, horses and buffaloes. We summarize recent studies into the characteristics of low susceptibility regarding protein structure, and biochemical and genetic properties.

Keywords: Transmissible spongiform encephalopathy; Low susceptibility; Dog; Rabbit; Horse; Buffalo; PRNP; SPRN

Transmissible spongiform encephalopathy (TSE), or prion disease, is an invariably fatal neurodegenerative disease detected in a wide range of mammalian species, including Scrapie in goats (Capra hircus) and sheep (Ovis aries); bovine spongiform encephalopathy (BSE) in cattle (Bos taurus); chronic wasting disease (CWD) in elaphure (Elaphurus davidianus) and moose (Alces americanus); feline spongiform encephalopathy (FSE) in cats (Felis catus); transmissible mink encephalopathy (TME) in minks (Mustela vison); and Creutzfeldt-Jakob disease (CJD), variant Creutzfeldt-Jakob disease (vCJD), fatal familial insomnia (FFI), Gerstmann-Straussler-Scheinker syndrome (GSS) and Kuru in humans (Homo sapiens) (Collins et al, 2004; Prusiner, 1982). Humans and other animals infected with TSE are clinically and pathologically characterized with neuronal progressive vacuolation, stellate cell gliosis, spongiform lesions in gray matter, amyloid deposition and eventually disastrous degeneration and death (Prusiner, 1998). No effective treatments have been found and the World Health Organization has named TSE and AIDS as two major health problems of the 21st century.

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Figure 1 Amino acid sequences of PrP in 16 mammals (data from GenBank)

Human (NM_000311.3), chimpanzee (NM_001099093.3), Rhesus (NM_001047152.1), deer (AY330343.1), elk (EU082291.1), mouse (NM_011170.3), rat (NM_012631.2), pig (NM_001008687.1), sheep (NM_001009481.1), goat(JF729302.1), rabbit (NM_001082021.1), dog (NM_001013423.1), cat (EU341499.1), horse(NM_001143798.1), cattle (NM_181015.2), buffalio (K.C.1). 137634.
converts into the disease-associated isoform (PrPSc). Although the primary structures of PrPC and PrPSc are the same, their secondary structures are quite different. PrPC is enriched with α-helix (42% are α-helix, 3% are β-fold), whereas PrPSc is enriched with β-fold (43% are β-fold, 30% are α-helix) and is protease resistant (McKinley et al., 1983; Pan et al., 1993; Prusiner, 1982). The massive intracellular accumulation of PrPSc induces formations of oligomer and amyloid fibrils, and eventually neuronal degeneration (Barron et al., 2007; Caughey et al., 2009).

PrP plays vital roles in the pathological process of TSE. Knockout and low expression of PrP effectively abolishes or reduces susceptibility to TSEs, respectively (Brandner et al., 1996; Büeler et al., 1993), whereas high expression is associated with susceptibility and a shortened incubation time for disease development (Manson et al., 1994).

TSE susceptibility is species-specific. Previous studies show high susceptibility in hamsters (Mesocricetus auratus) to TSE, as they can be infected by various PrPSc virus strains isolated from human, cattle, goats, mice (Mus musculus) and minks (Bessen & Marsh, 1992; Gibbs & Gajdusek, 1973; Kimberlin & Walker, 1977; Thomzig et al., 2006). Similar high susceptibility is also found in mice (Chandler, 1961; Gibbs & Gajdusek, 1973; Hill et al., 2000; Lasmézas et al., 1997; Thomzig et al., 2006). However, rabbits could not be infected by PrPSc strains isolated from human, goats and mice (Barlow & Rennie, 1976; Gibbs & Gajdusek, 1973). During the outbreak of BSE in the UK, infections in humans and several species of feline were reported, but no infection was found in dogs (Canis familiaris) or horses (Equus caballus) (Aldhous, 1990; Kirkwood & Cunningham, 1994). Collectively, species with confirmed susceptibility to TSE include humans, rhesus monkeys (Macaca mulatta), hamsters, mice, minks, elaphures, moose, goats, sheep, cattle and raccoons (Procyon lotor) (Imran & Mahmood, 2011). Only a few species, such as dogs (Canis familiaris), rabbits (Oryctolagus cuniculus) and horses (Equus caballus) have been recognized as TSE resistant (Fernandez-Funez et al., 2011; Yuan et al., 2013; Zhang, 2011a). Interestingly, although more than 190,000 cattle were infected by BSE, and buffaloes (Bubalus bubalis) and cattle are closely related, no buffalo has been reported with BSE infection (http://www.oie.int) and are of low susceptibility to BSE (Zhao et al., 2012). In this review, based on TSE susceptibility, animals have been classified into TSE susceptible animals and TSE low susceptible animals.

The pathological mechanisms of TSE are yet to be clarified. Although highly susceptible animals are important to understanding this disease, studies on animals with low susceptibility provide a new angle from which to examine TSE. Here, we review recent research developments on protein structures, biochemical characteristics and genetic features of four animals (dogs, rabbits, horses and buffaloes) with low susceptibility to TSE.

**Dogs**

During the outbreak of BSE in the UK, several species of feline were reportedly infected, including cheetahs (Acinonyx jubatus), pumas (Puma concolor) and cats (Kirkwood & Cunningham, 1994). Since 1990, about 100 cats and 29 captive felines, including 15 cheetahs, four lions (Panthera leo), three leopard cats (Prionailurus bengalensis), three pumas, three tigers (Panthera tigris) and one Asian golden cat (Catopuma temminckii), have been diagnosed with FSE (Imran & Mahmood, 2011). The presumed infection source was PrPSc-contaminated food; however, dogs and cats are provided similar food and no dogs were reported with TSE (Imran & Mahmood, 2011; Kirkwood & Cunningham, 1994; Wopfner et al., 1999). With further laboratory cell experiments, dogs have been recognized as a species with low TSE susceptibility. For example, when Madin-Dabney canine kidney cells (MDCK) were infected with brain tissue homogenates from CJD patients or RML prion strain isolated from scrapie animals, although the biosynthesis and processing of PrPSc in MDCK are similar with those in N2aPK1 cells of murine neuroblastoma, which are highly susceptible to TSE, no PrPSc was found in MDCK. When infected MDCK were used to infect N2aPK1 cells, no PrPSc was found in N2aPK1 cells either (Ploymenidou et al., 2008; Zhang & Liu, 2011).

The gene polymorphism of PRNP is correlated with TSE susceptibility (Westaway et al., 1994). In humans, at least 30 mutations of PRNP are intertwined with TSE susceptibility (Lloyd et al., 2011). In dogs, the amino acid residue 187 and 229 of the PrP sequence are histidine and glycine, respectively, whereas, they both are arginine in cats (Wopfner et al., 1999). No FSE-related polymorphic site was found by screening encoding sequences of PRNP in 609 animals (including 15 FSE infected cases) and 29 species from 22 genera of the Order Carnivora, but Stewart et al. (2012) did notice that amino acid residue 163 in all canines is either aspartate or glutamic acid, indicating this locus may have some
connection to TSE susceptibility.

The three-dimensional structure of PrP<sup>C</sup> may be another tool in resolving the puzzle of TSE susceptibility (Lin & Wen, 2011). To understand the structural differences of PrP<sup>C</sup> in animals with low and high susceptibility to TSE, Lysek et al (2005) carried out a study on nuclear magnetic resonance (NMR) structures of PrP<sup>C</sup> in dogs (canine PrP<sub>C</sub>, cPrP<sub>C</sub>), cats (feline PrP, fPrP<sub>C</sub>), pigs (sus scrofa PrP, scPrP<sub>C</sub>) and goats (ovine PrP, ovPrP<sub>C</sub>). Their overall three-dimensional structures are quite close, consisting of a N-terminal (constituted of about 100 amino acid residues in random coil) and a globular domain in the C-terminal (including three α-helixes and a pair of short, reverse paralleled β-folds constituting about 100 amino acid residues). The globular domain in the C-terminal is species-specific, e.g., four amino acids (Asp159Asn, Arg177His, Lys185 Arg and Gly229Arg) are different between cPrP<sub>C</sub> and fPrP<sub>C</sub>; Asp-159 and Arg-177 in cPrP<sub>C</sub> make it unique in potential distribution; in fPrP<sub>C</sub>, scPrP<sub>C</sub> and ovPrP<sub>C</sub> same positive potential distribution patterns are observed in their C-terminals.

The conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup> is critical in TSE pathogenesis. Besides PrP<sup>C</sup> and PrP<sup>Sc</sup>, the existence of the third state-β intermediate state, consisting mainly of β-fold in circular dichroism (CD) (Hornemann & Glockshuber, 1998), has raised attention due to its capability in introducing TSE (Collinge & Clarke, 2007). Khan et al (2010) claimed that in different species, the propensities in forming the β intermediate state are one of the vital factors influencing TSE susceptibility. Using dual wavelength CD, Khan et al (2010) compared the structures of the globular domain in high-susceptible (hamsters and mice) and low-susceptible species to TSE (rabbits, horses and dogs) and found that under inducing conditions with different pH values and urea concentrations, the propensities of forming the β intermediate state vary in different species. At pH 7.0, urea concentrations have no effect on PrP<sup>C</sup> and no β intermediate state is observed in all five species; at pH 5.0, the structure of hamster PrP<sup>C</sup> (hPrP<sub>C</sub>) is most unstable and easily forms the β intermediate state; at pH 4.0, the PrP<sup>C</sup> in all five species is unstable and easily forms the β intermediate state, the lowest concentration of β intermediate state occurs in dogs (Figure 2). The propensities in forming the β intermediate state (hamsters > mice > rabbits > horses > dogs) can also be adopted in evaluating

Figure 2 Propensities of conversions of PrP<sup>C</sup> into the β intermediate state in different species at pH 7.0 (A), 5.0 (B), 4.5 (C) and 4.0 (D) and different concentrations of urea (modified from Khan et al, 2010)
species’ susceptibilities to TSE (Fernandez-Funez et al., 2011). Using molecular kinetic methods, Zhang & Liu (2011) found stable molecular structures of wild ePrP\(^{C}\) under both neutral and acidic pH conditions, and in the neutral condition the salt bridge between D177 and R163 improves structural stability. These studies help to explain the mechanism of low TSE susceptibility in dogs and general TSE pathogenesis.

**Rabbits**

No cases of spontaneous TSE infection in rabbits have been reported to date. In 1973, Gibbs & Gajdusek failed to infect rabbits with either brain tissues from human CJD or Kuru patients or brain tissues from scrapie animals and minks with TME. In 1976, Barlow & Rennie failed to infect rabbits with ME7 strains isolated from animals with scrapie. By constructing rabbit PrP\(^{F}\) (RaPrP\(^{F}\)) over-expressed murine neuroblastoma tumor cell lines, Vorberg et al (2003) confirmed that RaPrP\(^{C}\) can neither be infected by RML strains nor convert into the PrP\(^{Sc}\). The *in vivo* experiments conducted by Fernandez-Funez et al (2010) support the view that rabbits are TSE resistant species. Fernandez-Funez et al (2010) expressed full length PrP of hamsters, mice and rabbits in drosophilae devoid of endogenous PrP, and found that cavernous transformation and isomers similar with PrP\(^{Sc}\) can only be found in the brains of transgenic drosophilae expressing shPrP\(^{C}\) and mouse PrP\(^{C}\) (moPrP\(^{C}\)), but not in drosophilae expressing RaPrP\(^{F}\). Moreover, Bellotti & Chiti (2008) reported that TSE correlates with the deposition of amyloid fibrils. Zhou et al (2011) found that Ficoll 70 and dextran 70 significantly accelerate the deposition of amyloid fibrils. Zhou et al (2011) found stable molecular structures of wild cPrP\(^{C}\), indicating that several amino acid residues in RaPrP\(^{C}\) can prevent the replication of isomers of PrP\(^{Sc}\) (Vorberg et al, 2003). About 33% of the different amino acids in moPrP\(^{C}\) and RaPrP\(^{C}\) features a unique charge that Ficoll 70 and the second α2-loop and the C-terminal of the third β-helix (165-172). The epitope consisting of the α2-β2 loop and the C-terminal of the third α-helix is considered capable of recognizing protein X and general TSE pathogenesis.
regulating progression of TSE (Kaneko et al, 1997). Protein dynamics analysis shows that RaPrPC has a constructively highly ordered β2-α2 loop (Wen et al, 2010a) which may function as a species barrier for TSE dissemination (Lin & Wen, 2011). However, compared with wild RaPrPC, S173N and I124V mutations affect the interactions of the β2-α2 loop with the third α-helix, and thereafter decrease the stability of the entire construct (Wen et al, 2010a, b).

In addition, when the salt bridges between D202-R156 and D178-R164 were removed in hPrPC and moPrPC, although secondary protein structures remained intact, the helix structures of RaPrPC were destroyed, indicating that salt bridges are important to the stability of RaPrPC (Zhang, 2009, 2010, 2011a).

Recently, Joaquin Castilla’s research group has raised questions about the view that rabbits are resistant to TSE. They amplified rabbit brain homogenates using serial automated protein misfolding cyclic amplification (saPMCA) and then inoculated this in vitro novel PrP into the brains of three other rabbits. One rabbit was found with TSE symptoms 766 days after inoculation even although no exogenous PrPSc was involved. Then the brain homogenates from this infected rabbit could 100% infect RaPrPC over-expressed transgenic mice. Therefore, Chianini et al (2012) claims that rabbits are not TSE resistant. Furthermore, using saPMCA, when the amplified proteins from mixtures of rabbit brain homogenates and BSE prion strains were inoculated into the brains of RaPrPC over-expressed transgenic mice, the resultant strains similar to BSE prion strains were discovered (Vidal et al, 2013). Fernández-Borges et al (2012) claims that in vivo infective experiments are imperfect when forming the conclusion that rabbits are resistant to TSE, especially when supported only by the observation that rabbits can not be infected with TSE naturally.

**Horses**

As there is no reports of horses being naturally infected with TSE, horses are recognized as low susceptibility species (Zhang, 2011a). Relative to dogs and rabbits, fewer studies have looked at low susceptibility in horses. Studies on the conversion of PrPSc into the β intermediate state show that under unstable conditions at pH 4, the PrPSc of hamsters, mice, rabbits, dogs and horses can convert into the β intermediate state, but the lowest level of β intermediate state is found in horses (Figure 2), indicating that equus caballus PrPC (ecPrPC) is relatively stable (Khan et al, 2010). Structural NMR on ecPrPC found two horse-specific amino acid alterations in its β2-α2 loop (Ser-167 and Lys-173, respectively), among which, S167 affects the highly ordered solution structure of the β2–α2 loop and may influence the low susceptibility of horses to TSE (Pérez et al, 2010). However, when amino acid residue 167 in moPrPC was mutated from asparagine into ecPrPC-specific serine (MoPrP\textsuperscript{D167S}), although MoPrP\textsuperscript{D167S} and ecPrPC share similar NMR structures and their β2–α2 loops in solutions are both relatively highly ordered, spongiform lesions were found in mice expressing MoPrP\textsuperscript{D167S} and neural diseases can be induced with the accumulation of PrPSc in the brain (Sigurdson et al, 2011). Therefore, the ordered state of the β2–α2 loop in solution alone does not fully explain different susceptibilities to TSE (Lin & Wen, 2011). Moreover, as the salt bridge in RaPrPC stabilizes protein structures, similar salt bridges consisting of GLU196-ARG156-HIS187, ARG156-ASP202 and GLU211-HIS177 are also found in ecPrPC (Zhang, 2011b). The structures of ecPrPC and cPrPC are stable under both neutral and acidic conditions (Zhang, 2011a). A common phenomenon found among RaPrPC, ecPrPC and cPrPC, is the salt bridge ASP177-ARG163 connects with the β2–α2 loop of PrP and is probably correlated with TSE susceptibility (Zhang, 2011a). Nevertheless, the low susceptibility of horses to TSE requires further work.

**Buffalo**

BSE was initially found in the UK in 1986, rapidly spread to over 25 countries, and caused major economic losses (Harman & Silva, 2009; Wells et al, 1987). BSE can also infect humans via the food chain and cause human vCJD (Collinge et al, 1996; Hill et al, 1997). The multiple pathogenic pathways of TSE, including spontaneous mutant, inheritance and infection (Nicholson et al, 2008), may explain why even after meat and bone meal was strictly forbidden, more than 15 000 BSE infected cattle were found in the UK (http://www.oie.int). Worldwide, there were over 190 000 taurus cattle, 1 Bos indicus, and 1 Bos indicus × Bos taurus cross reported with BSE infections (data of OIE, Novakofski et al, 2005; Seuberlich et al, 2006). Although buffaloes and cattle are quite close phylogenetically, no case of BSE infected buffalo was ever reported, suggesting that genetic factors
are crucial to BSE susceptibility (Zhao et al., 2012). The expression level of PrP is closely correlated with BSE susceptibility. Studies show that the PRNP gene of cattle has two indel (insertion and deletion) polymorphisms (a 23-bp indel in putative promoter, and a 12-bp indel in intron 1). These indel polymorphisms affect gene expression (Msalya et al., 2011; Sander et al., 2005) and eventually BSE susceptibility (Haase et al., 2007; Juling et al., 2006; Sander et al., 2004). Studies on polymorphisms in buffalo in Anatolia (Oztabak et al., 2009), Pakistan (Imran et al., 2012), Indonesia and Thailand (Uchida et al., 2014) show significant differences in frequency distributions between buffaloes and cattle. Recently, genotyping analysis on Chinese buffalo showed that the distribution frequencies of BSE susceptibility related to genotypes and alleles, including the 23-bp deletion allele (D23) and 12-bp deletion allele (D12), were significantly lower than those of healthy cattle and BSE infected cattle, indicating that the low PrP expressed in buffalo may influence BSE susceptibility. Our later experiments proved that in tissue of the cerebellum, brain stem, mesenteric lymph nodes and bronchial lymph nodes, the expression of PrP is lower in buffalo than in cattle (submitted data).

Although PRNP play a vital role in the pathogenesis of TSE, the underlying pathological mechanisms of TSE remain unclear. Some propose that other than prions, there may be other factors or proteins regulating the pathogenesis and pathological process of TSE (Daude & Westaway, 2011; Watts et al., 2007). The SPRN (shadow of prion protein) gene and its encoded protein Shadoon (Sho) have drawn lots of attention due to their roles in the pathogenesis of TSE. Comparative genomics analysis indicates that Sho is a newly discovered member of the prion protein family. Sho has been found in mammals such as mice and humans and is highly conserved from fish to mammals (Premzl et al., 2003). Sho and PrP C have a lot in common regarding structure and expression (Wang et al., 2014). In PrP Sc infected animal brains or nervous cell, with increasing PrP Sc expression, the level of Sho decreases dramatically (Watts et al., 2007, 2011; Westaway et al., 2011). Beck et al. (2008) reported that the insertion of a base (heterozygous) within the encoding area of SPRN induces a frame-shift mutation which is correlated with vCJD. So, it is highly possible that Sho regulates the process of TSE by functioning as an inhibitory factor (Daude & Westaway, 2011). Our analysis of differences in the genetics and expression of SPRN between buffalo and cattle show that in the hydrophobic domain (HD) within the encoding area, cattle have a 12-bp indel polymorphism which induces insertion/deletion of four amino acids. However, this phenomenon was not observed in buffalo (Zhao et al., 2012). The HD of Sho not only protects against physiological stressors in nervous cell, but also helps Sho to interconnect with PrP C (Wang et al., 2010). This interconnection is a prerequisite of Sho regulating the pathogenesis of disease (Wang et al., 2010). The exploration of the indel polymorphism within the Sho HD structural area is critical in fully understanding underlying mechanisms of TSE. Our luciferase reporter and immuno-blotting experiments confirm that compared to cattle, buffaloes have higher promoter activity and higher Sho expression, consistent with our prediction that buffaloes have more transcription factor binding sites than cattle (Zhao et al., 2012). These findings suggest that the low susceptibility of buffalos to BSE is probably attributable to significant genetic differences in SPRN.

**Further Research**

Joaquin Castilla’s research group denies there are TSE resistant mammals, and believes that with improvements in detection any species can be found to be at risk of TSE infection (Fernandez-Borges et al., 2012). However, from available data, TSE susceptibility does vary between species and we can classify animals as high susceptibility species and low susceptibility species. Scientists worldwide have applied various techniques to the study of TSE pathogenesis and have mainly focused on the genetic polymorphism of PRNP, expression levels of Prnp/PrP, three-dimensional structure and stability of PrP C and dynamics of PRNP. However, the pathogenesis of TSE remains unclear. Lin & Wen (2011) claim that the three-dimensional structure of PrP Sc and the physiological function of PrP C are keys to resolving this puzzle but these two research directions have proved extremely difficult, even after two decades of attention. With breakthroughs in novel technologies and methods however, progress is likely. Studies on TSE low susceptibility molecules and newly discovered SPRN (Wang et al., 2014) provide important clues about the formation of PrP Sc and our understanding of TSE pathogenesis. Due to similarities in Sho and PrP C regarding structure and function, especially their important roles in the pathogenesis and development of TSE, exploration of the biological functions of Sho and its regulatory effect on TSE will be vital.
References


Courageot MP, Daude N, Nonno R, Paquet S, Di Bari MA, Le Dur A, Kumming Institute of Zoology (CAS), China Zoological Society


Khan MQ, Sweeting B, Mulligan VK, Aarslan PE, Cashman NR, Pai EF, Chakrabarty A. 2010. Prion disease susceptibility is affected by β-


