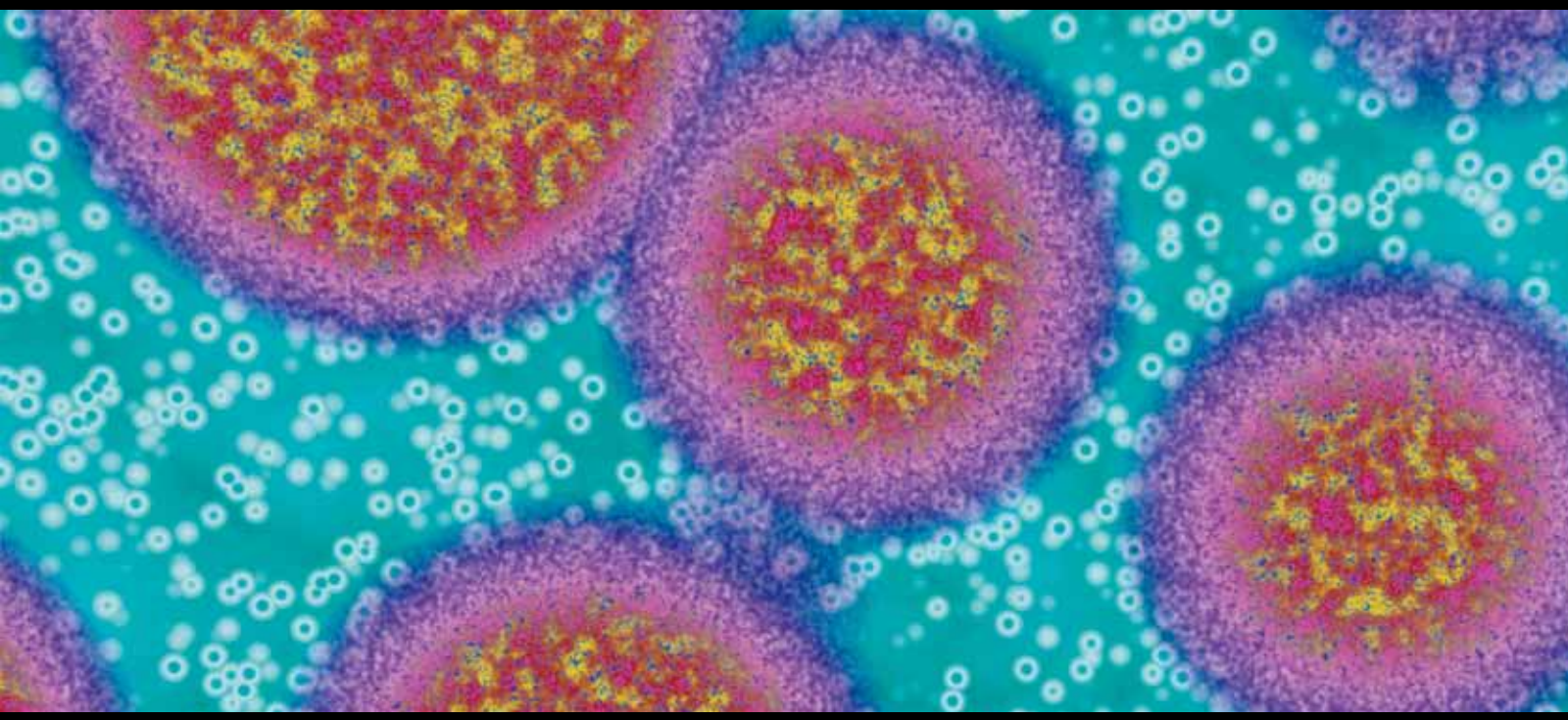
International Journal of Cell Biology

Protein Misfolding and Neurodegenerative Diseases

Guest Editors: Alessio Cardinale, Roberto Chiesa, and Michael Sierks



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Editorial Board

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Editorial

Protein Misfolding and Neurodegenerative Diseases

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This special issue includes fifteen reviews and two original research articles by leading scientists in the fields of neuropathology, biochemistry, and cell biology, dealing with the role of protein aggregation and prion-like propagation of protein misfolding in neurodegenerative diseases.

In the review article "*Breaking the code of amyloid-β oligomers,*" available at the following link: http://www.hindawi.com/journals/ijcb/2013/950783/, S. E. Lesnè outlines the "oligomeric" view of the amyloid hypothesis in Alzheimer's disease (AD), discussing how structurally different amyloid-β (Aβ) oligomers may contribute to the pathogenesis, and the controversial role of the prion protein (PrP) in Aβ toxicity. He stresses the need to thoroughly characterize the oligomeric Aβ assemblies for dissecting the disease mechanisms and designing specific, effective therapies.

Tau oligomers may also play an important neurotoxic role in AD. In the research article "*Trimeric tau is toxic to human neuronal cells at low nanomolar concentrations,*" available at the following link: http://www.hindawi.com/journals/ijcb/2013/260787/, H. Tian et al. show that two nonphosphorylated human recombinant tau splice variants are neurotoxic at low nanomolar concentrations. They provide evidence that trimeric but not monomeric or dimeric tau is responsible for the toxicity. In the review article "*The innate immune system in Alzheimer's disease,*" available at the following link: http://www.hindawi.com/journals/ijcb/2013/576383/, A. Boutajangout and T. Wisniewski focus on the potential roles of the triggering receptor expressed on myeloid cells 2 protein (TREM2) and Toll-like receptors (TLRs) in AD. They give an overview of TREM2 functions and its involvement in phagocytic and anti-inflammatory pathways. They also review the critical roles of TLR4 and 9 in the innate immune response, the interplay of these pattern recognition receptors, and highlight the importance of microglia-mediated innate immunity in AD pathogenesis.

Several articles deal with the cellular processes involved in protein folding and quality control and how their corruption may trigger neurotoxicity. In the review article "*Disulfide bonding in neurodegenerative misfolding diseases,*" available at the following link: http://www.hindawi.com/journals/ijcb/2013/318319/, M. F. Mossuto discusses the role of disulfide bond formation; in the review article "*Role of protein misfolding and proteostasis deficiency in protein misfolding diseases and aging,*" available at the following link: http://www.hindawi.com/journals/ijcb/2013/638083/, K. Cuanalo-Contreras et al. review the involvement of the unfolded protein response (UPR), the ubiquitin proteasome system (UPS), autophagy, and aggresome formation in neurodegenerative diseases and aging. In the review article "*ER dysfunction and protein folding stress in ALS,*" available at the following link: http://www.hindawi.com/journals/ijcb/2013/674751/, S. Matus et al. specifically focus on the role of UPR in amyotrophic lateral sclerosis (ALS), and in the research article "*Early delivery of misfolded PrP from ER to lysosomes by autophagy,*" available at the following link: http://www.hindawi.com/journals/ijcb/2013/560421/, C. J. Cortes et al. provide experimental evidence of a role of autophagy in the early quality control of misfolded PrP. In the review article "*S-Nitrosation and ubiquitin-proteasome system interplay*

in neuromuscular disorders," available at the following link: http://www.hindawi.com/journals/ijcb/2014/428764/, S. Rizza et al. give an overview on the mechanisms regulating S-nitrosation and its implication in redox signaling and neurodegeneration. They provide evidence that S-nitrosation is involved in UPS and suggest links with the pathogenesis of neuromuscular disorders and neuropathies.

In the review article "*Convergence of synapses, endosomes, and prions in the biology of neurodegenerative diseases,*" available at the following link: http://www.hindawi.com/journals/ijcb/2013/141083/, G. K. Gouras critically discusses the roles played by synapses and cellular proteolytic systems in generation, accumulation, and prion-like propagation of neurodegenerative disease-specific proteins. He stresses the need to define the physiological functions of the misfolding proteins as well as to understand the cell biology of the synapses and endocytic/exocytic pathways in neurons better.

The review article "*Prion protein misfolding, strains, and neurotoxicity: an update from studies on mammalian prions*" by I. Poggiolini et al. available at the following link: http://www.hindawi.com/journals/ijcb/2013/910314/ is a comprehensive review of the prion diseases, looking at the latest information on current knowledge of the mechanisms of PrP conversion and the molecular basis of prion strains. The review article "*From prion diseases to prion-like propagation mechanisms of neurodegenerative diseases*" by I. Acquatella-Tran Van Ba et al. available at the following link: http://www.hindawi.com/journals/ijcb/2013/975832/, summarizes the history of prion diseases, from the development of the prion concept to the production of synthetic prions, and discusses recent hypotheses on the mechanisms of *de novo* prion generation. The review article "*Synaptic dysfunction in prion diseases: a trafficking problem?*" by A. Senatore et al. http://www.hindawi.com/journals/ijcb/2013/543803/, reviews recent data pointing to intracellular PrP misfolding in synaptic dysfunction and suggests a new model of synaptotoxicity that could explain the phenotypic heterogeneity of prion diseases.

The review article "*Infectivity versus seeding in neurodegenerative diseases sharing a prion-like mechanism*" by N. Fernández-Borges et al. available at the following link: http://www.hindawi.com/journals/ijcb/2013/583498/, provides a critical appraisal of the current evidence supporting prion-like mechanisms in AD, frontotemporal dementia and other tauopathies, Parkinson's disease, and ALS, discussing crucial differences between these disorders and "real" prion infections. Along this line, the review article "*Prions ex vivo: what cell culture models tell us about infectious proteins*" by S. Krauss and I. Vorberg available at the following link: http://www.hindawi.com/journals/ijcb/2013/704546/, reviews the data on the cellular propagation of different protein aggregates, demonstrating that not all the typical characteristics of prions are shared by other misfolding proteins.

In the review article "*Identification of misfolded proteins in body fluids for the diagnosis of prion diseases*" available at the following link: http://www.hindawi.com/journals/ijcb/2013/839329/, F. Properzi and M. Pocchiari give an up-to-date and comprehensive overview of the assays for detecting pathological forms of PrP in body fluids, highlighting the technological progress made in recent years. They stress the need to validate these diagnostic tools in blood samples and the importance of understanding prion metabolism in blood for effective diagnosis. The review article "*Small-molecule theranostic probes: a promising future in neurodegenerative diseases*" by S. Aulíc et al. available at the following link: http://www.hindawi.com/journals/ijcb/2013/150952/, reviews the potential diagnostic and therapeutic activity of small molecules that bind and influence the aggregation of several misfolding proteins, and the review article "*Gene-based antibody strategies for prion diseases*" by A. Cardinale and S. Biocca available at the following link: http://www.hindawi.com/journals/ijcb/2013/710406/, presents an overview of the application of intracellular antibody technology (intrabodies) in prion diseases. They concisely review the concept of intrabodies and provide recent information on *in vitro* and *in vivo* studies. They stress the importance of targeting the actual neurotoxic species in prion diseases and improving the *in vivo* stability and efficacy of vectored antiprion antibody fragments.

Despite major progress in our understanding of the pathogenesis of neurodegenerative diseases and the role of protein misfolding, effective treatments are still lacking. We hope that this special issue will help broaden the view of the problem and stimulate further research in the field.

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Review Article

S-Nitrosation and Ubiquitin-Proteasome System Interplay in Neuromuscular Disorders

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Protein *S*-nitrosation is deemed as a prototype of posttranslational modifications governing cell signaling. It takes place on specific cysteine residues that covalently incorporate a nitric oxide (NO) moiety to form *S*-nitrosothiol derivatives and depends on the ratio between NO produced by NO synthases and nitrosothiol removal catalyzed by denitrosating enzymes. A large number of cysteine-containing proteins are found to undergo *S*-nitrosation and, among them, the enzymes catalyzing ubiquitination, mainly the class of ubiquitin E3 ligases and the 20S component of the proteasome, have been reported to be redox modulated in their activity. In this review we will outline the processes regulating *S*-nitrosation and try to debate whether and how it affects protein ubiquitination and degradation via the proteasome. In particular, since muscle and neuronal health largely depends on the balance between protein synthesis and breakdown, here we will discuss the impact of *S*-nitrosation in the efficiency of protein quality control system, providing lines of evidence and speculating about its involvement in the onset and maintenance of neuromuscular dysfunctions.

1. Redox Modifications and Cell Signaling

The main molecular mechanism underlying signal transduction in eukaryotic cells relies on the regulation of protein function by posttranslational modifications. The transient and reversible attachment of reactive moieties on specific residues is able to induce a plethora of effects on protein function, thereby giving rise to a dynamic interplay of protein interactions capable to convey signals within the cell. Among these, redox signal is highly specific and represents a prerogative of sulfur-containing residues. In particular, cysteine is more versatile than methionine in forming adducts because of its capability to be present under numerous oxidation states [1]. This feature allows cysteine reacting with many oxidant species, such as hydrogen peroxide, glutathione disulfide (GSSG), or nitric oxide (NO) moiety, generating in such a way the reversible *S*-hydroxylated (SOH), *S*-glutathionylated (SSG), or *S*-nitrosated (SNO) derivative, respectively [2]. Among them, glutathionylation or, widely, disulfide bond formation is the most stable cysteine oxidative modification. This is the reason why, physiologically, both *S*-hydroxylated and *S*-nitrosated proteins usually convert in *S*-glutathionylated (in the presence of high concentrations of GSH, as normally occurs inside the cells) or, widely, disulfide adducts. Therefore, hydrogen peroxide and NO can indirectly induce cysteine oxidation to disulfide. It should be also reminded that, similarly to hydrogen peroxide, NO overproduction has been reported being associated with irreversible sulfhydryl oxidation, such as sulfinylation (SO_2H) or sulfonylation (SO_3H) of metalloproteases [3]. However, these last modifications likely imply the production of peroxinitrite ($ONOO^-$), a more dangerous and more oxidant reactive nitrogen species (RNS) generated by the reaction between NO and superoxide anion ($O_2^{\bullet -}$), which has been copiously reported being involved in protein tyrosine nitration (see below).

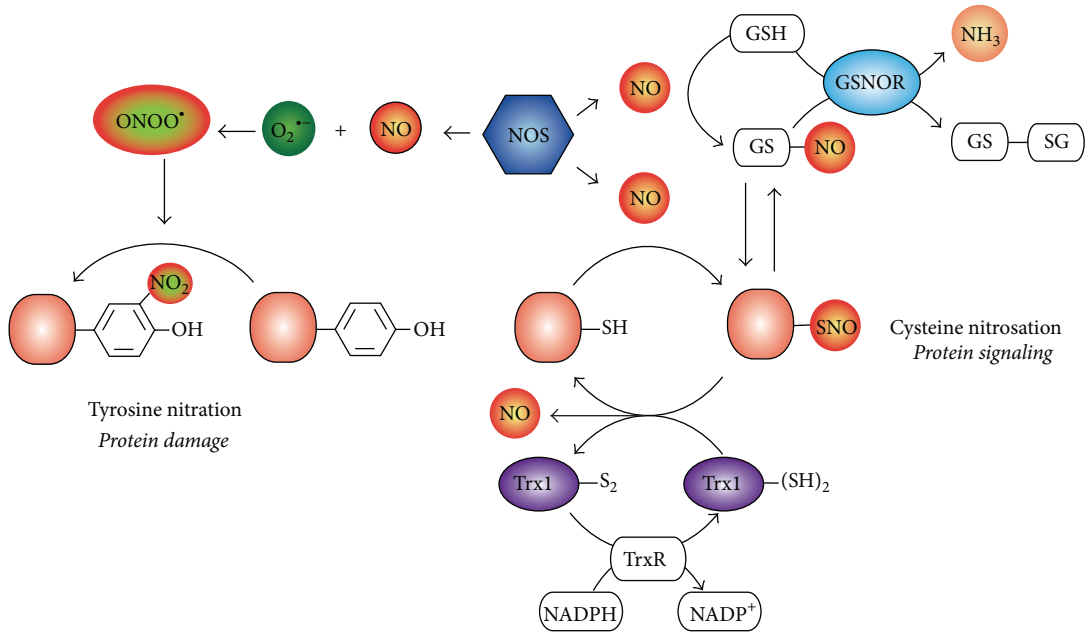

Figure 1: Tyrosine nitration versus *S*-nitrosation. Nitric oxide (NO), produced by NO synthase (NOS), can affect protein structure and function in different ways. Here only posttranslational modifications directly modifying protein residues are shown, tyrosine nitration (right) and cysteine *S*-nitrosation (left). The former adduct is irreversible (so far, no denitrating enzyme has ever been found) and responsible for protein damage occurring mostly upon the overproduction of NO. Indeed, under this condition (called nitrosative stress) NO can rapidly react with superoxide anion ($O_2^{\bullet -}$) to form peroxinitrite ($ONOO^-$) which is the main harmful radical species inducing tyrosine nitration. Conversely, upon physiological production of NO, reactive cysteines of both redox-sensitive proteins and glutathione (GSH) can undergo *S*-nitrosation, thereby generating their *S*-nitrosothiol derivatives, Prot-SNOs and *S*-nitrosoglutathione (GSNO), respectively. Prot-SNOs and GSNO are in equilibrium by transnitrosation reactions; therefore, the GSNO catabolizing enzyme, GSNOR reductase (GSNOR), by regulating GSNO levels also impacts on protein nitrosation extent. Thioredoxin 1 (Trx1) also participates in protein denitrosation by means of its vicinal thiols that reduce Prot-SNO and oxidize to an internal disulfide bridge, whose further reduction is catalyzed by Trx reductase (TrxR) and ensured by reducing equivalents provided by NADPH. Although both GSNOR and Trx1 concur to modulated protein *S*-nitrosation, it should be reminded that the former enzyme completely reduces GSNO to glutathione disulfide (GSSG) and ammonia (NH_3), whereas the latter releases the NO moiety of Prot-SNOs as NO itself or nitroxyl anion (HNO), which are species still capable to target protein substrates.

1.1. Implication of NO in Cell Signaling. Nitric oxide is a gaseous and membrane-diffusible radical molecule produced by the class of NADPH-dependent enzymes NO synthase (NOS) [4, 5]. The first evidence of NO involvement in signal transduction goes back to 1983 when it was demonstrated that cGMP-mediated regulation of blood vessel tone depended on the direct binding of NO to the heme iron (Fe-nitrosylation) of guanylyl cyclase [6, 7]. Since then, increasingly data provided further and indisputable lines of evidence pointing out this pathway being, in fact, implicated in many other functions, such as immune response [8], neurotransmission [9], and mitochondrial respiration [10]. Indeed, physiologically NO can bind to free iron within any heme-containing protein with a free ligand position [11]. By this reaction, and also by binding the copper binuclear centers in a noncompetitive manner [12], NO can regulate cytochrome *c* oxidase activity and, more widely, it can tune mitochondrial respiration.

Alongside these findings, the involvement of NO in redox signaling was emerging and progressively assuming distinctive signatures. Over those years, it became clear, indeed, that *S*-nitrosation is the main reversible posttranslational modification induced by NO able to regulate different classes of proteins [13–15]. The discovery of denitrosating enzymatic systems, namely, those dependent on thioredoxin 1 (Trx1) and *S*-nitrosoglutathione reductase (GSNOR) activities, which actively participate the *S*-nitrosothiol-to-sulfhydryl (SNO-to-SH) reduction [16–19] (Figure 1), definitively sealed the importance of *S*-nitrosation in cell physiology and human health (Table 1).

Actually, NO can also trigger irreversible modifications to proteins, such as the formation of nitrotyrosine. However, this is an event occurring only under certain conditions (nitrosative stress) that do not deal with signaling but just represents marker of damage. In particular, when NO is overproduced and no longer neutralized, it can react with oxygen-derived radical and nonradical species (ROS) thereby generating more dangerous RNS, for example, $ONOO^-$ [20, 21] (Figure 1).

As previously mentioned, protein *S*-nitrosothiols (PSNOs) generation is specific, redox-mediated, and reversible [22] depending on several factors, such as the environmental hydrophobicity conditions and the steric hindrance, as well as the net charge and the presence of oxygen [2, 13]. In addition, it has been reported that the specificity of *S*-nitrosation can also depend on the presence of an *S*-nitrosation motif [23] roughly characterized by

Table 1: Examples of pathological conditions associated with alterations in Prot-SNOs.

Protein-SNO	Pathology	Reference
Dynamin-related protein 1	Alzheimer disease	Cho et al., 2009 [86]
Protein disulfide isomerase	Alzheimer disease Parkinson disease	Uehara et al., 2006 [98]
X-linked inhibitor of apoptosis	Alzheimer disease Parkinson disease	Nakamura et al., 2010 [30]
Parkin	Parkinson disease	Chung et al., 2004 [53]
Peroxiredoxin-2	Parkinson disease	Fang et al., 2007 [97]
Ryanodine receptor 2	Heart failure	Gonzalez et al., 2007 [99]
O^6-alkylguanine-DNA alkyl transferase	Cancer	Wei et al., 2010 [62]
Ryanodine receptor 1	Duchenne/limb-girdle muscular dystrophy	Bellinger et al., 2009 [76] Andersson et al., 2012 [100]

acid-base residues surrounding the target cysteine [24]. Nevertheless, many other factors deeply impact on the identity of specific proteins undergoing *S*-nitrosated, such as cellular localization and compartmentation of NO production [25]. In addition, the evidence that NO moiety can be also transferred among proteins or low-molecular-weight *S*-nitrosothiols (e.g., *S*-nitrosoglutathione, GSNO), adds further complexity to the regulation of protein *S*-nitrosation [26].

1.2. Transnitrosation and Denitrosation. The major determinant for NO transfer (transnitrosation) is the difference between the redox potential of the two interacting cysteine residues [27]. This aspect takes more importance if transnitrosation does not occur with low-molecular-weight thiols, but takes place between proteins. Indeed, this reaction is responsible for the propagation of many NO-mediated cell signaling pathways and its significance has been highlighted quite recently in many physiopathological processes, such as (i) NO exchange between hemoglobin and the anion exchanger 1, which mediates NO release from erythrocytes [28]; (ii) transnitrosation among thioredoxin (Trx), caspase-3 and the inhibitor of apoptosis (IAPs) proteins, which is involved in the regulation of cell death by apoptosis [27, 29–31]; (iii) glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-mediated *S*-nitrosation of nuclear proteins, which contributes to cell death and accounts for the pathogenesis of several neurodegenerative diseases [32, 33]; (iv) transnitrosation between the neuronal-specific cyclin dependent kinase 5 (Cdk5) and dynamin-related protein 1 (Drp1), which plays a pivotal role in mitochondrial dysfunction typical of neurodegenerations [26, 34].

PSNOs levels are counterbalanced by denitrosation systems, the most important of which are the glutathione (GSH)/GSNOR and the Trx1/Trx reductase (TrxR) couples [2, 18, 35, 36] (Figure 1). In the light of what mentioned above, only a specific subset of proteins is *S*-nitrosated, resulting in the selective modulation of specific signaling pathways. In this scenario, it is plausible that the propagation or modulation of cell signals by *S*-nitrosation often implies a crosstalk with signaling modalities mediated by other mechanisms of posttranslational modification [37]. In the last decades, the discovery of *S*-nitrosation of both protein kinases and phosphatases suggested the influence of this modification in a wide range of signal transduction pathways mediated by phosphorylation/dephosphorylation [38–40]. This aspect is of great importance if one considers that *S*-nitrosation can convert into *S*-glutathionylated/disulfide adduct and that this is a well-known mechanism driving signal transduction mediated by phosphorylative cascades [41]. Likewise, it has emerged that *S*-nitrosation may also operate in the nucleus on epigenetic mechanisms of transcriptional regulation, in particular by interfering with histone acetylation status [42]. Ubiquitination of proteins, which represents the pivotal reaction underlying protein turnover and quality control, might be also affected by *S*-nitrosation because many enzymes involved in ubiquitination process have critical cysteines, which have been reported undergoing oxidation [43–45] or, actually, sumoylation [46]. *S*-nitrosation could therefore have deep implications in a number of pathophysiological conditions involving ubiquitin-proteasome system (UPS).

2. Dual Role of *S*-Nitrosation in Ubiquitin-Proteasome System

2.1. Ubiquitination. Ubiquitination is an enzymatic post-translational modification process occurring on proteins, based on the ligation of ubiquitin (an 8.5 kDa protein) on a target protein lysine residue (monoubiquitination). This first step may be followed by the formation of ubiquitin chains through the attachment of additional ubiquitin moieties to one or more of the seven lysine residues within conjugated ubiquitin (polyubiquitination). The covalent attachment of ubiquitin on lysine residues requires the coordinated reaction of three enzymes. The first reaction is accomplished by the ubiquitin-activating enzymes (E1), which promote ubiquitin adenylation required for its covalent binding to the cysteine residue located at the E1 active site. Ubiquitin-charged E1 enzymes next transfer ubiquitin to the cysteine of the ubiquitin-conjugating enzymes (E2) that, in concert with a wide class of enzymes known as ubiquitin ligases (E3) needed for target recognition, finally transfer ubiquitin on lysine residues of specific substrates [47]. Protein ubiquitination is the major mechanism underlying protein turnover and

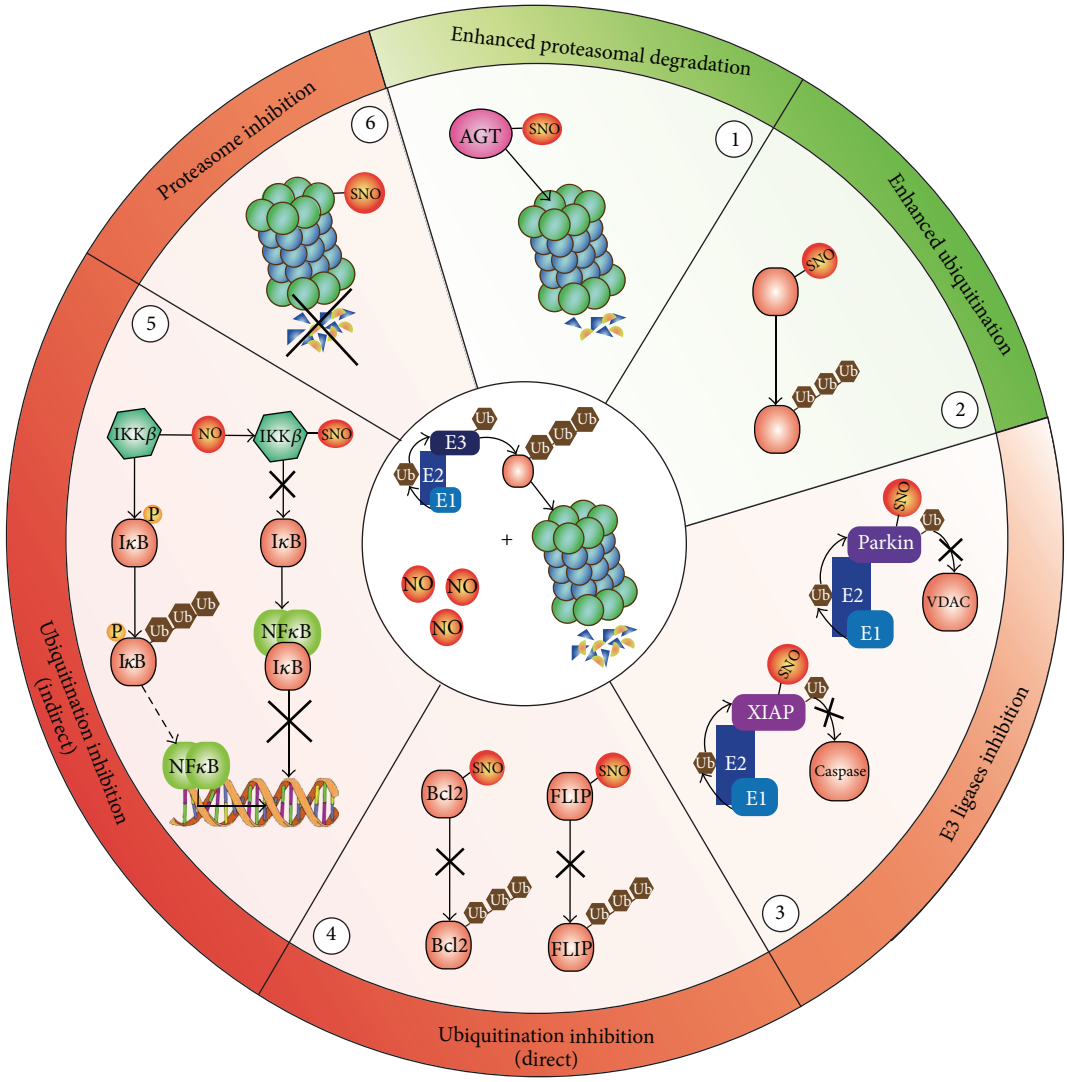

FIGURE 2: *S*-nitrosation-induced cellular effects on ubiquitin-proteasome system. Nitric oxide (NO) can target each one of the three components indispensable for protein ubiquitination and subsequent degradation: the ubiquitinating machinery (E1, E2, and E3 enzymes), the protein substrate, and the proteasome (center of the circle). *S*-nitrosation can enhance the degradation rate of the protein substrate (point 1, e.g., O^6-alkylguanyl-DNA alkyltransferase, AGT) or its ubiquitination (point 2). Conversely, *S*-nitrosation can (i) inhibit ubiquitin ligase activity of several E3s, such as Parkin and XIAP (point 3), (ii) affect ubiquitination directly, by changing protein structure, as demonstrated for Bcl2 and FLIP (point 4), or indirectly, by inhibiting enzyme activities of proteins acting as positive modifiers of ubiquitination (e.g., IKKβ, point 5), and (iii) directly impair protasome activity (point 6). *Red ring:* inhibitory effects; *green ring*: activating effects.

quality control, as it is responsible for the redirection of damaged/unfolded proteins towards the proteasome to be degraded. In addition, it mediates the recognition of damaged organelles, or protein aggregates, by means of adaptor proteins (i.e., p62/sequestosome) that are also implicated in autophagy-mediated degradation [48]. This further function emphasizes the paramount role of ubiquitination in cellular homeostasis, as it is at the crossroads between different degradative pathways (both proteasome and autophagy mediated). Although ubiquitination was initially shown to drive protein degradation, it is now commonly accepted that the nature of ubiquitination (monoubiquitination versus polyubiquitination), as well as the type of interubiquitin linkages in polyubiquitin chains, mediates different response in cellular processes and signaling, including antigen processing [49], apoptosis [50], cell cycle [51], DNA transcription, and repair [52].

2.2. Inhibitory Effects of S-Nitrosation. Nitric oxide can affect ubiquitin conjugation steps and proteasomal degradation of ubiquitinated proteins at different level (Figure 2). For instance, the catalytic site of ubiquitinating enzymes (E1-E2-E3) contains a cysteine residue that it is now arising could be susceptible to *S*-nitrosation [53–55]. *S*-nitrosation has been also shown to inhibit E3 ligase activity in RING (really interesting new gene) finger motif-containing proteins, modulating in such a way the downstream signaling

cascades. Notably, RING E3s do not have recognizable active sites that define the "canonical" enzymes. Instead, they have large binding interfaces and act as scaffold proteins bringing together the participant E2 and substrate proteins. Therefore, *S*-nitrosation might affect the interacting properties of this class of E3 ligase. One example is the RING finger E3 ligase parkin, whose mutations have been demonstrated being implicated in Parkinson's disease etiopathogenesis. *S*-nitrosation of parkin inhibits its activity [54], thereby resulting in enhanced accumulation of protein aggregates, as well as impairment of autophagy-mediated removal of damaged mitochondria (mitophagy) [2] (Figure 2). Likewise, *S*-nitrosation of the RING finger E3 ligase X-linked IAP (XIAP) has been reported to inhibit ubiquitin-mediated proteasomal degradation of caspase 3, thereby resulting in the promotion of cell death by apoptosis [30, 55] (Figure 2).

Beside the effects on ubiquitin conjugating system, NO can directly interfere with the proteasome protein complex. In vascular smooth muscle cell, *S*-nitrosoglutathione (GSNO) exposure revealed that the 20S catalytic core of the 26S proteasome contains 10 cysteines which undergo *S*-nitrosation, thus resulting in the inhibition of all three catalytic activities of the complex (chymotrypsin-, trypsin-, and caspase-like) [56] (Figure 2). Since the 26S proteasome is responsible for the time-dependent degradation of cell cycle proteins (e.g., cdk2, cdk4, cyclins, and the cyclin-dependent kinase inhibitors p21 and p27) [57], the inhibition of its activity by *S*-nitrosation can affect cell cycle progression and proliferation. The effects of *S*-nitrosation-mediated modulation of protein turnover can also directly impact on target proteins, such as in the cases of the key apoptosis regulatory protein Bcl2 and the antiapoptotic FLICE inhibitory protein (FLIP), whose *S*-nitrosation inhibits their ubiquitination and proteasomal degradation and finally leads to apoptosis suppression [58, 59] (Figure 2). Furthermore, *S*-nitrosation may indirectly inhibit ubiquitination *via* the regulation of alternative post-translational modifications, such as in the case of the NO-mediated activation of nuclear factor κ-light-chain enhancer of activated B cells (NF-κB). *S*-nitrosation of IκB kinase (IKKβ) inhibits its kinase activity, making it unable to phosphorylate the inhibitor of NF-κB (IκB) which, in turn, does not undergo ubiquitination and degradation *via* the proteasome, leaving NF-κB unable to translocate into the nucleus and to induce transcription [60, 61] (Figure 2).

2.3. Activating Effects of S-Nitrosation. While many observations argue for *S*-nitrosation being a posttranslational modification that negatively affects UPS efficiency, there is a literature supporting the hypothesis that it can also indirectly enhance ubiquitin-mediated protein degradation. In regards to this aspect, it should be reminded that polyubiquitination-mediated degradation of some proteins relies upon the conversion of N-terminal domain-located asparagine, glutamine or cysteine residues into arginine. This is required in order to allow recognition by E3 ligases of proteins being degraded (the so called *N-rule*) [62]. Arginylation of the N-terminal cysteines seems to be facilitated by *S*-nitrosation thereby suggesting the involvement of this modification in the turnover of many substrates.

The propensity of a number of proteins to undergo ubiquitination after *S*-nitrosation is not so unusual. An exhaustive example is provided by the key DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT), whose *S*-nitrosation has been reported to induce its massive proteasomal degradation and to negatively affect DNA repair [63] (Figure 2).

3. Ubiquitin-Protein System and Protein *S*-Nitrosation in Neuromuscular Diseases

3.1. Muscular Atrophies and Myopathies. Skeletal muscle atrophy can be defined as wasting or decrease in muscle mass owing to injury, lack of use, or disease. Muscle atrophy arises either from damage to the nerves that supply the muscles (neuromuscular disease) or disease of the muscle itself (musculoskeletal disease). The causes of atrophy rely on genetic mutations, such as in amyotrophic lateral sclerosis and muscular dystrophies, or are derived from systemic diseases, such as diabetes, cancer, and metabolic inflammation [64]. Nevertheless, the totality of atrophic conditions shares an imbalance between protein synthesis and degradation, resulting in reduced protein synthesis and increased protein breakdown, which in turn leads to reduced muscle mass and muscle fiber size. Indeed, independently of the etiology, muscle atrophies are commonly identified by the upregulation of the same set of genes, the so-called atrophy-related genes, or *atrogenes*, among which atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1) are well documented. These genes belong to the family of E3 ligase enzymes, which are responsible for the massive protein breakdown occurring in these diseases. In skeletal muscle atrophy, the central role of the ubiquitin-proteasome pathway has been characterized through the pioneering studies on gene expression profile independently performed by the research groups of Goldberg and Glass [65, 66]. In particular, they revealed that atrogin-1/MAFbx and MuRF1 are the two muscle-specific ubiquitin ligases upregulated in different models of muscle atrophy and responsible for the increased protein degradation *via* the UPS.

Another interesting common point of muscular atrophies is the occurrence of nitroxidative stress [67, 68]; indeed, accumulation of nitrotyrosine adducts has been detected in models of disuse-induced atrophies, as well as genetic-based dystrophies [69]. In skeletal muscle, the maintenance of a fully functioning fiber requires the correct assembly of the dystrophin glycoprotein complex (DGC). It is composed by several transmembrane and peripheral accessory proteins which are highly expressed in the sarcolemma and constitute a critical link between the cytoskeleton and the extracellular matrix [70]. It has been reported that DGC participates in cell signaling through the involvement of nNOS, which is predominant muscular isoform of NOS found to be associated to the complex *via* the alpha-syntrophin [71]. One possible mechanism underlying the overproduction of NO in muscle cell under atrophic conditions is the dislocation

Table 2: Role and targets of NO in neuromuscular dysfunctions.

NO adduct	Protein	Reference
S-NO Cys-3635	Ryanodine receptor 1	Bellinger et al., 2009 [76]
Tyr-NO?	NFkB	Suzuki et al., 2007 [71]
Tyr-NO?	FoxO3	Suzuki et al., 2007 [71]
S-NO Cys-553/558	Transient receptor potential cation channel	Yoshida et al., 2006 [85]
S-NO Cys-61/65	Myogenin	Martínez-Moreno et al., 2008 [80]

of nNOS from the DGC underneath the sarcolemmal membrane, followed by its redistribution into the cytosol where it produces NO [72]. The majority of congenital dystrophies depends on mutations in any of the complex components [73]. Interestingly, the dislocation of nNOS occurs in many types of dystrophies, such as Duchenne muscular dystrophy [70], which is characterized by the complete ablation of dystrophin, and in autosomal recessive limb girdle muscular dystrophy (AR-LGMD), where mutations of sarcoglycan proteins seem to be the main causative events of the pathology [74]. Furthermore, dislocation of nNOS from the DGC occurs also in rat models of disuse- or denervation-induced atrophy, indicating that this mechanism could underlie, at least in part, the pathology of muscular disorders [72]. More recently, it has been also demonstrated that nNOS dislocation induces force reduction, which is typical feature of dystrophin-null mouse models, by means of still not elucidated mechanisms putatively involving tyrosine nitration and also *S*-nitrosation [75]. The first evidence of *S*-nitrosation involvement in this class of pathologies involves the *S*-nitrosation, and the subsequent hyperactivation, of the Ca^{2+} release channel ryanodine receptor 1 (RyR1). Such a modification leads to a chronic Ca^{2+} leakage from sarcoplasmatic reticulum [76, 77] and triggers mitochondrial fragmentation underlying muscle atrophy [78]. Moreover, NO has been reported being involved in the activation of Forkhead box O (FoxO) 3a transcription factor (FoxO3a). Although the molecular mechanisms underlying this process are not well established yet, the increase of intracellular NO levels within the cell seems to be capable of mediating FoxO3a activation and nuclear translocation, thereby inducing skeletal muscle atrophy by upregulating MuRF1 or atrogin-1/MAFbx [79, 80]. In this context, it is of note to remind that also myogenin, a protein involved in myofiber differentiation and development of functional muscles, has been reported undergoing *S*-nitrosation, reasonable at the level of Cys61 and Cys65 [81]. This modification profoundly impacts on myogenin ability to bind DNA at the promoter regions to activate downstream gene expression (e.g., caveolin-3) and finally result in muscle atrophy (Table 2).

3.2. Neuropathies. Many diseases affecting muscle health and function also induce peripheral neuropathies as side effects. Indeed, denervation or peripheral nerve injuries (e.g., those characterized by partial loss of fibers or myelin in the nerve) strongly contribute to muscle wasting [82]. Besides the already mentioned muscular dystrophies, also cancer and diabetes, as well as aging-related cachexia, show alterations of nerve physiology associated with NO dysbalance and PSNOs increase [72, 83]. Moreover, it has been reported that NO overproduction and *S*-nitrosation could be directly associated with the transduction pathways underlying fatigue and myalgia deriving from muscle wasting [84], which are typical features of skeletal muscle atrophic states. In particular, recent lines of evidence argue for *S*-nitrosation of the transient receptor potential vanilloid 1 and ankyrin 1 (TRPV1 and TRPA1, resp.), two polymodal ion channels of peripheral sensory dorsal root ganglia, being the principal event underlying the sensitivity of noxious stimuli impinging on peripheral nociceptors [85, 86] (Table 2).

As above reported for correct muscle maintenance, a balanced ratio between protein synthesis and degradation is important also for neuronal viability. Actually, an efficient removal of unfolded or damaged proteins and organelles is crucial to prevent neuronal death and to preserve axonal integrity. In regard to this aspect, several proteins have been reported playing a pivotal role in neurodegenerative diseases when *S*-nitrosated. Drp1 is a case in point, as its mitochondrial translocation and GTPase activity seem to be enhanced when the protein undergoes *S*-nitrosation at Cys644 [87]. This *gain-of-function* modification—which has been found associated with Alzheimer's disease and pathological conditions affecting central nervous system (Table 1)—alters mitochondrial dynamics process by increasing mitochondrial fragmentation and finally contributes to neuronal cell demise. We readily refer to other comprehensive and more focused reviews dissecting in detail this aspect [88], while attempting here to deal with how *S*-nitrosation of proteins involved in ubiquitination process can impact on peripheral nervous system physiology. Among them, the ubiquitin E3 ligase TRIM2 (tripartite motif containing protein 2), which is involved in the regulation of axonal specification and polarization [89], has been very recently proposed to be neuroprotective [90]. In particular, Ylikallio and colleagues reported that *TRIM2* mutations that result in the complete loss of the protein are associated with childhood onset of axonal neuropathy leading to muscle mass reduction. Mouse models of TRIM2 deficiency recapitulate the human phenotype due to an aberrant axonal accumulation of neurofilaments that are no more ubiquitinated and degraded *via* the proteasome [91]. Although no evidence on possible redox reactions, namely, *S*-nitrosation, have been provided yet on TRIM2, it is plausible that its occurrence could inhibit TRIM2 activity, as already demonstrated for many other members of the ubiquitin E3 ligase superfamily, thereby allowing speculating that the existence of an *S*-nitrosated form of TRIM2 could correlate with the onset of axonopathy and muscle atrophy-associated peripheral neuropathy. Likewise, the mitochondrial ubiquitin E3 ligase MITOL has been demonstrated to regulate mitochondrial dynamics, as well as to counteract the toxicity of polyglutamine-containing protein ataxin 3 [92] and mutant superoxide dismutase 1 [93], which are the main causes of Machade-Joseph disease and amyotrophic lateral sclerosis, respectively. Very recently it has been indicated that MITOL

undergoes *S*-nitrosation and loss of activity [94], thereby resulting in mitochondrial aggregation and neuronal cell death. Among the large amount of substrates, MITOL also regulates the turnover of microtubule-associated protein 1B-light chain 1 (LC1) that, intriguingly, is ubiquitinated by MITOL and then subjected to proteasome-mediated degradation only when *S*-nitrosated [94]. Thus modified, indeed, LC1 translocates to the cytoskeleton, stabilizes microtubules and, consequently, freezes organelle transport. Therefore, under moderate (physiological) NO concentration, MITOL is required to maintain intracellular traffic by promoting LC1 degradation. Conversely, under nitrosative/toxic conditions, such as upon *N*-methyl-d-aspartate (NMDA) rector chronic activation, MITOL is inactivated, resulting in LC1 accumulation and mitochondrial dysfunction typical of neurodegenerative disease [94].

4. Future Research Perspectives and Therapeutic Strategies

On the basis of what previously described, excessive *S*-nitrosation seems to play a detrimental role in neurological disorders mostly due to its direct inhibitory effect on ubiquitin E3 ligases involved in the maintenance of cellular homeostasis (e.g., MITOL and TRIM2). Moreover nitrosative stress has been indicated to negatively affect muscle function and to induce muscular atrophy, owing to an excessive activation of the UPS (e.g., by means of atrogene induction *via* FoxO). In accordance with the above reported results, *S*-nitrosation has been also demonstrated being deeply implicated in sensitivity to nociceptive stimuli due to its impact on TRP ion channels. Altogether, these observations correlate with recent lines of evidence indicating that the sulfhydryl-containing molecule *N*-acetylcysteine (NAC) reduces pain and ameliorates muscle performance [95, 96], protects dystrophic myofibers against eccentric muscle damage, and contrasts abnormal calcium influx [97]. Being NAC a well-known antioxidant and denitrosating agent, this evidence suggests that nitrosative stress might represent a condition underlying or contributing to some pathological features of skeletal muscle disorders. Along this line, it has been demonstrated that pharmacological inhibition or genetic ablation of nNOS [75] reverts neuromuscular pathological phenotypes; however, these approaches have still not allowed discriminating whether tyrosine nitration or cysteine *S*-nitrosation is the principal mediator of neuropathy and myopathy induced by NO overproduction. Undoubtedly, the use of different NO donors does not represent a good model to unravel this issue. Indeed, their delivery of NO, which recapitulates a burst more than a persistent, and physiological, flux, has so far produced still questionable results. Cellular and mouse models of "genetically altered" *S*-nitrosation (e.g., GSNOR downregulating or knock-out models) could be of help in the next future to evaluate the specific contribution of different NO-mediated protein modifications: nitrationversus *S*-nitrosation. Figuring out this issue would open new avenues for the pharmacological treatment aimed at the restoration of a correct neuromuscular physiology for pathologies whose prognosis, on the contrary, is characterized by a progressive and irreversible loss of motion and cognitive abilities accompanied by chronic pain.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Salvatore Rizza and Costanza Montagna equally contributed to this work.

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Review Article

Prion Protein Misfolding, Strains, and Neurotoxicity: An Update from Studies on Mammalian Prions

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Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a group of fatal neurodegenerative disorders affecting humans and other mammalian species. The central event in TSE pathogenesis is the conformational conversion of the cellular prion protein, PrP^{C}, into the aggregate, β-sheet rich, amyloidogenic form, PrP^{Sc}. Increasing evidence indicates that distinct PrP^{Sc} conformers, forming distinct ordered aggregates, can encipher the phenotypic TSE variants related to prion strains. Prion strains are TSE isolates that, after inoculation into syngenic hosts, cause disease with distinct characteristics, such as incubation period, pattern of PrP^{Sc} distribution, and regional severity of histopathological changes in the brain. In analogy with other amyloid forming proteins, PrP^{Sc} toxicity is thought to derive from the existence of various intermediate structures prior to the amyloid fiber formation and/or their specific interaction with membranes. The latter appears particularly relevant for the pathogenesis of TSEs associated with GPI-anchored PrP^{Sc}, which involves major cellular membrane distortions in neurons. In this review, we update the current knowledge on the molecular mechanisms underlying three fundamental aspects of the basic biology of prions such as the putative mechanism of prion protein conversion to the pathogenic form PrP^{Sc} and its propagation, the molecular basis of prion strains, and the mechanism of induced neurotoxicity by PrP^{Sc} aggregates.

1. Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are rapidly progressive neurodegenerative disorders that affect many species of mammals. In humans, they comprise Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), kuru, Gerstmann-Sträussler-Scheinker disease (GSS), and the recently described variably protease-sensitive prionopathy (VPSPr), whereas natural TSEs in animals include scrapie of sheep and goats, bovine spongiform encephalopathy (BSE), and chronic wasting disease (CWD) in deer and elk.

Prion diseases belong to the growing group of disorders that are attributed to misfolding and ordered aggregation of proteins, which include Alzheimer's disease, Parkinson's disease, systemic amyloidosis, and many others. In prion disease, in particular, the cellular prion protein, PrP^{C}, after partial misfolding, converts into a partially protease-resistant disease-associated isoform, PrP^{Sc}, which aggregates in the brain and forms deposits that are associated with the neurodegenerative changes.

Distinguishing features of prion diseases among these disorders, however, are their wide phenotypic spectrum, the multiple apparent ethiologies (e.g., sporadic, genetic, and acquired), and the transmissibility between individuals, a characteristic which has allowed the early development of experimental models. This has led to the important discovery that mammalian prions occur, like conventional infectious agents, in a variety of different strains: these are defined as natural isolates of infectious prions characterized by distinctive clinical and neuropathological features, which are faithfully recapitulated upon serial passage within the same host genotype. The different strains of the TSE agent or prion are believed to be the main cause of TSE phenotypic diversity. In addition, the host variability in the gene encoding PrP^{C}

(*PRNP*), as determined by polymorphisms or mutations, also modulates the disease phenotype. In this review, we focus on three fundamental aspects of the basic biology of prions, which, despite the significant recent advances, remain unsolved. They include the molecular mechanisms of PrP^{C} to PrP^{Sc} conversion, the role of PrP^{Sc} in strain determination, and the mechanism of PrP^{Sc} aggregate-induced neurotoxicity. Due to the space constraint and the main expertise of the authors, emphasis is given to evidence obtained from the study of naturally occurring diseases, particularly in humans, and from animal models.

2. PrP^{C}-PrP^{Sc} Conversion

2.1. Structural Changes Associated with PrP^{C} to PrP^{Sc} Conversion. Understanding the structural features of PrP^{Sc} remains a key issue to gain the ultimate insight into the molecular basis of prion formation and propagation. Unfortunately, the insoluble nature of PrP^{Sc} has hampered most efforts to determine its structure by preventing the use of high-resolution techniques such as NMR or X-ray crystallography. Therefore, only partial structural information is available from low-resolution approaches such as Fourier transform infrared spectroscopy (FTIR), electron microscopy (EM), immunoassays, fiber X-ray diffraction, and limited proteolysis [1–9]. Full-length PrP^{C} encompasses a poorly definite domain at the N-terminal end of the protein (which spans ~100 residues), a globular domain in the central portion (residues 125–228), and a short flexible C-terminal domain, ending with the GPI anchor (residues 229-230/231) [10]. The globular domain is composed of three α-helices and two antiparallel β-sheets, separated by short loops and kept together in their final tertiary structure by interactions between the exposed amino acidic lateral chains that are in close contact with each other when the protein is correctly folded [10]. The conversion of PrP^{C} into the pathological conformer PrP^{Sc} is characterized by a significant increase of β-sheet secondary structure. Indeed, FTIR and circular dichroism (CD) spectroscopy experiments indicate a dramatic difference in the secondary structure between the two isoforms. While PrP^{C} contains 47% α-helix and 3% β-structure, PrP^{Sc} holds 17–30% α-helix and 43–54% extended β-structure, the range being partially due to the multiple forms and lengths of PrP^{Sc} [2, 11].

Taking advantage of the available low-resolution structural information and constraints about PrP^{Sc} and of computational techniques, different theoretical models have been proposed to describe the putative PrP^{Sc} structure. The β-helical model is based on fiber X-ray diffraction and computer modeling techniques and proposes that the segment ~90–175 forms a four-stranded β-sheet core organized in a β-helical configuration, whereas helices α2 and α3 would retain their native conformation [3]. An alternative "spiral" model is based on molecular dynamics simulations and indicates that during PrP^{C} conversion a longer single β-strand is generated from the elongation of the two native β-sheets. The newly formed β-strand would interact with other PrP molecules and, in turn, lead to polymerization [12]. In both models the basic subunit of the oligomers is considered a trimer. According to the authors who have proposed the spiral model, however, the β-helical model is in disagreement with several critical constraints: notably, it would not fit within the unit cell packing dimensions of the EM data for which it was modeled and would be inconsistent with antibody mapping studies, enzyme cleavage sites, and fibril disaggregation profiles [12]. Furthermore, the results of recent deuterium exchange experiments on brain-derived PrP^{Sc} showed that the region from residue ~90 to the entire C-terminus displays slow exchange rates that are typical for a structure consisting of a continuum of β-strands [13]. These findings from Surewicz's group appear inconsistent with both the "β-helical" and the "spiral" models, which are assuming an incomplete conversion of the α-helical structures into β-sheet and add further controversy to the issue. Of course, current models do not rule out the possibility that there are other structures that would satisfy the experimental constraints. Indeed, given that in mammals more than a dozen of different prion strains are documented, a higher structural heterogeneity is expected and should be explained.

2.2. Effects of PRNP Mutations. Several mutations in the PrP gene (*PRNP*) account for the genetic or familial form of human prion disease, in which the conversion of PrP^{C} into PrP^{Sc} is thought to occur spontaneously, triggered by the mutation. About forty mutations linked to familial CJD, GSS, FFI, or other atypical phenotypes have been identified to date [15]; they have been linked to a plethora of effects at both structural and clinicopathological levels. Based on their position in the gene, their effect, and the type of residue replaced, *PRNP* mutations can be classified in several groups: N-terminal or C-terminal mutations, missense, insert, or STOP-codon mutations, salt bridge-affecting, polar mutations, and hydrophobic or GPI-signal-peptide mutations [16].

Based on *in vitro* studies it has been proposed that disease-linked mutations increase the likelihood of PrP^{C} misfolding by thermodynamically destabilizing the protein [17–20]. However, this cannot be taken as a general mechanism because individual mutations differently (or barely) affect PrP^{C} stability. Besides influencing the stability of PrP^{C}, mutations may also alter its surface properties, thus triggering an abnormal interaction with other not yet identified cofactors, or causing an aberrant trafficking and accumulation inside the cell [16].

Atomic structural details, obtained using solution-state NMR spectroscopy, are available only for a few pathological human (Hu) PrP mutants. Based on the structural comparison of the folded domain (residues 125 to 228) of HuPrP carrying the CJD-linked E200K or V210I [21] mutations and the GSS-linked Q212P [22] mutation, it has been proposed that pathological mutants affects the aromatic and hydrophobic interactions between residues clustered at the interface of the β2-α2 loop and the C-terminal half of the α3 helix. The disruption of these interactions and the consequent exposure to the solvent of the hydrophobic core may represent a common effect of the three mutants, which has led to the proposal that the early stage of prion conversion

possibly involves the critical epitope formed by the β2-α2 loop and the α3 helix. Similar findings have been obtained with the X-ray crystal structure of both F198S and D178N mutants [23] and molecular dynamics experiments [24, 25].

HuPrP pathological mutants were also explored in several murine models. In particular, various transgenic (Tg) mouse models overexpressing mutated PrP constructs (or wild-type PrP) were developed in order to determine whether PrP is *per se* sufficient to give rise to disease and generate infectivity. In an early controversial study Hsiao and colleagues reported that Tg mice overexpressing the mutated PrP P101L, an homologous of the P102L substitution associated with the GSS syndrome in humans, spontaneously develop a clinical-pathological phenotype which propagated disease in inoculated Tg 196 mice expressing lower levels of mutant protein, suggesting that pathogenic PrP gene mutations resulted in the spontaneous formation of PrP^{Sc} and *de novo* production of prions [26]. Subsequent studies, however, have shown that the Tg 196 mice also spontaneously develop the disease in late life as a consequence of PrP overexpression, making the apparent prion propagation observed in this model more accurately characterized as disease acceleration rather than transmission [27]. Remarkably, disease transmission of brain extracts from Tg animals overexpressing the P101L mutation neither occurred to wild-type nor to Tg mice expressing MoPrP-P101L from two transgene copies that do not develop disease spontaneously in their natural lifespan [27], which is in full agreement with a previous study from Manson's group showing that *PRNP* gene-targeted 101LL mice expressing MoPrPP101L failed to develop the neurodegenerative disease spontaneously [28].

In line with the concept expressed above, several subsequent studies reported that Tg mice overexpressing PrP mutants often develop neuropathological features reminiscent of human TSEs, although in most cases the inoculation of their brain extracts in wild-type animals neither reproduced the main feature of the disease nor generated infectivity [29–34].

Results contradicting this general observation, however, have also been reported. Transgenic mice moderately overexpressing a mutant mouse PrP carrying two point mutations (170N and 174T) that are found as normal variants in the rigid loop of elk PrP spontaneously develop spongiform encephalopathy and PrP plaque deposition in the brain [35]. Repeated subpassages in Tg20 mice showed transmission of disease to wild-type mice and propagation of protease-resistant PrP^{Sc}. Similarly, Lindquist and collaborators were able to generate knock-in mice expressing the mouse equivalent of the PrP mutation (i.e., D178N-M129) associated with FFI. These mice developed *de novo* prion diseases with neuropathological traits similar to FFI that was transmissible to wild-type mice carrying the same 3F4 epitope [36]. A very similar result has been recently obtained by the same group using knock-in mice carrying the mouse equivalent of the most common human mutation (i.e., E200K) associated with genetic CJD. These mice developed the hallmark features of CJD, namely, spongiosis and proteinase K (PK)-resistant PrP aggregates. Furthermore, brain extracts from these mice caused a transmissible neurodegenerative disease after intracerebral inoculation in WT mice [37]. Finally, infectious prions were also reported to form spontaneously, even before the onset of the clinical symptoms, in chimeric mouse/human transgenic mice (called TgMHu2M), also expressing the CJD-linked E200K mutation [38]. Thus, according to these four studies, the introduction of a single (or two) amino acidic change(s) in *PRNP* in a critical position can cause remarkably different neurodegenerative diseases and may be sufficient to create distinct protein-based infectious prions.

Tg mice lines expressing human *PRNP* mutations were also used to study the effect of the mutation on disease susceptibility. Transgenic mice carrying the P101L mutation in PrP had remarkable differences in incubation time compared with wild-type littermates, following inoculation with several prion strains from human, hamster, sheep, and murine sources, suggesting a critical role for the structurally "flexible" region of PrP in agent replication [30]. In another study, Asante and collaborators [29] studied mouse lines homozygous for the human PrP102L, 129 M or for human PrP200 K, 129 M transgenes both expressed on *PRNP* null background. Although both lines did not develop spontaneous neurodegeneration, they showed a different susceptibility to inherited prion diseases. While PrP102L, 129 M were permissive to homotypic P102L prions and not to sCJD prions, PrP200 K, 129 M showed a similar susceptibility to both the E200K *inoculum* and classical sCJD prions [29]. Tg mice lines carrying *PRNP* mutations have also been used to unveil molecular pathways that are activated by the expression of mutant PrP, which may lead to neuronal dysfunction. In a recent study Senatore et al. shed light on the effects of insertional mutants on synaptic transmission [39]. Using Tg mice expressing a PrP insertional mutation linked to familial prion disease [31], they pinpointed the existing relationship between the early motor behavioral abnormalities and the impaired glutamatergic neurotransmission in cerebellar granule neurons. In particular, they showed that the misfolded mutant PrP undergoes an aberrant intracellular trafficking causing the intracellular accumulation of the voltage gated calcium channel $\alpha 2\delta$-1 subunit, which results in the disruption of the cerebellar glutamatergic neurotransmission [39].

2.3. Cellular Cofactors Featuring in PrP^{C} Conversion and Prion Propagation. Several lines of evidence suggest that different classes of cofactors, possibly acting as chaperones, can influence PrP^{C} conversion and prion propagation [40, 41]. To date, two types of cofactors, lipids and polyanions, have been implicated (Table 1), although their precise mechanism of action remains unclear. Among linear polyanions, glycosaminoglycans (GAGs) and sulfated polysaccharides such as pentosan polysulfate or heparan sulfate were shown to influence prion conversion *in vitro* [42–45] (Table 1) possibly by facilitating the formation of PrP^{C}-PrP^{Sc} complexes through multiple simultaneous interactions with several PrP molecules [42].

Most significantly, host-encoded RNA was shown to facilitate the prion-seeded conversion of PrP^{C} to PrP^{Sc} *in*

Table 1: Cofactors enhancing PrP^C conversion *in vitro*.

Cofactor	Experimental setting		Results	Refs.
Pentosan polysulfate (PPS)	Cell-free conversion assay	Hamster and mouse [^{35}S] GPI(-) PrP^C seeded with brain derived PrP^{res} from infected hamsters (263 K) and mice (87 V)	(i) PPS increases the rate of formation and the yield of [^{35}S] PrP^{res} (ii) PPS facilitates conversion of both Mo and SHa [^{35}S] GPI(-) PrP^C at different temperatures	[44]
Heparin	Cell-PMCA	Cell lysates plus exogenously expressed HuPrP seeded with sCJD, vCJD, and hamster-adapted scrapie 263 K	(i) Both low and high molecular weight heparin enhance PMCA efficiency (ii) Seed-dependent effect of heparin on amplification efficiency	[45]
Sulfated dextran compounds	PMCA	PrP^{Sc} derived from BSE-infected cattle brain diluted in PrP^C substrate	(i) Enhanced BSE PrP^{Sc} amplification (ii) Amplified PrP^{Sc} induce lesions typical of prion disease in TgBoPrP	[135]
Synthetic poly (A) RNA	PMCA	Normal and diluted scrapie brain homogenate	(i) Stochastic *de novo* formation of PrP^{Sc} molecules from unseeded purified substrates (ii) Both amplified Sc237 or 139H PrP^{Sc} and *de novo* PrP^{Sc} molecules cause scrapie in inoculated Syrian hamsters	[136]
Phosphatidylethanolamine (PE)	PMCA	recPrP substrate with a $recPrP^{Sc}$ seed	(i) Generation of infectious prions (ii) PE supports prion propagation using PrP molecules from multiple animal species	[55]
RNA from normal mouse liver plus POPG	PMCA	Normal mouse brain homogenate seeded with recPrP	(i) *In vitro* generated $recPrP^{res}$ (ii) $recPrP^{res}$ propagates its PK-resistant conformation to endogenous PrP^C (iii) $recPrP^{res}$ causes *bona fide* prion disease in wild-type mice	[50]

vitro [46–50]. However, whether RNA acts as a mere catalyst of the PrP misfolding process or, alternatively, is associated with the infectious particle and contribute to determine the prion strain specificity is still unsolved. A recent study showed that the requirement of RNA for *in vitro* amplification of PrP^{Sc} is species dependent, with only hamster-derived PrP^{Sc} being largely dependent on the presence of RNA, whereas mouse-derived PrP^{Sc} is not [51]. Another study showed similar RNA-dependent amplifications of six hamster prion strains [52]. DNA and phospholipids have also been implicated as cofactors modulating prion replication *in vitro*. The polymerization of the mouse recombinant PrP (rPrP) was enhanced in presence of nucleic acids and sequence-specific DNA binding to rPrP converted it from a α-helical conformation to a soluble, β-sheet enriched isoform similar to that found in the fibrillar PrP^{Sc} state [53, 54]. Unlike RNA, the essential membrane phospholipid phosphatidylethanolamine (PE) was described as a highly promiscuous cofactor that can promote prion propagation using rPrP molecules from different mammalian species [55]. Critical questions, which are still far from being fully answered, concerns the role, if any, of cofactors in modulating prion infectivity and the specific properties of prion strains. Preliminary data on *in vitro* reconstitute prions seem to indicate that the presence of cofactors enhances *in vivo* prion infectivity, whereas the data collected to date on the issue of strains appear to be inconsistent. For example, while the use of PE as unique cofactor in the propagation process allowed the adaption of two different native prion strains into the same unique output strain, suggesting that a single cofactor is able to force the conversion of different strains into a single strain having its own phenotypic features [56], in another study it was found that replication under RNA-depleted conditions does not modify RML prion strain properties [57].

2.4. Cellular Sites of PrP^{Sc} Formation. Being PrP^C a GPI-anchored protein, it mainly localizes in lipid rafts of cellular membranes where it can interact *in trans* with a variety of signaling molecules, including caveolin-1, Fyn, and Src tyrosine kinases [58], or with other cell-surface proteins as NCAM [59], stress-inducible protein 1 [60–62], vitronectin, lipoprotein receptor-related protein 1 [63, 64], or reelin [65].

Several lines of evidence suggest that lipid rafts are critically involved in the conversion of PrP^C into the pathological form PrP^{Sc}. Using immortalized neuroblastoma cells ScN2a, chronically infected by the Rocky Mountain Laboratory (RML) prion strain, Naslavsky et al. showed that PrP^{Sc} is attached to lipid rafts [66] and that the amount of the abnormal protein inversely correlates with sphingomyelin levels [67]. Furthermore, using thin-layer chromatography and mass spectrometry, it has been found that the insoluble aggregates of N-terminally truncated PrP^{Sc} (i.e., PrP 27–30) contain small amounts of two host sphingolipids, galactosylceramide and sphingomyelin [68], which also supports the

localization of PrP^{Sc} in rafts. Other data pointing to a raft-mediated conversion include the observations that depletion of cellular cholesterol or the replacement of PrP^{C} GPI-anchor with the transmembrane and cytosolic domain from nonrafts proteins diminished or prevented the formation of PrP^{Sc} [58]. More recent studies, however, highlighted the possibility that lipid rafts favour the conversion by bringing together PrP^{Sc} and PrP^{C}, rather than by triggering PrP^{C} refolding [69]. Indeed, given their role in PrP^{C} folding and stabilization of its conformation, lipid rafts may even prevent PrP^{C} transconformation. According to this view the conversion would occur only after PrP^{C} exits in these domains. Finally, other studies also suggested that lipid rafts do not provide the environment in which PrP^{C}-PrP^{Sc} refolding occurs, but rather promote PrP^{Sc} aggregation and fibrillization once the pathogenic misfolded protein has been produced elsewhere (reviewed in [70]).

Concerning the precise cellular site of conversion of PrP^{C} to PrP^{Sc}, early studies pointed to the cell surface [71], which appears a plausible location particularly for the case of transmitted prion diseases, or to the endocytic pathway [72–75]. Subsequent studies further underlined the potential role in the conversion process of intracellular compartments such as the endosomal or lysosomal pathways, or even the ER [76–79]. Evidence for the conversion of PrP^{C} to PrP^{Sc} occurring shortly after internalization, during an endocytic process, is indeed numerous. After treatment of both scrapie-infected Syrian hamster brain and ScN2a cell lines with guanidine-hydrochloride, which allows epitope unmasking in native PrP^{Sc}, the abnormal protein was primarily described intracellularly [75], where it was found to accumulate in lysosomes. In another study, using cryo-immunogold electron microscopy, PrP^{Sc} was found to be concentrated in early/recycling endosomes of neuritis of prion infected hippocampal neurons [77, 79]. Similarly, in three different neuronal cell lines infected with different prion strains more than 25% of PrP^{Sc} has been observed to colocalize with a marker for the early recycling compartment. Classic studies have also shown that PrP^{Sc} accumulates intracellularly as an N-terminal truncated form, which is generated after proteolytic cleavage in both endosomes and lysosomes [73, 74]. Supporting evidence for the role of endosomes or lysosomes in PrP^{C} conversion is also provided by the observations that an acidic pH triggers the conformational change of PrP^{C} to a PrP^{Sc}-like form and that the lowering of the temperature to 18°C, supposedly by slowing the rate of PrP^{C} endocytosis, reduces PrP^{Sc} formation (reviewed in [80]).

Finally, the main cellular site of PrP^{C} and PrP^{Sc} location was also found to differ depending on the investigated cell line. In ScN2a cells, for example, PrP^{C} and PrP^{Sc} colocalize in the late-endosomial compartments, whereas in scrapie-infected hypothalamic (GT1—7) cells PrP^{Sc} is present in an additional vesicular compartment which is flotillin-1-positive [81].

As a whole, the data collected indicate that in most infected cell lines the conversion event occurs either on the cell surface or along the endocytic pathway, with PrP^{Sc} ultimately mainly accumulating in lysosomes. Nevertheless, other cellular sites might be also involved depending on the cell type, the prion strain, or the disease etiology.

3. PrP^{Sc} and the Strain Phenomenon

The first demonstration of prion strains was obtained after transmission of distinct scrapie isolates [82]. When these sheep brain extracts were passaged to goats, a drowsy syndrome developed in some animals, while others had a scratching syndrome. A variety of scrapie strains were subsequently identified after passage through inbred mouse lines [83]. Properties that differentiate the strains are the length of incubation time following inoculation, the type and distribution of lesions (neuropathologic profile), and the pattern of intracerebral deposition of PrP^{Sc} [83–85]. The wide variety of scrapie strains has been traditionally seen as the major challenge to the protein only hypothesis [86, 87]. While in classical infectious diseases different strains of the agent are associated with variations in their nucleic acid genomes, the prion hypothesis implicates that PrP^{Sc} itself would encode the phenotypic properties of the strains.

Kascsak et al. [88] originally documented that the relative proportion of PrP^{Sc} glycoforms, the so called "glycoform ratio," was associated with strain variability and could be used to differentiate strains of the scrapie agent when isolated in inbred mice. At about the same time, mouse strains ME7 and 139A scrapie associated fibrils (SAF) were shown to differ from hamster strain 263K SAF in terms of morphology, sedimentation rate, and sensitivity to PK digestion [89]. Noteworthy, these distinctive PrP^{Sc} physicochemical properties were initially considered an effect of the scrapie agent on PrP rather than an evidence for a role of PrP^{Sc} itself in strain determination. Indeed, the idea that the molecular basis of strain variation may lie in the structure of PrP^{Sc}, as predicted by the prion hypothesis, was fully embraced only after Bessen and Marsh found that two strains of transmissible mink encephalopathy (TME), transmitted to inbred Syrian hamsters, give rise to PrP^{Sc} molecules with distinct electrophoretic mobility and degree of resistance to protease digestion [90]. The two TME strain-specific PrP^{Sc} have been subsequently propagated *in vitro* through non-genetic mechanisms [91], which has further strengthened the view that the self-propagation of distinct PrP^{Sc} conformers may represent the basis of the prion strain phenomenon.

Experiments of FFI transmission to Tg mice gave additional support to the idea that the diversity of prion strains is enciphered in the PrP^{Sc} structure [92]. Brain homogenates from subjects affected by FFI, which contained a PrP^{Sc} fragment after PK digestion (PrP^{res}) of 19 kDa, and from subjects with sporadic CJD (sCJD) or a genetic CJD (gCJD) subtype linked to the E200K-129M haplotype (CJDE200K-129M), which contained a PrP^{res} fragment with a relative molecular mass of 21 kDa, were inoculated to syngenic mice. The endogenous PrP^{res} recovered in the affected animals consistently and precisely replicated the size of the corresponding human PrP^{res}.

In 1998 Safar et al. [93] introduced the conformation-dependent immunoassay (CDI), which measures the extent of epitope exposure after GndHCl denaturation and is therefore assumed to measure indirectly the relative percent of PrP^{Sc} β-sheet and α-helical content. Eight mouse-passaged scrapie strains were analyzed for strain-specific differences in secondary structure [93]. By plotting the ratio of antibody binding to the denatured/native proteins as a function of the concentration of PrP^{Sc}, the authors observed that each strain occupies a unique position, suggesting a distinct conformation.

FTIR spectroscopy has also been used to measure the secondary structure of both PK-treated and full-length PrP^{Sc}. Caughey and colleagues have originally compared the conformations of PrP^{Sc} in the HY, DY, and 263K hamster TSE strains and found striking differences in their secondary structures [1]. Similarly, another team [94, 95] has subsequently found strain-specific differences in secondary structure, temperature stability, and hydrogen-deuterium exchange characteristics between purified PrP^{Sc} preparations obtained from three scrapie strains and the classical BSE strain after passage in hamster.

More recently, the issue of the relationship between PrP^{Sc} conformational stability and strain-specific properties, such as incubation time and *in vitro* replication efficiency, has been addressed. In 2006 Legname et al. [96] reported that a reduced resistance to GndHCl denaturation, indicative of a reduced conformational stability, correlates with a shorter incubation time in mouse adapted prion strains. Similarly, the stability of PrP^{Sc} aggregates both in terms of resistance to GndHCL induced denaturation and thermostability was inversely correlated with the capacity to induce a rapidly lethal disease [97]. The provided explanation for these observations is that a decrease of PrP^{Sc} stability increases PrP^{Sc} aggregate fragmentation resulting in an increase in agent replication that produces a correspondingly shorter incubation period and a more aggressive disease. The relationship between the stability of PrP^{Sc} aggregates and PrP^{Sc} replication investigated *in vitro* using the protein misfolding cyclic amplification (PMCA) paradigm [52] also supports a link between PrP^{Sc} conformational stability and fragmentation rate of PrP^{Sc} aggregates. Other data, however, suggest a more complex picture, especially *in vivo*, where additional factors, related to cellular processing, may also play a significant role. In apparent contrast with what was observed in mice, Ayers et al. [98] found that hamster-adapted scrapie strains with a short incubation period were more efficiently replicated, had a more stable conformation, and were more resistant to clearance from the soma of neurons than those with a longer incubation time which, in contrast, predominantly accumulated in glial cells. These results suggest that the progression of prion disease is also influenced by the balance between replication and clearance of PrP^{Sc} in neurons.

A potential new perspective to the study of PrP^{Sc} properties and their relationship to prion strains was opened by the characterization of the so-called "sensitive PrP^{Sc}" ($sPrP^{Sc}$), an isoform of abnormal PrP which is fully degraded at a PK activity comparable to that necessary to digest PrP^{C}, despite maintaining other properties that are specific for PrP^{Sc} [99–101]. Evidence for $sPrP^{Sc}$ being a biologically relevant species originally came from the study of PrP^{Sc} properties in naturally occurring prion diseases. Indeed, a fully PK-sensitive PrP^{Sc} has been detected in various phenotypically atypical variants of both human and animal prion diseases [102–107]. Furthermore, according to some studies [100, 108], $sPrP^{Sc}$ represents an invariable and quantitatively significant component of prions, contributing up to 90% of the whole PrP^{Sc} signal even in classic TSEs such as sCJD and classical scrapie. Recent studies have also found a correlation between the relative amount of $sPrP^{Sc}$ with strain-specific properties such as the incubation period after inoculation or the clinical duration of the disease [109, 110]. We also recently looked for $sPrP^{Sc}$ in purified detergent-insoluble PrP^{Sc} sCJD preparations [111]. At variance with the findings above, however, our results showed that, irrespectively of the human prion strain, this slowly sedimenting $sPrP^{Sc}$ represents a relatively minor component of abnormal PrP not exceeding 10% of total detergent-insoluble PrP^{Sc}. Thus, this significant discrepancy, which may depend at least partially on methodological aspects or data interpretation [111], needs to be further explored and explained.

Although not essential for prion propagation [112], PrP glycosylation of asparagine residues at positions 181 and 197 represents another factor likely contributing to the diversity of mammalian prions. Indeed, differences in ratios of di-, mono-, and unglycosylated PrP^{Sc} have been detected among phenotypic subtypes of both human and animal TSEs and are commonly used to differentiate specific strains [113–116]. This is consistent with the notion that glycosylation is critical in determining and maintaining conformation and interaction of glycoproteins [117, 118]. However, it is at present unclear whether glycans affect the backbone conformation of PrP^{Sc} molecules or rather modulate the interaction of these molecules by introducing specific steric constraints or by forming crucial intermolecular contact sites between PrP^{Sc} monomers [119]. In a recent elegant study Cancellotti et al. [120] have demonstrated that the passage in Tg mice expressing a PrP partially or completely lacking the N-glycan moieties affected the phenotypic characteristics of at least one TSE agent strain. Given that these changes could be successfully retained on passage in wild-type mice, it has been concluded that infectious properties of a TSE strain can be altered by posttranslational changes to host PrP, possibly as the result of the selection of mutant TSE strain.

Taken together all these pieces of evidence provide strong support to the argument that different PrP^{Sc} conformers encipher the prion "strains." Nevertheless the direct proof for this contention is not yet available. Until a higher resolution picture of PrP^{Sc} provides the precise molecular-level details surrounding the puzzling phenomenon of prion strains and the conformational adaptability of PrP observed upon cross-species transmission, questions and alternative interpretations of the data will remain. For example, we cannot yet be sure of whether the distinctive properties of

PrP^{Sc} directly reflect the tertiary conformation of monomers or are determined by interactions between PrP^{Sc} and other molecules acting as cofactors. PrP^{Sc} is extracted from the brain in a highly aggregated state and the heterogeneity in size of the PK digested protein core may well reflect the quaternary rather than the tertiary structure of the molecule. Similarly, the extent of conversion of each glycoform of PrP^{Sc}, which ultimately determines the glycoform ratio of PrP^{res}, may also represent a signature imparted by another molecule that interacts with PrP. Finally, the central question that still remains to be answered is how an identical primary sequence can drive different tertiary conformations in the prion protein, if no other informational molecule exist. Even more difficult to explain in terms of PrP^{Sc} structural plasticity are other two fundamental aspects of the biology of prions, the so-called "species barrier," that is, the phenomenon for which a strain must adapt to a new species host with a typical delay in incubation time, or even the loss of infection ability in that species, and, above all, the fact that prion strains, like conventional infectious agent strains, incur in spontaneous "mutations." The latter phenomenon is often explained with the quasispecies hypothesis [121], which predicts that PrP^{Sc} with different conformations may be present at low levels in an infectious inoculum and that the variant most suitable for replication in a particular host is selected to become the dominant component of the population [122, 123]. However, evidence for large numbers of conformations is still lacking nor is it clear whether the required multiple conformations would be plausible in terms of thermodynamic stability.

3.1. PrP^{Sc} Characterization and Strain Variation in Natural Hosts: CJD, FI, GSS, and VPSPr. Five major clinicopathological phenotypes of human prion disease are currently recognized. These are CJD, FI, GSS, PrP-cerebral amyloid angiopathy, and VPSPr (phenotypic features of each form are reviewed in [15, 124–128]). The vast majority of human prion cases belong to CJD and occur in a sporadic fashion and worldwide. Only a small proportion of CJD cases are associated with *PRNP* mutations, in the form of familial or more properly genetic CJD (gCJD). Secondary CJD associated with inadvertent medical transmission is termed iatrogenic CJD (iCJD), and the only known zoonotic form of CJD, which is associated with exposure to BSE, is termed variant CJD (vCJD). VPSPr is a very recently described rare sporadic phenotype resembling GSS, FI can either occur sporadically or in a familiar form (FFI) associated with the D178N-129 M *PRNP* haplotype, while GSS and PrP-CAA phenotypes are tightly associated with mutations in the *PRNP* gene. In CJD, the prototype of human prion diseases, the characterization of PrP^{Sc} after PK treatment has led to the discovery of two major fragments of protease-resistant PrP^{Sc} (PrP^{res}). The largest of these peptides, named type 1, has a relative electrophoretic mobility of 21 kDa and a primary PK cleavage site at residue 82 while the smallest, or type 2, has a relative molecular mass of 19 kDa and a primary cleavage site at residue 97 [114, 115, 129] (Figure 1). Based on the analysis of a large series of 300 sCJD cases it was shown that the two different PrP^{res} types can be associated with each of the three possible *PRNP* genotypes determined by the polymorphic codon 129 (methionine, M, or valine, V) and that the six different possible combinations between these two molecular variables significantly correlate with the clinicopathological heterogeneity of sCJD [130]. Intriguingly, the two PrP^{res} types were also detected in the genetic and acquired forms of CJD, including vCJD, thus independently from the apparent etiology of the disease, that is, sporadic, inherited or acquired by infection [114, 129, 131], suggesting that the same prion strains are contributing to all forms of human TSEs. Furthermore, PrP^{res} types 1 and 2 were also found to cooccur in the same brain in about one-third of all sCJD cases [130, 132–134]. The results obtained in large series of cases indicate that the deposition of either type 1 or 2, when concurrent, is not random and is always characterized by the coexistence of phenotypic features previously described for the "pure" subtypes, a finding which strongly suggests that these cases harbour a mixture of prion strains.

The identification of an excess of pathological phenotypes (i.e., at least six) with respect to PrP^{res} types 1 and 2 dichotomy has prompted further attempts to identify PrP^{res} properties that would correlate with each disease phenotype. Using a standardized high buffer strength for brain homogenization, PK digestion at pH 6.9 with a high enzyme concentration, and long running gels, Notari et al. [137] showed that distinctive PrP^{res} properties can indeed be found in sCJD phenotypes sharing the same PrP^{res} type. For example, (i) PrP^{res} type 2 from MV cases shows a unique doublet band that differs from PrP^{Sc} type 2 in MM and VV cases, and (ii) type 1 PrP^{res} from VV cases migrates faster than type 1 PrP^{res} from MM1 and MV1 samples when PK digestion is performed at pH under 7.2 (Figure 1(a)).

A further fine tuning of the PrP^{Sc} signature associated with each CJD-associated strain has been obtained with the discovery that PrP^{Sc} aggregates include PrP^{res} C-terminal fragments with a relative mass of about 12 and 13 kDa (PrP-CTF12/13), in addition to PrP 27–30 (Figure 1). These fragments originate from the cleavage of PrP^{Sc} at residues 162–167 and 154–156 and vary in relative abundance among sCJD subtypes; in particular the peptide CTF-13 is present in significant amount in MM1 cases and is particularly abundant in VV1 subjects, whereas all PrP^{res} type 2-associated sCJD subtypes but the MM 2T, as well as vCJD, show only traces of this fragment [138]. Notari et al. [138] also identified a novel C-terminally truncated PrP^{res} fragment showing an apparent molecular mass of either ~18.5 kDa (when associated with type 1) or ~17 kDa (when associated with type 2). This fragment shares the primary N-terminal sequence with either type 1 or type 2 but lacks the very end of the C-terminus together with the GPI anchor (PrP_{AF} 18.5-17) (Figure 1). Finally, a fragment with an apparent molecular mass of about 16 kDa, which is only generated in partially denaturing conditions (DCF 16), has been detected in sCJD MM1/MV1 (Figure 1). Epitope mapping indicates that the fragment has an intact C-terminal end and is truncated in the region between residue 112 and residue 144. Taken together, these data suggest that each sCJD subtype can be associated with a specific profile of PrP^{res} fragments (PrP 27–30, PrP_{AF}

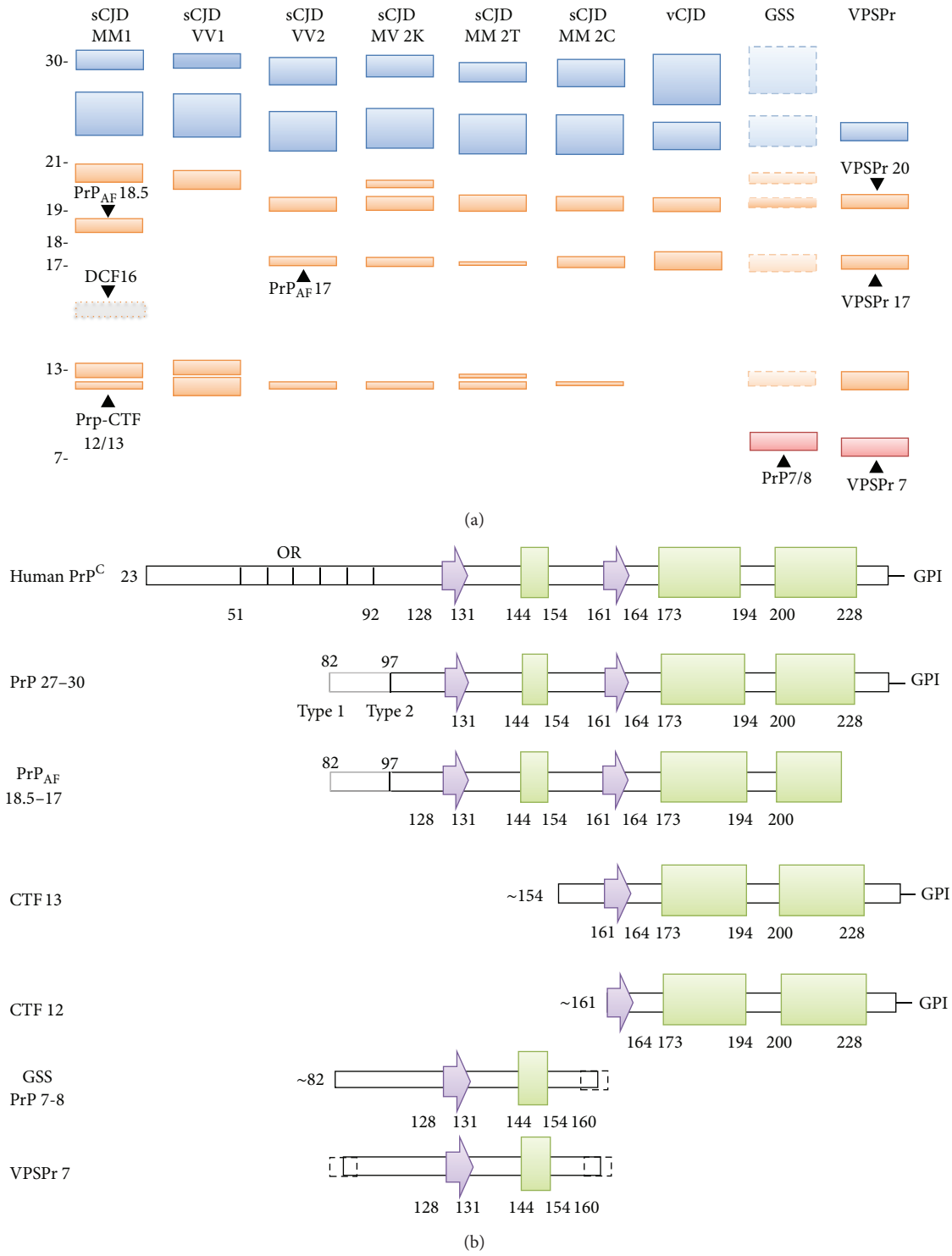

FIGURE 1: (a) Schematic representation of the spectrum of PrP^{res} fragments observed in human prion diseases and their electrophoretic profile. The unglycosylated forms of all PrP^{res} fragments with the glycosylation sites in their sequence are indicated in orange, while the fragments lacking these sites are shown in red. Among the glycosylated peptides, only the mono- and the diglycosylated forms of PrP^{res} 27–30 (18–21 kDa range) fragments are shown (in blue). The DCF16 fragment, which is generated only in partially denaturing conditions is labeled with a dotted line and a gray color. For GSS, the fragments that have been described only associated with specific *PRNP* mutations (e.g., P102L or A117V) are shown with dotted lines and in transparency. Molecular weights are indicated on the left in kDa. (b) Diagrams of the secondary structural elements of human PrP^{C} and of the PrP^{res} fragments observed in human prion diseases. Arrows are representative of β-strands and rectangles of α-helices and OR indicates the octapeptide repeats region. The secondary structure numbering has been derived from pdb (Protein Data Bank) id 2LSB (human PrP).

18.5-17, DCF 16, PrP-CTF12/13), possibly reflecting subtype-specific structural characteristics of the protein aggregate [138] (Figure 1).

PrP^{Sc} from different prion strains can also be typed through its glycoform ratio, that is the ratio between the three differently glycosylated isoforms of PrP^{res} 27–30 (i.e., diglycosylated, monoglycosylated, and unglycosylated) (Figure 1). In the large majority of CJD cases, PrP^{res} glycosylation is characterized by an overrepresentation of the monoglycosylated form [115, 130]. A rather grossly major distinction with diagnostic relevance has been introduced to distinguish the above-described "pattern A" from "pattern B" characterized by a predominance of the fully glycosylated form, the latter being found in vCJD [114, 116] or in gCJD and FFI linked to the E200K or D178N mutations, respectively [129, 139]. However, finer significant differences in PrP^{res} glycoform ratio have also been described among CJD subtypes with either "pattern A" or "pattern B" using either mono- or two-dimensional gel electrophoresis [130, 140].

Besides the strain typing approaches based on the analysis of the PrP^{res} fragments generated by PK cleavage and glycoform ratio, other approaches have focused on PrP^{Sc} detergent solubility and aggregate size, degree of protease-resistance, and conformational stability [141–143]. Kobayashi et al. [142] studied PrP^{Sc} aggregation in MM 1 and MM 2T sCJD (sFI) cases and found that the former has a larger aggregation size than that of the latter, a result which they also confirmed in case with the cooccurrence of PrP^{Sc} types 1 and 2. More recently, Saverioni et al. [111] have analyzed PrP^{Sc} protease resistance and aggregate size across the whole spectrum of human prions (all sCJD subtypes, sporadic FI (sFI), vCJD, and VPSPr) and found that the strain-specific PrP^{Sc} sensitivity varies over a 100-fold range of PK concentration and that these differences stem from both PrP^{Sc} aggregate stability and size.

Preliminary data on the conformational stability of PrP^{Sc} in CJD subtypes have also become available. Conformational stability assay (CSA), which measures the progressive loss of PrP^{Sc} PK-resistance after exposure to increasing concentration of GndHCl, showed that sCJDMM1 PrP^{Sc} is more stable than sCJDMM 2C PrP^{Sc} [143]. The same result was obtained with the conformation stability and solubility assay (CSSA), which measures the increase in solubility of PrP^{Sc} after exposure to increasing concentrations of GndHCl. [144]. Finally, both sCJDMM1 and VV2 PrP^{Sc} showed a higher stability than vCJD PrP^{Sc} in the conformation dependent immunoassay (CDI), which evaluates the increase in epitopes exposure after GndHCl denaturation [145].

According to Kim et al. [109] $sPrP^{Sc}$ concentration and stability is in close correlation with the disease progression rate. This, in turn, would reflect the association between the strain-specific amount and stability of $sPrP^{Sc}$ conformers and the efficiency in initiating the replication process *in vitro* [110].

Preliminary data obtained in three sCJD variants seem to suggest that both levels and stability of $sPrP^{Sc}$ are good predictors of the progression rate in sCJD and that small oligomers of protease-sensitive conformers of PrP^{Sc} may govern conversion potency. In particular, when $sPrP^{Sc}$ is less stable than $rPrP^{Sc}$, as in sCJDMM1 and VV2, the difference in stability would correlate with less accumulated $sPrP^{Sc}$ and a shorter duration of the disease, whereas when $sPrP^{Sc}$ conformers are more stable than $rPrP^{Sc}$, as in sCJD MM2, it would correlate with more accumulated $sPrP^{Sc}$ and a longer disease duration [110]. $sPrP^{Sc}$ oligomers, smaller in size than $rPrP^{Sc}$ polymers, may be the most powerful in triggering *in vitro* amplification due to an increased surface availability for recruiting PrP^{C} molecules for conversion. So, the strain in which these $sPrP^{Sc}$ conformers are most abundant would be the most efficient in amplification assays. In this regard, it is noteworthy that PMCA requires a sonication phase aiming to reduce the aggregation size of the seed. Although stimulating and sound with the current view of the biology of prions, the scenario depicted above must be taken with caution and definitely awaits confirmation by further investigations.

In addition to classical CJD variants and FI, human prion diseases include GSS and the recently described VPSPr. GSS is a familial disease which has been linked to missense, stop-codon, or insertional mutations in *PRNP*. The clinical phenotype in GSS is most commonly characterized by a progressive cerebellar syndrome, accompanied by extrapyramidal and pyramidal signs and cognitive decline, which may evolve into severe dementia [124]. However, a clinical variability, with either cognitive decline anticipating ataxia and rigidity or spastic paraplegia as a presenting symptom, has been observed. Neuropathological features associated with GSS disease vary substantially but always include PrP-positive multicentric amyloid plaques in the cerebellum and the cerebral cortex with or without associated spongiform change. Pioneering studies in GSS showed that purified amyloid preparations and the PrP^{res} obtained by *in vitro* proteolysis mainly comprise atypical unglycosylated 7-8 kDa PrP fragments with ragged N and C termini, primarily composed of mutant PrP, which are lacking in classic TSEs such as CJD and FI (Figure 1) [102, 146–151]. In keeping with the significant phenotypic heterogeneity of the disease, however, it was also shown that the western blot profile of PrP^{res} in GSS may comprise additional PrP^{res} fragments of higher molecular weight, including the CJD-associated PrP^{res} type 1 (Figure 1) [102, 146]. More specifically, GSS affected subjects carrying the most common GSS mutation (P102L) may either show a rapidly progressive CJD-like phenotype with both spongiform changes and amyloid plaques correlating with the cooccurrence PrP^{Sc} type 1 and the 8 kDa fragments or show a more slowly progressive "pure" GSS phenotype correlating with the presence of amyloid plaques and the 8 kDa PrP fragment [102, 146]. Finally, GSS associated PrP^{Sc} has also been reported to be unusually protease sensitive, at least in a subgroup of cases [102, 105, 106, 152]. Interestingly, when compared with CJD PrP^{Sc}, this increased proteolytic sensitivity of PrP^{Sc} does not correlate with a distinct aggregate sedimentation profile, suggesting that it is not due to a lower size of aggregates but rather to differences in their conformation [105, 106].

VPSPr is a recently described atypical variant of sporadic human prion disease, clinically characterized by language deficits, cognitive impairment, motor signs, especially Parkinsonism and ataxia, and an average longer clinical course than CJD [104, 153–156]. The disease can apparently affect all 3 codon 129 genotypes, although this genetic variability affects both susceptibility and phenotypic expression [104]. Pathologically, VPSPr is characterized by the spongiform change, which is especially seen in neocortical and subcortical regions of the cerebrum, such as the striatum and thalamus, and PrP-positive amyloid microplaques in the cerebellar molecular layers [104, 153].

Despite the clear differences in the clinicopathological phenotype between VPSPr and GSS, the characterization of PrP^{Sc} physicochemical properties has highlighted strong similarities which have led to the hypothesis that the former may represent the sporadic variant of the latter [153]. Indeed, PrP^{res} in VPSPr shows a striking, ladder-like, electrophoretic profile comprising at least 4 bands, including a prominent one migrating at about 8 kDa (Figure 1). Furthermore, the abnormal PrP shows a variable degree of PK-resistance according to the codon 129 genotype; it is highly protease-sensitive in subjects with VV, whereas it shows a degree of resistance comparable to some sCJD types in subjects MV or MM at codon 129 [104, 111]. A very recent study also demonstrated that VPSPr shares PrP^{Sc} features with a known familial CJD linked to a valine to isoleucine mutation at residue 180 of PrP (fCJDV180I), exhibiting similar patterns of glycosylation and protease cleavage [157].

3.2. Transmission Studies with Human Prions. The first characterization of the transmissible, strain-related properties of human sporadic prion isolates was accomplished in transgenic mice. Inocula from a single sFI (i.e., MM 2T) case produced disease characteristics that differed from those induced by sCJD MM1 as well as from genetic CJD cases carrying the E200K-129M or the V210I-129M haplotypes [158]. Preliminary data concerning the transmission properties of other sCJD subtypes became available a few years later [159, 160], but only recently the reevaluation of the National Institutes of Health series of prion disease transmitted to non-human primates [131, 161] and more comprehensive experimental transmissions to transgenic mice [153, 154] have substantially clarified the issue of the extent of strain variation in sporadic human prion disease and provided answers to the crucial question of how the current classification relates to different strains of sCJD. The results of these studies indicate that, besides the MM 2T variant already mentioned above, four out of five of the other neuropathologic and molecular "pure" types of sCJD defined by the classification of Parchi et al. [130, 133] behave indeed as different strains of agent. Most importantly, sCJD MM1 and MV1 isolates have identical transmission properties, which significantly differ from those of sCJD VV2 or MV 2K. Furthermore, both the sCJD MM 2C and sCJD VV1 subtypes behave differently from each other and from the other isolates after transmission [162]. However, at variance with the sCJD MM1/MV1 and VV2/MV 2K strains, only single cases of sCJD MM 2C, MM 2T, and VV1 have been examined, with the assumption that transmission characteristics of a single case will be representative of the particular subgroup. Thus, the results obtained for these rare subtypes, although clear and somehow expected, await confirmation [131, 162, 163]. Familial and acquired forms (except for vCJD; see below) are likely linked to the same pool of strains isolated from sCJD. For example, inocula from carriers of E200K and V210I mutations affected by the MM1 CJD phenotype showed the same transmission properties of sCJD MM1 inocula when propagated in Tg mice, non-human primates, or bank voles [92, 131, 160]; similarly, experimentally transmitted kuru reproduced the same clinico-pathological and biochemical features of VV2 and MV 2K sCJD [131]. Finally, similar properties have been observed by FFI and sFI prions when propagated into Tg mice [34, 158]. In contrast to prions propagated in classical CJD and kuru, the transmission properties of vCJD prions are strikingly distinct and have established vCJD as a distinct human prion strain [164, 165]. The vCJD prions transmit disease to wild-type mice far more efficiently than any other form of human prion disease [164–166] and in transgenic mice faithful propagation of the vCJD phenotype is dependent upon homozygous expression of human PrP 129 methionine [165, 167–170]. Transgenic mice homozygous for human PrP 129 valine show a pronounced transmission barrier to vCJD prions and propagate a distinct clinical-pathological phenotype [165, 167–169, 171]. As a consequence, the possibility that the BSE-vCJD strain may be associated with other human pathological phenotypes besides that observed in subjects carrying MM at codon 129 should not be dismissed.

With the significant exception of the GSS P102L associated with spongiform changes and PrP^{Sc} type 1, which shows CJD-like transmission properties, GSS variants have been more difficult to transmit to animals than CJD or FFI [161, 172]. This has led to the suggestion these GSS phenotypes are not true prion diseases (e.g., TSEs) and are better designated as nontransmissible proteinopathies. In more recent studies, however, the use of transgenic mice carrying GSS mutations such as A117V or the mouse equivalent of P102L has led to the finding that brain tissue from GSS patients carrying the corresponding mutation could induce a pathological phenotype into these mice, although with some significant differences between the two models [173, 174]. More specifically, in the first, the inoculation of brain extracts from a GSSP102L patient with no spongiform change caused almost no clinical disease but induced striking PrP-amyloid deposition in brains of several recipient mice; extracts of those brains failed to transmit neurological disease on further passage but again induced PrP-amyloid plaques in recipient mice [173]. In the second study, instead, the transmission of a more typical TSE phenotype, including the deposition of classic protease-resistant PrP^{Sc} 27–30, has been obtained in 117VV HuPrP transgenic mice challenged with A117V prion isolates [174]. Thus, especially according to this latter result, GSS may also be considered a true prion disease, although much less prone than CJD to transmit, possibly because it is characterized by the formation of less stable PrP^{Sc} aggregates.

3.3. PrP^{Sc} Properties and Strain Variation in Natural Hosts: Scrapie, BSE, and CWD

3.3.1. Scrapie. Biochemical typing of natural scrapie isolates has been largely based on the assessment of PrP^{res} electrophoretic mobility, glycoform ratio, and epitope mapping of PK-cleavage sites using different monoclonal antibodies. The use of other approaches such the analyses of PrP^{res} protease-resistance and conformational stability of PrP^{res} has been, so far, limited.

Despite the known diversity of classical scrapie strains that have been isolated in wild-type mice [83] or hamster [175, 176], the identification of strain-specifc PrP^{res} signatures in sheep with natural scrapie has proved to be challenging [177–182]. Indeed, the molecular signature of most isolates of classical scrapie comprises an unglycosylated PrP^{res} with a "high" (h-type) molecular mass (i.e., in the range of human PrP^{res} type 1 and including the epitope recognized by the N-terminal P4 antibody), whereas only a few cases show a PrP^{res} profile with a "low" (l-type) electrophoretic mobility (i.e., in the range of human PrP^{res} type 2 and not labeled by P4), similar to that seen in BSE or experimental scrapie strain CH1641 (Figure 2). Similarly, PrP^{res} glycoform ratios did not clearly differ from those found in cattle-BSE and did not reveal distinct subgroups of classical scrapie [180–185] (Figure 2). Some evidence for a strain-related heterogeneity of PrP^{Sc} associated with classical natural scrapie isolates derives from CDI analysis. It has been shown that PrP^{Sc} extracted from sheep with the VRQ/VRQ *PRNP* genotype has higher levels of PK-sensitive PrP^{Sc} than the PrP^{Sc} associated with ARQ/ARQ [108]. Furthermore, the two isolates propagated in mice are associated with two PrP^{Sc} with distinct conformational stability, with the PrP^{Sc}-VRQ inocula being more sensitive to denaturation than the other [186].

The unusual scrapie isolates with a l-type PrP^{res} profile, designated as CH1641-like, have for some time posed a diagnostic challenge because of the similarities with the PrP molecular properties of experimentally transmitted BSE to sheep. Immunoblot assays have shown that they share a migration pattern similar to the unglycosylated PrP^{res} fragment but have different levels of diglycosylated PrP^{res} [178] (Figure 2). More recently, however, it has been found that the PrP^{res} associated with the CH1641-like isolate clearly differs from BSE-PrP^{res} by the presence of an additional band at approximately 14 kDa, which is specifically recognized by the C-terminal antibody SAF84 (Figure 2) [187]. This additional PrP^{res} fragment was also observed after transmission in a transgenic mouse model (TgOvPrP4) of both the natural CH1641-like isolate [188, 189] and the CH1641 experimental scrapie isolate that was originally isolated from a British scrapie case and maintained by serial transmissions in sheep [190]. Unlike CH1641 this PrP^{res} fragment was not detected in the scrapie strains with h-type PrP^{res} (C506M3, Chandler, and 79A), arguing that PrP^{res} 14 kDa preferentially associates with l-type PrP^{res} [189]. Intriguingly, both l-type and h-type PrP^{res} were detected in the brain of TgOvPrP4 infected with some scrapie isolates [187], which strongly suggests that the two phenotypes found in mice could be the result of the cooccurrence of two strains in these sheep. Indeed the possible existence of a mixture of strains from a single scrapie case, which can only be separated by biological cloning, has been documented following bioassay in mice or hamsters [191, 192].

In 2003 an atypical scrapie strain (Nor98) was described in five sheep from Norway [193]. Scrapie cases similar to Nor98 were later detected in other European countries [194–196] and in the United States [197]. Western blotting analysis of Nor98-affected brain extracts has allowed the identification of a peculiar PrP^{res} electrophoretic profile consisting of multiple protein bands including a prominent band of relatively low molecular mass that was initially reported to migrate around 12 kDa [186].

In particular, the use of different mAbs raised against epitopes located in the middle and in the C-terminal regions of PrP has allowed the identification of two previously unrecognized fragments, respectively, designated as Nor98-PrP7 and PrP-CTF14 (Figure 2). Nor98-PrP7 is a PK resistant N- and C-terminally truncated fragment with a molecular weight of 7 kDa which is not affected by PNGase F treatment, while PrP-CTF14 is a C-terminal fragment migrating at 14 kDa after deglycosylation. Interestingly, both fragments showed an increased protease sensitivity when compared to PrP^{Sc} in classical scrapie, suggesting that the PrP^{Sc} associated with the two diseases have a different conformation [198].

The intracerebral inoculation of a panel of atypical/Nor98 scrapie isolates into mice overexpressing the ovine prion protein (Tg338) suggests that a single prion strain is responsible for atypical scrapie [199]. Using a set of PrP-specific monoclonal antibodies two distinct C- and N-terminally ragged PK-resistant PrP^{res} fragments of approximately 8 kDa and 5 kDa which are differently truncated at their C-termini were detected, thus confirming the complexity and the specificity of the molecular PrP^{res} phenotype of these atypical scrapie isolates [199] and its similarities with some human TSE variants such as GSS-P102L and VPSPr (Figures 1 and 2) [200].

3.3.2. Bovine Spongiform Encephalopathy (BSE). On the basis of the electrophoretic profiles of the unglycosylated band of PrP^{res}, three different BSE phenotypes are currently recognized: the classical BSE (C-type) and two atypical BSE variants showing, respectively, a lower (L-type) and a higher (H-type) relative molecular mass of PrP^{res} in comparison to the c-type [113, 181, 201, 202] (Figure 2(a)).

Early evidence suggested that BSE was caused by a prion strain characterized by an efficient ability to overcome the species barrier and with a PrP^{res} signature featuring a lower relative molecular mass compared to the PrP^{res} associated with classic scrapie (and CWD) and a marked predominance of the high molecular weight glycoform [164, 203].

In 2004, however, a distinct phenotype of bovine amyloidotic spongiform encephalopathy (BASE or L-type) [113], correlating with a PrP^{res} showing a slightly lower electrophoretic mobility than the PrP^{res} of the C-type and a predominant monoglycosylated isoform, was found [201]. The

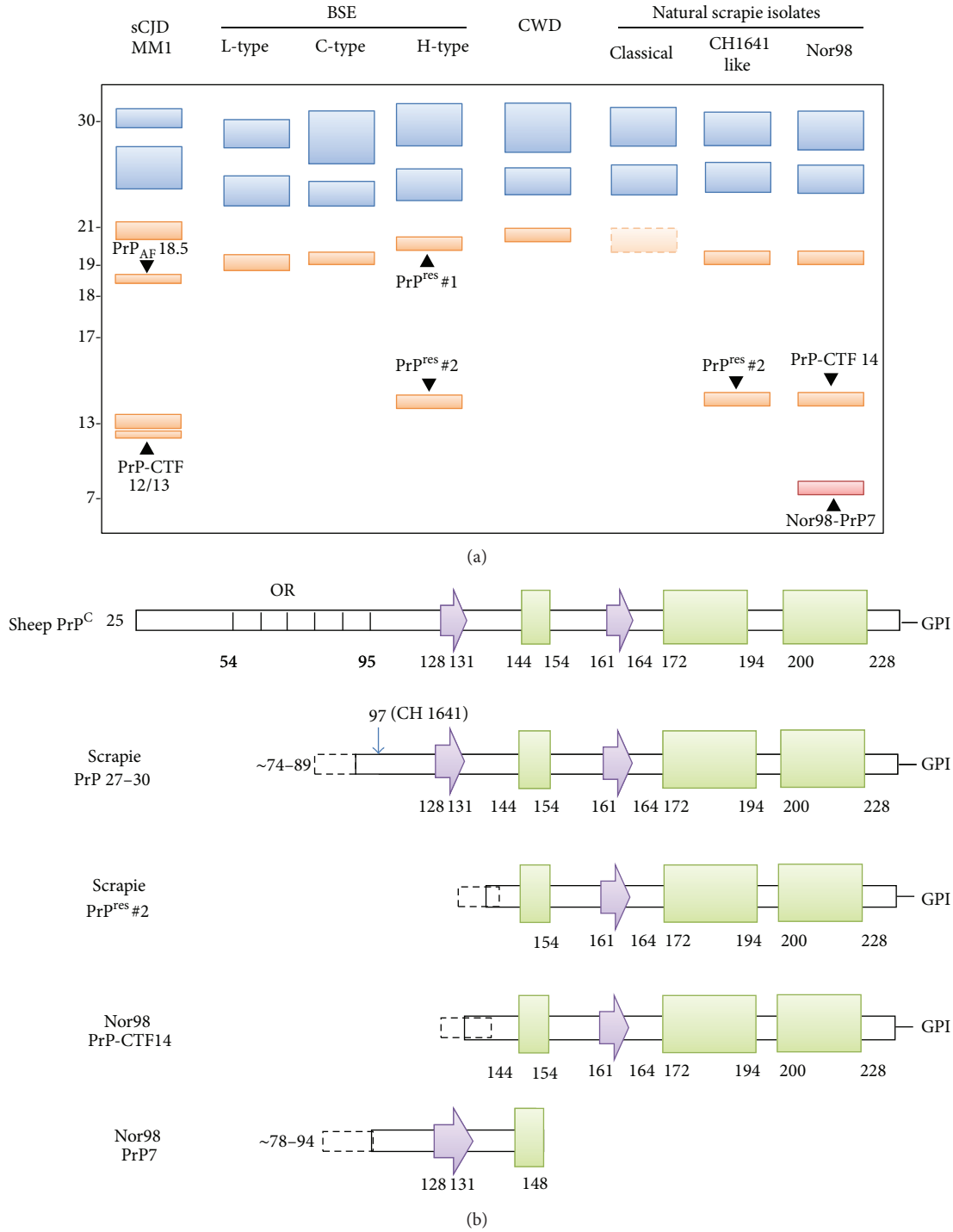

FIGURE 2: (a) Schematic representation of the spectrum of PrP^{res} fragments observed in animal prion diseases and their electrophoretic profile. The unglycosylated forms of all PrP^{res} fragments with the glycosylation sites in their sequence are indicated in orange, while the fragments lacking these sites are shown in red. Among the glycosylated peptides, only the mono- and the diglycosylated forms of PrP^{res} 27–30 (18–21 kDa range) fragments are shown (in blue). To facilitate the comparison with human forms, the profile of MM1 sCJD associated PrP^{res} is shown; note that the unglycosylated band of sCJDMM1 PrP^{res} has the same electrophoretic mobility of that of CWD as reported by Xie et al. [14]. (b) Diagrams of the secondary structural elements of sheep PrP^{C} and of the PK-resistant PrP fragments observed in classical and atypical Nor98 scrapie. Arrows are representative of β-strands and rectangles of α-helices and OR indicates the octapeptide repeats region. The secondary structure numbering has been derived from pdb (Protein Data Bank) id 1XYU (sheep PrP).

evidence that BSE and BASE are caused by two distinct prion strains is supported by transmission experiments showing that the inoculation of BSE or BASE brain homogenates in transgenic mice (Tgbov XV) causes two distinct phenotypes [204]. Noteworthy, BASE was also shown to convert into the classical BSE strain upon serial transmission to inbred mouse lines, which has raised the hypothesis that BSE originated from BASE [205].

Intriguingly also the H-type BSE, first described by Biacabe et al. in 2004 [201], can recapitulate most of the phenotypic features of classical BSE after cross-species transmission experiments in wild-type mice [206]. Compared with the C-type, the H-type strain is characterized by an extended N-terminus of PrP^{res} and by the presence of two distinct PrP^{res} cleavage products, $PrP^{res\#1}$ (19–30 kDa), showing a slightly higher electrophoretic mobility than the PrP^{res} of the C-type, and $PrP^{res\#2}$ (14–24 kDa), characterized by a more C-terminal cleavage [207] (Figure 2(a)). This typical H-type PrP^{res} banding pattern was also described in a BSE case associated with a *PRNP* mutation (E211K) [208].

3.3.3. Chronic Wasting Disease (CWD). CWD, like scrapie, is a prion disease mainly transmitted via an environmental route [209]. Although the horizontal transmission of CWD among cervids by direct or indirect contacts is remarkably efficient, its transmission to different species has yet to be fully clarified [210–212].

The PrP^{res} electrophoretic profiles of CWD-affected animals and of sCJDMM1 have led to the observations that they share some similarities as shown by the conformational stability assay and by the observation that in both samples the unglycosylated PK-resistant isoform migrates at 21 kDa, thus indicating a similar conformation of the PK resistant cores. However, the two PrP^{res} do not display a similar glycoform profile with a prevalence of the diglycosylated isoform in the CWD PrP^{res}, as observed in BSE and in vCJD [14] (Figure 2(a)). The same electrophoretic and glycoform profiles were also observed in two different CWD strains (CWD1 and CWD2) which were identified after the inoculation of different CWD isolates in Tg mice expressing cervid PrP (Tg (CerPrP)$1536^{+/-}$) [213].

Interestingly the PrP^{res} immunoblot analysis of white-tailed deers orally inoculated with the CWD agent revealed that in Q95H/G96S animals the unglycosylated fragment migrates at lower molecular weight and the level of PK-resistance seems to be reduced, suggesting the generation of a PrP^{res} with different properties which the PrP^{res} generated in the other infected cervids [214].

4. Role of PrP^{Sc} in Prion Toxicity and Neurodegeneration

The understanding of the mechanisms of toxicity resulting from misfolding and ordered aggregation of proteins involved in prion disease and many others neurodegenerative diseases remains an open question and a research priority. Indeed, in none of these diseases are the mechanisms of toxicity completely clear. While a large body of evidence indicates that the misfolded protein aggregates are the cause of the neurodegeneration, many studies link this toxicity to the existence of various intermediate structures, likely in the oligomeric state, prior to the fiber formation and/or their specific interaction with membranes [215, 216]. Indeed, in prion diseases it is well established that, in the absence of GPI-linked PrP^{C}, PrP^{Sc} is innocuous, suggesting that PrP oligomers and fibrils are not toxic *per se* [217], and that PrP^{C} may act as mediator of the toxic signal. Furthermore, the importance of certain physicochemical properties of the protein fragments forming the aggregate, such as size and glycosylation state, has also been highlighted by studies in prion disease, which uniquely comprise a wide range of disease phenotypes allowing for extensive molecular and clinicopathological correlations [125].

4.1. Insights from Studies on Naturally Occurring and Experimentally Transmitted Prion Diseases. From the study of affected brains we have learned that the events that are triggered by prion neuroinvasion and that result in neurodegeneration may vary significantly both in terms of resulting histopathology and speed of the neurodegenerative process. In humans the clinical course of a prion disease may range from a few weeks to at least one decade, and evidence from experimental transmissions and acquired prion diseases indicate that a similar heterogeneity likely characterize also the preclinical phase.

Histopathologically, while most prion diseases, including CJD, BSE, CWD, and most of scrapie cases (i.e., the classic transmissible spongiform encephalopathies or TSEs), are characterized by the triad of spongiform change, gliosis, and neuronal loss, some rare but very informative variants such as FI, GSS, or PrP-CAA may show very subtle or even absent spongiform change or be characterized by prominent extracellular amyloid plaques accumulating either in the neuropil or around blood vessels. Most significantly, in contrast to CJD, in which the abnormal PrP^{Sc} aggregates mainly consist of full-length protein together with GPI-anchored, N-terminal fragments truncated between residue 82 and residue 104 [129], in GSS or PrP-CAA affected patients the abnormal PrP plaque amyloid that accumulates is composed primarily of truncated internal PrP fragments (e.g., residues 82–153) that lack the GPI anchor and the glycosylated moiety [102, 146, 152]. In this respect, GSS patients carrying the P102L mutation can be considered a "quasinatural" experimental model. Indeed, while in some of these patients pure GSS histopathological features correlate with the presence of the GPI-anchorless PrP fragment, in others mixed CJD/GSS features (e.g., widespread spongiform changes cooccurring with amyloid plaques) correlate with the deposition of both types of PrP^{Sc} forms (e.g., GPI-anchored and glycosylated N-terminal PrP^{Sc} fragment + truncated internal PrP fragments lacking the GPI anchor). These observations strongly support the idea that PrP fragments have different neurotoxicities and cause distinct lesions as a consequence of their different properties, such as aggregation propensity [102]. In particular, the longer duration of illness in GSS patients can be explained postulating that the short GPI-anchorless

PrP^{res} fragments have a higher tendency toward aggregation and plaque formation and thus provide a relative protection with less neuronal dysfunction than the 21- or 19-kDa PrP^{res} glycosylated fragments or full length PrP^{Sc} associated with CJD that form more diffuse and smaller deposits. Consistent with this hypothesis is also the observation that, among the GSS P102L patients, those showing the mixed CJD/GSS phenotype have, on average, a significantly shorter course [102].

More recently, the evidence obtained from studies on CJD and GSS patients has received strong support from a transgenic mouse model expressing anchorless PrP [218]. In these Tg44 mice scrapie infection results in an unusual type of slow fatal prion brain disease distinguished by widespread deposition of PrP^{Sc} amyloid in the CNS [219] and in extraneural sites such as heart, brown fat, white fat, and colon [220, 221]. In the CNS of infected Tg44 mice the gray matter vacuolation typical of prion diseases is minimal, and PrP^{Sc} is primarily deposited as perivascular amyloid [219]. In this model, most of the typical clinical and neuropathological characteristics of scrapie are either absent or greatly reduced, despite the accumulation of brain PrP^{Sc} to levels comparable to those in scrapie-infected wild-type mice. This reduced brain damage could be due either to a need for anchored PrP^{C} on brain cells for toxicity induced by PrP^{Sc} and/or to a lower pathogenicity of PrP^{Sc} amyloid plaques compared to the more dispersed, amorphous, and membrane-associated PrP^{Sc} deposits seen in most other prion diseases. These findings highlight the role of GPI anchor in TSE pathogenesis [222]. It is likely that the anchoring of PrP^{Sc} aggregates to membranes by the GPIs could distort its local structure, composition, flexibility, fluidity, dynamics, integrity, and, hence, functionality. The results of several elegant EM studies corroborate these observations by showing that in all the naturally occurring TSEs of animals, as well as in experimental scrapie models of mice, there are a number of distinctive membrane changes, including membrane microfolding, membrane clefts, and abnormal endocytosis of dendrites, which are both directly linked to PrP^{Sc} and appear to be unique to prion diseases [223, 224]. These changes, however, were absent from Tg mice expressing only anchorless PrP and other Tg mice developing large amyloid plaques composed of abnormal prion protein [225].

While a definite progress has been made in understanding the divergent molecular pathology between classic TSEs and the "anchorless" PrP-amyloidosis, much less is known about the molecular basis of the different "neurotoxicity" associated with the various prion strains. Indeed, differences in the molecular and cellular pathology that correlate with the severity of the clinical phenotype have also been observed among classic TSEs such as sCJD. Subjects affected by the most common sCJD variant (e.g., the MM1 subtype), for example, do not accumulate higher amounts of PrP^{Sc} or develop more severe histopathological changes than the other sCJD variants despite their very rapid clinical course, sometimes lasting less than a month [115, 226]. Similarly, in a recent study in which we have correlated the amount of PrP^{Sc} deposition with the extent of microglial activation across the whole spectrum of sCJD subtypes, including the MM 2T or FI, we found that the degree of microglial activation differs significantly between disease subtypes and, above all, it does not correlate with the overall amount of PrP^{Sc} accumulation (Strammiello R and Parchi P, unpublished). Intriguingly, the most significant difference in the ratio between PrP^{Sc} amount and HLA-DR load was seen between two subtypes, the MM 2C and the MM 2T, sharing the average disease duration, codon 129 MM genotype, and PrP^{Sc} type 2. Overall, these data add to previous observations indicating that many critical properties of prions, including neurotoxicity, appear unrelated to the overall amount of PrP^{Sc} deposition. Furthermore, they indicate that there are strain-related differences in the apparent "neurotoxicity" associated with PrP^{Sc} deposition that must be addressed.

Another intriguing and largely unexplained issue of prion pathology concerns the regional specificity. In this respect, the study of FI, which is by far the most peculiar disease phenotype among those characterized by a "classic" PrP^{Sc} 27–30 deposition, has been very informative. The histopathological hallmark of FI, especially of the familial form linked to the D178N-129M *PRNP* haplotype, is a severe neuronal loss in the medial thalamic and inferior olivary nuclei [126]. These changes develop early since they are found in all affected subjects, irrespectively of the disease duration; furthermore they are found associated with amounts of PrP^{Sc}, which are at least tenfold lower than those detected in other sCJD subtypes where the neuronal loss in the thalamus is rarely so severe. In contrast, in the neocortex and, to a lesser extent, in the limbic cortex and the striatum of FFI patients, the amount of PrP^{Sc} increases with the duration of symptoms and eventually accumulates in significantly higher amounts than in the thalamus [227, 228]. Furthermore, the higher extent of PrP^{Sc} deposition correlates with the appearance of spongiform changes rather than with the degree of neuronal loss which remains milder than in the thalamus.

In conclusion, significant differences in the "neurotoxicity" associated with PrP^{Sc} deposition are also seen among classic TSE subtypes. However, in contrast with GSS, no significant data have been collected to explain how PrP^{Sc} may mediate these heterogeneous effects.

4.2. Insights from Studies on Animal Models. The first studies documenting the progression of neurodegeneration in prion disease dates back half a century and preceded the discovery of the prion protein. At that time experimental transmissions in primate and murine models already established that the appearance of spongiform change precedes neuronal loss and reactive astrogliosis [229]. It was later found that PrP^{Sc} deposition almost invariably represents the earliest event of the pathological cascade, which is immediately followed by microglial activation and the appearance of spongiform change. It was also found that the conversion of PrP^{C} into PrP^{Sc} is critical to the neurotoxicity associated with prion diseases since neither loss of PrP^{C} function nor deposition of PrP^{Sc} in absence of PrP^{C} expression is sufficient

to cause the prion-associated pathology [230, 231]. Having established the central role of both PrP^{Sc} and PrP^{C} in prion pathogenesis, the critical issue has progressively become the search for a link between PrP^{Sc}, neurotoxicity, and infectivity. Although the temporal and anatomical correlation between PrP^{Sc} formation and the development of infectivity and neuropathological changes is often obvious in prion disease, the overall correlation between PrP^{Sc} levels, infectivity, and neurotoxicity can be weak or even absent. For example, transgenic mice expressing some mutant forms of PrP^{C} that lack certain domains spontaneously develop neurological disorders, but no infectivity and bona-fide PrP^{Sc} are associated with prion protein aggregates accumulated in brain tissue of these animals [27, 232]. On the other hand, mice expressing GPI-anchorless prion protein show high levels of infectious PrP aggregate deposits, but reduced neurodegeneration compared to prion-infected wild-type mice [218]. Finally, there are subclinical infections in which there is abundant PrP^{Sc} but little symptomatology, for example, after inoculation of hamster prions into mice [233, 234]. Thus, it appears that infectious and neurotoxic forms of PrP could represent distinct molecular species, a view which is also supported by a recent study showing that prion propagation in brain proceeds via two distinct phases. More specifically, it has been shown that a clinically silent exponential phase, which rapidly reaches a maximal prion titre and is independent by PrP^{C} expression, is followed by a plateau phase, which determines time to clinical onset in a manner inversely proportional to prion protein concentration [235]. Notably, however, the same data would also fit the model of PrP^{C}-mediated PrP^{Sc} toxicity (see below), without requiring the existence of a toxic PrP as a distinct entity [236].

Despite this largely unsolved complexity, as for other protein aggregation diseases, PrP^{Sc} oligomers currently attract most of attention and appear to be the preferred researcher's candidate to explain both prion toxicity and infectivity. However, while there appears to be little doubt that infectious prion particles consist of small PrP oligomers, it is much less clear whether oligomers, and if so which oligomers, are involved in prion toxicity. As far as the mechanism of mediated toxicity is concerned, current evidence supports the view that small oligomers formed on membrane-bound GPI-PrP^{C} may act by compromising the integrity of cellular membranes or, more likely, by mediating a neurotoxic signal triggered from the extracellular milieu by PrP^{Sc}. Alternatively, PrP^{C} may disrupt the endosomal compartment after being internalized [237]. Lines of evidence suggesting that PrP^{Sc} neurotoxicity may involve impairment of the normal physiological activity of PrP^{C} have also been gathered, especially from the study of mutant forms of PrP that produce spontaneous neurodegeneration in transgenic mice without the formation of infectious PrP^{Sc} (reviewed in [238]). For example, Tg (PrPΔ32–134) mice, which express an N-terminally truncated form of PrP, spontaneously develop a neurodegenerative phenotype that is stoichiometrically reversed by coexpression of wild-type PrP, but only partially rescued by coexpression of a PrP^{C} isoform carrying an insert mutation. The rescuing effect of wild-type PrP would implicate a molecular target for PrP, which is presumably a receptor or another cell-surface complex capable of transducing a signal to the interior of the cell. Based on these evidences, Harris and collaborators [239] have proposed that PrP^{Sc} (or other toxic forms of PrP), by interacting with the same putative membrane target, may subvert a normal function of PrP^{C} to generate a neurotoxic signal. Although of significant interest, the proposed mechanism is in apparent contrast with the dominant mode of inheritance of familial prion diseases. Furthermore, the connection between the neurotoxic mechanisms activated by artificial mutants and those operative in "natural" prion diseases of humans and animals remain to be demonstrated. Whatever the nature and the mechanism of action of the toxic molecular species, there is a growing body of data to show that it is the synapses that are the first or most susceptible component of the neuron to succumb in the disease process rather than the death of the cell soma. Compromised synaptic function is currently thought to underlie the earliest symptoms in several neurodegenerative diseases, and loss of synapses, spines, and dendrites is thought to precede the loss of neuronal cell bodies [240–243]. Using an engineered mouse model Mallucci and collaborators [244] have shown that the block of PrP^{Sc} formation by knocking out PrP^{C} in prion-infected mice during the course of disease prevented neuronal loss and progression to clinical disease. PrP knockout produced both long-term survival and neuroprotection and the disappearance of early spongiform change, thus indicating that spongiosis is a predegenerative change occurring in neurons which may represent an early morphological marker of functional impairment [244].

Using the same model, this group of researchers has recently demonstrated that the decline of synapse number and transmission is associated with an abrupt loss of synaptic proteins [245]. PrP replication and the consequent rise of PrP levels during disease would cause a sustained induction of the cellular unfolded protein response (UPR). Rising levels of unfolded proteins in the ER would cause the phosphorylation of PERK-P, followed by that of eIF2a, which ultimately causes a reduction of new protein synthesis. The resulting chronic blockade of protein synthesis would lead to synaptic failure, spongiform changes, and, ultimately, neuronal loss. Based on these findings, it has been proposed that the key trigger to prion neurodegeneration is the continued, unchecked activation of the UPR due to the rising levels of PrP during disease, with fatal repression of translation rates.

4.3. The Role of Microglia in PrP^{Sc} Clearing and Prion Disease-Associated Neurodegeneration. A major theme in studies of the role of microglia in neuropathology is the dichotomy between their contributions to neurodegeneration *versus* neuroprotection. Prion diseases are not an exception to this theme. Lines of evidence indicate that PrP^{Sc} can be efficiently cleared from the brain and that phagocytosis by microglia represents a prominent clearing mechanism [246, 247]. On the other hand, it has also been shown that activated microglia may assume an aggressive phenotype and

release inflammatory cytokine fostering neuronal apoptosis and neurodegeneration [248].

Recent studies have contributed to shed some light into the molecular events regulating microglial activation during prion infection. In murine prion disease, the microglia was shown to activate early in the disease process, even in the absence of widespread histologically detectable PrP^{Sc} deposits [249]. This activated phenotype, which has been referred to as anti-inflammatory or benign, shows low levels of inflammatory cytokines and readily detectable levels of TGF-β and PGE2 [241, 250]. While there is no evidence that the enhanced levels of PGE2 are detrimental, nor that TGF-β is injurious, this situation may significantly worsen in the presence of systemic inflammation. Indeed, when mice were challenged systemically with endotoxin to mimic an intercurrent infection, this maneuver led to a dramatic switch in the microglial phenotype with an aggressive inflammatory cytokine profile and increased neuronal apoptosis [248]. This concept of rapid switching of the microglia phenotype is of course entirely in keeping with what is known about the degree of plasticity of the cells of the macrophage lineage. Systemic inflammation has a profound impact on a number of other animal models of neurological disease [251] and accelerates cognitive decline in Alzheimer's patients [252].

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Research Article

Early Delivery of Misfolded PrP from ER to Lysosomes by Autophagy

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Prion diseases are linked to the accumulation of a misfolded isoform (PrP^{Sc}) of prion protein (PrP). Evidence suggests that lysosomes are degradation endpoints and sites of the accumulation of PrP^{Sc}. We questioned whether lysosomes participate in the early quality control of newly generated misfolded PrP. We found PrP carrying the disease-associated T182A mutation (Mut-PrP) was delivered to lysosomes in a Golgi-independent manner. Time-lapse live cell imaging revealed early formation and uptake of GFP-tagged Mut-PrP aggregates into LysoTracker labeled vesicles. Compared with Wt-PrP, Mut-PrP expression was associated with an elevation in several markers of the autophagy-lysosomal pathway, and it extensively colocalized with the autophagosome-specific marker, LC3B. In autophagy deficient ($ATG5^{-/-}$) mouse embryonic fibroblasts, or in normal cells treated with the autophagy-inhibitor 3-MA, Mut-PrP colocalization with lysosomes was reduced to a similar extent. Additionally, 3-MA selectively impaired the degradation of insoluble Mut-PrP, resulting in an increase in protease-resistant PrP, whereas the induction of autophagy by rapamycin reduced it. These findings suggest that autophagy might function as a quality control mechanism to limit the accumulation of misfolded PrP that normally leads to the generation of PrP^{Sc}.

1. Introduction

Prion diseases, such as Creutzfeldt-Jakob disease (CJD) of humans and bovine spongiform encephalopathy (BSE) of cattle, are transmissible neurodegenerative disorders linked to the accumulation of a misfolded isoform (PrP^{Sc}) of the host-encoded glycophosphatidylinositol (GPI)-linked prion protein (PrP^{C}) [1]. As a membrane protein, PrP^{C} follows the secretory pathway to its destination on the outer leaflet of the plasma membrane where it ultimately follows the endocytic pathway for degradation in lysosomes. Mutations of the PrP gene linked to familial prion disease promote the misfolding of PrP that may delay its exit from the endoplasmic reticulum, leading to impaired delivery to the plasma membrane and an alternative pathway for degradation.

Autophagy is an evolutionarily conserved lysosomal degradation pathway usually activated under low nutrient conditions which acts to sequester and deliver cytoplasmic material, including organelles, toxic metabolites, or intracellular pathogens, to the lysosome for degradation and/or recycling [2]. This process is highly regulated by a series of autophagy-related gene products or Atg proteins [3, 4]. Key proteins include Atg6 and its mammalian homolog Beclin-1, which participate in the formation of the double layered isolation vacuole [5], Atg8 and its mammalian homolog, the cytosolic microtubule associated protein 1 light chain 3 (MAP-LC3) that is incorporated into autophagosome membranes [6], and Atg12 and Atg5, which are required for autophagosomal membrane nucleation and are targeted to autophagosomes via a ubiquitin-like conjugation system [7]. In the recent years, autophagy has been shown to function in the elimination of several neurodegenerative-linked proteins [8–10], including PrP [11–13].

We recently found that chronic administration of the autophagy-inducing agent rapamycin to transgenic Tg(PrP-A116V) mice that model genetic prion disease, reduced the total load of misfolded PrP, prevented PrP amyloid plaque deposition in their brains, and significantly delayed disease onset [13]. These results support autophagy as a mechanism to limit the production of misfolded PrP; however, the cellular pathway by which misfolded PrP is eliminated has not been defined. To begin to address this question, we studied the possible role of autophagy in the cellular trafficking of a familial CJD-associated PrP mutant (T183A) [14, 15], well known to undergo intracellular aggregation and accumulation [16–20]. Our findings suggest that autophagy functions as an early quality control mechanism to limit the *de novo* generation of misfolded pathogenic PrP.

2. Materials and Methods

2.1. Plasmids/Cell Culture. The T182A mutation was introduced into a mouse sequence wild type (Wt) PrP containing pSP72 plasmid, using Quick Change (Stratagene) and the following PCR primers: GACTGCGTCAATATCGCCATCAAGCAGCACACG (T182A sense), CGTGTGCTGCTTGATGGCGATATTGACGCAGTC (T182A anti). An additional set of Wt and T182A mutant PrPs was engineered with the human/hamster monoclonal antibody (mAb) 3F4 epitope, to allow selective detection of recombinant mouse PrP in mouse neuroblastoma N2a cells that express moderate levels of endogenous PrP. The PrP-GFP construct, a gift of David Harris (Boston University, Boston, MA), was also used to generate Wt and T182A PrPs lacking the 3F4 epitope. All constructs to be expressed in mammalian cells were ligated into the $pCB6^{+}$ vector under the control of the CMV promoter, using standard molecular biological protocols. Cell lines were purchased from ATCC and grown in recommended media at 37°C and 5% CO_2. For transfections, Lipofectamine 2000 (Invitrogen), following manufacturer's protocol, was used. For stable transfections, cells were selected and maintained in 200 μg/mL G418 (Sigma) added to the media. Reagents used were bafilomycin (BafA1) 0.05–0.1 μM (Sigma), brefeldin A (BFA) 5–10 μg/mL (Sigma), 3-methyladenine (3-MA) 2–10 mM (Sigma), rapamycin 1–20 μM (Sigma), leupeptin 100 μM (Sigma), and E-64 20 μM (Sigma). Endo H (New England Biolabs) digestions were performed according to the manufacturer's instructions. To ensure the results were not specific to a single cell type, we employed three cell types for these studies: mouse neuroblastoma N2a, HeLa, and COS-7 cells. Of this group, N2a cells best approximate neurons, making them most useful for the fractionation studies and other functional studies such as assessing autophagy upregulation. However, because of their small cytoplasm, they were not ideal for imaging studies. For these, HeLa cells, with their larger cytoplasm, and which have been used extensively in autophagy-related studies elsewhere, were primarily used. COS-7 cells were also used in the extended time lapse live imaging studies, as these are large cells with a flat architecture, and they provide the best option for time-lapse imaging. Because they lack endogenous PrP expression, they were also employed in many of the biochemical assays.

2.2. Antibodies. The D13 human F(ab) anti-mouse PrP antibody (InPro) was used to detect recombinant mouse PrP expressed in HeLa cells, and the 3F4 mAb (gift of Richard Kascsak, Staten Island, NY) was used in N2a cells expressing recombinant PrP engineered with the 3F4 epitope tag (residues 109 and 112 as Met). Cy5 conjugated anti-human or anti-mouse antibody (Jackson ImmunoResearch) was used as the secondary antibody for immunofluorescence. Primary antibodies against giantin and secondary antibodies AlexaFlour 488 anti-mouse, anti-rat, and anti-rabbit were from Molecular Probes. For Western blots, HRP-conjugated anti-human (D13) or anti-mouse (3F4) IgG (Pierce) was used. Primary antibodies LAMP-1, BECN-1, and tubulin, in addition to secondary anti-mouse IgM and anti-mouse IgG secondary antibodies, were purchased from Santa Cruz Biotechnology. LC3B polyclonal rabbit primary antibody and phosphorylated mTOR and Atg12 polyclonal antibodies were from Cell Signaling.

2.3. Immunolocalization. In most cases, cells were grown on glass coverslips, fixed with ice cold methanol for 5 minutes, blocked in 2% BSA in PBS, probed (overnight at 4°C in a wet chamber) with appropriate primary antibody dilutions (1 : 50–1 : 200) in PBS, and incubated for 1 hr at 23°C with secondary antibody (1 : 200) in PBS, and appropriate washing throughout. Coverslips were mounted with antifade medium (Vectashield) and stored in the dark (4°C). Specimens were imaged on an Olympus DSU spinning disk confocal microscope system. For double-immunostaining studies, sequential scanning as well as secondary antibodies with distinct emission spectra (Cy5 and Alexa488) was used to eliminate fluorescence crosstalk between color channels. For LC3 staining and colocalization with GFP-tagged PrP, cells were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, washed with PBS, permeabilized in 0.1% Triton X-100 for 5 minutes, washed in PBS, blocked in 2% BSA for 1 h, and then incubated at 4°C overnight with rabbit anti-LC3 antibody (Cell Signaling) at 1 : 200. Following a wash, cells were incubated with secondary antibodies and DyLight 649-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, Lab, Inc.) (1 : 200) at room temperature for 3 h, and then washed with PBS. For staining nuclei, cells were incubated with 10 μg/mL DAPI (Sigma-Aldrich) for 1 min. Cells were analyzed using NIH ImageJ software. Li's method for the estimation of colocalization was determined using an ImageJ software JACOP plugin. Li's intensity correlation quotient (ICQ) provides an overall index of whether the staining intensities of two channels are associated in a random, dependent, or segregated manner. It ranges from 0.5 (completely dependent correlation) to −0.5 (completely segregated staining) [21].

2.4. Live Cell Imaging. For short term visualization, LysoTracker DND-99 (Molecular Probes), at a final concentration of 50 nM, was added to media 30 minutes prior to imaging. For time-lapse imaging, the concentration of LysoTracker was reduced to 15–30 nM to prevent lysosomal dye overload. Confocal photomicroscopy was performed on an Olympus IX81 DSU spinning disk confocal microscope equipped with

a 100x (NA 1.45) oil objective, a 14-bit chilled EM-CCD camera (Hamamatsu C9100-12), Ludl filter changers/shutters, and a stage microincubator with 5% CO_2 (Harvard Apparatus). Z stacks were collected every 1 micrometer over the cell volume once, for steady state imaging, or every 10 min for 5 to 16 h, to capture protein expression, using Slidebook software. Multiple fluorophore acquisitions used sequential capture to avoid crosstalk.

2.5. Electron Microscopy. Cells were postfixed with 4% paraformaldehyde (PFA) plus 1.25% glutaraldehyde for 60 min, rinsed with 0.1 M sodium cacodylate buffer, and fixed with 1% OsO_4 in sodium cacodylate (pH 7.4, 1 h at 4°C). Osmicated cells were rinsed with maleate buffer and then en bloc stained with 1% uranyl acetate in maleate buffer, pH 6.0, for 1 h. Cells were dehydrated in graded ethanol solutions and then embedded in Spurr's resin. Cells were thin sectioned, stained with lead citrate and uranyl acetate, and examined using a FEI Tecnai F30 electron microscope.

2.6. Subcellular Fractionation. N2a cells from two 100 mm dishes were homogenized using a ball-bearing homogenizer with a 12 μm clearance in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM $MgAc_2$, and a protease inhibitor cocktail (Roche) at a final concentration of 1 vol of cell pellet per 5 volumes of homogenizing medium. Postnuclear supernatants were fractionated by top loading on a step sucrose gradient comprised of 1 mL of 2 M sucrose, 4 mL of 1.3 M sucrose, 3.5 mL of 1.16 M sucrose, and 2.0 mL of 0.8 M sucrose. All solutions contained 10 mM Tris-HCl, pH 7.4, and 1 mM $MgAc_2$. The gradients were centrifuged for 2.5 h at 100,000 ×g in a Beckman SW41Ti rotor. Twelve 1 mL fractions were collected from the top of each gradient and assayed by Western blot.

2.7. Western Blotting. As previously described [22], confluent cells were lysed in ice cold lysis buffer (mM: 20 Tris-HCl, pH 8.0, 150 NaCl, 1 EDTA; 0.5% Triton X-100, 0.5% Na-DOC), protein concentration determined with BCA assay (Pierce), and 30–50 μg total protein subjected to SDS PAGE. Proteins were transferred to PVDF membranes (BioRad), probed with primary antibodies (described in text) overnight, washed and incubated with HRP-conjugated secondary antibodies (1 : 5000 dilution; 60 min), and developed with West Pico ECL (Thermo Scientific, Rockford, EL) chemiluminescence reagent for 5 min. Blots were exposed using a BioRad XRS Image Documentation system, and densitometry was assessed by ImageJ.

2.8. Solubility Assay. Cells were lysed in lysis buffer, cleared at 1500 ×g for 1 minute, then centrifuged at either 16,000 ×g (Figure 4) or 100,000 ×g (Figure 5) for 1 hour at 4°C. The supernatant was separated and the pellet was washed, centrifuged, and resuspended in the original starting volume of lysis buffer. Equal volumes of reconstituted pellet and supernatant were submitted for Western analysis. Fractions of soluble and insoluble PrP were estimated as the densitometric fraction of the sum of the supernatant and pellet fraction signals, as described in the text.

2.9. PIPLC Release Assay. Confluent cells were transiently transfected with either Wt- or Mut-PrP and expressed overnight in 150 mm plates (~2.6×10^7 cells). At the begining of the experiment the cells were washed 5 times with Opti Pro SFM (serum free media) and then incubated with 3 mL of the same media with or without PIPLC at 1 U/mL (Sigma, Cat number p-8804) at 4°C for 90 min. The release medium was collected and cleared of detached cells by centrifugation at 900 rpm for 3 min. Total protein was precipitated from the media by the addition of 4 volume of cold methanol and centrifugation at 12,000 ×g for 10 min at 23°C. The pellet was dried and resuspended in 30 mL of 1X SDS loading buffer, all of which was subjected to SDS-PAGE and immunoblotting. The cells on the plates were rinsed twice with PBS and harvested by the addition of lysis buffer for Western analysis.

3. Results

3.1. PrP-T182A Is Aberrantly Trafficked. The alanine substitution of threonine at residue 182 of mouse PrP (183 in human PrP) disrupts the N-X-T consensus sequence for the first of two N-linked glycosylation sites, which eliminates the diglycosylated fraction of PrP and results in its propensity to aggregate and undergo aberrant trafficking [16]. Several investigators have shown that PrP-T182A does not traffic to the plasma membrane [19, 20, 23, 24], which was confirmed in our hands, using three separate cell lines, including HeLa, N2a, and COS-7 cells. Representative examples of studies replicated in at least two cell types are presented. First, PrP-T182A (hereafter referred to as Mut-PrP) transiently expressed for 16 h produced only non- and monoglycosylated PrP that were sensitive to Endoglycosidase H (N2a data, using 3F4 tagged PrPs is presented), supporting a lack of complex glycosylation and limited trafficking to cis-Golgi (Figure 1(a)); second, phosphatidylinositol-phospholipase C (PIPLC) treatment, which cleaves PrP from its GPI anchor, effectively released surface-bound Wt-PrP, but not Mut-PrP, into the culture media (COS-7 cell data, using nontagged PrPs, presented) (Figure 1(b)); and third, immunofluorescence confocal microscopy showed Wt-PrP to be consistently present on the plasma membrane, whereas Mut-PrP was consistently absent (N2a data presented). However, both Wt-PrP and Mut-PrP colocalized with ER (protein disulfide isomerase, PDI) and Golgi (giantin) markers although we questioned whether Mut-PrP colocalized more completely with ER and less completely with Golgi than did Wt-PrP (Figure 1(c)). To assess a potential difference in steady state cellular distribution, we performed sucrose gradient cell fractionation on N2a cells stably expressing Wt- or Mut-PrP (Figure 1(d)). The vast majority of Wt-PrP was present within fraction 6, consistent with Golgi (GM130), whereas Mut-PrP was found in heavy and light fractions although it was concentrated primarily within the heavier fractions, especially fractions 8 and 10 that cofractionated with the ER marker calnexin (Figure 1(d)). The presence of Mut-PrP in lighter fractions is not surprising, based on its cycling within the cis-Golgi. The apparent discontinuous enrichment in fractions 8 and 10 suggests they might be compartmentalized as aggregates in variably sized vesicles.

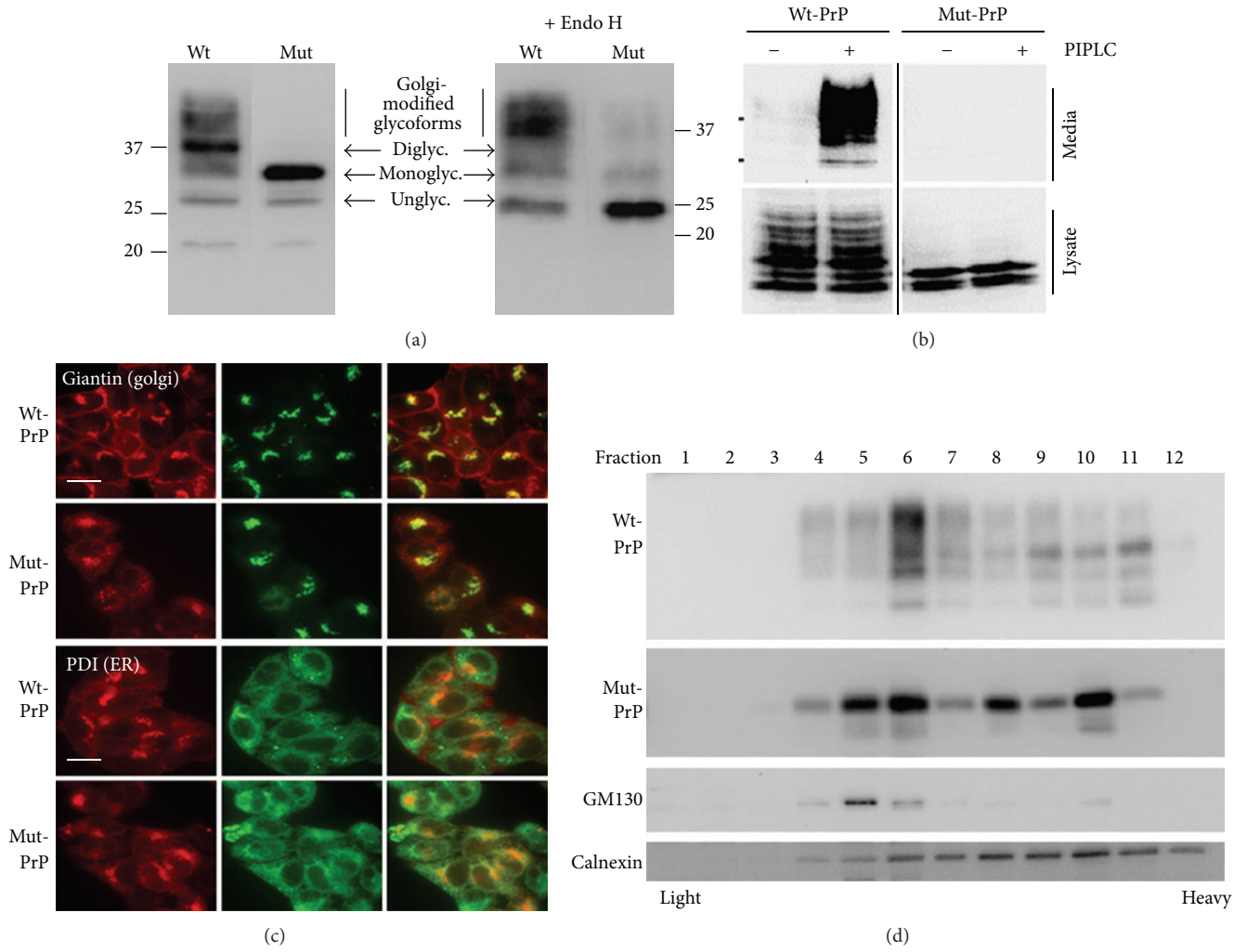


Figure 1: Aberrant trafficking of PrP-T182A. (a) Western blot of whole cell lysates prepared from N2a cells transiently expressing either Wt- or Mut-PrP carrying the 3F4 epitope (i.e., PrP(3F4)) for 16 h, before and after digestion with Endoglycosidase H (Endo H), followed by probing with 3F4 mAb. Wt-PrP has two N-linked glycosylation sites at positions 180 and 196, producing un-, mono-, and diglycosylated PrP. The T182A mutation disrupts the N-X-T glycosylation consensus site, resulting in loss of diglycosylated PrP. The lower molecular weight fractions of Wt-PrP and the core-glycosylated fraction of Mut-PrP are sensitive to Endo H, whereas higher molecular weight, Golgi-modified glycoforms, is resistant. (b) Western blot of Wt- and Mut-PrP recovered from the media (top) or lysates (bottom) of COS-7 cells transiently expressing PrPs for 16 h and then incubated for 90 min at 4°C in OptiPro media in the absence (−) or presence of PIPLC (+). Wt-PrP was detected in the media of PIPLC treated cells, whereas Mut-PrP was not (top panels), although the expression of each was evident in cell lysate preps (bottom panels), as revealed by D13 F (ab) anti-mouse-PrP antibody. (c) Immunofluorescence confocal microscopy of N2a cells expressing either Wt- or Mut-PrP(3F4) (red), costained for giantin or PDI (green). In contrast to Wt-PrP, Mut-PrP is consistently absent from the plasma membrane although it does label the ER and Golgi, indicating limited trafficking to at least cis-Golgi. Scale bars = 12 μm. (d) Sucrose gradient subcellular fractionation of N2a cells stably expressing Wt- or Mut-PrP(3F4) shows that Mut-PrP is distributed primarily in heavier fractions that correspond with the ER marker calnexin, compared with Wt-PrP that cofractionates primarily with the Golgi marker GM130. PrP is detected with 3F4 mAb.

To establish whether Mut-PrP is delivered to lysosomes despite the lack of plasma membrane localization, we performed indirect immunofluorescence confocal microscopy using HeLa cells following 16 h of transient expression of Wt- or Mut-PrP. HeLa cells provide an advantage to N2a cells, as they have a larger cytosol to facilitate the imaging of lysosomes and their colocalization with PrP. Lysosome-associated membrane protein-1 (LAMP-1) was found to colocalize with both Wt- and Mut-PrP, supporting the delivery of Mut-PrP to lysosomes (Figure 2(a)). To more conveniently assess its presence in lysosomes, we performed live imaging in HeLa cells expressing a GFP-tagged PrP (PrP::GFP) in the presence of LysoTracker Red, a fluorescent marker that preferentially accumulates within acidic vesicles that primarily include lysosomes (Figure 2(b)). The insertion of GFP between the last residue of mature PrP (position 230) and the GPI anchor does not alter its trafficking and permits live cell imaging [25]. To estimate the degree of the colocalization of

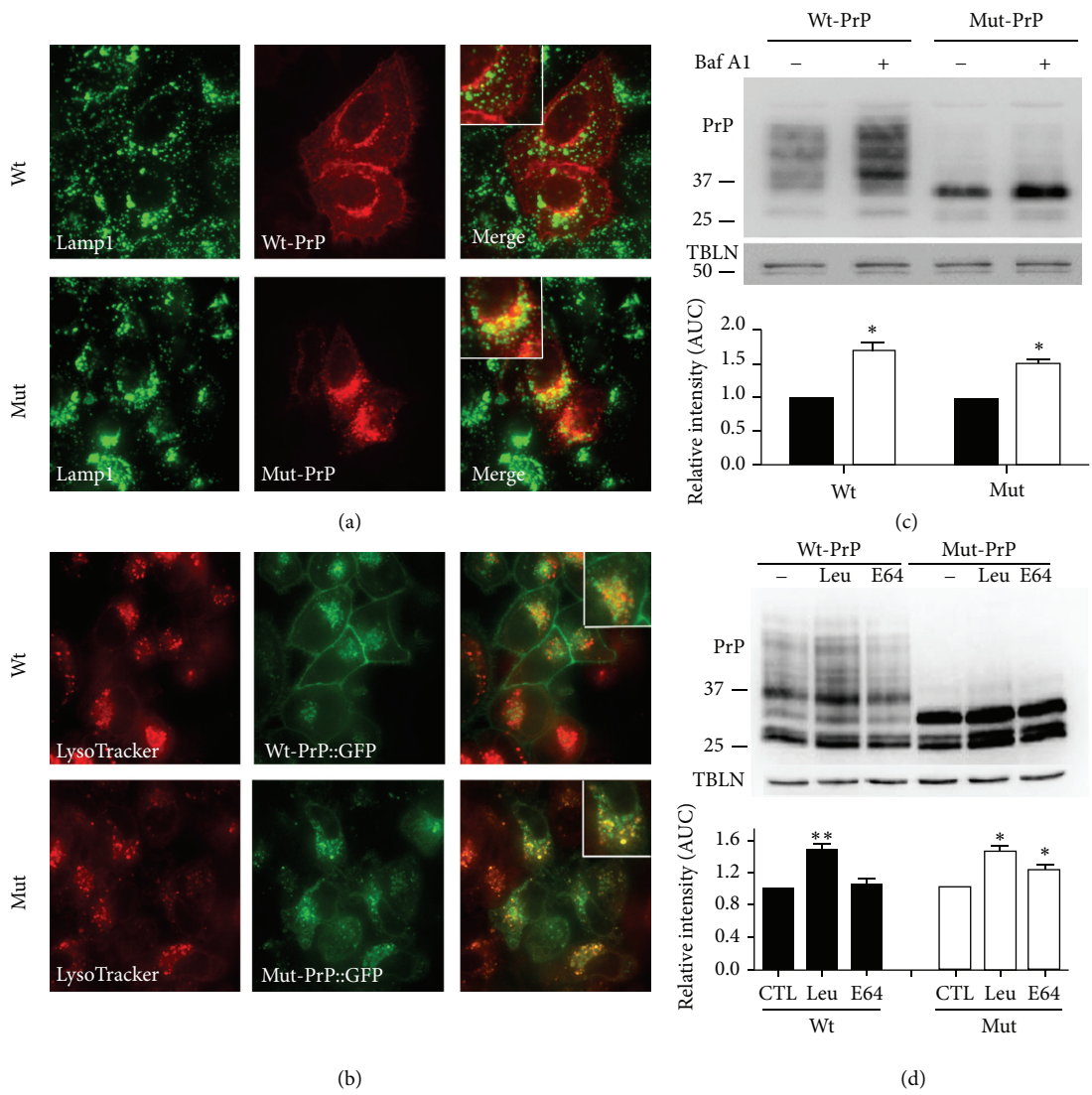


Figure 2: Mut-PrP in lysosomes. (a) Indirect immunofluorescence confocal microscopy of fixed HeLa cells after 16 h expression of Wt- or Mut-PrP, detected with anti-mouse-PrP D13 F(ab) antibody. Red (PrP) and green (LAMP-1) channels are displayed separately to the left of merged images. Yellow indicates colocalization. (b) Live cell fluorescence microscopy of HeLa cells transiently expressing Wt-PrP::GFP or Mut-PrP::GFP in the presence of 50 nM LysoTracker Red shows comparable results with indirect immunoflourescence. (c) Western blot of total lysates of COS-7 cells transiently expressing Wt- or Mut-PrP after 16 h incubation with vehicle (−) or 0.1 μM Bafilomycin A1 (Baf A1) added to the media. PrP is detected with the D13 antibody. Graphic representation of densitometric signal of Western blot from 3 experiments is displayed below. (d) as in (c), but following incubation in the absence (−) or presence of 100 μM Leupeptin (Leu) or 20 μM E64, probed with D13. α-tubulin (TBLN) as a loading control in (c) and (d). Molecular weight markers (kDa) on the left. Densitometric quantification of blots in (c) and (d) is shown in graphs as relative intensity compared to untreated condition. $^{*}P < 0.05$, $^{**}P < 0.005$ (Student's paired t-test, $n = 3$ experiments each).

PrP with LysoTracker, the raw images from red and green channels were applied to Li's intensity correlation quotient (ICQ), using an ImageJ (NIH) plugin. The ICQ ranges from −0.50 to +0.50, the former representing a completely random association and the latter representing a highly dependent association. We found that Wt-PrP::GFP intensely labeled the plasmalemma and colocalized sparsely with intracellular LysoTracker (ICQ of 0.19 ± 0.011) (Figure 2(b), top row), whereas Mut-PrP::GFP was undetectable on the plasmalemma and accumulated intracellularly as puncta that colocalized extensively with LysoTracker (ICQ of 0.48 ± 0.02, $P < 0.001$, student's t-test, $n = 15$ cells) (Figure 2(b), bottom row). Because the pattern of colocalization with LysoTracker was similar to that observed with LAMP-1, we considered it to be a surrogate marker for lysosomes although we recognize that other acidic vesicles may be labeled.

To establish whether Mut-PrP undergoes active degradation in lysosomes, we assessed the effect of lysosome inhibitors on the steady state level of Mut-PrP. Here, we used COS-7 cells, which conveniently lack endogenous PrP

expression allowing the expression of non-3F4-tagged mouse PrPs. Cells were transiently transfected and allowed to express Wt- or Mut-PrP for 16 h prior to incubation for 6 hours with the vacuolar-type ATPase inhibitor Bafilomycin A1 (Baf A1) (Figure 2(c)), E-64, or leupeptin, inhibitors of resident lysosomal enzymes cathepsins B and L (Figure 2(d)). In addition to the expected increase in Wt-PrP, a significant increase in the steady state level of Mut-PrP was seen with each inhibitor, suggesting that it undergoes some degree of degradation in lysosomes.

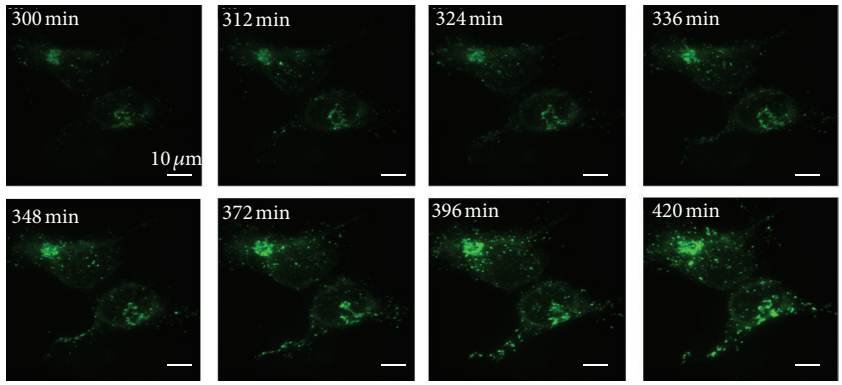
3.2. Mut-PrP Follows a Direct Intracellular Route to Lysosomes. The absence of Mut-PrP on the plasmalemma and its colocalization with LAMP-1/LysoTracker positive vesicles support an intracellular route to lysosomes rather than the canonical endocytic pathway from plasmalemma to lysosomes. In support of this, we carried out time-lapse confocal microscopy of GFP-tagged PrP to track its course. COS-7 cells, based on their large size and flat architecture, features that facilitate time lapse imaging, were used for these studies. Cells were transiently transfected for 3 h, at which time the transfection media was replaced with fresh media and time-lapse imaging started. Mut- and Wt-PrP expression was detectable by 5 h (300 min) in some cells, but between 6 and 8 h in the majority of cells (90% of 120 cells surveyed). Thus, in some experiments, time-lapse imaging was delayed until 5 to 7 h, for efficiency. The general pattern of the expression of Mut-PrP was initially studied. As with other cell types, no plasma membrane localization could be appreciated in COS-7 cells. Surprisingly, at the earliest appearance of signal, Mut-PrP formed puncta that were distributed throughout the cell. With observation up to 12 h, the puncta grew in number, intensity, and size (Figure 3(a) and supplement Video S1 in Supplementary Materials available online at http://dx.doi.org/10.1155/2013/560421).

To visualize the intracellular delivery of Mut-PrP to acidic compartments, a similar paradigm as above was followed, but with 30 nM LysoTracker Red added to the media 30 min prior to imaging at ~7 h (Figure 3(b) and supplement Video S2). Within 8 h (480 min) following transfection, yellow puncta signifying the colocalization of Mut-PrP with LysoTracker Red positive vesicles were detected (Figure 3(b), arrows). Continued imaging revealed an increase in the number and size of yellow puncta over time, suggesting ongoing delivery and accumulation of Mut-PrP within acidic vesicles. In contrast to Mut-PrP, Wt-PrP appeared primarily as a diffuse signal although a small number of puncta that colocalized with LysoTracker Red positive vesicles appeared at a much later time point at ~750 min after transfection. Importantly, these puncta were observed after Wt-PrP was visualized on the plasmalemma at ~600–650 min (Figure 3(c) and supplement Video S3). Thus, these findings support the concept that Wt-PrP follows an indirect pathway that leads first to the plasma membrane and then lysosomes via the canonical endocytic pathway, whereas Mut-PrP is delivered to lysosomes via a faster, more direct intracellular route that does not involve the plasmalemma.

3.3. The Golgi Is Not Essential for This Pathway. It is well established that lysosomal targeting of ER-synthetized proteins occurs from the trans-Golgi network (TGN) via a mannose-6-phosphate receptor-mediated pathway [26]. However, based on its sensitivity to Endo H, PrP carrying the T182A mutation does not traffic beyond the mid-Golgi stack, making that route unlikely. This is supported by our findings and prior work from others that show this PrP mutant is retained in the ER [27], suggesting that it may not pass ER quality control. However, to determine whether the Golgi participates in this pathway, we assessed whether chemical disruption of Golgi by Brefeldin A (BFA) impairs the colocalization of Mut-PrP with LysoTracker labeled vesicles. To first confirm effective disruption of Golgi, we treated HeLa cells, transiently expressing either Wt-PrP::GFP or untagged Wt-PrP, with 5 μg/mL of BFA for 3 hours, and assessed the effect on the trafficking and glycosylation of PrP. Functional disruption of Golgi was noted by, (1) the absence of Wt-PrP::GFP labeling on the plasma membrane (Figure 4(a)), (2) the presence of intracellular reticular staining pattern, consistent with ER retention and, (3) the absence of high molecular weight glycoforms normally added in the Golgi (Figure 4(b), asterisk).

We then assessed the effect of BFA on PrP colocalization with LysoTracker Red. HeLa cells were transfected with Wt- or Mut-PrP::GFP and allowed to express for 5.5 h (the earliest time point at which PrP signal was detected in our time lapse studies), at which time BFA or vehicle was added for 3 h. LysoTracker Red was added for 30 min, and cells were visualized by confocal fluorescence live cell microscopy at ~9 h after transfection (Figure 4(c)). In cells treated with BFA, Mut-PrP colocalization with LysoTracker not only persisted, but also increased slightly ($P < 0.05$) relative to cells with intact Golgi (Figure 4(d)). Interestingly, Golgi disruption also produced a small, but statistically significant, increase in Wt-PrP colocalization with LysoTracker (Figure 4(d)). We considered that, in the absence of functionally intact Golgi, Wt-PrP might be retained in the ER and, because of the general propensity of PrP to misfold, it too may aggregate within the ER lumen and follow the same pathway to lysosomes as Mut-PrP. In support of this, we found the detergent insoluble fraction of Wt-PrP was significantly increased in BFA-treated cells (Figures 4(e) and 4(f)). Interestingly, BFA did not significantly increase the already elevated fraction of insoluble Mut-PrP (Figure 4(f)). This provides further support that Mut-PrP undergoes limited trafficking to the Golgi and has a high propensity to aggregate in the ER independent of the Golgi. Overall, these findings suggest that (1) an intact Golgi is not required for the intracellular delivery of Mut-PrP to lysosomes; (2) the ER is the primary site of origin of Mut-PrP that accumulates in lysosomes; and (3) aggregation of PrP, induced by its retention within the ER, might trigger its intracellular transport to lysosomes.

3.4. Mut-PrP Expression Activates Autophagy. Because autophagic vesicles have been reported by some to originate from ER membranes [28, 29] and Mut-PrP is retained within the ER, we considered autophagy as a possible mechanism for intracellular delivery of Mut-PrP to lysosomes. To initially



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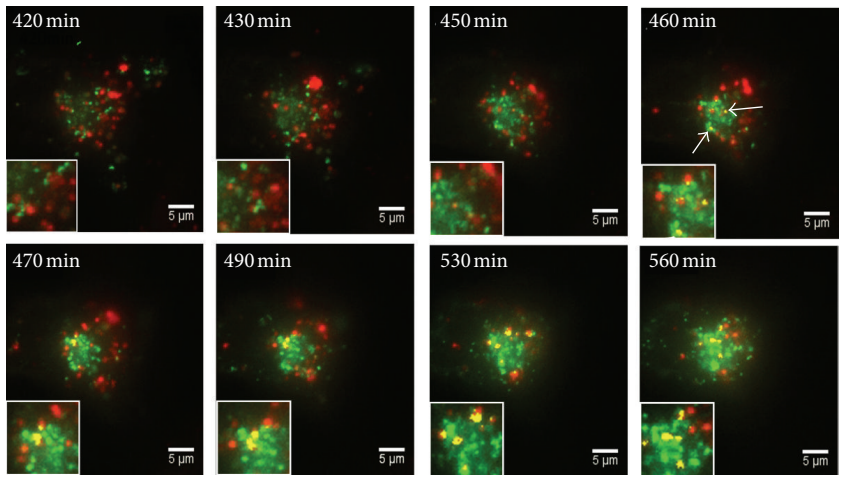

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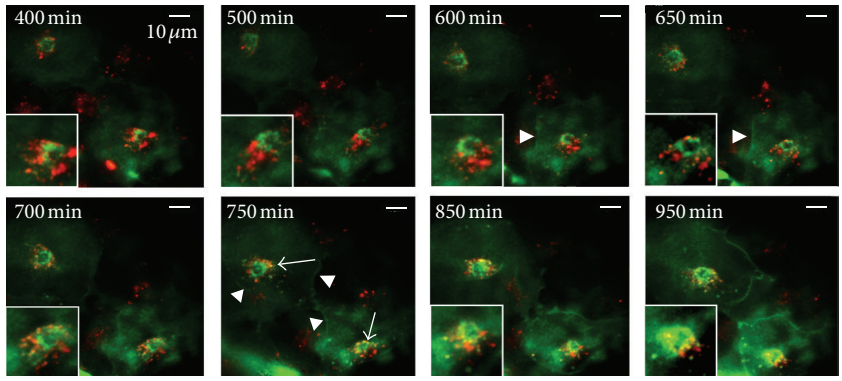

(c)

Figure 3: Time-lapse photomicroscopy of PrP::GFP trafficking to lysosomes. (a) At $T = 0$ COS-7 cells were transfected with Mut-PrP::GFP over 3 h, the media were replaced, and imaging started. (a) displays frames of live video collected from $T = 300$ min (5 h) to 420 min. Images were extracted as individual frames from supplement Video S1, using ImageJ. (b) Mut-PrP::GFP, as in (a), but in the presence of 30 nM LysoTracker Red. Frames were collected between $T = 420$ (7 h) and 560 min. Arrows indicate the first appearance of yellow puncta, signifying Mut-PrP delivery to LysoTracker Red positive vesicles. Images were extracted from supplement Video S2. Magnified insets, approximately 2x original image. (c) Wt-PrP::GFP in the presence of LysoTracker Red imaged as in (b) for Mut-PrP, but with a time window of 400 to 950 min, to capture PrP entry into lysosomes. Wt-PrP::GFP is detected on the plasmalemma (arrowheads) prior to its colocalization with LysoTracker Red positive vesicles (arrows), at a much delayed time point than Mut-PrP (750 versus 460 min). Images extracted from supplement Video S3. Magnified insets 4x original image, to compare with (b).

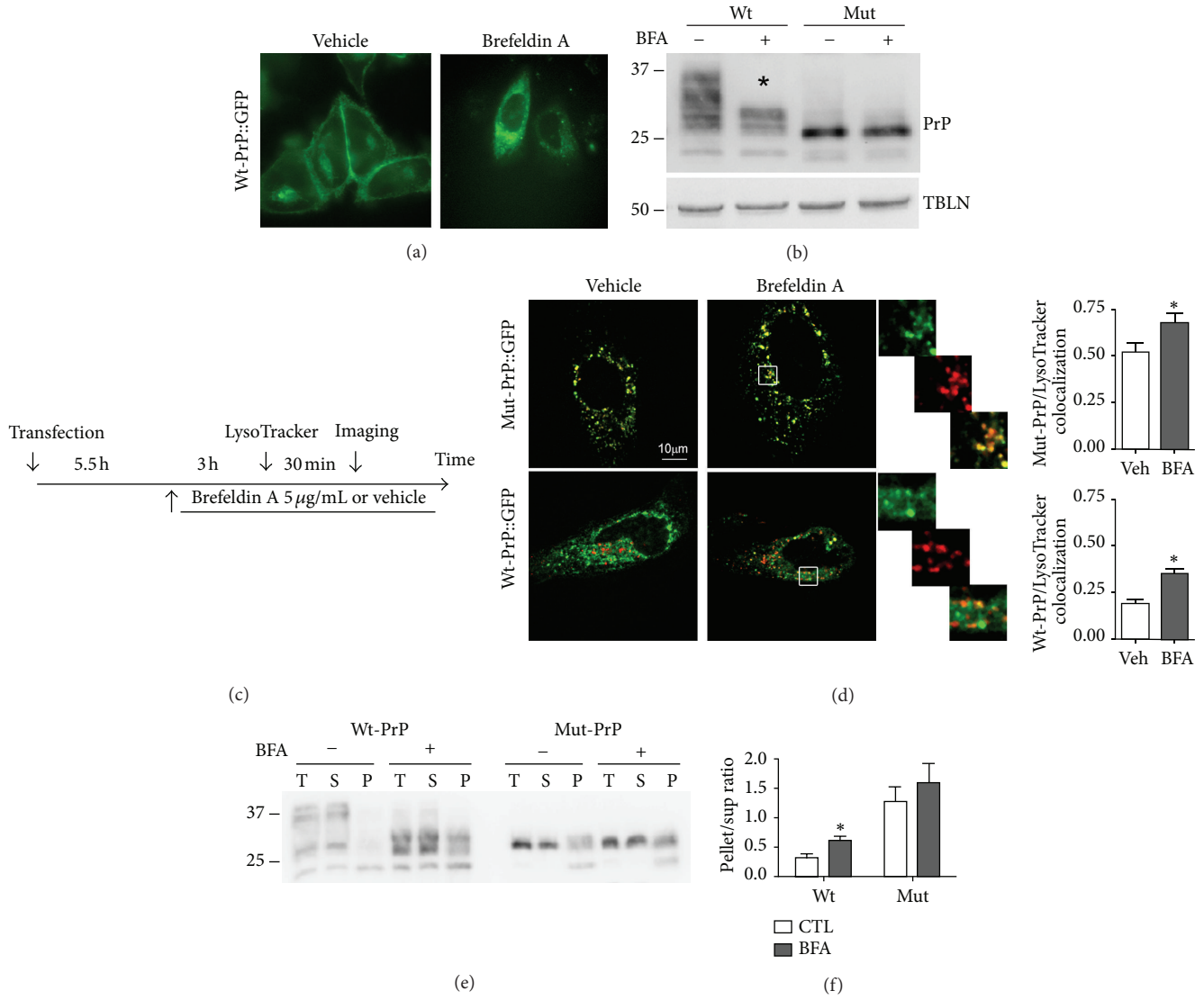

Figure 4: An intact Golgi is not essential for the delivery of Mut-PrP to LysoTracker positive vesicles. (a) Golgi disruption by Brefeldin A (BFA) (5 μg/mL for 3 h) was confirmed by the absence of Wt-PrP signal on the plasmalemma of HeLa cells expressing Wt-PrP::GFP, compared with vehicle-treated cells sampled at the same time point. (b) Functional disruption of Golgi by BFA was biochemically confirmed by the absence of complex glycans (*) on Wt-PrP, following Western blot of total cell lysates, compared with vehicle-treated (−) cells. Mut-PrP does not acquire complex sugars and as such is unaffected by BFA. PrP was detected with D13 antibody. (c) Experimental paradigm; times are relative to the completion of the transfection protocol. BFA (5 μg) was added at 5.5 hours, at the earliest detection of PrP signal, LysoTracker Red (50 nM) was added 30 minutes prior to the start of imaging at 9 hours, and images were collected by 10 hours. (d) Immunofluorescence of live cells expressing GFP-tagged Mut-PrP after treatment with vehicle or BFA. Green (PrP::GFP) and red (LysoTracker) channels are displayed as merged. Separate channels for the boxed areas of BFA treated cells are magnified and displayed to the right of the merged images. Correlation coefficient analysis revealed a statistically significant increase in colocalization of PrP and LysoTracker after BFA treatment for both Wt-PrP and Mut-PrP. Wt-PrP veh = 0.1891 ± 0.0194, BFA = 0.3505 ± 0.01997 (n = 28 cells, $P < 0.001$); Mut-PrP veh = 0.5112 ± 0.05329, BFA = 0.6710 ± 0.05438 (n = 23 cells, $P < 0.05$). (e) BFA treatment increases the insoluble fraction of PrP. N2a cells stably expressing 3F4-tagged Wt- or Mut PrP were treated with BFA (5 ug/mL for 3 h) or vehicle, and the insoluble fraction of PrP was separated by centrifugation at 16,000 ×g, and equal volumes of the total (T), supernatant (S), and pellet (P) fractions were loaded and subjected to Western blot using the 3F4 mAb. (f) Densitometric quantification of (e), using Image One (BioRad) software. The fraction of insoluble Wt-PrP was significantly increased after BFA treatment ($P < 0.05$, n = 4), while the increase in insoluble Mut-PrP did not reach significance (n = 4).

screen for autophagy activation, we compared our stable N2a cell lines stably expressing Wt- or Mut-PrP, with respect to the steady state levels of several markers of the autophagy-lysosome pathway, including Beclin-1, an autophagy initiator and the mammalian homologue of Atg6, free Atg12 and Atg12 bound to Atg5, which are targeted to autophagosomes, and LAMP-1. Compared with Wt-PrP expressing cells, the level of each of these markers was greater in cells expressing

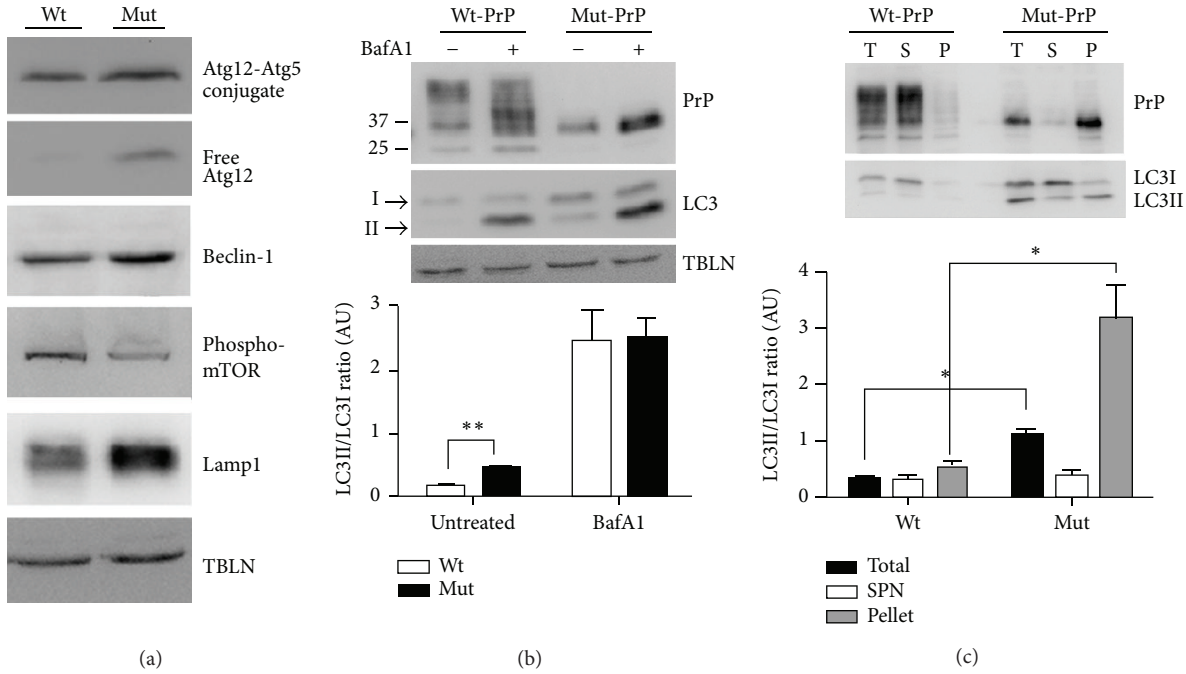

FIGURE 5: Mut-PrP expression activates autophagy. (a) Western blot of total lysates of N2a cells stably expressing Wt- or Mut-PrP engineered with the 3F4 epitope and probed for Atg12 (free and conjugated with Atg5), Beclin-1, phosphorylated mTOR, and LAMP-1. Loading was normalized for total protein. Tubulin (TBLN) is presented as a loading control. (b) Western blots of total lysates prepared from cells in (a) that were treated for 6 h with vehicle or 50 nM Bafilomycin A1 to limit LC3 degradation and probed for PrP with 3F4 mAb, LC3, and tubulin. PrP was detected by 3F4 mAb. The LC3II/LC3I ratio was measured by densitometry of each fraction, using Image One (BioRad) software from ECL developed Western blots, and displayed in the graph below ($^{**}P = 0.002$, student's t-test, $n = 3$ experiments). (c) Representative blots of total (T), supernatant (S), and pellet (P) fractions of cells in (a), following lysis and separation by centrifugation at 100,000 ×g for 1 h, were probed for PrP and LC3. LC3II/LC3I ratios for each fraction are presented in the graph below ($^{*}P < 0.05$, student's t-test, $n = 3$).

Mut-PrP. In addition, the phosphorylated (activated) mammalian target of rapamycin (mTOR), which is normally suppressed during autophagy, was reduced in Mut-PrP cells relative to Wt-PrP expressors (Figure 5(a)).

Microtubule-associated protein 1 light chain 3 (MAP1-LC3), the homolog of Atg8 in yeast, referred to as LC3 in mammals, is specifically incorporated into autophagosomes [6, 30]. During autophagy LC3I, a 16 kDa protein is cleaved, lipidated, and incorporated into autophagosome membranes as LC3II, a 14 kDa protein. Thus, an increase in the LC3II/LC3I ratio has been used as a marker for autophagy induction [31]. We found this ratio to be significantly higher in cells expressing Mut-PrP compared to those expressing Wt-PrP ($P = 0.002$) (Figure 5(b)). An increase in LC3II might also result from impaired autophagic flux, resulting either from impaired fusion of the autophagosome with the lysosome or impaired degradation of LC3II within the lysosome. To indirectly test for this, we treated some cells with the vacuolar-type H(+)ATPase inhibitor Bafilomycin A1, which alkalinizes lysosomal compartments leading to impaired degradation of LC3II and/or reduced fusion of autophagosomes with lysosomes [32, 33]. Cells were incubated with 50 nM Bafilomycin A1 added to media for 6 h, prior to lysis and Western blotting. This resulted in a marked increase in the level of LC3II in both cell lines, suggesting that autophagic flux is not obviously impaired in Mut-PrP expressors (Figure 5(b), graph).

To determine if the increased LC3II was associated with the misfolded/aggregated fraction of Mut-PrP, we separated detergent soluble and insoluble fractions of these cells, by centrifugation at 100,000 ×g, and compared the LC3II/LC3I ratios within each fraction (Figure 5(c)). As expected, Mut-PrP was primarily confined to the insoluble fraction, confirming that it favors the misfolded/aggregated state. In addition, the LC3II/LC3I ratio was not only significantly greater in Mut-PrP expressing cells (Figure 5(c), black bars) but also was the highest within the insoluble fraction (Figure 5(c), grey bars), suggesting that LC3II within the autophagosome membrane may become incorporated with the aggregated PrP cargo it surrounds, similar to that recently reported with synuclein [34].

3.5. Ultrastructural Markers of Autophagy in Mut-PrP Expressing Cells. A hallmark of autophagy is the presence of ultrastructural markers, notably double membrane vesicles characteristic of autophagosomes. We carried out transmission electron microscopy (TEM) on cells transfected with Wt- or Mut-PrP to document the presence of these markers. We studied two cell lines using two methods of PrP expression. COS-7 cells were transiently transfected with Wt-PrP

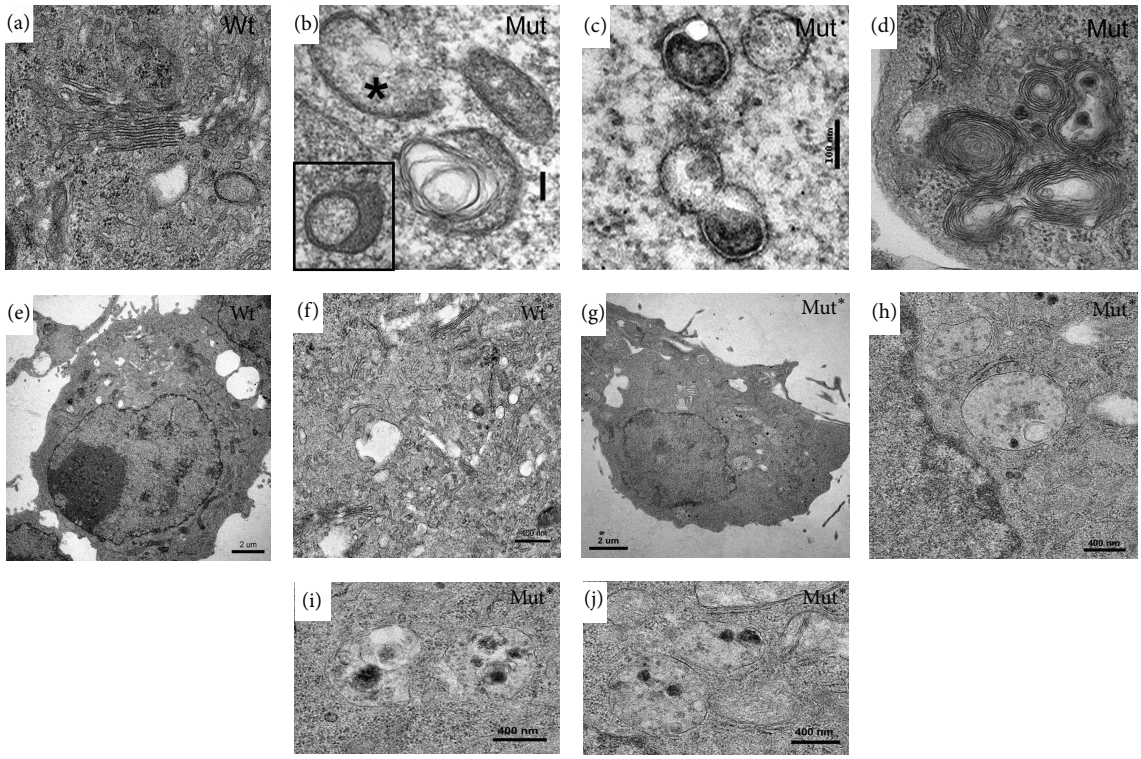

Figure 6: Ultrastructural markers of autophagy with Mut-PrP expression. Transmission electron micrographs of transiently transfected COS-7 cells ((a)–(d)) and stably transfected N2a cells ((e)–(j)). TEM of COS-7 cells expressing Wt (a) or Mut- ((b)–(d)) PrP for 24 h. Normal appearing ultrastructure present in cells expressing Wt-PrP (a), and abnormal membranes characteristic of isolation membranes ((b), asterisk), double membrane autophagosomes (c), and myelinoid (multilamellar) bodies (d) were evident in the majority of cell sections sampled from Mut-PrP expressing cells. (e)–(j) Mouse neuroblastoma N2a cells stably expressing Wt-PrP ((e)–(f)) or Mut-PrP ((g)–(j)). Mut-PrP expressing cells displayed an accumulation of multivesicular bodies and autophagy-related structures consistent with late stage autophagosomes and/or autolysosomes. These structures were largely absent in cells expressing Wt-PrP.

(Figure 6(a)) or Mut-PrP (Figures 6(b)–6(d)), and N2a cells were stably expressing Wt-PrP (Figure 6(e)) or Mut-PrP (Figures 6(f)–6(j)). Although this was not a quantitative study, when compared with cells transiently expressing Wt-PrP (Figure 6(a)), double bilayer membrane vesicles measuring 0.1 to 0.4 μm in diameter, characteristic of autophagosomes (Figures 6(b) and 6(c)), isolation membranes (Figure 6(b), asterisk), and myelinoid (multilamellar) bodies (Figures 6(b) and 6(d)), known features of autophagy [31, 35], were readily apparent in cells expressing Mut-PrP (Figures 6(b)–6(d)). In N2a cells stably expressing Mut-PrP, the majority (70%) of surveyed cells ($n > 25$) displayed multivesicular bodies (MVBs) (Figure 6(h)) and autophagosomes/autolysosomes that contained cargo (Figures 6(h), 6(i), and 6(j)). Such structures were sparse or absent in cells stably expressing Wt-PrP (Figures 6(e) and 6(f)). These observations agree with our data in Figure 5, which supports an induction of the autophagy pathway in Mut-PrP expressing cells.

3.6. Mutant PrP Colocalizes with LC-3 Labeled Vesicles. We next assessed whether Mut-PrP could be detected within autophagosomes. To facilitate the study, we generated HeLa cell lines stably expressing Wt- or Mut-PrP::GFP, to which we applied simultaneous direct and indirect immunofluorescence to detect PrP::GFP and LC3, respectively (Figure 7). In Wt-PrP expressing cells, we found LC3 staining to be diffusely distributed throughout the cytosol, whereas in cells expressing Mut-PrP, its distribution was more polarized and overlapped closely with the intracellular distribution of Mut-PrP (Figures 7(a), 7(b), and 7(e)). Pretreatment of cells with Bafilomycin A1, to inhibit autophagosome fusion and degradation in lysosomes, did not alter the colocalization pattern of either Mut-PrP or Wt-PrP, despite significant enhancement of LC3 staining (Figures 7(c), 7(d), and 7(f)). Quantification of PrP colocalization with LC3, using Li's ICQ, was also similar in the presence or absence of Bafilomycin A1 and averaged 0.11 for Wt-PrP/LC3 and 0.45 for Mut-PrP/LC3 (Figures 7(e) and 7(f)), with a score of 0.5 representing maximum overlap of signal. This strongly suggests that Mut-PrP is specifically and closely associated with autophagosomal structures.

3.7. Autophagy Inhibition Impairs Delivery of Mut-PrP to LysoTracker Labeled Vesicles. Our findings thus far suggested that Mut-PrP might induce autophagy as a mechanism for its delivery to lysosomes for degradation. To confirm the dependence of this pathway on autophagy, we determined if induction and/or inhibition of autophagy could modify the delivery of PrP to acidic vesicles labeled by LysoTracker Red. The relative colocalization of PrP::GFP with LysoTracker was, therefore, assessed following 16 h incubation

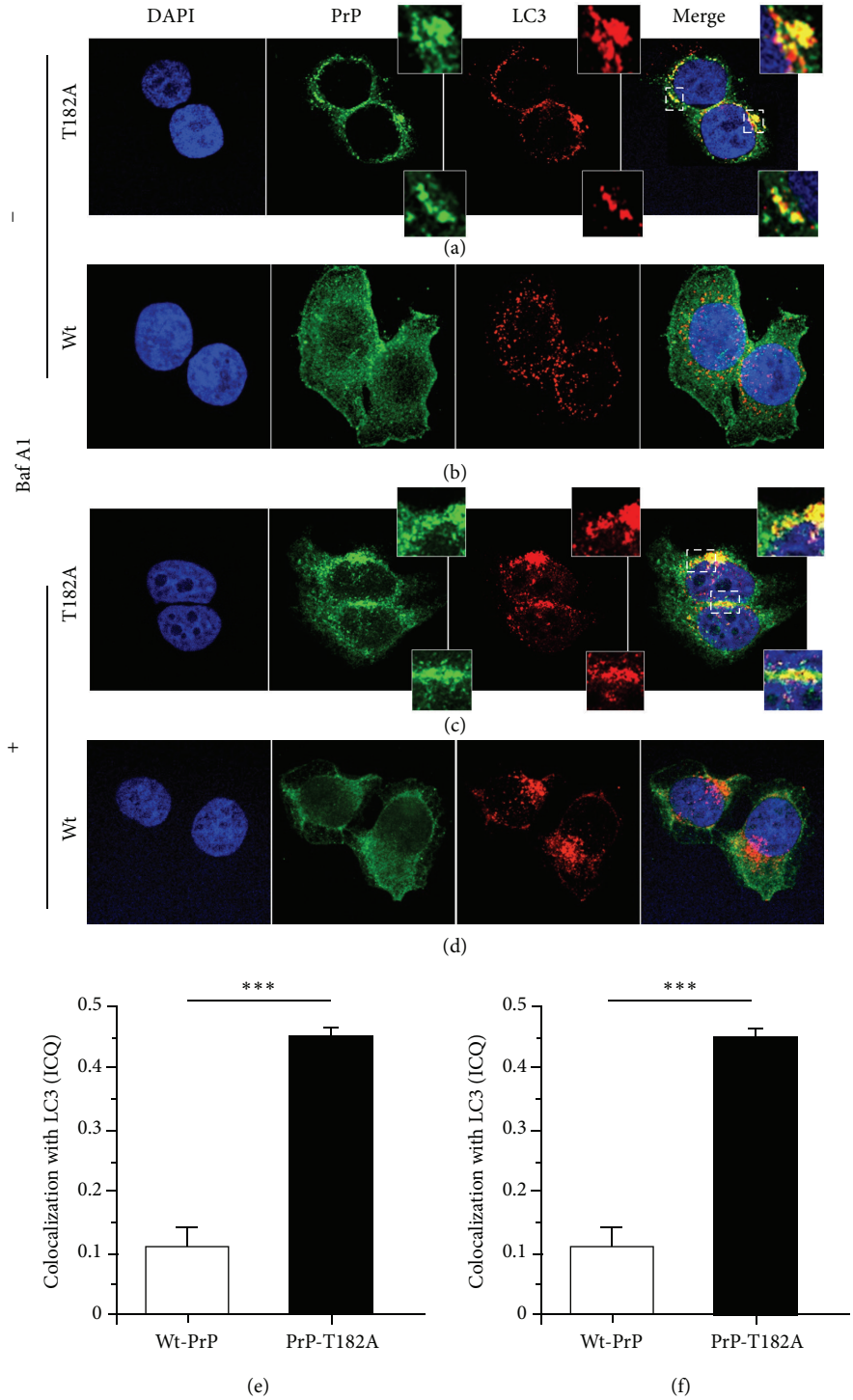

Figure 7: Mut-PrP colocalizes with autophagosomes. Confocal immunofluorescence of HeLa cells stably expressing GFP-tagged Mut- (a, c) or Wt- (b, d) PrP before (–) and after (+) incubation for 6 h with 50 nM Baf A1. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 5 min, then stained with anti-LC3B antibody and DyLight 649-conjugated Affini-Pure goat anti-rabbit IgG, prior to inverted confocal fluorescence microscopy imaging using a Marianas Yokogawa type spinning disk (original magnification, x100). Li's method (NIH Image J plugin) was applied to determine the degree of colocalization of PrP::GFP (green) and LC3B (red). Graphs display the intensity correlation quotient (ICQ), which ranges from −0.5 (no correlation of signals) to 0.5 (complete correlation), between LC3B and Wt- or Mut-PrP in the absence (e) or presence (f) of BafA1. (***$P < 0.0001$). There were no differences in any values between those treated with BafA1 and control cells. Actual values are (e) BafA1(−) Wt-PrP = 0.110 ± 0.06, Mut-PrP = 0.450 ± 0.023, (f) BafA1(+) Wt-PrP = 0.117 ± 0.038, Mut-PrP = 0.447 ± 0.015, $n = 10$ cells each.

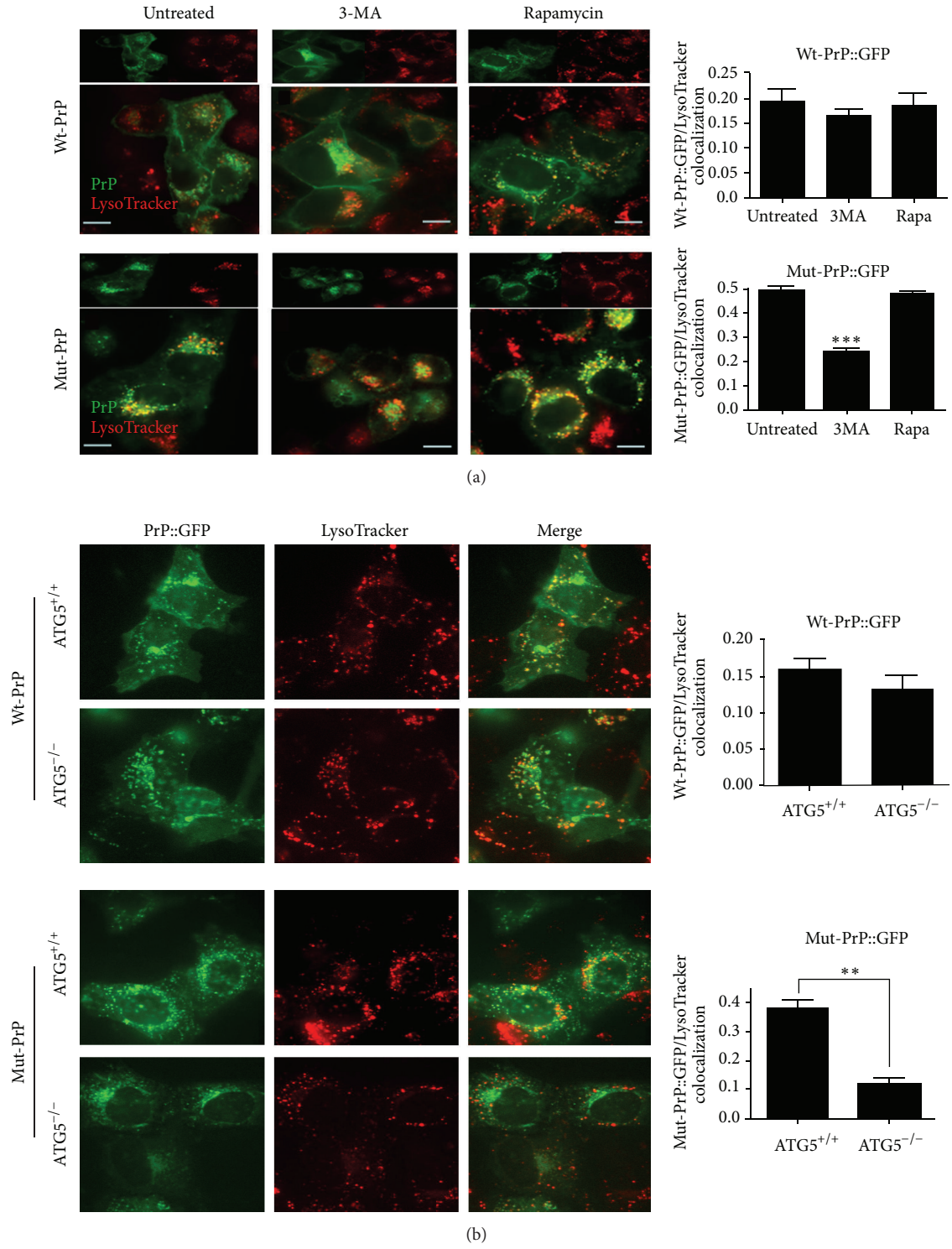

Figure 8: Autophagy participates in the delivery of Mut-PrP to LysoTracker labeled vesicles. (a) Three hours after transfection, HeLa cells expressing GFP-tagged Wt-PrP or Mut-PrP were treated for 16 h with 2 mM 3-MA, 2 μM rapamycin, or fresh media alone (untreated), followed by incubation with 50 nM LysoTracker Red for 30 min prior to live cell confocal fluorescence microscopy. Individual channels are displayed above merged images. Yellow indicates colocalization. Scale bars = 10 μm. Graphic display of Li's correlation coefficient between Mut-PrP and LysoTracker for each treatment is shown to the right ($^{***}P < 0.001$, student's paired t-test, difference from untreated, $n = 5$ each). (b) Normal ($ATG5^{+/+}$) and autophagy-deficient ($ATG5^{-/-}$) mouse embryonic fibroblasts (MEFs) transiently transfected with GFP-tagged Wt-PrP or Mut-PrP were allowed to express for 16 h prior to confocal microscopy, performed 30 min following the addition of 50 nM LysoTracker Red. Single 0.5 μm slice images are shown. Each channel is displayed to the left of the merged image. Graphic display of Li's correlation coefficient between PrP and LysoTracker Red in WT MEFs compared with $ATG5^{-/-}$ MEFs is to the right ($^{**}P < 0.005$, student's t-test, $n = 5$ each).

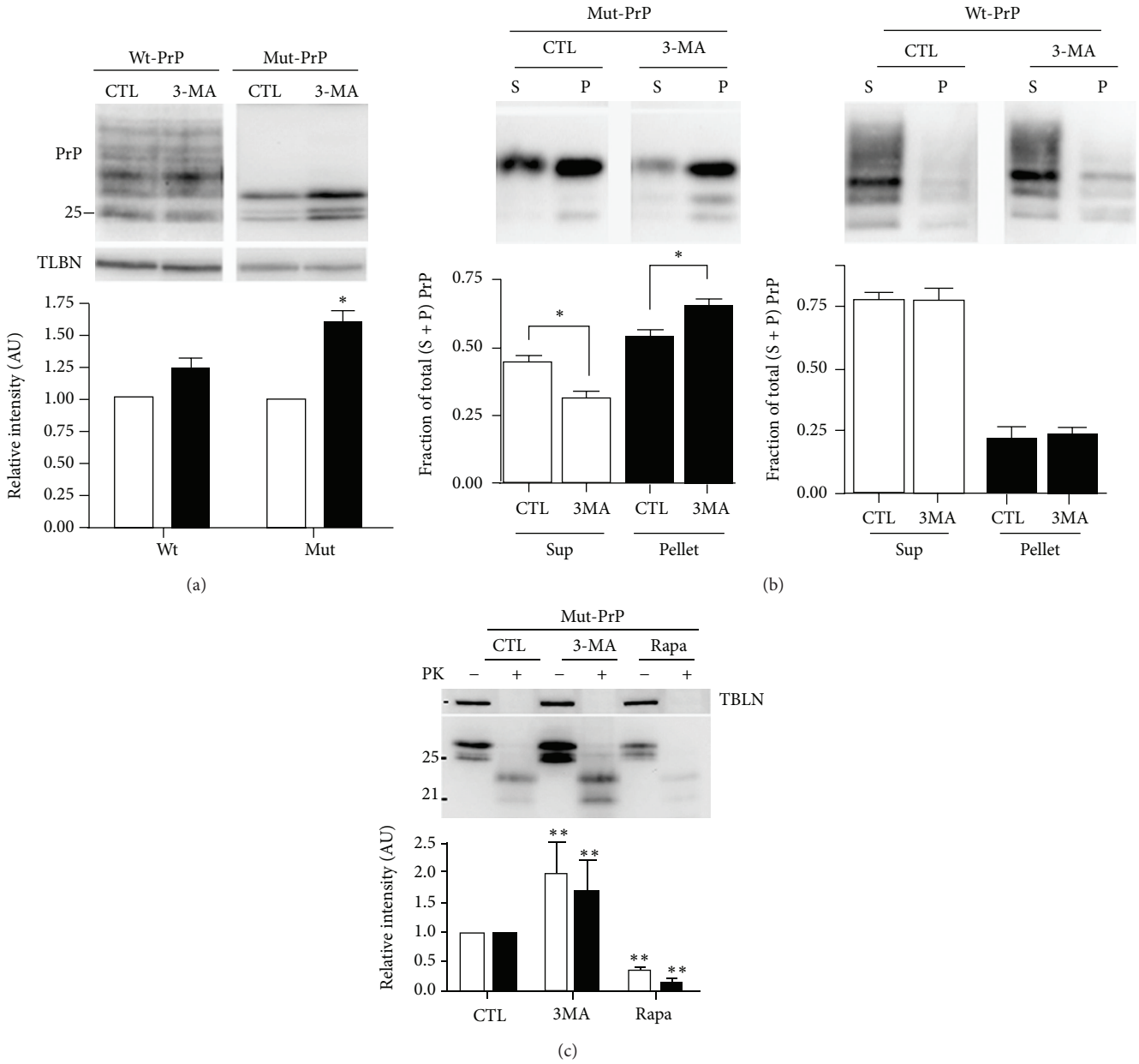

Figure 9: Autophagy is selective for insoluble/aggregated PrP. (a) Representative Western blot of total lysates from COS-7 cells transiently expressing Wt- or Mut-PrP (3F4) after 16 h of incubation with 5 mM 3-MA. The corresponding graph displays the relative densitometry of PrP signal before (open bars) and after (filled bars) 3-MA treatment ($^{*}P < 0.05$, student's paired t-test, $n = 3$). Tubulin (TBLN) is a loading control. (b) Representative Western blots of supernatant (S) and pellet (P) fractions of cells transiently expressing Mut or Wt PrP after 16 h of vehicle (CTL) or 10 mM 3-MA, and the corresponding graph from 3 experiments each. The insoluble (pellet) and soluble (sup) fractions of Mut-PrP are presented as the densitometric signal of each fraction over the sum of the two fractions (sup + pellet) ± SEM ($^{*}P < 0.05$, 2-way ANOVA, Bonferroni posttest). (c) Cells transiently expressing Mut-PrP were treated for 6 h with 10 mM 3-MA or 20 μM rapamycin, harvested in lysis buffer, and digested with 5 μg/mL Proteinase-K (PK) for 15 min at 37°C. Densitometry quantified as the intensity of each fraction (i.e., total PrP (open bar) or PK-resistant PrP (filled bar)), relative to their respective untreated normalized control fractions ($^{**}P < 0.005$ ANOVA, Bonferroni post test, difference from CTL, $n = 3$ experiments).

with 2 mM 3-methyladenine (3-MA), a commonly used inhibitor of autophagy [36], or 2 μM rapamycin, to induce autophagy [37]. For live cell imaging, we transiently transfected HeLa cells to avoid the confounding effect of the long half-life of Mut-PrP. Neither the distribution of Wt-PrP nor its colocalization with LysoTracker was significantly altered by either treatment although a small, but nonsignificant, reduction in colocalization following 3-MA was noted (ICQ = 0.19 ± 0.11 (CTL), 0.16 ± 0.06 (3-MA), 0.18 ± 0.11 (rapamycin), $P = 0.653$ for CTL versus 3-MA treated) (Figure 8(a), top row). In contrast, 3-MA significantly reduced Mut-PrP colocalization with LysoTracker, compared

with untreated cells [ICQ = 0.48 ± 0.022 (CTL), 0.23 ± 0.048 (3-MA)] (Figure 8(a), bottom row). Interestingly, rapamycin did not enhance the already prominent colocalization of Mut-PrP with LysoTracker (Figure 8(a) bottom row and graph).

As an alternate method to assess the functional role of autophagy in the trafficking of Mut-PrP to LysoTracker labeled vesicles, we employed an autophagy-deficient mouse embryonic fibroblast (MEF) cell line that lacks Atg5 (i.e., $ATG5^{-/-}$ MEFs), a key protein in the initiation of Atg5/Atg7-dependent macroautophagy [38] (Figure 8(b)). As in non-MEFs, Wt-PrP::GFP colocalized at a relatively low level with LysoTracker in wild type (WT) MEFs (ICQ 0.16 ± 0.06). Interestingly, in agreement with the 3-MA data in HeLa cells, a slight but nonsignificant reduction in the colocalization of Wt-PrP with LysoTracker was observed in $ATG5^{-/-}$ MEFs (ICQ 0.13 ± 0.07, $P = 0.782$, $n = 15$, student's t-test), again suggesting that a small fraction of Wt-PrP might follow this route. As in the HeLa cells, Mut-PrP expressed in WT MEFs colocalized with LysoTracker to a greater extent than Wt-PrP (ICQ 0.38 ± 0.1), whereas, in $ATG5^{-/-}$ MEFs, the reduction in colocalization was greater than that measured in 3-MA treated HeLa cells (ICQ 0.12 ± 0.06, $P = 0.005$). Interestingly, a small but detectable level of Mut-PrP did continue to colocalize with LysoTracker in $ATG5^{-/-}$ MEFs, supporting a recent finding that these cells may have an Atg5-independent autophagy pathway [39] or that a secondary mechanism for delivery to lysosomes exists.

3.8. Autophagy Is Selective for Misfolded PrP. To confirm that autophagy plays a functional role in the delivery of intracellular Mut-PrP to lysosomes, we assessed whether inhibiting autophagy alters the steady state levels of Mut-PrP. To test this, we again used COS-7 cells because they lack endogenous PrP and transient transfection was used to affect treatments early in the expression of PrP. Thus, 8 h from the transfection of Mut- or Wt-PrP, cells were treated with 5 mM 3-MA overnight (16 h). This resulted in significantly increased levels of Mut-PrP, but not Wt-PrP ($P < 0.05$) (Figure 9(a)). Because we noted earlier that LC3II partitioned with the insoluble fraction of Mut-PrP (see Figure 5), we questioned whether inhibiting autophagy with 3-MA primarily affects the insoluble fraction of Mut-PrP on the way to the lysosome. To assess this, cells were treated as described above and then detergent lysed and centrifuged at 16,000 ×g, to separate soluble and insoluble PrP. Comparing the ratio of soluble to insoluble PrP fractions revealed a significant shift of Mut-PrP, but not Wt-PrP, from the soluble to insoluble fraction (Figure 9(b)), suggesting autophagy primarily functions to eliminate the misfolded/aggregated fraction of PrP.

Based on these results, we questioned whether autophagy directly affects the *de novo* generation of pathogenic Proteinase-K resistant Mut-PrP^{Sc}. The T182A mutant of PrP is well known to develop partial resistance to PK when expressed in cultured cells [19, 23]. Since 3-MA inhibits the clearance of misfolded/insoluble PrP, the fraction predicted to be the precursor to PrP^{Sc}, we considered that autophagy acts to reduce the spontaneous generation of PK-resistant Mut-PrP. Thus, COS-7 cells were transfected as described above and treated for 6 h with either 10 mM 3-MA or 20 μM rapamycin, followed by the digestion of lysates with 5 μg/mL PK for 15 min at 37°C. In the presence of 3-MA, an increase in the level of Mut-PrP was accompanied by a proportional increase in PK-resistant PrP, whereas rapamycin reduced PK-resistant PrP to negligible levels (Figure 9(c)).

4. Discussion

Here we show for the first time the autophagy features in the intracellular trafficking and the degradation of newly synthesized misfolded/aggregated mutant PrP. This was revealed using an extensively studied PrP mutant linked to familial CJD and known to display aberrant trafficking and intracellular accumulation. We found that the T182A Mut-PrP was not localizable to the plasma membrane, it rapidly formed intracellular aggregates, and it colocalized with LysoTracker/LAMP-1 positive vesicles in a Golgi-independent manner, sooner, and to a greater extent, than Wt-PrP. Mut-PrP expression was also associated with an elevation in several markers of the autophagy-lysosomal pathway, the presence of ultrastructural markers of autophagy, and it extensively colocalized with the autophagosome-specific marker, LC3B. As a functional correlate, we found the delivery of Mut-PrP to lysosomes was profoundly impaired in autophagy-deficient $ATG5^{-/-}$ MEFs and in normal cells treated with the autophagy inhibitor 3-MA. The latter also selectively impaired the degradation of the insoluble fraction of Mut-PrP, leading to an increase in the amount of PK-resistant Mut-PrP.

Autophagy has been shown to play a role in several long-lived proteins associated with neurodegenerative disease, including huntingtin [9], synuclein [10], and beta-amyloid (Aβ) [8]. In general, autophagy appears to function as an alternative means to clear cytoplasmic protein aggregates too large to fit within the pore of the proteasome, which supports the idea that impaired autophagy might contribute to the development of neurodegenerative disease [40]. Based on our current findings with a Mut-PrP prone to aggregation and retention in the ER [19], autophagy also appears to function as an early quality control mechanism that links the ER to lysosomes. Such a route has been suggested with other aggregation-prone proteins, including α1-antitrypsin Z mutant [41, 42], vasopressin [43], and dysferlin [44]. Although we did not address the potential role of the proteasome, it was previously reported that the T182A mutant of PrP is not significantly affected by proteasome inhibition [23], which is likely a consequence of its propensity to rapidly misfold and aggregate within the ER, thereby limiting its ability to unfold and be retrotranslocated to the cytosol. Thus, another outlet, such as autophagy, is required for its elimination.

Ashok and Hegde [45] reported that some disease-linked mutated PrPs traffic to lysosomes from the trans-Golgi, although the mechanism of transport has not been defined. Despite misfolding in the ER, the C-terminal domain mutations of PrP they studied were found to traffic to

the Golgi prior to their degradation in Bafilomycin A1 sensitive compartments (most likely lysosomes). Although similar to our proposed pathway, the PrP mutants they studied were not recognized by ER quality control and, importantly, they reached the trans-Golgi, as defined by their Endo H resistance, a key distinction from the Endo H sensitive T182A mutant that does not traffic beyond the mid-Golgi stack. Our finding that Golgi disruption by Brefeldin A did not prevent delivery of Mut-PrP to acidic compartments further supports a Golgi-independent process in its delivery to lysosomes.

This report is not the first to link autophagy to PrP or prion disease. Oh et al. [46] found that cultured hippocampal neurons lacking PrP ($Prnp^{-/-}$) displayed increased levels of LC3II and autophagy-related EM ultrastructures relative to $Prnp^{+/+}$ neurons, and the reintroduction of PrP^{C} into the cells reverted them to the wild type phenotype. They suggested that the reduction in endogenous PrP^{C} that occurs in prion disease, as a result of its conversion to PrP^{Sc}, could activate autophagy. While our studies do not rule out autophagy induction during later stages of prion disease, when PrP^{C} levels could be significantly reduced, we show that autophagy might function as an early quality control mechanism to eliminate misfolded/aggregated PrP that accumulates in the ER. As such, this process may be especially important in genetic prion diseases, to limit the accumulation of mutated PrP that has a high propensity to spontaneously misfold in the ER.

Although the mechanism for induction of autophagy has not been determined, we hypothesize that ER stress induced by accumulation of PrP aggregates within the ER is the trigger. This is supported not only by the results with Mut-PrP, but also by the finding that Brefeldin A, which limits Wt-PrP exit from the ER and increases its insoluble fraction, promoted trafficking of PrP to lysosomes. Interestingly, Brefeldin A is a powerful inducer of ER stress and the unfolded protein response (UPR), suggesting a link between UPR and autophagic delivery of PrP to lysosomes. Prior work supports the induction of ER stress and the unfolded protein response (UPR) in cell culture models of prion disease and in brain samples derived from patients with CJD [47, 48], in addition to mice infected with prions [49]. Importantly, recent evidence also links the induction of autophagy with ER stress-induced UPR [50, 51]. Bernales et al. [52] found that DTT-mediated induction of the UPR in yeast caused ER swelling and the accumulation of autophagic vesicles delimited by ribosome-studded ER membranes. We hypothesize that a similar process in mammalian cells might lead to the sequestration of accumulating PrP aggregates within the ER. This is further supported by a recent work suggesting that the ER membrane is the origin of at least some types of mammalian autophagic isolation membranes [29].

In addition to quality control of misfolded secretory proteins, the autophagic pathway we outline here could have a secondary function specific to prion disease, which is to limit the *de novo* generation of PrP^{Sc}. We found that inhibiting autophagy increased the absolute level of insoluble PrP, suggesting that normally or partially folded PrP^{C} may be recruited to form misfolded/aggregated PrP if the pool of misfolded PrP is not efficiently removed by autophagy. Because misfolded/aggregated PrP is thought to contribute directly to the generation of PrP^{Sc}, autophagy could then act to limit the *de novo* generation of PrP^{Sc}. This is underscored by the finding that the inhibition of autophagy in cells expressing Mut-PrP resulted in a proportionate increase in the overall level of PK-resistant PrP, while induction of autophagy reduced it. This compares well with the work of others, who found that induction of autophagy using several agents, including lithium, trehalose, imatinib (i.e., Gleevec), and rapamycin, reduced the levels of PrP^{Sc} in chronically prion-infected cell lines, and in some cases, *in vivo* [11, 12, 53, 54]. Thus, as with poly-Q expanded mutant huntingtin protein [55], autophagy might play a protective role in prion disease. In fact, we confirmed that the protective effect of rapamycin in Huntington disease mouse models [56] also translated to an improved survival and reduced plaque deposition in our Tg (PrP-A116V) mouse model of genetic prion disease [13].

While these data suggest that the manipulation of the autophagy pathway be an important therapeutic target for many neurodegenerative proteinopathies, it is important to be cautious when designing autophagy therapeutics targeting such diseases. Recent reports indicate that autophagy induction could also result in neurotoxic effects, especially in disorders in which autophagy flux (full progression through the autophagy pathway), and not autophagy initiation, is impaired. Such flux impairments have been reported for Huntington disease and Alzheimer's disease [57, 58]. It is noteworthy that prions and misfolded mutant PrP, including this T182A PrP mutant, have been reported to be released into the media of cultured cells, and, as such, have the potential to infect neighboring cells. In fact, PrP-T182A was found to accumulate intracellularly, possibly within lysosomes, in heterologous cells exposed to secreted material [23]. Our results in general, and especially the finding that Brefeldin A treatment enhanced colocalization of PrP-T182A within acidic vesicles labeled by LysoTracker, suggest two important principles: first, the delivery of the mutant PrP to this compartment is primarily via an intracellular pathway, since Brefeldin A should effectively block the secretory pathway, and second, the secretion of this protein is likely a result of MVB release via exosomes, as reported for *bona fide* prions in prion-infected cultured cells [59, 60], or alternatively, directly from lysosomes via lysosomal exocytosis [61]. It is, therefore, cautioned that autophagy induction in prion disease, with little or no concomitant increases in lysosomal clearance of PrP, could conceivably result in enhanced prion spread. Although our prior results in Tg(PrP-A116V) mice and the current results presented here in cultured cells, proved beneficial, caution is still advised. More research into genetic prion disease, the role played by autophagy plays in such disorders, as well as the similarities and differences to acquired prion diseases, is needed.

5. Conclusion

Our studies provide for an early quality control mechanism that employs autophagy to eliminate newly synthesized

misfolded aggregated PrP from the ER that might otherwise contribute to the formation of pathogenic PrP^{Sc}. This model provides new opportunities to understand the nature of prion generation and propagation, in addition to other proteins that may utilize this pathway. Whether manipulating autophagy can alter the course of human prion disease will be of a great interest in the design and development of possible treatments, especially in the case of genetic prion disease.

Conflict of Interests

The authors have no financial conflict of interests with the commercial entities mentioned within this paper.

Acknowledgments

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Review Article

Synaptic Dysfunction in Prion Diseases: A Trafficking Problem?

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Synaptic dysfunction is an important cause of neurological symptoms in prion diseases, a class of clinically heterogeneous neurodegenerative disorders caused by misfolding of the cellular prion protein (PrP^C). Experimental data suggest that accumulation of misfolded PrP^C in the endoplasmic reticulum (ER) may be crucial in synaptic failure, possibly because of the activation of the translational repression pathway of the unfolded protein response. Here, we report that this pathway is not operative in mouse models of genetic prion disease, consistent with our previous observation that ER stress is not involved. Building on our recent finding that ER retention of mutant PrP^C impairs the secretory trafficking of calcium channels essential for synaptic function, we propose a model of pathogenicity in which intracellular retention of misfolded PrP^C results in loss of function or gain of toxicity of PrP^C-interacting proteins. This neurotoxic modality may also explain the phenotypic heterogeneity of prion diseases.

1. Introduction

Prion diseases, also known as transmissible spongiform encephalopathies, are progressive and invariably fatal degenerative disorders of the central nervous system (CNS) that affect humans and other animals [1]. Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome, and fatal familial insomnia (FFI) are the most common forms in humans; scrapie of the goat and sheep, bovine spongiform encephalopathy (BSE), and chronic wasting disease of deer and elk are the best-known examples of prion zoonoses [2]. Widespread neuronal loss, astrocytosis, spongiform change (vacuolation of the neuropil in the gray matter), and in some cases amyloid plaques are key neuropathological findings in prion diseases, which in humans usually present with loss of motor coordination and other motor abnormalities, dementia, and neurophysiological deficits [3].

Similarly to other progressive neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, frontotemporal dementia, and the tauopathies, prion diseases can arise sporadically or be genetically inherited; however, they can also be acquired by infection [4]. This is dramatically illustrated by kuru, a prion disease of the Foré-speaking people of Papua New Guinea, which used to be transmitted among women and children by ritual cannibalism [5]. Other forms transmitted by infection are variant CJD (vCJD) due to consumption of BSE-infected meat products and iatrogenic CJD in recipients of cadaveric sources of human growth hormone or dura mater grafts or blood transfusions from asymptomatic donors who subsequently died from vCJD [6, 7].

The infectious agent (prion) is scrapie prion protein (PrP^{Sc}) [8]. This is a conformationally altered isoform of the cellular prion protein (PrP^C), a glycosylphosphatidylinositol (GPI)-anchored cell surface glycoprotein of uncertain function expressed at the highest level by neurons in the CNS [9–11]. Like most membrane-associated proteins, PrP^C is cotranslationally translocated into the endoplasmic reticulum (ER), where it undergoes oxidative folding and facultative N-linked glycosylation. After transit in the Golgi, PrP^C is delivered to the cell surface, where it resides in lipid rafts. Cell surface PrP^C can be released into the extracellular space or internalized to an endosomal compartment, from which

it is either recycled to the plasma membrane or diverted to lysosomes for degradation [12].

PrP^{C} and PrP^{Sc} have identical amino acid sequences but distinct conformations and biochemical properties. PrP^{C} has a predominant α-helix content and is soluble in detergents and protease-sensitive. In contrast, PrP^{Sc} is rich in β-sheets, tends to form detergent-insoluble aggregates, and shows variable degrees of resistance to proteinase-K (PK) digestion [13, 14].

PrP^{Sc} propagates by imprinting its aberrant conformation onto endogenous PrP^{C} molecules [8]. This conversion starts on the cell surface [15] and proceeds within the endocytic compartment [16, 17]. It probably involves a process of nucleated polymerization in which oligomers of PrP^{Sc} serve as seeds that recruit and stabilize abnormal conformations of PrP^{C}, followed by fragmentation of the PrP^{Sc} polymers into new propagation-competent oligomers [18, 19].

Genetic prion diseases, including familial CJD, GSS, FFI, and PrP-cerebral amyloid angiopathy (PrP-CAA) are linked to point mutations or insertions in the *PRNP* gene encoding PrP^{C} [20]. These diseases are thought to arise because of an intrinsic tendency of the mutant PrP^{C} molecules to misfold and aggregate, eventually acquiring the PrP^{Sc} structure. Sporadic prion diseases, including the majority of CJD cases, sporadic fatal insomnia, and the recently described variably protease-sensitive prionopathies [21], are believed to be due to spontaneous misfolding of wild-type PrP^{C}, at a low frequency or to rare somatic *PRNP* mutations.

Prion diseases vary widely in their clinical presentation. CJD is a subacute spongiform encephalopathy mostly involving the cerebral cortex, striatum, and cerebellum and recognized clinically by dementia and motor abnormalities. FFI is characterized clinically by sleep alterations and autonomic dysfunction and neuropathologically by severe degeneration of the anterior ventral and mediodorsal nuclei of the thalamus [22]. GSS is a slowly progressive ataxia with PrP amyloidosis mainly in the cerebellum and basal ganglia. PrP-CAA is a slowly progressive dementia with PrP-amyloid deposits in blood vessels of the CNS [23, 24].

The reason for this variability is not known. Brain tissues from patients with different prion diseases contain pathological forms of PrP with variable degrees of protease resistance and/or distinct PK cleavage sites, suggesting that different conformational isoforms of PrP may have specific neurotoxic properties.

Only recently have we begun to understand how abnormally folded PrP causes neuronal dysfunction and degeneration. Experimental evidence indicates a dissociation between prion infectivity and pathogenicity and suggests that abnormal forms of PrP, structurally different from PrP^{Sc}, are the actual trigger of the neurodegenerative process [25]. Nerve endings are the initial targets of the toxic PrP species, which perturbs normal synaptic function and morphology. Beyond this step, when functional recovery is still possible [26], synaptic loss and neuronal death are irreversible stages of the pathogenic process.

2. Starting from the End: Neuronal Death in Prion Diseases

The observation that neurodegeneration in prion diseases occurred in the absence of a typical tissue inflammatory response [27, 28] suggested the involvement of programmed cell death (PCD), rather than necrosis. PCD is an active process requiring activation of gene expression and protein synthesis and is morphologically and biochemically distinguishable from necrosis. There are many types of PCD, but only apoptosis and autophagy have been consistently reported in natural and experimental prion diseases.

2.1. Apoptosis. Apoptosis is morphologically characterized by shrinkage of the cell, condensation of the chromatin, blebbing of the plasma membrane, and fragmentation of the nucleus, without significant morphological alterations of other subcellular organelles. In the endstage, small membrane-bound cell fragments (apoptotic bodies) are formed, that are rapidly ingested by phagocytic cells without inducing an inflammatory reaction. Biochemically, apoptosis involves internucleosomal cleavage of genomic DNA and in mammals is regulated by the Bcl-2 (B-cell lymphoma protein 2) family of proteins, Apaf-1 (apoptotic protease-activating factor 1), and the cysteine protease caspase family [29].

The first clue to apoptosis in prion disease was nuclear fragmentation and internucleosomal DNA cleavage in primary neurons exposed to PrP106-126, a synthetic peptide used to model prion-induced neuropathology [30]. Analysis of brains from scrapie-affected sheep, CJD and FFI patients, and experimentally prion-infected rodents identified cells with fragmented nuclei, DNA cleavage, and caspase activation, confirming the involvement of PCD [31–45]. In addition, transgenic Tg(PG14) mice expressing a mutant PrP carrying a nine-octapeptide repeat insertion associated with a genetic prion disease showed massive apoptosis of cerebellar granule neurons (CGNs) [46]. Finally, morphological and biochemical features of apoptosis were seen in hypothalamic GT1 cells, primary CGNs, and cerebellar organotypic cultures infected with scrapie [47–49].

Several studies investigated whether blocking the apoptotic program could prevent or ameliorate prion pathology. Transgenic overexpression of the antiapoptotic Bcl-2 protein or targeted deletion of the proapoptotic gene Bax (Bcl-2-associated X protein) did not prevent neuronal loss and neurological disease in prion-infected mice [50, 51], neither did genetic ablation of caspase-12, a proposed mediator of ER stress-induced cell death [52]. Bax deletion rescued CGNs in Tg(PG14) mice but did not prevent the synaptic degeneration and the progressive neurological disease that develop in this model [53]. These results indicated that targeting Bcl-2 family-dependent or ER stress-related apoptotic pathways was not enough to prevent neurodegeneration and suggested that additional (or alternative) mechanisms could be operative in prion diseases leading to synaptic loss and neuron demise.

2.2. Autophagy. Macroautophagy (hereafter referred to as autophagy) is a physiologically regulated catabolic pathway that despatches cytoplasmic material, like long-lived proteins and organelles, to the lysosomes for degradation. It is a multistep process in which part of the cytoplasm is initially enclosed in a double-membraned structure to form the autophagosome, also called autophagic vacuole. The autophagosome then fuses with lysosome to form an autolysosome, where the captured material is degraded by lysosomal hydrolases. Autophagosomes can also fuse with early endosomes or multivesicular bodies (late endosomes) to form amphisomes, which then fuse with lysosomes for degradation [54].

Autophagic cell death is presumed to result from excessive levels of cellular autophagy. Morphologically there is degradation of organelles with preservation of cytoskeletal elements until late stages and, like apoptosis, it does not instigate a tissue inflammatory response. Recent data point to a close interplay between autophagy and apoptosis, with the former acting as an inhibitor of the apoptotic program or occurring upstream of apoptosis [55, 56].

A number of studies have brought to light a possible role of autophagy in prion diseases. Abundant autophagic vacuoles and multivesicular bodies were seen in synaptic terminals, neuritis, and neuronal cell bodies in the CNS of prion-infected rodents, CJD, GSS, and FFI patients [57–64]. However, in contrast to a putative disease-promoting activity of autophagy, its pharmacological induction slightly prolonged survival of prion-infected mice [65–67] and significantly delayed the onset and progression of neurological illness in a Tg mouse model of GSS [68]. This beneficial effect was attributed to enhanced clearance of the pathological PrP isoform [65, 68, 69]. A recent report confirmed an increase in autophagic flux in prion-infected cells but found that PrP^{Sc} undergoes lysosomal degradation independently of the autophagic route [70].

Additional studies are necessary to clarify whether autophagy serves a protein quality-control function against misfolded PrP and if its failure or overactivation contributes to neurodegeneration [71]. It will be important to identify the signaling pathways that activate autophagy in prion disease and test the effect of genetic interference in animal models. It will also be essential to investigate the interplay between autophagy and apoptosis, as therapeutic inhibition of PCD depends on understanding how one process controls the other. However, blocking neuronal death might not be sufficient to halt prion disease. Growing evidence, in fact, suggests that synaptic failure, rather than the actual death of neurons, is the primary cause of neurological dysfunction in prion disorders.

3. Synaptopathy in Prion Diseases: Correlation between Symptoms and Synaptic Failure

The relation between synaptic pathology and neurological deficits has been extensively studied in mice intrahippocampally injected with Me7 or 87V prions. In these models, there is a progressive decrease in the number of synapses in the *stratum radiatum* with degeneration of the presynaptic compartment and loss of dendritic spines, well before death of CA1 pyramidal neurons [72–76]. Concomitant with this initial synaptic pathology, there are abnormalities in hippocampal synaptic plasticity, which parallel alterations in spontaneous ethological behaviors such as open field activity, burrowing, and nesting [77, 78]. A similar pathological sequence is seen in mice intrahippocampally injected with RML prions, in which defects in presynaptic hippocampal function and degeneration of synapses parallel deficits in recognition memory, burrowing, and nesting and precede loss of pyramidal cells [79, 80]. Thus synaptic dysfunction and degeneration are important determinants of the early behavioral abnormalities in prion-infected mice.

Disruption of synaptic connectivity is an important correlate of symptomatology also in human prion diseases. Neuropathological analyses in humans are of necessity restricted to the terminal phase of the illness when there is often extensive loss of neurons in addition to synaptic degeneration. However, cases of genetic prion disease linked to octapeptide repeat insertions have been described that show widespread synaptic loss but preservation of nerve cells, supporting the idea that the neurological deficits correlate with loss of neuronal processes rather than cell bodies [81]. This is corroborated by experiments in Tg(PG14) mice, indicating that synaptic disruption is the major determinant of neurological illness (see Section 5) [53, 82].

What causes synaptic failure in prion disease? Abnormal PrP deposition is extracellular in most forms of prion disease, often occurring as diffuse protease-resistant "synaptic-like" deposits in perineuronal structures throughout the neuropil [83]. Therefore, a common assumption is that synaptic loss is due to a direct toxic effect of accumulated PrP. In the Me7 model, neither the magnitude nor the spatial pattern of PrP^{Sc} deposition correlates with the number of synapses lost [73, 75]. Moreover, in 87V-infected mice, alterations in synaptic morphology in the hippocampus occur before PrP^{Sc} deposition [39]. In Tg(PG14) mice, protease-resistant PrP, as detected by immunocytochemistry, accumulates in the molecular layer of the cerebellum in a synaptic-like pattern [46]; however, immunoelectron microscopy demonstrates that PG14 PrP accumulations are not truly synaptic in their localization [84]. Thus, in both infectious and genetic models, synaptic degeneration cannot be readily explained by a toxic effect of deposited PrP.

It may be argued that soluble rather than deposited forms of PrP^{Sc} are the actual synaptotoxic species. Monomers and soluble oligomers of recombinant PrP have been generated *in vitro* that are toxic to neurons in culture and after intracerebral injection in mice [85–87]. However, it remains to be seen whether similar forms of soluble PrP are generated in prion disease and play any role in synaptic dysfunction.

An alternative explanation is that synaptic failure is the consequence of PrP^{C} misfolding within neurons. Recent data point to a crucial role of PrP accumulation in the ER.

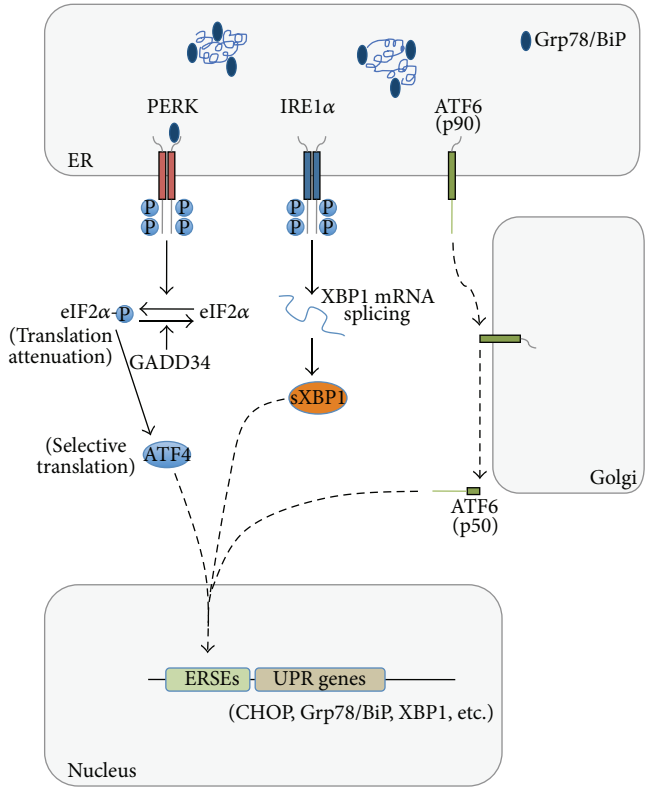

Figure 1: UPR signaling pathways in mammalian cells. The UPR is mediated by three ER-resident transmembrane proteins that sense ER stress through Grp78/BiP binding/release to their luminal domains and/or through direct interaction with unfolded proteins. The kinase PERK (double-stranded RNA-activated protein kinase-like ER kinase) is activated by dimerization and phosphorylation. Once activated, it phosphorylates eIF2α (eukaryotic translation initiation factor 2). This inhibits protein translation, reducing the overload of misfolded proteins. This pathway also selectively enhances translation of ATF4 (activating transcription factor 4) that induces the expression of CHOP. In ER-stressed cells, IRE1α (inositol-requiring transmembrane kinase and endonuclease) multimerizes and autophosphorylates, setting in motion its RNAse activity. Activated IRE1α initiates the unconventional splicing of the mRNA encoding the transcriptional factor XBP1 (X-box-binding protein 1) to produce sXBP1, a more stable form of XBP1 with a potent transactivator domain that enhances transcription of genes involved in protein folding, secretion, and ER-associated degradation. Another ER stress sensor is ATF6 (activating transcription factor 6). This is a type II ER transmembrane protein whose cytosolic domain contains a bZIP transcriptional factor. ATF6 is transported to the Golgi where it is processed within the transmembrane domain to release the cytosolic domain, which translocates to the nucleus and induces expression of the ER chaperone Grp78/BiP and XBP1. GADD34, a protein phosphatase upregulated by the PERK pathway, dephosphorylates eIF2α to restore global protein synthesis. ERSEs: ER stress responsive elements.

4. An ER Stress-Mediated Mechanism of Synaptic Dysfunction in Prion-Infected Mice

Moreno et al. discovered a molecular mechanism underlying synaptic failure in RML-infected mice [80]. They found that PrP accumulation in the hippocampus was associated with activation of the translational repression pathway of the unfolded protein response (UPR). The UPR is an adaptive signal transduction cascade that is activated when misfolded proteins accumulate and aggregate in the ER; it involves a tripartite signaling that enhances the folding capacities in the ER, improves misfolded protein disposal through ER-associated degradation, and reduces the rate of protein synthesis and translocation into the ER lumen (Figure 1) [88, 89]. The signal for repression of protein synthesis is triggered by the autophosphorylation of the ER-associated kinase PERK, which phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α). This inhibits protein translation, reducing the overload of misfolded proteins. Phosphorylation of eIF2α also activates ATF4, a transcription factor that induces expression of CHOP. ATF4 and CHOP cooperate to restore mRNA translation by upregulating target genes encoding functions in protein synthesis [90]. If the adaptive UPR effectively reduces the unfolded protein load, restoration of protein synthesis promotes cell survival. However, if protein synthesis increases before restoration of proteostasis, a signal is activated that promotes apoptotic cell death [90].

Moreno et al. found a progressive increase in PERK and eIF2α phosphorylation in the hippocampus of RML-infected mice, in parallel with accumulation of PrP^{Sc} and rising levels of total PrP [80]. They reported a decline in protein translation with a sudden drop in the levels of pre- and postsynaptic proteins, such as the SNARE proteins SNAP-25 and VAMP-2, the NR1 subunit of the N-methyl-D-aspartate

receptors (NMDARs), and PSD-95. This was associated with a deficit in hippocampal synaptic transmission and abnormal burrowing behavior. Lentivirally mediated overexpression of GADD34, a specific eIF2α-P phosphatase, reduced eIF2α-P levels and restored protein synthesis, rescuing the synaptic transmission defect and the behavioral abnormalities. The same effects were seen upon neuron-specific PrP^C silencing by RNA interference [80], suggesting that accumulation of misfolded PrP^C in the neuronal ER was the proximate cause of UPR and PERK-mediated translational repression. Thus, intraneuronal PrP^C misfolding during prion infection would ultimately lead to synaptic failure by reducing the levels of proteins essential for synaptic transmission. Another study suggested that hyperactivation of calcineurin due to calcium release from the stressed ER could also contribute to neuronal dysfunction in prion disease [91].

Our findings in mouse models of genetic prion disease are consistent with the idea that ER retention of misfolded PrP affects synaptic function but that ER stress is not involved.

5. Alterations in Voltage-Gated Calcium Channel Activity Underlie the Neurotransmission Deficit Associated with Motor Impairment in Mutant PrP Mice

Tg(PG14) mice develop a progressive neurological illness characterized clinically by ataxia and neuropathologically by cerebellar atrophy due to loss of synaptic endings in the molecular layer and massive apoptosis of CGNs [46, 92]. To test whether blocking the apoptotic program could prevent neurodegeneration and motor dysfunction, we crossed Tg(PG14) with Bax knockout mice. Bax deletion efficiently rescued CGNs but had no effect on the development of ataxia and synaptic loss [53]. This suggested that disruption of synaptic connectivity in the cerebellum was vital in the Tg(PG14) disease and prompted us to test whether abnormalities in neurotransmission could be detected before neurodegeneration, in parallel with the onset of motor dysfunction.

We found that the motor behavioral deficits in Tg(PG14) mice emerged before synaptic loss and were associated with defective glutamatergic neurotransmission in CGNs due to impaired calcium influx through voltage-gated calcium channels (VGCCs) [82]. The same functional changes were seen in CGNs of Tg(CJD) mice that express the mouse PrP homologue of the D178N/V129 mutation linked to genetic CJD and develop motor abnormalities in the absence of granule cell death [82, 93]. Thus, in two different mouse models of genetic prion disease, the onset of motor behavioral abnormalities was dissociated from neuron demise and correlated with defective glutamatergic transmission in CGNs due to alterations in VGCC activity.

6. ER Retention of PG14 PrP Is Not Associated with an ER Stress Response

Analysis of PG14 PrP metabolism and localization in CGNs showed that this mutant misfolds soon after synthesis in the ER, is delayed in its biosynthetic maturation, and accumulates abnormally in this organelle [94–96]. This suggested that intracellular accumulation of mutant PrP might be critical in neuronal dysfunction, possibly due to activation of an ER stress response [25]. However, molecular biology, biochemical and immunohistochemical analyses of brain tissues, and primary CGNs from the mutant mice found no increase in the expression of UPR-regulated genes [97] or activation of the PERK/eIF2α translational repression pathway (Figures 2 and 3). There were also no changes in the amounts of synaptic proteins, as the levels of synaptophysin, SNAP-25, the synaptic vesicle fusion protein synaptotagmin I, and the secretory vesicle chaperone CSPα were not affected in Tg(PG14) at the onset of the cerebellar deficit [82]. Thus, in contrast to RML-infected mice where alterations in synaptic function correlate with ER stress-induced translational repression [80], the neurotransmission defect in Tg(PG14) mice was not associated with a decrease in protein synthesis as a consequence of ER stress. Moreover, calcineurin activity was decreased rather than induced in the Tg(PG14) cerebellum [98], arguing against an involvement of ER stress-induced calcium release and calcineurin hyperactivation in synaptic dysfunction.

How might the lack of an ER stress response be explained despite demonstrable mutant PrP misfolding and retention in this organelle? A reasonable explanation is that PG14 PrP never accumulates in the ER to a high enough level to trigger the UPR. We did in fact find that although it was delayed in its biosynthetic maturation, PG14 PrP eventually escapes the ER quality control system of the cell and is trafficked to post-ER compartments [94–96].

In the next section, we describe the mechanism by which impaired trafficking of PG14 PrP alters VGCC function. Our studies brought to light an alternative, UPR-independent modality by which intracellular PrP^C misfolding affects synaptic proteostasis.

7. ER Retention of Mutant PrP Causes Inefficient Synaptic Targeting of VGCCs

How could misfolding of mutant PrP in the ER alter VGCC function? First, we asked whether intracellular PrP retention was responsible for the VGCC defect. We found that PG14 PrP molecules with a deletion in the hydrophobic core (HC) between residues 114 and 121 had less tendency to misfold and accumulate in transport organelles and were more efficiently delivered to the cell surface than their full-length counterparts [99], providing a model for assessing the role of intracellular retention. We compared the effect of HC-deleted and full-length PG14 on neuronal calcium dynamics and found that the calcium response in CGNs expressing HC-deleted PG14 PrP was similar to that of the wild-type controls [82]. This suggested that misfolding and ER retention of mutant PrP were necessary to induce the VGCC defect.

Because our data pointed to a role of intracellular PrP retention, we hypothesized that PG14 PrP interacted with VGCCs in transport organelles, interfering with their trafficking towards the plasma membrane. VGCCs are heteromeric proteins consisting of the pore-forming $Ca_V\alpha_1$

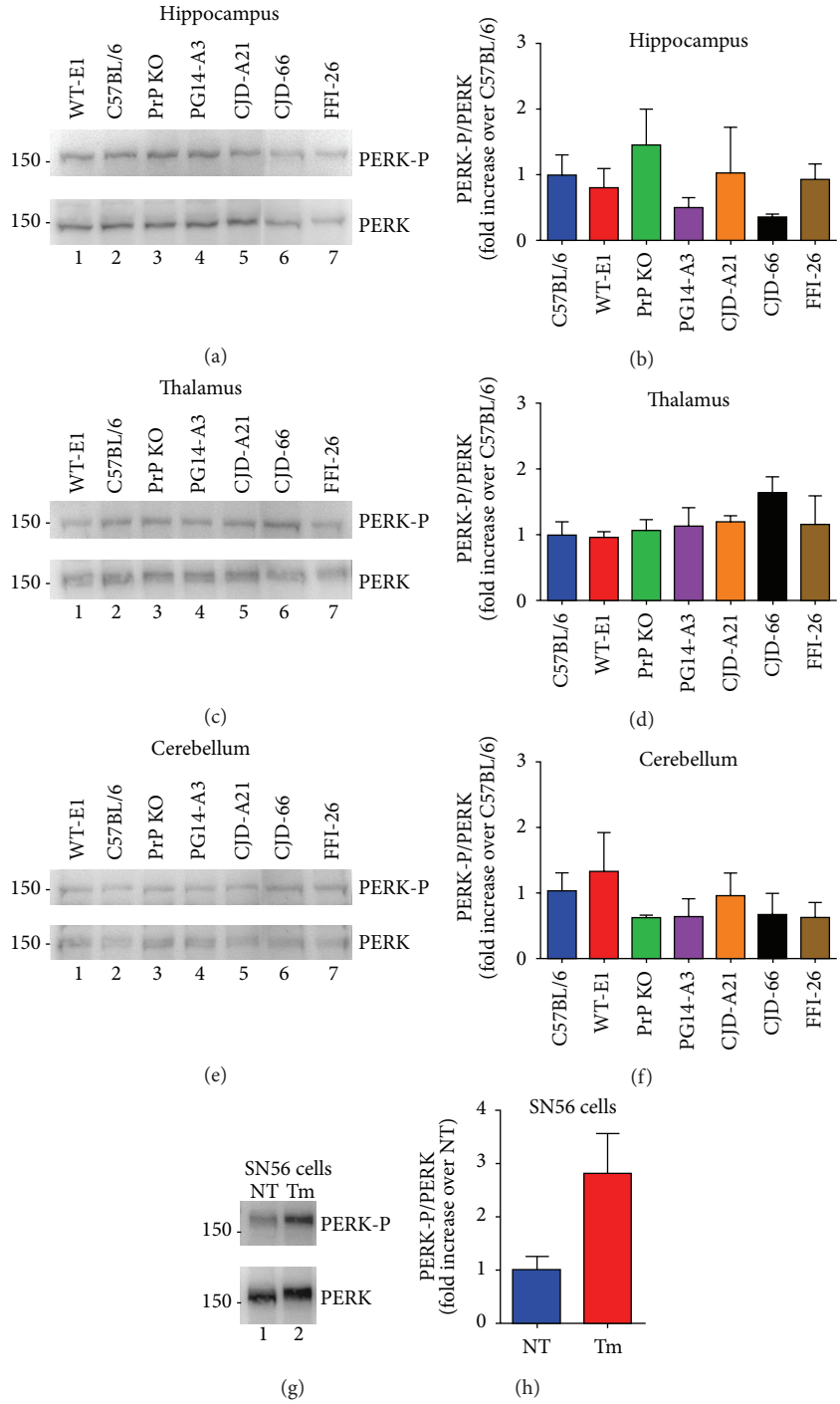

Figure 2: Phosphorylation of PERK is not increased in the brains of mutant PrP mice. Phosphorylation of PERK was evaluated in brain extracts of the following mice: C57/BL6J (PrP level 1X), PrP KO (C57BL/6J/*Prnp*$^{0/0}$, European Mouse Mutant Archive, Rome, Italy; EM: 01723), Tg(WT-E1$^{+/+}$) overexpressing 3F4-tagged wild-type PrP at ~4X, Tg(PG14-A3$^{+/-}$) expressing 3F4-tagged PG14 PrP at ~1X, Tg(CJD-A21$^{+/-}$) expressing 3F4-tagged D177N/V128 PrP at ~1X, Tg(CJD-66$^{+/-}$) expressing untagged D177N/V128 PrP at ~2.5X, and Tg(FFI-26$^{+/-}$) mice expressing untagged D177N/M128 PrP at ~2.5X. These mice were originally generated on a C57BL/6J X CBA hybrid and then bred with C57BL/6J/*Prnp*$^{0/0}$ mice ([92, 93] and manuscript in preparation). Proteins were extracted from the hippocampus, thalamus, and cerebellum of mice of the indicated strains/genotype ((a)–(f)) or from SN56 cells ((g) and (h)), using a lysis buffer containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM $MgCl_2$, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, and 125 mM sucrose, supplemented with Phos-STOP and protease inhibitors (Roche) [80]. Protein extracts (50 μg) were analyzed by Western blot with anti-PERK-P and antitotal PERK antibodies (1 : 1000; Cell Signaling) ((a), (c), (e), and (g)). Molecular mass markers are in kilodaltons. Phosphorylation levels were quantified by densitometric analysis of Western blots and expressed as the -fold increase over the level in C57BL/6 mice ((b), (d), (f), and (h)). Tunicamycin (Tm) treated HeLa cells were analyzed at 2 hours as control for UPR activation. Each value is the mean ± SEM of three animals of 300–350 days of age or from three independent cell preparations.

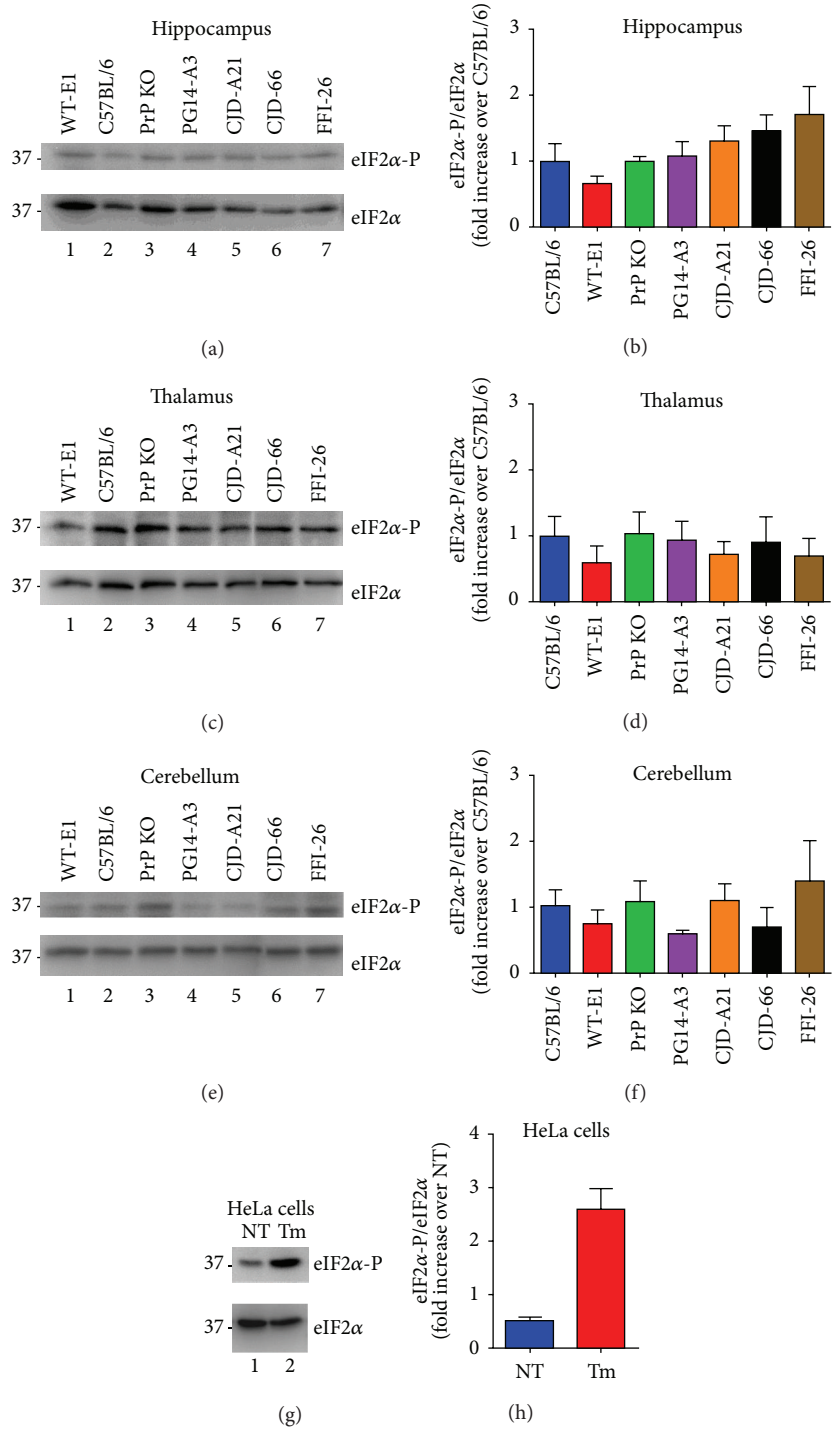

Figure 3: Phosphorylation of eIF2α is not increased in brains of mutant PrP mice. The same brain protein extracts (20 μg) as in Figure 2 ((a)–(f)) or lysates of HeLa cells ((g) and (h)) were analyzed by Western blot with anti-eIF2α-P and antitotal eIF2α antibodies (1 : 1000; Cell Signaling). Molecular mass markers are in kilodaltons. Phosphorylation levels were quantified by densitometric analysis of Western blots and expressed as the -fold increase over the level in C57BL/6 mice ((b), (d), (f), and (h)). Tunicamycin (Tm) treated HeLa cells were analyzed at 2 hours as control for UPR activation. Each value is the mean ± SEM of three animals of 300–350 days of age or from three independent cell preparations.

subunit, which governs the biophysical and pharmacological properties of the channel, and the auxiliary $\alpha_2\delta$ and $Ca_V\beta$ subunits, which regulate the cellular trafficking and activity of $Ca_V\alpha_1$ [100]. Glutamate release from CGNs is mainly governed by P/Q-type channels made of the $Ca_V\alpha_{1A}$, $\alpha_2\delta$-1, and $Ca_V\beta_4$ subunit isoforms. The $\alpha_2\delta$ subunits play a vital role in intracellular trafficking of the pore-forming $Ca_V\alpha_1$ subunits and boost calcium current amplitude by increasing the number of channels on the cell surface [101, 102]. Thus, retention of $\alpha_2\delta$ in secretory organelles due to interaction with mutant PrP could impair VGCC delivery and function at presynaptic sites.

Our studies confirmed this. We found a physical interaction between $\alpha_2\delta$-1 and PrP (both wild-type and mutant) by co-immunoprecipitation, and the two proteins colocalized in transfected cells. We also observed that $\alpha_2\delta$-1 and $Ca_V\alpha_{1A}$ were weakly expressed on the cell surface and localized intracellularly in mutant PrP-expressing cells, indicating impaired secretory transport. Finally, we found smaller amounts of $\alpha_2\delta$-1 and $Ca_V\alpha_{1A}$ in cerebellar synaptosomal fractions of Tg(PG14) mice and reduced colocalization with synaptic markers, consistent with inefficient targeting of the channel complex to axonal terminals of granule neurons [82].

Thus, owing to ER retention of mutant PrP, $\alpha_2\delta$-1 accumulates intracellularly, impairing delivery of the VGCC complex to synapses. This negatively affects depolarization-induced calcium influx and glutamate release, leading to alterations of cerebellar synaptic transmission and motor control.

8. Other Possible Pathological Consequences of Mutant PrP Interactions in the ER

The observation that the synaptic delivery of VGCCs is impaired in neurons expressing mutant PrP due to interaction with $\alpha_2\delta$-1 suggests that the secretory transport of other PrP-interacting cargoes may also be impaired. Possible candidates are the $\alpha_2\delta$-2 and $\alpha_2\delta$-3 isoforms, which share fairly high sequence identity with $\alpha_2\delta$-1, and have been identified as potential PrP interactors in proteomic screening [103]. Different $\alpha_2\delta$ isoforms are expressed in functionally distinct neurons of the brain, so an impairment of their trafficking resulting from sequestration by mutant PrP may affect VGCC function and neurotransmission in different neural circuits, accounting for the complex symptomatology of genetic prion diseases.

Other proteins involved in neurotransmission, whose secretory transport could be altered by mutant PrP, are the NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs). These ligand-gated ion channels are composed of combinations of distinct subunits whose assembly is finely tuned in the ER [104]. PrP interacts physically with the NR1 and NR2D subunits of NMDARs and the GluA1 and GluA2 subunits of the AMPARs, and these interactions are important for normal neuronal physiology and survival [105–108]. It will be interesting to see whether the cellular trafficking and synaptic localization of NMDARs and AMPARs are impaired in neurons expressing mutant PrP and explore any functional consequence.

9. Possible Alterations of Secretory Transport in Nongenetic Forms of Prion Disease

The evidence that misfolding of mutant PrP in the ER affects synaptic transmission by impairing membrane delivery of VGCCs raises the question whether a similar mechanism is operative in nongenetic forms of prion disease. In sporadic prion diseases, PrP^C is believed to misfold spontaneously at a low frequency. This could preferentially occur during biosynthesis in the ER lumen, where the oxidative folding of the nascent PrP^C polypeptide may be affected by perturbations of ER homeostasis. Consistent with this, treatment of neuroblastoma N2a cells with several ER stressors caused the formation of a misfolded PrP^C isoform that was more prone to PrP^{Sc} conversion [109].

In prion diseases acquired by infection, exogenous PrP^{Sc} induces conversion of PrP^C on the cell surface or within an endocytic compartment, rather than in the ER [15–17]. However, stimulation of PrP^C retrograde transport toward the ER increases PrP^{Sc} levels in prion-infected N2a cells, suggesting that ER-localized PrP^C may also misfold [110]. In addition, PrP^{Sc} replication perturbs ER calcium homeostasis [111], and this could favor misfolding of newly synthesized PrP^C [109]. Thus, several mechanisms may trigger misfolding and ER retention of PrP^C in nongenetic prion diseases, potentially interfering with secretory transport of VGCCs and perhaps other PrP-interacting proteins. Intriguingly, VGCC activity is impaired in scrapie-infected GT1 cells [112], but whether this is due to defective transport of the channel to the plasma membrane remains to be established.

Although we have emphasized the role of PrP^C misfolding in the neuronal ER, protein trafficking may also be impaired by the accumulation of PrP in other compartments of the secretory pathway. We did in fact find that PrP carrying the FFI mutation accumulates in the Golgi of N2a cells and that its expression is associated with an alteration of the GDI/Rab11 pathway governing post-Golgi vesicular trafficking [113]. A recent report indicates that post-Golgi trafficking is also impaired in prion-infected N2a cells [114].

Finally, misfolded PrP accumulation may alter the secretory transport of PrP-interacting proteins also in nonneuronal cells. For example, PrP^C interacts with the $\alpha 2$ and $\beta 2$ subunits of Na^+/K^+-ATPase in glial cells, and this interaction is involved in regulating glutamate-dependent release of lactate from astrocytes [105]. Lactate released from astrocytes is taken up by neurons and is an important energy source, at least during high neuronal activity. Thus, any impairment of $\alpha 2/\beta 2$-ATPase transport in mutant or prion-infected astrocytes could contribute to neuronal dysfunction.

10. From Synaptic Dysfunction to Neuronal Death: Role of Intracellular PrP Retention in the Phenotypic Heterogeneity of Prion Diseases

Does intracellular PrP accumulation ultimately lead to neuronal cell death? Persistent UPR in the hippocampus of

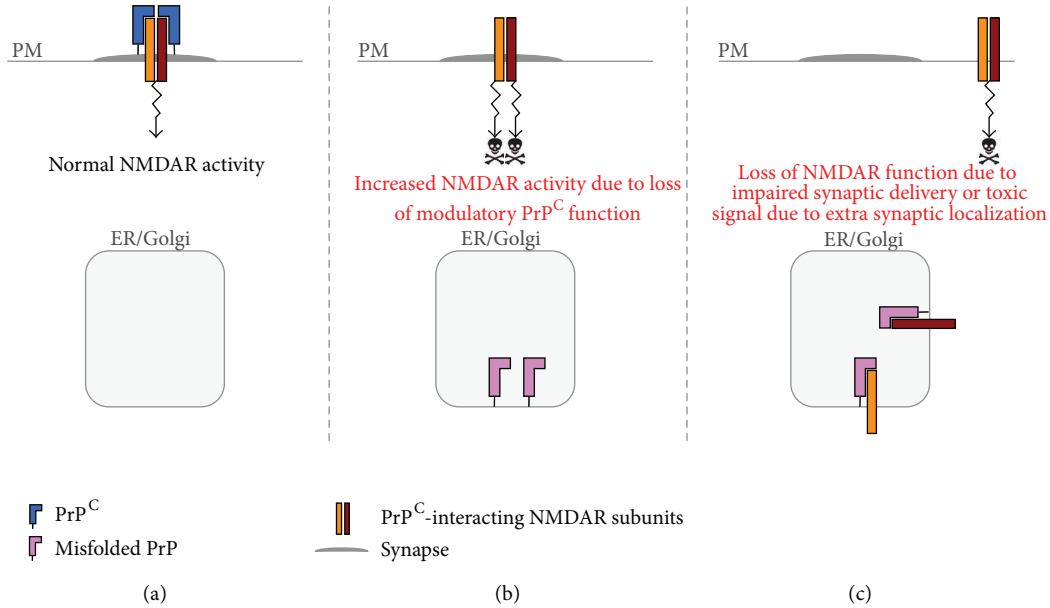

Figure 4: A role for intracellular PrP retention in NMDAR dysfunction. (a) PrP^C on the plasma membrane (PM) attenuates NMDAR activity by associating with the NR2D subunit. (b) Owing to PrP^C misfolding in transport organelles (ER/Golgi), PrP^C is retained intracellularly. This results in increased NMDAR activation, potentially triggering neurotoxicity. (c) Intracellular retention of misfolded PrP^C with NR2D and NR1 subunits results in impaired delivery of NMDARs to the cell surface or their abnormal targeting to extrasynaptic sites, leading to loss of NMDAR function and/or activation of neurotoxic stimuli.

RML-infected mice might kill neurons through activation of the ATF4/CHOP apoptotic pathway [80]. However, degenerating hippocampal neurons in these mice do not show morphological features of apoptosis, suggesting that this may not be the actual effector mechanism of cell death [80]. In addition, PERK and eIF2α are not activated in brains of individuals with sporadic, infectiously acquired, or genetic prion disease, not even in those brain areas with the most pronounced neuropathological changes [115].

We would like to offer an alternative explanation for how intracellular accumulation of misfolded PrP might kill neurons. We propose that neuronal death in prion diseases may result from a functional perturbation of proteins that physiologically interact with PrP^C, either because of sequestration in transport organelles or because their normal activity on the cell surface is corrupted in the absence of PrP^C (Figures 4 and 5).

In addition to $\alpha_2\delta$ subunits, whose functional impairment could lead to apoptotic cell death [116, 117], other PrP-interacting proteins whose abnormal function could mediate neurotoxic effects are the glutamate receptors. PrP^C attenuates activation of NMDARs through its interaction with the NR2D subunit, thereby protecting neurons from glutamate-induced excitotoxicity [106] (Figure 4(a)). This neuroprotective function could be lost with intracellular PrP retention, making neurons more susceptible to excitotoxic stimuli (Figure 4(b)). In addition, misfolded PrP could sequester NR2D or NR1 (the other PrP^C-interacting NMDAR subunit [108]) in transport organelles, reducing NMDAR plasma membrane delivery or interfering with their correct targeting to the synaptic membrane (Figure 4(c)). This could also result in neuronal damage, since synaptic NMDAR activation promotes survival, while activation of extrasynaptic NMDAR signals causes stress and death [118]. Intracellular retention of GluA1 and GluA2 with misfolded PrP [105, 107] might also be involved. For example, sequestration of GluA2 may result in AMPARs lacking this subunit, which are more permeable to calcium, potentially exacerbating excitotoxic phenomena [119]. Consistent with a role of excitotoxicity in PrP-mediated neurodegeneration, a neurotoxic mutant PrP was recently seen to sensitize neurons to glutamate-induced cell death [120].

Retention of misfolded PrP in the secretory pathway might also indirectly affect PrP^C-mediated signaling functions. There is increasing evidence, in fact, that PrP^C serves as a cell surface scaffold for a variety of signaling modules that control neuronal differentiation and survival [121] (Figure 5(a)). These prosurvival signals may be lost or corrupted in case of misfolding and intracellular retention of PrP^C, eventually triggering neuronal death (Figures 5(b) and 5(c)). Thus, prion disease pathogenesis may result from toxic activities engaged by intracellular PrP^C misfolding in conjunction with loss of PrP^C function on the cell surface.

Could this model of toxicity explain the heterogeneous clinical presentation of prion diseases? Ion channels, glutamate receptors, and signaling complexes are generally made of different subunit isoforms, which are expressed in functionally distinct neurons of the brain. PrP^C may preferentially interact with specific isoforms, inducing functional abnormalities only in certain types of neurons. For example,

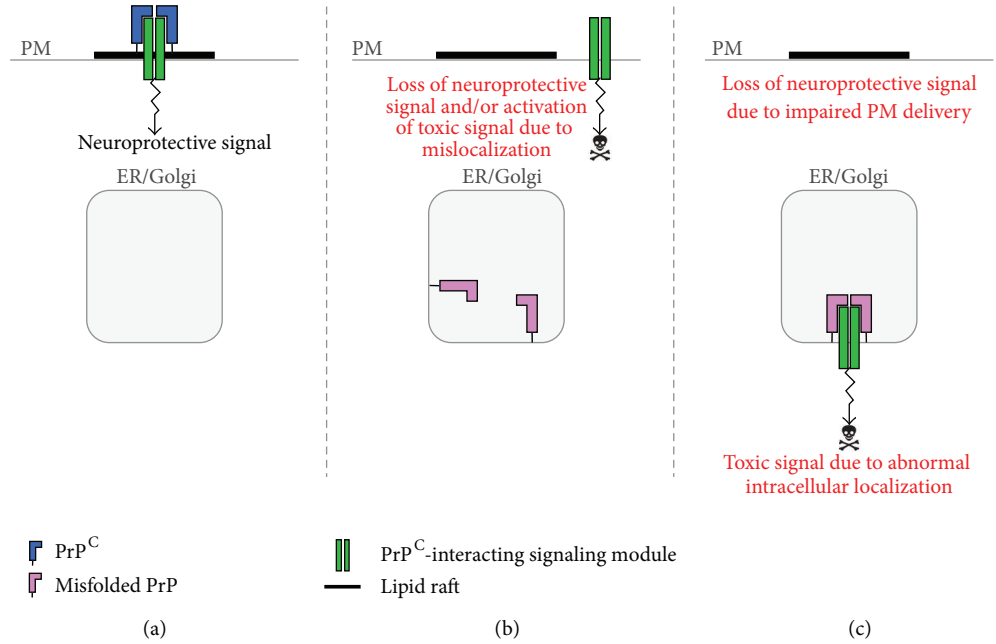

FIGURE 5: Theoretical model for how intracellular retention could perturb PrP^C-dependent signaling. (a) PrP^C acts as scaffold molecules that keep a prosurvival signaling complex in lipid rafts of the plasma membrane (PM). The lipid raft localization would be essential to activate neuroprotective signaling. (b) Owing to retention in transport organelles (ER/Golgi), PrP^C function is lost and the signaling complex localizes in nonraft regions of the PM, losing its neuroprotective activity and potentially eliciting a neurotoxic signal. (c) Misfolded PrP sequesters the signaling module in intracellular compartments, leading to loss of neuroprotective function on the cell membrane. Intracellular retention might also cause the complex to function abnormally and generate a toxic signal.

PrP^C co-immunoprecipitates with the NR2D but not the NR2B subunit of NMDARs [106]. Therefore, PrP^C misfolding may specifically affect neurons expressing NR2D.

Then too, different misfolded variants of PrP may differ in their interacting properties. PG14 and D178N PrPs are structurally different [122] and have different ability to interact with the GluA2 subunit of AMPARs [107]. PG14 co-immunoprecipitates with GluA2 as does wild-type PrP, whereas D178N PrP does not [107]. This suggests that intracellular retention of PG14, but not D178N PrP, may impair GluA2 trafficking. Thus, different misfolded forms of PrP may have different effects on neuronal function—hence on the clinical presentation of disease—depending on whether they lose or maintain the ability to interact with their molecular partners.

11. Summary and Conclusions

PrP^C misfolding has long been known to play a key role in the pathogenesis of prion diseases, but only recently have we started elucidating the neurotoxic mechanisms. Experimental studies have indicated a dissociation between prion infectivity and neurotoxicity, and the assumption that PrP^{Sc} is both infectious and pathogenic is being progressively replaced by the view that noninfectious PrP species are the actual neurotoxic culprits [25, 123, 124]. There is also a great deal of experimental data against the idea that extracellular aggregates of misfolded PrP are intrinsically neurotoxic, indicating instead that neuronal degeneration is triggered by conformational conversion of endogenous PrP^C [79, 125–127]. Finally, synaptic dysfunction is emerging as the primary determinant of neurological illness, so therapeutic interventions should aim at preventing synaptic damage, in addition to blocking neuronal death.

The observation that several mutant PrPs acquire abnormal conformations soon after synthesis in the ER and are delayed in their biosynthetic maturation and secretory transport suggested that intracellular accumulation could be crucial in neuronal dysfunction [25]. This is now supported by our demonstration that mutant PrP impairs the synaptic delivery of VGCCs through a physical interaction with $\alpha_2\delta$-1 in transport organelles, leading to alterations in neurotransmission [82]. In this review, we argue that other channels or signaling complexes could gain neurotoxic functions because of misfolding and retention of mutant PrP in the secretory pathway and that similar mechanisms may also be operative in nongenetic prion diseases. The neurotoxic modality that we propose might also explain the clinical heterogeneity of prion diseases, since different pathological conformations of PrP may selectively impair the trafficking and activity of different proteins, preferentially expressed in specific types of neurons.

In conclusion, emerging evidence points to a key pathogenic role of PrP^C misfolding in the secretory pathway. Impairment of secretory protein trafficking may be a major cause of neuronal dysfunction and degeneration in prion

diseases and perhaps in other neurodegenerative disorders caused by intracellular accumulation of misfolded proteins.

Authors' Contribution

A. Senatore and E. Restelli contributed equally to this work.

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Review Article

Role of Protein Misfolding and Proteostasis Deficiency in Protein Misfolding Diseases and Aging

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The misfolding, aggregation, and tissue accumulation of proteins are common events in diverse chronic diseases, known as protein misfolding disorders. Many of these diseases are associated with aging, but the mechanism for this connection is unknown. Recent evidence has shown that the formation and accumulation of protein aggregates may be a process frequently occurring during normal aging, but it is unknown whether protein misfolding is a cause or a consequence of aging. To combat the formation of these misfolded aggregates cells have developed complex and complementary pathways aiming to maintain protein homeostasis. These protective pathways include the unfolded protein response, the ubiquitin proteasome system, autophagy, and the encapsulation of damaged proteins in aggresomes. In this paper we review the current knowledge on the role of protein misfolding in disease and aging as well as the implication of deficiencies in the proteostasis cellular pathways in these processes. It is likely that further understanding of the mechanisms involved in protein misfolding and the natural defense pathways may lead to novel strategies for treatment of age-dependent protein misfolding disorders and perhaps aging itself.

1. Introduction

Multiple and complex biological processes occur simultaneously in living cells. These processes must be strictly regulated in order to allow an optimal equilibrium and function. Proteins are key macromolecules, which perform a vast array of functions within living organisms, including replicating genetic material, catalyzing metabolic reactions, maintaining the cellular structure, participating in cellular signaling, immune responses, cell adhesion, cell cycle, responding to stimuli, and transporting molecules from one location to another. Life depends on the proper function of thousands of proteins, which in turn depends upon the acquisition of the correct, biologically functional folding of the protein. The cellular processes responsible for the synthesis, folding, and turnover of proteins are known as protein homeostasis or proteostasis [1]. The proteostasis network controls protein concentration, subcellular location, folding through molecular chaperone systems and folding enzymes, protein degradation mediated by the proteasome, lysosome, and autophagy, among others. Defects of proteostasis may commonly lead to aberrant folding, aggregation, and accumulation of proteins resulting in cellular damage and tissue dysfunction.

2. Protein Misfolding in Disease

Currently there are at least 30 different human diseases reported to be associated with protein misfolding, where at least one particular protein or peptide misfolds and accumulates into a well-organized fibrillar structure often called amyloid [2]. The list includes various neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease (PD), Huntington disease (HD), Transmissible Spongiform Encephalopathies (TSE), and Amyotrophic Lateral Sclerosis

(ALS) as well as diverse systemic disorders, such as familial amyloid polyneuropathy (FAP), Type II Diabetes (T2D), secondary amyloidosis, and dialysis-related amyloidosis [3].

Histopathological, genetic, and biochemical studies have provided compelling evidence for protein misfolding and aggregation as the critical event in the pathogenesis of PMDs. The relationship between protein misfolding and aggregation in PMDs first came from postmortem histopathological studies. Protein aggregates usually occur in the organs and regions essentially injured by each disorder [4]. Mutations in the genes that encode the protein component of fibrillar aggregates are genetically associated with inherited modes of many PMDs [4, 5]. The familial forms usually have an earlier onset and higher severity than sporadic cases and are associated with a more extensive burden of protein aggregates [5]. The development of transgenic animal models containing mutant forms of the human genes encoding the fibrillar protein is another evidence for the key contribution of protein misfolding to disease pathogenesis [6]. Several pathological and clinical characteristics of PMDs have been observed in transgenic models in which protein aggregates were produced. Finally, many *in vitro* studies have shown that misfolded oligomers and aggregates composed by different proteins acquire a cytotoxic activity, leading to cell death and tissue damage [7]. However, the mechanisms of cytotoxic and the molecular species responsible for cell damage are still unknown. Taken together these findings support the idea that the common cause of PMDs is the accumulation of misfolded protein aggregates. However, the final proof of this hypothesis would be to cure the disease in humans by arresting or reversing the formation and accumulation of misfolded protein aggregates. With a couple of exceptions, this goal has not been achieved so far [8].

3. Protein Misfolding in Aging

A major risk factor for most PMDs, in particular neurodegenerative diseases, is aging [9]. This finding suggests that aged cells and tissues are more prone to form and accumulate misfolded aggregates. Surprisingly, a putative role of misfolded proteins in the progressive decline of cellular and tissue functioning during natural aging has not been studied in detail. Nevertheless, recent reports have indicated that there is a widespread accumulation of insoluble proteins during aging in different species [9, 10]. Interestingly, there was a substantial overlap between the age-dependent insoluble proteins identified in worms and yeast [10–12]. Several of them have been implicated in PMDs [10]. However, it is unknown whether the age-dependent accumulation of insoluble proteins is a cause of cellular dysfunction resulting in aging or a consequence of the progressive decline of proteostasis. However, the fact that selective knockdown of these aggregation prone proteins increased lifespan of *C. elegans* suggests that accumulation of insoluble proteins may not be a mere consequence of aging [10]. Furthermore, compounds which are known to bind to protein aggregates or stimulate proteostasis lead to increase in lifespan when administered in *C. elegans*, supporting the role of protein aggregates in aging [13, 14]. Specific stimulation of cellular pathways involved in the removal of protein aggregates had similar positive effect in the longevity of *C. elegans*, *Drosophila melanogaster*, and *Mus musculus* [15–18].

Aggregation prone sequences or particular mutations may stimulate protein aggregation during aging or PMDs. However, irrespective of the cause of misfolding, stimulation of proteostasis, aiming either to prevent misfolding or to degrade aggregated proteins, may be beneficial against aging or PMDs. In this review we will summarize the role of proteostasis, including alterations in clearance mechanisms, such as proteasome, unfolded protein response, and autophagy, in disease and aging (Figure 1). As reviewed below, there is compelling evidence for the involvement of proteostasis deficiency in disease as well as natural aging.

4. The Unfolded Protein Response and Its Role in PMDs and Aging

The endoplasmic reticulum (ER) is one of the major cellular organelles involved in protein homeostasis. Almost one-third of the total cellular proteins utilize the ER to attain their folded and posttranslationally modified active state [19]. Although the ER is well equipped to handle synthesis and folding of significantly high amount of proteins, genetic or environmental alterations are known to stress out the ER leading to misfolding and accumulation of proteins [20, 21]. The main mechanism by which ER combats against protein misfolding is known as the unfolded protein response (UPR) [22]. At the molecular level, UPR consists of activation of three different transmembrane proteins, including ATF6 (activated transcription factor 6), PERK (double stranded RNA activated protein kinase—like ER kinase), and IRE1α (inositol-requiring transmembrane kinase and endonuclease) [22]. While activated PERK blocks protein translation by phosphorylating eukaryotic translation initiation α (eIF2α), activated ATF6 (p50ATF6) acts as transcription factor to induce expression of ER-resident chaperones like BiP. When activated, IRE1 alternatively splices XBP1 mRNA. The spliced gene product induces transcription of different genes involved in the ER-associated degradation (ERAD) pathway [22]. The main goals of the UPR are to (i) shut down further protein synthesis to reduce the overload of the ER, (ii) induce ER-resident chaperones to prevent misfolding, and (iii) activate ER-associated degradation (ERAD) (IRE1α pathway) system to shed off misfolded protein burden using the proteasome. While temporary stress is effectively handled by the UPR, chronic stress leads to continuous accumulation of misfolded protein beyond the capacity of the UPR resulting in ER-induced suicidal response [21].

Many studies have reported the activation of the UPR in neurodegenerative diseases associated with protein misfolding which we reviewed previously [23]. Although the location of the protein aggregates in different diseases may be different, they may ultimately lead to production of chronic ER stress. In particular for PD, ALS, and TSEs, disease specific aggregates were found in the lumen of ER in the respective experimental models [24–26]. ER stress mediated

cytotoxicity was also observed when cells were exposed to aggregated proteins of different sources [27–30]. Supporting this view, Aβ mediated cytotoxicity was exacerbated in cell lines compromised in specific UPR activation pathways, including PERK or XBP1 [31, 32]. Although activation of UPR in neurodegenerative disorders associated with protein aggregation is very well established, the effect of individual UPR pathways is quite complex and can be disease specific. For example, reduced expression of PERK in an ALS mouse model has been shown to accelerate the disease onset [33], leading to the idea that stimulation of PERK/eIF2α pathway might alleviate protein aggregate mediated ER stress. However, in a mice model of TSEs, PERK/eIF2α mediated sustained translational inhibition led to neuronal death which could be reversed by reinitiating the translation process [34]. Deletion of XBP1, which is the central executor of IRE1 pathway, did not influence prion disease progression in animal models [35]. However XBP1 deficiency delayed ALS and HD disease onset and progression in respective mice model by activating autophagic response [36, 37].

Interestingly, decline in UPR function has been shown to occur naturally during aging [38–40]. The expression level of some crucial players in the UPR, like the chaperone BiP, PDI, PERK kinase, and eIF2α, decreases during aging. These abnormalities shift the balance of ER stress response towards destructive pathways during aging. An optimum degree of ER stress, mild enough just to activate the protective UPR response, may be beneficial against accumulation of misfolded proteins, but a sustained and chronic activation of the UPR might have deleterious consequences [20, 41].

5. The Ubiquitin Proteasome System and Its Role in PMDs and Aging

The ubiquitin proteasome system (UPS) is the predominant cytoplasmic cellular network responsible for the degradation of short-lived, damaged, and abnormal proteins [42, 43]. Thus it plays a crucial role in the maintenance of cellular dynamics. Altered proteins tagged with ubiquitin are recognized by the proteasome for proteolytic degradation. A detailed mechanism of the ubiquitinylation process has been described elsewhere [42]. Proteasome is a multisubunit, barrel-shaped complex composed of 20S catalytic core particle and two 19S regulatory particles located at the edges of the core forming the 26S proteasome [44].

Compelling evidence has shown impaired proteasome function in neurodegenerative disorders associated with protein misfolding [45, 46]. Supporting this view, when proteasome function was decreased in adult rats using synthetic inhibitors, the animals presented Parkinson-like symptoms and degeneration of the substantia nigra pars compacta [47]. However, those results were not reproducible by a different group [48]. In autosomal recessive PD, genetic mutations in the gene encoding an ubiquitin ligase, involved in proteasomal degradation (Parkin), lead to its loss of function resulting in accumulation of damaged proteins and consequent neuronal injury [49, 50]. In a cellular model expressing truncated tau protein, reduction of proteasomal activity resulted in

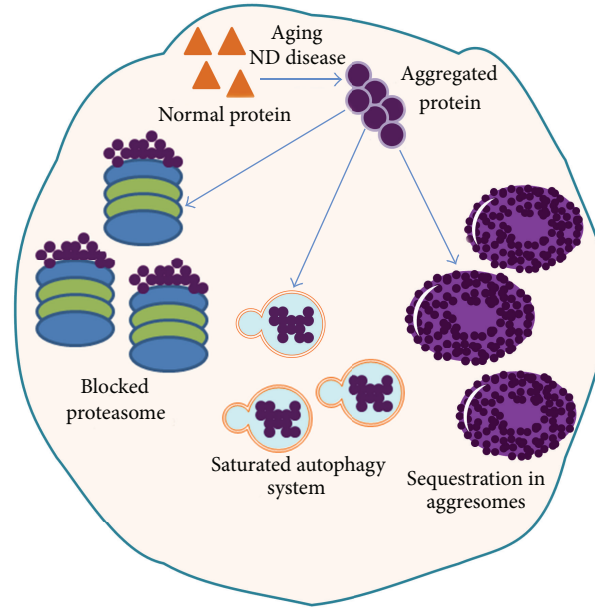

Figure 1: Protein aggregates formed during aging and PMDs impair diverse cellular clearance mechanisms.

increase in protein aggregation. Conversely, using chemical activation of proteasome by geldanamycin, it was possible to accelerate the degradation of this intracellular misfolded protein [51]. Since optimum proteasome function is crucial for cellular homeostasis, a compromised proteasome immediately became a target in PMDs. Furthermore it was suggested that a proteasome with diminished function could eventually promote more aggregate formation leading to cellular toxicity [52]. One limitation for the proteasome to clear protein aggregates is that the large size and proteolytic stability of misfolded aggregates pose difficulties for them to enter into the proteasome chamber that has a pore size of 13 angstroms. In a recent study aggregated β-sheet-rich PrP was shown to decrease proteasome activity by blocking the opening of 20S proteasome [53]. Similar observation was made in case of AD where ubiquitinated and aggregated tau has been found to bind the substrate recognition site of the proteasome, leading to a steric hindrance in the entry site of the catalytic core [54]. This problem often results in jamming the proteasome, which may have deleterious consequences for proteostasis [43]. In fact, aggregates formed by different proteins have been shown to directly inhibit proteasome activity [55–58].

Generation of transgenic mice with impaired proteasome activity is extremely difficult due to the crucial function of proteasome during development. However, a conditional inactivation of an ATPase subunit (Rpt2) of the proteasome in substantia nigra's neurons showed accumulation of α-synuclein positive, Lewy body-like deposits followed by severe neurodegeneration [59]. Supporting further the key role of proteasome in PMDs, it has been demonstrated that a motor neuron specific deletion of ATPase subunit (Rpt3) leads to accumulation of TDP43 and FUS proteins, followed by progressive loss of motor neuron resembling

ALS pathology [60]. In an attempt to study the effect of chronic proteasome inhibition, a mice model overexpressing proteasome antagonist UBB^{+1} (mutant form of ubiquitin B) was generated. Strikingly, just 20% of proteasomal inhibition was enough to produce an AD-like behavioral deficit in this mouse model [61].

Decrease in proteasomal activity with aging has also been widely reported [62–65]. An age-related decrease in proteasome activity weakens cellular capacity to remove damaged proteins and favors the development of diseases [65]. It has been recently found that a transgenic mouse model exhibiting decreased chymotrypsin-like proteasome activity had a shortened lifespan [66]. These transgenic mice accumulate damaged and oxidized proteins and presented premature aging signs as well as aggravated age-related metabolic disorders [66]. Another example that links proteasomal activity with aging came from studies performed on the longest-lived rodent, *Heterocephalus glaber*, better known as naked mole rat. The lifespan of this organism is about 30 years and they remain healthy during the major part of their life. Analysis of the three different catalytic activities of the proteasome, in comparison to mice, revealed that there is a three- and sixfold increase in trypsin-like and chymotrypsin-like proteasome activity, respectively, which promotes a highly efficient protein turnover and clearance of misfolded and damaged proteins [67]. *Heterocephalus glaber*, as well as other species including *Homo sapiens*, accumulates with age an intracellular fluorescent yellowish pigment called lipofuscin, which is a general marker of aging and is resistant to removal by degradation [68, 69]. It is mainly composed by oxidized and crosslinked proteins and in a minor extent by lipids and sugars. Similar to the protein aggregates produced during neurodegenerative disorders, lipofuscin has also been shown to inhibit proteasome and reduce the rate of protein degradation [70].

Preserving a balanced proteasome activity during chronological aging might be an interesting strategy to elongate lifespan and prevent age-related degenerative disorders associated with protein misfolding. Many studies have been done using model systems to evaluate the effect in aging and disease of genetic manipulation of diverse components of the proteasome (Table 1). Ectopic expression of the non-ATPase subunit (Rpn11) of the 19S regulatory particle has been shown to maintain the integrity of the proteasome and to suppress polyglutamine induced toxicity in *Drosophila melanogaster* [15]. Furthermore, Rpn11 overexpression in the adulthood was enough to significantly extend the mean lifespan [15]. Similar extension of lifespan was observed when Rpn6 subunit expression was elevated in a mutant form of the nematode *Caenorhabditis elegans* [16]. When overexpressed in wild type worms, Rpn6 had a positive effect in lifespan under mild stress. Conversely, silencing the same subunit resulted in a decreased longevity and less resistance to stress conditions [16]. The same group also reported that the homolog of RPN-6 subunit in *Homo sapiens*, PSMD-11, is naturally overexpressed in human embryonic stem cells (hESC) that do not exhibit replicative senescence, leading to a high level of proteasomal activity [71]. It seems that the PSMD11/RPN6 subunit stabilizes the interactions between the 20S and 19S proteasome resulting in a higher efficiency of proteasome assembly [72]. Taken together this evidence strongly suggests that there is a fundamental role of proteasome activity in aging and degenerative diseases associated with protein misfolding.

6. Autophagy and Its Role in PMDs and Aging

Besides of the UPS, autophagy is another clearance mechanism to degrade damaged organelles and proteins [73, 74]. It involves the lysosomal degradation system and is implicated in multiple conserved pathways that regulate metabolism and longevity [75, 76]. Normally, it is activated under stress conditions, e.g., starvation, as a protective mechanism to ensure survival and cellular homeostasis by protein turnover [77]. Autophagy is classified in three different types according to the mechanism used for the capture and degradation of substrates: chaperone mediated autophagy (CMA), microautophagy, and macroautophagy [74]. In CMA, proteins that contain the pentapeptide KFERQ are recognized by the chaperone heat shock cognate protein 70 and transported to the lysosome for its hydrolysis [78]. Microautophagy refers to a process in which some portions of the cytosol are trapped directly by the lysosome without the intervention of chaperones [79]. Macroautophagy involves sequestration of damaged organelles or large protein aggregates into cargo vesicles known as autophagosomes that transport the contents to the lysosome for its degradation [80]. Although autophagy is considered to be an adaptive process, current studies suggest that a basal level of autophagy is always active and is involved in protein quality control [81–83].

A crucial role for autophagy in neurodegenerative disorders associated with protein misfolding has been recently recognized [84]. Accumulation of autophagic vacuoles has been found in different inherited forms of neurodegenerative diseases [85, 86]. Aggregation prone proteins related to AD (tau), PD (α-synuclein), and HD (polyQ-expanded huntingtin) are known substrates for autophagy. Furthermore, enhanced autophagy has been shown to reduce polyQ-expanded huntingtin aggregates and toxicity in different models including cells, *Drosophila*, and mice [87–90]. Inhibition of autophagy has also been reported to exacerbate protein aggregation and toxicity in these models. Similar results were obtained in *Drosophila* overexpressing AD specific mutant of tau, strengthening the involvement of autophagy in the clearance of disease specific protein aggregates [87]. In a mouse model of AD (expressing human Aβ), heterozygous deletion of beclin 1 (a protein that participates in the regulation of autophagy) resulted in a reduction of autophagy which, in turn, generated an exacerbated AD pathology, including extra- and intracellular Aβ deposition and neurodegeneration [91]. Partial recovery of autophagy by lentiviral administration of beclin 1 reduced the AD pathology. Similarly, improved clearance of Aβ aggregates was observed in mouse model of AD when autophagy was stimulated by administration of the antihistamine drug

Table 1: Genetic modulation of proteasome in different models and its effect on aging and disease.

Subunit deficiency/overexpression	Function	Phenotype	Model	Reference
Rpt2 inactivation	ATPase	Ubiquitin and α-synuclein positive Lewy like intraneural inclusion in neurons and neurodegeneration	*Mus musculus*	[59]
Rpt3 inactivation	ATPase	TDP43, FUS accumulation, basophilic inclusion bodies in neurons, locomotor impairment, loss of neurons	*Mus musculus*	[60]
β5t deletion	Chymotrypsin-like proteolytic activity	Shortening of lifespan, accumulation of polyubiquitinated and oxidized proteins, aggravated age-related metabolic disorder	*Mus musculus*	[66]
Rpn11 overexpression	Deubiquitination of the proteasome substrate	Extension of lifespan, suppression of polyQ induced toxicity	*Drosophila melanogaster*	[15]
Rpn6 overexpression	Stabilizing the interaction between CP and RP	Extension of lifespan under mild stress condition	*Caenorhabditis elegans*	[16]

Latrepirdine [92]. Even in the absence of any disease specific proteins, central nervous system specific reduction of autophagy by conditional deficiency of *Atg7* (autophagy target gene 7) resulted in loss of pyramidal neurons in hippocampus, cortex, and Purkinje cells in the cerebellum [93]. Similar results were obtained by other groups when they genetically reduced autophagy by selective deficiency of *Atg5* or *Atg17*/FIP200 in the neurons [94, 95]. The mechanism that distinguishes nutrient dependent adaptive autophagy from basal autophagy, involved in protein quality control, is still a mystery. HDAC6 (ubiquitin-binding deacetylase, histone deacetylase-6) was identified as the central component of basal autophagy which is not involved in the autophagy activation [96]. This protein appears to play an important role in the fusion of autophagosome to lysosome where the aggregated proteins are degraded. Interestingly, HDAC6 inactivation resulted in accumulation of protein aggregates and neurodegeneration [96]. Transport of autophagosomes to lysosome is governed by the dynein motor. Motor neuron disease specific mutation in the dynein has been shown to reduce removal of aggregated proteins. Furthermore, this mutation in dynein machinery enhanced mutant huntingtin aggregation and toxicity in fly and mouse models of HD [97].

Several studies reported reduced expression of different autophagy related genes, including Atgs, in aging [98], resulting in reduced autophagy and in turn accumulation of lipofuscin. Loss-of-function mutations in Atg1, 7, and 18 and beclin 1 have been shown to reduce lifespan in *C. elegans* [98]. The fact that even in case of normal human brain aging Atg5 and 7 and beclin 1 are downregulated suggests altogether a crucial role of autophagy in aging [98]. The first hint suggesting that an enhanced autophagy may increase lifespan came from the finding that caloric restriction, which elongates lifespan in almost all species tested, induces autophagy [99]. Even more importantly, prevention of autophagy abolishes the effect of caloric restriction in different experimental models. Analysis of the genes that are upregulated during caloric restriction showed that the LIPL-4 lipase in worms may have a role in the observed longevity extension by a mechanism that possibly activates autophagic response through the production of ω-6 polyunsaturated fatty acids [100]. Interestingly, ω-6 polyunsaturated fatty acids have been found to induce autophagy and increase lifespan even in conditions that do not resemble caloric response. Moreover, in human epithelial cells autophagy was activated when the medium was supplemented with ω-6 polyunsaturated fatty acids [100]. These results suggest that autophagy may be a crucial target for lifespan extension.

One of the evolutionary conserved pathways in eukaryotes that regulate autophagy is the target of rapamycin (TOR) serine-threonine kinase [101]. TOR can associate with distinct proteins and form two different complexes, TORC1 and TORC2. When TOR is active, it triggers anabolic processes that include increase in protein synthesis along with a reduction of autophagy [101]. Rapamycin, a compound discovered and isolated from a soil sample of the Chilean Easter Island, inhibits TORC1 signaling. It binds to the protein FKBP12, forming a complex that subsequently binds and inhibits TOR, leading to activation of autophagy [102]. Cumulative evidence coming from studies using rapamycin suggests a possible role of autophagy in longevity, aging, and neurodegeneration. A study performed in *C. elegans* demonstrated that rapamycin treatment as well as genetic knockdown of TORC1 signaling increased stress resistance and autophagy and had positive impact in health and lifespan [103]. The effect of rapamycin and caffeine on TOR inhibition and lifespan was also tested using *Schizosaccharomyces pombe* as a model organism. An increased longevity and decreased aging rate was observed with both compounds. However, caffeine seems to inhibit TOR at a transcriptional level rather than by a direct interaction with TOR [104]. Moreover, when encapsulated rapamycin was administered in the diet of 600-day-old mice, an extension in the median and maximal lifespan was observed [18, 105]. However, one

has to be cautious while interpreting the effect of rapamycin on aging. Rapamycin also suppresses inflammation, which has positive effect on lifespan. A recent study gives insight into the role of TORC1 in the regulation of autophagy and aggregated proteins in *Saccharomyces cerevisiae* [106]. Proteins that become insoluble with age are sequestered into autophagic bodies, visible by light microscopy in aged cells. By stimulation of the autophagic machinery, using nitrogen starvation, an increase was observed in the amount of these cargo vesicles [12]. Insoluble protein accumulation was observed when TORC1 was inhibited genetically and pharmacologically, suggesting that the protein transition to insolubility and sequestration in autophagic vesicles is an intermediate process before autophagic degradation and that is regulated by TORC1 [12]. However, it was also shown that insoluble protein accumulation is not necessarily dependent on autophagic activation, indicating that TORC1 regulates both processes using different mechanisms and that most likely they act together to eliminate damaged proteins [12].

7. Aggresomes and Their Role in PMDs and Aging

Aggresomes are cytoplasmic inclusion bodies that sequester aggregated proteins [106]. Formation of aggresomes appears to be a protective response when there is an excessive accumulation of misfolded proteins that cannot be cleared by canonical mechanisms like UPS or autophagy. Aggresomes may be formed as a transient mechanism to respond to impaired proteostasis under these conditions [107]. Damaged proteins are transported through the cytoskeleton to the centrosome or the microtubule organizing center with the help of accessory proteins, where aggregated proteins reach a high local concentration leading to the formation of aggresomes. It is believed that aggresomes act as a cytoprotective method preventing the interaction of aberrant proteins with normal cellular molecules. Evidence also indicates that there is recruitment of UPS and lysosomes, suggesting that aggresomes can be digested by these two proteostasis mechanisms [108–110].

To study the role of the aggresome formation in PMDs, a yeast model expressing polyQ polypeptides was developed. In this model when aggresome formation was prevented, there was an increased proteotoxicity supporting the view of a protective mechanism [111]. It also seems that there are recognition signals, like proline-rich domains, that may target some proteins to the aggresomes. Another signal that is believed to be involved in the formation of aggresomes is the ankyrin-like repeat in synphilin 1, a protein related to PD [112]. When proteasome inhibitors were administered to cells expressing synphilin 1, the formation of aggresomes was promoted, suggesting that proteasomal inhibition may be a signal that triggers aggresome formation [112].

When aggresomes are formed in mitotic cells, the distribution of the aggregates becomes asymmetrical upon cell division, causing one cell to "inherit" more damaged proteins than the other [113]. Apparently the aggresomes are segregated to the daughter cells through a mechanism involving the microtubule organizing center. It has been suggested that asymmetrical distribution of cellular components, causing one cell to receive more damage than the other, leads to differential aging [114]. A similar phenomenon was observed in *D. melanogaster* neuroblasts expressing the N-terminal fragment of human huntingtin that under experimental conditions formed aggresomes [115]. Neuroblasts divided to give rise to another neuroblast and a ganglion mother cell. Neuroblasts are short-lived cells that die during the embryogenesis process whereas ganglion mother cells divide in two cells that survive during all the fly lifespan. Analysis of the segregation of the aggresomes showed that the inclusion body was always inherited to the short-lived cell (neuroblast) and the ganglion mother cell did not receive the damaged proteins. These results suggest that the formation and segregation of aggresomes could have implications for the processes of cellular differentiation and aging [115].

8. Concluding Remarks

It is estimated that by 2050 there will be 2 billion people aged over 60 years old. Increased vulnerability of cells to physiological and environmental stress due to loss of protein homeostasis raises disease susceptibility with aging. Therefore the process of aging itself will greatly increase the onset of different diseases. All neurodegenerative diseases and most PMDs are strictly associated with aging, suggesting a link between protein misfolding and aging. Recent studies indicate that protein misfolding and aggregation of a widespread range of proteins naturally occur with time in different species. In case of diseases associated with protein misfolding, genetic mutations drastically increase the aggregation propensity of specific proteins, leading to accelerated accumulation of protein aggregates. Initially, the clearance machinery takes care of it. However, with time, the clearance capacity is compromised either due to aging or by a direct inhibitory activity of protein aggregates, resulting in the disruption of cellular homeostasis. This generates a death cycle in which protein misfolding promoted by aging defects leads to further damage of the clearance machinery, which in turn produces more accumulation of misfolded aggregates, getting to the point that these structures cause cellular toxicity, tissue dysfunction, and disease. Boosting up the clearance machinery by genetic and pharmacological tools showed beneficial effect on lifespan and protection against neurodegenerative disorders associated with protein misfolding in different animal models. It is important to keep in mind that an optimum activity of the clearance machinery is crucial to maintain steady-state level of different proteins in the cell. Therefore, an imprudent stimulation of clearance may be harmful as well. Thus, further studies are required to understand the specific mechanism of protein misfolding, the involvement of the clearance machinery, and the development of therapeutic strategies to combat the accumulation of misfolded protein aggregates and their beneficial effect in disease and aging.

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Review Article

ER Dysfunction and Protein Folding Stress in ALS

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Amyotrophic lateral sclerosis (ALS) is the most frequent paralytic disease in adults. Most ALS cases are considered sporadic with no clear genetic component. The disruption of protein homeostasis due to chronic stress responses at the endoplasmic reticulum (ER) and the accumulation of abnormal protein inclusions are extensively described in ALS mouse models and patient-derived tissue. Recent studies using pharmacological and genetic manipulation of the unfolded protein response (UPR), an adaptive reaction against ER stress, have demonstrated a complex involvement of the pathway in experimental models of ALS. In addition, quantitative changes in ER stress-responsive chaperones in body fluids have been proposed as possible biomarkers to monitor the disease progression. Here we review most recent advances attributing a causal role of ER stress in ALS.

1. Introduction

Several neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS), share common features, among them the presence of abnormal protein aggregates and the inclusions containing specific misfolded proteins. The presence of these abnormal protein aggregates has been temporally and spatially correlated with the activation of stress signaling pathway emerging from the endoplasmic reticulum (ER), a cellular reaction named the "unfolded protein response" (UPR). In the last years, ER stress levels and UPR activation in neurodegenerative diseases have been extensively studied. In this review, we focus on recent findings placing ER stress as a key component of neurodegeneration in ALS and discuss the different mechanisms by which the UPR may impact disease progression and the therapeutic potential of manipulating this signaling pathway in ALS.

2. Amyotrophic Lateral Sclerosis

ALS is a progressive and deadly adult-onset motoneuron disease characterized by muscle weakness, spasticity, atrophy, paralysis, and premature death [1, 2]. The pathological hallmark of ALS is the selective degeneration of motoneurons in the spinal ventral horn, most of brainstem nuclei, and cerebral cortex. ALS has an average age of onset around 50 years and estimated incidence of 1-2 cases per 100,000 individuals [1]. ALS is presently incurable with a mean survival time of 1–5 years from diagnosis, often resulting in fatal respiratory dysfunction. The majority of ALS patients lack a defined hereditary genetic component and are considered sporadic (sALS), while approximately 10% of cases are familial (fALS) [1]. The most common genetic causes of fALS are the recently defined hexanucleotide repeat expansion in the intronic region of *C9orf72* and the mutations in the gene encoding cytosolic superoxide dismutase 1 (*SOD1*), which together

account for around 50% of fALS cases. Many other disease-causative genes have been identified, including TAR DNA-binding protein (TARDBP or TDP-43), fused in sarcoma (FUS/TLS), vesicle-associated membrane protein-associated protein B (VAPB), among others [1, 3]. All of these mutations trigger the aggregation of the affected protein, which is associated in part with a gain of neurotoxic activity and possibly neuroinflammatory processes. Overexpression of human fALS-linked SOD1 and TDP-43 mutants in mice recapitulates essential features of the human pathology, provoking age-dependent protein aggregation, paralysis, motoneuron degeneration, and muscle atrophy (reviewed in [2, 4]). Studies in these mouse models of ALS have revealed valuable information about the molecular bases of the disease and, in particular, how the presence of these mutant proteins can trigger ER stress.

Since the same groups of neurons are affected in sALS and fALS leading to a similar pathology, it is predicted that therapies in mutant ALS genetic models may translate to sporadic ALS. In fact, accumulation of misfolded oligomers or protein inclusions containing wild-type (WT) TDP-43, FUS, or SOD1 has been recently shown to be a prominent histopathological feature of sALS (see examples in [5, 6]). Different pathogenic mechanisms have been proposed in ALS including neuroinflammation, glial activation, neuronal trafficking problems, excitotoxicity, mitochondrial dysfunction, and oxidative stress (reviewed in [2, 4]). Interestingly, accumulating evidence from several laboratories points towards a key role of alterations of protein homeostasis in the disease process, in both sALS and fALS (reviewed in [7–9]). In this context, ER stress is emerging as an interesting target for the development of prototypic treatments to ALS. In the next sections, we provide a comprehensive update of the work implicating ER stress to ALS pathogenesis.

3. ER Stress and UPR Signaling: An Overview

The ER is the first compartment where secreted and membrane proteins are synthesized and folded. For this process, a large and efficient network of chaperones, foldases, and cofactors are expressed at the ER to promote folding and prevent abnormal aggregation of proteins. The ER also operates as a major intracellular calcium store and plays a crucial role in the synthesis of lipids. A number of stress conditions can interfere with the function of this organelle and cause abnormal oxidative folding at the ER lumen, resulting in a cellular condition termed "ER stress" [10]. ER stress engages the unfolded protein response (UPR), an integrated signal transduction pathway that reestablish homeostasis by increasing the protein folding capacity and quality control mechanisms of the ER [11]. Conversely, chronic ER stress results in apoptosis of irreversibly damaged cells through diverse complementary mechanisms [12].

The UPR is activated by three main stress sensors, including PKR-like ER kinase (PERK), inositol-requiring transmembrane kinase/endonuclease (IRE1), and activating transcription factor 6 (ATF6). IRE1 is an ER located kinase and endoribonuclease conserved from yeast to humans. Upon UPR activation, IRE1 initiates the splicing of the mRNA encoding the transcriptional factor X-Box-binding protein 1 (XBP1), converting it into a potent activator of multiple UPR-responsive genes (termed XBP1s) [13–15]. XBP1s control the expression of genes involved in protein folding, secretion, protein quality control, and ER-associated degradation (ERAD) [16, 17]. IRE1α also regulates other signaling events including the downstream activation of JNK, modulating apoptosis and autophagy levels. In addition, IRE1 is able to degrade a subset of mRNA through its RNAse activity on a tissue specific manner (reviewed in [18]).

The activation of the stress sensor PERK reduces protein translation into the ER by phosphorylating eukaryotic initiation factor 2 alpha (eIF2α), which in turns contributes to decrease the misfolded protein overload [19]. The phosphorylation of eIF2α also allows the expression of activating transcription factor 4 (ATF4), a key factor that upregulates a subset of UPR-targeted genes involved in amino acid and redox metabolism, autophagy, protein folding, and apoptosis [20–22] (reviewed in [11, 23]). Among them, CHOP is a key mediator of apoptosis under ER stress [11, 23], which may operate by controlling the expression of several pro-apoptotic members of the BCL2 family of proteins (i.e., BIM and PUMA) in addition to GADD45 [24]. Sustained PERK signaling also contributes to apoptosis by enhancing oxidative stress and by resuming protein synthesis after prolonged ER stress [25–27].

ATF6 is activated at the ER and then translocates to the Golgi apparatus where it is processed, releasing the cytosolic domain that acts as a transcription factor [11]. ATF6 controls a subset of UPR-targeted genes related to protein folding and quality control mechanisms [28, 29]. Overall, UPR signaling responses integrate information about the nature and intensity of the stress stimuli to modulate the expression of a large spectrum of partially overlapping target genes that orchestrate adaptation to stress or trigger cell death programs [12].

4. ER Stress Signaling in sALS

The involvement of ER stress in sporadic ALS can be inferred from correlative studies in human postmortem tissue. Many reports have identified the upregulation and activation of the three main UPR signaling branches, in addition to the description of elevated levels of ER chaperones and cell death signals linked to ER stress [30–34] (see examples in Figure 1). Ilieva et al. showed enhanced phosphorylation of eIF2α and increased levels of the ER foldase PDIA1 along with elevated levels of oxidized proteins in spinal cord of sporadic ALS patients [32]. We also reported the upregulation of the ER foldase ERp57 in sALS and fALS, in addition to the expression of XBP1s and ATF4 [31]. Other groups also described the upregulation of CHOP in sALS [30, 33] (Figure 1(d)). In line with the aforementioned observations, augmented levels of PERK, ATF6, and IRE1 have been found [30] (Figure 1(c)).

Additional support for the importance of ER stress in ALS pathogenesis comes from ultrastructural studies [35, 36]. Oyanagi et al. detected distended and fragmented ER cisternae in the affected cells of the anterior horn of the spinal cord [35]. In a recent study, Sasaki observed an

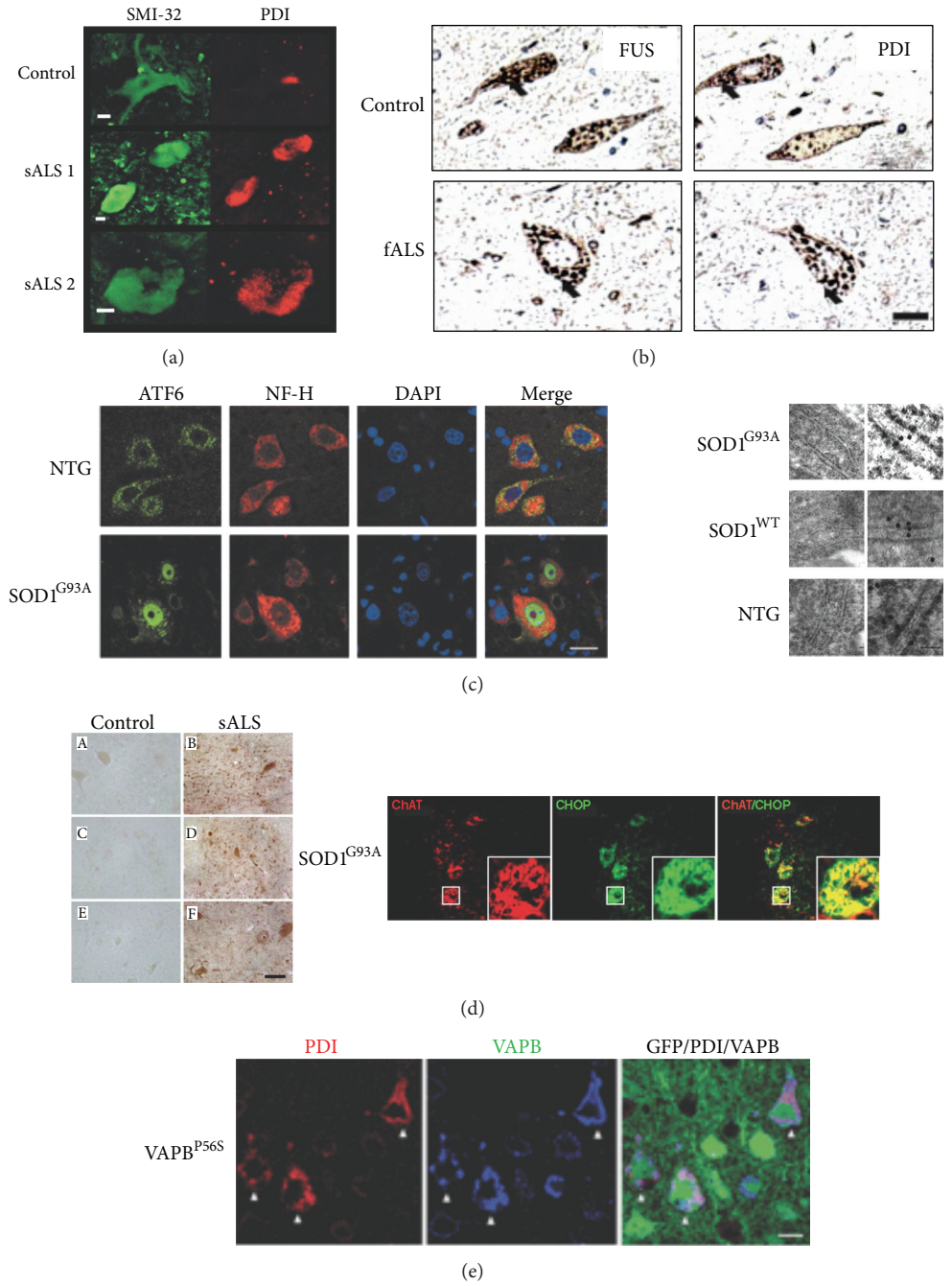

FIGURE 1: UPR activation in ALS human samples and mouse models of the disease. Several examples of published data indicating the activation of the UPR in SALS human samples and animal models. (a) Immunostaining of spinal cord motoneurons with the neurofilament marker SMI-32 showing PDIA1 (PDI) overexpression in samples from two sporadic ALS patients (sALS) compared with healthy subjects. Scale bar, 10 μm (from Atkin et al. [30]). (b) Immunohistochemistry of spinal cord section from a familial ALS patient (fALS) with a FUS mutation. The colocalization of FUS protein (left panel) and PDIA1 (PDI) protein (right panel) is indicated with black arrows. Scale bar, 40 μm (from Farg et al. [92]). (c) Left panel, immunodetection of the UPR sensor ATF6 (green), neurofilament (NF-H, red), and DAPI (blue) in spinal cord sections from SOD1^{G93A} mutant mice and nontransgenic control animals (NTG). Scale bar, 20 μm. Right panel, SOD1 protein detection in ER lumen by immunoelectron microscopy in SOD1^{G93A} mutant, SOD1 wild-type (SOD1^{WT}), and nontransgenic (NTG) mice. Scale bar, 50 nm (from Kikuchi et al. [38]). (d) Left panel, CHOP positive cells detected in spinal cord sections from human sporadic ALS (sALS) patient. Control tissue in (A), (C), and (E). Pictures derived from cervical spinal cord ((A) and (B)), thoracic spinal cord ((C) and (D)), and lumbar spinal cord ((E) and (F)). Scale bars, 65 μm. In the right panel, immunolocalization of CHOP (green) in anti-ChAT (red) positive spinal cord motoneurons from SOD1^{G93A} mutant mice. Scale bar: 40 μm. The areas with a box are shown at higher magnification. Scale bar 10 μm (from Ito et al. [33]). (e) Immunostaining of corticospinal motor neurons from 3-month-old VAPB^{P56S} transgenic mice. Transgene detected with GFP (green), PDIA1 (PDI) (red staining), and VAPB (blue staining). Arrowheads show neurons with accumulation of PDI and VAPB. Scale bar: 20 μm (from Aliaga et al. [50]). Copyright authorization was obtained from each journal for all images.

increased immunostaining for GRP78 (BiP) in affected but also normal-appearing motor neurons from sporadic patients [36]. Strikingly, a detailed examination of ER in normal-appearing motor neurons by electron microscopy revealed dilated ER lumen containing amorphous or granular material [36]. Additionally, ribosome-free membranous structures extending from the ER membrane, electron-dense material resembling Bunina bodies, Hirano bodies, and honeycomb-like structures were observed in patient samples only [36]. Together, these biochemical and morphological evidence correlate the development of ALS with the markers of ER stress.

5. UPR Activation in Experimental Models of ALS

Several laboratories have also shown the occurrence of ER stress in most cellular and animal models of fALS associated with mutations in FUS, TDP-43, SOD1, VAPB, and Ataxin-2 (see examples in [37–50]) (Figure 1). Moreover, in addition to ALS, disturbances in the function of the ER are thought to contribute to cell loss in a number of important human diseases including Parkinson's, Huntington's, and Alzheimer's disease [7, 51]. In this section, we discuss mostly *in vivo* validations of a functional involvement of ER stress in ALS.

In an elegant study from Caroni's group, a systematic transcriptomic analysis was performed using laser dissection of a group of neurons that die early (vulnerable motoneurons) during the course of the disease and a second group that is resistant in a mutant SOD1 model of ALS [52] (Figure 4). This study showed that only affected motoneurons of fALS mouse models were selectively prone to undergo early and chronic ER stress, which was the main molecular signature identified using gene expression profile analysis. Moreover, these changes were detected even before the earliest denervation in asymptomatic animals [52]. In support of this idea, several recent publications suggest that "stressful events" are occurring at the intracellular and intercellular level long before the locomotor defects and the protein aggregation are observed. For example, spinal cord neurons from neonatal SOD1 transgenic mice show hyperexcitability [53, 54], which would be one of the earliest abnormalities found so far.

In addition to UPR markers, Saxena et al. also observed that ALS vulnerable neurons specifically engage stress-management pathways such as protein ubiquitination and hypoxia-related genes, several weeks before this happens in resistant motoneurons [52]. Furthermore, activation of the UPR in vulnerable motoneurons coincides with the activation of microglia [52] (Figure 4(a)). It is unclear whether resistant motoneurons are protected due to differential disease stress inputs (differential degree of stress) or due to particular cellular mechanisms that generate increased resistance to cellular stress. In conclusion, regardless of the cause of motoneuron stress, it is becoming evident that modulation of protein folding stress or the proteostatic capacity of motoneurons may represent a potent therapeutic target to delay the symptomatic phase of ALS. In this context, the use of gene therapy or small molecules to reinforce the stress response capacity is becoming an interesting tool for disease intervention (Figure 4(c)).

In order to understand the contribution of ER stress and the UPR to ALS, many groups have manipulated UPR components and studied the evolution of the disease (Figure 2). The deficiency of the ER stress-related proapoptotic genes *ask1*, *puma*, or *bim* delays ALS in mouse models, possibly by rescuing motoneuron viability [37, 55, 56]. We investigated if deficiency of the transcription factor XBP1 could have an impact on ALS progression by crossbreeding a conditional knockout mouse for XBP1 in the nervous system [57] with transgenic mouse overexpressing mutant SOD1 [31]. Unexpectedly, despite predictions that deletion of this important UPR component would enhance the severity of experimental ALS (i.e., impaired adaptation to ER stress), we observed that the SOD1 mutant offspring that were knockout for XBP1 in the nervous system had delayed disease onset. These effects were associated with reduced accumulation of mutant SOD1 aggregates *in vivo* and in cell culture models and enhanced autophagy levels [31]. In agreement with this concept, we and others have recently reported that the pharmacological activation of autophagy can improve the survival and disease signs of mouse models of ALS, an effect associated with the clearance of abnormal protein aggregates [58, 59]. These findings can be contrasted with the unexpected results obtained from the treatment of the mutant SOD1 mice with another autophagy inductor, rapamycin, in which an accelerated progression of the disease was observed [60]. These results may be explained by the fact that the rapamycin target, mTOR (mammalian target of rapamycin), is involved in diverse cellular processes such as regulation of mRNA translation, cell metabolism, and inflammation, among others [61]. Despite these divergent results, autophagy represents an interesting target for future therapeutic development.

Other studies have validated a functional contribution of the UPR to ALS with unexpected results (Figure 2). Remarkably, a treatment of mutant SOD1 transgenic mice with salubrinal [52], a small molecule that enhances eIF2α phosphorylation [62], led to significant protection against experimental ALS progression [52]. Consistent with this report, *perk* haploinsufficiency ($perk^{+/-}$ mice) exacerbated the severity of experimental ALS, decreasing life span. This phenotype was associated with exacerbated neuronal loss and enhanced mutant SOD1 aggregation [43]. In this study, however, the loss of one *perk* allele did not decrease the induction of ATF4 at the early symptomatic stage and only partially reduced ATF4 levels at the end stage of the disease [43]. In agreement with this observation the levels of ATF-4 target genes, such *chop* and *bip* were not altered in $PERK^{+/-}/SOD1^{mutant}$ mice [43]. These studies suggest that the effects attributed to *perk* haploinsufficiency in ALS pathogenesis are mostly related to the inhibition of protein translation through eIF2α phosphorylation and not due to ATF4 induction.

We also have recently reported the impact of targeting the transcription factor ATF4 in ALS *in vivo* using a full knockout model. Unexpectedly, ATF4 deficiency reduced the probability of the birth of mutant SOD1 mice, suggesting

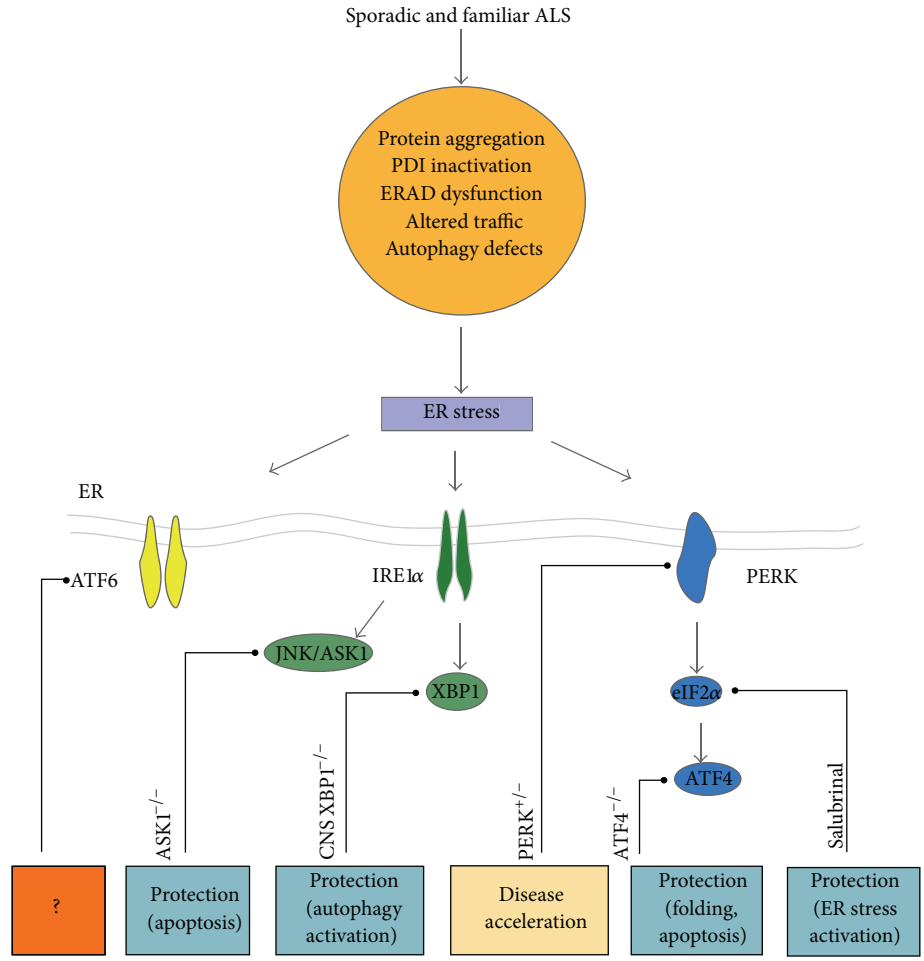

Figure 2: Targeting the UPR in ALS. Different factors may induce ER dysfunction in ALS. For example, abnormal protein aggregation/misfolding, PDIs inactivation by nitrosylation, ERAD dysfunction, altered vesicle traffic, and/or autophagy defects represent conditions that could induce ER stress and lead to an adaptive stress response known as the unfolded protein response (UPR) at early disease stages. The manipulation of different UPR components has revealed a functional contribution of distinct ER stress signaling events in preclinical models of ALS. Genetic targeting of ASK1 ($ASK1^{-/-}$), a downstream signaling component of IRE1α, protects against the development of experimental ALS decreasing motor neuron death in the spinal cord of mutant $SOD1^{G93A}$ mice [56]. The deletion in the CNS of the transcription factor XBP1 (CNS $XBP1^{-/-}$) increases the survival of the mutant $SOD1^{G86R}$ mice, associated with reduced accumulation of mutant SOD1 aggregates *in vivo* and enhanced autophagy levels [31]. PERK haploinsufficiency ($PERK^{+/-}$) enhanced the severity of experimental ALS, associated with elevated levels of neuronal loss and mutant SOD1 aggregation [43]. The deletion of the transcription factor ATF4 ($ATF4^{-/-}$) in the $SOD1^{G86R}$ mutant mice delays the appearance of the symptoms and the extended animal survival. These effects were associated to changes in the ER protein folding network and apoptotic genes [63]. In a pharmacological strategy, the treatment of mutant SOD1 mice with a small molecule that selectively induces eIF2α phosphorylation, salubrinal, protects against disease progression [52]. No manipulation of ATF6 in animal models of ALS has been described.

that the UPR may even contribute to mitigating pathological stress during development in this model [63]. On the other hand, the ATF4 knockout/mutant SOD1 transgenic mice that were born showed delayed disease onset and prolonged life span [63]. Consistent with the role of ATF4 in apoptosis, its deficiency completely ablated the induction of BIM and CHOP in mutant SOD1 mice, in addition to induced quantitative changes in the protein homeostasis network. Conversely, ATF4 deficiency enhanced mutant SOD1 misfolding at the end stage of the disease. Thus, PERK signaling may have differential and contrasting effects on ALS pathogenesis, in which eIF2α phosphorylation affords protection whereas ATF4 induction may trigger motoneuron apoptosis.

Although the activation of UPR has not been entirely described in animal models expressing TDP-43 mutant proteins [64], in a recent study, the use of drugs to alleviate ER stress showed significant protection against the neurotoxicity induced by mutant TDP-43 in worm and zebrafish models of ALS [65]. The treatment of these animal models with salubrinal or guanabenz, two drugs that sustain eIF2α phosphorylation by different mechanisms [62, 66], reduced toxicity and improved motility of worms and fishes expressing mutant

TDP-43 [65]. These results, together with those obtained from pharmacological intervention of eIF2α in mutant SOD1 mouse models, support the idea that ER stress is a main event in ALS. In summary, these studies illustrate the complex nature of UPR signaling in ALS, clearly demonstrating that targeting specific components of the pathway may have distinct consequences on disease progression [12]. These studies have identified some of the components of the UPR as a potential target to treat ALS.

6. A Role of the Glia and Oligodendrocyte UPR in ALS?

The extracellular environment can influence motoneuron fate in the context of ALS as depicted by the interplay between motoneurons and the glia. For example, it is possible to induce ALS pathology in mice overexpressing mutant SOD1 in nonneuronal cells [67]. In cellular assays, supernatant derived from astrocytes/motoneuron cocultures of mutant SOD1 transgenic mice can trigger neuronal death of wild-type neuronal cultures. The toxic factors released from mutant SOD1 primary cells are able to induce hyperexcitability and subsequent cell death [68].

Several studies have shown that the expression of mutant SOD1 in astrocytes or microglia regulates the progression of ALS (see examples in [69–71]). A recent study showed that UPR activation also takes place in these glial cells [72]. ER stress markers can be observed particularly in microglia even at early stages of the disease. These results support the idea that UPR may have a broad impact on noncell autonomous aspects of ALS [72].

Recent reports suggest that oligodendrocytes may also play a relevant role in ALS. Extensive degeneration was reported in the gray matter oligodendrocytes in the spinal cord of mutant SOD1 mice prior to the appearance of disease signs [73]. Similar results were observed in ALS human postmortem tissue [74]. Although new oligodendrocytes were formed, they did not mature and were unable to mediate remyelination. Of note, great advances have been obtained in understanding the role of ER stress in oligodendrocytes in models of multiple sclerosis, where inflammatory reactions trigger demyelination and motoneuron degeneration [75, 76]. IFN-γ-dependent activation of the PERK pathway in oligodendrocytes was protective in a mouse model of multiple sclerosis [77]. Moreover, salubrinal also protected against disease progression in the same model [78]. A recent paper confirmed the protective role of PERK pathway against cytotoxic events using a temporally controlled activation of PERK in oligodendrocytes of an experimental model of multiple sclerosis [79]. Similarly, we have recently reported a reduced locomotor recovery in ATF4 or XBP1 knockout models after a spinal cord injury. In addition, gene therapy to deliver active XBP1 into the spinal cord had a significant impact on motor recovery after spinal cord injury which was associated with enhanced oligodendrocyte survival [80]. This is an important finding considering the close relationship of glia and neurons and a possible coordinated/associated stress response between both cell types. These results support the notion that modulating the UPR in a non-cell autonomous manner may also represent an interesting strategy to attenuate ALS progression. This idea remains to be tested.

7. The PDI Family of Proteins and ALS

At the early stages of the UPR activation, the folding capacity of the ER is increased through the up-regulation of the ER chaperons such as BiP/Grp78, Grp94, calreticulin (CRT), calnexin (CNX), and several members of the protein disulfide isomerase (PDI) family [81]. These events reduce ER stress levels by enhancing the folding capacity of the ER or by removing terminally misfolded proteins through ER-associated degradation (ERAD) [82]. In the last years, the role of ER resident chaperons and foldases, in particular some members of the PDI family, has gained an important place in the ALS field. Here we discuss most relevant data revealing a participation of these proteins in the ALS.

A recent genetic screening revealed associations of PDIA1 intronic variants as a risk factor to develop ALS [83]. However, no mechanistic studies were provided to determine the possible impact of these genetic alterations on the disease. PDIs are a large protein family comprised of 21 known members of the thioredoxin superfamily, classified based on sequence and structural homology (reviewed in [84]). Most PDIs have a foldase function and catalyzed disulfide bond formation and, as we will discuss later, can also inhibit protein aggregation and modulate cell viability. Of note, several PDI family members have been involved in neurodegenerative disease such as Parkinson's disease, Alzheimer's Disease, prion-related disorders, and Huntington's disease (review in [85]). Importantly, a proteomic analysis of spinal cord tissue of mutant SOD1 mice reporting PDIA1 and ERp57 (also known as Grp58 or PDIA3) as major up-regulated proteins was the first study suggesting a possible participation of PDIs in ALS [86]. These results were later confirmed by independent study [87].

Mutant SOD1 has been shown to accumulate in the ER *in vivo* [38, 86]. In addition, the translocation of SOD1 to microsomal fractions has been reconstituted *in vitro* with purified components [88]. Mutant SOD1 is also secreted to the extracellular space through a classical Golgi-dependent mechanism [41]. Atkin et al. reported a physical interaction between the wild-type and mutant SOD1 and PDIA1 *in vivo* [86]. They also showed a colocalization of PDIA1 with mutant SOD1 inclusions. This was also observed in spinal cord samples from ALS patients [32, 89]. Similarly, mutant SOD1 was shown to interact with the ER chaperone BiP in the spinal cord of mutant SOD1 transgenic mice [38]. At the functional level, PDIA1 overexpression in cell culture reduced mutant SOD1 aggregation, ER stress, and also induced cell death [90]. In contrast, the inhibition of PDI with the antibiotic bacitracin [91] increased mutant SOD1 inclusions [86], suggesting that PDIA1 prevents the formation of SOD1 aggregates. Similarly, TDP-43 positive inclusions have been shown to colocalize with PDAI1 in sALS samples [89]. ALS-linked FUS mutant has been also shown to induce ER stress, colocalizing with PDIA1 in cell culture and spinal cord tissue from sALS and fALS cases, in addition to animal models of the disease [92]. Moreover, a physical association between

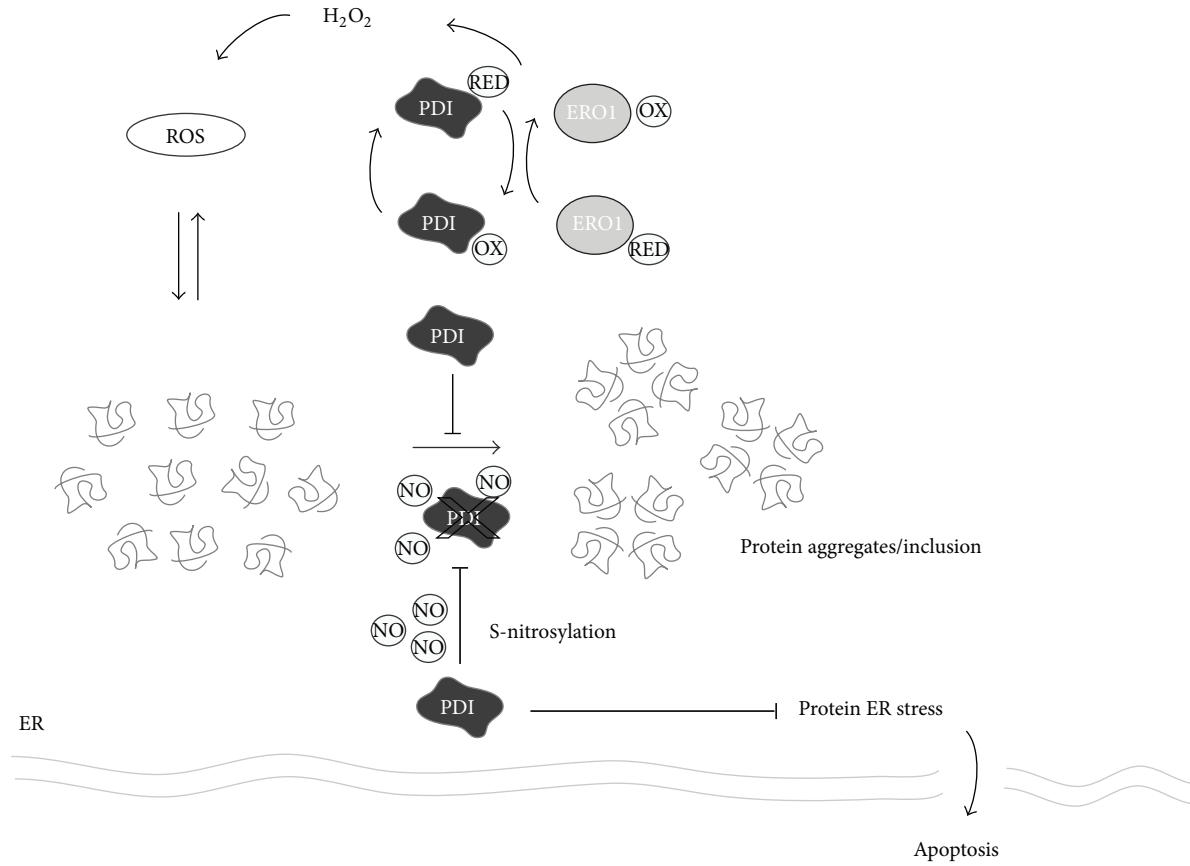

Figure 3: Possible role of protein disulfide isomerases (PDIs) in ALS. PDIs are up-regulated in diverse models of ALS in addition to tissue from patients. They also have been suggested as possible biomarkers to monitor disease progression using body fluids. PDIA1, in the figure PDI, colocalized with protein inclusions containing FUS, TDP43 or SOD1 in human tissue and mouse models of ALS. The exact contribution of PDIs to ALS is currently a matter of debate. PDIs could have a protective role through decreasing protein aggregation and global ER stress. S-nitrosylation appears to inactivate PDI and contribute to ER stress. Reactive oxygen species (ROS) produced from redox folding (disulfide bond formation) may also contribute to the generation of protein aggregates.

mutant FUS and PDIA1 was reported [92]. It is still unknown if the manipulation of PDI levels will a_ect the progression of experimental ALS *in vivo*.

Modification and inactivation of PDIA1 were also reported in spinal cord tissue from sALS and mouse models of the disease [90]. Similar observations were also described before in brain tissue derived from Parkinson's and Alzheimer's disease patients [93]. It was proposed through cell culture studies that PDI nitrosylation may contribute to the disease by inhibiting the protective roles attributed to these foldases. This abnormal modification of PDI could result from altered nitric oxide synthase activity found in mouse models of the disease [94]. Although PDIs are thought to have a neuroprotective activity, one report suggested that PDIA1 and ERp57 may actually have a pro-apoptotic activity in models of Alzheimer and Huntington's disease [95]. Accordingly, UPR activation in microglia correlated with an increase of PDIA1 protein and neurotoxicity [72]. These data suggest that future therapeutic manipulation of the UPR should examine in more detail its impact on glial cells.

The formation of disulfide bonds by PDIs inside the ER requires specific redox conditions and fine balance between the oxidized and reduced states of PDIs [96–100]. The ER is an extremely oxidizing environment compared with the cytoplasm, and the maintenance of its redox state relies on PDI activity of the formation of the disulfide bonds. The generation of disulfide bonds is highly regulated and involves the enzyme ERO1, which is an important oxidase for disulfide formation [101]. The perturbation of the redox status of the ER is deleterious for the proper cell function and there are tight mechanisms to buffer the possible redox fluctuations [102]. We have recently described that ATF4 deficiency alters the redox status of the cell and also the ER as measured by monitoring H_2O_2 levels, a subproduct of the PDI/ERO1 cycle [63]. Of note, the treatment of motoneuron cells with the antioxidant trolox is able to revert the enhanced aggregation of mutant SOD1 observed after knocking down ATF4. In addition, overexpression of ERO1 also modulated mutant SOD1 aggregation [63], suggesting that the manipulation of ER redox state can impact the misfolding of mutant SOD1. Taken together, these data suggest that PDIs may play a significant role in ALS by affecting different aspects of cell physiology including protein aggregation, cell survival, and the redox status of the ER (Figure 3).

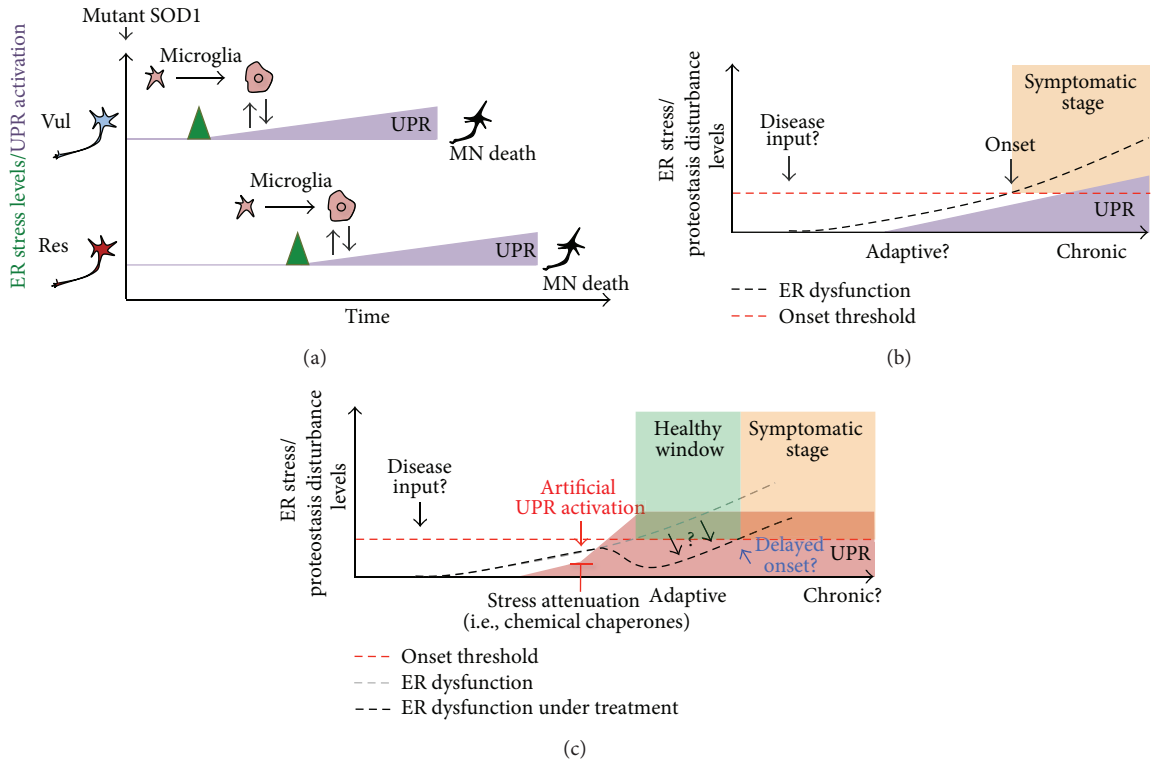

Figure 4: ER stress and the selective neuronal vulnerability in ALS. (a) Schematic representation of ER stress levels (green label) and UPR activation (purple label) in the two subgroups of neurons that have been identified in mutant SOD1 mouse models of ALS: one population that dies early (vul, vulnerable, blue) and another that dies later during disease progression (res, resistant, red). Activation of stress markers is a common feature detected in Vul and Res motoneurons. However, vul motoneurons express these stress markers earlier than de res neurons. The UPR is activated in both subgroups of neurons. UPR activation also correlates with microglial activation in both groups. It is not known what determines the resistance of Res cells in the disease. (b) Time-course of ER stress levels and UPR activation in familial ALS models. ER stress and protein disturbance increase during ALS progression ("ER dysfunction," black dashed line). During the presymptomatic stage of the disease, UPR activation might represent an adaptive response that attenuates ER stress levels. Over time, the stress condition exceeds the capacity of the cell to manage protein folding stress and pro-apoptotic pathways are activated. This shift "onset threshold" in UPR signaling regulation could be associated with motoneuron dysfunction/loss and the onset of the disease. During the symptomatic stage, a strong and chronic UPR activation occurs. (c) Possible therapeutic approaches to modulate the UPR in ALS. An earlystage preventive treatment may modulate UPR levels to enhance the adaptive capacity of motoneurons and reduce ER stress levels or other proteostasis disturbances. This may delay disease onset and disease evolution "healthy window". The therapeutic approaches include gene therapy to deliver active UPR components and the use of smallmolecules that selectively activate specific UPR signaling branches (pharmacologic approaches) or act as chemical chaperones to alleviate global ER stress. This reduction in ER stress levels in motoneurons could also be achieved by modulating glial UPR.

8. ER Stress Signaling in sALS: Novel Biomarker for Disease Prognosis?

Early studies have shown that several ER chaperones can be secreted to the extracellular space upon stress [103]. Recently, PDIA1 levels have been reported to be up-regulated in the cerebrospinal fluid (CSF) of ALS patients [30]. Interestingly, Vijayalakshmi et al. showed the induction of ER stress in spinal motor neurons exposed to CSF of sporadic ALS patients [34]. This fact suggests that measuring stress factors in CSF may represent an interesting tool to monitor ALS disease progression. There is a current need for biomarkers of ALS to assess, on a quantitative manner, disease prognosis and the efficacy of clinical trials.

In a recent proteomic screening searching for biomarkers in blood samples from sALS patients, the up- regulation of the ER stress-responsive chaperones PDIA1, ERp57, and other chaperones was observed [104]. Similar changes were also seen in mononuclear cells from blood of mutant SOD1 mice. It was demonstrated that TDP-43, cyclophilin A, and ERp57 are strongly associated with disease course in a longitudinal study in ALS patients and control subjects, ERp57 having the best score [104]. These two studies open the interesting possibility of monitoring stress signatures to diagnose and monitor progression of ALS.

9. Perspective

ER dysfunction is currently viewed as a relevant factor driving diverse diseases of the nervous system, representing an important niche for drug discovery. Due to the fact that the type, intensity, and temporality of ER stress stimuli determine

how the UPR integrates information towards controlling cell fate, this pathway offers interesting targets to modulate both cell survival and death mechanisms. Depending on the disease context, targeting strategies may involve attenuation of ER stress levels, inactivation of pro-apoptotic components of the UPR, or the enhancement of UPR signaling responses toward adaptation to stress (Figure 4). The scenario in ALS is very complex. Genetic and pharmacological manipulation of the pathway in preclinical models of the disease supports the idea that the UPR may contribute to both cell viability of stressed cells and also the elimination of motoneurons when there is irreversible damage. More research is needed to understand the consequence of manipulating the UPR to validate the pathway as a target. For such step, it is essential to define the optimal targets to alleviate ER stress in ALS. Importantly, it is becoming clear that sporadic and familial ALS, regardless of the specific genetic alteration, may converge into alterations on ER function, offering unique therapeutic opportunities. The fact that mutations in PDIA1 gene were recently described in ALS patients suggests a causative role of proteostasis defects at the ER. Supporting this notion, mutations in two important proteins involved in the degradation of misfolded proteins, Ubiquilin1 [105] and p62 [106], have been found in ALS cases. Predicting and defining the possible side effects of manipulating the UPR at the systemic levels remains an important subject for future validation of the pathway as a drug target and move forward into the development of human therapies.

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Review Article

Small-Molecule Theranostic Probes: A Promising Future in Neurodegenerative Diseases

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Prion diseases are fatal neurodegenerative illnesses, which include Creutzfeldt-Jakob disease in humans and scrapie, chronic wasting disease, and bovine spongiform encephalopathy in animals. They are caused by unconventional infectious agents consisting primarily of misfolded, aggregated, β-sheet-rich isoforms, denoted prions, of the physiological cellular prion protein (PrP^{C}). Many lines of evidence suggest that prions (PrP^{Sc}) act both as a template for this conversion and as a neurotoxic agent causing neuronal dysfunction and cell death. As such, PrP^{Sc} may be considered as both a neuropathological hallmark of the disease and a therapeutic target. Several diagnostic imaging probes have been developed to monitor cerebral amyloid lesions in patients with neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, and prion disease). Examples of these probes are Congo red, thioflavin T, and their derivatives. We synthesized a series of styryl derivatives, denoted theranostics, and studied their therapeutic and/or diagnostic potentials. Here we review the salient traits of these small molecules that are able to detect and modulate aggregated forms of several proteins involved in protein misfolding diseases. We then highlight the importance of further studies for their practical implications in therapy and diagnostics.

1. Introduction

Neurodegenerative diseases are a medical, social, and economic problem of paramount importance in developed countries. Besides the fact that their etiology is generally unknown, developing therapeutic and diagnostic interventions for diseases of the central nervous system (CNS) is further complicated by the impermeability of the blood brain barrier (BBB). Thus, Alzheimer's disease (AD) and prion diseases are still not curable with drugs, and only in 2012 [1–3] positron emission tomography (PET) imaging probes have been included in the AD diagnostic armamentarium.

In recent years, the close cooperation between drug delivery/treatment and molecular imaging disciplines has resulted in a relatively new branch of knowledge, known as theranostics. The term theranostics was coined to indicate the concomitant therapeutic and diagnostic properties in a single agent. The purpose of theranostics is to optimize the efficacy and safety of therapy, as well as to streamline the entire drug development process. Several exciting examples of theranostic systems have now been reported in the literature for the treatment of cancer [4], atherosclerosis [5], and gene delivery [6], but very few examples are reported in the neuropathological field, especially in the prion field. In our recent work [7] we detailed the development of a small molecule with fluorescent properties that is able to simultaneously detect and inhibit Aβ and PrP^{Sc} plaques in *in vitro* studies. The progress to date in the design and utilization of these compounds is discussed herein.

2. Human Prion Diseases

The term prion (pronounced "pree-on") is the acronym for proteinaceous infectious particle. The prion hypothesis was put forward in 1982 to explain the surprising transmission mechanisms of this unconventional protein [13, 14]. The discovery that proteins can behave like infectious agents to transmit disease is a milestone in biology. In fact, what sets prions apart, as proposed by Prusiner [14], is that the actual infectious principle consists merely of protein and is capable

of replicating and transmitting infections without the need for informational nucleic acids. Over the past decade, there has been renewed interest in proteins causing neurodegeneration since they may all act as prions (i.e., amyloid-β, α-synuclein). This hypothesis has profoundly influenced the development of diagnosis methods and effective therapies for the corresponding diseases.

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), occur in both humans and animals. Prion diseases are a group of rapidly progressive disorders characterized by a defined spectrum of clinical abnormalities. The number of human and animal diseases recognized as TSEs has increased steadily in recent years. They all share similar hallmarks such as the spongiform degeneration of the brain and variable amyloid plaque formation (PrP^{Sc}). In fact, PrP^{Sc} is the disease-associated isoform of the endogenously expressed prion protein (PrP^{C}), which may be present as amyloid deposits. The first cases of human prion disease, Creutzfeldt-Jakob disease (CJD), were reported in the 1920s [15, 16]. Since then, different forms of human TSEs have been described that can appear as sporadic, inherited, or iatrogenic disorders; they include CJD, Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), and kuru. In animals, several TSEs have been reported, including scrapie in goats and sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and transmissible mink encephalopathy.

Here we review some crucial points of human prion disorders.

2.1. CJD. CJD presents as a sporadic, hereditary (familial), or acquired (iatrogenic or BSE-related) illness. Approximately 85% of all CJD cases occur sporadically, geographically, and ubiquitously, with an incidence rate of 0.5–2 cases per one million people per year. The median age of onset is the seventh decade (64 years) equally affecting men and women [17]. The clinical progression typically occurs over a few weeks. Around 70% of those afflicted die in less than 6 months. Typically, CJD presents with progressive dementia and cerebellar degeneration, characterized by spongiosis, neuronal loss, astrogliosis, and a clinical syndrome accompanied by dementia, memory loss, ataxia, and myoclonus. Early symptoms, present in approximately one-third of the cases, include fatigue, insomnia, depression, weight loss, headaches, general malaise, and ill-defined pain sensations. In addition to mental deterioration and myoclonus, frequent additional neurological features include extrapyramidal signs, cerebellar ataxia, pyramidal signs, and cortical blindness [18]. Homozygosity for methionine (Met) or valine (Val) at position 129 of the human PrP gene (*PRNP*) has been identified as a predisposing factor in the majority of sporadic and iatrogenic CJD cases [19, 20].

2.2. Familial CJD. Familial CJD (fCJD), representing 5–15% of all CJD cases, is classified into many haplotypes based on *PRNP* mutations present in the open reading frame and codon 129 on the mutant allele [21]. The majority of fCJD cases (>70%) have been associated with codon 200 mutations (E200K) [22–24] or with a codon 178 mutation (D178N) in the *PRNP* gene [25–27]. The symptoms of the familial form of CJD vary depending on the type of PrP mutation involved [28].

2.3. Iatrogenic CJD. Iatrogenic CJD (iCJD) is a very rare disease resulting from neurosurgery, corneal grafting, human dura mater implants, and the use of human growth hormone (hGH) and pituitary derived gonadotropin (hGNH). Iatrogenic CJD was first recognized in 1974 in a US patient who received a corneal transplant from a donor later proven to have died from CJD [29]. Worldwide, at least 226 cases of iCJD, including 26 US cases, have been associated with administration of contaminated human growth hormone (hGH) from cadavers. Of 74 UK cases reported from 1979 till 2011, 65 individuals received human-derived growth hormone, the other 8 individuals received infected dura mater implants, and one case of iCJD was reported after receiving human gonadotropin. In April 2013 one case of probable iCJD was reported (http://www.cdc.gov/eid) after treatment for 23 months with commercial cadaveric hGH when the patient was 6 years old. At the age of 33, 26.5 years (range 25.5–28 years) after the midpoint of commercial cadaveric hGH treatment, dizziness and gait imbalance developed, causing a fall. Seven months after the fall, he entered a state of akinetic mutism; he died 9 months after symptom onset. A small number of additional cases, known as variant CJD, are caused by secondary infection transmitted by transfusion of blood products. No new sources of disease have been identified, and current practices, which combine improved recognition of potentially infected persons with new disinfection methods for fragile surgical instruments and biological products, should continue to minimize the risk for iatrogenic disease until a blood screening test for the detection of preclinical infection is validated for human use [30].

2.4. Variant CJD. In 1995 and early 1996, a small number of remarkably young CJD patients were diagnosed in the United Kingdom. Due to its similarity to sCJD, this human disease was termed new variant of CJD (vCJD) [31]. In contrast to sCJD, the median age of onset of the disease in vCJD patients is 28 years (sCJD 64 years) and the clinical course is prolonged (median 14 months, sCJD 6 months). The appearance of vCJD in the United Kingdom and the experimental evidence that vCJD is caused by the same prion strain responsible for BSE raised the possibility of a vCJD epidemic [32, 33]. Laboratory transmission studies in transgenic mice showed that the characteristics of vCJD, including incubation period and neuropathological changes, are very similar in BSE and vCJD [34]. The favored hypothesis for transmission of BSE to humans is a dietary exposure to prion-contaminated bovine tissue (likely CNS) in the 1980s [35]. Variant CJD is difficult to distinguish from other neurological disorders, hence a definitive diagnosis has relied on neuropathology. It has been shown that vCJD can be diagnosed by PrP^{Sc} immunostaining on a tonsil biopsy [36]. The majority of vCJD cases have been recognized in individuals homozygous for Met at codon 129 in the *PRNP* gene [37]. However, Peden et al. reported a case of a patient who was heterozygote at

codon 129 of PRNP, suggesting that susceptibility to vCJD infection is not confined only to the Met homozygous PRNP genotype [38]. The human genotype at codon 129 of the *PRNP* gene is known to be a key determinant in human TSEs. This polymorphism modulates phenotype and disease susceptibility to acquired or sporadic prion infection [39]. The large majority of individuals affected by prion diseases are homozygous at codon 129 for either Met or Val [19, 20, 40]. The prevalence of Met/Met is only 39% in the normal Caucasian population, whereas the frequency for Met/Val is about 50% and for Val/Val 11% [19]. In some reports the protective effect of *PRNP* codon 129 heterozygosity is seen in some of the inherited prion diseases [41, 42].

2.5. GSS. GSS is a rare form of prion disease and occurs at a rate of one per 100 million people per year worldwide [43]. In contrast to CJD, GSS is almost always described in a familial context. Only a few sporadic cases resembling GSS have been reported so far [44]. The syndrome was first described in 1928 by the Austrian neurologist Josef Gerstmann (1887–1969), followed by a more detailed report in collaboration with his colleagues Ernst Sträussler and Ilya Scheinker [45]. Most patients show the first symptoms in the fourth or fifth decade of life. Investigations have shown that missense mutations are present in the *PRNP* gene of GSS patients. To date, a variety of mutations have been identified, and the most common is at codon 102 (P102L) [46]; others reported are 105 (P105L) [47], 114 (G114V), 117 (A117V) [48], 131 (G131V) [49], 180 (V180I), 187 (H187R), 198 (F198S) [50], 202 (D202N), 212 (Q212P), and 217 (Q217R) [42]. The STOP mutations reported are Y145STOP-129 M [51, 52], Q160STOP, Y226STOP, and Y227STOP [53] (Figure 1). In addition, several insertional mutations have been described that occur in the N-terminal octapeptide repeat region of PRNP [54, 55].

2.6. FFI. Fatal familial insomnia (FFI) was first described in 1986 in a 53-year-old man [56]. Since then, it has been reported in several European countries [57–59], Australia [60], and Japan [61]. The occurrence of FFI is associated with the same codon 178 mutation (D178N) also observed in a subtype of familial CJD [62]. The phenotype caused by the D178N mutation depends on a polymorphism at codon 129. The Met 129-Asn 178 allele segregates with FFI, while the Val 129-Asn 178 allele segregates with fCJD [25]. Recently, the first cases of a sporadic form of fatal insomnia (sFI) have been reported in a 44-year-old man and a 58-year-old woman [63–65]. FFI and sFI have similar disease phenotypes. Both disorders have clinical features of disrupted sleep (loss of sleep spindles, slow-wave sleep, and enacted dreams during rapid-eye-movement sleep), autonomic hyperactivation, and motor abnormalities (myoclonus, ataxia, dysarthria, dysphagia, and pyramidal signs). PET shows pronounced thalamic and limbic hypometabolism that become more widespread in later stages. Neuropathological assessment reveals severe neuronal loss and astrogliosis of the anterior medial thalamus and inferior olives, with later cerebral cortical and cerebellar involvement [66].

2.7. Kuru. Kuru ("trembling with fear") is the prototype of human spongiform encephalopathy. It is restricted to the Fore people living in the Eastern Highlands of New Guinea, where prions were transmitted by ritualistic cannibalism [67]. The disease occurred mostly in children and women, because they consumed the brain of deceased family members. In 1959, the local government banned the cannibalistic practice.

3. Prion Conversion: The "Protein-Only" Hypothesis

The central molecular event in the replication of mammalian prions is the self-propagating conformational conversion of PrP^{C} to the misfolded PrP^{Sc} form. This postulate is known as the "protein-only hypothesis" [14]. In recent decades several efforts have been made to understand the mechanism of PrP^{C} to PrP^{Sc} conversion. Two models have been proposed, known as (i) template-directed refolding model and (ii) seeded-nucleation model.

(i) The template-directed refolding model postulates a direct interaction between PrP^{Sc} and PrP^{C}, which is induced to convert into more PrP^{Sc}. A high-energy barrier might prevent the spontaneous conversion of PrP^{C} to PrP^{Sc}. In this model the critical step in the conversion is the formation of a dimer between PrP^{Sc} and PrP^{C} or a partially destabilized folding intermediate of PrP^{C} denoted by PrP^{*}. Eventually PrP^{Sc} acts as a template that catalyzes the refolding of PrP^{C} to a thermodynamically more stable PrP^{Sc} conformation (Figure 2(a)).

(ii) The "seeding" or nucleation-polymerization model states that PrP^{C} and PrP^{Sc} are in a reversible thermodynamic equilibrium. So, only if several monomeric PrP^{Sc} molecules (less stable than PrP^{C}) are mounted in a highly ordered seed can more monomeric PrP^{Sc} be recruited and eventually aggregated to form amyloid. In such a crystal-like seed, PrP^{Sc} becomes stabilized. The rate-limiting step in this mechanism is not the conformational conversion itself but the nucleation step. Fragmentation of PrP^{Sc} aggregates increases the number of nuclei, which can recruit more PrP^{Sc} and thus seems to replicate the agent. In sporadic prion diseases, fluctuations in the local PrP^{C} concentration might (exceptionally rarely) trigger spontaneous seeding and self-propagating prion replication (Figure 2(b)).

In this transition, the primary structure of PrP does not change, but the secondary and tertiary structures in PrP^{Sc} are considerably different from those in PrP^{C}.

3.1. Physiological Functions of PrP^{C}. It is still unclear whether the toxicity of PrP^{Sc} represents a gain of function [68] or whether loss of function of PrP^{C} is responsible for neuropathological changes induced by prions [69]. One thing is certain—PrP^{C} has to be expressed in CNS to permit the

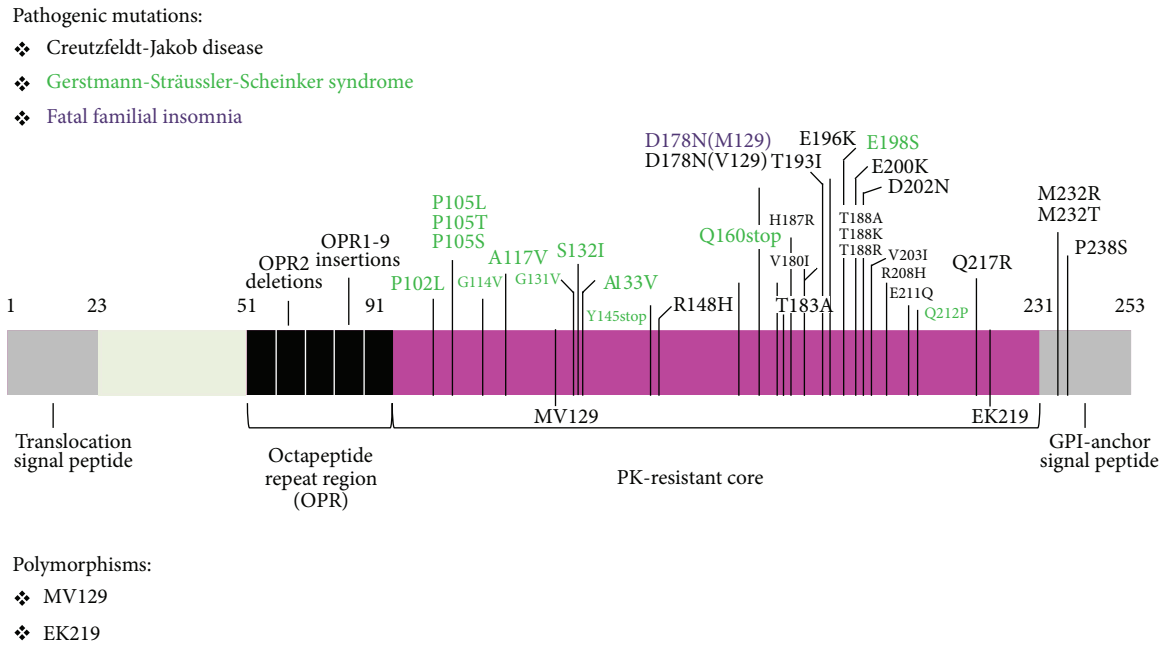


Figure 1: Pathogenic mutations and polymorphisms in the human PrP. The pathogenic mutations associated with human prion diseases are shown above the human PrP coding sequence. These consist of 1, 2, or 4–9 octapeptide repeat insertions (OPR1-9) within the octapeptide repeat region between codons 51 and 91, a 2 octapeptide repeat deletion (OPR2), and various point mutations causing missense or stop amino-acid substitutions. Point mutations are designated by the wild-type amino acid preceding the codon number, followed by the mutant residue, using single letter amino-acid nomenclature. Polymorphic variants are shown below the PrP coding sequence.

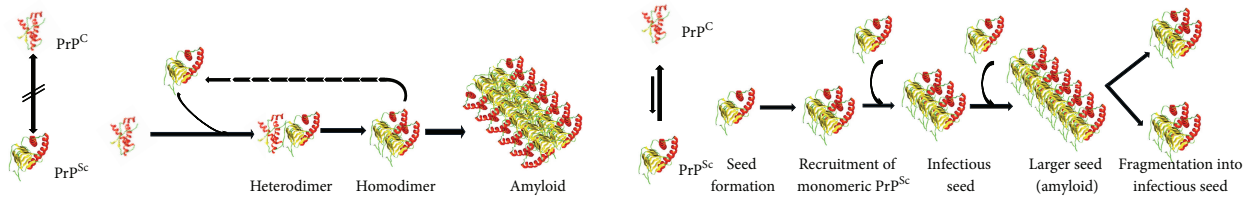


Figure 2: (a) The "template-assistance model" [8] and (b) the "seeding nucleation model" [9].

conversion into PrP^{Sc}, since the infection of PrP-deficient mice, *Prnp*$^{0/0}$ [Zürich I] [70] or *Prnp*$^{-/-}$ [Edinburgh] [71], was not successful. As predicted by the protein-only hypothesis, these mice were entirely resistant to prion infections [72]. The ubiquitous presence of PrP^{C} supports the notion that PrP has a generalized cellular function in brain tissue. Several experimental studies [73–75] suggest that PrP^{C} could play a role in synaptic structure, function, and maintenance. Defining the function of PrP^{C} remains one of the main challenges in prion biology, and it is an absolute requirement also for comprehending TSEs attributed to the posttranslational PrP^{C} to PrP^{Sc} conversion.

The focus of this review is not the physiological form of PrP, rather the pathologic PrP^{Sc} scrapie isoform.

4. Diagnosis of TSEs

Unfortunately, confirming a clinical diagnosis of TSEs has historically been difficult, as conventional laboratory tests have been ineffective in detecting them. For example, the cerebrospinal fluid most often appears normal, except for an increase in tau and 14-3-3 proteins. Both of these biomarkers support the CJD diagnosis with a sensitivity of 92% and specificity of 71% [76]. Brain MRI is increasingly useful in identifying sCJD cases. High signal abnormalities in the basal ganglia and/or cortical ribbon on diffusion weighted imaging (DWI) and fluid attenuated inversion recovery (FLAIR) sequences have recently been added to the diagnostic criteria for probable sCJD (Figures 3(a) and 3(b)) [77]. Moreover, neuroimaging with MRI is useful to exclude other causes of subacute neurologic illnesses. Generally, few imaging abnormalities are seen, for example, generalized atrophy in some cases; in less than 10% of sCJD cases, hyperintensity of the basal ganglia may be seen in T2-weighted images. In vCJD, putaminal hyperintensity on T2-weighted images is a common finding [78]. In FFI, PET may detect thalamic hypometabolism although in other prion diseases PET generally shows nonspecific cortical hypometabolism. A helpful

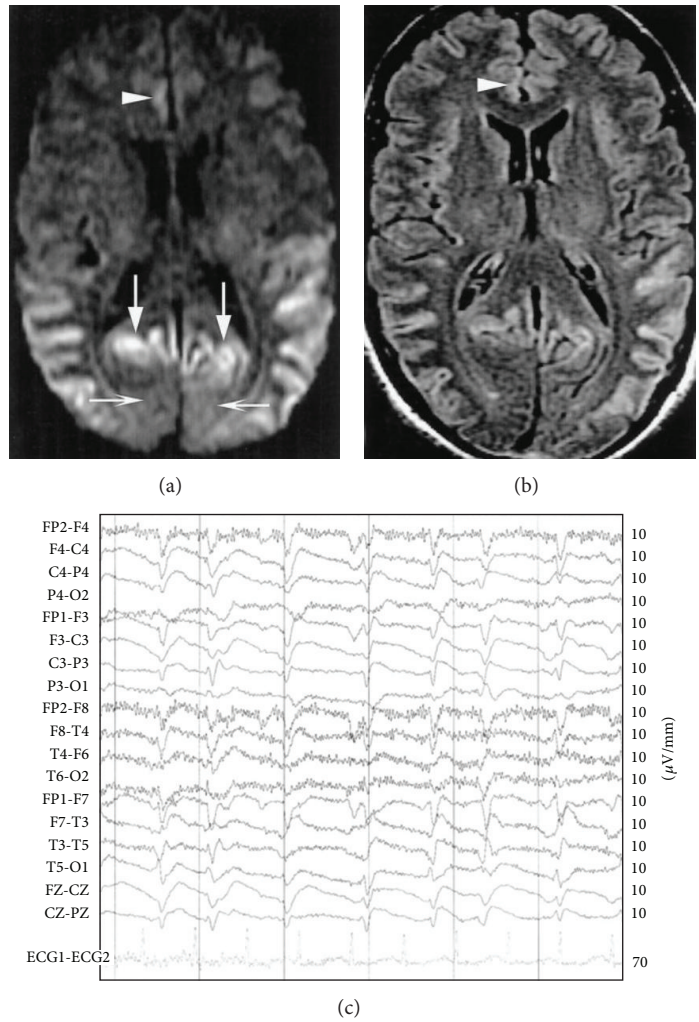


Figure 3: (a) A 50-year-old man with definite sCJD. Axial DWI shows pathologic hyperintensity in bilateral posterior temporoparietal neocortex. Cortex along parietal-occipital fissure is abnormally hyperintense (vertical arrows), but primary visual region is spared (horizontal arrows). Note asymmetric abnormal hyperintensity in right cingulum (arrowhead). Striatum is uninvolved. (b) FLAIR image at same level shows more subtle pathologic hyperintensity in all abnormal regions on DWI, as shown in cingulate cortex (arrowhead) [10]. (c) Definite sCJD (MM1); total duration: 10 months; EEG at 6 weeks: typical (used to classify case as probable); source: http://www.eurocjd.ed.ac.uk.

test is the electroencephalogram (EEG), which measures brain wave activity (Figure 3(c)). The EEG often shows a characteristic abnormal pattern, typically observed in later stages of the disease, but this technique does not confirm a TSE diagnosis. A definite diagnosis of prion disease, as with any dementia, can be made only by pathologic confirmation following biopsy or autopsy. Since the definitive antemortem detection of PrP^{Sc} in biopsy specimens is discouraged, because it is invasive and poses risks to health care personnel, unfortunately the last option is autopsy and the analysis of postmortem tissue of infected patients [79].

Prion diseases are generally characterized by widespread neurodegeneration and therefore exhibit clinical signs and symptoms of cognitive and motor dysfunction. In addition, infectious prions propagate by forming amyloid plaques, which are considered as the main hallmark of the disease and serve as a main diagnostic criterion. Since PrP^{Sc} is partially resistant to digestion with proteinase K (PK), this characteristic feature has been used to identify infected samples. Other biochemical characteristics useful to differentiate PrP^{C} from PrP^{Sc} are insolubility in nonionic detergents and high content of β-sheet secondary structure. The assays for the detection of PrP^{Sc} test brain tissue, where the greatest concentrations of prions are found during the terminal stage of disease. Standard histopathological and immunohistochemical techniques are used to view the tissue microscopically and identify characteristic vacuoles, plaques, or other abnormal features and staining associated with prion diseases. The standard confirmatory test is the Western blot after PK digestion.

4.1. Western Blot. This technique takes advantage of the partial PK resistance of the scrapie form. Treating PrP^{Sc} with PK results in the removal of only 90 amino acids from the N-terminus (Figure 1). The remaining PrP "core" is denoted by

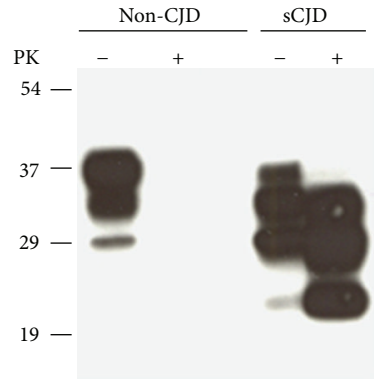


Figure 4: Detection of PK-sensitive PrP^{Sc}. (a) Conventional Western blot of PrP treated with or without PK. No PrP was observed after PK treatment in the samples from non-CJD. The PK-resistant PrP27–30 was indicated in the sample from sCJD. Samples were digested with 50 μg/mL proteinase K for 1 hour at 37°C, completely hydrolyzing PrP^{C}. Proteinase digestion cleaves ~90 amino acids from the amino terminus of PrP^{Sc} to generate PrP27–30. Blot is developed with anti-PrP mouse monoclonal antibody 3F4 [11].

PrP^{RES} (PrP, proteinase resistant). Limited protease digestion of PrP^{Sc} often produces a smaller, protease-resistant molecule of approximately 142 amino acids, referred to as PrP 27–30. Under the same conditions, PrP^{C} and some forms of PrP^{Sc} are completely hydrolyzed. Although resistance to limited proteolysis has proven to be a convenient tool for detecting PrP^{Sc}, not all PrP^{Sc} molecules are resistant to protease digestion (denoted by sensitive PrP^{Sc}, $sPrP^{Sc}$) [80–84]. Figure 4 shows the typical Western blot profile of infected/uninfected and PK digested/PK not digested brain homogenates.

4.2. Conformation-Dependent Immunoassay (CDI). Another test useful for the detection of prions is the conformation-dependent immunoassay (CDI). This diagnostic test simultaneously measures specific antibody binding to denatured and native forms of PrP [82]. In 1998, Prusiner et al. described this assay as not only able to measure very low levels of PrP^{Sc} but also capable of discriminating among a wide variety of prion strains. In 2002, the same authors [85] reported that CDI is capable of measuring the disease-causing isoform (PrP^{Sc}) in bovine brainstems with sensitivity similar to that of the end-point titrations in transgenic (Tg) mice expressing bovine PrP (BoPrP). Prion titers were ~10^7 ID_{50} units per gram of bovine brainstem when measured in Tg BoPrP mice, a figure ~10 times greater than that determined by bioassay in cattle and ~10,000 times greater than that determined by bioassay in wild-type mice. This immunoassay provides important information about the tertiary and secondary structure of PrP^{Sc}, which is strain dependent. Results from CDI should be correlated with those from optical spectroscopic techniques such as time-resolved fluorescence spectroscopy (FRT) and circular dichroism (CD) spectroscopy. The ability to assay features of the tertiary and secondary structure of PrP^{Sc} in crude homogenates opens several new areas of investigation, including determination of PrP^{Sc} structure in various tissues as well as in different regions of the CNS for a variety of prion strains. In 2005, the sensitivity of the assay was improved by selectively precipitating the PrP^{Sc} with $Na_2H[PW_{12}O_{40}]$ [86].

4.3. Protein Misfolding Cyclic Amplification (PMCA). As reported by several groups, sustained propagation of PrP^{Sc} (largely in the CNS) results in the accumulation and deposition of the pathogenic protein. Therefore, the conversion into PrP^{Sc} can be reproduced *in vitro* using a technique named protein misfolding cyclic amplification (PMCA) which was pioneered by Soto and colleagues [87]. PMCA allows propagation of PrP^{Sc} *in vitro* from very small amounts of undetectable seeding material to quantities sufficient for detection by Western blot or plate-based immunoassays. For example, using brain-derived PrP^{C} as a substrate, as little as 1 μg/mL of PrP^{Sc} can be detected [88]. This ultrasensitive method has been previously applied to identify prions in a wide range of tissue and fluids from scrapie-infected sheep (blood, feces, saliva, and milk) where only small amounts of the infectious agent reside [89–93]. Given its unique ability to detect prions in readily accessible tissue and at preclinical stages of the disease, PMCA is a viable preclinical test for prion diseases.

4.4. Amyloid Seeding Assay (ASA). Back in 2004, Legname et al. [94] reported the production of synthetic prions via *in vitro* conversion of recPrP [94]. Under different conditions they were able to obtain two different forms of β-sheet enriched structures (β-oligomer PrP^{Sc}-like and recPrP aggregates in fibrillar amyloid form). The polymerization process was monitored by simply applying thioflavin (ThT) to the reaction mixture. This dye shows strong increase of fluorescence upon binding to β-sheet-rich structures like amyloid aggregates. Importantly, in this work authors discovered that the addition of a seed of prefolded amyloid to the fresh reaction substantially shortens the fibrillation process (called lag phase). This experiment shows that recPrP fibrils can be induced by seeding, defining the technique as amyloid seeding assay (ASA). Later in 2007, the authors reported that the ASA detected PrP^{Sc}, the sole component of the prion, in brain samples from humans with sporadic Creutzfeldt-Jakob disease as well as in rodents with experimental prion disease [95]. Using the ThT assay, they found that many prion strains are capable of seeding the polymerization of recPrP

into amyloid, demonstrating that this seeding property can be used as an assay to detect prions in biological samples [95].

4.5. Real-Time Quaking-Induced Conversion Assay (RT-QUIC). The development of *in vitro* techniques, such as PMCA and ASA, has generated the potential for sensitive detection of prions. Quaking-induced conversion assay (QUIC) is another PrP^{Sc} amplification assay similar to ASA [96]. This *in vitro* PrP^{Sc} amplification technique employs soluble recombinant PrP (rPrP-sen) as a substrate, which is seeded with PrP^{Sc} and then subjected to intermittent automated shaking. This technique can be performed more easily than PMCA, which requires repeated sonication. Previous studies have shown that QUIC assays correctly discriminate between normal and scrapie-infected CSF samples in both hamster and sheep prion disease models [97, 98]. More recently, a more refined QUIC assay, known as real-time quaking-induced conversion assay (RT-QUIC), was designed [99]. RT-QUIC offers sensitivity similar to the *in vivo* bioassay in hamsters but is roughly 50–200 times faster and much less expensive. RT-QUIC allows the detection of ≥1 fg of PrP^{Sc} in diluted Creutzfeldt-Jakob disease (CJD) brain homogenate [99]. These findings indicate the promising enhanced diagnostic capacity of RT-QUIC in the antemortem evaluation of suspected CJD [100]. Moreover, Gmitterová et al. reported that the ELISA assay, which measures all 14-3-3 isoforms, was very useful in PrP^{Sc} detection [101]; however, this system is not commercially available. Therefore, according to the World Health Organization, diagnosis of prion diseases is usually based on medical history, symptoms (myoclonus, depression), and diagnostic tests, for example, MRI scans and EEGs.

5. Compounds That Target PrP^{Sc}

The presence of PrP^{Sc} deposits is considered a hallmark for prion diseases and serves as a main diagnostic criterion. At the same time it represents a therapeutic target for pharmacological intervention. In fact, treatment investigations target mostly the accumulation of PrP^{Sc} in the brain. Dozens of drug candidates for TSEs have been reported to date, but only very few proved to be effective in *in vivo* studies. The two most promising compounds, quinacrine and pentosan polysulphate, have largely been dismissed as ineffective in patients [102, 103]. A number of compounds have shown antiprion activity in numerous studies using prion inhibitory assays in cell culture [104–107]. These compounds include sulfated polysaccharides, for example, pentosan polysulphate [108], Congo red and other azo dyes [109], amphotericin B and analogues [110], anthracyclines [111], phthalocyanines and porphyrins [112], phenanthridine derivatives [113], inorganic ions, branched polyamines, antagonists of the N-methyl-D-aspartate receptor, such as memantine [114], and acridine derivatives, such as quinacrine [115–117]. Immunotherapeutic approaches are also being attempted for prion infection, with various levels of success [106, 118, 119]. In addition, further methods have recently been reported in the screening of large compound collections *in vitro* [113, 120, 121].

6. Diagnosis of Alzheimer's Disease

Whereas prion diseases are a rare form of neurodegenerative diseases leading to dementia, Alzheimer's disease (AD) is the most common one.

The pathological features of AD include neuritic plaques composed of amyloid-β peptide (Aβ) fibrils, neurofibrillary tangles of hyperphosphorylated tau (NFT) protein, and neurotransmitter deficits. Although there has been a rapid increase in the understanding of the etiology, genetics, and underlying pathophysiological mechanism for AD during recent years, there is still no cure for the disease. Therapy is mainly symptomatic as it aims to replace the neurotransmitter deficits. In the quest for disease-modifying treatments, many drug development programs pursue strategies directly related to amyloid or tau. Indeed, these extracellular plaques and deposits of Aβ and intracellular NFT became over the years the pathological hallmark of AD and drug targets. Despite a robust support for the importance of both, most efforts have focused so far on developing antiamyloid agents to be used in the early stages of the disease. A prerequisite for the early treatment of the disease would be early detection of AD plaques. Therefore, several strategies have been developed for the imaging of amyloid, namely, radiolabeled amyloid-β peptide (Aβ) antibodies and peptide fragments, small molecules for PET and SPECT imaging, and compounds for MRI.

Several research groups have adopted the small-molecule approach to develop substances suitable for amyloid imaging. Some of the most promising compounds are derivatives of Congo red, thioflavin T, stilbene, and FDDNP. Some of them, like [^{18}F]FDDNP and [^{18}F]TZDM, have been reported to have affinity for diffuse plaques or Aβ_{1-42}-positive plaques [122, 123]. Notably, FDDNP has been reported to label also PrP plaques in brain sections [124]. However, these compounds have some limitations in their practical use as probes for *in vivo* imaging, because of their delayed washout and nonspecific accumulation in the brain white matter [125]. Nonspecific binding of imaging probes leads to high background activity and low contrast images of target structures, resulting in difficult early detection of plaque deposits. Therefore, some basic criteria need to be followed to obtain a small-molecule probe for amyloid plaques (Table 1). Table 1 lists the criteria of an ideal imaging compound for the detection of amyloid in brains of living patients with AD.

The visualization of amyloid plaques in the brains of living patients with AD would greatly aid the assessment of efficacy for antiamyloid therapy. To date, a number of groups have worked on MRI [126, 127] and PET [12, 128, 129] probes for amyloid plaques. Notably, the PET ligand Pittsburgh compound B ([^{11}C-]PIB, or 6-OH-BTA-1) has shown promise in early clinical trials and is currently used in a number of human studies [130, 131]. Other groups reported the development of the new near infrared fluorescent (NIRF) ligands for Aβ [132, 133]. Due to the short physical half-life of carbon-11 (20.4 minutes), recently, great efforts have focused on the development of Aβ plaques tracers radiolabeled with fluorine-18, a radioisotope with a considerably longer half-life (109.4

Table 1: Ideal properties for a diagnostic small molecule.

(i) Stable *in vivo*
(ii) Moderately lipophilic
(iii) Entering the brain in sufficient amounts and retained in the brain
(iv) Low uptake of metabolites to brain
(v) Detection of plaques (imaging properties)
(vi) High specificity for amyloid deposits, low nonspecific bonding

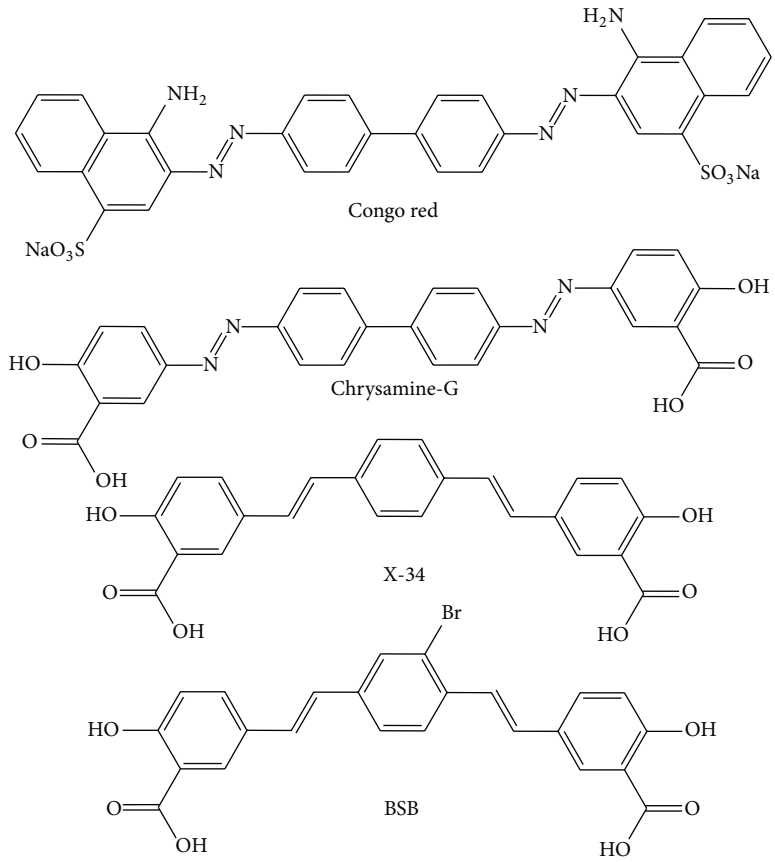
minutes). Some of them, like 4-(N-methylamino)-4′-(2-(2-(2-[^{18}F]fluoroethoxy)ethoxy)ethoxy)-stilbene ([^{18}F]BAY94-9172, florbetaben, with Ki = 2.22 ± 0.54 nM) [1, 2] and 2-(3-[^{18}F]fluoro-4-methylaminophenyl)benzothiazol-6-ol ([^{18}F]GE-067, flutemetamol, Ki = 0.74 ± 0.38 nM) [3], had already been reported under clinical trials. In April 2012, (E)-4-(2-(6-(2-(2-(2-[^{18}F]fluoroethoxy)ethoxy)ethoxy)pyridin-3-yl)vinyl)-N-methylaniline ([^{18}F]AV-45, florbetapir, Ki = 2.87 ± 0.17 nM) [134, 135] had been approved by the US Food and Drug Administration (FDA) as a radioactive diagnostic agent indicated for brain imaging of Aβ plaques in patients who are being evaluated for AD and other causes of cognitive impairment. Although autopsy remains the only positive way to diagnose Alzheimer's disease, being able to identify the Aβ plaques *in vivo* is a major step forward.

Because the biologic role of β-amyloid peptides is uncertain, researchers are also investigating alternative targets of intervention at various stages of progression. Ongoing efforts by the research community to qualify biomarkers in clinical trial designs and methods for enriching study populations with patients with early-stage Alzheimer's disease reflect important FDA priorities. Despite our growing understanding of the relationship between various disease-based biomarkers and the clinical course of Alzheimer's disease, it remains unclear whether the effect of a drug on one or more such biomarkers can actually predict a meaningful clinical benefit.

7. Amyloid Dyes and Their Derivatives

As mentioned above, candidate probes have primarily been derived from amyloid dyes such as Congo red (CR) and thioflavin T (ThT) [125]. Among all amyloid-staining compounds, CR provides historically the most standardized way of staining amyloid plaques and is still employed in postmortem histological analysis of AD brains, as the binding is specific [136]. Here we review a few aspects of Congo red, thioflavin T, and their derivatives.

7.1. Congo Red (CR). Congo red (Scheme 1) was invented in 1884, by the young German chemist Paul Böttiger (Böttiger, P. Deutsches Reichs Patent 28753, August 20, 1884). He created the first "direct" dye that did not require additional substances for fixation to the textile fibers [137]. The mechanism of interaction of CR with amyloid fibrils is not well understood. Some studies suggest that the origin of the specific binding of CR to amyloid-β aggregates is due to the combination of electrostatic interactions between the negatively charged CR's sulfonate groups with the positively charged amino-acid residues in the β-sheet structures [138, 139]. However, it is even generally believed that CR's binding depends on the secondary configuration of the fibril, consisting predominantly of cross-β-sheets [140]. Unexpectedly, recent investigations indicate that the dye also possesses the capacity to interfere with processes of protein misfolding and aggregation. This is possible by stabilizing native protein monomers or partially folded intermediates, while reducing the concentration of more toxic protein oligomers [141]. In fact, CR is able to block Aβ aggregation and toxicity in rat hippocampal neuron culture [142, 143], in HeLa and PC12 cells [144], and in human macrophage culture [145]. Although the effect of CR in transgenic mouse models of AD has not been investigated so far, CR exerted a positive effect on other experimental models, such as *Drosophila melanogaster.* Feeding with 5% w/v CR from the embryonic stage resulted in marked survival prolongation, and further histological analysis showed the reduction in the amount of Aβ aggregates and preservation of brain and retinal tissue [146]. Back in 1992, Caughey and Race [147] reported that CR suppresses even PrP^{Sc} accumulation and inhibits scrapie agent replication (in interval going from 1.4 μM to 42 μM) in cell culture studies (on mouse neuroblastoma cells, N2a), showing that the accumulation of PrP^{Sc} remained suppressed even after CR removal. In *in vivo* studies, CR has been observed to exert an ameliorative effect in animals experimentally infected with two different prion strains (263K and 139H) [148, 149]. Dosages of 0.1 and 10 mg CR (i.p.) did not have any effect, while higher CR dosages (10 mg once a week or 5 mg twice a week) induced a small increase in incubation time in i.c. scrapie-infected mice. A cumulative weekly dose of 75 mg of CR distributed over six days (12.5 mg) had a considerable effect, with incubation times extended almost to 14 days. In the i.p. infected animals, the lower dosages of 1 mg and 10 mg of CR produced a similar extension of incubation time. In a second trial of the same study, CR in dosages of 25 mg per day was given over 6 days, 1 or 2 weeks before inoculation, at the day of infection, or 1, 2, 3, or 4 weeks later. The maximal effect was achieved if treatment was initiated on the same day of scrapie infection. Treatments started 2 weeks before or 2 weeks after infection were less effective and almost ineffective if started at 3 and 4 weeks after infection. Thus, the timing of CR treatment is crucial for beneficial effect. On the other hand, other *in vitro* experiments, either with Aβ [150] or PrP^{Sc} [151], showed that at low concentrations CR can promote the protein aggregation. Hence, the effect of CR on fibril formation can be either inhibitory or stimulatory depending on its concentration. At low concentrations, CR binding populates generation of partially folded, aggregation-prone forms of proteins (oligomers and protofilament intermediates) resulting in accelerated fibril formation. At higher concentrations, however, CR inhibits fibril arrangement supporting the denatured state, which is much less prone to aggregation. Since CR is toxic (highly carcinogenic due to its benzidine structure) and is not able to cross the BBB, derivatives have been developed and made suitable for antemortem and *in*



Scheme 1: Chemical structures of Congo red and its derivatives, potential *in vivo* imaging agents for β-amyloid plaques.

vivo visualization and quantification of brain amyloids. Here we report some examples of CR derivatives able to inhibit some of these aggregated proteins. Chrysamine-G, X-34, and BSB were the most promising derivatives of CR dye.

7.2. Chrysamine-G. Chrysamine-G (CG) is the most intensively examined compound among structural analogues of CR. In this derivative, naphthalenesulfonic acid groups are exchanged for salicylic acid groups with a retained interdistance of 19–20 Å. Its smaller size, as compared to CR, and the higher lipophilicity allow it to cross the intact BBB when injected in a dose of 1 mg/kg in mice [139]. Most importantly, CG appears to be less toxic than CR, since the administration *in vivo* (10 mg/kg–100 mg/kg via i.p.) did not induce any notable behavioral effects in mice during an observation period of up to 72 h [139]. When incubated with human postmortem brain tissue homogenates, [^{11}C]CG showed the labeling of amyloid angiography and significantly higher binding in the frontal, temporal, and parietal cortices of AD patients in comparison to those of age-matched controls [152]. CG appeared to be a more potent Aβ inhibitor than CR, with effective concentrations of the latter being in the range of 2–20 μM. This finding is in agreement with higher binding affinities of CG than CR to synthetic Aβ (Ki of 0.37 μM and 2.8 μM, resp.). Chrysamine-G even attenuated Aβ_{25-35}-induced toxicity in PC12 cells, validated as a decrease in MTT reduction in the concentration range of 0.2–2 μM [153].

7.3. X-34. X-34 (1,4-bis-(3-carboxy-4-hydroxyphenylethenyl)-benzene) is a highly fluorescent CG derivative, whose structure consists of a central benzene ring, where the two diazo bonds (N=N) were replaced by alkene bonds (C=C). Most importantly, naphthalenesulfonic acids of CR are substituted by salicylic acids; as for CG, this change results in higher lipophilicity and better BBB penetration capacity. This compound has shown promising staining properties of the β-sheet structures of amyloid plaques and cerebrovascular amyloid in AD autopsy of brain tissue [154].

7.4. BSB. In 2000, Skovronsky and colleagues reported the synthesis of another CR derivative, BSB [(trans, trans)-1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy)styrylbenzene], demonstrating its high binding affinity for Aβ aggregates *in vitro* (Ki = 0.4 μM) [155]. Like X-34 and CG, BSB specifically labels senile plaques in postmortem AD brain sections. The authors even observed that BSB permeates living cells in culture and binds specifically to intracellular Aβ aggregates. After i.c. injection in living transgenic mouse models of AD amyloidosis, BSB labels plaques composed of Aβ with high sensitivity and specificity. Lastly, BSB crosses the BBB and labels numerous AD-like plaques throughout the brain of the transgenic mice after i.v. injection. Thus, the authors concluded that BSB is an appropriate starting point for future efforts to generate an antemortem diagnostic tool for AD. In 2004, Ishikawa et al. [156] hypothesized the application of

BSB in the prion field. The authors found that BSB bound to compact plaques of PrP^{Sc}, not only in the brain specimens of certain types of human TSEs but also in the brains of TSE-infected mice, when the probe was injected intravenously. The compound was also able to inhibit abnormal PrP^{Sc} formation in a cellular model of TSE with IC_{50} value of 1.4 μM. Furthermore, in an additional experimental mouse model, the intravenous injection of 1 mg BSB prolonged the incubation period by 14% [156]. The efficacy was only observed against the RML strain. Hence, this compound is promising not only as imaging probe but also for therapeutic purposes in TSEs caused by certain strains.

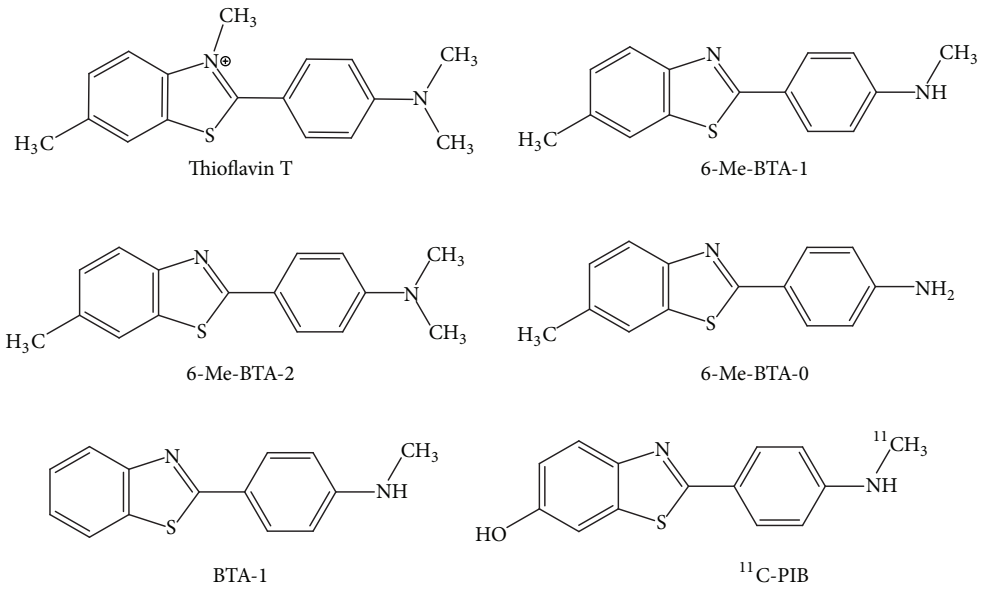
8. Thioflavin T (ThT)

Thioflavin T (ThT) is another dye useful in the analysis of aggregated amyloid proteins (Scheme 2), and it is widely used even to examine fibrillation kinetics *in situ*. In 1959, Vassar and Culling first described the use of the benzathiole dye thioflavin T as a potent fluorescent marker of amyloid in histology [157], demonstrating the potential of fluorescent microscopy for amyloid fibril diagnosis. They noted that ThT is selectively localized in amyloid deposits, thereupon exhibiting a dramatic increase in fluorescent brightness. In fact, the binding to amyloid deposits is slightly weaker than with CR (Ki in the sub- and low μM range), but it exhibits a green fluorescence that becomes more than 1000 times brighter upon binding to amyloid plaques [158]. Afterwards, Naiki et al. and LeVine [158–163] were among the first to characterize the fluorescence spectra and binding properties of ThT. They showed that, upon binding of fibrils, ThT displays a dramatic shift of the excitation maximum (from 385 nm to 450 nm) and the emission maximum (from 445 nm to 482 nm) and that ThT fluorescence originates only from the dye bound to amyloid fibrils [159–161]. These studies showed that dye binding is linked to the presence of the cross-β structure of fibrils. However, the lack of an atomic resolution structure of amyloid fibrils complicates the elucidation of the binding mode. Unfortunately, ThT possesses the disadvantage of containing a charged group, the positively charged quaternary nitrogen of the benzothiazolium group (Scheme 2), which would likely limit the permeation of the BBB of this compound. However, the ability of ThT to specifically recognize and bind with modest affinity to amyloid has allowed it to serve as an excellent starting scaffold for derivatization and elaboration to generate a number of amyloid stains and clinical reagents, included for use in medical imaging of amyloid in living patients [12, 164, 165].

8.1. 6-Me-BTA-0, 6-Me-BTA-1, and 6-Me-BTA-2. In 2001, Klunk et al. [165] showed that removing the charge from ThT affected the amyloid-binding properties of ThT derivatives. In that work the authors reported the synthesis of three ThT derivatives, 6-Me-BTA-0, 6-Me-BTA-1, and 6-Me-BTA-2, all of which were 600-fold more lipophilic than ThT. They found that the binding to $A\beta_{1-40}$ fibrils presented higher affinity (Ki = 20.2 nM) than ThT (Ki = 890 nM). These uncharged ThT derivatives stained both plaques and neurofibrillary tangles (NFT) in postmortem AD brain, showing some preference for plaque staining. Furthermore, they examined whether an uncharged, lipophilic derivative of ThT would enter the brain in amounts sufficient for imaging by PET. That compound, designed as [N-methyl-^{11}C]6-Me-BTA-1, entered the brain at levels comparable to those commonly used by neuroreceptor imaging agents (0.223 %ID-kg/g or 7.61 %ID/g at 2 min after-injection) and showed good clearance of free and nonspecifically bound radioactivity in normal rodent brain tissue (brain clearance $t_{1/2}$ = 20 min). In contrast, the 6-Me-BTA compounds did not display the classic shift in excitation and emission spectra when bound to Aβ that has been well documented for ThT.

8.2. BTA-1 and 6-OH-BTA-1. One year later, the same group [166] showed that the derivative without the methyl group in position 6 of benzothiazole moiety, denoted by BTA-1 or (2-[4′-(methylamino)phenyl]benzothiazole (Scheme 2), had more promising characteristics than the previously reported compounds. This molecule presented high affinity for the amyloid plaques (Ki = 11 nM for $A\beta_{1-40}$), and the intravenous injection of [^{11}C-] labeled BTA-1 in wild type mice resulted in high brain uptake (12.9 %ID/g at 2 min after-injection). Importantly, [^{11}C]BTA-1 is characterized by relatively rapid egress of radioactivity from normal brain tissue. Amyloid deposits were imaged with multiphoton microscopy in the brains of living PS1/APP transgenic mice following the systemic injection of unlabeled BTA-1. The authors concluded that the [^{11}C]BTA-1 was a promising radioligand for further development as a PET amyloid-imaging agent for AD.

In 2004, this uncharged ThT derivative was taken into consideration also by Ishikawa et al. as PrP^{Sc} inhibitor and as a molecule able to label PrP deposition in TSE brains [156]. Using a well-known PrP^{Sc} inhibition assay in cell culture on ScN2a, the authors found that BTA-1 had a promising inhibitory activity (IC_{50} = 4 nM) and low toxicity, since no apparent changes were observed up to 10 μM of treatment. Next, they assessed its utility as diagnostic imaging tool for PrP plaques using the histopathological specimens from human TSE cases. They found that it was able to fluorescently label most of the PrP plaques in the cerebral cortices of GSS cases and of variant CJD cases, whereas it was not able to stain PrP plaques of sporadic CJD cases. Similar results were observed when the postmortem brains of Tg7 mice infected with the 263K strain were used, considering that it stained the plaque type of PrP in the cerebral white matter between cortex and hippocampus. Due to the absence of positive charge and its capability to cross the BBB, they even performed *in vivo* experiments using Tg7 mice infected with 263K strain. A bolus injection of BTA-1 labeled PrP plaques in the white matter between cortex and hippocampus of the affected brains. Faint cerebrovascular labeling was occasionally observed at 4 h after the injection, but not at 18 h or later. Moreover, no significant labeling was observed in uninfected transgenic mice. Similar results were observed in Tga20 mice infected with RML strain, although labeled PrP plaques were less frequently observed. Even the 6-hydroxy BTA-1 derivative (also called PIB or 6-OH-BTA-1) inhibited PrP^{Sc} formation in ScN2a cells with an IC_{50} in the nanomolar



Scheme 2: Chemical structures of thioflavin T and its derivatives, useful for potential *in vivo* imaging for β-amyloid plaques.

range; more importantly, it has been selected for the first human trial of a benzothiazole amyloid-imaging agent [131]. In their latter report, Rowe et al. analyzed 16 patients with diagnosed mild AD and 9 controls. Their results demonstrated that PET imaging with the [^{11}C-]PIB tracer provided quantitative information on amyloid deposits in living individuals with AD. Thanks to the favorable radiotracer profile, PIB has become the most commonly used PET amyloid agent, adopted in more than 40 research centers worldwide (Figure 5). However, a recent study highlighted that [^{11}C-]PIB PET does not detect PrP-amyloid in prion disease patients, including variant Creutzfeldt-Jakob disease [167].

8.3. NIAD-4. Another emerging approach for *in vivo* detection of aggregated proteins is optical imaging through special near-infrared (NIR) fluorescent contrast agents. In 2005, following the rules reported in Table 1, Nesterov et al. designed a small molecule known as [[5′-(4-hydroxyphenyl) [2,2′-bithiophen]-5-yl]methylene]-propanedinitrile, or simply NIAD-4 (Scheme 3) [133]. The binding studies with artificially aggregated amyloid protein assays revealed that NIAD-4 binds to the same site as BTA-1 with a Ki of 10 nM. This affinity is much higher than that of ThT (Ki = 580 nM) and is close to that of high-affinity amyloid-binding compounds like PIB (Ki = 4.3 nM) [168]. Nesterov et al. [133] studied the specificity of NIAD-4 binding to Aβ by *in situ* histochemical staining of fixed sections from transgenic mouse brain. Brain sections were obtained from aged APP transgenic mice with AD-like pathology. The brain sections were labeled with a NIAD-4 (10 μM) solution in DMSO/propylene glycol for 15 min at room temperature. *In vitro* fluorescence imaging showed high-specificity labeling of NIAD-4, which revealed the exact position and size of the aggregated Aβ deposits. The authors [133] showed also the *in vivo* Aβ binding of NIAD-4 in aged APP transgenic mice. Mice were prepared with cranial windows to allow direct monitoring of the brain surface and then administered 10 μM of 2 mg/kg NIAD-4 solution by i.v. injection. Red fluorescence imaging using multiphoton microscopy showed that the agent readily crossed the BBB and labeled specifically both the plaques and cerebrovascular amyloid angiopathy. A radiolabeled version of NIAD-4 may also be advantageous for PET or SPECT imaging.

8.4. BF-168. Several stilbene derivatives have been synthesized as compounds for the probing of amyloid plaques [169]. Stilbene shows binding to Aβ aggregates in the nanomolar range [170]. Similar series of imaging probes were reported in [171], describing *in vitro* and *in vivo* properties of some styryl-based derivatives of ThT. The most promising one was 6-(2-fluoroethoxy)-2-[2-(4-methylaminophenyl)ethenyl]benzoxazole (BF-168) (Scheme 3). In AD brain sections, BF-168 selectively binds senile plaques and recognizes Aβ_{1-42}-positive diffuse plaques as well as neuritic plaques. Intravenous injection of BF-168 in PS1/APP and APP23 transgenic mice resulted in specific *in vivo* labeling to both compact and diffuse amyloid deposits in the brain. In addition, ^{18}F-radiolabeled BF-168 intravenously administered to normal mice showed abundant initial brain uptake (3.9 %ID/g at 2 min after injection, a sufficient level for brain imaging probe) and fast clearance ($t_{1/2}$ = 24.7 min, indicating fast brain washout) sufficient for the compound to be a PET imaging probe. Furthermore, autoradiograms of brain sections from APP23 transgenic mice at 180 min after intravenous injection of [^{18}F]BF-168 showed selective labeling of brain amyloid deposits with little nonspecific binding. These findings strongly suggest that styrylbenzoxazole derivatives are promising candidate probes for PET and SPECT imaging for early detection of amyloid plaque formation in high-risk AD patients in presymptomatic stage [171]. Additionally, this new styrylbenzoxazole compound clearly labeled PrP^{Sc} plaques in brain specimens from human

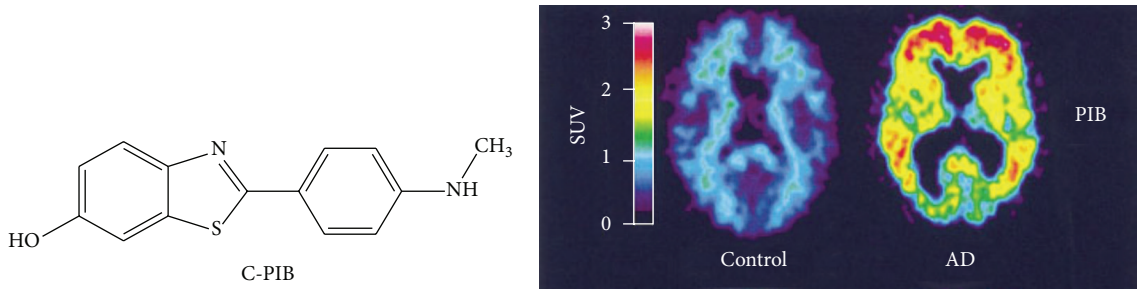
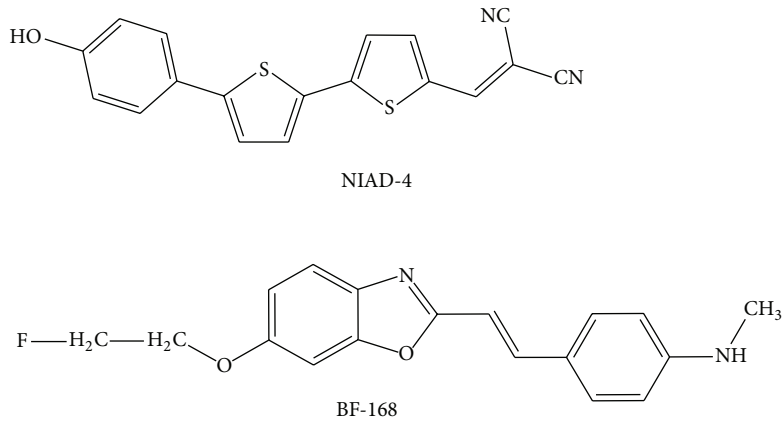


Figure 5: PET imaging of human AD brain using [^{11}C-] PIB [12]. PIB standardized uptake value (SUV) images show a marked difference between PIB retention in AD patients and healthy control subjects. PET images of a 67-year-old healthy control subject (left) and a 79-year-old AD patient. The left column shows lack of PIB retention in the entire gray matter of the healthy subject.



Scheme 3: Chemical structures of potential *in vivo* imaging agents for β-amyloid plaques.

TSEs (sCJD and vCJD) [172]. BF-168 also inhibited abnormal PrP formation in TSE-infected cells with $IC_{50} = 0.4$ nM in ScN2a cell line model and prolonged the lives (~11.4%) of mice infected intracerebrally with TSE when the compound was administered intravenously at the preclinical stage. Even though their efficacy depends on the pathogen strain, these derivatives are a new class of compounds with potential as both therapeutic drugs and imaging probes for TSEs.

8.5. G8. Meanwhile, other styryl derivatives have been studied [129, 169, 173, 174]. Li et al. [175] tested a group of styryl-based neutral compounds as potential *in vivo* imaging agents for β-amyloid plaques. The most promising one in this work was designed as STB-8 (Figure 6(a)), and its use in *ex vivo* and *in vivo* imaging experiments on an AD transgenic mouse model showed excellent BBB permeability and specific staining of the β-amyloid plaques (Figure 6(b)) [175].

A similar chemical scaffold was reported in our recent work [7]. The compound (E)-6-methyl-4-amino-2-styryl-quinoline or G8 is a small molecule (Figure 6) with the proper features to potentially diagnose, deliver therapy, and monitor response to therapy in protein misfolding diseases. These features include compound fluorescent emission in the NIR region and the ability to interact with both Aβ and prion fibrils, staining them with high selectivity. Moreover, the compound possesses an antiaggregation property against $A\beta_{1-42}$ using the well-known ThT-based fluorimetric assay [176] and prolongs the lag phase of PrP^{Sc} formation in fibrillation assay [177]. At a concentration of 50 μM, G8 delayed fibril formation, extending the lag phase to ≥70 h (control: 59 h). A similar profile was found for GN8, an antiprion drug candidate (Figure 6) for which a specific binding with PrP has been experimentally shown [178]. With such a good *in vitro* profile, we treated the ScGT1 and ScN2a cell lines with the compound, and the viability was quite good. At 1 μM concentration, G8 showed a very low toxicity, with cell viability above 90% if compared with nontreated cells, while at a 10 μM concentration it still showed a tolerable toxicity, with a residual 60% cell viability not different from that of drug candidate GN8. Starting from these nontoxic concentrations, we treated the cells to evaluate their inhibitory activity, and we found that the compound possessed a submicromolar capability to inhibit PrP^{Sc} ($EC_{50} = 0.5 \pm 0.1$ μM), greater than GN8 ($EC_{50} = 1.5 \pm 0.1$ μM) in our system. To confirm the labeling of PrP^{Sc} aggregates in living cells, fluorescent staining with G8 was carried out using the same ScGT1 and ScN2a cell models. We found that 0.025% of G8·HCl (0.84 mM) was sufficient to observe many fluorescent spots in the treated cells examined by fluorescent microscopy (Figure 6(c)). Importantly, no spots were observed in the

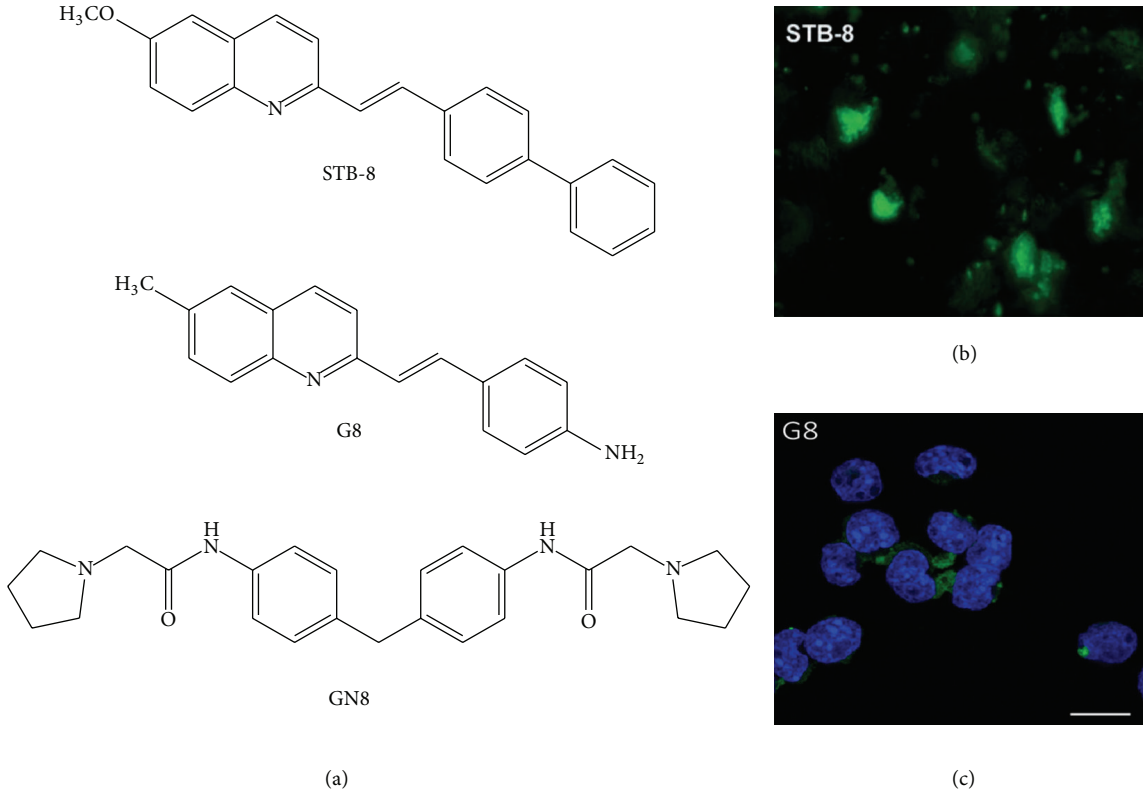


Figure 6: (a) Chemical formula of the three promising antiprionic compounds and *in vitro* staining with (b) STB-8 compound (β-amyloid plaques) and (c) G8 compound (PrP^{Sc} deposits).

uninfected cells, confirming a specific binding. Furthermore, the staining pattern was consistent with that observed with 0.025% thioflavin S (ThS), a common PrP^{Sc} dye. A further experiment proved that G8·HCl (0.25 mM) distinguishes the abnormal, aggregated, and PK-resistant PrP^{Sc} isoform from the normal, PK-sensitive PrP^{C} isoform. Thus, after eliminating PrP^{C} through a PK digestion step, the previous fluorescence-staining pattern was observed. We primarily used the FITC filter set for these studies, but we confirmed the staining by employing the ThS one, which is within the NIR optical window. G8 was able to cross the BBB in an *in vitro* model, such as parallel artificial membrane permeability assay (PAMPA, Pe $23.1 \pm 1.9\ 10^{-6}\ cm^{-1}$).

From a medicinal chemistry perspective, G8 offers peculiar advantages: (1) a lower molecular weight than previous sensors [179] and (2) a small-molecule scaffold that is easily amenable to further manipulation to improve fluorescence response and amyloid-binding properties. Most importantly, with respect to the previously reported NIR amyloid sensors [132, 133, 180–182] it offers the advantage of a concomitant promising antifibrillar profile (*in vitro* and in a cellular context), together with a low toxicity. If these distinctive properties are confirmed *in vivo*, G8 is likely to become the first purposely designed therapeutic and diagnostic (theranostic) tool for prion diseases and AD.

9. Conclusion

All the efforts made to date to develop rapid, accurate, and highly sensitive antemortem tests to detect prions early in the course of the disease have failed. Most tests still involve PK digestion, and the specificity and sensitivity of tests that do not use PK require further validation. Nevertheless, neuroimaging shows promise as a future clinical diagnostic tool for neurodegenerative diseases. Continued expansion of scientific imaging tools has been essential toward a new standard strategy that links established *in vitro* and cell culture experimental assays to imaging studies for living subjects. In fact, over the last few years the rapid development of different compounds suitable for visualizing aggregated β-sheet-rich proteins has led to the first promising *in vivo* studies of the amyloid ligands, such as PIB [12]. Florbetapir is the first radioactive dye for brain imaging of amyloid plaques to be approved by the FDA. With its introduction into the clinical practice, we are now effectively entering the era of neurodegenerative disease imaging.

Our hope is that our own G8 molecule [7] will confirm *in vivo* the results obtained *in vitro*. Molecular imaging in living subjects offers distinct advantages when compared with conventional *in vitro* and cell culture research techniques in biology. Therefore further work on promising imaging compounds is necessary to access *in vivo* studies. The use of these compounds could represent a good approach to detect

and treat neurodegenerative disorders such as Alzheimer's disease and prion diseases. As the term theranostics is derived from the words therapeutics and diagnostics, the final application of theranostics is combining disease diagnosis and therapy. This combination in a single molecule enables real-time feedback on the biodistribution and the target site accumulation of the compound. The concurrent delivery and readout of efficacy can be exploited to tailor treatment regimens for specific treatment groups.

Effective treatments for devastating disorders such as Alzheimer's disease and prion diseases are urgently needed, as the world's population continues to age. We are confident that purposely-designed theranostics might soon become powerful tools to combat them.

Acknowledgment

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Review Article

Convergence of Synapses, Endosomes, and Prions in the Biology of Neurodegenerative Diseases

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Age-related misfolding and aggregation of disease-linked proteins in selective brain regions is a characteristic of neurodegenerative diseases. Although neuropathological aggregates that characterize these various diseases are found at sites other than synapses, increasing evidence supports the idea that synapses are where the pathogenesis begins. Understanding these diseases is hampered by our lack of knowledge of what the normal functions of these proteins are and how they are affected by aging. Evidence has supported the idea that neurodegenerative disease-linked proteins have a common propensity for prion protein-like cell-to-cell propagation. However, it is not thought that the prion-like quality of these proteins/peptides that allows their cell-to-cell transmission implies a role for human-to-human spread in common age-related neurodegenerative diseases. It will be important to better understand the molecular and cellular mechanisms governing the role of these aggregating proteins in neural function, especially at synapses, how their propagation occurs and how pathogenesis is promoted by aging.

1. Synapses

The brain is particularly vulnerable to degenerative diseases of ageing. Aberrant aggregation of proteins/peptides is the common theme among these diseases. Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common age-related neurodegenerative diseases, while other less common, albeit devastating, neurodegenerative diseases include Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), prion diseases, and frontotemporal dementia (FTD). Although the specific protein aggregates and selective cellular vulnerabilities differ, shared disease mechanisms are increasingly apparent among neurodegenerative diseases and next to aberrant protein aggregation also include anatomically selective cell-to-cell propagation. Major themes of research on these diseases have included therapeutic neurotransmitter replacement, most successful with dopamine for PD, elucidating the biology of aberrant protein misfolding, and trying to understand how ageing promotes the development of these diseases. More recently, synapses have moved more to the center of research on these diseases [1, 2]. Neurites (axons and dendrites) and synapses are a unique feature of neurons and play fundamental roles in brain function. Furthermore, the aggregation-prone proteins linked pathologically and genetically to neurodegenerative diseases are normally present particularly at synapses. For example, the PD-linked protein α-synuclein is known to normally reside primarily in presynaptic compartments [3, 4], although, as the name indicates, a nuclear role also characterizes this protein that aggregates in the distinctive cytoplasmic Lewy bodies and Lewy neurites that characterize PD and the related Lewy body dementia (LBD).

An important role at synapses for the AD-linked β-amyloid (Aβ), and the amyloid precursor protein (APP) from which it is derived, is also increasingly becoming apparent (Figure 1). APP is transported down axons and dendrites to synapses [2, 5], where the proteases that generate Aβ are also localized [6]. The precise processing and trafficking of APP and Aβ in pre- versus postsynaptic compartments and how these relate to the mechanism of synaptic damage in AD remain to be elucidated. Evidence supports that Aβ

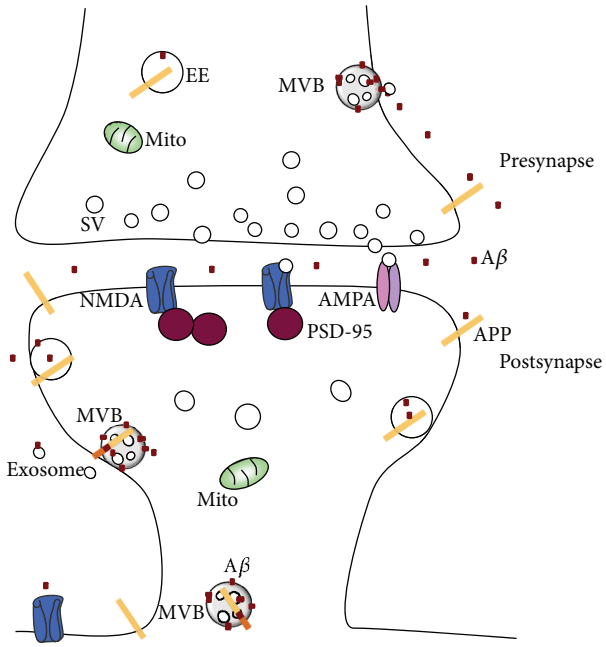

Figure 1: Schema of synaptic biology relating to Alzheimer's disease. APP is present in endosomes, including early and late/MVB endosomes, as well as at the cell surface. Aβ is associated with MVBs and other endosomes, as well as being secreted from the cell surface, also via exosomes. The relative proportions of Aβ peptides and APP processing in the pre- versus postsynapse remain uncertain. The cellular mechanism(s) of Aβ transmission from or to the pre- and postsynapse is also not yet clear. MVB: multivesicular body; EE: early endosome; SV: synaptic vesicle.

accumulation in synapses alters synaptic function by altering important synaptic proteins and receptors [2].

A major hurdle for research on neurodegenerative diseases has been that the normal physiological roles and functions of the aggregation-prone proteins have been difficult to ascertain. A potential reason for this could very well be that synapses are so complex and are only gradually being elucidated. Although mouse knockout studies do not support that loss of function of the disease-linked proteins is the salient issue in these various diseases [7, 8], it is nevertheless possible that their propensity to aggregate is a feature that makes these proteins normally important at synapses. It is further possible that a better understanding of the normal function of neurodegenerative-linked proteins at synapses will be important in order to uncover better therapeutic targets and devise more effective therapies for these diseases.

Synaptic activity and plasticity are of central importance in the brain and at synapses, and it has become clear that neurodegenerative disease-linked proteins are modulated by synaptic activation [9, 10]. The major nonneuronal cells of the brain, the astrocytes and microglia, are also increasingly linked to synaptic function and thereby might impact the pathophysiology of these diseases that appear to initiate at synapses. Modulation of synapses has also been shown to directly impact synapse damage in the brain of transgenic mouse models of neurodegenerative diseases [11].

2. Endosomes

The endosome-lysosome system and the ubiquitin proteasome system (UPS) play many essential roles in cells and are increasingly implicated in neurodegenerative diseases of ageing [12]. In neurons, these systems, best known for their role in protein degradation, are also important for the normal function of synapses [13]. The diversity of rare genetic neurodegenerative storage diseases of childhood linked to aberrant protein or lipid accumulation in the endosome-lysosome system supports the potential disease relevance of this system also in the common age-related degenerative diseases of the brain [14]. The endosome-lysosome system is involved in many central functions, including cellular internalization, degradation, and release. In Down syndrome (DS), characterized by trisomy of chromosome 21, which invariably leads to age-related AD-like pathology and dementia, abnormal endosome enlargement has long been known to precede the characteristic neuropathological amyloid plaques and tau tangles [15].

The related autophagy system is intimately linked with the endosome-lysosome system and is important for the engulfment and degradation of larger subcellular structures, including whole organelles. Autophagy is a prominent neuropathological feature of neurodegenerative diseases [16], where autophagic vesicles, limited by double or multilamellar membranes, accumulate in neurons and their processes. Autophagic vesicles are particularly abundant in dystrophic neurites in AD brain and transgenic mouse models of AD. Autophagic vesicles are thought to form from the formation of double membranes in the cytoplasm. Autophagic vesicles are thought to subsequently fuse with endosomes or lysosomes followed by degradation of their contents by lysosomal proteases. Although autophagy is considered a mechanism of normal cellular degradation, autophagy is rarely seen on electron micrographs of normal brain (personal observations). The autophagy system is further implicated in neurodegenerative diseases of ageing, since inhibition of the mammalian target of rapamycin (mTOR), which is well known to induce autophagy, was found to prolong the lifespan in lower organisms and mammals [17]. More recent studies show that pharmacological mTOR inhibition by rapamycin is protective in transgenic mouse models of Alzheimer's disease [18, 19]. A challenge for the development of treatments of neurodegenerative diseases based on mTOR inhibition is that mTOR is part of a central signaling system regulating many cellular functions. For example, mTOR is important in synaptic plasticity [20].

Neurites can extend considerable distances and synapses are thus at a greater distance from their cell bodies than are components of other cells. Moreover, lysosomes do not normally reside in neurites and synapses. Therefore, protein degradation at synapses depends on retrograde transport via endosomes back to the cell body. Late endosomes can take on some limited lysosomal functions, but compared to lysosomes they are much less efficient at degradation, particularly of aggregation prone insoluble proteins. Endosomes also play important roles in the regulated delivery, recycling, and degradation of receptors at synapses that are important for

synaptic plasticity [21]. Interestingly, the inner vesicles that characterize the late endosomal multivesicular body (MVB, alternatively called multivesicular endosome) are released as exosomes, and increasing evidence suggests that exosomes may be important in the release and propagation of neurodegenerative disease-linked proteins [22]. Several of these disease-linked proteins have been localized to endosomes. For example, AD-linked Aβ peptides were shown to normally localize and then accumulate and aggregate particularly in MVBs of dystrophic neurites and synapses of AD transgenic mouse models and human AD brains, where they associated with localized subcellular pathology, even prior to extracellular amyloid plaques [23–25].

The UPS is also increasingly linked with neurodegenerative diseases of ageing. For example, mutations in Parkin represent an example of a UPS-linked protein (component of a ubiquitin ligase) that is mutated in familial forms of PD. Moreover, the UPS and the endosome-lysosome system are linked, since transmembrane proteins at the cell surface, including APP and neurotransmitter receptors at synapses, can be ubiquitinated and routed via the endocytic pathway to the lysosomes for degradation [13, 26]. AD-linked Aβ normally localizes to and with AD pathogenesis preferentially accumulates at the outer limiting membranes of MVBs [23] where the endosomal sorting complexes required for transport (ESCRT) reside, which is involved in targeting ubiquitinated transmembrane proteins for degradation. Experimental evidence supports that accumulating Aβ42 associated with MVBs impairs MVB sorting by disrupting the UPS [26]. Altered regulation of synaptic protein trafficking from early aberrant protein/peptide accumulation near synapses might lead to the earliest synapse dysfunction in neurodegenerative diseases [2]. Interestingly, mutations of CHMP2b, a component of ESCRTIII, have been linked with familial forms of FTD [27]. Although FTD, the 2nd most common cause of dementia before the age of 65, is not genetically linked with Aβ/APP, it has been linked to familial mutations in tau, the main constituent of the other characteristic neuropathology of AD, the neurofibrillary tangles. Moreover, cells release tau, which interestingly is also stimulated by synaptic activity [28]. Release of tau may potentially occur via MVBs and exosomes. Cell-to-cell propagation of tau has recently become a hot topic in the field of neurodegenerative diseases [29].

The endocytic pathway is also central to cholesterol and lipid uptake and trafficking in neurons, which are implicated in AD [30, 31] and are important in injury and synapse remodeling in neurons. The lipoprotein carrier apolipoprotein E4 (ApoE4) polymorphism is the major genetic risk factor for typical late onset AD. Cholesterol traffics via the endocytic pathway into cells, and together with the endoplasmic reticulum (ER) and mitochondrial-ER membranes, this interconnected pathway that regulates cellular cholesterol metabolism has been related to Aβ [32]. Recent research supports that apoE, generated mainly by astrocytes, is important for regulating neuronal Aβ [33, 34]. Interestingly, the apoE receptor, low-density lipoprotein receptor (LDLR) related protein (LRP), is routed from the plasma membrane to the limiting membrane and then internal vesicles of MVBs and interacts with APP via Fe65 [35]. As noted previously, Aβ normally localizes to MVBs and with AD pathogenesis accumulates at MVBs, and upon release from the cell, MVB inner vesicles are then called exosomes. Thus, it is conceivable that apoE biology intersects with Aβ/APP in endosomes and might even modulate propagation of secreted misfolded proteins via exosomes and/or protein degradation via the MVB-lysosome pathway. In addition, endosomes have a lower pH, which is known to promote aggregation and amyloid formation of misfolding proteins, including Aβ and PrP [36–38]. Finally, evidence consistent with leakage of endosome-lysosome contents by Aβ has been reported [39, 40]. Consistent with such leakage, immunoelectron microscopy showed marked accumulation of Aβ42 directly associated with outer membrane disruption of MVBs in AD transgenic mice [23]. Thus, multiple lines of evidence point to an important role of the endosomal-lysosomal system and in particular of endosomes at neurites and synapses in common neurodegenerative diseases of ageing.

3. Prions

Prions are the unusual proteins that are best known for their ability to propagate disease between members of a species and between different species [41]. Prion diseases have therefore been classified among infectious diseases. Formerly classified as atypical "slow virus" diseases, this nomenclature was abandoned, since they propagate as proteins and in contrast to viruses lack nucleic acids. However, clinically and pathologically, prion diseases are most similar to common neurodegenerative diseases, such as AD. Prions can form fibrillar aggregates with amyloid-like characteristics. The normal role of the prion protein is also poorly understood [8], although it is expressed at particularly high levels in the brain and is thought to primarily localize to synapses. Analogous to AD and PD, the majority of prion diseases are sporadic, without a clear infectious or genetic cause. Less common familial forms of prion disease with mutations in the prion protein exist. Although the term prion is widely associated with fear, based on its infectivity from highly publicized outbreaks of prion disease, prion proteins are normal proteins that can even provide protective functions [42, 43]. Thus, prions, like amyloids [44], should not be only viewed in a negative light, and PrP likely plays important physiological roles, which potentially may be particularly relevant at synapses. The mere evolutionary presence of PrP, which is conserved in mammals, as well as some deficits noted in PrP deficient mice [45], supports a physiological role for PrP. A more recent development in research on neurodegenerative diseases is the surprising realization that other disease-linked aggregation prone proteins, such as Aβ [46, 47], α-synuclein [48, 49], and tau [29], can also propagate in experimental systems.

Despite the many years of research on prion propagation, the cellular mechanism(s) of propagation still remain(s) unclear. The recent surge of research on this topic particularly in AD, PD, ALS, and HD will undoubtedly lead to important new insights. Some overlooked earlier papers have provided clues to cellular mechanisms of propagation in these

diseases. For example, a literature from the 1990s showed that exogenously added extracellular AD-linked Aβ1–42 peptides induced a marked upregulation of newly generated Aβ42 within the treated cells [50]. Moreover, it was shown that extracellular Aβ1–42 failed to alter synapses in the absence of APP or in neurons where de novo generation of Aβ was inhibited [10]. Similarly, depletion of endogenous PrP protects against scrapie-induced PrP pathogenesis [51, 52]. While neurons generate Aβ from APP within neurons, secreted Aβ can also be internalized by neurons [53]. More recent evidence points to synapses as selective sites of neuron-to-neuron spread of Aβ [54]. One could speculate that release of exosomes might be particularly prominent near synaptic terminals, although it was estimated that only about 1% of released Aβ was associated with exosomes [55]. Overall, the cell biology of such synapse-associated endocytic and exocytic pathways in neurons is less well understood than of other cells.

A challenge in considering prion-like cell-to-cell propagation is to explain where and how the initial pathological conformation of disease-linked peptide forms and what determines the anatomical selectivity of spread by various disease-linked proteins. Recent evidence supports the surprising scenario that in the setting of aberrant intracellular protein aggregation, the secretion of AD-linked Aβ is actually impaired [56]. In general, the release of more toxic soluble oligomers appears to be at much lower levels than those of monomeric proteins; for example, the concentration of Aβ oligomers is about 1% of the monomeric forms in cerebrospinal fluid. In addition, aggregation of misfolded proteins begins in a selected population of neurons. Thus, it follows that if abnormal aggregation can initially arise spontaneously in one anatomical region, it might be possible that other vulnerable cells might also have de novo appearance of abnormal aggregated conformations rather than a prior requirement for propagation from other cells. It is also possible that despite the reductions in the normal secretion of monomers, cell-to-cell transmission of more aggregated oligomers may be the driving force in disease propagation, which even at low levels might still act as the nidus to drive further aggregation in the recipient cells.

There is no convincing evidence that proteins linked to the common age-related neurodegenerative diseases can spread from person to person as was recently highlighted in a study of patients who had received growth hormone [57]. Thus, neuroscientists need to contribute to reducing the excessively alarmist view that is linked with the term prion in the public. Actual prion diseases are remarkably rare in humans and even the highly publicized outbreaks were relatively small in scale. For example, the number of human cases of variant Creutzfeldt-Jakob disease cases per year associated with the outbreak of bovine spongiform encephalopathy in Great Britain peaked in the year 2000 with 28 deaths (http://www.promedmail.org/direct.php?id=20120809.1236446). Yet, this overall low incidence of human cases should not take away from the importance of proper safety precautions associated with avoiding contamination with prions or in efforts to prevent prion outbreaks in animals and man.

4. Conclusion

Neurodegenerative diseases of ageing are a growing disease epidemic that is placing an increasing financial and emotional toll on societies. Our slow progress in developing treatments that will eventually slow down or even halt the progression of these debilitating diseases likely hinges on a better understanding of the complexity of the ageing brain, cell biology, and synapses. Rare gene mutations or more common polymorphisms have provided new clues in our understanding of the biochemical pathways that determine these important diseases. Synapses are turning out to be potentially critically vulnerable sites prone to diseases of protein misfolding. The major degradative organelles, the lysosomes, localize to the cell body of neurons and are thus removed from distal neurites and synapses in which various endosomal organelles provide diverse functions, including secretion and degradation. Cellular degradation systems, such as the endosome-lysosome system and the UPS, may be particularly vulnerable to the development of age-related dysfunction of the nervous system and thereby might predispose to aberrant protein aggregation with ageing. Major contributors to the ageing process, including mitochondrial dysfunction, cardiovascular disease, and inflammation, likely impact the declining function of these important cellular degradation pathways. It will be critical to better define the cellular and biochemical pathways implicated in neurodegenerative diseases as well as to elucidate the normal biology of synapses. It is also possible that the aggregation-prone properties of misfolding proteins linked to neurodegenerative diseases hinge on their normal role at synapses and their propensity to aggregate. Furthermore, it will be important to better define the more precise cellular mechanisms leading to cell-to-cell propagation of neurodegenerative disease-linked proteins.

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Review Article

Prions *Ex Vivo*: What Cell Culture Models Tell Us about Infectious Proteins

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Prions are unconventional infectious agents that are composed of misfolded aggregated prion protein. Prions replicate their conformation by template-assisted conversion of the endogenous prion protein PrP. Templated conversion of soluble proteins into protein aggregates is also a hallmark of other neurodegenerative diseases. Alzheimer's disease or Parkinson's disease are not considered infectious diseases, although aggregate pathology appears to progress in a stereotypical fashion reminiscent of the spreading behavior ofmammalian prions. While basic principles of prion formation have been studied extensively, it is still unclear what exactly drives PrP molecules into an infectious, self-templating conformation. In this review, we discuss crucial steps in the life cycle of prions that have been revealed in *ex vivo* models. Importantly, the persistent propagation of prions in mitotically active cells argues that cellular processes are in place that not only allow recruitment of cellular PrP into growing prion aggregates but also enable the multiplication of infectious seeds that are transmitted to daughter cells. Comparison of prions with other protein aggregates demonstrates that not all the characteristics of prions are equally shared by prion-like aggregates. Future experiments may reveal to which extent aggregation-prone proteins associated with other neurodegenerative diseases can copy the replication strategies of prions.

1. Prions—Infectious Agents Composed Predominately of Protein

Prion diseases or transmissible spongiform encephalopathies (TSEs) are invariably fatal neurodegenerative diseases that are associated with severe spongiform vacuolation and nerve cell loss [1]. Animal and human TSEs are infectious diseases that either naturally spread between individuals of the same species or have been accidentally transmitted through food contaminants, blood and medical products, or during surgery [1]. Animal prion diseases include scrapie in sheep and goats, bovine spongiform encephalopathy, and chronic wasting disease in elk and deer [1]. TSEs in humans also occur sporadically or are of familial origin. Human prion diseases manifest as Creutzfeldt-Jakob disease, fatal familial insomnia, Gerstmann-Sträussler-Scheinker syndrome and Kuru. Familial prion diseases are associated with dominantly inherited mutations in the coding region of the cellular prion protein. Many prion strains have also been successfully adapted to laboratory animals. Prion strains can be propagated in the same inbred mouse lines, where they produce phenotypically distinct neurological diseases [2]. Interestingly, prion strains selectively target specific brain regions, while leaving others unaffected [3, 4]. During the course of the disease, the cellular prion protein PrP^{C} misfolds and aggregates in the affected brain areas, leading to accumulation of abnormal PrP (termed PrP^{Sc}) intracellularly or extracellularly. PrP^{Sc} purified from brains of diseased animals is closely associated with prion infectivity, arguing for a causal relation between the conformational state of the protein and its infectious properties [5]. According to the prion hypothesis [5] and compelling new evidence [6–8], prions constitute a class of proteinaceous

infectious agents composed almost exclusively of protein without coding nucleic acid. For propagation, PrP^{Sc} acts as a template that catalyzes the conformational conversion of the cellular prion protein PrP^{C}. The existence of prion strains that can propagate in the same inbred mouse lines has posed a conundrum to the prion hypothesis. Accumulating evidence now argues that prion strains are encoded by different PrP conformers capable of faithfully replicating their specific structure in the affected hosts. The strain-specific biological and biochemical signatures are likely enciphered by conformational variants of PrP^{Sc} [9–11].

The precursor of PrP^{Sc}, PrP^{C}, is a natively folded protein, with an unstructured amino terminal domain. Mature PrP^{C} is a glycosylphosphatidylinositol- (GPI-) anchored membrane protein that is abundantly expressed in the central nervous system but also in other tissues. The exact function of PrP^{C} has not been elucidated, but several studies suggest it has a cytoprotective function in neurons [12]. PrP^{Sc} fundamentally differs from PrP^{C} in its biophysical properties. PrP^{Sc} has a high β-sheet content, is insoluble in nonionic detergents, and its globular domain (approximately amino acid residues 89–230) is resistant to proteinase K (PK) [13]. Treatment of histological specimen or tissue lysates with PK is used routinely to identify prions in biological samples. It is important to note, however, that the exact PrP conformer with infectious properties has not been defined. Recent data argue that infectious PrP propagated *in vivo* and in cell culture can also be PK-sensitive, adding another layer of complexity to the characterization of infectious proteins [10, 14]. For simplicity, we will refer to infectious PrP molecules as PrP^{Sc}.

Propagation of prions is thought to occur through a process of nucleation-dependent polymerization, in which a seed of aggregated PrP^{Sc} templates the conformational conversion of its soluble homotypic isoform. The initial step of seed or oligomer formation is a slow and rate limiting process. In a subsequent elongation step, monomeric protein is recruited into the structurally ordered β-pleated fibrils, so-called amyloid. In a third step, secondary nucleation events such as filament fragmentation produce additional seeds that elongate and multiply [15]. Flow field-flow fractionation has recently been used to separate prions by size, demonstrating that particles composed of 14–28 monomers exhibit the highest infection properties *in vivo* [16]. Two lines of evidence argue that aggregate shearing is crucial for prion multiplication. First, aggregate fragmentation is an essential step in the so-called protein misfolding cyclic amplification (PMCA) developed by Saborio and colleagues [17]. In this assay, PrP^{Sc} present in brain homogenate serves as a template that is mixed with substrate PrP^{C} present in normal, uninfected brain homogenate. Consecutive steps of incubation and sonication catalyze PrP^{Sc} growth and segregation, leading to an exponential increase of prion polymers with infectious properties. Second, protein aggregate fragmentation is crucial for the propagation of prion-like protein aggregates in yeast and filamentous fungi [18, 19]. Prions in lower eukaryotes are protein-only epigenetic elements of inheritance that replicate by a seeded polymerization/fragmentation process similar to mammalian prions. Interestingly, yeast prions are fragmented by chaperone Hsp104 in conjunction with additional chaperones [20, 21]. Hsp104 has no homologue in mammalian cells, and the *in vivo* mechanism of mammalian prion fragmentation is so far unknown.

2. PrP^{Sc} Formation in Cell Culture

The establishment of prion cell culture models has greatly enhanced our understanding of the cellular mechanisms of prion formation. However, even decades after the first successful prion infection *ex vivo*, many aspects of prion replication still remain elusive. The most puzzling observation is that the susceptibility of a given cell line can only be determined empirically. Most PrP^{C} expressing cell lines are refractory to mammalian prion infection for unknown reasons [22, 23]. Prion strains also demonstrate an exquisite host cell tropism not only *in vivo* [3, 4] but also in tissue culture cells [24–29]. Prion propagation *ex vivo* is not restricted to neuronal cells, and also epithelial cells or fibroblast cell lines are permissive to certain strains. Many prion strains from various origins have never been successfully propagated in cell culture [30]. These observations suggest that so far unidentified strain and host cell specific factors control the replication of prion strains. Because of the usually low infection rates, most studies have been performed with previously established cell lines persistently infected with prion strains RML or 22L. In the following sections, we will briefly review basic findings on the uptake, the initiation of an infection, and the propagation of prions in permanent cell culture models.

Uptake of PrP^{Sc} is an early step of prion infection and is independent of PrP^{C} expression [31]. PrP^{Sc} uptake is also observed in nonpermissive cell lines and thus not indicative of a productive infection [31, 32]. PrP^{Sc} formation in cell culture is initiated once PrP^{C} has been translocated to the cell surface [33]. Substantial amounts of newly formed PrP^{Sc} are already detectable within minutes to hours post exposure [34, 35]. Importantly, transient PrP^{Sc} formation has been demonstrated in resistant cell lines, arguing that initial seeding of endogenous PrP^{Sc} does not necessarily lead to a successful prion infection *ex vivo* [34]. The continuous presence of PrP^{Sc} over multiple cell passages is indicative of a productive infection of the cell culture. Abnormal prion protein accumulation is routinely detected by western blot analysis of cell lysates treated with PK (50 μg/mL, 37°C, 1 hr) or by indirect immunofluorescence in fixed, prion infected cells following antigen retrieval by harsh denaturants such as guanidinium hydrochloride [36], formic acid [35] or partial proteolysis by proteinase K [36, 37]. In cultured cells, PrP^{Sc} is mainly confined to vesicles of the endocytic pathway, including early endosomes, recycling endosomes, and lysosomes [37–40]. Lipid rafts [41–43] and/or endocytic recycling compartments [40, 44] likely constitute sites of PrP^{Sc} formation. PrP^{Sc} produced in cell cultures has a half-life time of approximately 30 hrs [45]. Both lysosomes and autophagosomes have been implicated in PrP^{Sc} clearance [46–48]. Importantly, with one

exception, prions in permanent cell lines do not induce visible morphological or pathological changes [49].

3. Sustained Propagation of Mammalian Prions in Culture

Vertical spreading from mother to daughter cells is a prominent feature of mammalian prions in tissue culture (Figure 1(a)) [50]. Under the right cell culture conditions, mammalian prions can be propagated *ex vivo* indefinitely. The mouse neuroblastoma cell line N2a infected with RML/Chandler strain in the late 1980's [51, 52] has been distributed throughout the world and still serves as the prototype cell line for studying cellular aspects of prion propagation. Cell division affects the aggregate load of the cell, diluting the number of infectious particles by half (Figure 2). The continuous prion propagation in cell culture implies that proper fragmentation and partitioning mechanisms are in place for seed multiplication. It is possible that large PrP^{Sc} aggregates are segregated by mechanical force, for example, during endocytosis, thereby producing smaller prion entities. Alternatively, unidentified cofactors catalyze the fragmentation of larger prion aggregates. Prion propagation in mammals is confined to the cell surface or endocytic vesicles, suggesting that cofactors necessary for aggregate fragmentation reside in the same cellular compartments. It is tempting to speculate that cellular quality control mechanisms are also exploited by mammalian prions to disassemble high molecular weight prion aggregates into smaller infectious entities. However, the disaggregase Hsp104 necessary for production of infectious prion entities in yeast does not exist in mammalian cells. Several chaperones have been identified that interact with or regulate PrP folding and misfolding in mammalian cells [53–55], but their contribution to prion propagation is unclear. Incomplete clearance of prion particles, for example, via lysosomes or autophagy, could potentially contribute to prion particle fragmentation. This hypothesis is supported by the observation that infection of autophagy-deficient mouse embryonic fibroblasts with mammalian prions was significantly enhanced by ectopic expression of autophagy-related protein ATG5 [56].

4. Prion Infection Spreads to Adjacent Cells

Prions *ex vivo* are not only transmitted to progeny cells, but they also spread to neighboring cells (Figure 1(a)). Dissemination of mammalian prions *in vitro* involves at least two independent routes. Horizontal transmission of prions induces a prion phenotype in the recipient cells that spreads again both vertically and horizontally. In some cell culture models, prions were secreted into the cell culture supernatant [29, 49, 63]. Several studies demonstrated that prions are often associated with exosomes released by the donor cells. Exosomes containing PrP^{Sc} have been shown to efficiently initiate prion propagation in recipient cells [64–68]. How exosomes make contact with the recipient cells and how incorporated prions then induce infection is currently unknown. Direct proximity between donor and recipient cells drastically increased the infection in other cell culture models [69]. In some instances, prions travel through cytoplasmic bridges, so-called tunneling nanotubes that form transiently between cells [70]. These data suggest that prions can utilize several distinct routes for efficient cellular spreading. So far, it is unclear if the observed differences in spreading mechanisms are due to different cell culture models or strain-specific dissemination strategies. Of note, horizontal transmission of prions in cell culture is generally much less efficient than vertical transmission to daughter cells [50].

5. Not All PrP Aggregates Are Infectious

The conformational transition of cellular prion protein to a misfolded, aggregated isoform is believed to be the underlying principle of prion formation, but PrP expression levels, mutations, impairment of the cellular quality control mechanisms, and some chemicals also trigger formation of PrP aggregates in cell culture. PrP^{C} expressed in cell culture is usually soluble, but overexpression increases the amount of insoluble protein (unpublished results). Prion proteins harboring familial mutations expressed in cell culture are often more abundant in detergent insoluble fractions and exhibit slightly enhanced PK resistance [71–76]. Some mutant PrP molecules linked to inherited prion diseases are retained in the cytosol, where they can aggregate into detergent-insoluble, partially PK-resistant assemblies following proteasome impairment [77]. Importantly, the moderate increase in PK resistance of PrP mutants expressed in tissue culture systems has so far not translated into infectious properties. It is possible that pathogenic mutations destabilize PrP and make it more prone to aggregate, and those misfolded proteins become refolded into an infectious PrP^{Sc} isoform in a secondary event during the very long incubation time *in vivo* [71, 74]. Of note, misfolding and aggregation are not confined to PrP with familial mutations, and replacements within the PrP coding sequence can alter PrP processing and increase PrP protease-resistance [78]. Truncated versions of PrP lacking the signal peptide and the GPI anchoring signal undergo spontaneous aggregate formation in the cytosol of mammalian cells [79]. Imbalances in cellular proteostasis can also alter the cellular localization of PrP and influence its solubility. Proteasome impairment increases the fraction of cytosolic PrP and triggers aggregation, but infectious properties of those aggregates have not been reported [80, 81]. Chemical compounds can alter the trafficking, cellular localization, and aggregation state of PrP^{C} [82–84]. The most important implication from these studies is that PrP aggregates induced in cell culture by mutations or chemicals or triggered by changes in protein homeostasis have not acquired conformations that are self-propagating.

6. Recombinant Prions Induce Chronic Infections in Permissive Cell Cultures

Recently, it was shown that a synthetic prion strain, induced by the inoculation of β-sheet rich amyloid fibrils first into transgenic and subsequently into wild-type mice, was capable of replicating in mouse neuroblastoma cells with high fidelity

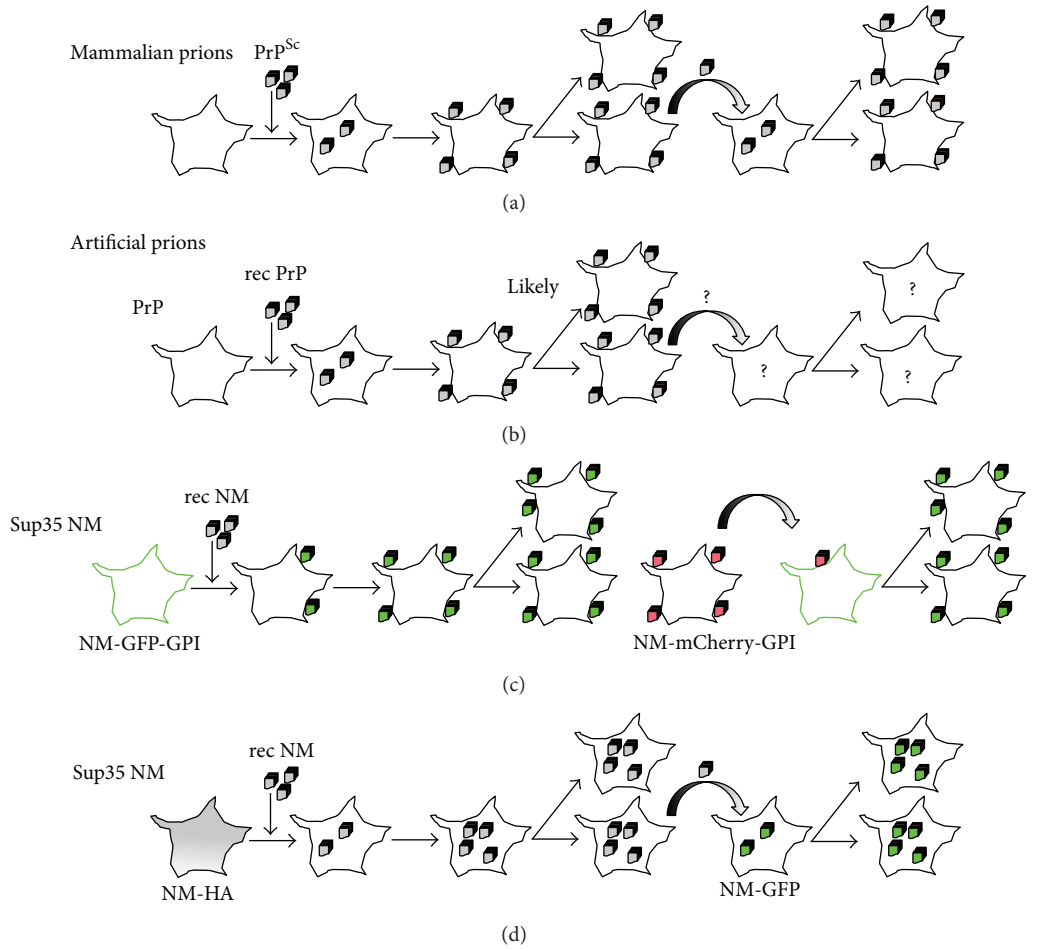

Figure 1: Induction of a PrP or Sup35 NM prion phenotype in mammalian cells. (a) Mammalian prions can infect selected cell cultures. Exposure of permissive cells to brain homogenate containing PrP^{Sc} leads to infection of cells. Prions persist mainly through stable inheritance of PrP^{Sc} aggregates by daughter cells. Prions also spread to adjacent cells by cell-to-cell contact or exosomes and induce productive infection in recipient cells. (b) Artificial prions produced *in vitro* from recombinant prion proteins. PrP aggregates derived from PrP and minimal components by PMCA are infectious to some cell lines. The increase in PrP^{Sc} over time suggests that prions propagate, likely by vertical transmission to daughter cells. Spreading to adjacent cells has not been studied so far. (c) Recombinant NM fibrils produce a heritable NM aggregation phenotype in N2a cells expressing a GPI-anchored NM-GFP fusion protein. When N2a cells that spontaneously form mCherry-tagged NM-GPI aggregates are cocultured with NM-GFP-GPI expressing cells, they induce NM aggregation in neighboring cells. (d) Cytosolically expressed NM-HA is soluble but can be induced to aggregate upon addition of recombinant NM fibrils. The NM aggregate phenotype is transmitted vertically and horizontally.

[85]. New studies now demonstrate that synthetic prions derived from minimal components *in vitro* have the capacity to directly infect cell cultures (Figure 1(b)) [17, 62]. Synthetic prions produced by PMCA from recombinant mouse PrP mixed with synthetic anionic phospholipids and total liver RNA induced a sustained infection of murine SN56 cells (Figure 3(a)) [17]. Liver RNA could also be replaced by synthetic polyA RNA, leading to the formation of recombinant prions which are capable of chronically infecting a subclone of CAD cells [62]. The *ex vivo* propagation of recombinant synthetic prions produced by PMCA is surprising, given the restricted cell tropism of most prion strains. It is possible that the chosen cell lines generally represent highly susceptible substrates for prions [29, 86]. Future experiments may reveal if recombinant prions have an equally restricted host cell spectrum like natural prion strains.

7. The Mammalian Cytosol Supports Propagation of Infectious Protein Aggregates

Prion diseases are the only known protein misfolding diseases that arise by aberrant folding of a GPI-anchored precursor protein. The strong association of PrP^{Sc} with membrane fractions and the attachment of PrP^{C} to the outer leaflet of the cell membrane via a GPI moiety have led to the hypothesis that GPI-anchorage of amyloidogenic proteins might be key for

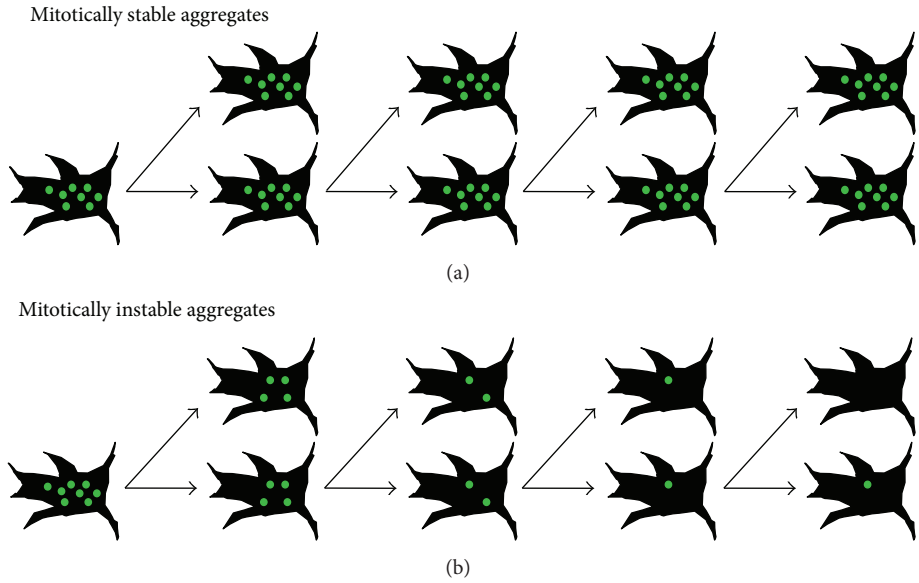

Figure 2: Mitotic stability of aggregate phenotypes is indicative of aggregate fragmentation. Dividing cells are a good model system to study propagation propensities of protein aggregates. (a) Proteins that can propagate as prions are mitotically stable in tissue culture, meaning that they are bidirectionally transmitted to daughter cells. Generation of infectious seeds that can self-propagate must be at least as fast as cell division. Prion aggregates must also be capable of escaping effective cellular clearance mechanisms. PrP^{Sc}, NM derived prions [57, 58], and some SOD1 mutants [59] fulfill these criteria. (b) Many protein aggregates are mitotically instable. Unidirectional segregation during cell division might represent an evolutionary conserved mechanism to protect a subset of the progeny cells from toxic effects of protein aggregates. Relatively poor mitotic stability has been reported for polyQ aggregates [60, 61].

the infectious properties of protein aggregates. The influence of the GPI anchor on prion-like properties of aggregation-prone proteins has recently been studied by tethering the yeast prion domain of the translation termination factor Sup35 to the N2a cell membrane via a GPI anchor [87]. The fusion protein of the prion domain NM with the GPI anchor either remained soluble or spontaneously aggregated, potentially dependent on its expression level or the fused fluorescent protein ([87] and unpublished results) (Figure 1(c)). Fluorescently labeled recombinant NM fibrils prepared *in vitro* were efficiently taken up by the cells and induced aggregation of the membrane-tethered soluble NM-GFP-GPI. Once switched into the aggregated state, this conformation was faithfully inherited by daughter cells. Coculture of cells expressing spontaneously aggregating NM-mCherry-GPI with cells expressing soluble GFP-tagged NM-GPI also induced a self-perpetuating prion phenotype in the latter.

Recent findings using the same Sup35 NM domain expressed in the cytosol of the same cell line, however, argue that cell membrane attachment via a GPI moiety is not a general requirement for infectious properties of protein aggregates in tissue culture (Figure 1(d)) [57]. In this experimental setting, the antibody epitope-tagged Sup35 NM domain was stably expressed in the cytosol as a soluble protein [79]. Spontaneous NM aggregation was not observed, not even under oxidative stress conditions [58]. Exposure of cells to *in vitro* produced fibrils from recombinant NM (Figure 3(b)) induced aggregation of the endogenous NM. Once induced, the aggregation state of NM was remarkably stable over multiple cell passages without any obvious loss of aggregate-bearing cells. NM aggregation could also be induced horizontally by direct transfer of NM aggregates from donor to acceptor through cell contact. The induced NM aggregation state in the acceptor cells was again heritable, strongly arguing that NM aggregates fulfill all criteria for prions in cell culture.

8. Prion-Like Properties of Proteins Associated with Neurodegenerative Diseases

Several neurodegenerative diseases are accompanied with intra- or extracellular deposition of amyloidogenic protein assemblies. While their primary sequences are diverse, aggregated proteins share a similar structure, consisting of an ordered arrangement of β-sheets [88–90]. Often, pathology begins locally, then progresses to other areas of the brain, reminiscent of the pathology spreading observed in prion diseases [91]. In contrast to prion diseases, neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), or Huntington's disease (HD) are not considered infectious diseases. Exciting research of the last years has demonstrated that aggregate pathology can spread to interconnected brain areas in several neurodegenerative disease models [91], suggesting that even nonprion protein aggregates somehow propagate and spread in tissue. These findings have led to the hypothesis that protein aggregates associated with neurodegenerative diseases share characteristic features of prions. Recent research has focused extensively on establishing

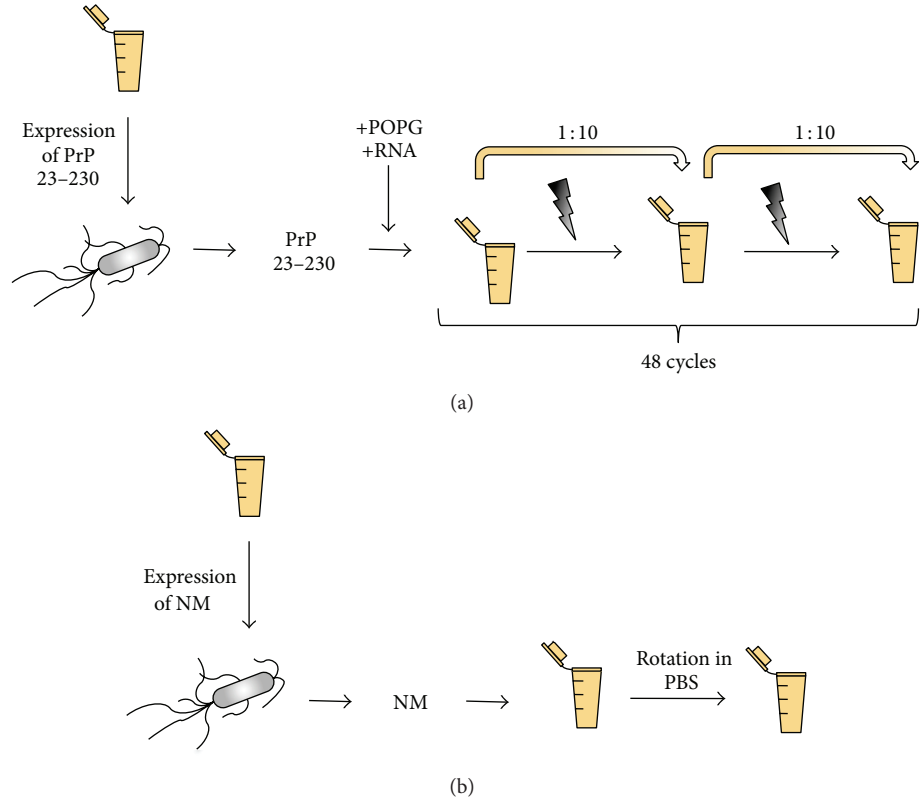

Figure 3: Generation of infectious protein polymers from minimal components *in vitro*. (a) For the generation of infectious PrP aggregates, purified recombinant murine PrP comprising amino acid residues 23 to 230 is mixed with lipid POPG and total liver RNA or synthetic polyA RNA. In a so-called protein misfolding cyclic amplification reaction (PMCA), intermittent pulses of sonication shear aggregates and increase the number of seeds. Preformed seeds are subsequently elongated and fragmented by serial dilution of samples into a new reaction buffer (supplemented with recombinant PrP and cofactors) and PMCA. Synthetic prions generated by this method have been shown to chronically infect SN56 and CAD cells [8, 62]. (b) The formation of Sup35 NM fibrils does not require additional cofactors. Purified recombinant NM is diluted to a concentration of 10 μM in phosphate buffered saline and left at room temperature overnight. Formation of NM fibrils is expedited by agitation. Addition of these fibrils to N2a cells stably expressing soluble NM induces a heritable NM prion phenotype [57, 58].

cellular models for unraveling prion-like phenomena associated with neurodegenerative disease-related proteins. These studies convincingly demonstrated that aggregation-prone proteins can move between cells and seed protein aggregation in recipient cells. It is, however, a misconception that intercellular transmission and seeding of aggregates equal propagation. Propagation of prions—at least *in vitro*—is controlled by the rate of aggregate elongation, fragmentation, and degradation [15]. "Propagation" implies that transmitted seeds induce a self-templating aggregation state that will ultimately increase the number of protein aggregates per cell. Indeed, mammalian prions have found efficient ways to replicate, they produce enough seeds per cell that sustain the cellular clearance and then infect progeny and neighboring cells. This remarkable propagation efficiency is easily revealed in mitotically active cells. Here, constant cell division exponentially reduces the prion load, unless dilution and clearance effects can be compensated for by high rates of prion multiplication. The persistence of protein aggregates in dividing cells is, thus, indicative of how efficient a protein aggregate can propagate. In the following chapter we will discuss cell autonomous and noncell autonomous aggregation behaviors of the intracellular proteins Huntingtin (Htt), Tau, α-synuclein, and SOD1 in cell culture. We will particularly focus on the differences and similarities of PrP and other neurodegenerative disease-related proteins based on (i) their spontaneous aggregation propensities, (ii) their ability to aggregate upon addition of exogenous fibrillar seeds, (iii) their sustained propagation as induced aggregates, and (vi) their transmission in cell culture models. Of note, protein aggregates usually form in postmitotic neurons *in vivo*. However, mitotic stability *ex vivo* indicates how easily protein aggregates can propagate in a cellular environment.

9. Aggregation of Htt Polypeptides Encoded by Htt Exon 1

HD is a monogenic disease caused by expansion of CAG repeats in exon 1 of the Htt gene, resulting in an elongated polyglutamine (polyQ) region in the mutant protein [92]. Thus, the etiology of prion diseases crucially differs from that of HD. Mutant Htt undergoes proteolytic cleavage, giving

rise to aminoterminal fragments (~the first 100–150 amino acid residues) that comprise the polyQ tract. Aggregated polyQ fragments are found in brains of HD patients and mouse models [93]. Therefore, HD pathogenesis is frequently modeled with proteins encoded by Htt exon 1.

(i) Aggregation Propensity When Expressed in Cell Culture. Normal nonmutant Htt protein (polyQ proteins with 6–35 glutamine residues) is usually soluble when expressed *ex vivo*. Expression of polyQ polypeptides causes spontaneous aggregate formation in a broad variety of cell types, including nonneuronal and neuronal cells lines as well as cultures of primary neurons [92]. Aggregation kinetics, subcellular localization, and shape of the aggregates are affected by repeat lengths, presence of Htt carboxy terminal regions, expression level, expression kinetics, and cell type [94–111].

(ii) Uptake and Seeding. Similar to PrP^{Sc}, recombinant polyQ peptides are internalized by cultured cells and can seed polymerization of a soluble Htt reporter protein. This has been shown by adding recombinant, fluorescently labeled polyQ fibrils to COS7, HEK293, CHO, HeLa, and N2A cells [60]. Interestingly, polyQ fibrils appeared to access the cytosol by direct penetration of the cell membrane. Internalized recombinant fibrils induced coaggregation of ectopically expressed soluble Htt fragments with nonmutant glutamine stretches.

(iii) Sustained Propagation. One hallmark of PrP^{Sc} is its stable inheritance in permanent cell cultures. For Htt, the mode of inheritance by daughter cells seems less stable. Asymmetric inheritance has been suggested based on the observation that big aggresome-like structures of polyQ aggregates are transmitted to only one daughter cell [61]. Interestingly, the number of cells with induced polyQ aggregates declined exponentially upon cell division, until reaching a low, but apparently persistent steady-state level of approximately 4% above background (Figure 2(b)) [60].

(iv) Cell-to-Cell Transmission. Coculture experiments of donor HEK cells expressing GFP-HttQ71 with cells expressing soluble HttQ25 revealed very inefficient aggregate induction in recipient cells, suggesting that Htt aggregates might not be transferred between cells at high rates [60]. Cell-to-cell transmission of polyQ proteins has also been demonstrated in cocultures of human H4 glioma and HEKT cells transfected with Htt exon-1 Q103 fused to halves of the Venus fluorescent reporter by bimolecular fluorescence complementation [99]. Importantly, in this experimental setup, no direct evidence for transfer of Htt aggregates has been provided, since the transferred protein species could also be monomeric or oligomeric. To date, it has not been shown if cell-to-cell transfer of polyQ aggregates induces ongoing replication of aggregates capable of propagating vertically and horizontally like prions.

10. Tau Aggregation in Cell Culture

Neurofibrillary tangles (NFTs) are a pathological hallmark of more than 20 so-called Tauopathies, including Alzheimer's disease (AD) and frontotemporal dementia. Accumulated hyperphosphorylated Tau protein aggregates into inclusions called paired helical filaments (PHF), which are the main component of NFTs. Tau is a microtubule-associated protein that stimulates and stabilizes microtubule assembly. Tau mutations cause familial neurodegenerative diseases. Tau is a naturally unfolded, highly soluble protein that exists as six isoforms [112, 113]. Alternative splicing generates Tau isoforms with three or four repeat domains (RD). The repeat domains are involved in microtubule binding and fibrillization. The mechanisms that trigger Tau conversion remain elusive. In AD brains, Tau is hyperphosphorylated, with a three- to four-fold increased phosphorylation level. Upon hyperphosphorylation, Tau dissociates from the microtubules and aggregates into the neurofibrillary tangles, resulting in microtubule destabilization and neurotoxicity [114, 115].

(i) Aggregation Propensity When Expressed in Cell Culture. Because of its high solubility, Tau—despite hyperphosphorylation—does not spontaneously aggregate upon overexpression in most cell lines [116, 117]. Spontaneous aggregation of overexpressed Tau constructs has been achieved using mutated full-length Tau and truncated Tau forms comprising the approximately 132 amino acid residue long aggregation-prone four-RD region [116, 118, 119].

(ii) Uptake and Seeding. Preformed recombinant aggregates derived from RD Tau or full-length Tau can be taken up by a variety of permanent cells and primary neurons [118, 120–122]. In primary neurons and HeLa cells, recombinant Tau was taken up by fluid-phase endocytosis [122]. Uptake of Tau appears to depend on its aggregation state, as monomeric or long fibrillar Tau is not internalized [122]. Similar to PrP^{Sc}, seeding properties of internalized Tau fibrils were shown. By applying preformed recombinant full-length or Tau RD fibrils to neuronal precursor cells, HEK cells, or primary neurons, formation of intracellular Tau aggregates by recruitment of soluble Tau was induced [118, 119, 123]. Importantly, recombinant Tau fibrils could also induce endogenous mouse Tau inclusions in wild-type hippocampal neurons, arguing that seeding does not require Tau overexpression [121].

(iii) Sustained Propagation. The propagation propensity of induced Tau aggregates in cell culture has not been elucidated so far. It is unclear if the transmitted aggregates also trigger a self-catalyzed propagation of Tau aggregates. If Tau aggregates faithfully replicate upon induction like prions, they should be maintained upon continuous culture.

(iv) Cell-to-Cell Transmission. Both spontaneously formed Tau RD aggregates and exogenously induced Tau full-length aggregates are transmitted from donor to acceptor cells in coculture experiments [118, 119]. Coculture of cells expressing differently tagged, spontaneously aggregating Tau RD variants revealed double stained inclusions, suggesting that Tau RD variants were exchanged between cells. This appears to depend on the release of fibrillar Tau RD species directly into the culture medium [119]. Transmission of HA-tagged Tau RD from the donor to a recipient cell line expressing both

CFP- and YFP-tagged Tau RD variants increased fluorescent resonance energy transfer (FRET), suggesting that transmitted Tau RD increased endogenous Tau RD interaction and aggregate formation in the recipient. Of note, these experiments have been performed with nonphysiological, spontaneously aggregating Tau mutants, and the seeding of full-length Tau by cell-to-cell transmission has yet to be demonstrated.

11. Alpha-Synuclein Aggregation in Cell Culture

Parkinson's disease (PD) is characterized by deposition of the intracellular α-synuclein in dense Lewy bodies or Lewy neurites. α-synuclein is a 14 kDa protein that plays a role in synaptic neurotransmitter vesicle trafficking and releasing [124]. Increased α-synuclein expression and mutations in the coding region trigger misfolding and aggregation of α-synuclein into oligomers and amyloid fibrils *in vivo* [125, 126]. Post-translational modifications such as phosphorylation, oxidation, nitration, and carboxy terminal truncation have been observed *in vivo* and could affect α-synuclein fibrillization [127].

(i) Aggregation Propensity When Expressed in Cell Culture. Recent studies in different mammalian cell lines suggest that endogenously or ectopically expressed human α-synuclein exists predominately as an unfolded monomer [128]. In some cell culture models, tetrameric or oligomeric species have been identified [129–132]. Overexpression of wild-type α-synuclein in diverse cell lines does not generally induce inclusion body formation [133], but mutant and/or truncated α-synuclein forms intracellular aggregates in some but not all cellular models [134]. Most often, α-synuclein aggregation is induced by treatment of the cells with oxidative stress and/or increased calcium levels [135–139]. α-synuclein aggregation in cell culture can also be promoted by coexpression of mutant leucine-rich repeat kinase, a protein linked to familial PD [140], or by treatment with mitochondrial inhibitor rotenone [141].

(ii) Uptake and Seeding. α-synuclein seeds prepared from recombinant protein *ex vivo* can be taken up by a variety of different cells [142–146]. Endocytosis has been proposed as a possible route for internalization of fibrillar recombinant human α-synuclein [142, 146]. In some instances, uptake of fibrils into the cells by physiological routes failed, and fibrils had to be introduced via cationic lipid transfection [145]. Variations in uptake efficiencies might be due to differences in fibril preparation, as has been shown for different α-synuclein oligomers [143, 144]. Exogenous α-synuclein oligomers or fibrils can seed the formation of intracellular inclusions in different permanent and primary cell models [143–146]. Overexpression of wild-type or mutant α-synuclein [146] is not generally required for noncell autonomous aggregate induction, but aggregation kinetics appear to be faster under α-synuclein overexpression conditions [144, 145].

(iii) Sustained Propagation. So far, sustained propagation of induced α-synuclein aggregates in cell culture has not been reported. Future experiments will be necessary to evaluate if α-synuclein aggregates, once triggered, gain self-propagating properties that allow them to continuously propagate over multiple passages. In analogy to mammalian prions, this would require that aggregate growth and fragmentation exceed the loss of aggregates by cellular clearance or cell division.

(iv) Cell-to-Cell Transmission. Coculturing experiments with cells producing α-synuclein aggregates with recipient cells revealed transfer of α-synuclein between cells with subsequent aggregate induction in the recipient cell [147, 148]. The exact mechanism of aggregate transfer has not been elucidated. Low amounts of soluble and aggregated forms of α-synuclein have been shown to be released by cells, potentially by unconventional endocytosis or packaged into exosomes [149–152].

12. Superoxide Dismutase 1 Aggregation in Cell Culture

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that usually occurs sporadically but can also be of genetic origin. During the course of the disease, motor neurons in spinal cord, brain stem, and brain degenerate [153]. More than 140 mutations in the copper/ zinc superoxide dismutase (SOD1), an antioxidant enzyme, are linked to approx 2% of familial ALS cases (http://alsod.iop.kcl.ac.uk/). Misfolding of mutant SOD1 and cytoplasmic inclusion of body formation underlies disease pathology [154].

(i) Aggregation Propensity When Expressed in Cell Culture. Active SOD1 is located in the cytoplasm in the form of a homodimer. Overexpression of wild-type SOD1 usually does not lead to inclusion body formation in cell culture [155]. Still, misfolded states of the wild-type or mutant SOD1 overexpressed in cell culture could be revealed by antibody detection of normally buried antibody epitopes [156]. The aggregation propensities of various SOD1 mutants differ in cell culture [59, 155, 157].

(ii) Uptake and Seeding. Aggregated recombinant SOD1 can be taken up by neuroblastoma cells [59], and primary or permanent microglia cell cultures [158]. Uptake of aggregated SOD1 was decreased by either chemical inhibition of rafts and scavenger receptors, or by macropinocytosis inhibitors, indicating that uptake mechanisms might depend on the cell line or seed preparation [59, 158]. Recombinant mutant SOD1 aggregates added to the cell culture medium gained access to the cytosol of N2a cells, where they recruited the homotypic protein into small, dispersed aggregates [59].

(iii) Sustained Propagation. Unlike polyQ aggregates, seed-induced SOD1 aggregates exhibit a remarkable mitotic stability in cell culture [59]. The stable propagation of the SOD1 aggregation phenotype over multiple passages in cell culture is reminiscent of that of mammalian prions and suggests steady state levels of SOD1 aggregates.

(iv) Cell-to-Cell Transmission. Fluorescently tagged mutant SOD1 aggregates are efficiently taken up by N2a cells and subsequently transmitted to cocultured cells [59]. Direct cell contact between donor and acceptor cells was not required for transmission, suggesting that SOD1 was secreted into the cell culture medium. Secretion of endogenous SOD1 has been reported for a variety of different cell lines, including fibroblasts, neuroblastoma, motor neuron cell lines and primary spinal cord cultures [159–161]. It remains to be established if and how self-perpetuating SOD1 aggregates produced in cell culture spread horizontally and induce a SOD1 aggregation phenotype in the acceptor cells [59].

13. Concluding Remarks

Prions are proteinaceous infectious protein aggregates that have the capacity to enter the host cells and impose their abnormal conformational states onto their endogenous counterparts. Noncell autonomous aggregate induction through external seeds has recently also been demonstrated for a variety of different proteins associated with nonprion neurodegenerative diseases. The ability to invade cells and seed cytosolic aggregation appears to be a general feature of amyloidogenic proteins. Importantly, the aggregation state of a protein does not reflect its infectious properties and only a thorough investigation of its propagation *ex vivo* or *in vivo* can ultimately confirm infectious potentials. Prion replication proceeds through a process of seeded polymerization and secondary nucleation events by fibril fragmentation, and escape of those seeds from cellular clearance is crucial for prion maintenance. Mitotically active cells represent tractable models for studying aspects of prion fragmentation and clearance, as inefficient prion fragmentation or enhanced clearance result in rapid prion loss due to aggregate dilution by cell division. The fact that mammalian prions can successfully replicate mitotically active cells argues for a steady state between prion formation and prion reduction. The observed mitotic instability of other protein aggregates *ex vivo* could, thus, be due to inefficient fragmentation or enhanced clearance. In conclusion, the ability of a given protein aggregate to achieve a steady state of aggregate multiplication and reduction will ultimately affect its prion capacity.

Note. While in print persistent propagation of induced alpha-synuclein aggregates was reported in mouse neuroblastoma cell line N2a [162].

Abbreviations

AD: Alzheimer's disease
ER: Endoplasmic reticulum
GdnHCl: Guanidinium hydrochloride
GPI: Glycosylphosphatidylinositol
HA: Hemagglutinin antibody epitope
HD: Huntington's disease
Htt: Huntingtin
NFT: Neurofibrillary tangles
NM: Prion domain and middle domain of the yeast Sup35 translation termination factor
PD: Parkinson's disease
PHF: Paired helical filaments
PK: Proteinase K
PMCA: Protein misfolding cyclic amplification
polyQ: Polyglutamine
PrP: Prion protein
PrP^{C}: Normal cellular isoform of the prion protein
PrP^{Sc}: Disease-associated isoform of the prion protein
RD: Repeat domain of Tau
TSE: Transmissible spongiform encephalopathy.

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Review Article

From Prion Diseases to Prion-Like Propagation Mechanisms of Neurodegenerative Diseases

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Prion diseases are fatal neurodegenerative sporadic, inherited, or acquired disorders. In humans, Creutzfeldt-Jakob disease is the most studied prion disease. In animals, the most frequent prion diseases are scrapie in sheep and goat, bovine spongiform encephalopathy in cattle, and the emerging chronic wasting disease in wild and captive deer in North America. The hallmark of prion diseases is the deposition in the brain of PrP^{Sc}, an abnormal β-sheet-rich form of the cellular prion protein (PrP^{C}) (Prusiner 1982). According to the prion hypothesis, PrP^{Sc} can trigger the autocatalytic conversion of PrP^{C} into PrP^{Sc}, presumably in the presence of cofactors (lipids and small RNAs) that have been recently identified. In this review, we will come back to the original works that led to the discovery of prions and to the protein-only hypothesis proposed by Dr. Prusiner. We will then describe the recent reports on mammalian synthetic prions and recombinant prions that strongly support the protein-only hypothesis. The new concept of "deformed templating" regarding a new mechanism of PrP^{Sc} formation and replication will be exposed. The review will end with a chapter on the prion-like propagation of other neurodegenerative disorders, such as Alzheimer's and Parkinson's disease and tauopathies.

1. The Story of the Prion Protein That Was Mistaken for a Virus

Prion diseases and prion infectious agents [1] are among the most fascinating biological topics of the twentieth century and have been under the spotlight for the last 30 years, particularly due to the striking epidemic of bovine spongiform encephalopathy (BSE), which started in Great Britain in the mid-eighties and then spread to other European countries [2]. The transmission of the bovine prion agent to humans, possibly through consumption of prion-contaminated beef products, led to the emergence of a new human prion disease, named "variant" Creutzfeldt-Jakob disease (vCJD), in young people [3]. Recently, several cases of secondary human-to-human transmission of vCJD through transfusion of prion-contaminated blood [4–6] have raised doubts within the scientific community about the safety of blood products and highlighted the crucial need of diagnostic tests for prion detection in blood. Currently, the development of reliable blood tests and of therapies is the main mission of scientists working in the prion field.

Historically, the infectious agent that causes prion diseases was supposed to be an atypical virus belonging to the category of "slow viruses" [7, 8]. Then, in 1967, Pattison and colleagues reported [9] that the scrapie agent was resistant to heat and formaldehyde, two treatments that inactivate most viruses, thus introducing a doubt about the true nature of this infectious agent. In addition, in 1967, Alper and colleagues showed that the scrapie agent was also resistant to ionizing radiations and UV light irradiation that normally inactivates nucleic acids, suggesting that it was probably devoid of nucleic acids [10]. Based on these intriguing experimental data, Griffith suggested that the scrapie agent could be a protein that self-replicates through autocatalytic conformational changes [11]. This audacious hypothesis retained the attention of Stanley Prusiner who purified the scrapie agent

from the brain of scrapie-infected hamsters and reported that inactivation by physicochemical agents that destroy proteins abolished the infectivity of such purified preparations [12]. In 1982, he proposed the new term of "PRION," for "proteinaceous infectious only particle," to define this atypical agent. The revolutionary idea that a protein may act as a virus was unbelievable at that time and Stanley Prusiner had to struggle hard to convince the scientific community. His outstanding work on prions earned him the Nobel Prize of Medicine in 1997, although at that time the ground-breaking concept of proteinaceous infectious particles was not yet definitively proved.

2. Prion Diseases: The Revolutionary Concept of Pathogenic Misfolded Proteins

The prion protein (PrP) is the main component of prion agents and, remarkably, can fold into different (normal or pathogenic) conformations that are thermodynamically stable [13]. PrP^{C}, the normal cellular isoform, is mostly folded into α-helices [14] and is detergent-soluble and completely digested by proteinase K. Conversely, PrP^{Sc} (for scrapie form), the abnormally folded isoform, is mostly folded into β-sheets [15], which confer insoluble property in detergents, and is partially resistant to proteinase K. Indeed, PrP^{Sc} digestion by proteinase K produces an N-terminally truncated fragment that begins around residue 90 and is commonly called PrP27-30. PrP^{Sc} isoforms are the main constituent of amyloid plaques and of brain deposits in patients affected by CJD. For this reason, PrP^{Sc} is considered as the main disease marker and is the reference for the histopathological analyses carried out to diagnose prion diseases.

How can prions multiply? In the original "protein-only" hypothesis proposed by Griffith and Prusiner [1, 11], PrP^{Sc} can trigger the autocatalytic conversion of normal PrP^{C} into PrP^{Sc} and imprints its misfolded form to PrP^{C}, which in turn becomes pathological (Figure 1). This conversion process involves several PrP^{Sc} intermediates that are generated through a complex oligomerization mechanism and then self-assembled into protofibrils, which in turn grow into amyloid fibrils [16, 17]. Then, large fibrils can break naturally, producing small fragments, called seeds, that will propagate *de novo* the prion agent (seeding process) [18–20]. As both PrP^{C} and PrP^{Sc} are exposed at the cell surface and attached to the plasma membrane through a GPI anchor, they can propagate in tissues via cell-cell contacts [21, 22]. Many recent lines of evidence indicate that the most neurotoxic species within this replication cycle are the small soluble oligomers rather than the large amyloid fibrils, which would serve as "reservoirs" to trap small neurotoxic species [17, 23, 24].

3. Development of Animal Models to Study Prion Infectivity

The advent of molecular biology allowed the generation of cell (the neuroblastoma ScN2a cell line) and transgenic animal models [25–27] to investigate the molecular basis of prion replication, pathogenicity, and propagation. The crucial role of PrP was demonstrated using mice in which the gene coding for PrP (*Prnp*) was genetically ablated [28, 29]. These mice are resistant to prion inoculation and cannot propagate and replicate the infectious agent. Later on, *Prnp* knock out (using the *cre/lox* system) in the neurons of adult mice with early prion infection allowed demonstrating that the synaptic impairment, spongiosis, and behavioural deficits observed in these animals could be reversed [30]. Conversely, transgenic mice that harbour high copy numbers of a wild-type *Prnp* transgene develop a neurological syndrome that is similar in some aspects to prion disease, but they do not produce transmissible PrP^{Sc} unless they are inoculated with prions [31]. For decades, no animal model of sporadic prion disease was available in which prions formed spontaneously from wild-type PrP and could be transmitted to other animals. Interestingly, spontaneous development of transmissible spongiform encephalopathy was observed in transgenic mice that overexpress a mouse-elk PrP chimeric molecule harbouring the two point mutations S170N and N174T that induce a rigidity of the $\beta 2$-$\alpha 2$ loop region [32]. The disease could be transmitted by intracerebral inoculation of brain homogenates from ill mice to tga20 mice that overexpress wild-type PrP and from them to wild-type mice. These findings illustrate the importance of PrP $\beta 2$-$\alpha 2$ loop region. This region is rich in glutamine and asparagine residues, which are frequently encountered in amyloidogenic proteins, and may act as "hot spots" for protein aggregation. Recently, Watts et al. generated a transgenic mouse model, named Tg(BVPrP), that overexpresses wild-type bank vole (BV) PrP. These mice develop spontaneous CNS dysfunction between days 108 and 340 after birth that recapitulates the hallmarks of prion diseases [33]. Moreover, the disease could be transmitted to tga20 and wild-type mice by intracerebral injection of brain homogenates from ill Tg(BVPrP) animals. This is the first animal model showing that wild-type PrP can spontaneously form infectious prions *in vivo* and thus will be very useful for understanding the aetiology of sporadic prion diseases, such as sporadic CJD.

Several transgenic mice that overexpress the most frequent mutant PrP proteins, such as P101L PrP [34, 35] or PrP with a 9 octarepeat insertion in the N-terminus [36], were also generated. Although these mice succumb to spontaneous prion disease with various incubation times, they do not show all the biochemical and pathological features of prion diseases and often fail to transmit prion infectivity to wild-type animals. Recently, several transgenic mouse models of genetic prion diseases retained our interest:

(i) the Tg(PG14) transgenic mice that express a mutant PrP with 14 octapeptide repeats [37] and present a progressive neurological disorder with ataxia, PrP deposition, and massive loss of cerebellar granule cells. They also display the main biochemical properties of PrP^{Sc}, such as partial resistance to proteinase K, detergent insolubility and resistance to GPI-anchor cleavage by phospholipase;

(ii) the Tg(MHu2ME199K) mouse model of genetic CJD, which is caused by the E200K substitution in human

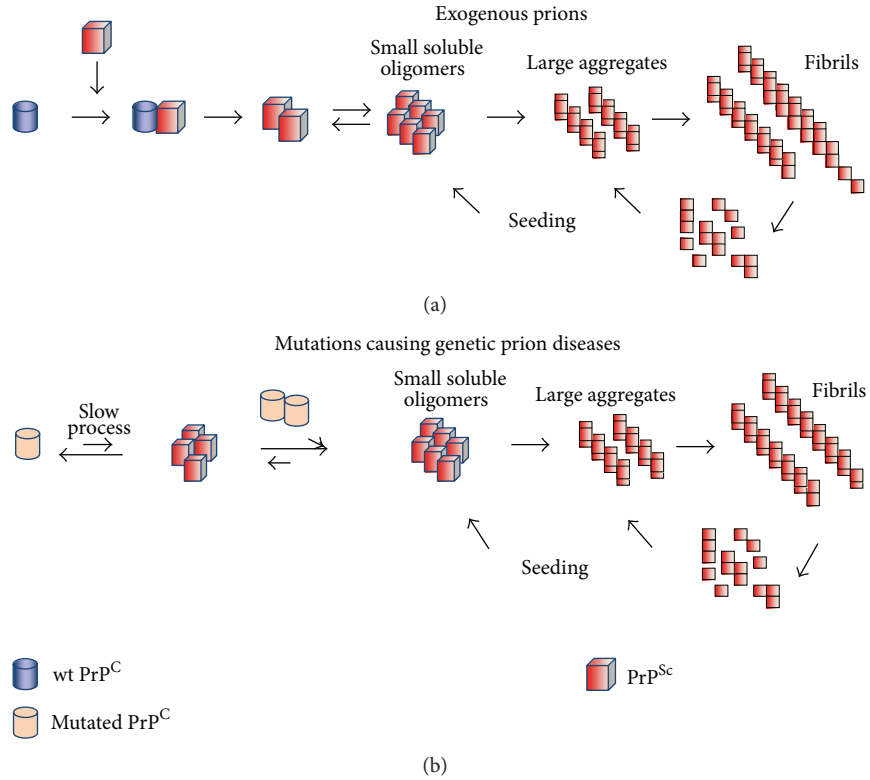

Figure 1: Proposed mechanisms of PrP^C conversion into PrP^{Sc}, with exogenous (a) or genetic (b) prions. Wild-type PrP^C is represented by a cylinder colored in blue, mutated PrP^C is outlined in blue, and PrP^{Sc} is represented by red square.

PrP. These animals express a chimeric mouse-human PrP harbouring the corresponding mouse E199K mutation (PrPMHu2ME199K) [38] and develop progressive neurodegenerative disease from 6 months of age. Histopathological analysis of their brain revealed the presence of astrocytic gliosis, spongiosis, and PK-resistant PrP deposits by western blotting. Importantly, brain extracts from Tg(MHu2ME199K) sick mice transmitted the prion disease to wild-type animals [38];

(iii) the knock-in Ki-3F4-FFI mice that express a mutant PrP (D177N-M129-3F4 tagged) associated with fatal familial insomnia (FFI), a genetic human prion disease [39]. These mice present several neurological features (atrophied cerebellum, enlarged ventricles, and thalamus abnormalities) that are similar to those seen in humans with FFI. Surprisingly, these animals display a protease-sensitive PrP (sPrP) isoform, like patients with FFI, and the disease is transmissible to control Ki-3F4-WT mice (wild-type *Prnp*) and to transgenic mice that overexpress wild-type PrP (tga20) [40]. The presence of PK-sensitive PrP^{Sc} in FFI mice supports recent findings showing that new PK-sensitive synthetic prions can be infectious [41], as well as the identification of a novel human prion disease called VPSPr (variably protease-sensitive prionopathy) [42], which is characterized by the presence of PrP^{Sc} with highly variable PK resistance. As for all neurodegenerative disorders, it is important to generate the most appropriate animal models of the genetic forms of the disease in order to develop pertinent therapeutic strategies.

4. Synthetic Mammalian Prions and Recombinant Prions: The Proof-of-Concept of the Protein-Only Hypothesis

Despite the compelling evidence in favour of the prion hypothesis, some sceptics argued that the definite proof could be obtained only by producing *in vitro* the infectious material used for intracerebral inoculation starting from pure normal PrP, a technical feat that seemed impossible for many decades. A strong advance was the finding that recombinant hamster PrP(90-231) (recPrP, which corresponds to human PrP27-30) purified from *E. coli* under reducing conditions at pH > 7 has a high β-sheet content and low solubility, like PrP^{Sc}. Conversely, recPrP refolding by oxidation to form a disulphide bond produced a soluble protein with a high α-helix content, similar to normal PrP^C [43]. The ability of recPrP to adopt either an α-helix- or β-sheet-rich conformation strongly suggests that the PrP sequence is intrinsically plastic. Some PrP domains may have a relatively open conformation which makes it susceptible to conversion into

PrP^{Sc} under appropriate physicochemical conditions [44]. However, inoculation of the β-sheet-rich recPrP isoform in mice did not transmit the disease. At the beginning of 2000, Baskakov et al. [19, 20] managed to partially solve the complex pathway of PrP assembly into amyloids. To study the kinetic pathway of amyloid formation, they used an unglycosylated recombinant PrP form that corresponds to the PK-resistant core of PrP^{Sc} and found that it can adopt two abnormal β-sheet-rich isoforms (β-oligomers and amyloid fibrils) via separate kinetic pathways. The tendency to generate either form is driven by the experimental conditions. Acidic pH (similar to the pH found in endocytic vesicles) favours the transition from α-monomers to β-oligomers, whereas neutral pH promotes amyloid fibril formation [20]. These multiple misfolding pathways and the generation of distinct β-sheet-rich isoforms might explain the difficulties to generate infectious prions *in vitro* from pure recombinant PrP. Then, Baskakov and Legname inoculated some amyloid fibrils from purified recombinant PrP(89-231) in the brain of Tg(PrP89-231) transgenic mice that express a truncated PrP variant corresponding to the PK-resistant core. After 580 days of incubation, all injected mice were sick and showed neurological symptoms reminiscent of prion diseases. Analysis of brain tissue sections revealed spongiosis, astrocytic gliosis, and the presence of PK-resistant PrP^{Sc} [45]. At the second passage, brain extracts from these mice were inoculated to both Tg(PrP89-231) and wt FVB mice. Both types of mice showed clinical signs and the biochemical features of prion disease after 150 days (FVB mice) and 250 days (Tg(PrP89-231) animals) of incubation. These findings indicate that a new prion strain can be generated from pure recombinant PrP designated "synthetic mammalian prions" and that it can induce a transmissible neurodegenerative disease in transgenic mice. Subsequent *in vivo* experiments with various synthetic prion strains obtained from recombinant PrP fibrils demonstrated that conformationally stable recombinant amyloids produced more stable prion strains with a longer incubation time, whereas more labile amyloids generated less stable strains with a shorter incubation time [46]. One major criticism to this work is that the recombinant fibrils were first injected in transgenic animals that overexpress PrP and not in wild-type mice. Thus, the inoculation of recombinant fibrils might have resulted in an acceleration of preexisting conditions produced by transgenesis as it is the case for transgenic mice that overexpress normal or mutated PrP [31, 36]. However, injection of recombinant hamster PrP (recPrPHa) fibrils in wild-type Golden Syrian hamsters provided strong evidence that fibrils can induce transmissible disease *de novo* [47], although 100% success rate was only achieved at the second passage and was correlated with the presence of PrP^{Sc} in the brain. The animals showed clear signs of transmissible spongiform encephalopathy (TSE), and the unique clinical course and neuropathological features suggested that a new prion disease was induced by recPrPHa fibrils. This new prion strain was designated as SSLOW due to the very long disease incubation time. These experiments are in strong favour of the protein-only hypothesis; however, it remains to be elucidated how recombinant PrP fibrils trigger the formation of transmissible PrP^{Sc}. The predominant hypothesis, which is based on the "template-assisted" mechanism of propagation, is that the preparation of recombinant PrP fibrils might have included some minute amount of PrP^{Sc} (Figure 2(a)) that was responsible for the disease. This could also explain the long incubation time and the lower than 100% transmissibility at the first passage. However, recent work by Makarava et al. [48, 49] suggests a new templating mechanism, called "deformed templating" (Figure 2(b)). Three different inocula with conformationally distinct amyloid states (0.5 M fibrils, 2 M fibrils and S fibrils) were prepared *in vitro* from purified recPrPHa [49]. After inoculation in mice, no signs of prion infection were found in animals injected with 2 M and S fibrils that are reminiscent of PrP^{Sc}, whereas the less stable 0.5 M fibrils induced a pathogenic process that eventually led to transmissible prion disease. Using the protein misfolding cyclic amplification (PMCA) technique, they showed that the 0.5 M recPrPHa fibrils used to inoculate wild-type animals did not contain classical PrP^{Sc}. However, these fibrils gave rise to an atypical proteinase K-resistant PrP (PrPres) that was detected using a modified PMCA procedure. This atypical transmissible PrPres has a structure that resembles that of amyloid seeds and was observed during the asymptomatic stage of the disease before the emergence of the classical PrP^{Sc} form [49]. This work provides evidence that apparently non-infectiou amyloid fibrils with a structure different from that of PrP^{Sc} can lead to transmissible prion disease and suggests a new mechanism of prion conversion through "deformed templating." In this model (Figure 2(b)), recombinant PrP fibrils, which have a structure that is significantly different from that of PrP^{Sc}, can progressively acquire a new folding pattern and adapt to the template of the classical PK-resistant PrP^{Sc} [48, 49].

An alternative approach to demonstrate the protein-only hypothesis was explored by Wang et al. [50] who created recombinant prions by PMCA using recombinant mouse PrP (purified from *E. coli*) in the presence of synthetic phospholipids and total liver RNA. The recombinant prions obtained in these conditions showed all the features of the pathogenic PrP isoform, especially the protease resistance and transmissibility in wild-type CD-1 mice that succumbed to prion disease in about 150 days. This experiment provides strong evidence in support of the protein-only hypothesis because prions with high infectivity titre could be generated *in vitro* from well-defined components. It also illustrates the key role of lipids and RNA as cofactors to facilitate PrP conversion. Recently, Deleault et al. identified phosphatidylethanolamine as the single cofactor required to facilitate the conversion of recombinant PrP into infectious recombinant PrP^{Sc} during PMCA [51, 52].

5. Extending the Prion Concept to Other Neurodegenerative Diseases: The Prionopathy World

During the last decades, many publications have shown that neurodegenerative disorders as diverse as Alzheimer's,

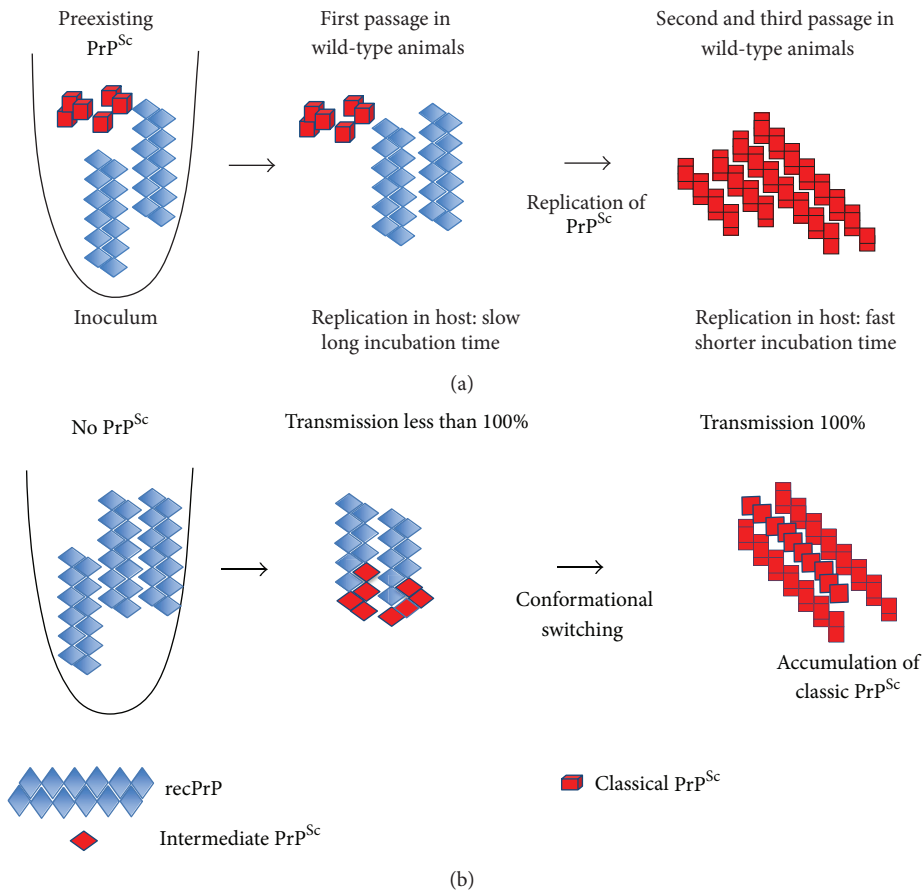

Figure 2: Schematic representation of two possible mechanisms of *de novo* propagation of prions from recPrP fibrils. (a) In this model, the preparation of recPrP fibrils contains very small amounts of classical PrP^{Sc}. A long incubation time is required to amplify and propagate *in vivo* this minute amount of PrP^{Sc}. (b) The second model ("deformed templating" mechanism) hypothesizes that there is no classical PrP^{Sc} in the fibril preparation and that recPrP fibrils can be converted into PrPres (PrP^{Sc}-like structures) with low efficiency. After several passages, these PrP^{Sc}-like structures progressively adopt the structural features of classical PrP^{Sc}. Schema adapted from Makarava et al., 2011 [48].

Parkinson's, Huntington's, and Creutzfeldt-Jakob disease share a common pathogenic mechanism involving the aggregation and deposition of misfolded proteins. Although the type of aggregated proteins is disease specific (Table 1), they all share a "prion-like" mechanism of cell-cell propagation, with similar pathways of protein aggregation that involve oligomeric species leading to fibril formation and amyloid deposition. For instance, several studies have investigated the putative prion-like mechanism involved in the transmission of misfolded amyloid beta (Aβ) (Table 1) by inoculating brain extracts from patients with Alzheimer's disease (AD brain tissues) in several animal models. In marmoset, a non-human primate, amyloid plaques were induced 6-7 years after inoculation of AD brain tissue [53]. These plaques were composed of aggregated Aβ peptides similar to those found in the host, and Aβ deposition was not restricted to the injection site, suggesting diffusion of the newly formed aggregates. The experiment could be successfully reproduced in Tg2576 transgenic mice that express the β-amyloid precursor protein (APP) with the Swedish mutation corresponding to the familial form of AD. Specifically, intracerebral injection of AD brain tissue in these animals led to a peculiar distribution of Aβ deposits [54, 55]. Five months after injection, the Aβ aggregates were localized only in the ipsilateral side, whereas after 12 months senile plaques and vascular deposits were detected in both hemispheres, suggesting spreading of the aggregates. The use of other transgenic mouse models (APP23 and APP/PS1 animals) [56] showed that the brain Aβ deposit profiles vary with the host and the brain extracts used to induce amyloidosis, similar to what was observed with different prion strains [56–58]. Altogether these results clearly indicate that inoculation of brain extracts containing preformed Aβ seeds accelerates the formation of new Aβ deposits *in vivo*, in transgenic mice and non-human primates. They also support the hypothesis of a transmissible origin of AD. Remarkably, Stohr and coworkers induced cerebral β-amyloidosis by inoculating purified Aβ aggregates derived from brain or aggregates composed of synthetic Aβ peptides in Tg(APP23:*Gfap*-luc) mice [65]. Monitoring of Aβ deposition in live Tg(APP23:*Gfap*-luc) mice by using bioluminescence imaging showed that Aβ aggregates self-propagate as prions.

Similar findings were reported concerning the induction of Tau aggregates [59] in the brain of transgenic mice that

Table 1: Prion model of induction described for neurodegenerative diseases.

Disease	Normally folded protein "Precursor"	Abnormally folded protein "Prion form"	Protein aggregates detected	Seeding inoculum	Prion-like propagation in mammals	References
CJD/scrapie	PrP^{C}	PrP^{Sc}	PrP^{Sc} deposits plaques	Various mammalian prions and recPrP fibrils	WT and Tg mice Non-human primates	[32, 33, 37–40] [45–50]
Alzheimer (AD)	Amyloid precursor protein (APP)	Amyloid beta peptides Aβ	Aβ plaques	Human AD and Tg mice brain extracts blood	Marmosets TgAPP2576 TgAPP23, TgAPP/PS1	[53–58]
Tauopathies	Tau	Tau aggregates	Neurofibrillary tangles (NFTs)	Tg(HuTauP301S) brain extracts	Tg(wt Tau)	[59, 60]
Parkinson (PD)	α-Synuclein	α-Synuclein aggregates	Lewy bodies	Human preformed α-Syn fibrils	(i) Fetal tissue grafts in human PD patients (ii) Tg (α-SynA53T) and WT mice	[61, 62] [63, 64]

express wild-type human Tau after intracerebral injection of brain extracts from old Tg(HuTauP301S) mice containing insoluble Tau aggregates. Neurofibrillary tangles, neuropil threads, and coiled bodies could be visualized not only in neurons but also in oligodendrocytes of the injected animals. In addition, mouse Tau can coaggregate with human Tau P301S, indicating cross-species seeding [60].

The hallmark of Parkinson's disease (PD) is the presence in the brain of Lewy bodies and Lewy neurites that contain high amounts of aggregates of misfolded α-Synuclein (α-Syn). In the dual-hit hypothesis proposed by Hawkes and coworkers, PD originates in the nose and foregut after inhalation/ingestion of an unknown neurotropic pathogen and then aggregates spread throughout the nervous system with a stereotypic pattern following unmyelinated axons [66]. This theory is based on extensive postmortem analyses of patients with PD that identified the olfactory bulb and enteric plexus of the stomach as early sites of Lewy pathology, and also on evidence of olfactory and autonomic dysfunction as early nonmotor PD symptoms [66]. Based on this hypothesis, Luk et al. [63] have stereotaxically injected preformed recombinant α-Syn fibrils in the cortex and striatum of Tg(α-SynA53T) mice that express human α-Syn harbouring the A53T mutation related to familiar PD and showed that α-Syn aggregates can spread in the tissues with a prion-like mechanism of propagation. Similarly, a single intrastriatal inoculation of synthetic α-Syn fibrils in wild-type nontransgenic mice led to the cell-to-cell transmission of pathologic α-Syn and Parkinson's-like Lewy pathology in anatomically interconnected regions. Accumulation of toxic aggregates in these mice triggered a progressive loss of dopamine neurons in the *substantia nigra* and a reduced dopamine levels culminating in motor deficits [64]. Remarkably, experiments of transplantation of fetal cells in PD subjects showed the presence of Lewy body-like inclusions 14 years after grafting that stained positively for α-Syn and ubiquitin and had reduced immunostaining for dopamine transporter [61]. This result was confirmed in other PD subjects transplanted with fetal mesencephalic dopaminergic neurons (11–16 years) who developed α-Syn-positive Lewy bodies in grafted neurons [62]. These results suggest a host-to-graft disease propagation mechanism with implications for cell-based therapies [61, 62].

6. Conclusion

These last years have been marked by the end of the controversy about the protein-only hypothesis concerning prion diseases. In addition, a growing number of studies have shown that other amyloidogenic proteins implicated in various neurodegenerative disorders can propagate *in vivo* with a prion-like mechanism. We witness the opening of a new field of research in neurodegenerative disorders [67, 68], and the lessons learned from prion diseases will help scientists develop new strategies for diagnostic and therapeutic approaches for other neurodegenerative disorders.

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Review Article

The Innate Immune System in Alzheimer's Disease

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Alzheimer's disease (AD) is the leading cause for dementia in the world. It is characterized by two biochemically distinct types of protein aggregates: amyloid β (Aβ) peptide in the forms of parenchymal amyloid plaques and congophilic amyloid angiopathy (CAA) and aggregated tau protein in the form of intraneuronal neurofibrillary tangles (NFT). Several risk factors have been discovered that are associated with AD. The most well-known genetic risk factor for late-onset AD is apolipoprotein E4 (ApoE4) (Potter and Wisniewski (2012), and Verghese et al. (2011)). Recently, it has been reported by two groups independently that a rare functional variant (R47H) of TREM2 is associated with the late-onset risk of AD. TREM2 is expressed on myeloid cells including microglia, macrophages, and dendritic cells, as well as osteoclasts. Microglia are a major part of the innate immune system in the CNS and are also involved in stimulating adaptive immunity. Microglia express several Toll-like receptors (TLRs) and are the resident macrophages of the central nervous system (CNS). In this review, we will focus on the recent advances regarding the role of TREM2, as well as the effects of TLRs 4 and 9 on AD.

1. Introduction

Alzheimer's disease is the most common cause of dementia globally [1]. AD is characterized by the presence of amyloid β (Aβ) deposits in the forms of parenchymal amyloid plaques and congophilic amyloid angiopathy (CAA) as well as aggregated tau protein in the form of neurofibrillary tangles (NFT). These lesions are associated with neuronal loss and synaptic damage, which produce the cognitive dysfunction which characterizes AD. Mutations in three genes have been shown to cause early-onset AD (EOAD): the amyloid precursor protein (APP), Presenilin 1 (PS1), and Presenilin 2 (PS2) [2, 3]. Mutations associated with these genes affect <1% of all AD patients and are not informative regarding the causes of the much more common late-onset AD (LOAD) [2, 3]. Inheritance of the apolipoprotein E (ApoE4) allele is the major genetic risk factor that is associated with late onset AD [4, 5]. Several environmental factors are known as risk factors for LOAD including, aging, head trauma, type 2 diabetes, hypertension, hypercholesterolemia, and vascular pathology [6].

Recently, two independent groups of investigators have identified a rare variant in the gene encoding the triggering receptor expressed on myeloid cells 2 protein (*TREM2*), which is predicted to result in a R47H substitution that causes an ~3-fold increase in the susceptibility to LOAD. Although the odds ratio of Trem2 R47H is comparable to the presence of a single copy of ApoE4, this variant has a population frequency of only ~0.3% [7–9]. TREM2 is located on chromosome 6p21.1 and encodes five exons. It is a transmembrane glycoprotein, made up of an extracellular immunoglobulin-like domain, a transmembrane domain, and a cytoplasmic tail, that couples with DAP12 for its signaling function [10].

TREM2 is expressed in microglia, macrophages, osteoclasts, and dendritic cells. TREM2 was initially identified as a phagocytic receptor of bacteria [11]. It recognizes anionic lipopolysaccharides (LPS) on the surface of bacteria, via signaling through DAP12, triggering their phagocytosis. Other pattern recognition receptors which have been shown to play a critical part in macrophage/microglial function and have a role in AD-related pathology are the Toll-like receptors (TLRs) [12–14]. In this review, we will focus on the potential roles of TLRs and TREM2 in AD.

2. TREM2 in AD

The *TREM2* gene encodes 5 exons that code for a 693 pb DNA which translated into 230 amino-acids called TREM2 [15–17]. In the normal brain, TREM2 is highly expressed in white matter, hippocampus, and neocortex, while low levels are found in the cerebellum of the human brain [18]. This distribution of TREM2 expression is consistent with the distribution of AD-related pathology. In AD animal models, it has been reported that TREM2 is upregulated in amyloid plaque-associated microglia, such as in aged APP23 mice [19], a result which was confirmed by another group [20]. In AD model TgCRND8 mice TREM2 mRNA was increased, correlating with the rise in Aβ levels [7].

TREM2 is a phagocytic receptor of bacteria [11] and forms a receptor signaling complex with the TYRO protein tyrosine kinase binding protein (TYROBP, also called DAP12), that triggers phagocytosis and the release of reactive oxygen species [21]. TREM-2 is defined as an innate immune receptor expressed on the cell surface of microglia, macrophages, osteoclasts, and immature dendritic cells [22]. Microglia play a key role in the immune response in the central nervous system (CNS) and are the resident innate immune cells responsible for the early control of infections. TREM2 is known to have anti-inflammatory properties; it suppresses inflammatory responses by repression of cytokine production and secretion [23]. TREM2 reduces macrophage activation and inhibits cytokine production in response to both TLR2 and TLR4 ligands zymosan and LPS [24, 25]. Conversely reduction of *TREM2* expression by either RNA interference or by targeted gene deletion amplified inflammatory cytokine responses by macrophages following stimulation of multiple different TLRs including TLR2, 4, and 9 [26]. Hence, it has been speculated that TREM2 has a protective role in AD pathogenesis; its anti-inflammatory properties could reduce inflammation-induced innocent bystander neuronal damage [8, 16, 17]. In addition to the anti-inflammatory roles of TREM2, it is also known to effect phagocytosis of damaged/apoptotic cells. TREM2 interacts with endogenous ligands on neurons, leading to the direct removal of damaged cells [27]. In various models of multiple sclerosis increased microglial expression of TREM2 is associated with increased phagocytosis and a promotion of a M2-like activation state of microglia, which is thought to have protective effects [28, 29]. The removal of damaged or apoptotic neurons mediated via TREM2 could promote tissue repair in response to AD-related pathology. This TREM2 mediated phagocytic activity also has been linked to an enhanced ability of microglia to clear Aβ and amyloid plaques in vitro and in AD model APP23 Tg mice [20]. Microglia are well known to have the potential to acquire a broad array of cytotoxic and cytoprotective functional states; TREM2 appears to be important in the regulation of this balance in relation to AD pathology.

In 2013, Jonsson et al. performed whole genome sequencing on 2261 Icelandic individuals and found that a rare mutation (rs75932628-T, frequency of 0.63%), predicted to result in a TREM2 R47H substitution, was associated with an increased risk of AD (odds ratio 2.92). Subsequently, this association was replicated in cohorts from the USA, Germany, The Netherlands, and Norway [9]. Concurrently, Guerreiro et al. confirmed the link between LOAD and the R47H variant by meta-analysis of three imputed data sets of genomewide association studies (EADI, GERAD and ANM) [7]. They also found six additional variants (Q33X, Y38C, T66M, D87D, R98W, and H157Y) that were present in affected cases and not in controls, which could be related to AD pathology. Three of these variants (Q33X, Y38C, and T66M) had been previously reported in the homozygous state to be associated with a frontotemporal dementia-like syndrome (without AD-related plaques and NFT) [30]. The critical role of TREM2 for neuronal health is highlighted by patients with autosomal recessive disorder with near complete loss of TREM2 function called polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL or Nasu Hakola disease) [31–33]. Affected individuals have a progressive inflammatory neurodegenerative disorder with formation of multiple bone cysts. They typically present in the second decade of life with psychiatric symptoms and/or bone fractures, which is followed by a worsening dementia, leading to death in the 4th or 5th decades of life. The TREM2 mutation associated with this disorder (Q33X) has been identified, as discussed previously, in the heterozygous state with an increased risk of LOAD [7]. Patients with a partial loss of function of Colony-stimulating factor 1 receptor (CSF1R), which like TREM2 is a microglial receptor that signals via DAP12, have a corticobasal syndrome called hereditary diffuse leukoencephalopathy with spheroids [34]. Neither the latter disorder nor PLOSL is associated with amyloid plaques or NFT pathology; however, there is increased microgliosis along with neurodegeneration, highlighting the likely importance of the balance between microglial phagocytic and inflammatory pathways in AD.

3. Relationship between Tau and Trem2

Neurofibrillary tangles (NFT) are one of the pathological hallmarks of AD, which are formed by the intracellular, neuronal accumulation of abnormally aggregated and hyperphosphorulated tau protein. NFT deposition correlates better with the degree of dementia, compared to the amyloid plaque burden [35]. A number of studies have shown that increases of hyperphosphorylated tau protein (ptau) in CSF correlate with neuronal loss and is predictive of cognitive decline in AD [36–40]. A recent large GWAS study has shown that the Trem2 R47H variant has a strong association with both elevated CSF tau and ptau levels [41].

4. Toll-Like Receptors Structure

Toll was first identified as a receptor expressed by insects and was found to be essential for establishing dorsal-ventral orientation during embryonic development in Drosophila melanogaster [42] and for being important for defense against microbial infection [43, 44]. To date, 11 members of TLR family have been identified in humans and 13 in mice, which trigger both innate and adaptive immune responses [45–47]. Each TLR has at least one known binding ligand and/or adaptors except TLR10 [48, 49]. *TLR11*, *12*, and *1* that are not present in the human genome [50]. TLR 4 was the first mammalian homolog of Toll identified as a pattern recognition receptor required for adaptive immunity [43]. The TLRs are important for regulating microglial responses to Aβ. Fibrillar Aβ triggers microglia inflammatory cytokine production via TLR4-TLR6 heterodimers, whose assembly is regulated by CD36 [51]. Treatment of microglia with plaque material produces marked upregulation of TLR2, TLR4, TLR5, TLR7, and TLR9 mRNA [52].

5. Roles of TLR4 and TLR9

In addition to amyloid plaques and neurofibrillary tangles that characterized AD, inflammation is observed with the progression of the disease, which is linked to production of cytokines by activated microglia. Microglia can also play a neuroprotective role by clearing Aβ via increased phagocytosis and proteolytic degradation [53–55]. Microglia can activate both innate and adaptive immune response as well express several Toll-like receptor (TLRs). TLRs play a key role in the innate immune system. Innate immunity is the first line of defense against invading microbes [56]. When a pathogen invades the body, it typically possesses a pathogen-associated molecular pattern, better known as PAMP. These PAMPs are sensed by pattern-recognition receptors (PRRs) and one specific group of PRRs is the Toll-like receptors (TLRs) [57]. TLRs are critical for eliciting an innate immune response to invading pathogens and are also important for triggering the adaptive immune responses [58, 59]. TLR engagement on antigen-presenting cells (APCs) induces cytokine release and costimulatory molecule expression that primes cells for subsequent activation and expansion of antigen-specific T cells [58–61]. TLRs also recognize a variety of danger-associated molecular patterns, called DAMPs. TLRs are expressed in all glial cells including microglia, astrocytes, oligodendrocytes, and a limited repertoire in neurons.

TLR4 is the most actively investigated and characterized in relation to AD pathology. It recognizes microbial motifs of LPS (lipopolysaccharides) [62, 63]. The gene encoding *TLR4* in humans has 4 exons and is located on chromosome 9q32-q33, with 4 exons. TLR4 is mostly expressed in lymphocytes, monocytes, macrophages, and splenocytes [64, 65]. TLR4 has been found expressed in many other cells including epithelial [66], endothelial cells [67], and cancer cells [68, 69]. TLR4 expression on cerebral vascular endothelium increases following subarachnoid hemorrhage. TLR4 deficiency has been shown to be protective against ischemic damage and enhance neuronal survival in stroke mouse models [70].

TLR9 is a protein encoded by the *TLR9* gene which has been designated as CD289. TLR9 was originally identified as a receptor that could differentially recognize bacterial DNA versus mammalian DNA, based on the high frequency of hypomethylated CpG motifs in nonmammalian DNA [71, 72]. TLR9 recognizes intracellular pathogen-derived non-methylated CpG motifs of bacterial and viral DNA. TLR9 is expressed by several cells including dendritic cells, B lymphocytes, monocytes, and natural killer cells. TLR9 is expressed in the cytoplasm within the endoplasmic reticulum, as well as on other intracellular vesicles but not on the plasma membrane [73].

6. TLRs, TREM2, and Alzheimer's Disease

Numerous studies have shown that TLRs have a crucial role in immune surveillance and inflammatory responses in the central nervous system (CNS) [12]. TLRs expression was identified on glial cells in human postmortem AD [74]. TLRs are important in AD, specifically those expressed on microglia (TLRs 1–9) [75, 76]. It has been shown that TREM2 is coupled with the immunoreceptor tyrosine-based activation motif (ITAM) sequence containing signaling adapter, DAP12, which negatively regulates TLR responses in both macrophages and dendritic cells [26, 77]. Hence, it likely TREM2 can regulate phagocytosis and/or inflammatory responses mediated via TLRs in response to AD pathology. It has been reported that TLRs on the surface of microglia cells bind Aβ, which triggers downstream intracellular signaling cascades [78, 79]. In AD patients high expression of CD14 (coreceptor for TLR4) was observed in parenchymal microglia of the frontal and occipital neocortex, hippocampus, and around senile plaques. Immunoreactivity with CD14 was detected in some perivascular areas [80]. In AD brains, high expression of TLR2 and CD14 was detected in microglia associated with amyloid plaques [74]. In AD Tg models, such as APP23, high levels of CD14 were observed in the microglia detected in the cortex and hippocampus [81]. Increased TLR4 mRNA was reported in another AD Tg model TgCRND8 [82]. In addition, TLR4-deficient mice displayed increased diffuse Aβ and fibrillar Aβ deposits compared with control mice [78], suggesting that TLR4 signaling is involved in Aβ clearance [83]. Microglia deficient in TLR2, TLR4, or the coreceptor CD14 are not activated by Aβ and do not show a phagocytic response [84]. Transgenic AD mice lacking TLR4 have markedly elevated levels of diffuse and fibrillar Aβ. Furthermore, stimulation of microglial cells with TLR2-, TLR4-, or TLR9-specific agonists accelerates Aβ clearance both in vitro and in vivo [85]. It has been reported that TLR9-mediated pathways regulate inflammatory mediator expression in astrocytes. Neurons are thought to express intracellular TLRs, including TLR9, suggesting a role for TLRs during both physiological and pathological conditions [86]. The intracerebroventricular administration of CpG in AD model Tg2576 mice has been shown to ameliorate cognitive impairments [87]. Our group has shown that the administration of the TLR9 agonist CpG oligonucleotides (ODN) containing unmethylated CpG sequences to Tg2576 mice induced a reduction of cortical and vascular Aβ levels

without apparent toxicity and improved cognitive function [88]. We had previously shown that CpG stimulation is beneficial for the generation of an immune response to prion disease [89]. Several CpG DNA drugs have demonstrated good safety profiles in humans and have been tested in numerous clinical trials as antitumor, antimicrobial agents, and adjuvants in vaccines [90, 91]. Recently, we have shown that TLR9 stimulation with CpG in 3xTg AD mice with both amyloid plaque and NFT pathology greatly reduces both of these pathologies in association with cognitive benefits [92]. These results indicate that stimulation of the innate immune system through TLR9 with CpG ODN is an effective and safe method to reduce the amyloid burden and also tau-related pathology in AD model mice.

7. Conclusion

Studies conducted in the early 1990s have suggested the potentially critical role of microglia for both the formation and clearance of amyloid lesions [93–95]. Numerous studies on the relationship of TLRs to AD have shown that modification of these signaling pathways can have profound effects on AD-related pathology, through modification of the inflammatory state of microglia/macrophages. TLRs may either have a positive or negative impact on cellular responses during AD. Studies have shown that appropriate stimulation of TLR9 can ameliorate both Aβ and tau-related pathology. The recent finding by two large consortiums that a rare variant of *TREM2*, a gene which regulates phagocytosis and the activation state of microglia/macrophages, is linked to LOAD has further highlighted the important role of innate immunity in AD. This finding adds to prior data linking other genes that are associated with microglia function and a low increased risk of LOAD, such as CR1, CD33, and MS4A4A/MS4A6A [96]. These studies indicate that modification of microglial function in AD is an important therapeutic target.

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Review Article

Infectivity versus Seeding in Neurodegenerative Diseases Sharing a Prion-Like Mechanism

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Prions are considered the best example to prove that the biological information can be transferred protein to protein through a conformational change. The term "prion-like" is used to describe molecular mechanisms that share similarities with the mammalian prion protein self-perpetuating aggregation and spreading characteristics. Since prions are presumably composed only of protein and are infectious, the more similar the mechanisms that occur in the different neurodegenerative diseases, the more these processes will resemble an infection. *In vitro* and *in vivo* experiments carried out during the last decade in different neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's diseases (PD), and amyotrophic lateral sclerosis (ALS) have shown a convergence toward a unique mechanism of misfolded protein propagation. In spite of the term "infection" that could be used to explain the mechanism governing the diversity of the pathological processes, other concepts as "seeding" or "*de novo* induction" are being used to describe the *in vivo* propagation and transmissibility of misfolded proteins. The current studies are demanding an extended definition of "disease-causing agents" to include those already accepted as well as other misfolded proteins. In this new scenario, "seeding" would be a type of mechanism by which an infectious agent can be transmitted but should not be used to define a whole "infection" process.

1. Introduction

It has been some time since the old axiom, "one protein—one structure," became obsolete [1]. This is most obvious for prion scientists that try to understand how different protein structures can originate from the same primary sequence. The idea that proteins must acquire a specific and unique conformation has shifted due to biochemical and structural evidence from neurodegenerative diseases showing that different folding states of the same protein are essential in certain biological processes. This concept was nicely expressed by Batch and colleagues: "The misfolding and aggregation of proteins is often an accident waiting to happen. Consequently, organisms have developed sophisticated chaperone and quality-control systems to limit abnormal protein interactions and the accumulation of toxic aggregates" [2]. The ability of proteins to reach different isoforms has crucial consequences in the cell and in the whole organism as demonstrated in prion diseases, neurodegenerative pathologies where the prion protein (PrP) misfolding process is the key event.

We will review the behavior of different proteins implicated in several neurodegenerative diseases selecting those with a high impact on our society and comparing them to prion diseases as reference. The term "prion-like" will be used to describe molecular mechanisms that share similarities with the mammalian prion protein self-perpetuating aggregation and spreading characteristics. Since prions are presumably composed only of protein and are infectious, the more similar the mechanisms that occur in the different reviewed diseases are, the more these processes will resemble an infection.

2. *In Vitro* Propagation

Essentially, any protein could show a prion-like propagation if it were able to acquire a characteristic folding that could be induced to other surrounding proteins with identical or similar amino acid sequences. Thus, the biological information would be transferred protein to protein through a conformational change. Although PrP in transmissible spongiform encephalopathies (TSEs) is the most representative protein of this phenomenon, APP (amyloid precursor protein) and tau in Alzheimer's disease (AD) as well as in frontotemporal dementias (FTD), α-Synuclein in parkinson's diseases (PD) and superoxide dismutase 1 (SOD-1), and the 43KDa TAR (trans-activator regulatory) DNA binding protein (TDP43) in amyotrophic lateral sclerosis (ALS) are also involved in protein misfolding propagation. All these proteins are implicated in neurodegenerative diseases in which the pathogenic process had been associated with the presence of amyloid deposits predominantly composed by misfolded proteins [3–6]. Although these diseases share a neurodegenerative process that leads to death, the type of proteins involved, their localization and the manner in which they accumulate in amyloid fibers are not shared.

In vitro studies were and still are of vital importance for understanding the mechanisms governing misfolding propagation from one protein to another. The process by which a protein changes from its initial folding to a structure prone to amyloid formation was initially described by Jarrett and Lansbury. The so called "seeding," first observed in PrP conversion to amyloid fibrils, proved to be similar to the "one-dimensional crystallization" mechanism, a nucleation-dependent phenomenon that is common to other amyloid forming proteins such as β-amyloid (Aβ) [7]. The same *in vitro* studies also showed that propagation implies protein aggregation and that in some cases the aggregation that follows misfolding can occur spontaneously. Jarrett and Lansbury described the key parameters that characterize the dynamics of aggregation such as the lag phase and the aggregation rate [8]. Although their experiments were focused on PrP and β-amyloid, we now know that those parameters can be used in all the proteins mentioned above and that they all share the ability to propagate misfolding *in vitro*.

One of the first lines of evidence that showed a similar *in vitro* behavior for the prion protein and the β-amyloid peptide was provided by Come and collaborators. They pointed out that the β-amyloid peptide contains a C-ter region similar to PrP(96-111), which is necessary for amyloid formation and possibly responsible for protein aggregation initiation *in vivo*. These proteins showed in *in vitro* fibrillization experiments the existence of a kinetic barrier to amyloid formation expressed as a lag phase analogous to crystal growth. These results suggested the formation of an ordered nucleus, which is a rate-determining step for aggregation and is followed by rapid fibril growth. Seeding was also demonstrated by incubation with preformed fibrils, which shortened considerably the lag phase and is consistent with a nucleation-dependent mechanism [7, 8]. Other studies have confirmed the seeding phenomenon described previously for Aβ and tau [9–14] and well characterized by Stöhr and collaborators for PrP, showing that seed-enhanced growth could be achieved in homogeneous solution and could be enhanced by sonication. They proposed a mechanistic model of fibrillization that included the presence of several intermediate structures [15]. In a similar manner, a nucleation-dependent mechanism for α-synuclein fibrillogenesis was also described. As the previous one, it consists of an initial lag phase (nucleation) followed by a growth phase (elongation) and a constant state phase where the organized aggregate and the monomer are at an equilibrium [16]. The nucleation-dependent process may be the rate-limiting step in PD during the generation of Lewy body (LB) α-synuclein fibrils. Serpell and collaborators also showed the conformational change of α-synuclein from an α-helix structure to a β-sheet conformation during assembly *in vitro*. In this study, different types of recombinant α-synuclein were used: carboxy-terminally truncated human α-synuclein (1–87) and (1–120), wild-type human α-synuclein and the A53T mutant human α-synuclein. Surprisingly, wild-type and A30P mutant human α-synucleins showed slower rates of aggregation than the truncated proteins. The fibrils generated as a result of shaking were identical to the fibrils extracted from dementia with Lewy bodies and multiple system atrophy brains [17]. α-synuclein fibril formation, previously characterized by Spillantini and coworkers from the substantia nigra of idiopathic PD patients, had been reproduced *in vitro*. The labeled extracted structures corresponded principally to single filaments; however, small clusters of filaments were also observed. Fibrils showed a variable morphology: straight and unbranched with diverse length and width [6].

Interestingly, prions occur in the form of different strains that show distinct biological and physicochemical properties, even though they are encoded by prion proteins with the same amino acid sequence, albeit in presumably different conformations. Recent studies focused on polymerization/fibrillization of β-amyloid and tau also demonstrated the existence of structurally different aggregates visualized by electron microscopy (EM) and atomic force microscopy (AFM). These protein aggregates also behaved differently in cell culture [11–13, 18]. These studies can be considered pioneers identifying the strain phenomenon in AD and other tauopathies. Similarly, Wang and coworkers showed that tau fragments prepared by endogenous proteases aggregated spontaneously *in vitro* and propagated to tau fragments as well as to full-length tau in a similar way to the one described in prion propagation [19, 20].

A common feature in the neurodegenerative diseases is the existence of mutations in the proteins favoring (with some exceptions) protein misfolding. This *in vivo* event is responsible for the genetic and familial forms of several diseases and usually gives rise to a spontaneous early onset [21–26]. Many studies have proved that this occurrence can also be mimicked *in vitro*. Mutations in APP (occurring in early AD) alter mostly the processing of this precursor protein by secretases, leading to the release of greater amounts of Aβ peptide or the alteration in the ratio of Aβ types. Jarret and collaborators tested the *in vitro* aggregation kinetics of some of the most abundant variants of Aβ found in senile plaques showing distinct rate of amyloid formation from *in vivo* [27]. Around 10%

of tauopathies are familial forms of FTD and are associated with the presence of mutations. The wild-type and the most characteristic mutant forms of tau were studied by Frost and coworkers *in vitro*. Fibrils composed of mutant or wild-type tau showed different structures by FTIR (fourier transform Infrared spectroscopy). Seeding of wild-type tau with mutant fibrils led to a new structure different from that formed with wild-type tau seed, which can explain the phenotypic diversity of tauopathies [9]. A similar observation was described by Narhi and coworkers in Parkinson's disease-related studies. Despite that both wild-type and mutated α-synucleins can be used to *in vitro* assemble fibrillar aggregates with a cross-β-sheet conformation, aggregate formation is accelerated when mutated α-synuclein (characterized in PD patients) is used [22]. For instance, fibrils composed of α-synuclein A30P mutant acting as seed accelerate the nucleation-dependent fibrillization of the wild-type protein perpetuating the "A30P strain" properties generated *in vitro* [28]. Likewise, ALS-associated mutations that promote *in vivo* toxicity also accelerate *in vitro* aggregation of highly purified TDP-43. This protein, a pathological hallmark of ALS, is considered as inherently aggregation prone [29].

Although the tertiary and quaternary protein structures involved in aggregate formation among the diversity of neurodegenerative pathologies are likely different, the antibody recognition of a common aggregated structure suggests a similar oligomeric organization [30]. This fact is extremely interesting since it allows future studies on therapeutic approaches and predicts common propagation mechanisms of these diseases.

3. Cell-to-Cell Propagation

While *in vitro* misfolding protein propagation is not affected by protein localization or by the existence of cellular components/factors, it might be possible that *in vivo* propagation is impeded at the cellular level. In order to study the "prion-like" phenomenon that implies the spreading of the self-perpetuating protein aggregates also from cell to cell, a diversity of studies have been performed using cell cultures. Thus, misfolded tau protein was propagated cell to cell after seeding by pathological tau conformers leading to pathogenesis of Alzheimer-like tangles in cells [14, 18]. *In vivo*, cell-to-cell propagation was unequivocally demonstrated using a tau transgenic model in which overexpression of human tau P301L was restricted to the entorhinal cortex (EC-II) area. Tau proteins spread from neuron to neuron into different brain areas coaggregating with mouse endogenous tau, in a way similar to prions [31]. A recent study has demonstrated that soluble oligomeric Aβ can also be transmitted neuron to neuron depending on direct neuritic connections, following a prion-like intercellular spread. The authors of this study propose macroautophagy as a potential mechanism for disease spreading, similar to endolysosomal and lysosomal exocytoses described for prions [32].

There are also several lines of evidence that suggest α-synuclein as a candidate to be the pathogenic factor implicated in the prion-like spread of PD pathology. Freundt and coworkers showed neuron-to-neuron transmission of α-synuclein fibrils through axonal transport [33]. Fibrillar α-synuclein internalization in primary neurons followed by the transport of these fibrils to the cell bodies of second-order neurons was observed. Moreover, exogenous α-synuclein fibrils were an efficient seed for the formation of Lewy-body-like intracellular inclusions in cultured cells [34]. *In vitro* preformed fibrils were added into α-synuclein overexpressing cells and the formation of insoluble intracellular inclusions was evaluated. Aggregates very similar to PD Lewy bodies composed principally of β-sheet, hyperphosphorylated and polyubiquitinated α-synuclein were observed in subcellular localization [34].

The prion-like behavior in ALS was studied using cell cultures expressing ALS-causing mutant SOD-1 or TDP-43. The cells were seeded using *in vitro* preformed SOD-1 aggregates that penetrated through macropinocytosis. As a consequence, the pathological misfolding of the endogenous soluble mutant protein was triggered. Aggregates were effectively transferred to adjacent cells via exosomes or nanotubules and continued growing even after the misfolded proteins acting as seed were eliminated. This suggests a cyclical self-perpetuating behavior, mimicking what happens in prion diseases [35–37].

4. When the *In Vivo* Propagation Can Also Be Transmissible

The transmission ability from an individual to another is one of the main hallmarks of prion diseases. Transmissibility requires *in vivo* propagation, which should occur somewhere in the cell. Although the most probable place is the cytoplasmic membrane where GPI-anchored PrP is located, it is not restricted there because *in vivo* propagation has also been observed in models that express GPI-less secreted PrP in which misfolding and accumulation occur in the extracellular matrix [38]. Thus, before discussing the transmission capacity of these diseases, we should focus on their ability to spread throughout the central nervous system. In AD patients, one of the first changes observed in the brain is the deposition of Aβ plaques. Therefore, their apparition and spreading have been widely studied. One of the most detailed studies analyzed 83 brains from healthy as well as AD affected donors in different disease stages to correlate depositions with disease progression [39]. Aβ depositions initially appeared in basal portions of the isocortex, but as the disease progressed, the whole isocortical area became affected with a mild presence of plaques in the hippocampus, finally spreading to subcortical areas. The neurofibrillary tangles (NFT) and neuropil threads (NT) formed by tau resulted in much better indicators of disease progression, with a characteristic distribution pattern with much less variation among individuals than Aβ plaques. The latter were initially confined to a single layer of the transentorhinal region, followed by a severe involvement of the entorhinal and transentorhinal and finally arriving to isocortical destruction [39]. The degree of disease development could be measured through the amyloid spreading, being this behavior of deposit propagation characteristic also in some TSEs.

More recently, Seeley and collaborators used functional neural-network sensitive neuroimaging methods to analyze the atrophy patterns of those networks in brains of donors with 5 different dementias, including AD and FTPD. They demonstrated that as in the case of TSEs, where there are many lines of evidence of direct propagation of deposits along transsynaptic connections [40], other neurodegenerative diseases are also related to neural-network dysfunction. Each dementia syndrome would have its constant pattern of affected regions that would match different neural networks. This suggests that many disease-related proteins such as β-amyloid, tau, or α-synuclein are able to misfold, aggregate, and spread with specific brain networks [41].

A similar *in vivo* propagation was also proposed in Parkinson's disease. The disease may initiate in the periphery/enteric nervous system, accessing the central nervous system (CNS) through retrograde transport along neuronal projections from the gastrointestinal tract. After the Lewy pathology is transferred to CNS, it ascends from the lower brainstem through susceptible regions of the midbrain including the substantia nigra and the forebrain and finally spreads to the cerebral cortex at later stages of the disease. Alternatively, pathology may begin at the anterior olfactory structures spreading to midbrain and cerebral cortex. As the pathology progresses, the severity of the lesions in the susceptible regions and the clinical manifestations increase [42, 43].

Although *in vivo* propagation through the spinal motor neurons of the ALS-related misfolded proteins (SOD-1 and TDP-43) has been hypothesized, an *in vivo* prion-like propagation has not been demonstrated yet [37].

Despite the spreading mechanisms demonstrated by some of the mentioned misfolded proteins that resemble a prion-like propagation, their "labelling" as transmissible or infectious proteins would require verifying if this phenomenon can also be stimulated by their exogenous inoculation. As a result, pathological processes that would occur spontaneously could be accelerated or, in other cases, a disease that would not happen naturally could be initiated *de novo*.

Each of the neurodegenerative diseases described in this review is being studied over several animal models. Most of them are based on transgenic mouse models that overexpress the human disease-causing proteins. Overexpressed proteins carrying one or more mutations recreate the human disease with shorter progression times. The majority of those models reproduce the spreading of protein misfolding differing in propagation rates, affected areas, and the number or type of implicated proteins. Thus, they are excellent tools to study transmissibility of exogenous aggregates and to verify prion-like behavior based on the ability of propagation and self-perpetuation of the disease-related proteins.

One of the first many lines of evidence of Aβ transmissibility was described by Kane and coworkers in 2000. In this study, β-amyloid precursor protein transgenic mice (Tg2576) were intracerebrally infused with diluted supernatants of autopsy-derived neocortical homogenates from Alzheimer's patients. While Tg2576 mice develop β-amyloid deposits spontaneously at 9 months of age [44], the inoculated mice showed a significant reduction between 3 and 8 months in the β-amyloid plaque's onset. Wild-type or Tg2576 mice infused with healthy human brain did not show any Aβ deposition in the brain. As Tg2576 spontaneously favor this phenomenon it was considered as "seeding," far from the idea of "infection" designated for prions after inoculation [45].

Few years later different Alzheimer transgenic mouse models were also inoculated with autopsied Alzheimer's patient brain extracts. These studies show that β-amyloidogenesis is highly dependent on the expression of human APP of the different transgenic mouse models (the host) and on Aβ status (the agent). It was suggested that the variable seeding efficacy of these *in vivo* studies compared to *in vitro* studies was due to the occurrence of various Aβ conformations with partially distinct biological activities, comparable to prions [46].

In an attempt to recapitulate other prion features, the proteinase K resistance of Aβ extracts prepared from aged transgenic mouse brains was compared to that of synthetic fibrillar Aβ. As it has been described in TSE, a higher resistance of the brain-derived Aβ was observed. Surprisingly, PK digested derived Aβ retains the ability to induce β-amyloid deposition in APP23tg mice similar to what happens in prions [47].

The previous transmission studies were based on transgenic mouse models that, within the required time, spontaneously develop the same processes that are trying to be induced. This suggests that the newly formed β-amyloid depositions could be interpreted as an acceleration of the amyloidogenesis rather than a *de novo* induction. In order to address this, Morales and collaborators carried out new transmissibility experiments in which wild-type human APP overexpressing transgenic mice that do not develop spontaneous amyloidogenesis were inoculated with human Alzheimer's disease brain extracts. After long incubation times, the animals showed *de novo* β-amyloid depositions induced by contact with Aβ extracts, ruling out a seeding acceleration and suggesting an infectious prion-like propagation [48]. The use of this model has been very useful to demonstrate that the misfolded proteins can be *de novo* induced exogenously and refute the idea that the event can be explained as a simple seeding phenomenon. This is one of the first powerful lines of evidence demonstrating "infectivity" versus "seeding" mechanism. However, those animals overexpress the wt human APP and it could be argued that the seed was shortening a lag phase (likely longer than the animal lifespan) in the "primed" mice by overexpression.

The most efficient infection route for prion transmission is the intracerebral inoculation. Other parenteral inoculations (intravenous, intraperitoneal, etc.) although less efficient can also be used for prion transmission. Although previous successful Aβ inoculations were performed by direct intracerebral infusion of different preparations of β-amyloid, other peripheral routes were also used [49]. These results remind of those observed using some prion strains which, despite an efficient intracerebral transmission, fail on the intraperitoneal and/or oral route [50]. Recent peripheral inoculation experiments have shown for the first time that β-amyloid can be transmitted intraperitoneally when enriched β-amyloid extracts are used. This fact recapitulates prion transmission studies where barriers can be overcome

by improving mouse models, enriching inocula, extending expected incubation times, or increasing the number of animal passages [51].

While most of the animal models used to study Aβ propagation were based on transgenic mice, a rat model has also been used to evaluate the pathological effect of exogenous Aβ extracts intracerebrally inoculated. This model as well as the one used by Morales and colleagues is characterized by the absence of spontaneous Aβ deposition. Both studies concluded that β-amyloid deposits can be generated *de novo* as in a genuine prion infection [48, 52].

Another very interesting model is the marmoset, a New World monkey that naturally develops Aβ plaques when aged. A 20-year experiment confirmed that misfolding protein deposits onset can be reduced following intracerebral inoculation of exogenous β-amyloid [53].

The prion-like propagation phenomenon is not limited to Aβ peptide in AD. Tau protein follows similar mechanisms of fibrillization both in AD and in FTPD and other tauopathies. Prion-like *in vivo* seeding and spreading of tauopathies has been demonstrated using two different transgenic mouse lines, ALZ17 and P301S. While the first transgenic line expresses human wild-type tau and does not develop spontaneous tau aggregates, the second one expresses human P301S mutant tau (linked to a familial form of FTPD) and shows abundant tau inclusions. When ALZ17 mice were inoculated intracerebrally with brain extracts from aged P301S animals, assembly of wild-type human tau into filaments was observed, as well as spreading of pathology from the site of injection to adjacent brain regions [54]. In a similar way, Sydow and colleagues showed that tau pathology can be triggered in an inducible transgenic mouse model expressing aggregation prone human tau mutant (containing a ΔK280 mutation, associated with FTDP that aggregates rapidly *in vitro*) along with endogenous mouse tau. This model developed mixture tangles composed of both human and murine tau rapidly after induction of human tau mutant. The mixture of tau species turned richer in mouse tau when the expression of human tau was switched off. The interspecies coaggregation ability of tau reminds of another well described characteristic of prions [10].

Interactions between both Aβ and tau observed in transgenic models support the amyloid cascade hypothesis of AD and suggest that polymerized Aβ forms trigger a cascade of events leading to the formation of tau NFT. Transgenic mouse models expressing human tau mutant P301L, which is aggregation prone, were inoculated with Aβ-containing brain extracts purified from aged APP23 mice. A strong tau deposition even in regions far from injection site was induced. When both transgenic mouse models were crossbred, an induction of early tau deposition greater than the one induced in older simple tau transgenic mice was observed. However, the Aβ depositions were similar to those observed in the simple APP23 transgenic mice, confirming strong parallelism with prion diseases [55].

The first evidence of the similarity between Parkinson's and prion diseases had a different origin compared to the transmission studies conducted in Alzheimer's disease. Nevertheless, it was unquestioned to ascribe to α-synuclein a prion-like behavior in terms of its ability to be propagated *in vivo* and to be transmitted among individuals. Fourteen years after transplantation of human fetal neurons in a PD patient it showed postmortem pathological changes typical of PD in the grafted neurons located in the putamen. Numerous grafted nigral neurons showed aggregated Lewy-body-like structures with α-synuclein and ubiquitin [56]. This study was the first evidence that the propagation of a misfolded protein in PD could be explained through a prion-like mechanism. Similar studies confirmed that transplanted dopaminergic neurons developed PD pathologic changes as a consequence of their proximity to the already affected neurons from the PD patient [57, 58]. Grafted cells contained posttranslationally modified and aggregated α-synuclein suggesting that aggregation and deposition in transplanted dopaminergic neurons were caused by the misfolded α-synuclein in the host brain, which was transmitted to grafted cells [58]. In all these studies the "seeding" process was carried out in an opposite direction than previously shown in the transmission experiments of AD. The remaining misfolded α-synuclein in PD patients was able to self-propagate using fresh α-synuclein from grafted naïve tissues.

The transplantation studies done in human were nicely replicated in different mouse models. Thus, the injection of mouse cortical neuronal stem cells into the hippocampus of transgenic mice overexpressing human α-synuclein triggered the direct transmission of α-synuclein from host to grafted cells as was previously described in the prion field [59, 60]. An *in vitro* coculture model was used to demonstrate that α-synuclein was transmitted via endocytosis to neighboring neurons forming LB-like juxtanuclear inclusions. A failure of the protein quality-control systems, especially lysosomes, promoted the accumulation of transmitted α-synuclein and related to inclusion formation demonstrating cell-to-cell transmission of α-synuclein aggregates [61].

More recently, two different models showed that extracellular α-synuclein was taken up by cells through endocytosis and interacted with intracellular α-synuclein. The first model was created using a viral vector to engineer rat nigral neurons to overexpress human α-synuclein that subsequently was transported to the striatum. Rat ventral mesencephalic neurons were grafted into the striatum of these mice showing a frequent transfer of α-synuclein from the rat brain to grafted dopaminergic neurons [62]. The second model was based on the use of grafted wild-type mouse embryonic mesencephalic neurons in the striatum of mice overexpressing human α-synuclein. Six months after grafting, the presence of intracellular human α-synuclein immunoreactive punctae was observed in few grafted cells [63].

In a similar way to previous transmission studies using prions or AD as a source of "propagative agent," young TgM83 mice (transgenic mouse of synucleinopathy expressing human A53T mutated α-synuclein) were inoculated intracerebrally with brain homogenates from older TgM83 mice affected by the synucleinopathy. Prion-like acceleration of α-synucleinopathy was observed through the presence of both α-synucleins hyperphosphorylated and aggregated, together with a decrease on the survival time of mice. By contrast, there was no evidence of α-synucleinopathy in α-synuclein

knockout mice suggesting an important role for α-synuclein protein in the transmission of pathology from affected to unaffected areas, as what happens with PrP during prion propagation [64]. A similar experiment using the same model is described later showing again that the intracerebral inoculation of pathological α-synuclein initiates a rapidly progressive neurodegenerative disease. Animals were injected with brain homogenates of aged symptomatic animals showing abundant LB-like α-synuclein pathology into the neocortex and striatum of young healthy animals. Abundant α-synuclein lesions were widespread throughout the CNS 90 days after injection. In the same study, α-synuclein preformed fibrils (PFFs) previously generated *in vitro* from human wild-type full-length α-synuclein were also inoculated. *In vitro* PFFs were also able to initiate and propagate α-synuclein pathology in the same manner as TgM83 sick mice [65].

The last pieces of evidence showing a prion-like behavior in PD were obtained using wild-type mice. Animals were injected in the dorsal striatum with synthetic murine α-synuclein fibrils initiating a cell-to-cell transmission of pathologic α-synuclein and PD-like Lewy pathology in anatomically interconnected regions. This LB accumulation produced a progressive loss of dopamine neurons in the substantia nigra reducing dopamine levels and therefore generating a progressive performance deterioration of impaired motor coordination and balance [66]. The efficient PD-like pathology transmission was unlikely due to the use of murine fibrils in a wild-type model since the human preformed α-synuclein fibrils and sarkosyl-insoluble α-synuclein purified from PD patient brains also efficiently induced LB-like pathology with abnormal phosphorylated α-synuclein-positive structures. Nevertheless, the percentage of mice developing PD pathology was lower using human α-synuclein than using mouse α-synuclein as an inoculum, evidencing a prion-like species barrier phenomenon [67].

Triggering a disease in wild-type animals after the exogenous inoculation of any kind of "agent" should be considered the definitive probe of "agent" transmission independently of the mechanism by which the animals develop the disease.

There is still no evidence in animal models that would make certain that the ALS-related proteins (SOD-1 and TDP-43) can be propagated or transmitted *in vivo* following a prion-like mechanism.

5. Why Talk about "Seeding" or "*De Novo* Induction" When It Is Being Described as an Infection?

During the last decade, research groups working on different neurodegenerative disorders have carried out *in vitro* and *in vivo* experiments that have been previously performed to study prion diseases. As a consequence, a convergence among this type of diseases toward a unique mechanism of misfolded protein propagation have been observed. This fact is triggering the use of new common therapeutic approaches (bexarotene, Anle138b, etc.) or common diagnostics tools [30, 68, 69].

Despite the data already accumulated, there are crucial studies that should be implemented or performed in a more prion-like style. Strain and species barrier phenomena are still two important prion features on which the next studies in neurodegenerative disorders should be focused for a complete recapitulation of prion diseases.

It is surprising that among all the reviewed articles discussing prion-like mechanisms, none of them mention "infectivity" as a mechanism to understand the diversity of the pathological processes. On the other hand, both "seeding" and "*de novo* induction" are concepts frequently mentioned to describe the *in vivo* propagation and transmissibility of misfolded proteins, an identical *in vivo* process that in the prion field is considered "infection". There are several reasons why this might be explained. (i) It took more than 30 years for most of the prion community to agree with the "protein only hypothesis" [70]. (ii) Although "infectious" is not synonymous of "contagious," both terms could be considered equal in certain contexts. Thus, the assumption that common neurodegenerative diseases should be considered as infectious diseases could be perceived as unnecessarily frightening to the population, and (iii) the lack of a wider definition for "infectivity" able to encase all the disease-causing agents.

Far from conventionalisms and if we assume that Koch's postulates must be adapted to accommodate etiologically atypical diseases [71], we should extend the definition of "infectious agent" to include parasites, bacteria, virus and viroids, prions, and, why not, other misfolded proteins. Thus, we would suggest the following definition: "Infection is a process by which a self-propagating agent that exogenously penetrates or is generated spontaneously causes disease or damage as a consequence of its intrinsic capacity to make identical or similar copies of itself through a diversity of mechanisms requiring or not exogenous components." According to this and trying to answer the question opened from the title of this review, "seeding" would be a type of mechanism by which an infectious agent can be transmitted but should not be used to define a whole "infection" process.

Conflict of Interests

The authors declare no competing financial interests.

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Research Article

Trimeric Tau Is Toxic to Human Neuronal Cells at Low Nanomolar Concentrations

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In Alzheimer's disease (AD), tau aggregates into fibrils and higher order neurofibrillary tangles, a key histopathological feature of AD. However, soluble oligomeric tau species may play a more critical role in AD progression since these tau species correlate better with neuronal loss and cognitive dysfunction. Recent studies show that extracellular oligomeric tau can inhibit memory formation and synaptic function and also transmit pathology to neighboring neurons. However, the specific forms of oligomeric tau involved in toxicity are still unknown. Here, we used two splice variants of recombinant human tau and generated monomeric, dimeric, and trimeric fractions of each isoform. The composition of each fraction was verified chromatographically and also by atomic force microscopy. The toxicity of each fraction toward both human neuroblastoma cells and cholinergic-like neurons was assessed. Trimeric, but not monomeric or dimeric, tau oligomers of both splice variants were neurotoxic at low nanomolar concentrations. Further characterization of tau oligomer species with disease-specific modifications and morphologies is necessary to identify the best targets for the development of biomarker and therapeutic development for AD and related tauopathies.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia, characterized by progressive cognitive impairment, cerebral atrophy, and neuronal loss, with death generally occurring four to eight years after diagnosis [1]. Two pathological hallmarks of AD, extracellular neuritic plaques primarily composed of amyloid beta (Aβ) and intracellular neurofibrillary tangles (NFTs) primarily composed of tau protein, were originally identified in 1907 by Dr. Alois Alzheimer [2]. While great strides have been made in understanding the mechanisms that promote aggregation of Aβ and tau into the hallmark plaques and tangles, comparatively little progress has been achieved in halting or curing the disease. Analysis of familial AD cases implicated production of Aβ as a primary factor in progression of AD, leading to the rise of the amyloid cascade hypothesis which states that Aβ misfolding and aggregation initiates AD pathogenesis and triggers other effects such as tau phosphorylation, aggregation, and tangle formation [3]. The amyloid hypothesis had dominated the field for more than a decade and has driven numerous clinical studies for therapeutic interventions including several immunization studies targeting Aβ [4–6]. However failure of several clinical trials targeting Aβ has cast doubt on its relevance as a therapeutic target [7]. Increasing evidence indicates that tau also plays an important role in the progression of AD. Tau misfolding and aggregation can take place independently of amyloid formation [8], and in many cases the presence of tau lesions is associated with AD without presence of Aβ aggregates [9]. Clearance of Aβ plaques without reducing soluble tau levels is insufficient to ameliorate cognitive decline in double transgenic mice overexpressing Aβ and tau P301L [10]. These results among many others indicate that oligomeric tau may be an important therapeutic target for AD.

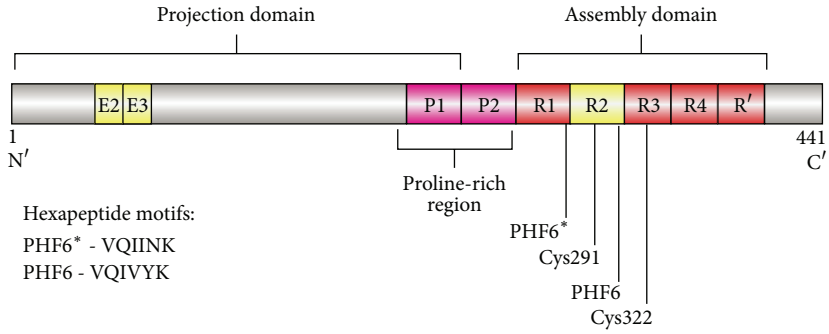

Figure 1: Tau protein structural features in linear diagram. A full-length tau protein with 441 amino acids (tau441 or tau 2N4R) is shown. Alternative splicing showed in yellow rectangles results in a total of six isoforms, denoted by either their total number of amino acids or the number of N′-terminal exons (Ns) and microtubule-associated repeats (Rs).

Tau in its monomeric form is a microtubule-associated protein crucial for microtubule assembly [11, 12] and stabilization [13]. Six major tau isoforms can be generated by alternative posttranscriptional splicing of exon 2 and exon 3 on the N-terminal projection domain and of exon 10 (Repeat 2) on the assembly domain (Figure 1). Tau contains three or four similar repeats in the microtubule-binding domain (MBD) that binds to and helps promote microtubule stability and function. For example, Repeat 2 and Repeat 3 contain hexapeptide motifs of PHF6* and PHF6, respectively (Figure 1). These motifs increase the tendency to form β-sheet structures that can interact with tubulins to form microtubules and also facilitate self-assembly to generate oligomeric and higher-order aggregates [14, 15]. Tau isoforms with or without the second microtubule-binding repeat can aggregate, but only the isoforms with the second repeat can form extended oligomeric forms mediated by disulfide linkages due to the additional cysteine in the second repeat (Figures 1 and 2). Therefore, in this study we utilized tau isoforms containing the second repeat unit to study the role of tau aggregation in neurotoxicity.

Hyperphosphorylation of tau is required for the release of tau from microtubules and its mislocalization to the somatodendritic compartment enabling tau to self-associate into oligomers and higher-order aggregates. However, the hyperphosphorylation of tau is not directly related to its toxicity but rather a mechanism to regulate its interaction with tubulin to stabilize microtubules and to regulate transport along microtubules. Expression of exogenous tau in mature hippocampal neurons leads to blockage of transport along microtubules and degeneration of synapses that can be rescued by phosphorylation of tau by kinase MARK2 to unblock the microtubule tracks [16]. Significantly, tau in the extracellular space is reported to be less phosphorylated than intracellular tau [17, 18] and more toxic in its dephosphorylated state [17]. Extracellular oligomers of recombinant full-length human tau protein were shown to be neurotoxic in mice and impair memory consolidation [19], and similar work at other labs has shown similar effects with recombinant tau oligomers and tau oligomers composed of hyperphosphorylated tau from AD brain. Thus, the hyperphosphorylation of tau associated with disease may

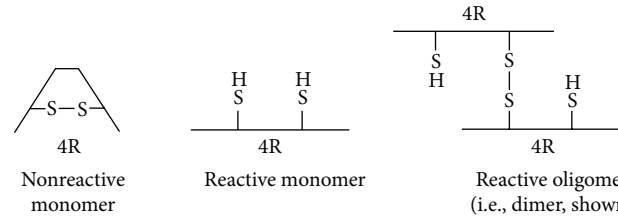

Figure 2: Schematic of nonreactive monomer, reactive monomer, and reactive oligomer. Reactivity implies the ability to form an intermolecular disulfide linkage. Intramolecular disulfide linkage causes formation of nonreactive tau monomer. The free thiols in a reactive monomer allow formation of an intermolecular or intramolecular disulfide linkage. Reactive oligomer has one or more free thiols readily forming disulfide linkage with reactive monomeric tau for the oligomer extension purpose.

be a causal factor in tau self-association into oligomers, but the hyperphosphorylation of tau in and of itself may not be the basis for the toxicity of extracellular tau oligomers.

Neurofibrillary tangles (NFTs) have traditionally been correlated with neuronal loss [20] and considered to be key intracellular indicators of AD. Approaches for targeting tau aggregation have focused on inhibiting hyperphosphorylation and fibril formation, reducing total tau levels, or stabilizing microtubules [21]. However, accumulating evidence suggests that soluble oligomeric rather than insoluble fibrillar tau species are neurotoxic and play an important role in the onset and progression of AD [21–24]. Although NFTs are a hallmark feature of AD, they can exist in AD neurons for up to 20 to 30 years [25] before postmortem confirmation and therefore are less likely to induce immediate toxicity in AD brain [26]. In animal models of tauopathy, the presence of NFTs does not correlate well with neuronal loss and memory deficits [27]. Reduction in neuronal loss and improvement in memory performance are observed despite an increase in NFTs [28]. In addition, the presence of NFT pathology does not localize well with areas of neuronal loss [29–31], synapse loss or dysfunction in the hippocampus along with microglial activation occurs well before the presence of NFTs [32]. In contrast, oligomeric tau was implicated in numerous studies

as playing a key role in AD progression [33–35] and to be a primary initiator of neurotoxicity and neurodegeneration [36]. Oligomeric tau has been identified in early stages of neuronal cytopathology in AD and closely correlates with hyperphosphorylation on microtubule-binding sites [24]. Tau oligomers can propagate endogenous tau pathology throughout the brain similarly to prions, demonstrating their neuronal toxicity [37]. The presence and concentrations of two tau oligomers (140 kDa and 170 kDa) correlate with memory loss in various age rTg4510 mice [33]. Oligomeric tau also induces synaptic and mitochondrial dysfunction [19]. Although tau is predominantly intracellular, the role of extracellular tau is gaining attention as extracellular oligomeric tau can have acute effects on long-term potentiation in hippocampal slices and can transmit pathology to healthy neurons [37]. Detection of oligomeric tau levels in human CSF and blood is also a promising AD diagnostic biomarkers along with total and hyperphosphorylated tau levels [38]. Because of the important role of oligomeric tau in AD and the recognition of the importance of extracellular tau in disease, it is critical to identify the key toxic tau species in disease etiology. Here we show our studies of the extracellular neurotoxicity of monomeric, dimeric, and trimeric forms of two four-repeat recombinant human tau variants to help identify the key tau species involved in the onset and progression of AD.

2. Material and Methods

2.1. Recombinant Human Tau (rhTau) Preparation and Purification. rhTau was purified as monomers from bacterial (BL21 DE3) clones with tau constructs in the pET21B and pET29a vectors. Standard methods were used to grow and induce the protein with 1 mM IPTG. Pelleted cells were lysed with CelLytic B lysis buffer, lysozyme, benzonase, and protease inhibitors according to the manufacturer's protocol (Sigma Aldrich, St. Louis, MO). Cation exchange (GE Healthcare Life Sciences) was used for the first step of purification with SP-Sepharose resin for both tau constructs, and 300 mM NaCl in 25 mM Tris-HCl pH 7.4 was used to elute tau protein. Amicon Ultra Centrifugal Devices (Millipore) were used to buffer-exchange the protein preparations into 50 mM Tris-HCl pH 7.4. Protein concentration was determined using a BCA assay (Thermo Fisher Scientific). Tau oligomers were generated by incubating tau monomers at a concentration of 5 μM in 50 mM Tris buffer pH 7.4 with 100 mM NaCl at 37°C overnight. The monomeric and oligomeric species were resolved by 6% PAGE, eluted, and buffer-exchanged into 50 mM Tris-HCl. Fractions were analyzed by nonreducing SDS-PAGE to minimize degradation of oligomeric proteins and silver staining to enhance the signal and to verify the purity of tau variants. Protein concentration was determined using the BCA assay.

2.2. Height Distribution Analysis. AFM sample preparation and imaging were performed as described previously [39–44]. Aliquots of 10 μL 0.50 μM purified tau variants in 50 mM Tris-HCl buffer were deposited on separate mica pieces for imaging using MultiMode AFM Nanoscope IIIA system (Veeco/Digital instruments, Santa Barbara, CA) which was set in tapping mode and equipped with silicon AFM probes (VISTA probes, Nanoscience Instruments). Height distribution analysis of the different tau samples was fit to a normal distribution probability model using Gwyddion 2.20. All detectable protein molecules were assumed to be spherical and the height values approximate their diameters.

2.3. Cell Culture and Treatments. SH-SY5Y human neuroblastoma cell lines (American Tissue Culture Collection) were cultivated in tissue culture flask (Falcon by Becton Dickinson Labware). Cells were grown in a medium containing 44% v/v Ham's F-12 (IrvineScientific), 44% v/v MEM Earle's salts (IrvineScientific), 10% v/v denatured fetal bovine serum (FBS) (Sigma Aldrich), 1% v/v MEM nonessential amino acids (Invitrogen), and 1% v/v antibiotic/antimycotic (Invitrogen). Media were renewed once every two to three days. The cells were passaged to a new flask when they were confluent in the flask. For toxicity studies, the SH-SY5Y cells were seeded in a 48-well cell culture cluster plate (Costar by Corning Incorporated) with 5×10^4 cells/well in 300 μL fresh medium. Each experiment was conducted in triplicate. Cell density was estimated by reading a fixed volume on a hemocytometer. After growth in a 37°C incubator for 24 hours, the tissue culture media were replaced with fresh serum-free media for the neurotoxicity test on nondifferentiated cells. To investigate tau toxicity on cholinergic neurons, a duplicate set of the cultured cells was induced into cholinergic-like phenotype by incubation with retinoic acid at a final concentration of 10 μM for 3 to 5 days [43, 45–47]. The cultivated nondifferentiated and cholinergic-like neurons were treated with monomeric, dimeric, and trimeric variants of 1N4R and 2N4R at final concentrations of 2.26 nM, 4.50 nM, 11.15 nM, and 15.50 nM. A PBS negative control was used as a standard for subsequent LDH assay analysis. Cultures were incubated with tau species at 37°C and sampled at 3, 18, 24, and 48 hour time points by harvesting 30 μL/well aliquots of culture supernatant.

2.4. LDH Assay. The LDH protocol is adapted from a commercial kit (Sigma Aldrich) based on the generic protocol of Decker and Lohmann-Matthes [48]. The LDH assay was performed as described previously [40]. Absorbance was measured at 490 nm (reference wavelength 690 nm). Relative absorbance values were calculated by subtracting the reference values from the values obtained at 490 nm. LDH% values greater than 150 are considered toxic.

2.5. Statistical Analysis. The relative absorbance values of all samples were normalized to those of controls which were set as 100% for each independent experiment. Group mean values were analyzed by one-way ANOVA with $P < 0.05$ standard and LSD post hoc significant differences test. All analyses were performed with SPSS 21.0 (IBM Corp., Armonk, NY).

3. Results

3.1. rhTau Aggregate Analysis. We expressed recombinant human tau in a bacterial host system to eliminate any post-translational phosphorylation of tau and therefore remove any potential effects that phosphorylation may have on tau aggregation or loss of function. The resulting nonphosphorylated human recombinant tau (NPrhTau) monomers contain reactive cysteine groups with free thiols, facilitating the formation of intramolecular disulfide bonds to make stable nonreactive monomers and the formation of intermolecular disulfide bonds to produce tau oligomers and higher-degree aggregates (Figure 2). The polymerization reaction is controlled by incubation time and protein concentration. The nonreactive monomeric, dimeric, and trimeric forms of both the 2N4R and 1N4R splice variants generate stable aggregate morphologies with defined size profiles dependent on the degree of oligomerization and length of the splice variant as evidenced by SDS-PAGE (Figure 3) and AFM height distribution analysis (Figure 4). The oligomer heights increment for each additional monomeric tau unit is fixed within a certain isoform, which is 0.5 nm for 1N4R variants and 1.0 nm for the 2N4R variants (Figure 4). The size of each respective 2N4R species is also larger than the corresponding 1N4R species (Figures 3 and 4) as expected given that tau 2N4R contains the extra N-terminal insert compared with the 1N4R variants.

3.2. Extracellular rhTau Induced Neurotoxicity Test. While neither the monomeric or dimeric forms of tau from either the 1N or 2N splice variants displayed detectable toxicity, the trimeric form of both variants exerted marked toxicity toward nondifferentiated (Figure 5(a)) and retinoic acid induced cholinergic-like neurons (Figure 5(b)) with LDH values well above the toxic threshold of 150 at low nanomolar concentrations (11.15 nM, and 15.50 nM). The full-length 2N4R trimeric tau form displayed significantly higher toxicity than the 1N4R trimeric form toward nondifferentiated neurons (Figure 5(a)), although the effect is diminished in the cholinergic-like neurons (Figure 5(b)). When trimeric tau was added to nondifferentiated SH-SY5Y cells, an increase in toxicity was observed with time at the highest concentrations for both the 1N4R (Figure 6(a)), and 2N4R (Figure 6(b)) trimeric variants. However, when trimeric tau was added to the cholinergic-like neurons, the toxicity of the 1N (Figure 6(c)) and 2N (Figure 6(d)) variants was relatively consistent over the first 24 hours, but increased after 48 hours. Both variants of trimeric tau showed increased toxicity toward the cholinergic-like neurons compared to the nondifferentiated neurons at short incubation times (Figure 7(a)) but the reverse was observed at longer incubation times (Figure 7(b)).

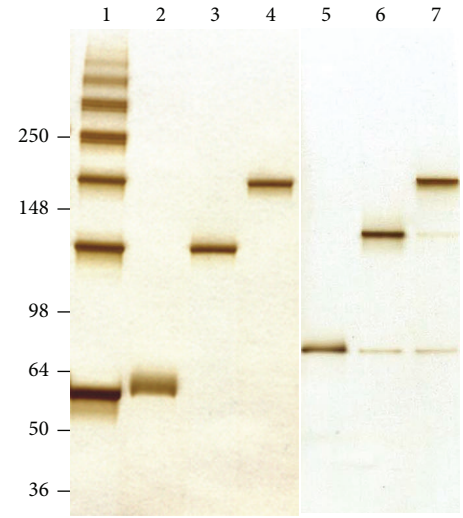

Figure 3: Recombinant human tau (rhTau) monomeric and oligomeric species production and purification. rhTau 1N4R enriched with disulfide-mediated tau oligomers (lane 1) was used for the purification of monomeric, dimeric, and trimeric tau species (lanes 2–4), 2N4R purified monomeric, dimeric, and trimeric species (lanes 5–7).

4. Discussion

While the amyloid cascade hypothesis [49] has dominated studies into the etiology of AD over the last decade or more, the importance of tau in the onset and progression of AD is steadily becoming more apparent. Tau pathology has been observed in the absence of Aβ deposits in children and young adult cases, and tau aggregates in the entorhinal-hippocampal regions precede the onset of Aβ pathology [8, 9]. Numerous studies have shown that various oligomeric forms of Aβ are toxic to neurons and can impair cognitive performance [50, 51], thus implicating their potential role as valuable biomarkers for diagnosing AD [42, 52, 53]. Similar to the important role of various soluble oligomeric Aβ species in AD, different soluble oligomeric forms of tau may also play a critical role in AD, also causing neuronal loss and cognitive dysfunction [19, 54, 55]. Therefore to facilitate diagnoses and therapeutic treatments for AD, it is important to identify the key tau species involved in the onset and progression of the disease. Given that tau has multiple splice variants and posttranslational modification sites, we attempted to simplify the complex diversity of tau forms by focusing on two nonphosphorylated human recombinant tau isoforms, 1N4R and 2N4R. These two four-repeat (4R) isoforms of tau both have all four repeats of the microtubule-associated domains and are more prone to form the aggregates readily phosphorylated by brain protein kinases than those with only three repeats (3R) [56] due to the presence of Repeat 2 with a microtubule-affinity enhancing hexapeptide motif [14, 15] and an additional cysteine that forms disulfide linkages to stabilize the aggregates.

The most disease-relevant tau material to use to study toxicity of extracellular tau forms would be well characterized tau oligomers purified from AD cerebrospinal fluid (CSF) using methods to preserve their posttranslational modifications, including phosphorylation, glycation, ubiquitination, aggregation, and truncation. Preparations from several non-AD and AD cases would be necessary to understand the significance of the results. Here we performed an initial study

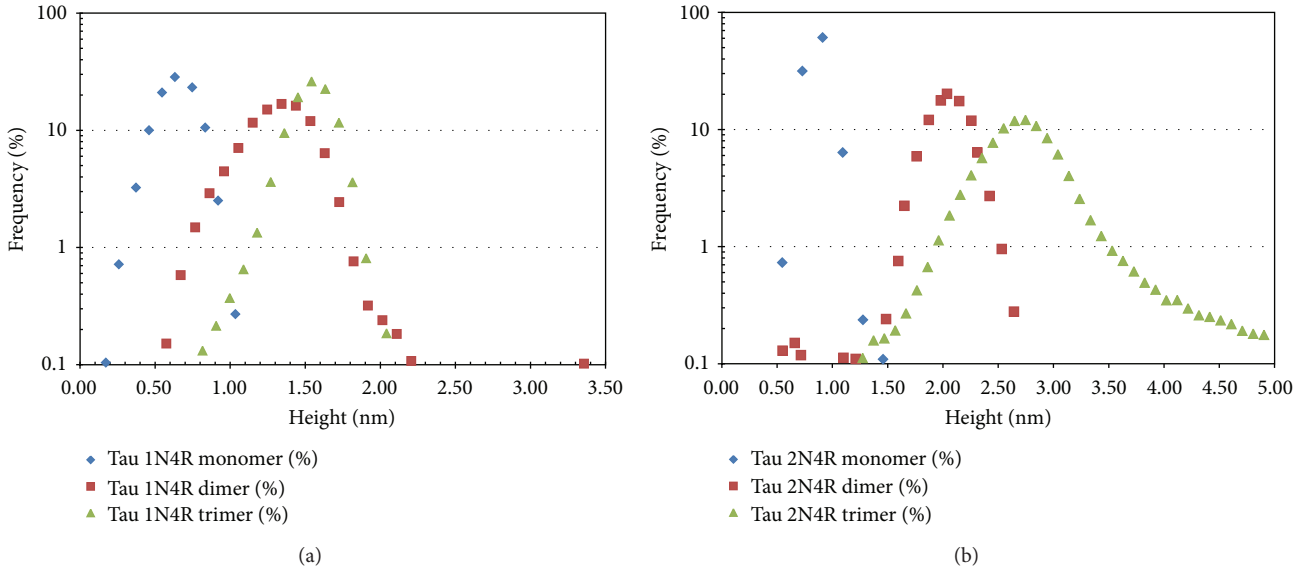

Figure 4: Plots of height distribution of monomeric, dimeric, and trimeric fractions of rhTau 1N4R (a) and tau 2N4R (b). The height value of each particle was measured using Gwyddion. The numbers of particles falling in continuous size ranges were calculated and normalized into count percentages. The peak values give an approximate value for each tau species particle size. As expected, high-degree oligomers are larger than low-degree oligomers within the same isoform, and corresponding oligomeric aggregates from the longer isoform are larger than aggregates from the shorter isoform.

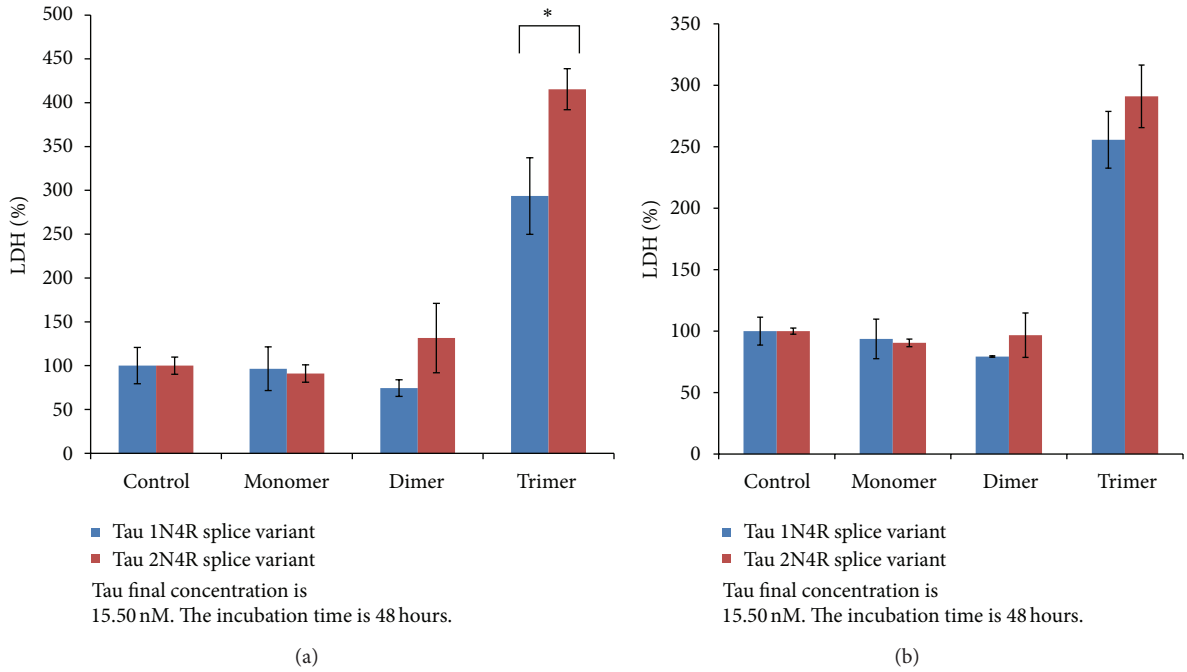

Figure 5: Neurotoxicity of extracellular 15.5 nM monomeric, dimeric, and trimeric forms of 1N4R and 2N4R tau variants toward (a) nondifferentiated human neuroblastoma cells (SH-SY5Y) and (b) Retinoic-acid-differentiated SH-SY5Y cells was measured after 48-hour incubation using an LDH assay. For both four-repeat tau isoforms, trimeric form is more neurotoxic than monomeric and dimeric forms ($P < 0.001$) on either neuron type. Full-length trimeric rhTau is more neurotoxic than 1N4R trimeric rhTau. ($P < 0.05$).

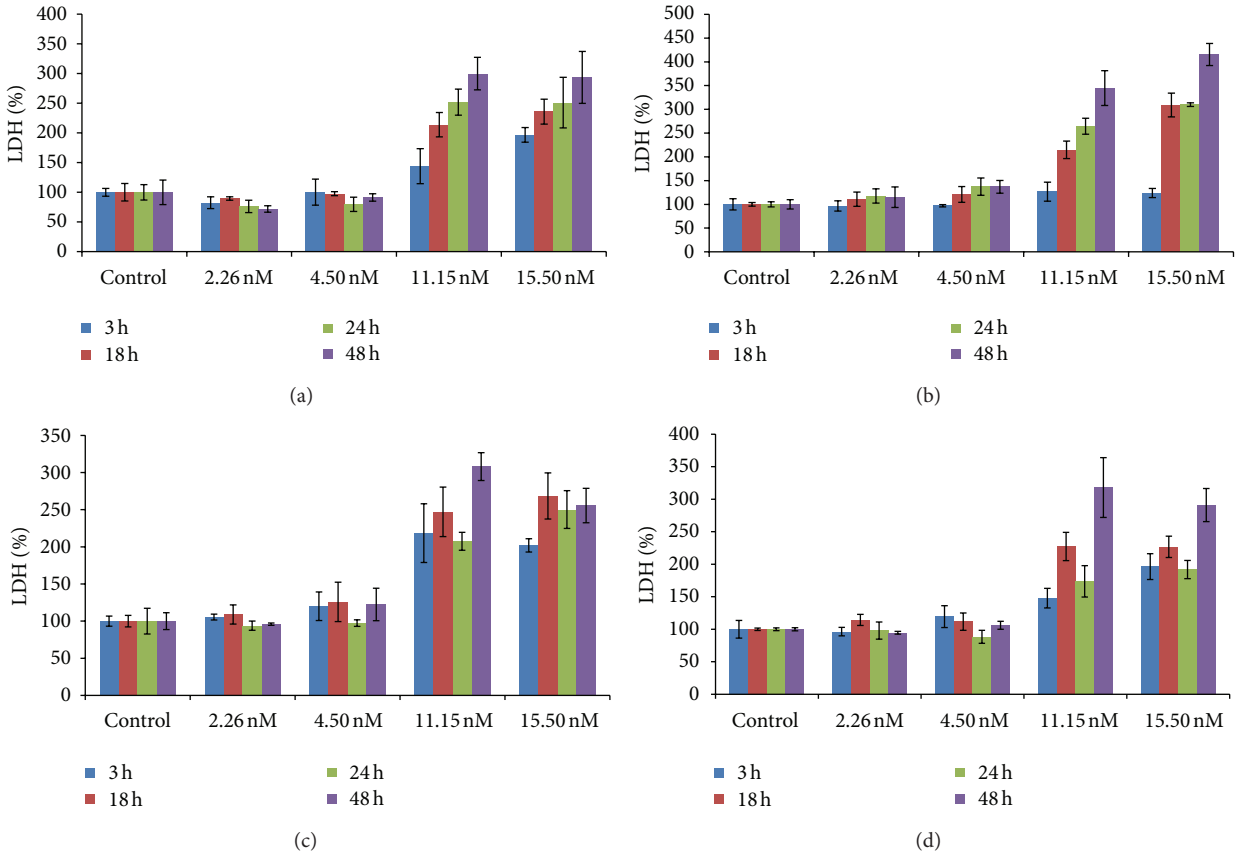

Figure 6: Time and concentration dependence of neurotoxicity induced by trimeric rhTau (1N4R and 2N4R) toward neuroblastoma cells measured by LDH assay. Nondifferentiated SH-SY5Y cells incubated with (a) 1N4R tau and (b) 2N4R tau; retinoic-acid-differentiated SH-SY5Y cells incubated with (c) 1N4R tau and (d) 2N4R tau.

focused specifically on unmodified tau protein oligomers and control monomer to specifically understand the relevance of oligomer structure to extracellular toxicity.

We determined the toxicity of the different tau variants using both nondifferentiated and cholinergic-like neuroblastoma cell lines to determine how aggregate size and cell phenotype affected toxicity. Cholinergic cells are particularly vulnerable in AD with significant neuronal loss in the nucleus basalis of Meynert (NBM), that is, the hippocampus and the cortex [57]. NBM is enriched in cholinergic cells and undergoes degeneration and a significant decrease of acetylcholine production in AD [58]. Decreased levels of acetylcholine and a number of other cortical cholinergic markers lead to clinical dementia and impairment in cognitive function [58], indicating that cholinergic cells are particularly vulnerable in AD. Here we show that trimeric, but not monomeric or dimeric, tau is toxic to neuronal cells at low nanomolar concentrations and that the full-length 2N tau variant is more toxic than the shorter 1N variant to nondifferentiated neurons (Figure 5). Both trimeric tau variants cause toxicity to both nondifferentiated SH-SY5Y cells and retinoic acid induced cholinergic-like neurons when tau was applied extracellularly at nanomolar levels (Figure 6). However, the cultured cholinergic-like neurons show increased susceptibility to trimeric tau induced toxicity at short incubation times compared with similar nondifferentiated neurons (Figure 7(a)), perhaps partially accounting for the increased vulnerability of cholinergic-like neurons in AD. Since the nondifferentiated cells were equally susceptible to trimeric tau induced toxicity at longer incubation times (Figure 7(b)), these results suggest that toxicity of extracellular trimeric tau is not dependent on receptors or proteins specifically associated with cholinergic cells but that toxicity might be facilitated by them. Our results are consistent with a recent study showing that low molecular weight (LMW) misfolded tau species exclusive of monomeric tau can be endocytosed by neurons and transported both anterogradely and retrogradely to induce endogenous tau pathology in vivo while fibrillar tau and brain-derived filamentous tau cannot be endocytosed [59]. This suggests that tau toxicity may be spread through cells in certain brain regions by endocytosis of trimeric and larger oligomeric forms of tau and that this uptake is facilitated in cholinergic neurons.

Neuronal toxicity of oligomeric tau may share similar properties to that of oligomeric Aβ where the critical feature involved in neuronal toxicity is the aggregation state of the protein more than posttranslational modifications [23, 60]. While there are a wide variety of tau variants that occur

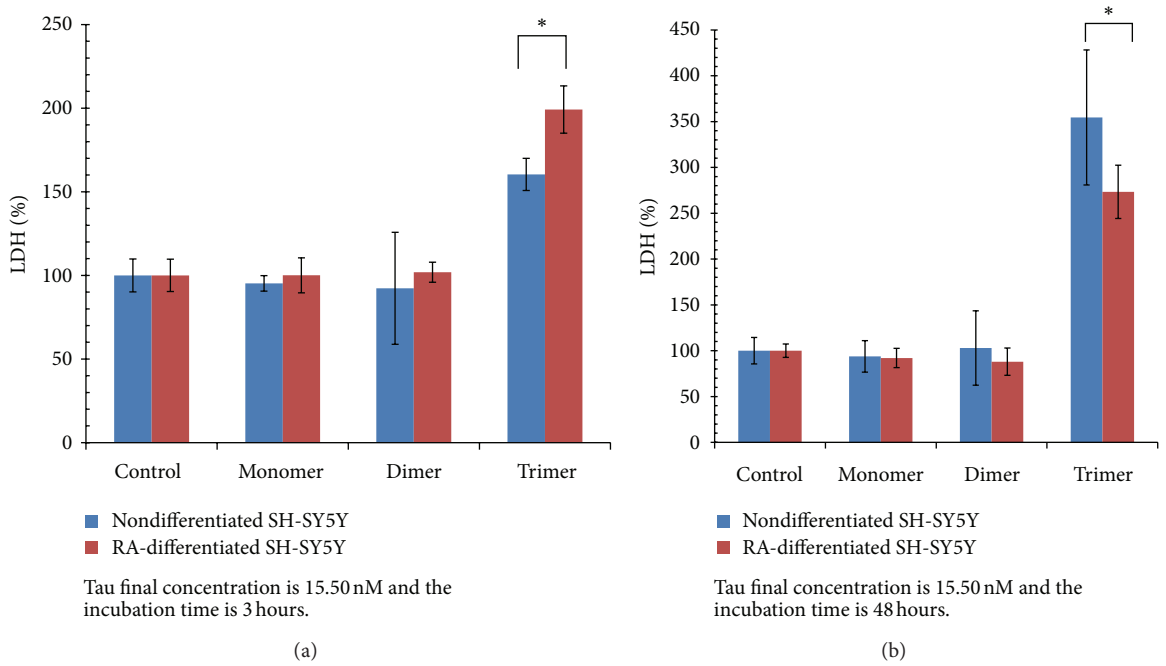

Figure 7: Comparison of rhTauinduced neurotoxicity toward nondifferentiated SH-SY5Y cells and retinoic-acid- (RA-) differentiated SH-SY5Y cells. The data combine toxicity results of 15.5 nM monomeric, dimeric, and trimeric forms of both 1N4R and 2N4R tau variants. (a) After 3 hours-incubation, RA-differentiated SH-SY5Y cells are more vulnerable to extracellular trimeric rhTau toxicity than nondifferentiated SHSY-5Y cells are ($P < 0.05$). (b) After 48-hours incubation, nondifferentiated SH-SY5Y cells are more vulnerable to extracellular trimeric rhTau toxicity than RA-differentiated SH-SY5Y cells ($P < 0.05$).

in vivo including different posttranslational modifications, splice variants, and aggregated species, this study begins to more systematically probe the role of selected tau variants in AD. Further studies are needed to determine the contribution of splice variants and AD-specific posttranslational modifications found in extracellular tau to the toxicity of the tau variants and to how these tau variants affect other neuronal models including primary neurons or induced pluripotent stem cells. Well characterized reagents that can selectively identify specific tau variants and morphologies will be useful for these further studies.

Conflict of Interests

Eliot Davidowitz, Patricia Lopez, and James Moe are employees of Oligomerix, Inc., 3960 Broadway, New York, NY 10032, USA. Partial funding for this work was provided by Oligomerix, Inc. There is no other conflict of interests.

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Review Article

Breaking the Code of Amyloid-β Oligomers

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Departing from the original postulates that defined various neurodegenerative disorders, accumulating evidence supports a major role for soluble forms of amyloid proteins as initiator toxins in Alzheimer's disease, Parkinson's disease, frontotemporal dementias, and prion diseases. Soluble multimeric assemblies of amyloid-β, tau, α-synuclein, and the prion protein are generally englobed under the term oligomers. Due to their biophysical properties, soluble amyloid oligomers can adopt multiple conformations and sizes that potentially confer differential biological activities. Therein lies the problem: with sporadic knowledge and limited tools to identify, characterize, and study amyloid oligomers, how can we solve the enigma of their respective role(s) in the pathogenesis of neurodegenerative disorders? To further our understanding of these devastating diseases, the code of the amyloid oligomers must be broken.

1. Commentary

For a century, the cardinal features of Alzheimer's disease (AD), amyloid plaques and neurofibrillary tangles, were thought to underlie this chronic neurological disorder. However, based on the evidence accumulated over the past ten to fifteen years, the toxicity of these lesions has been questioned. Instead, the emerging soluble aggregation-intermediate forms of amyloid-beta (Aβ) and tau proteins, which compose plaques and tangles, are now believed to underlie the synaptic and neuronal losses observed in AD. Studies focusing on oligomeric Aβ assemblies [1–4] have paved the way for other amyloid proteins including tau [5], alpha-synuclein [6–8], and the prion protein PrP [9] in the field of neurodegenerative disorders. This principle simply revolutionized our understanding of AD, Parkinson's disease, frontotemporal dementias, and prion diseases, opening new avenues for therapeutic strategies.

In what might seem like an all rosy affair, this paradigm shift also contributed to complicating even more the putative sequence of biological events responsible for these diseases. In AD, the classical view of the amyloid hypothesis postulated that amyloid plaques are altering the physiological function of neurons, which in turn disrupts tau biology leading to the demise of the cell [10]. The modern view of the amyloid hypothesis suggests the involvement of a multitude of endogenous bioactive Aβ molecules [11] that include Aβ dimers, trimers, Aβ^*56, annular protofibrils, and amyloid plaques, as opposed to a single culprit (i.e., plaques). This notion appears to be consistent with the myriad cell surface receptors and signaling pathways that have been described as specifically activated by putative endogenous soluble Aβ oligomers [11]. If this scenario was not entangled enough, numerous studies aiming at elucidating the function of oligomeric Aβ (oAβ) use oligomeric preparations of synthetic Aβ peptides whose folding conformation and posttranslational modifications might not accurately reflect to these found in biologically relevant systems (i.e., brain, cerebrospinal fluid, blood, and primary neurons). In the end, this increased complexity of the problem coupled with a lack of adequate experimental descriptions of the oAβ used and specific detection tools (e.g., antibodies specific to a single Aβ assembly) renders interpretation and comparison of the observed phenomena between different research groups arduous [12] and impedes on our progress to better understand the role of Aβ oligomers in AD.

A clear example of this issue plaguing our field is illustrated by the controversial debate surrounding the role of the cellular form of the prion protein (PrP^C) in mediating the deleterious effects of oligomeric Aβ. In 2009, Lauren and colleagues reported that PrP^C was acting as a receptor for synthetic Aβ oligomers also called Aβ-derived diffusible ligands (ADDLs) [13, 14]. ADDLs have been characterized by denaturing electrophoresis (SDS-PAGE), transmission electron microscopy (TEM) and size-exclusion chromatography (SEC) coupled with static light scattering (SLS) [14], but each technique generated inconsistent and contradicting results. First, ADDLs ran as an undefined smear ranging from ~25 to 200 kDa using SDS-PAGE followed by western blotting with the sole 6E10 antibody detecting $A\beta_{1-16}$. Additional bands were detected as putative monomers, trimmers, and tetramers in the ADDL preparation but since these same immunoreactive bands were also detected in freshly resuspended synthetic Aβ peptides, they are likely a result of the presence of SDS in the experimental conditions. SDS is known to artificially alter the electrophoretic migration of synthetic Aβ [15]. TEM revealed that ADDLs contained spheroidal structures of various sizes; the most abundant form appeared to correspond to 5-6 nm spheroids. It is important to note that short filamentous structures were also clearly visible possibly corresponding to protofibrils. Finally, liquid phase chromatography coupled with SLS revealed the presence of only two elution peaks under nondenaturing conditions, a broad trailing peak detected shortly after the void volume containing Aβ molecules of ~500 kDa mass and a well-defined sharp peak corresponding to monomeric Aβ peptides. The authors concluded that the preparation of ADDLs used was approximately made of an assembly composed of 50 to 100 Aβ monomers [14]. Based on the aforementioned data, it seems reasonable to conclude that these ADDLs are not stable under denaturing conditions as previously reported [16] and that the exact composition of the synthetic Aβ oligomers used remains inconclusive. Despite the apparent inconsistency of the observations characterizing the oAβ used in this study, PrP^C appeared to be necessary to mediate the inhibition of long-term potentiation (LTP) induced by oAβ [14].

As expected, this study stimulated several independent groups to reproduce these findings using various sources and preparations of Aβ [17–20]. A team led by Gianluigi Forloni first reported that PrP^C was not required to mediate the cognitive impairments induced by synthetic Aβ oligomers [17]. Synthetic Aβ peptides were prepared to generate ADDLs following the same groundwork established by William Klein and his colleagues at Northwestern University [13, 21]. Analyses using atomic force microscopy (AFM) and SEC defined the ADDLs and obtained and confirmed the presence of mixed structural species (i.e., spherical assemblies and protofibrils) by AFM and the presence of two elution peaks following SEC (a sharp peak close to or within the void volume and a smaller peak containing putative Aβ monomers). While these elements could suggest at first glance that the ADDLs generated at Yale and at the Mario Negri Institute are similar, it bears to mention here that the columns used in both studies greatly differed (a sequential connection of Superdex 200, Superdex 75, and Superdex peptide, 10/30, HR SEC columns for the Yale group and a single Superdex 75 column for the Italian group) raising the possibility that in fact both ADDL preparations were different.

To further demonstrate the involvement of PrP^C in Aβ-induced deficits, the role of PrP^C was examined in middle-aged APPPS1ΔE9 transgenic mice used to model Alzheimer's disease [22] expressing or deficient for the *Prnp* gene [23]. Gene deletion of *Prnp* had no apparent effect on soluble and insoluble monomeric Aβ levels as measured by western blot analyses using 6E10 despite a ~20% reduction in amyloid burden, indicating potential discrepancies in Aβ measurements and quantification. Behaviorally, ablation of *Prnp* resulted in rescuing synaptic loss, APP-induced premature mortality, and spatial learning and memory compared to APPPS1 mice [23]. Puzzlingly, CA1 LTP was not altered in APPPS1ΔE9 hippocampal slices, possibly suggesting that the endogenous Aβ species responsible for LTP inhibition are not present or that these mice might develop homeostatic compensations in response to synaptic injury induced by Aβ. In addition to the apparent inconsistency in the Aβ levels, the nature and characterization of the Aβ molecules in 12-month-old APPPS1 and APPPS1x$Prnp^{-/-}$ were not mentioned, begging the question as to whether the same Aβ species initially found to interact with PrP^C are the same as the hypothesized Aβ oligomers present *in vivo*.

A few months later, two independent studies published at the same time challenged the conclusions that PrP^C is a mediator of Aβ toxicity [18, 19]. PrP^C was not found to be required for Aβ-induced synaptic deficits in hippocampal slices transfected with a carboxyl terminal domain of the amyloid precursor protein APPct100 and for ADDL-induced LTP inhibition [19]. In the former paradigm, it is unknown whether oligomeric Aβ species are present in APPct100-expressing slices [19, 24], and if they were, the information pertaining to their characterization was not discussed [19]. In the second experimental condition, hippocampal slices were incubated with synthetic Aβ oligomers. Although the method used to generate ADDLs was identical to the one used by Lauren and coworkers, gene deletion of the *Prnp* gene failed to rescue the LTP inhibition induced by ADDLs. It is important to note that the characterization of the Aβ oligomers formed only included one western blot analysis with an unspecified antibody following SDS-PAGE and revealed the presence of a poorly resolved smear ranging from ~35 to ~180 kDa and monomers. In addition, the concentration at which the mixtures were used (1 μM) was greater than those used by the original study (20–200 nM), possibly adding an additional confounding factor when comparing the experimental designs. Due to the absence of data describing the aggregation state of the Aβ used in these paradigms, it is difficult to conclude that the results presented invalidate the findings of the initial study by Laurén et al. [14].

The role of PrP^C in mediating Aβ-induced LTP deficits was investigated in hippocampal slices of 2 to 4-month-old $APPPS1_{L166P}$ mice [25] that were genetically manipulated to

express 2, 1, or 0 copies of the *Prnp* gene [18]. Contrary to earlier findings [23], LTP was impaired in an age-dependent fashion in $APPPS1_{L166P}$ slices, but *Prnp* copy numbers did not influence the observed LTP deficits [18]. Neither full-length APP and carboxyl-terminal fragments of APP CTFα and CTFβ nor soluble $A\beta_{42}$ levels were altered by *Prnp* genotypic differences indicating that PrP^C does not alter APP/Aβ metabolism in this mouse model. Despite these rigorous analyses of APP derivatives, the exact nature and relative abundance of soluble Aβ assemblies present in 4-month-old $APPPS1_{L166P}$ mice were not addressed.

In light of these disparate observations, *Nature Neuroscience* published an editorial in April 2011 entitled "State of Aggregation" which reiterated the critical need to clearly describe the initial state of the protein, its source, and its stoichiometry in order to maximize the success of independent groups that want to reproduce observed phenomena.

Shortly thereafter, Freir and colleagues confirmed that PrP^C is required for LTP inhibition induced by ADDLs and by protein lysates of AD brain tissue containing Aβ [26]. A major reason as to why this study stood out relies on the fact that synthetic oAβ preparations were carefully characterized by SEC, analytical ultracentrifugation, electron microscopy, and by SDS-PAGE and that all techniques produced results that were intrinsically consistent. SEC and AUC analyses of ADDLs and biotinylated ADDLs (bADDLs) confirmed the presence of 2 peaks reminiscent of these described by Laurén et al. However, leading and trailing shoulders in the SEC elution peaks were observed suggesting the presence of species ranging from 90 to 400 kDa in the mixture, which was confirmed by AUC. Astutely, the authors also noticed that the addition of a biotin residue to Aβ artificially enriched the abundance of high-molecular weight species compared to unbiotinylated ADDLs. Using EM, both spherical and short filamentous structures were observed consistent with the profile obtained in the original study [14]. Finally, SDS-PAGE followed by 6E10 immunoblotting analyses confirmed that ADDLs are not SDS resistant and predominantly migrate as experimental artifacts as Aβ monomers, dimers, trimers, and tetramers following denaturation [15]. When this mixture was applied to hippocampal slices, LTP was inhibited in wild-type but not *Prnp*-deficient mice. Altogether, based on these biophysical observations, PrP^C appears to be mediating the inhibition of LTP induced by one or several unidentified synthetic Aβ oligomers. More importantly, a similar rescue of LTP inhibition was observed in $Prnp^{-/-}$ slices when Tris-buffered saline (TBS) soluble protein extracts from an AD brain were applied. Biochemical analysis of TBS fractions from AD and control brains by immunoprecipitation/western blotting revealed the presence of putative SDS-stable Aβ dimers (~7 kDa) and monomeric Aβ in AD TBS extracts, while no Aβ species were detected in control TBS lysates. It is difficult to determine whether other Aβ assemblies were present as there was substantive nonspecific background in the "no protein" condition ranging from 18 to 80 kDa and because only one antibody was used to detect Aβ (presumably 6E10).

Integrating the observations from the studies mentioned above, it seemed reasonable at the time to conclude that PrP^C is required for the inhibition of LTP induced by a mixture of soluble brain-derived Aβ species.

After two years of intense investigation, we still did not know the answers to the most crucial questions related to oAβ if one aims to use this knowledge to develop diagnostic and therapeutic tools: (1) which endogenous Aβ assembly is binding to PrP^C? (2) Where is this interaction occurring? (3) When do endogenous oAβ engage PrP^C? (4) How does PrP^C mediate the deleterious effect(s) of oAβ?

We sought to answer these questions combining *in vivo* experiments using human, transgenic mouse brain tissues and *in vitro* paradigms using primary neurons derived from various mouse lines [27]. To ascertain the relevance of the study, all soluble Aβ species were purified from human AD brain tissue or conditioned media of transgenic cortical neurons in liquid phase experiments (i.e., immunoaffinity capture in suspension followed by SEC) and characterized by immunoprecipitation/western blot using a panel of 4 antibodies detecting the N-terminal region (6E10), the central domain (4G8), or the C-termini of Aβ (40- and 42-end specific antibodies Mab2.1.3 and Mab13.1.1, kind gifts from Pritam Das, Mayo Jacksonville). In a reproducible fashion, we isolated endogenous Aβ monomers, dimers, trimers, Aβ^*56, and protofibrillar species migrating at ~175–180 kDa in absence of any additional detectable Aβ species using our panel of Aβ antibodies. Of note, we also used the oligomer-specific antibody A11 [28] to further confirm the nature of human Aβ^*56 (data not shown). Moreover, none of the purified soluble Aβ species displayed aberrant migration profiles induced by SDS-PAGE analysis (i.e., apparent monomers, dimers, trimers, and tetramers comigrating in the same lane), and all soluble Aβ captured were eluted at the predicted molecular weight during SEC, arguing against the possibility that the assemblies detected are gel artifacts. Finally, putative Aβ dimers and trimers could be found in the conditioned medium of primary mouse cortical neurons expressing the Swedish mutant form of human APP disproving that these apparent Aβ oligomers are induced by lysis or the presence of detergents. Because we thoroughly characterized and documented the initial or current state of the endogenous oAβ present in our biological specimens, we believed we could address the who/where/when/how. Briefly, we identified that PrP^C formed a complex with Fyn/Caveolin-1 in AD brain tissues and that Aβ dimers were the only low-molecular oligomer that coimmunoprecipitated with this complex. Using 84 human brain specimens from the Religious Orders Study (ROS), we also demonstrated that both PrP^C and active Fyn (phosphorylated at Y416, pFyn) proteins were abnormally elevated in AD compared to age-matched controls and that Fyn activation was correlated to PrP^C expression levels [27]. We next applied a mixture of oAβ purified from AD brain tissue containing Aβ monomers, dimers, trimers, Aβ^*56, and protofibrils onto protein extracts enriched in membrane proteins derived from control subjects with no detectable Aβ species. Upon PrP^C pulldown, only Aβ dimers were visibly captured further validating the coimmunoprecipitation findings previously obtained using AD brain.

To determine where oAβ could interact with PrP^C, we performed triple-labeling immunofluorescence colocalization experiments using sections from AD and control brain and confocal imaging and image reconstruction. Soluble Aβ was identified as punctae along the neuronal processes, colocalized with PrP^C at dendritic spines in AD but not control brain tissue, which accounted for ~22% of oAβ present at dendritic spines labeled with Fyn. Although the data were slightly higher (~36%), analyses performed on Tg2576 primary cortical neurons expressing Aβ monomers, dimers, and trimers generated similar results. Importantly, pFyn was also observed to colocalize with Aβ and PrP^C most notably at synaptic varicosities traditionally considered to reflect alterations in microtubule organization. Since tau is a microtubule-associated protein and believed to mediate Aβ-induced deficits, we analyzed tau phosphorylation status and cellular localization when PrP^C/Fyn/oAβ were engaged into forming an active complex. Consistent with the synaptic varicosities, tau was hyperphosphorylated at Y18, a well-known target phosphorylation site for Fyn [29], and abnormally accumulated at postsynaptic sites reminiscent of phenomena associated with synaptic dysfunction [30, 31].

It then appeared that Aβ dimers could bind to PrP^C engaging the activation of Fyn at dendritic spines, but knowing when this pathological event took place remained unknown. To address this question, we examined the role of aging on oAβ in $APPPS1_{L166P}$ mice. In 2-month-old $APPPS1_{L166P}$, Aβ monomers and apparent Aβ trimers were readily detected albeit at low levels. In contrast, very abundant Aβ monomers and putative Aβ dimers and trimers were observed at 14 months of age. These results were consistent with earlier reports considering that Aβ dimers are associated with plaques [4, 32] and that amyloid deposition occupies ~10% of the neocortical areas at 8 months in $APPPS1_{L166P}$ mice [25]. Further supporting the hypothesis that Aβ dimers activate the PrP^C/Fyn complex, Fyn activation was remarkably elevated in aged $APPPS1_{L166P}$ mice while undetectable in young animals [27]. In addition, the electrophoretic migration pattern for oAβ did not appear to differ substantially between $APPPS1_{L166P}$ mice expressing PrP^C and $APPPS1_{L166P}$ mice deficient for *Prnp* ($APPPS1_{L166P}$x$Prnp^{-/-}$). As predicted by our hypothesis, Fyn phosphorylation was reduced by ~50% at 14 months of age in $APPPS1_{L166P}$x$Prnp^{-/-}$ mice suggesting that oAβ, and presumably Aβ dimers induced the activation or PrP^C/Fyn in aged $APPPS1_{L166P}$ mice when amyloid burden is well established.

Finally, we sought to establish how PrP^C mediated the effects of oAβ. To this end, we applied isolated Aβ monomers, dimers, trimers, Aβ^*56, and protofibrils at equimolar concentrations (5 nM) onto primary cortical neurons. After 60 minutes, only Aβ dimers and trimers induced Fyn phosphorylation. Since Aβ trimers did not appear to interact with PrP^C based on our coimmunoprecipitations, our results pointed to Aβ dimers as the major soluble endogenous Aβ ligand for PrP^C *in vitro*. These findings were also in agreement with our *in vivo* data showing that *Prnp* gene deletion partly abolished Fyn activation in aged $APPPS1_{L166P}$ mice. Tau, known to mediate Aβ-induced deficits [33], was hyperphosphorylated at Y18 in neurons treated with Aβ dimers and trimers. In aged $APPPS1_{L166P}$ mice, removing both copies of *Prnp* diminished tau hyperphosphorylation by ~40% and missorting by ~65% compared to $APPPS1_{L166P}$x$Prnp^{+/-}$ mice. In contrast, overexpressing PrP^C in $APPPS1_{L166P}$ mice ($APPPS1_{L166P}$xtga20) led to an ~60% increase in tau phosphorylation at Y18 and 80% in tau missorting to the postsynaptic density. Accompanying this apparent potentiation of the PrP^C/Fyn pathway activation in old $APPPS1_{L166P}$xtga20 mice, the expression of postsynaptic but not presynaptic proteins including the postsynaptic scaffold protein PDS-95 was reduced by ~35% adding weight to the suggestion that increasing PrP^C expression was potentiating Aβ dimer-induced toxicity *in vivo*.

The publication of our study was preceded by a few months by a study from the Strittmatter group whom reported that oAβ binds to postsynaptic PrP^C to activate Fyn and impair neuronal function [34]. Here, synthetic oAβ were used as previously described [14] as well as TBS-soluble extracts from individuals diagnosed with AD. Despite using 4 antibodies to identify PrP^C-oAβ complexes (namely, 2454, 82E1, NU-4, and AB5306) on immobilized PrP molecules, the characterization of the species detected with these antibodies in both preparations was not documented thereby hampering our ability to "put clothes on the emperor" to borrow the expression employed by Benilova et al. [12].

Instead, I am convinced that we, as a field, need to dedicate more efforts into better defining what oligomeric amyloid species are employed if we want to leapfrog towards a more comprehensive knowledge of the disease. I think we can do better than describing "a subset of peptide with deleterious actions on neurons and synapses."

A recent study from the Ashe and Lesné groups [32] provides support to the need of distinguishing oligomeric forms of Aβ from each other as opposed to considering them as a pool of molecules triggering the same biological effect. If correct, the findings suggest that the mixture of soluble Aβ species present in the continuum of aging AD is evolving contrasting with the determined mixture of synthetic oAβ preparations. Specifically, Aβ^*56 was most prominent in preclinical phases of AD, Aβ trimers were elevated in early symptomatic phases (i.e., mild-cognitive impairment), and Aβ dimers dominated in late symptomatic phases of AD. If longitudinal studies can confirm these changes, knowing the pathophysiological function of each Aβ oligomer in the brain could be crucial in designing therapeutic interventions. Such vision could be envisioned particularly at a time when personal medicine is emerging and when our population is aging very quickly.

In addition, another important advance in our knowledge of AD will be to decipher where each oligomeric Aβ assembly is coming from, that is, intracellularly or extracellularly [35].

For these reasons, I believe we should encourage better characterization of the soluble forms of Aβ we use experimentally and pursue initiatives to develop new reagents specific to each oligomeric Aβ assembly (which might also allow us to identify the formation and location of Aβ

oligomers *in situ*) in the hope that together we can soon break the code of the Aβ oligomer enigma.

Conflict of Interests

The author has no conflict of interests in relation to this paper.

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Review Article

Gene-Based Antibody Strategies for Prion Diseases

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Prion diseases or transmissible spongiform encephalopathies (TSE) are a group of neurodegenerative and infectious disorders characterized by the conversion of a normal cellular protein PrP^{C} into a pathological abnormally folded form, termed PrP^{Sc}. There are neither available therapies nor diagnostic tools for an early identification of individuals affected by these diseases. New gene-based antibody strategies are emerging as valuable therapeutic tools. Among these, intrabodies are chimeric molecules composed by recombinant antibody fragments fused to intracellular trafficking sequences, aimed at inhibiting, *in vivo*, the function of specific therapeutic targets. The advantage of intrabodies is that they can be selected against a precise epitope of target proteins, including protein-protein interaction sites and cytotoxic conformers (i.e., oligomeric and fibrillar assemblies). Herein, we address and discuss *in vitro* and *in vivo* applications of intrabodies in prion diseases, focussing on their therapeutic potential.

1. Introduction

Prion diseases or transmissible spongiform encephalopathies (TSE) are a group of fatal neurodegenerative disorders comprising Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI) and kuru in humans, chronic wasting disease in cervids, bovine spongiform encephalopathy in cattle, and scrapie in sheep [1]. Prion diseases together with others, as Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD), are also termed conformational or misfolding diseases, because they are characterized by protein misfolding and accumulation of intracellular and/or extracellular aggregates [2]. In the case of TSE, experimental evidence points to the conversion of the normal cellular prion protein (PrP^{C}), into the misfolded and pathogenic form (PrP^{Sc}), as the key event in the pathogenesis [3, 4]. A template-based self-propagating process underlines the generation of infectious prions. Therefore, molecules that interfere with PrP^{C}/PrP^{Sc} conversion and/or neutralization serve as potential therapeutic candidates for prion diseases. Moreover, cellular cofactors, as nonproteinaceous chaperones or RNA, may play a crucial role in the generation of infectious prions and could be considered as additional therapeutic targets [5].

There are as yet no effective treatments for TSE, and immunobased approaches are emerging as important therapeutic strategies against these pathologies [6, 7]. A distinguishing feature of antibodies is that they can be specifically selected against conformational protein species including pathological conformations (misfolded monomers, oligomers, and/or fibrils), which represent major therapeutic targets of misfolding diseases. This unique property makes antibodies promising molecules against prion disease and for conformational disorders in general. Among immunotherapeutic approaches, the gene-based intracellular antibody or intrabody technology is a potentially useful platform for the treatment of neurodegenerative diseases [8].

This approach is based on the expression of chimeric molecules composed of specific recombinant antibodies or antibody fragments fused to intracellular trafficking sequences to be active not only in different subcellular compartments but also in the extracellular milieu [9]. This allows one to target a wide variety of neurotoxic conformers derived from misfolding-prone proteins that are generated in the

cytoplasm (i.e., tau, α-synuclein), the nucleus (i.e., huntingtin), or the secretory compartment, inside or outside the cell (i.e., APP, β-amyloid peptides, prion [10–14]). Due to this definition, we consider intrabodies all recombinant antibody fragments which are engineered for a gene-based immunotherapy protocol. Many intrabodies against proteins involved in the pathogenesis of Alzheimer's, prion, Huntington's, and Parkinson's diseases have been generated and successfully applied in cellular and animal models of these diseases [8, 15]. The availability of effective *in vivo* gene delivery systems into the brain remains a major hurdle for the clinical application of intrabodies, but the growing development of new gene carriers and delivery systems holds great promise for the next future.

2. Intrabodies

Intrabodies are chimeric recombinant antibody fragments engineered to block or modulate the function of target proteins. The approach is a valuable tool to inhibit, *in vivo*, the function of a wide range of selected antigens both at intracellular and extracellular levels [9]. Since the first report in mammalian cells [16], intrabodies have found applications as therapeutics in infectious diseases, in cancer and in neurodegenerative disorders [8, 17, 18]. Unlike other gene-based technologies, they operate at a posttranslational level and can be selected against a precise epitope of target proteins, including protein conformers, protein-protein interaction sites, posttranslational modifications, and also nonprotein antigens.

Antibodies, as most secreted proteins, have a transient hydrophobic leader sequence which directs them through the secretory compartment. By in frame fusion with intracellular trafficking sequences, antibodies have been targeted to the cytoplasm, nucleus, endoplasmic reticulum (ER), plasma membrane [19, 20], and, more recently, also towards the degradative compartment [21]. Examples of targeting signals successfully used for intrabody application are as follows. The cytoplasmic expression of intrabodies is obtained by removal of the leader sequence of secretion (leader-less) and the nuclear targeting by adding to the leader-less antibody fragments one or more nuclear localization sequences (NLS) [22]. A signal peptide of 11 aminoacids, derived from Engrailed homeoprotein, that allows cytoplasmic single chain variable fragments (scFvs) to be secreted in the absence of classical secretion signals, has been recently identified [23]. Fusion of such a nontraditional secretion sequence to an anti-α-synuclein scFv enables the secretion of the intracellular target antigen [24]. Intrabody targeting to the degradative pathways is obtained by addition of a proteasomal targeting signal, such as the PEST motif of the ornithine decarboxylase [21, 25].

In order to prevent the appearance of receptors or resident proteins on the plasma membrane or to inhibit the secretion of selected proteins, ER-retained intrabodies are designed with a leader sequence at the N-terminus and a retention peptide, KDEL, at the C-terminus [22]. Retention in the trans-Golgi is achieved with a trans-Golgi retention signal [26]. Intrabodies targeted to the plasma membrane can be obtained by fusing a single chain antibody fragment with a receptor transmembrane domain [27].

2.1. Choosing an Intrabody Format. The recombinant antibody format more widely used for intrabodies is the single-chain Fv fragment (scFv) and consists of the variable domains of the immunoglobulin heavy (VH) and light (VL) chains linked with a flexible polypeptide which prevents dissociation. Other types of antibody fragments have been successfully engineered and used as intrabodies. These are the recombinant bispecific and tetravalent antibody fragments (intradiabodies) and the single domain fragments [28, 29]. The intradiabodies are made of two scFvs linked through the second and third heavy chain constant domains [30]. Single-domain fragments are composed of one variable domain (such as VL or VH chains) and are the smallest functional antibody fragments expressed as intrabodies [31, 32]. They derive from naturally occurring homodimeric heavy-chain antibodies (VHHs) present in the immune system of camelids and have excellent properties of solubility, stability and expression in mammalian cells. These molecules are easily produced, are much smaller in size, and can be engineered into new reagents with enhanced therapeutic efficacy. Due to their smaller size, they can potentially target cryptic epitopes. Recent studies have demonstrated, by intracarotid and intravenous injections into live mice, that basic VHHs (with an isoelectric point ≥ 9) are capable of crossing the brain blood barrier (BBB) *in vivo* and diffusing into the brain tissue [33]. Interestingly, a camelid antiprion antibody, which abrogates PrP^{Sc} replication in prion infected neuroblastoma cells, is able to transmigrate across the BBB and cross the plasma membrane of neurons, demonstrating a potential use for treatment of prion diseases [34]. Another interesting new recombinant scFv format specifically designed to cross the BBB and directed against the pathologic form of prion has been generated. In this scFv, the linker peptide was substituted with a cell-penetrating peptide (CPP) derived from penetratin. Most of the purified antiprion scFv-CPP intravenously injected was localized in brain cells, demonstrating its capacity to enter the CNS [35].

For clinical applications of intrabodies, generation of humanized and/or human-derived antibody domains offers obvious potential advantages. Improved strategies for *in vitro* selection of fully humanized recombinant antibodies directly from human antibody-display libraries through the creation of large natural or synthetic repertoires of antibody fragments are progressively developing [36, 37].

3. Intrabodies against Prionoses

Intrabodies can be specifically selected against conformational epitopes of amyloidogenic proteins involved in the pathogenesis of misfolding diseases. These conformational intrabodies can inhibit different stages of the aggregation process through (i) stabilization of the native state molecule, (ii) inhibition of the oligomerization process, (iii) neutralization of potentially toxic oligomeric species, (iv) inhibition of fibril formation, and (v) disruption and clearance of preformed aggregates.

It is widely accepted that the cause of prion diseases is the conformational change of the cellular prion protein, PrP^C, from a globular to a protease-resistant β-sheet rich form, PrP^{Sc}. Interaction between these two species raises the conversion rate and leads to generation of potentially infectious particles [4]. Therefore, blocking PrP^C/PrP^{Sc} interaction is a major therapeutic target. Intrabodies can be used to halt this pathological interaction by different modes of action: (a) direct binding to one of the two molecular species [38, 39], (b) trapping PrP^C in the ER [14], and (c) rerouting PrP^C to the proteasome degradation pathway [40]. In particular, rerouting native proteins in precise intracellular locations is a unique property of intrabodies. In the case of misfolding prone antigens, diverting amyloidogenic plasma membrane proteins from the site of aggregation or diverting them to the degradative pathway is an attractive way to block cytotoxicity [41]. It is worth noting that the 37 kDa laminin receptor (LRP/LR) is another potential identified therapeutic target for prion diseases. Thus, LRP/LR has been shown to be a receptor of the pathogenic prion PrP^{Sc} and to play an important role in prion propagation and pathogenesis [42].

3.1. In Vitro Studies. Intrabody applications against prion disorders have been reported by several groups in prion-infected cell culture systems. Our group generated and stably expressed ER-retained antiprion KDEL-8H4 scFv fragments in a neuronal cell line susceptible to scrapie infection. Their intracellular expression causes a marked impairment of prion maturation and translocation towards the membrane compartment, with a strong reduction in membrane PrP^C levels. As a consequence, formation and accumulation of the pathogenic scrapie species, PrP^{Sc}, in 139A prion strain infected cells are impaired [14]. A subsequent *in vivo* study showed that mice, intracerebrally injected with a lysate derived from KDEL-8H4 expressing cells infected with scrapie, neither developed scrapie clinical sign nor brain damage, demonstrating effective treatment [43]. The secretory version of the same intrabody (Sec-8H4), able to recognize PrP^C in the secretory pathway, strongly inhibits PrP^{Sc} accumulation in 139A scrapie strain infected cells. By analysing its mode of action, it was found that PrP^C total level is markedly reduced due to a selective rerouting of PrP^C to the proteasome pathway. Notably, Sec-8H4 intrabody impairs the secretion of endogenous prion molecules associated to exosomes-like vesicles, a potential spreading route for prion infectivity [40]. Drastic reduction of PrP^{Sc} accumulation was also reported by coculturing a human rhabdomyosarcoma cell line secreting antiprion scFv (6H4) fragments with chronically scrapie infected neuroblastoma cells [44]. More recently, other studies demonstrated the inhibitory effect of cell-mediated secretion of antiprion scFv fragments. The antiPrP^C scFvT2, derived from a new mouse monoclonal antibody which recognizes a discontinuous epitope of prion protein, was reported to inhibit scrapie accumulation by co-culturing a neuroblastoma scFvT2-secreting cell line with prion infected cells [45]. Recombinant antiprion 3S9scFv secreted by transfected HEK293T prevented prion accumulation in both 22L prion-infected N2aC24L1-3 and in Chandler prion-infected N2aC24Chm cells in a dose-dependent manner [46].

Another passive immunotherapy approach to treat prion disease has been attempted in cell culture by testing the monovalent version of the antiprion D18scFv through direct addition to scrapie-infected cells or by infection with lentiviral or recombinant adeno-associated viral (rAAV) vectors. Direct addition of D18scFv in scrapie-infected GT1 cells resulted in reduction of proteinase K- (PK-) resistant PrP^{Sc} level in a concentration-dependent manner. By comparing two viral transducing systems, lentiviral vectors were more efficient than rAAV in transferring of the anti-PrP D18scFv gene and in interfering with PrP^{Sc} accumulation in both ScGT1 and ScN2a cells [47].

3.2. In Vivo Applications. To evaluate the therapeutic effect of antiprion intrabodies *in vivo*, recombinant adeno-associated viral vectored scFvs have been applied in prophylactic TSE paradigms. rAAV is considered an ideal delivery vector in gene therapy, especially for transducing neurons in various regions of the brain [48]. The safety profiles of rAAV and its high efficiency of gene transduction have rendered this vector a valuable delivery vehicle for treating brain pathologies, including neurodegenerative disorders. Serotype 2 of rAAV (rAAV2) is one of the most commonly used vectors for brain delivery and is currently under evaluation in rAAV-based phase I/II clinical trials of Parkinson's and Alzheimer's diseases [49–51]. This vector has been employed for *in vivo* application of intrabodies in prion diseases. By using a combinatorial phagemid library of human scFvs, Wuertzer et al. identified four different PrP-specific scFv fragments, evaluated their affinity by surface plasmon resonance analysis, and assessed *in vivo* their therapeutic potential [38]. Mice were initially intracerebrally injected bilaterally into the thalami and striata with rAAV2 antiprion scFvs and, 1 month later, subjected to intraperitoneal inoculation with RML prions. Analysis of disease severity indicated that rAAV2 D18scFv, an antibody fragment specific for the putative region of PrP^C-PrP^{Sc} interaction and with the highest affinity for PrP^C compared to the other scFvs, was the most effective and significantly delayed the onset of clinical signs compared to infected mice in the control group. Although all mice injected with antiprion intrabodies succumbed, rAAV2 D18scFv expressing mice demonstrated significantly extended incubation periods compared to control mice (250 days ± 8 SD versus 199 days ± 1 SD). A significant decrease of PK-resistant PrP^{Sc} burden was also observed both in white matter tracts and gray matter parenchymal regions of the brains injected with this intrabody, 27 weeks after infection, when all parameters of disease severity were assessed. Since it has to be yet established whether the accumulation of misfolded prion correlates with pathological changes and survival, as previously argued [52], the analysis of the amount of PK-resistant PrP^{Sc} at the terminal stage of the disease would be informative. Analysis of the other antiprion scFvs used in this study suggests that there is no correlation between their

affinity and *in vivo* efficacy. However, the specificity of PrP^C epitope recognized by these scFvs should also be considered.

More recently, D18scFv was engineered into the rAAV9 vector for intracerebral injection into scrapie-infected mice [39]. rAAV9 serotype has major advantages with respect to rAAV2 including higher neuronal transduction efficiency, intracerebral diffusion, and trans-BBB neurotropism [53–55]. One month after rAAV9 D18scFv injection, mice were intraperitoneally inoculated with RML prion strain. Behavioural analysis of infected mice showed that bilateral administration of rAAV9 D18scFv into hypothalamus, thalamus, and hippocampus delays the onset of neurological symptoms compared to untreated mice (187 ± 7 days versus 166 ± 5 days). Evaluation of survival time indicated an extended time with respect to control mice, even though this result was not statistically significant. Furthermore, neuropathological assessment at early stage of disease revealed that mice expressing the antiprion scFv have lower levels of spongiosis and gliosis compared to controls, as well as PK-resistant PrP^{Sc}, mostly in the thalamus, hippocampus, and caudate/putamen nuclei. This diminished level of PrP^{Sc} was also evident in mice sacrificed at the terminal stage of disease, when, differently, neuropathological changes were similar both in rAAV9 D18scFv and sham-injected mice. This result corroborates the hypothesis that severity of neuropathology and survival time are not correlated with the amount of scrapie burden, as already suggested [52].

As mentioned before, LRP/LR is a prion receptor and passive immunotransfer of the anti-LRP/LR scFv partly reduces scrapie burden in the spleen [56]. Microinjection of scrapie-infected mice with rAAV serotype 2 vectors encoding for anti-LRP scFv-N3 and -S18 resulted in the reduction of peripheral PrP^{Sc} propagation, without a significant prolongation of incubation times and survival [57]. It is not clear whether the difference in results between rAAV study described by Wuertzer et al. 2008 [38] and this study is due to the different choice of target (PrP^C versus LRP/LR) or different route of infection (intraperitoneal versus intracerebral). The fact that Zuber et al. observed reduction in PrP^{Sc} level not associated with a prolongation of incubation times and survival is a further evidence that scrapie accumulation does not automatically correlate with disease progression and infectivity.

Another interesting study reported the generation, by infection with lentiviral vector, of a stable Ra2 microglial cell line secreting the antiprion 3S9scFv/GFP [46]. In order to evaluate the prophylactic antiprion effect of *ex vivo* gene transfer of 3S9scFv, using brain-engraftable microglial cells, Ra2 cell line was intracerebrally injected at 1 and 3 weeks before brain inoculation of mouse-adapted Chandler prion strain. Analysis of survival time showed that mice injected with 3S9scFv/GFP-Ra2 microglial cells survive longer than corresponding controls expressing GFP alone, although the effect was slight (~10 days). Assessment of PK-resistant PrP^{Sc} levels revealed no differences between antiprion scFv expressing mice and controls. In a therapeutic perspective, 3S9scFv/GFP-Ra2 microglial cells were also intracerebrally injected 7 or 13 weeks after infection with Chandler or 22L scrapie prions. In Chandler scrapie-infected mice, survival times were similar in both experimental groups, while in mice inoculated with 22L prions, there was an effect when 3S9scFv/GFP-Ra2 cells were injected 7 weeks but not 13 weeks after infection. Also in this case, the amount of PK-resistant PrP^{Sc} was not affected by the expression of antiprion scFv. It is noteworthy that authors failed to detect the injected 3S9scFv/GFP-Ra2 cells in the brain of mice at the terminal stage of disease, arguing that the low antiprion effect reported could be ascribed to a short lifetime of this cell line and/or to low scFv expression level.

4. Conclusions

In the last 20 years, several antibodies have entered the biopharmaceuticals market for the treatment and diagnosis of various pathologies, including neurodegenerative diseases. Different *in vitro* studies demonstrated that antiprion antibody fragments prevent prion propagation and scrapie accumulation. Notwithstanding this fact, *in vivo* applications of these antibodies, both as passive immunization and gene-based strategy (intrabodies), failed to demonstrate a complete protection of scrapie infected animal models, even though they were able to reduce cerebral PK-resistant PrP^{Sc} and delay the onset of the disease. One concern is the choice of the therapeutic target molecule (prion monomer, oligomers, PK-sensitive or PK-resistant PrP^{Sc} or LRP/LR). So far, although different intrabodies directed to PrP^{Sc}, and specific oligomeric species have been generated, the *in vivo* therapeutic efficacy still remains to be demonstrated. Another issue is the *in vivo* stability of viral vectored recombinant antibody fragments and/or the downregulation of antibody expression due to neurotoxic effect of PrP^{Sc} on antibody-expressing neurons. Both these factors could significantly decrease the therapeutic efficacy of antiprion intrabodies.

The requirement of more effective and safety *in vivo* gene delivery systems into the brain remains a crucial issue for clinical application of intrabodies against neurodegenerative diseases. Currently, viral vectors (rAAV) are the main choice for *in vivo* delivery of intrabodies, but the growing development of synthetic gene carriers (nanomaterials) or new Trojan-horse approaches hold great promise for developing effective therapeutic antibody delivery strategies against prion diseases.

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Review Article

Identification of Misfolded Proteins in Body Fluids for the Diagnosis of Prion Diseases

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Transmissible spongiform encephalopathy (TSE) or prion diseases are fatal rare neurodegenerative disorders affecting man and animals and caused by a transmissible infectious agent. TSE diseases are characterized by spongiform brain lesions with neuronal loss and the abnormal deposition in the CNS, and to less extent in other tissues, of an insoluble and protease resistant form of the cellular prion protein (PrP^{C}), named PrP^{TSE}. In man, TSE diseases affect usually people over 60 years of age with no evident disease-associated risk factors. In some cases, however, TSE diseases are unequivocally linked to infectious episodes related to the use of prion-contaminated medicines, medical devices, or meat products as in the variant Creutzfeldt-Jakob disease (CJD). Clinical signs occur months or years after infection, and during this silent period PrP^{TSE}, the only reliable marker of infection, is not easily measurable in blood or other accessible tissues or body fluids causing public health concerns. To overcome the limit of PrP^{TSE} detection, several highly sensitive assays have been developed, but attempts to apply these techniques to blood of infected hosts have been unsuccessful or not yet validated. An update on the latest advances for the detection of misfolded prion protein in body fluids is provided.

1. Introduction

There are several forms of Transmissible Spongiform Encephalopathy (TSE) diseases or prion diseases affecting humans and different species of farm and wild animals (i.e., sheep, cattle, and cervids). Some of them have an apparently spontaneous occurrence (i.e., sporadic and genetic TSEs; some forms of atypical bovine spongiform encephalopathy or scrapie), while others are linked to the consumption of prion-contaminated food as in the variant Creutzfeldt-Jakob disease (CJD) [1], feedstuff in bovine spongiform encephalopathy (BSE) [2], or medical and surgical devices in iatrogenic CJD [3]. Transmission of variant CJD via blood transfusion and possibly plasma-derived factor VIII from asymptomatic donors [4] indicates that prion infectivity is present in blood months or years before clinical onset. Thus, the occurrence of epidemics in farm animals and episodes of human cases linked to prion infection pose serious public health issues that are often difficult to solve [5]. An eloquent example is given by the yet unexplained discrepancy between mortality (176 death from 1995 to June 2013) [6] and estimated prevalence data (1 in 4,000 to 1 in 10,000 people) of variant CJD in the British population [7]. This incongruity is causing great concerns because healthy and infected donors who are not promptly identified might transmit disease by blood transfusion, surgical instruments, or plasma-derived products.

The only validated surrogate marker of infection is the abnormally misfolded isoform of the cellular prion protein (PrP^{C}) despite intensive but disappointing search for the identification of other disease-specific biomarkers in easily accessible tissues or body fluids [8, 9]. Misfolded PrP (PrP^{TSE}) accumulates in the CNS and other tissues of infected hosts assuming different conformations that are related to the strain of prions [10]. PrP^{TSE} is easily detected by western blot or immunohistochemistry methods after removing the cellular isoform (PrP^{C}). Most anti-PrP antibodies, in fact, do not distinguish between PrP^{TSE} and PrP^{C} requiring the removal of the cellular isoform for achieving disease-specific signals. This is usually realised by pretreating samples with

proteases (usually proteinase K) that partially digest PrP^{TSE} but completely remove PrP^{C}. The use of proteinase K (PK), however, removes fractions of poorly aggregated misfolded PrP^{TSE} that is usually present in blood [11] and likely other body fluids decreasing the chance of detection. Finally, it is still debated whether PrP^{TSE} is unequivocally associated with prion infectivity as there are occasions in which PrP^{TSE} is either not associated with infectivity [12] or absent in infected hosts [13]. Despite these limits, PrP^{TSE} remains the best available choice for confirming the diagnosis of prion diseases and for the identification of prion-associated infectivity in tissues and body fluids. Moreover, the profile that assumes PrP^{TSE} in western blot, reflecting different pathological conformations, is of great help for making a correct molecular diagnosis of sporadic CJD and for differentiating sporadic from variant CJD [14].

In the last 15 years several methods have been developed for increasing the sensitivity of PrP^{TSE} detection with the aim of finding a reliable assay for an early diagnosis of prion diseases in easily accessible tissues or body fluids. An overview of these developments is the objective of this work.

2. Protein-Misfolding Cyclic Amplification (PMCA)

In 2001, Saborio and colleagues [15] developed a novel protocol for the in vitro amplification of the misfolded prion protein based on the principle that disaggregated PrP^{TSE} incubated in the presence of a large excess of PrP^{C} produces novel PrP^{TSE}. Disaggregation of fibrils requires a sonication step, which can be repeated several times, in a cyclic process, to allow sensitive detection of the misfolded PrP of the original seed. The protein-misfolding cyclic amplification (PMCA) was originally developed using hamster brain homogenate and has since been shown to be an efficient method for the amplification of brain PrP^{TSE} of other species including mouse, sheep, cattle, bank voles, cervids, and humans [16–23]. In human samples, the amplification of PrP^{TSE} is strongly influenced by the correct matching of methionine/valine in the 129 residue of PrP, suggesting that this polymorphic site of the protein is important for the amplification of PrP misfolding by the PMCA assay [24–26].

Ten cycles of sonication are sufficient to increase the sensitivity of standard western blots from 6–12 picograms to 0.3–0.5 picograms of brain PrP^{TSE} and, with an improved automated protocol which enables a substantial increase in the number of amplification cycles, up to femtogram levels [27]. PMCA is therefore a promising platform for prion diagnosis in body fluids (blood, urine, and CSF) where the level of PrP^{TSE} is estimated in the range of picograms per mL.

The group led by Soto reported the first successful identification of PrP^{TSE} in blood (buffy coat) of scrapie affected hamsters with 89% sensitivity and 100% specificity [27] and positive signals in 50% of samples taken in the preclinical stage of disease as early as 20 days after intraperitoneal 263K scrapie injection [28]. The detection of PrP^{TSE} in blood of preclinical scrapie-infected hamsters is consistent with data on infectivity detection in blood [8].

Since then, automated PMCA revealed the presence of PrP^{TSE} in plasma fractions [29], urine [29–31], and cerebral spinal fluid (CSF) [32] of scrapie-diseased hamsters with sensitivity ranging from 50 (plasma) to 100 percent (CSF) (Table 1). In the CSF samples from scrapie-infected hamsters, PMCA was performed by using a further improved protocol (rPrP-PMCA) in which PrP^{C} was replaced by recombinant PrP (recPrP), allowing a sensitivity greater than that observed with previous PMCA protocols [32].

Other than in hamster models, PrP^{TSE} was amplified from blood leukocytes of both naturally [20, 34] and experimentally scrapie-infected sheep [33] where PrP^{TSE} bands were detected as early as 90 days postinoculation and correlated with infectivity titres [33]. On leukocytes of naturally scrapie-infected sheep, PrP^{TSE} was detected in all tested animals with 100% specificity by using an enhanced (i.e., addition of poly-A PMCA) protocol [20].

Attempts to detect PrP^{TSE} in blood of other species such as cattle with BSE and cervids with CWD produced negative or controversial results [34, 37, 58]. In patients with various forms of prion diseases, the detection of PrP^{TSE} by PMCA was not attempted (or results were not published) in sample of blood, blood derivatives, plasma, urine, or CSF despite amplification of PrP^{TSE} was successfully reported in human brain samples taken from both sporadic and variant CJDs [16, 24–26].

Finally, PMCA amplification of PrP^{TSE} in samples from body fluids, other than blood, taken from prion-infected hosts was successfully achieved in a variety of species and included saliva and urine in sheep with scrapie [36, 36]; saliva, urine, and CSF in cervids with CWD [38, 58]; and CSF and saliva in cattle with BSE [37]. A list of prion-infected body fluids analysed by PMCA with the obtained sensitivity and specificity is shown in Table 1.

In conclusion, PMCA has certainly been a breakthrough for detection of minute amount of PrP^{TSE} that are likely present in body fluids and therefore is a candidate method for developing sensitive tests for the diagnosis of prion diseases in animals and humans. Moreover, the amplified product of PMCA retains the PrP^{TSE} signature of the original seed allowing the molecular diagnosis of CJD in humans and scrapie in sheep with important public health implications. In the last 10 years, PMCA has frequently been modified by addition of poly-A [20] or sulfated dextrans [37], by the use of recombinant PrP instead of brain PrP^{C} [32], or by coupling with sensitive immunoassays [34] that have on one side improved the sensitivity of PrP^{TSE} detection but, on the other hand, made the comparison of data produced by different laboratories difficult. PMCA coamplifies infectivity together with PrP^{TSE} [59, 60] mimicking the disease-specific pathogenic event but requiring safety precautions in diagnostic laboratories. Finally, PrP^{TSE} bands may appear in control preparations after several PMCA cycles [61]. This finding, whether related to *de novo* formation of PrP^{TSE} [20, 60, 61] or cross-contamination of samples [22], raised concern

Table 1: Detection of misfolded PrP in body fluids.

Assay[1]	Species	Body fluid	Assay sensitivity (brain dilution)[2]	Inoculation route	Time (dpi)	Starting volume (mL)	Number of tested samples (treated/controls)	Sensitivity (%)	Specificity (%)	Reference
PMCA	*Hamster*	*Blood (bc)*	10^{-12} (fg)	ip	14	1	5/5	0	100	[28]
					20	1	6/4	50	100	
					40	1	10/5	60	100	
					60	1	5/4	40	100	
					70	1	5/5	20	100	
					80	1	5/5	0	100	
					90 (c.s.)	1	10/10	80	100	
			10^{-12} (fg)	ic	68 (c.s.)	1	18/12	89	100	[27]
			10^{-12} (fg)	ic	60	1.5	4/3	100	100	[29]
					68 (c.s.)	1.5	3/3	100	100	
		Blood (plasma)	10^{-12} (fg)	ic	60	1.5	4/5	50	100	[29]
					68 (c.s.)	1.5	4/5	67	100	
		Urine	10^{-12} (fg)	ic	68 (c.s.)	0.02	7/4	86	100	[29]
				oi	155 (c.s.)	0.02	7/4	67	100	
		CSF	(—)	ip	90 (c.s.)	(—)	5/5	80	100	[30]
			(ag)	ic	68 (c.s.)	0.002	6/14	100	100	[32]
		Blood (wbc)	10^{-8}	oi	30	10^{4}	1/2	0	100	[33]
					60	10^{+4} (cells)	1/2	0	100	
					90	10^{+4} (cells)	1/2	100	100	
					130	10^{+4} (cells)	1/2	100	100	
					190 (c.s.)	10^{+4} (cells)	1/2	100	100	
	Sheep		10^{-9}	ni	c.s.	10^{+7} (cells)	10/8	100	100[3]	[20]
		Blood (plasma)	10^{-8}	ni/oi[4]	b.c.s.	0.5	6/7	100	100	[34]
					c.s.	0.5	9/7	100	100	
		Urine	10^{-8}	ni/oi[4]	c.s.	0.5	4/4	100	100	[35]
		Saliva	(fg)	ni	360	(—)	12/20	100	88	[36]
	Cattle	*Saliva*	10^{-11} (fg)	oi	c.s.	0.005	3/1	33	100	[37]
		CSF	10^{-11} (fg)	oi	c.s.	0.005	3/1	33	100	
	Cervids	*Blood (plasma)*	10^{-8}	oi	b.c.s.	0.5	2/1	100	100	[34]
					c.s.	0.5	3/1	100	100	
		Urine	10^{-8}	oi	c.s.	0.5	5/2	100	100	[35]
		CSF	10^{-5}	ni	c.s.	0.025	4/31	75	100	[38]

Table 1: Continued.

Assay[1]	Species	Body fluid	Assay sensitivity (brain dilution)[2]	Inoculation route	Time (dpi)	Starting volume (mL)	Number of tested samples (treated/controls)	Sensitivity (%)	Specificity (%)	Reference
QUIC	*Hamster*	*Blood (plasma)*	4×10^{-14} (ag)[5]	ic	10	0.5	1/11	100	100	[39]
					30	0.5	3/11	100	100	
					80 (c.s.)	0.5	9/11	100	100	
		CSF	10^{-9}	ic	c.s.	0.002	2/2	100	100	[40]
		Nasal lavage	10^{-9}	ic	c.s.	0.004	8/2	100	100	[40]
	Sheep	*CSF*	(fg)	ni	c.s.	0.005	1/1	100	100	[41]
		Spiked vCJD blood (plasma)	4×10^{-14} (ag)[5]	ni	c.s.	0.5	4/4	100	100	[39]
	Human	*sCJD CSF*	10^{-9} (fg)[6]	ni	c.s.	0.005	16/14	87.5	100	[42]
			5×10^{-6} (0.1 pg)	ni	c.s.	0.015	67/51	87	100	[43]
		gCJD CSF (E200K)	10^{-9} (fg)[6]	ni	c.s.	0.005	22/1	87	100	[44]
		gCJD CSF (V203I)	10^{-9} (fg)[6]				2/1	100	100	
		GSS CSF	10^{-9} (fg)[6]				20/1	78	100	
		FFI CSF	10^{-9} (fg)[6]				12/1	100	100	
ICE	*Human*	*CJD Blood (bc)*	(—)	ni	c.s.	10	9/6	55	100	[45]
sFIDA	*Sheep*	*Blood (plasma)*	(—)	ni	c.s.	(—)	10/5	100	100	[46]
	Cattle	*CSF*	(—)	oi	c.s.	0.02	6/6	50	100	[47]
Ligand-based IA	*Sheep*	*Blood (wbc)*	(—)	ni	b.c.s.	5	23/129	56	100	[48]
		Blood (wbc)	(—)	ni	c.s.	5	80/129	55	100	
		Blood (wbc)	(—)	oi	c.s.	5	7/129	71	100	
Metal matrix	*Human*	*vCJD blood*	$10^{-10(5)}$	ni	c.s.	(—)	21/190	70	100	[49]
		sCJD blood					27/190	0	100	
EP-vCJD	*Human*	*Spiked vCJD blood*	$5 \times 10^{-7(5)}$	ni	c.s.	(—)	nd/1000	100	100	[50]
		vCJD blood		ni	c.s.	(—)	3/20000	0	98	[51–53]
CDI	*Human*	*vCJD blood*	$10^{-5(5)}$	ni	c.s.	(—)	7/4	100	100	[54]
		sCJD blood (wbc)	0.5 pg	ni	c.s.	10^{+6} cells	24/27	0	100	[55]
MPD	*Sheep*	*Blood (serum)*	1 ID/mL[7]	ni	c.s.	0.2	13/1	100	100	[54]
	Primates	*Blood (plasma)*		ni	c.s.	0.2	8/4	100	100	[56]
	Human	*sCJD Blood (plasma)*		ni	c.s.	0.2	5/5	100	100	
MDS	*Hamster*	*Blood (plasma)*	10^{-8}	(—)	c.s.	0.078	9/9	100	100	[57]

[1]The assay specification does not include protocol variations.

[2]Assay sensitivity is indicated as the end point dilution of scrapie brain homogenates. In brackets, estimates of the PrP^{TSE} amount provided by the authors.

[3]Spontaneous formation of misfolded PrP was reported in some control samples after increased cycles of sonication.

[4]Authors used samples from both naturally and experimentally infected sheep.

[5]Dilution of variant CJD brain into healthy human blood.

[6]Dilution of sporadic CJD into artificial CSF.

[7]Measured on previously titered terminal plasma pool from mice experimentally infected with the Fukuoka-1 strain of GSS.

dpi: days postinoculation; fg: femtograms; ag: attograms; bc: buffy coat; wbc: white blood cells; c.s.: clinical symptoms; b.c.s.: before clinical symptoms; ic: intracerebral; ip: intraperitoneal; oi: oral inoculation; ni: natural infection; CSF: cerebral spinal fluid; CJD: Creutzfeldt-Jakob disease; GSS: Gerstmann-Sträussler-Scheinker syndrome; FFI: fatal familial insomnia.

for the reliability of PMCA in diagnostic applications. This inconvenience, however, is easily settled by using low PMCA cycles and appropriate technical tips to avoid possible prion contamination [22].

3. Quacking Induced Conversion (QuIC)

A spin-off of the PMCA method was obtained by substituting sonication with automated tube shaking for the conversion of recPrP substrates [62]. The novel "quacking induced conversion" (QuIC) protocol enables the amplification of 1 femtogram of PrP^{TSE} of scrapie hamster brain homogenate within one day, reducing the complexity and timing of misfolding amplification. Hamster recPrP promotes the conversion of brain misfolded proteins of other species such as sheep with scrapie and humans with sporadic and variant CJDs, regardless of the primary sequence of the PrP^{TSE} seed [41]. Some spontaneous PK-resistant fragments of less than 12 kD are occasionally observed in unseeded control samples [32], but they wane out by reducing the incubation time of the reaction [41].

One of the most significant improvements of misfolding amplification methods was achieved when western blots were replaced by a real-time fluorescent colour reaction (real-time QuIC) [40, 42]. This novel read-out system, based on a fluorescent amyloid-sensitive thioflavin dye (ThT) [63], allowed the implementation of the whole QuIC procedure to a high-throughput 96-well format. The real-time QuIC (RT-QuIC) is an efficient quantitative method for the detection of minute amount of PrP^{TSE} with estimates of the 50% seeding dose (SD_{50}) of hamster scrapie brain in the same order of magnitude of infectious doses (LD_{50}) [40].

RT-QuIC protocol has been adapted to the detection of brain PrP^{TSE} of other species such as CWD-infected deer, scrapie-infected sheep, and sporadic CJD patients by using species-specific recPrP [40, 42, 64]. Full-length human recPrP and both truncated and full-length hamster recPrPs are efficient substrates for the amplification of PrP^{TSE} in sporadic CJD brain irrespective of the 129 codon phenotypes [43, 64]. A note of disappointment is that the efficacy of variant CJD brain in seeding RT-QuIC reaction is consistently lower than sporadic CJD samples [64].

The presence of PrP^{TSE} in the CSF by the QuIC assay was initially revealed by Atarashi and colleagues [62] in 263K-scrapie infected hamsters and Orrú and colleagues [41] in scrapie-infected sheep. In 2010, Wilham and colleagues [40] revealed the presence of PrP^{TSE} in the CSF of 263K scrapie-infected hamsters by RT-QuIC and estimated a titre of about 10^{-2} SD_{50} per μL. CSF samples from control animals did not revealed any presence of PrP^{TSE} indicating a high specificity of the assay. These encouraging results on the CSF of scrapie-infected host promoted further studies in patients with various forms of prion diseases. A blinded experiment was initially performed on 30 CSF samples of definite sporadic CJD patients provided by the Australian National CJD Registry and 155 controls (25 suspected CJD cases and 130 neurological controls) achieving 87.5% specificity and 100% sensitivity. CJD cases were positive irrespectively of 129 codon genotypes [42]. Similarly, McGuire and colleagues [43] screened CSF samples from sporadic CJD patients provided by the National Creutzfeldt-Jakob Disease Research & Surveillance Unit, Edinburgh, UK, including all three 129-codon genotype and obtaining 99% specificity and 94% sensitivity. In the same study, the specificity and sensitivity of the 14-3-3 protein, a surrogate marker currently used for the diagnosis of sporadic CJD, were 65% and 94%, respectively. An example of the RT-QuIC output in the CSF of a sporadic CJD patient is given in Figure 1.

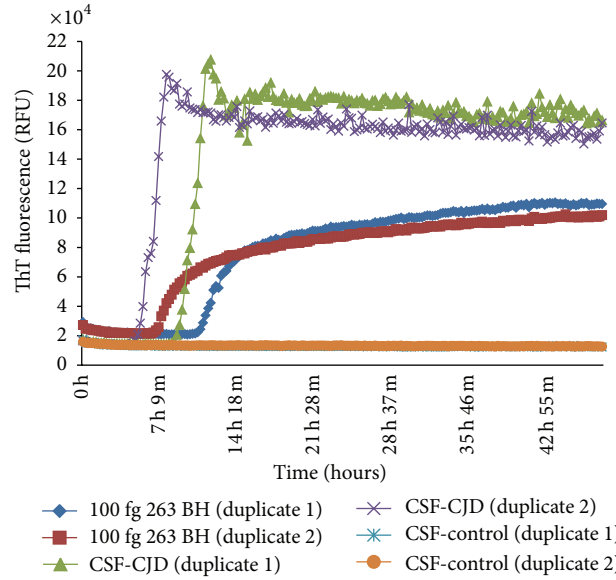

Figure 1: RT-QuIC reactions seeded with 15 μL of human CSF samples from one Italian sporadic CJD patient (12076) and one non-CJD control (13004). 100 fg of 263K prion-infected hamster brain homogenate were used to seed positive control reactions. Each sample was processed in duplicate.

Finally, Sano and colleagues [44] reported that the RT-QuIC assay on the CSF of patients with genetic prion diseases has 78% sensitivity in GSS, 100% in FFI, 87% in E200K genetic CJD, and 100% in V203I genetic CJD, suggesting that the RT-QuIC assay for the detection of PrP^{TSE} in the CSF might become a valid method for improving the diagnosis of patients with a clinical suspicion of human prion disease.

Besides CSF, the RT-QuIC assay revealed PrP^{TSE} in nasal lavages from hamsters infected with the transmissible mink encephalopathy (TME) hyper strain [40] and, by using immunoaffinity beads coupled with the conformational 15B3 anti- PrP^{TSE} antibody (enhanced QuIC), in plasma of scrapie-infected hamsters [39]. The assay showed 100% sensitivity and specificity and was able to detect a positive signal long before the appearance of clinical signs of scrapie.

Finally, the application of 15B3-conjugated beads to the QuIC protocol and the use of a hamster-sheep chimeric recPrP as substrate in the reaction increased the sensitivity (up to attogram levels) and the speed of detection (28 hrs) of variant CJD brain misfolded proteins spiked into human blood [39]. Despite this good achievement there is still no

report on the use of the enhanced QuIC assay in human blood.

Overall, RT-QuIC methodology is a powerful platform for the detection and large-scale screening of misfolded PrP in both human and animals. Up to attogram levels of misfolded PrP can be detected and properly quantified within few hours by using high-throughput 96-well formats. The high levels of specificity obtained in a variety of tissues and species by using flexible recombinant substrates demonstrates the versatility of the novel method. It is of note that RT-QuIC PK-resistant products are reported to be noninfectious (quoted by [43]) and therefore likely more secure in large-scale screening diagnostic procedures. The two disadvantages of this assay are the relatively poor performance in amplifying PrP^{TSE} from variant CJD tissues [64] and the failure to reproduce the original PrP^{TSE} signature impeding the molecular diagnosis of sporadic CJD and the distinction between sporadic and variant CJDs.

4. Other Potential Assays for the Detection of Misfolded PrP in Blood

4.1. Immunocapillary Electrophoresis (ICE). The assay, originally developed by Schmerr and colleagues [65] and based on a competitive immunoassay with PrP fluorescent peptides, was soon proven efficient for the detection of PrP^{TSE} in blood of scrapie-infected sheep and elks with CWD [66]. However, these results were not confirmed in other laboratories using blood samples from CJD-infected chimpanzees or sporadic, iatrogenic, genetic, and variant CJD patients [45, 67]. It is therefore unlikely that this assay will be of any use for the diagnosis of human prion diseases.

4.2. Surface Fluorescence Intensity Distribution Analysis (Surface-FIDA). This assay consists in the immobilization of single PrP aggregates on a capture antibody coated surface that are then visualized by the concomitant binding with two anti-PrP fluorescent antibodies and a double-laser beam scanning system (surface-FIDA). The method discriminates aggregated PrP forms from monomeric PrP without the use of the proteinase K (PK) digestion step and therefore recognizes both PK-resistant and PK-sensitive PrP^{TSE}. Surface-FIDA enabled the counting of bovine and hamster PrP aggregates in brain homogenates and in bovine cerebrospinal fluid [47]. PrP aggregates were also blind-detected in blood of scrapie-infected sheep ($n = 15$) with high specificity and sensitivity [46], although it remains unsettle whether the detection of PrP aggregates correlates with infectivity. It is of note that spiking of blood plasma with PrP^{TSE} from brain was unsuccessful suggesting that the properties of PrP^{TSE} from brain are different from endogenous blood misfolded PrP [46].

4.3. Ligand-Based Immunoassay. Terry and colleagues [48] reported the detection of PrP^{TSE} in 55% of blood mononuclear cells (PBMC) obtained from scrapie-affected sheep ($n = 80$) and 71% of experimentally BSE-affected sheep by a modified polyanionic ligand assay of the IDEXX HerdCheck methodology [68]. The assay resulted positive also in a subset of scrapie-infected sheep several months before the onset of clinical signs suggesting that PrP^{TSE} can be detected in asymptomatic prion-infected hosts. However, the relatively low sensitivity observed in prion-infected sheep, the long timings of sample preparations, and the amount of blood volumes required for the purification of PBMC foretell that this assay would not be easily applicable to large-scale diagnostic scopes.

4.4. Solid-State Binding Matrix. The assay, based on the affinity that PrP^{TSE} has for stainless steel particles [69, 70], was adapted for the detection of misfolded PrP in blood of patients with various forms of CJDs [49]. The selective absorption of PrP^{TSE} on the metal matrix concentrates misfolded protein up to the point that the signal can be detected by an ELISA assay. Because of the selectivity of the metal matrix in binding only misfolded PrP, there is no need to pretreat samples with PK that likely removes a conspicuous fraction of PrP^{TSE} in blood. This method was initially tested on human blood spiked with vCJD brain homogenate where misfolded particles in up to the 10^{-10} brain dilution were detected. Subsequently, blood of variant and sporadic CJD patients was analysed on a blinded experiment including samples from patients with other neurological diseases and controls. Only samples that were reactive in two separate assays were scored as positive. About two-third of blood samples from variant CJD but none from sporadic CJD patients and neurological or nonneurological controls yield positive signals in both assays resulting in 100% specificity for variant CJD [49].

4.5. EP-vCJD Blood Screening Assay. In 2003, Paramithiotis and colleagues [71] reported the manufacture of an antibody directed against PrP epitopes that are exposed only upon protein misfolding and therefore specific to PrP^{TSE}. This conformational anti-PrP^{TSE} antibody was then used for the epitope-protection (EP) vCJD-screening assay, which was later implemented by Amorfix. The high-throughput assay achieved 100% sensitivity and specificity on 1,000 blinded human plasma samples, which included samples that were spiked with variant CJD-infected and normal brains [50]. In 2009, the specificity of the method was ascertained on a large-scale screening initiated in France in over 20,000 human blood samples [51]. Results showed that on the first run 486 samples were positive (97.6% specificity), 20 of which were then confirmed positive on a second screening [51]. The repeat-reactive samples were finally considered negative on a third screening [51]. Subsequently, Amorfix tested three variant CJD blood samples provided by the National Institute for Biological Standards and Control (NIBSC, UK) that resulted negative [52]. The sensitivity of the test was therefore further improved for the detection of 1 : 5,000,000 dilution of variant CJD-infected brain spiked into blood [52]. However, despite this enhanced sensitivity, the test was still unable to detect prions in blood of variant CJD patients, and it was

finally concluded that more research is required before the reevaluation of the assay [53].

4.6. Conformation-Dependent Immunoassay (CDI). In 1998, Safar and colleagues [10] developed an ELISA-formatted, dissociation-enhanced time-resolved fluorescence detection system based on specific antibody binding to epitopes that are accessible in PrP^{C} but that are unmasked only in denatured PrP^{TSE}. This method does not require PK treatment and is able to recognize both sensitive and resistant PK misfolded proteins and different PrP^{TSE} conformations. The assay, improved by incorporating a capture antibody, was able to discriminate PrP^{TSE} signature in different molecular forms of sporadic CJDs, iatrogenic CJDs and genetic TSEs [72] and detect up to a 10^{-5} dilution of PrP^{TSE} from variant CJD brain used for spiking human normal plasma [54, 73]. However, endogenous PrP^{TSE} was undetectable in white blood cells of sporadic patients by CDI [55], but we are not aware of its use in variant CJD blood.

4.7. Misfolded Protein Diagnostic Assay (MPD). This technique is based on a pyrene-labeled palindromic sequence of prion peptides that converts to β-sheets in the presence of PrP^{TSE} [56, 74]. This process induces an excimeric signal from the conjugated pyrenes that propagates to other peptides with the final goal to amplify the PrP^{TSE} signal. MPD assay detects PrP^{TSE} in brain of 263K scrapie-infected hamsters during the preclinical and clinical stages of disease [74] and in small volumes of plasma from prion-infected mice and sheep with sensitivity up to 1 infectious dose per mL [56]. The same assay discriminated in blinded small-scale experiments control plasma from that of patients with sporadic CJD and squirrel monkeys with experimental CJD with 100% specificity and sensitivity [56].

4.8. Multimer Detection System (MDS). This technique is a modified ELISA assay that recognizes only multimeric forms of PrP^{TSE} without using any pretreatment with proteinases, which might remove PrP^{TSE} forms likely present in body fluids [57]. This assay uses the same principle previously described by Pan and colleagues [75] and is based on the use of two monoclonal antibodies that share overlapping epitopes. Monomers (PrP^{C}) are captured by an antibody attached to the surface of a plate and are not detected by the second antibody due to the absence of any exposed epitopes. On the other hand, multimers (PrP^{TSE}) are easily recognized by the second antibody because they expose more copies of the same epitope. The assay was tested on plasma samples of nine scrapie-infected and nine control hamsters resulting in 100% specificity and sensitivity [57]. This simple assay, however, requires validation in other laboratories and more basic work for determining whether the multimeric forms detected by the MDS assay are related to infectivity.

5. Final Remarks

It is unquestionable that in the last 15 years there has been an outstanding progress in improving the detection of PrP^{TSE} for developing sensitive and specific diagnostic assays. These sophisticated and highly sensitive methods successfully detect up to attogram levels of PrP^{TSE} in body fluids of different species (Table 1). A major breakthrough is the development of the RT-QuIC technology for the detection of PrP^{TSE} in the CSF of patients with sporadic [43, 64] and genetic [44] TSEs that as soon as is validated by other groups will change the diagnostic criteria of human prion diseases.

Endogenous PrP^{TSE} has been identified in blood of scrapie-infected hamster by PMCA [27, 28] and RT-QuIC [39] assays and of patients with variant CJD by the solid-state binding matrix assay [49]. Despite these successful observations, however, there are no published reports on the application of either PMCA or enhanced RT-QuIC on blood samples of patients with any form of prion diseases suggesting that both assays still need substantial improvement before their use in the diagnostic setting. Although the development of the RT-QuIC technology for the detection of PrP^{TSE} in blood samples is more recent than PMCA, an extra impediment of the RT-QuIC assay might come from the interference of blood molecules with the ThT reading. On the other hand, the solid-state binding matrix assay might be a valid alternative for the development of a blood test for variant CJD, but the relative low sensitivity (71%) and the finding that some control samples resulted positive in one of the two runs [49] make the use of this assay a remote ambition.

What remains elusive is the reproducible detection of endogenous PrP^{TSE} in blood despite the successful identification of minute amounts of spiked brain PrP^{TSE} into healthy blood. It becomes more and more evident that the properties of PrP^{TSE} in brain are different from those in blood and that some components of blood both inhibit and interfere with PrP^{TSE} detection causing false positive and negative results and compromising the reproducibility of the assay [46, 51, 76]. A clear example is given by the failure of the EP-vCJD assay that had excellent and reproducible performances on spiked blood but then completely failed to identify positive and negative human blood samples [50–53].

These findings pose the question on whether the criteria delineated by the National Institute for Biological Standards and Control (NIBSC, UK) [77] for prion diagnostic assay validation in terms of satisfactory sensitivity and specificity on spiked blood and for the request of variant CJD blood samples are still relevant for defining the best condition of success of potential prion test in blood.

We think that the principles for assay validation and accessibility to variant CJD blood samples should rather focus on reproducible and large scale blinded studies on blood taken from animal models of prion diseases, such as scrapie or BSE in sheep followed by a large scale screening of healthy blood donors to ascertain a sufficient level of specificity.

Finally, our impression is that the research on prion detection in blood does not really need further sensitive assays

but rather requires further work aiming to the identification of interfering blood components and understanding prion metabolism in blood.

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Review Article

Disulfide Bonding in Neurodegenerative Misfolding Diseases

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In recent years an increasing number of neurodegenerative diseases has been linked to the misfolding of a specific protein and its subsequent accumulation into aggregated species, often toxic to the cell. Of all the factors that affect the behavior of these proteins, disulfide bonds are likely to be important, being very conserved in protein sequences and being the enzymes devoted to their formation among the most conserved machineries in mammals. Their crucial role in the folding and in the function of a big fraction of the human proteome is well established. The role of disulfide bonding in preventing and managing protein misfolding and aggregation is currently under investigation. New insights into their involvement in neurodegenerative diseases, their effect on the process of protein misfolding and aggregation, and into the role of the cellular machineries devoted to disulfide bond formation in neurodegenerative diseases are emerging. These studies mark a step forward in the comprehension of the biological base of neurodegenerative disorders and highlight the numerous questions that still remain open.

1. Introduction

Neurodegenerative misfolding diseases (NMD) are a group of diseases involving the misfolding of one or two proteins and their accumulation into aggregated species toxic to neurons, leading to a wide range of neurological symptoms (Table 1). Among these are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), prion-related disorders (PrDs), and amyotrophic lateral sclerosis (ALS). In each case a specific protein loses its functional structure to populate partially unfolded species that reorganize themselves into polymeric structures with different degrees of ordered structure, from oligomers to amyloid fibrils [1].

The fate of a protein depends on two major factors, its sequence and its cellular environment. From the sequence perspective, many studies have identified several features of the amino acid *sequence* of a protein that help predict its aggregation behavior, such as charge, hydrophobicity, patterns of polar and nonpolar residues, and tendency to form secondary structures [2, 3]. After peptide bond, the disulfide bond is the most common covalent link between amino acids in proteins. Disulfide bonds are known to stabilize proteins thermodynamically by decreasing the entropy of the unfolded state, to increase mechanical stability and to confine conformational changes [4]. From the cellular environment point of view, instead, in order to be formed, disulfide bonds need a highly efficient network of *enzymes* in particular cellular compartments, such as protein disulfide isomerases (PDIs), whose role has been revealed as central in many neurodegenerative disorders, being upregulated in many NMD and in some cases interacting directly with misfolded and aggregated proteins [5, 6].

Disulfide bonds are present in 15% of the human proteome and they are enriched in secreted proteins (65%), due to the need of greater protein stability in the absence of the accurate quality control systems present inside the cell. Interestingly they are present in 55% of the proteins involved in pathologic amyloid formation [7], suggesting an important role of disulfide bonds in the kinetics of aggregation and in the structure and toxicity of the formed aggregates. Do disulfide bonds have a role in neurodegenerative misfolding disorders? The analysis of a list of proteins that misfold and aggregate in neurodegenerative diseases (Table 1) revealed that only 25% of the proteins listed have disulfide bonds, a value closer to the general presence of disulfide bonds in the proteome (15%).

This distribution of disulfide bonds presence in proteins involved in misfolding diseases (Figure 1) shows that while

Table 1: Presence of disulfide bonds in proteins involved in neurodegenerative misfolding diseases.

Neurodegenerative disease	Protein	Cellular localization	Localization of deposits	Presence of SS
Prion-related disorders	Prion	Membrane-bound	Extra- and intracellular	Yes
Amyotrophic lateral sclerosis	SOD1	Cytosol	Intracellular	Yes
	TDP-43	Cytosol/nucleus	Intracellular	No
	FUS	Cytosol/nucleus	Intracellular	No
Alzheimer's disease (Tauopathies)	Aβ	Extracellular	Extracellular	No
	Tau	Cytosol	Intracellular	Yes
Parkinson's disease	Synuclein	Cytosolic, membrane-bound	Intracellular	No
	Synphilin-1	Cytoplasm	Intracellular	No
Huntington's disease	HTT	Cytosol	Intracellular	No
Spinal and bulbar muscular atrophy X-linked 1	Androgen receptor	Cytosol/nucleus	Intracellular	No
Spinocerebellar ataxias	Ataxins	Cytosol/nucleus	Intracellular	No

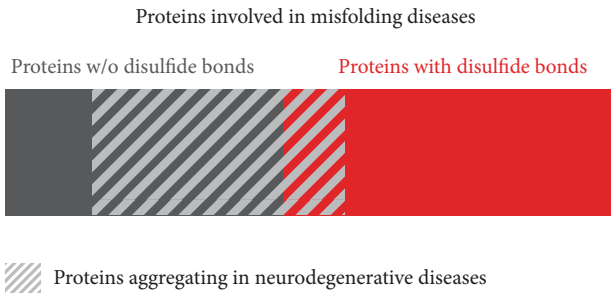

Figure 1: Disulfide presence (red) in proteins involved in misfolding diseases (solid) and in neurodegenerative misfolding diseases (patterned).

proteins without disulfide bonds mainly aggregate in and impair neurons, proteins with disulfide bonds are mostly involved in systemic pathologies. Do neurons produce less disulfide-bonded proteins? Are neurons more sensitive to intracellular aggregates formed by cytosolic proteins (mostly disulfide free) or to the stress caused by their presence? Interestingly, a well-known cytosolic and neurotoxic protein (huntingtin fragment with extended polyglutamine repeat) has been expressed in the endoplasmic reticulum, and surprisingly it did not form aggregates [8]. The authors suggest that polyglutamine aggregation is a property restricted to the nucleocytosolic compartment and that compartment-specific cofactors promoting or preventing the aggregation of pathological proteins could exist. Is there any difference in the aggregation and cytotoxic behavior of cytosolic and secreted proteins?

The questions about protein aggregation, disulfide bonds, and neurotoxicity are numerous, and the proposed answers are often controversial. Here we focus on two simple aspects of disulfide bonds and neurodegenerative diseases: the role of disulfide bonds in the stabilization of proteins involved in aggregation and neurodegeneration and the involvement of PDIs in neurodegenerative misfolding diseases.

2. Disulfide Bonds in Proteins Aggregated in Neurodegenerative Diseases

Proteins have evolved several structural and sequence-based strategies to avoid misfolding and potentially toxic aggregation such as stabilizing protein folding, controlling aggregation by gatekeepers residues, limiting β-propensity, hydrophobicity, and net charge [9].

Many studies on the effects of disulfide bonds on protein misfolding and aggregation revealed that (i) some disulfide bonds are positioned to prevent the population of aggregation prone conformation of some proteins (insulin, IAPP, β-lactoglobulin, and lysozyme) [3, 7, 10], (ii) disulfide may promote specificity in intermolecular association occurring by domain swapping [11], (iii) considering large protein structure datasets, regions of proteins involved in protein-protein interactions, and therefore often aggregation prone, was observed to be enriched in disulfide bonds [12], and (iv) in the human proteome, disulfide bonds are associated with sequences (both intra- and extracellular) of higher aggregation propensity [7]. These observations suggest that disulfide bonds have coevolved with protein sequences to minimize protein misfolding and propensity to form potentially toxic aggregates.

The involvement of disulfide bonds in proteins involved in neurodegenerative misfolding diseases is limited to the few proteins that have them, that is, prion, SOD1, and Tau. Nonetheless, the study of the role of disulfide bonds in protein stability and in the aggregation process has not led to a definitive scenario. Here, an overview of the open questions about the involvement of disulfide bonds in each one of the tree proteins involved in NMD is presented.

2.1. Prions. Prion-related disorders are neurodegenerative diseases characterized by the conversion of the prion protein PrP from its normal cellular structure (PrP^C) to a highly β-sheet, protease-resistant, scrapie conformation (PrP^{Sc}). The only PrP disulfide bond (Cys179–Cys214) stabilizes

the overall fold of the protein by connecting the two long C-terminal helices [13]. Whether the presence of the intact disulfide bond is required or not [14, 15] for aggregation, whether disulfide reshuffling occurs during PrP^{C} to PrP^{Sc} conversion *in vivo,* and whether intermolecular disulfide bonds play a role in stabilizing PrP^{Sc} aggregates [16] are still subject of some controversy [17].

2.2. SOD1. Amyotrophic lateral sclerosis (ALS) is the most common degenerative disease of the motor neuron system. The cause of ALS is unknown although 5–10% of cases are familial with a Mendelian pattern of inheritance [18]. About 20% of familial ALS cases share a mutation in the copper/zinc superoxide dismutase 1 (SOD1) gene [19]. SOD1 is a powerful antioxidant protein that metabolizes oxygen radicals that are produced by cellular metabolism. The active enzyme is a homodimer: each subunit contains four Cys residues at positions 6, 57, 111, and 146. An intramolecular disulfide bond between cysteine residues 57 and 146 is required for folding and stabilization of mature SOD1 [20]. Many familial ALS mutations render SOD1 more sensitive to intramolecular disulfide bond reduction [21], decreasing the apparent melting point below physiological temperature [22] and a corresponding increase in the population of their (A4V, L38V, G93A, L106V) unfolded states [23].

Intermolecular disulfide bonds have been identified in SOD1 aggregates in animal models of familial ALS [24, 25]. Experimental evidence suggested that even if disulfide cross-linking is not required for aggregation of mutant SOD1 [26], disulfide scrambling by intra- and intermolecular isomerization constitutes an important pathway for the aggregation of mutant SOD1 [27].

Despite the importance of disulfide bonds in SOD1 and ALS, it is not known how the intramolecular disulfide bond is formed in the reducing environment of the cytosol and how the thiol-disulfide status of SOD1 changes in the course of the disease.

2.3. Tau. Tauopathies are a group of neurodegenerative diseases, including AD, Pick disease, and corticobasal degeneration, characterized by the accumulation of insoluble Tau fibrils. Tau exists as six different isoforms that result from alternative splicing of mRNA [28], and the longest isoform of Tau has two Cys residues at positions 291 and 322 (numbering based upon the longest form) that can form both intra- and intermolecular disulfide bonds. Although several studies suggest that intermolecular disulfide bonds can promote Tau aggregation *in vitro*, there are lines of evidence that intramolecular disulfide bonds retard Tau aggregation *in vitro* [29]. The precise mechanisms underlying these observations remain unclear. Recent data suggest that intra- and intermolecular disulfide bonds could be one of the factors determining the range of pathological Tau isoforms [30].

2.4. Use of Disulfide Engineering in Aβ. In many cases a disulfide bond has been added to proteins that misfold and aggregate in neurodegenerative disorders. The most studied case is Aβ, where disulfide bonds have been engineered to allow the homogeneous population and stabilization of specific aggregated species (hairpin conformations, dimers, oligomers, and fibrils) and the subsequent characterization of their biochemical, structural, and biological properties [31].

3. Disulfide Bonding in Neurodegenerative Misfolding Diseases

In eukaryotic organisms enzyme-catalyzed disulfide bonds are formed in specialized cell compartments, such as the endoplasmic reticulum (ER) and the intermembrane space of the mitochondria [32].

The ER not only provides an environment suitable for disulfide formation, being very oxidizing with a low GSH : GSSH ratio, but it contains also many proteins dedicated to protein folding and correct disulfide bonding. Already during their translocation into the ER lumen, proteins are oxidized by protein disulfide isomerases (PDIs). The PDIs are maintained in an oxidized state by the flavoprotein Ero1, which transfers electrons directly onto oxygen generating H_2O_2 [33].

PDI was the first enzyme characterized to catalyze thiol-disulfide exchange reactions in the ER, but in recent years the number of PDI family members has been growing very quickly [34]. In humans the PDI family currently has 20 defined family members, such as ERp57, ERp72, P5, ERp44, PDIp, ERp29, ERp19, and ERdj5, defined by similarity to PDI and localization in the ER. They all contain at least one domain that is similar to one of the four thioredoxin domains of PDI, but a detailed characterization of their specific physiological function has not yet been performed.

PDIs are a class of proteins activated upon ER stress [35]. Since extensive protein misfolding and aggregation have been found to induce massive ER stress, the involvement of PDIs in neurodegenerative misfolding diseases has been the subject of intensive studies. PDI family members have been reported to be upregulated in many mouse models of protein misfolding diseases and in postmortem human samples from patients affected with neurodegenerative diseases [5, 6]. Particular attention has been given to the role of PDIs to the pathogenesis of many neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), prion-related disorders (PrDs), and amyotrophic lateral sclerosis (ALS). In some cases, moreover, PDIs have been also shown to directly interact with misfolded or aggregated proteins [36–39]. The results have been extensively described in recent reviews [5, 6, 40]. Here the major findings are reported and classified for pathology, to highlight which are the common features and the unclear aspects.

3.1. Prion-Related Disorders. Creutzfeldt-Jacob (CJD) disease was the first human brain disease shown to be associated with an upregulation of PDIs, both by a proteomic study of sporadic CJD brain tissue [41] and by the analysis of several human brain samples from patients affected with sCJD and vCJD [42]. In particular ERp57 levels correlate with the levels of prions misfolding and inversely correlate with the extent of neuronal damage in murine models of infectious scrapie prions [38, 43] suggesting that ERp57 prevents aggregation and neurotoxicity of prion protein [38].

Moreover, PDI can physically interact with misfolded prion [38, 39], and general inhibition of PDI activity with bacitracin causes the increase of aggregated prion species [39], consistently with the idea that intermolecular disulfide bond formation is also an important factor in prion misfolding [16].

3.2. Amyotrophic Lateral Sclerosis (ALS). Many studies report a link between PDI and the pathogenesis of ALS. PDI and ERp57 were identified as the two main proteins upregulated in the spinal cord of a symptomatic ALS transgenic mice [44], and in the spinal cord and CSF of human sporadic ALS patients [45, 46] where PDI also colocalizes with abnormal protein inclusions associated with sporadic ALS [47]. Interestingly, PDI colocalizes with inclusions in motor neurons of mutated SOD1 (G93A) mice [44] and in human ALS patients [45]. The finding that PDI overexpression in cell culture protects against mutant SOD1 neurotoxicity [48] further indicates that PDI has an important role in protection against mutant protein aggregation in ALS. Knocking down PDI increased the levels of mutant SOD1 aggregation, and its overexpression had the opposite effect [48]. Moreover, a small molecule mimicking the active site of PDIs decreases mutant SOD1 aggregation *in vitro* [48].

Besides physically interacting with SOD1 [44], moreover, PDI interacts also with FUS [49] and colocalizes with TDP43 in human ALS tissue [47].

Despite its demonstrated protective role, increased levels of PDI in ALS were not beneficial in patients. As previously shown for Parkinson's and Alzheimer's brain tissues [50], posttranslational modification of PDI by S-nitrosylation of the critical active site cysteine residues, leading to the inhibition of PDI enzymatic activity, has been observed also in spinal cord tissues of sporadic ALS patients and in transgenic SOD1G93A mice. PDI was found enriched and nitrated in the aggregates isolated from the spinal cord of an ALS mouse model [36, 51]. These modifications could explain the loss of protection by PDI in disease [48].

3.3. Parkinson's Disease (PD). Increased expression of PDIs family members has been observed in many studies on PD. PDI and ERp57 were found to be upregulated in two gene profile studies of PD cell culture models [52, 53]. PDIp is induced in a toxicological mouse model of PD and in brain tissue derived from PD patients [54]. PDI is overexpressed in α-synuclein transgenic mice [55]. The function of PDIs in PD *in vivo*, however, is still not clear.

PDI decreases the aggregation of the Parkinson's disease-associated synphilin-1 protein in neuroblastoma cells [50]. It also prevents the aggregation of α-synuclein in cell-free *in vitro* systems [56].

3.4. Alzheimer's Disease (AD). PDI levels are increased in the brain of AD patients, where PDI colocalizes with neurofibrillary tangles in cells containing neurofibrillary tangles [57, 58]. ERp57 is found in the cerebrospinal fluid of AD patients physically associated with Aβ [37], suggesting a role as a carrier protein that prevents the aggregation of the Aβ peptide.

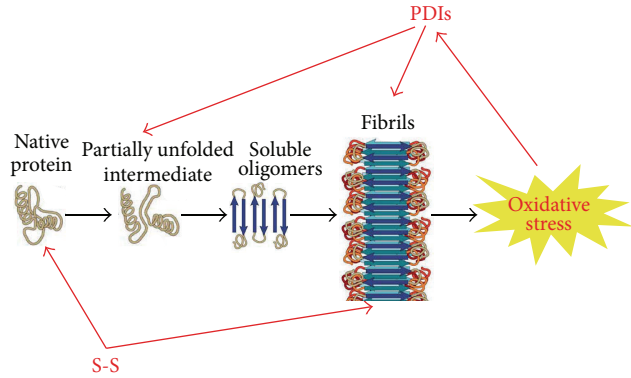

Figure 2: Schematic representation of the role of disulfide bonds (S-S) and disulfide bonding enzymes (PDIs) in the misfolding and aggregation of proteins involved in neurodegenerative misfolding diseases. Disulfide bonds stabilize the monomeric protein slowing down the population of aggregation-prone conformations. They also mediate in many cases the formation of aggregates by intermolecular disulfide bonds. PDIs, instead, are upregulated in the presence of protein aggregation as a general response to cellular stress and UPR activation. In some cases PDIs also interact specifically with the aggregating proteins or the aggregates. (Adapted from [1]).

4. Conclusions

Protein sequences have used different strategies to evolve and reach their functional folding in an efficient way, minimizing the risks of misfolding and (toxic) aggregation [9]. Disulfide bonds carry out this job by a double action: on one side, due to their covalent nature, they stabilize protein structure and functionality, on the other side a family of proteins (PDIs) has evolved, primarily designed to assist disulfide bonding but eventually devoted to promote protein folding and minimize protein misfolding, aggregation, and cell toxicity (Figure 2).

Intramolecular disulfide bonds stabilize the monomeric folding of prion, SOD1, and Tau retarding their aggregation. Intermolecular disulfide bonds, instead, have a more controversial role in pathogenesis. In many cases their presence is still under debate, although their importance in determining the biophysical and biological features of the formed aggregates and as a possible target for treatment.

Different potential roles of PDIs in neurodegenerative diseases are suggested by experimental findings:

(i) the upregulation of PDIs in animal models of NMD and in postmortem tissues from NMD patients can be part of the general UPR response to the ER stress driven by extensive protein misfolding in the cell;

(ii) the colocalization of PDIs with protein aggregates suggests a chaperone activity of PDIs aimed at limiting protein aggregation itself. If this chaperone activity acts in stabilizing monomers or also in disrupting aggregates has not been addressed so far;

(iii) the physical interaction of PDIs with the monomeric forms of several aggregating proteins indicates a role in modulation of the aggregation of specific disease-associated proteins by a direct association.

PDIs appear as a cellular strategy to avoid protein aggregation, but several questions remain unanswered. Which are the mechanisms of PDIs actions in affecting protein aggregation? And what about their effect on protein aggregates? Are different PDIs members specific for different substrates? Interestingly, some observations indeed indicate that some disease and protein specificity of PDI protection is likely to exist. PDI action, in fact, is not specific for amyloid-like aggregates: PDI overexpression inhibits the formation of Ig aggregation in a model of Russell Bodies formation [59] but does not decrease the number of inclusions formed by the variant of α1-antitrypsin [60].

Given the urgent need for novel therapies to treat neurodegenerative misfolding diseases, a solid understanding of disulfide bonding role in protein misfolding and aggregation could lead to the identification of new potential therapeutic targets.

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