Review article

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Amyloid and prions: some biochemical investigations of cerebral amyloidosis in mice

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Abstract

Prion-like transmission of protein aggregates or amyloid in several neurodegenerative diseases, such as Parkinson's disease, Huntington's disease and Alzheimer's disease, in addition to the transmissible spongiform encephalopathies (or prion diseases), has been proposed recently. This is a controversial idea and, in this paper, we consider what we mean by a "prion", and by "amyloid", and present some biochemical investigations of cerebral prion amyloidosis in mice.

Key words: *prion, amyloid, transmissible spongiform encephalopathy, mouse, strains, time-resolved fluoroimmunoassay, cerebral amyloidosis, Alzheimer's disease, Huntington's disease, Parkinson's disease.*

Introduction

Historically, a prion was defined as a "proteinaceous infectious particle" whose "main or sole component" was the prion protein (PrP) [31] and the name was originally essentially a synonym for the self-replicating agent which causes the progressive, transmissible spongiform encephalopathies (TSEs) of mammals. Infectious and transmissible (and contagious) can be used interchangeably in lay language to describe the spread of a disease pathology or a disease-causing agent from one cell or organism to another, usually by direct contact, or via air or water. In science, in this context, transmissible is used generically to describe that property of movement and replication of a non-host pathogen but it can also apply to the simple propagation of disease or disease pathology in the absence of a self-replicating agent.

Prion-like transmission of protein aggregates or amyloid in several neurodegenerative diseases, such as Parkinson's disease, Huntington's disease and Alzheimer's disease, in addition to the transmissible spongiform encephalopathies (or prion diseases), has been proposed recently [9,12,15,16]. Induced diseases such as experimental encephalomyelitis [26] or systemic amyloidosis [39] are examples of transmissible but non-infectious diseases, and they are not normally considered as prion diseases. But can they be regarded as "prion-like"? Perhaps, but it is not informative to lump these diseases, as well as AD, HD and PD, together with TSEs as "prion-like": we need a more stringent description than the historical definition of "prion" to guide our understanding. This better definition was provided by Reed Wickner in 1996 [36], and subsequently refined further [37].

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The original definition of a yeast prion highlighted the following properties:

- reversible curability;
- over-producing the (prion) protein increases the frequency of prion generation;
- the phenotype of (a) mutant (prion protein) resembles the phenotype of the prion; and it required the biochemical proof of a prion by demonstrating infectivity from an altered version of a synthetic or recombinant form.

These properties of a "prion" were distilled from the genetics of yeast but, apart from "reversible curability" which seems a long way away in terms of mammalian prion disorders, the revised concept of a prion is very similar and has four tenets:

- a prion is a chromosomally-encoded protein that exists as a conformational isomer of one of two covalently identical forms;
- the prion form can induce the other (normal or cellular) form to a new copy of the prion form;
- in both mammals and yeast, over-producing the protein will increase the frequency of prion generation; and
- that the effects of the prion form can be reproduced *in vivo* by a suitably re-folded recombinant or synthetic copy of the protein.

Nowhere in this definition is there a need for the prion isoform to aggregate for its function, or for those aggregates to have a distinct morphology or biochemistry such as an amyloid fibril or resistance to proteases, respectively. Several naturally-occurring prion diseases, such as bovine spongiform encephalopathy and classical scrapie in sheep and their laboratory rodent models, are associated with protease-resistant prion protein (PrP) which can be visualised by light microscopy as amyloid plaques and by electron microscopy of tissue extracts or tissue sections as part of a fibrillar structure [19,20]. However, there are model systems of prion diseases which produce high titres of infectivity in the presence of little or no protease-resistant prion protein [1], and the abnormal prions of natural cases of atypical scrapie in sheep have a very different biochemistry to those causing classical scrapie and BSE [17]. Baskakov and Breydo considered what might make a protein infectious [2] and they and others finally provided evidence meeting Wickner's fourth criterion in the definition of a prion almost 30 years after Prusiner's initial hypothesis (for a review see [27]), and protease-resistance is not a necessary feature of synthetic prions [11].

Prion diseases differ from other amyloid-associated protein misfolding diseases (e.g. Alzheimer's) because they are naturally transmitted between individuals and involve spread of protein conformational changes between tissues. Factors underlying these features of prion diseases are poorly understood although, of all protein misfolding disorders, only prion diseases involve the misfolding of a glycosylphosphatidyl-inositol (GPI)-anchored protein and GPI anchoring has been proposed as the key factor which modulates the propagation and spread of prion aggregates [25]. Using a GPI-anchored version of the amyloidogenic yeast protein Sup35NM (Sup35(GPI)) expressed in neuronal cells, Speare *et al.* found treatment of cells with Sup35NM fibrils induced the GPI anchor-dependent formation of self-propagating, detergent-insoluble, protease-resistant, prion-like aggregates of Sup35(GPI) [34]. While it can be inferred from these model studies that a GPI-anchor may enhance the transmissibility and pathogenesis of prion diseases relative to other protein misfolding diseases, apparently contrary evidence showing the lack of a GPI-anchor on the mammalian prion protein does not prevent amyloid formation and amplification of the level of infectious particles was found in a Tg PrP GPI-minus mouse challenged intracerebrally with various strains of murine-passaged scrapie [10].

These studies, with apparently contradictory indications, focus on the amyloid or aggregate nature of some prions although as we have previously noted "amyloid" is not part of the definition of a prion so it is also relevant here to look at the definition of "amyloid". The Wiktionary [21] defines amyloid as i) a waxy compound of protein and polysaccharides that is found deposited in tissues in amyloidosis; and ii) any of various starch-like substances. The name is derived from the Latin for starch (amylum), because of its comparable iodine-binding properties, and pre-dates the realisation that this material is protein. Various histological stains have been used to provide an operational definition of amyloids and, most widely, its binding to the polyaromatic dye Congo Red and characteristic light-green birefringence when visualised under polarized light has been diagnostic. Many neurological diseases have amyloid deposits in the brain as their distinguishing feature and years before the characterisation of the mammalian prion protein by Prusiner *et al.*, histological staining for amyloid plaques and vascular amyloid was a technique for the morphometric classification of different types or strains of experimental prion disease

in mice [13,38]. More recently, a broader biophysical definition of amyloid has come to include any polypeptide which polymerizes to form a cross-β structure (for a review of the structure and nomenclature of amyloids see [33]), *in vivo*, or *in vitro*.

Our interpretation of the current controversy about the prion-like nature of AD, HD and PD, and the non-TSE-like nature of some human and animal prion protein diseases [23,30], is that it could be clarified by a better operational definition of the prion form of the relevant proteins so that the definition covers both amyloid and non-amyloid components of prions and does not focus on "protease resistance" as its main operational criterion. As a first step towards that goal, we report here the detergent extractability and epitope occlusion of murine prion protein in the Fraser and Bruce experimental models of mouse prion cerebral amyloidosis.

Material and methods

All materials and methods used to produce the electron micrographs of murine amyloid in Fig. 1 are provided by Jeffrey *et al.* [23] and those used to determine the data presented for mouse survival time and amyloid score in Table I are to be found in the references in the Table footnotes. The materials and methods outlined below refer to those used to produce the data for Fig. 2 and the A' score of Table I.

Animals and prion strains

Frozen brain from terminally-affected mice (VM/Dk or SV/Dk strains) infected intracerebrally with a 20 μL dose of 10^{-2} (w/v)-inoculum of various prion strains (22L, 87V, 22A, ME7, 79A, 301V) were kindly supplied to us by Ms Angela Chong and Dr Robert Somerville, BBSRC Institute for Animal Health, Neuropathogenesis Unit, Edinburgh. 87V, 22A and 301V prions were produced by serial passage in *Prn-p*^{b} mice and ME7, 79A and 22L prions were produced by serial passage in *Prn-p*^{a} mice. Under these passage conditions, their characteristics of lesion profile and relative incubation period are stable and this encouraged us to compare our biochemical data with historical data in the literature on their amyloid content [5-8,14,28].

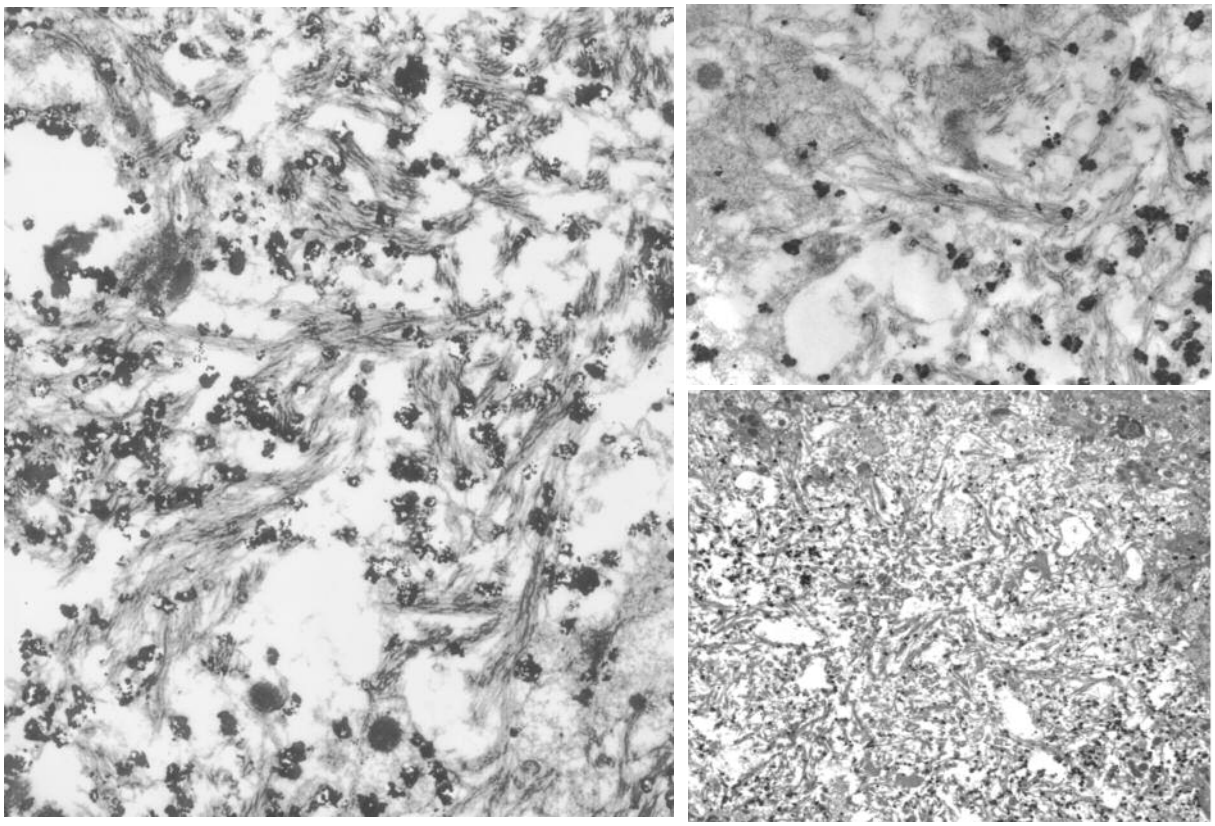

Fig. 1. Immuno-electron microscopy of murine scrapie plaques with anti-prion protein mAb, P2. Kindly provided by Dr Gillian McGovern

Table I.

Prion type	Mouse strain	*Prn-p*	Survival time (days)[a]	Amyloid score[a]	A' score[b]
ME7	C57Bl/6	a	161 (± 1)	13	26.7
ME7	VM/Dk	b	333 (± 2)	511	34.7
79A	C57Bl/6	a	152 (± 1)	0	6.5
79A	VM/Dk	b	293 (± 6)	0	11.3
22L	C57Bl/6	a	157 (± 2)	–	7.0
22L	VM/Dk	b	300 (± 1)	–	10.0
301V	C57Bl/6	a	300 (± 4)	–	22.7
301V	VM/Dk	b	120 (± 1)	–	18.7
22A	C57Bl/6	a	458 (± 4)	370	9.0
22A	VM/Dk	b	195 (± 1)	0	13.3
87V	VM/Dk	b	294 (± 4)	962	36.3

a) [4–7,13,27], b) this work

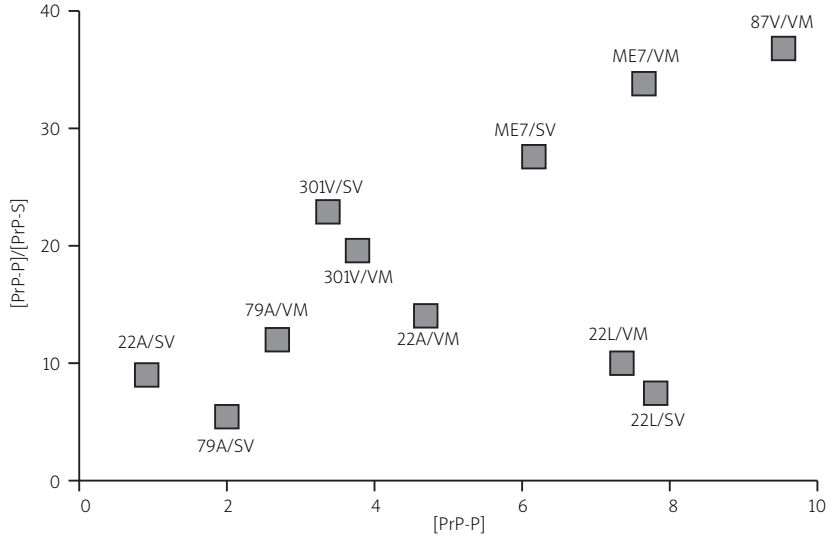

Fig. 2. PrP signal after denaturation of an "operationally-insoluble" or "pellet" fraction of mouse brain infected with different prion strains ([PrP-P]) against the ratio of that signal and the PrP signal after denaturation of an "operationally-soluble" or "supernatant" fraction from the same brain extract ([PrP-P]/[PrP-S]).

Biochemical procedures

Brain homogenisation and use of various detergents

For each prion strain/mouse strain combination, six whole mouse brains were processed and analysed individually as follows: brain (0.4 g) was homogenised at 20% (w/v) in 1.4M-Guanidinium chloride, 10mM-Tris-HCl, pH 7.4 at 4°C and then mixed with an equal volume of 1% (w/v) ZW3-14 detergent and allowed to stand for 15 minutes. The homogenate was spun at 8000 g for 15 minutes, and pellet and supernatant separated and sonicated in either 6M-GdnHCL, 1mM-Dithiothreitol (DTT), 0.5%-ZW3-14, pH 7.4 (pellet) or mixed with 3 volumes of 8M-GdnHCL and adjusted to 1 mM-Dithiothreitol (DTT), 0.5%-ZW3-14, pH 7.4 (supernatant).

The denatured pellet (P) or supernatant (S) was then diluted 1 : 50 into time-resolved fluoroimmunoassay buffer for measurement of total PrP content. Values were averaged for all brains of a particular strain/mouse line model (n = 6) and the standard error of the mean was ± 15%. The efficiency of extraction and denaturation by other detergents (Triton-X100, sodium dodecyl sulphate, octyl glucoside and NP-40), and chaotropic agents (guanidinium with different counter anions; and urea) on the assay signal was also evaluated (data not presented; but see [18]).

Time-resolved fluoroimmunoassay

The time-resolved fluoroimmunoassay for abnormal prion protein has been described previously in principle [3,32] and used a standard capture antibody: detection antibody sandwich format. For this investigation, we absorbed monoclonal antibody (mAb) FH11 (produced at the Institute for Animal Health, Compton and provided by Dr Chris Birkett and Mrs Ruth Hennion) to NUNC low fluorescence Maxisorp microtitre plates by overnight incubation with 0.2 mL antibody (~ 1 mg/mL in phosphate-buffered saline, pH7.4), aspiration, air drying and storage prior to use at 4°C. The mAb was raised against recombinant bovine PrP and cross reacts with the bovine sequence G^{49}NRYPPQGGGGWG and to a lesser extent to the mouse sequence G^{89}QGGGTHNQWNK (by Pepscan analysis, Birkett, unpublished). For detection mAb, we used mAb 6H4 purchased from Prionics SG, Zurich, Switzerland; this mAb has a wide species range (including mouse) and the epitope is in the C-terminal region of PrP (residues 145-160; Prionics SG, Zurich). This reagent was labelled with N-1(*p*-isothiocyanatophenyl)-diethylene-triamine N^1, N^2, N^3-tetraacetate chelated to europium and the conjugate characterised according to the manufacturer's protocol (Perkin-Elmer, Beaconsfield, UK; Eu-labelling kit, Cat. No. 1244-302). Typically, stock solutions were prepared and stored at 20 μg/mL immunoglobulin (11 Eu^{2+}/Ig) in Tris-HCl buffer (pH 7.8) containing sodium azide (0.1%), sodium chloride (0.9%) and bovine serum albumin (0.1%). Before use, the stock was diluted 1 : 100 v/v in Tween-40 assay buffer (Perkin-Elmer; Cat. No. 1244-106) and filtered through a 0.22 μm filter. The final concentration of detecting antibody was about 200 ng/mL and 200 μL (40 ng) was used per microtitre plate well.

Recombinant mouse prion protein (recPrP, sequence equivalent to the *Prn-p*^{a} allotype [35]) was used to calibrate the fluorescence signal in terms of a concentration of prion protein; the protein was produced in *E. coli* using a dual-origin vector based on pMG165, purified and supplied to us by Dr Alan Bennett, IAH, Compton). Standard curves were produced using recPrP in concentrations of guanidinium chloride and detergent equivalent to those in the samples; equivalent dose-response curves were produced in this assay format using the mouse *Prn-p*^{b} allotype (data not presented).

In brief, the assay protocol was as follows: dry coated microtitre plates were washed in assay buffer (Perkin-Elmer, Cat. No. 1244-106) containing 0.5%-Tween 40. Standard or sample (200 μL) in Tween 40-assay buffer was added to the wells and the plate incubated with shaking for 1h at room temperature. Subsequently, plates were washed 3× using Perkin-Elmer plate washer and tapped dry. Eu-labelled mAb (200 μL) was added to each well and the plates incubated 1h at room temperature with shaking. The plates were then washed 6× and dried. Enhancement solution (200 μL) was added to each well, incubated for 5 minutes and then the signal measured using the Perkin Elmer Victor II Plate fluorimeter. The concentration of recPrP equivalents in the detergent-extracted-brain pellet ([PrP-P]) and concentration of recPrP equivalents in the detergent-extracted-brain supernatant ([PrP-S]) were then determined from the recPrP standard curve.

Results & Discussion

The mammalian prion proteins (PrP) of transmissible spongiform encephalopathies (TSE) are GPI-linked plasma membrane polypeptides of ~230 amino acids. More than half the molecule is tightly folded in a three α-helices structure linked by short stretches of β-sheet and the rest, containing a metal binding His repetitive structure, adopts a flexible random-coil conformation in solution. In the wild-type mouse, there are two alleles of the prion protein gene, *Prn-p*^{a} and *Prn-p*^{b}, and they encode proteins differing in two amino-acids, at codons 108 and 189; *Prn-p*^{a} mice encode the allotype 108L/189T and *Prn-p*^{b} encodes the allotype 108F/189V [35].

Most of the data in the literature which we have mined as part of our meta-analysis of mouse prion amyloid pre-dates the discovery of the prion protein and it is summarized in Table I, with our more recent biochemical investigations of the detergent solubility and extractability of abnormal prion protein from the brains of terminally-affected, scrapie-infected mice. Fraser and Bruce used various strains of wild-type mice

for their studies, and defined their strains of prions in terms of histological lesion profiles at set levels of the brains and the relative ranking of survival time of a panel of mice differing at their *Sinc* (scrapie incubation time) locus [4]. Our biochemical investigations used Sinc congenic mice: VM/Dk (*Sinc*^{p7}) and SV/Dk (*Sinc*^{s7}) [produced by back-crossing the *Sinc*^{s7} allele of C57Bl/6 mice onto the VM/Dk background] [22].

In their studies on the aetiology of scrapie amyloid in mice, Fraser and Bruce relied on conventional dye stains such as Masson's trichrome or haematoxylin/eosin and their metric for amyloidosis was the number of amyloid structures per section visualized by light microscopy and the percentage of mice affected. They painstakingly evaluated the experimental parameters which affected their amyloid score and concluded that *Sinc* genotype and prion strain were the predominating factors (see references in Table I), although they also uncovered in their use of non-*Sinc* congenic mouse lines a contribution from other genes to their amyloid score [6]. *Sinc* congenic lines were later developed by Dickinson and colleagues, and *Prn-p* shown to be encoded within the *Sinc* locus [22]. The *Sinc*^{s7} mouse lines used by Fraser and Bruce are now known to encode the *Prn-p*^{a} allotype, and their VM/Dk *Sinc*^{p7} line encodes the *Prn-p*^{b} allotype [29].

During TSE pathogenesis in *Prn-p* congenic mice, neural cell membrane structures contort, glial cells proliferate and at the end stages of disease there is widespread deposition of conformationally-altered aggregates of PrP and other cell membrane debris in the brain. Figure 1 shows labelling of cerebral amyloid plaques in the brain of a *Prn-p*^{b} mouse terminally-affected by the 87V prion strain using gold-particle-tagged mAb P2 and electron microscopy; mAb P2 is specific of residues 60 to 80 of PrP [23]. Various pathological parameters such as vacuolation, degree of gliosis, amyloid deposition or distribution and type of PrP^{D} immunostaining have been used to categorise prions and compliment their biological properties such as the relative survival time of mice differing in their *Prn-p* (*Sinc/Prn-i*) allelism.

In this study, we have expanded the categorisation of different types of prion in *Prp-p* congenic mice to include the ease with which detergents extract and solubilise abnormal prion protein (PrP^{D}) from brain membranes and the amyloidic character of that PrP^{D} as determined by guanidinium chloride treatment and a version of Safar's conformation-dependent immunoassay (CDI) [32]. Figure 2 plots the PrP signal after denaturation of an "operationally-insoluble" or "pellet" fraction of mouse brain infected with different prion strains ([PrP-P]) against the ratio of that signal and the PrP signal after denaturation of an "operationally-soluble" or "supernatant" fraction from the same brain extract ([PrP-P]/[PrP-S]). We found that the ratio of the amounts of PrP in a detergent soluble form to its amyloidic or "insoluble" form (the amyloid coefficient, A') varied with prion type or strain but was mostly independent of the *Prn-p* genotype of the congenic mouse host.

For a single prion strain, the ranking of its incubation periods in *Prn-p*^{a} and *Prn-p*^{b} mice, and the corresponding A', correlated with [PrP-D]; similarly, Fraser and Bruce also reported their amyloid score correlated with the incubation period rather than the age at inoculation of mice for individual strains [8, Table I]. We found an exception in the example of the 22A prion where its [PrP-P] in *Prn-p*^{a} mice differed considerably from that in *Prn-p*^{b} and both this parameter, and A', were inversely correlated to their respective incubation periods. This disparity in biochemical properties of 22A in these congenic mice mimics the disparity of the histochemical characteristics of 22A in *Prn-p*^{a} (where it is re-categorised as 22F) and *Prn-p*^{b} mice [4] and adds weight to the use of A' as an independent biochemical parameter for categorising prion types.

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