Porcine prion protein amyloid

Per Hammarström and Sofie Nyström*

IFM-Department of Chemistry; Linköping University; Linköping, Sweden

ABSTRACT. Mammalian prions are composed of misfolded aggregated prion protein (PrP) with amyloid-like features. Prions are zoonotic disease agents that infect a wide variety of mammalian species including humans. Mammals and by-products thereof which are frequently encountered in daily life are most important for human health. It is established that bovine prions (BSE) can infect humans while there is no such evidence for any other prion susceptible species in the human food chain (sheep, goat, elk, deer) and largely prion resistant species (pig) or susceptible and resistant pets (cat and dogs, respectively). PrPs from these species have been characterized using biochemistry, biophysics and neurobiology. Recently we studied PrPs from several mammals in vitro and found evidence for generic amyloidogenicity as well as cross-seeding fibril formation activity of all PrPs on the human PrP sequence regardless if the original species was resistant or susceptible to prion disease. Porcine PrP amyloidogenicity was among the studied. Experimentally inoculated pigs as well as transgenic mouse lines overexpressing porcine PrP have, in the past, been used to investigate the possibility of prion transmission in pigs. The pig is a species with extraordinarily wide use within human daily life with over a billion pigs harvested for human consumption each year. Here we discuss the possibility that the largely prion disease resistant pig can be a clinically silent carrier of replicating prions.

KEYWORDS. prion, pig, amyloid fibril, misfolding, transmissibility, seeding, TSE, prion strain, strain adaptation

ABBREVIATIONS. PrP, prion protein; HuPrP, human prion protein; BoPrP, bovine prion protein; PoPrP, porcine prion protein; recPrP, recombinant prion protein; TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy; PSE, porcine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; vCJD, variant Creutzfeldt-Jakob disease
Mammalian prions are composed of misfolded aggregated prion protein (PrP) with amyloid-like features. Prions are zoonotic disease agents that infect a wide variety of mammalian species including humans. Mammals and by-products thereof which are frequently encountered in daily life are most important for human health. It is established that bovine prions (BSE) can infect humans while there is no such evidence for any other prion susceptible species in the human food chain (sheep, goat, elk, deer) and largely prion resistant species (pig) or susceptible and resistant pets (cat and dogs respectively). PrPs from these species have been characterized using biochemistry, biophysics and neurobiology. Recently we studied PrPs from several mammals in vitro and found evidence for generic amyloidogenicity as well as cross-seeding fibril formation activity of all PrPs on the human PrP sequence regardless if the original species was resistant or susceptible to prion disease. Porcine PrP amyloidogenicity was among the studied. Experimentally inoculated pigs as well as transgenic mouse lines overexpressing porcine PrP have, in the past, been used to investigate the possibility of prion transmission in pigs. The pig is a species with extraordinarily wide use within human daily life with over a billion pigs harvested for human consumption each year. Here we discuss the possibility that the largely prion disease resistant pig can be a clinically silent carrier of replicating prions.

**TRANSMISSIBILITY OF PrP AMYLOIDS**

Prions are self-replicating proteinaceous pathogens composed of prion protein, PrP. Albeit the exact mechanisms of prion toxicity is not known, prion diseases are believed to correlate with the rate of prion replication.\(^1,^2\) Prions are known to be composed of aggregates of misfolded PrP molecules stacked in a beta-pleated-sheet structure with noticeable similarities to amyloid fibrils.\(^3,^4\) Also known as scrapie associated fibrils first discovered by Merz.\(^5\)

Prion protein amyloid, APrP, is linked to a number of human prion diseases most notably inherited diseases such as Gerstmann- Straussler-Scheinker disease with mutations in the PrP sequence.\(^6\) Importantly, in humans infected by BSE, vCJD (bovine prions) or Kuru (human prions) the disease is associated with large amounts of amyloid plaque. Hence these diseases likely represent strains of prions with APrP conformational polymorphs which may mature to amyloid plaque. The significance of amyloid plaques in prion diseases has been subject to considerable discussions.\(^7,^8\)

Amyloid fibrils are defined by pathology as aggregates of misfolded protein forming insoluble non-branched fibrils stabilized by cross-beta-sheet secondary structure with tinctorial properties of Congo red affinity exhibiting birefringence under cross-polarized light.\(^9\) Amyloid fibrils are also ThT/ThS positive and represent a mature phase of misfolded protein amyloidogenesis.\(^10\) Amyloid fibril formation is a chemical reaction similar to crystallization where a preformed fibril functions as a nucleus, nidus, or seed that accelerates the process of further fibrillation of soluble precursor protein. It is well established that amyloid fibrils show extensive structural polymorphism and is similarly believed to manifest as different pathogenic states representing prion strains in prion diseases.\(^11\) The plethora of molecular structures of PrP in infectious and transmissible prions has recently been reviewed by Baskakov and co-workers.\(^8\) A consensus structural feature from several studies of APrP is a cross-beta-sheet structure of the C-terminal sequence stretching from a variable starting point from the N-terminus (residue 90–140) all the way toward the final residue 231 within PrP fibrils. Considerable variations in the alignment of inter- and intramolecular beta-strands, packing of sidechains as well as quaternary filament assemblies are believed to compose a vast variability defining the structure of prion strains.

Seeding studies to produce APrP in vitro can mimic the molecular concept of the mechanism of replicative prion transmission. Replication is sustained by access to soluble PrP substrate to feed the reaction of self-replicating APrP fibrils which is accelerated by fragmentation of fibrils.\(^2\) Recently we showed that
7 diverse mammalian PrPs are amyloidogenic under near native conditions \textit{in vitro}.\textsuperscript{12} Hence we should treat these PrPs as a class of proteins which with ease can transform into amyloid fibrils. We purified native folded recombinant full length prion proteins (recPrP) with human (HuPrP), bovine (BoPrP) and porcine (PoPrP) sequence (among several others) and subjected them to an \textit{in vitro} fibrillation assay NCCA (native condition conversion assay) employing near native conditions (50 mM phosphate (pH 7.4), 100 mM NaCl, 50 mM KCl, 37°C, vigorous shaking). In this assay all 3 protein sequences were readily converted into amyloid-like fibrils as displayed by ThT kinetics, Congo red birefringence, LCO fluorescence, and transmission electron microscopy.\textsuperscript{12} Furthermore, seeds (1%, i.e. 50 nM PrP on a monomer basis) from the end point of unseeded reactions were added to freshly prepared recPrP and in all instances the lag times were significantly reduced upon seeding (Fig. 1). Importantly cross-seeding efficiency was essentially refractory to seed-substrate sequence heterology (Fig. 1).

**FIGURE 1.** Recombinant prion protein from with human, bovine and porcine sequence was fibrillated \textit{in vitro} under near native conditions as described in Nyström & Hammarström (2015).\textsuperscript{12} The bars represent lag times in minutes for unseeded (black columns) and seeded with 1% preformed fibrils of all the included sequences (blue for human, green for bovine and pink for porcine).

**FIGURE 2.** Structure and sequence comparisons. (a) Overlaid NMR structures of human, bovine and porcine PrP (blue: HuPrP PDB code 1QM2; green: BoPrP PDB code 1DWY; pink: PoPrP PDB code 1XYQ). (b) Sequence alignment with residues unique for one of the 3 species indicated with color code as in a)
In line with the protein-only hypothesis of prion disease transmission, PrP sequence mismatches of diverse mammalian PrPs have been put forward as one likely modulator of the known species barriers for prion transmission. The overall 3-dimensional fold is well conserved as determined by NMR spectroscopy.\(^{13-15}\) (Fig. 2a). Sequence alignments of the human, bovine and porcine PrP sequences show that they are 88–93% pairwise identical. Between BoPrP, HuPrP and PoPrP there are 10 amino acid substitutions that are unique to human, 3 that are unique to bovine and 9 that are unique to porcine PrP (Fig. 2b).

**PORCINE PrP AMYLOIDOGENTICITY**

From our seeding data (Fig. 1 and reference.\(^{12}\)) it is evident that the nucleation dependent polymerization process of fibrillation of these PrPs is not directly dependent of sequence identity between seed and substrate. The main message therefrom is that pure mammalian PrPs are compatible to efficiently cross-seed each other. What mainly attracted our attention was the striking amyloidogenicity of porcine PrP. RecPoPrP spontaneously and rapidly converted \textit{in vitro} to amyloid fibrils, even faster than human or bovine sequence (Fig. 1).

Despite numerous attempts to infect pigs with prions, hogs appear largely resistant to prion disease even when challenged by prions intracerebrally.\(^{16}\) It is known that incubation time in human disease can stretch over 10 y if the contaminated material is of bovine origin and transmitted via the oral route. Incubation times can be somewhat shorter (6.5–8 years) if the route of transmission is secondary to BSE via blood transfusion.\(^{17,18}\) In Kuru, incubation periods are estimated to be between 4 and 40 y although no species barrier exists in this case.\(^{17}\) Since PrP misfolding appears to be causative in initiating TSEs this implicates that prion disease resistant species such as pigs could harbor replicating prions without showing symptoms. Hence, the notion of separated fibril formation and neurotoxicity signaling merits further studies also in putatively disease resistant species

**PRION STRAIN ADAPTATION AND POSSIBILITIES OF PORCINE PRION TRANSMISSION**

The need of time and serial passages to allow prion strain adaptation when jumping between species is a well-known fact within the prion field. Numerous transmission experiments using transgenic mice expressing a range of prion sequences from different species have been conducted over the last decades aiming at delineating the species barrier and how this is modulated depending on homology between donor and recipient species as well as prion strain/isolate properties. Importantly transmission has almost exclusively been defined as selection by clinical disease, \textit{i.e.}, based on neurotoxicity. Over 50 mammalian species have been sporadically afflicted by and experimentally or accidentally infected with prions with neurotoxic outcome.\(^{19}\) Evidence for harboring replicating and infectious prions in absence of clinical disease was shown by hamster-mouse experiments exhibiting established species transmission barriers in terms of neurotoxicity.\(^{20,21}\) Obviously there is no simple answer to the question of species barrier modulators and perhaps there is no such thing as a prion resistant species.\(^{22}\) Cross-species strain adaptation of BSE and scrapie in wild type mice was investigated by the Fraser group.\(^{23}\) A number of TSE isolates from cattle and sheep were inoculated into C57BL/6 wild type mice expressing endogenous mouse PrP at 1x level. Cattle BSE was transmitted in the first generation with incubation times spanning between 400 and 500 d (Fig. 3a). At third passage the life span of the experimental animals dropped to around 250 d. When using scrapie isolates, the initial inoculation resulted in clinical disease resulting in death between 350 and 850 d post inoculation depending on isolate. Some scrapie isolates failed to transmit to the experimental animals. By the third passage the life spans for all active scrapie isolates converged at around 200–250 d (Fig. 3a). These experiments demonstrated that prion strain adaptation is relatively rapid also following host species jump even when PrP expression levels are normal, if the initial inoculum is transmissible.
What about pigs? In several recent papers which in our view have not received sufficient attention the notion of prion resistant pigs was challenged by generation of transgenic mice with knocked out endogenous PrP and overexpressed PoPrP. Different lines of tgPoPrP mouse were proven to be susceptible to clinical disease triggered by a variety of prion strains, suggesting that the surrogate host species (mouse) and prion strain are more important than what PrP sequence it expresses for neurotoxicity to commence. In more detail, Torres and colleagues experimentally subjected transgenic mouse lines expressing porcine PrP to a number of different TSE isolates. Their studies demonstrate that prion infection is strain specific when porcine PrP is overexpressed (4x) and used as in vivo substrate. PoTg001 mice inoculated with classical scrapie, regardless of donor genotype, resisted prion disease both at first and second passage (Fig. 3b). On the other hand, Nor98 scrapie (Atypical scrapie) as well as BSE from both cattle and BoTg mouse model resulted in clinical disease in the PoTg001 mice. However, in the first generation, disease progression was
slow. Incubation time until death was as long as 600 d and the hit rate was low. This indicates that disease has barely developed by the time the mice reach their natural life span limit which in this study was set to 650 d. Already in the second passage the hit rate was 100 % and the incubation time was cut in half (Fig. 3b). No further shortening of incubation time was observed upon third passage. This shows that PoPrP is capable of forming infectious and neurotoxic prions in vivo if triggered by a compatible prion strain and if given enough time to develop. Both BSE and Nor98 rapidly adapts to the PoPrP host sequence, resulting in higher penetrance as well as in markedly shorter life span already in the second passage, well within the limits of normal life span for a mouse.

There are several crucial variables which impact the susceptibility of prion diseases and transmission studies. PrP sequence of host, PrP sequence of prion, prion strain, prion dosage, PrP expression level of host, host genetic background, route of transmission and neuroinvasiveness if peripherally infected. Importantly the PrP expression level corresponds to the rate of prion disease onset. This likely reflects 2 converging variables: a) PrP as a substrate to the prion misfolding reaction i.e. self-catalyzed conversion and b) PrP as a mediator of neurotoxicity through interactions with misfolded PrP within prions.

The non-homologous recPrPs presented here and in, easily adapt to each other and form amyloid fibrils in accordance with what is seen in vivo when inoculum composed of BoPrP used to challenge mice expressing PoPrP (Fig. 3b). A review of the literature showed that BSE strains have a high degree of penetrance in both experimental and accidental transmission. Over 50% of the species reported to be susceptible to prion disease were infected by a BSE strain. Recent data from our lab shows that the promiscuity of BoPrP fibrils holds true also in the case of recombinant in vitro experiments. When cross-seeding human, bovine, porcine, feline and canine PrPs with any of the other, the recBoPrP seed outcompetes the other seeds in all instances except when the HuPrP acted as substrate (Data not shown). In this case recPoPrP fibrils have the highest seeding efficiency (Fig. 1). These findings in combination with the Torres experiments implicate that a PoPrP substrate in vivo (in pigs) could adapt to an amyloidogenic prion strain of bovine or ovine prion disease and hence replicate in the new host.

For adaptation of experimental strains through multiple passages, donors are selected based on neurotoxicity (that is on TSE disease phenotype) not on basis of amyloid fibril formation. Hence the traits of transmissible amyloidyotypic prion strains may be largely unexplored if these strains require more time to transform to neurotoxic strains e.g. as proposed by Baskakov’s model of deformed templating. There is experimental evidence for BSE transmission into pig via parenteral routes, with an incubation period of 2–3 years, well within what is to be considered normal lifespan. For a breeding sow in industrial scale pig farming that is 3–5 y (Bojne Andersson, personal communication). In small scale and hobby farming both sows and boars may be kept significantly longer. Collinge and Clarke describe how prion titers reach transmissibility levels well before the prion burden is high enough to be neurotoxic and cause clinical disease. It is known that prion strains need time and serial passages to adapt. Knowing that pigs in modern farming are rarely kept for enough time for clinical signs to emerge in prion infected pigs it is important to be vigilant if there is a sporadic porcine spongiform encephalopathy (PSE) as has been seen in cattle (BASE) and sheep (Nor98). Hypothetically such a sporadic and then infectious event could further adapt and over a few generations have reached the point where clinical PSE is established within the time frame where pigs are being slaughtered for human consumption (Fig. 4).

USE OF MATERIALS DERIVED FROM PIG IN VIEW OF PORCINE PRP AMYLOID

The pig is the most versatile species used by humans for food and other applications. Over
1.5 billion pigs are slaughtered each year worldwide for human use. Besides juicy pork sirloin other parts from pig are used for making remarkably diverse things such as musical instruments, china, leather, explosives, lubricants etc. Pig offal is used for human medicine, e.g., hormone preparations such as insulin and cerebrolysin, in xenographs, sutures, heparin and in gelatin for drug capsules. While the late Carleton Gajdusek had strong views in diverse areas of prion biology, according to journalist Richard Rhodes, he was correct on his prediction on BSE prions (vCJD) in the blood supply (see text box above). An opinionated scientist can sometimes be ignored due to a judgment of character and Gajdusek was certainly provocative. Notwithstanding society should remain vigilant on the possibility that Gajdusek was also prophetic on porcine prions given the exceptionally wide spread use of pigs in everyday human life and medicine. As discussed previously it is currently not established what relations transmissible neurotoxic prion strains and amyloid morphotypic mature APrP strains have. Given the hypotheses that amyloidotypic PrP conformations can transmit with low neurotoxicity, it is interesting to reflect on possible implications. Pigs are slaughtered at 6–8 months of age. Because amyloid deposition is associated with old age, this is likely far too young for spontaneous development of APrP amyloid from PoPrP as well as other amyloidogenic proteins. From the perspective of seeded amyloidogenesis it is however a potential ideal case for highly transmissible titers of APrP (Fig. 4). In such a scenario the potential of porcine prions constitutes the perfect storm, clinically silent due to neurotoxic resistance and with high titers of transmissibility. When it comes to prions CNS material is most heavily infected. In addition, however, fat tissue (to make lard and tallow) is known to harbor extraordinary amounts of amyloid in systemic amyloidoses. Amyloid fibrils of misfolded large proteins (AA, AL, ATTR) are notoriously hydrophobic due to the abnormal exposure of hydrophobic residues which
normally in the folded structure being hidden in the protein core. The amyloid accumulation in fat tissue is likely a phase-separation from a rather hydrophilic environment in circulation toward the hydrophobic environment provided by adipocytes. Adipose tissue could in addition represent an in vivo environment well suitable for fibril formation. What about APrP?

In analysis of mice expressing Glycophosphatidylinositol, (GPI)-anchorless PrP, abdominal fat contains appreciable amounts of infectious prions in APrP isoform stained with ThS. Notably mice overexpressing anchorless PrP provides a silent carrier status for a long time prior to presenting symptoms and is severely afflicted by amyloid fibril formation following scrapie (RML) infection. Recall that this study showed that GPI-anchored PrP is needed to present clinical neurotoxicity. Evidently circulating anchorless-PrP (analogous to recPrP) is more amyloidogenic compared to GPI-anchored PrP and is poorly neuroinvasive. Amyloidosis is systemic in anchorless-PrP mice and is not limited to fat but is also found as extensive cardiac amyloid deposits. Interestingly cardiac APrP was recently reported in one BSE inoculated rhesus macaque which showed symptoms of cardiac distress prior to death from prion neurotoxicity. It is noteworthy that transgenic mice expressing PoPrP appear sensitive to strains with biochemical features of amyloidogenic prion strains i.e., BSE and Nor98. (Fig. 3b). We recently adopted the parallel in-register intermolecular β-sheet structural model of the APrP fibril from the Caughey lab to rationalize cross-seeding between various PrP sequences. It is tempting to use this structural model to speculate on the adaptation of mono-N-glycosylated PoPrP at the expense of double-N-glycolylated PrP in the original BSE inoculum reported in the Torres experiments. In this APrP model monoglycosylated PrP at N197 is structurally compatible while N181 is not, due to burial in the in-register intermolecular cross-beta sheet (Fig. 5).

It appears that amyloidotypic prion strains, APrP, are transmissible but associated with lower neurotoxicity compared to diffuse aggregated PrP associated with synaptic PrP accumulations. It is possible that the amino acid substitutions in PoPrP compared to HuPrP and BoPrP are important for neurotoxic signal transmission (Fig. 2b, 5). The main issue hereby is that transmissibility of APrP will remain undetected unless used for surveillance. AA amyloidosis is frequent in many animals (e.g. cattle and birds) but is exceptionally rare in pigs, suggesting that APrP should it reside in pig fat would be traceable using newly developed screening methods.
CONCLUDING REMARKS

Should the topic of porcine PrP amyloid be more of a worry than of mere academic interest? Well perhaps. Prions are particularly insidious pathogens. A recent outbreak of peripheral neuropathy in human, suggests that exposure to aerosolized porcine brain is deleterious for human health.43,44 Aerosolization is a known vector for prions at least under experimental conditions.45-47 where a mere single exposure was enough for transmission in transgenic mice. HuPrP is seedable with BoPrP seeds and even more so with PoPrP seed (Fig. 1), indicating that humans could be infected by porcine APrP prions while neurotoxicity associated with spongiform encephalopathy if such a disease existed is even less clear. Importantly transgenic mice over-expressing PoPrP are susceptible to BSE and BSE passaged through domestic pigs implicating that efficient downstream neurotoxicity pathways in the mouse, a susceptible host for prion disease neurotoxicity is augmenting the TSE phenotype.25,26 Prions in silent carrier hosts can be infectious to a third species. Data from Collinge and coworkers.21 propose that species considered to be prion free may be carriers of replicating prions. Especially this may be of concern for promiscuous prion strains such as BSE.19,48 It is rather established that prions can exist in both replicating and neurotoxic conformations.49,50 and this can alter the way in which new host organisms can react upon cross-species transmission.51 The naïve host can either be totally resistant to prion infection as well as remain non-infectious, become a silent non-symptomatic but infectious carrier of disease or be afflicted by disease with short or long incubation time. The host can harbor and/or propagate the donor strain or convert the strain conformation to adapt it to the naïve host species. The latter would facilitate infection and shorten the incubation time in a consecutive event of intra-species transmission. It may be advisable to avoid procedures and exposure without proper biosafety precautions as the knowledge of silence carrier species is poor. One case of iatrogenic CJD in recipient of porcine dura mater graft has been reported in the literature.52 The significance of this finding is still unknown. The low public awareness in this matter is exemplified by the practice of using proteolytic peptide mixtures prepared from porcine brains (Cerebrolysin) as a nootropic drug. While Cerebrolysin may be beneficial for treatment of severe diseases such as vascular dementia,53 a long term follow-up of such a product for recreational use is recommended.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed

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