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Prion Neurotoxicity: Insights from Prion Protein Mutants

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Abstract

The chemical nature of prions and the mechanism by which they propagate are now reasonably well understood. In contrast, much less is known about the identity of the toxic prion protein (PrP) species that are responsible for neuronal death, and the cellular pathways that these forms activate. In addition, the normal, physiological function of cellular PrP (PrP^C) has remained mysterious, hampering efforts to determine whether loss or alteration of this function contributes to the disease phenotype. Considerable evidence now suggests that aggregation, toxicity, and infectivity are distinct properties of PrP that do no necessarily coincide. In this review, we will discuss several mutant forms of PrP that produce spontaneous neurodegeneration in humans and/or transgenic mice without the formation of infectious PrP^{Sc}. These include an octapeptide insertional mutation, point mutations that favor synthesis of transmembrane forms of PrP, and deletions encompassing the central domain whose neurotoxicity is antagonized by the presence of wild-type PrP. By isolating the neurotoxic effects of PrP from the formation of infectious prions, these mutants have provided important insights into possible pathogenic mechanisms. These studies suggest that prion neurotoxicity may involve subversion of a cytoprotective activity of PrP^C via altered signaling events at the plasma membrane.

Introduction

Prion diseases are fatal neurodegenerative illnesses of man and animals. This group of disorders includes Creutzfeldt-Jakob Disease (CJD), kuru, fatal familial insomnia (FFI), Gerstmann-Sträussler syndrome (GSS), and new variant CJD in humans, as well as bovine spongiform encephalopathy in cattle, chronic wasting disease in deer and elk, and scrapie in sheep and goats (Prusiner, 2004). Patients affected with these disorders suffer from dementia and ataxia, and often display spongiform degeneration and amyloid deposition in their brains.

A wealth of evidence suggests that the central molecular event in prion diseases is the conformational conversion of PrP^C, a normal cell-surface glycoprotein, into PrP^{Sc}, an abnormal isoform that is infectious in the absence of nucleic acid (Aguzzi et al., 2008; Prusiner, 1998). The precise structural differences between the two PrP isoforms remain to be defined, although it is clear that PrP^{Sc} contains significantly more β-sheet and is more

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protease-resistant and aggregated than PrP^{C}. The conversion of PrP^{C} to PrP^{Sc} is thought to involve a templating mechanism in which the two forms physically interact.

Although we now have a detailed understanding of how prions propagate, the cellular mechanisms by which they kill neurons, and the toxic forms of PrP responsible, are poorly understood (Chiesa and Harris, 2001; Harris and True, 2006). Important insights into this issue have been obtained by analysis of PrP molecules carrying neurotoxic mutations. Several kinds of mutant PrP molecules induce spontaneous neurological illness in human beings or transgenic mice in the absence of infection from exogenous sources (Table 1 and Fig. 1). One category of such molecules are those carrying point or insertional mutations linked to human familial prion diseases. These mutants generally display PrP^{Sc}-like biochemical properties, and at least part of their pathogenicity is likely to depend on the toxic properties of the oligomeric protein aggregates that they form. A second category of mutations are those in the N-terminal signal sequence and hydrophobic domain that influence the membrane topology of PrP. A third category includes a series of deletion mutations encompassing the central region of PrP that endow the protein with a powerful neurotoxic activity suppressible by co-expression of wild-type PrP. Mutants in the last two categories are not aggregated or protease-resistant, and their effects are likely due to alterations in a physiological activity of PrP^{C}.

Importantly, none of these three categories of mutations is accompanied by the formation of infectious PrP^{Sc}. By isolating the neurotoxic effects of PrP from the propagation of infectious prions, these mutants have made it possible to focus on pathogenic mechanisms underlying the disease process. Some of these mechanisms turn out to be surprisingly similar to those associated with non-infectious neurodegenerative disorders such as Alzheimer’s disease.

In this article, we will discuss work form our laboratory utilizing each of these three categories of PrP mutants. As a prelude, we will first review what is currently known about the PrP forms and cellular pathways underlying prion neurotoxicity, as well as our current understanding of the physiological function of PrP^{C}.

Prion neurotoxicity: what is the toxic molecule?

What form of PrP is responsible for killing neurons? It has commonly been assumed that PrP^{Sc} itself is the primary cause of neurodegeneration, based on the temporal and anatomical correlation between the accumulation of this form and the development of neuropathological changes. However, there are a number of situations where this correlation is weak or absent. In several kinds of transmission experiments, for example, significant pathology and/or clinical dysfunction develop with little accumulation of PrP^{Sc} (Flechsig et al., 2000; Lasmezas et al., 1997; Manson et al., 1999). In addition, some familial prion diseases are not transmissible, and are not accompanied by the accumulation of protease resistant PrP (Brown et al., 1994b; Piccardo et al., 2001; Tateishi and Kitamoto, 1995; Tateishi et al., 1990; Tateishi et al., 1996). On the other hand, there are sub-clinical infections in which there is abundant PrP^{Sc} but little symptomatology, for example after inoculation of hamster prions into mice (Hill et al., 2000; Race et al., 2001).

Taken together, these situations argue that PrP^{Sc}, the infectious form of PrP, may not be the proximate cause of neuronal dysfunction and degeneration in prion diseases. Several alternative forms of PrP, distinct from both PrP^{C} and PrP^{Sc}, have therefore been hypothesized to be the primary neurotoxic species (designated PrP^{toxic}). In subsequent sections, we will discuss two possible candidates for PrP^{toxic} ($PG14^{spon}$ and ^{Ctm}PrP).

Prion neurotoxicity: what are the cellular pathways?

How do PrP^{Sc} or other toxic forms of PrP induce neuronal death? There is now a growing body of evidence that PrP^{C}, in addition to serving as a precursor of PrP^{Sc}, acts as a signal transducer or mediator of the neurotoxic effects of PrP^{Sc} (Harris and True, 2006). Support for this concept derives from several experimental situations in which expression of membrane-anchored PrP^{C} in target neurons is essential for conferring sensitivity to PrP^{Sc}-induced neurodegeneration or toxicity. First, *Prn-p*$^{0/0}$ neurons are resistant to the pathologic effects of PrP^{Sc} supplied from grafted brain tissue (Brandner et al., 1996) or from nearby astrocytes (Mallucci et al., 2003). Second, scrapie-inoculated mice expressing a glycolipid anchor-negative version of PrP develop minimal brain pathology and neurological dysfunction despite the accumulation of substantial amounts of PrP^{Sc} amyloid (Chesebro et al., 2005). Although interpretation of this experiment is complicated by the relatively low expression levels of anchorless PrP in these mouse lines, one possible implication is that PrP must be membrane-anchored to efficiently mediate a toxic signal (Aguzzi, 2005). Third, *Prn-p*$^{0/0}$ neurons in culture have been found to be resistant to apoptosis induced by exposure to the synthetic peptide PrP106–126, which has been used as a model for PrP^{Sc} (Brown et al., 1994a). Finally, a recent study has shown that PrP^{C} exerts a protective activity against cellular stress, and that PrP^{Sc} abrogates this activity by activating stress-related signaling cascades (Rambold et al., 2008).

Combined with evidence that PrP^{C} normally serves as a neuroprotective molecule (see below), these results taken together suggest a “subversion-of-function” hypothesis to explain prion-induced pathology (Harris and True, 2006): specifically, interaction with PrP^{Sc} (or other pathogenic intermediates) alters a normal, physiological activity of PrP^{C} in such a way that a neurotoxic stimulus is delivered. We will return to this mechanism later, when discussing the neurotoxicity of certain deleted forms of PrP.

Function of PrP^{C}

In order to specify how PrP^{C} function is altered or subverted in prion diseases, it is clearly essential to understand the normal, physiological role of the protein. Surprisingly, however, twenty-five years since PrP^{C} was first identified as an endogenous cellular protein, its physiological activity remains obscure (reviewed in Westergard et al., 2007). A variety of functions have been proposed, including roles in metal ion trafficking (Pauly and Harris, 1998), cell adhesion (Mange et al., 2002), and signal transduction (Mouillet-Richard et al., 2000). However, the evidence in favor of each of these hypothesized functions is not definitive.

Attempts to deduce the function of PrP^{C} from the phenotypes of PrP-null mice have been unrewarding. These mice display no major anatomical or developmental deficits, with the

exception of lines in which the gene encoding Doppel (a PrP paralog) is artifactually up-regulated (Büeler et al., 1992; Manson et al., 1994). Subtle phenotypic abnormalities have been described in PrP knock-out mice at the cellular or behavioral level, but some of these have been controversial, and in any case they have not provided an unambiguous clue to the biological function of PrP^{C} (Steele et al., 2007). A recent report suggests a role for PrP^{C} in embryonic cell adhesion, based on the phenotype of zebrafish in which expression of a PrP homologue has been knocked down (Malaga-Trillo et al., 2009). Whether this purported function is conserved in mammalian PrP remains to be determined.

Several intriguing lines of evidence have emerged recently suggesting that PrP^{C} may exert a cytoprotective activity, particularly against stresses (either internal or environmental) that initiate an apoptotic program (reviewed in Roucou et al., 2004; Roucou and LeBlanc, 2005). First, PrP over-expression rescues cultured neurons, some mammalian cell lines and yeast cells from pro-apoptotic stimuli, including Bax expression, serum withdrawal, and cytokine treatment (Bounhar et al., 2001; Diarra-Mehrpour et al., 2004; Kuwahara et al., 1999; Li and Harris, 2005; Roucou et al., 2005). Second, there is evidence that endogenous PrP protects cells against oxidative and pathologic stressors. For example, neurons cultured from *Prn-p*$^{0/0}$ mice display several subtle abnormalities related to increased susceptibility to oxidative stress (Brown et al., 2002). Moreover, after ischemic or traumatic brain injury, lesion size is larger in *Prn-p*$^{0/0}$ compared to wild-type mice (Hoshino et al., 2003; McLennan et al., 2004; Sakurai-Yamashita et al., 2005; Spudich et al., 2005). Finally, *Prn-p*$^{0/0}$ mice are more susceptible to kainate-induced seizures (Rangel et al., 2007) and their retinal photoreceptors are more prone to light-induced degeneration (Frigg et al., 2006).

To explore the cytoprotective activity of PrP^{C}, we recently attempted to reproduce several of the published assays for this activity (Christensen and Harris, 2008). In one set of experiments, we found that PrP over-expression had a minimal effect on the death of MCF-7 breast carcinoma cells treated with TNF-α, and on *Prn-p*$^{0/0}$ immortalized hippocampal neurons (HpL3–4 cells) subjected to serum deprivation. In a second set of assays, we observed only a small difference in viability between cerebellar granule neurons cultured from PrP-null and control mice in response to activation of endogenous or exogenous Bax. Although our results do not rule out a cytoprotective activity of PrP^{C}, they suggest that existing cell culture systems may be inadequate for modeling this activity.

Mutant PrP molecules associated with inherited prion diseases

Studies of mutant PrP molecules associated with inherited prion diseases have provided important clues to the molecular characteristics of PrP^{toxic}, and the mechanisms by which it is pathogenic. Approximately 10% of the cases of CJD and all cases of GSS and FFI are linked to dominantly inherited, germline mutations in the PrP gene on chromosome 20 (Mead, 2006). The mutations are presumed to favor spontaneous conversion of the protein to the PrP^{Sc} state without the necessity for contact with exogenous infectious agent. Point mutations occur in the C-terminal half of the PrP molecule, and are associated with either CJD, GSS or FFI. Insertional mutations, which are associated with a variable phenotype that can include features of CJD or GSS, consist of one to nine additional copies of a peptide repeat that is normally present in five copies in the N-terminal half of the protein.

A number of studies have used transfected cell lines to analyze the biochemical and cell biological properties of PrP molecules carrying disease-associated mutations (reviewed by Harris, 2003). These studies have revealed that some, but not all mutants, display PrP^{Sc}-like biochemical properties including aggregation propensity as well as resistance to proteases and a GPI-specific phospholipase (Lehmann and Harris, 1996; Priola and Chesebro, 1998). Correlating with these abnormal biochemical properties, some mutants display altered subcellular localization, including partial retention in the ER (Ivanova et al., 2001), or retrotranslocation into the cytoplasm with subsequent degradation by the proteasome (Lorenz et al., 2002). Interestingly, some disease-associated PrP mutants are identical to wild-type PrP in terms of their biochemical properties and cellular distribution (Harris, 2003), raising the possibility that these molecules are pathogenic, not because they misfold and aggregate, but because the mutation subtly alters a physiological activity normally associated with PrP^{C}. Unfortunately, expression in transfected cells of PrP molecules carrying disease-associated mutations is generally not cytopathic, making it difficult to analyze the neurotoxic mechanisms underlying these mutants. For this reason, we and others have turned to transgenic models of familial prion diseases.

A transgenic model of an octapeptide insertion: Tg(PG14) mice

Tg(PG14) mice express the mouse homologue of a nine-octapeptide insertional PrP mutant associated with a familial prion disease of humans (Duchen et al., 1993; Krasemann et al., 1995; Owen et al., 1992) (Fig. 1). These animals display a spontaneous, progressive neurodegenerative disease with symptoms of ataxia, cerebellar granule cell loss, gliosis, and PrP deposition (Chiesa et al., 2000; Chiesa et al., 1998). Tg(PG14) mice spontaneously accumulate in their brains an insoluble and weakly protease-resistant form of the mutant protein (Chiesa et al., 2003). This form (designated $PG14^{Spon}$) is highly neurotoxic, but is not infectious in animal bioassays. In contrast, when Tg(PG14) mice are inoculated with the RML strain of prions, they accumulate a different form of PG14 PrP (designated $PG14^{RML}$) that is highly protease-resistant and infectious in animal transmission experiments (Chiesa et al., 2003). Consistent with other published work, these studies emphasize that infectivity and pathogenicity are two distinct properties of PrP.

To gain insight into the molecular determinants of infectivity and pathogenicity, we have undertaken a biochemical characterization the biochemical properties of $PG14^{Spon}$ and $PG14^{RML}$. Our initial studies (Chiesa et al., 2003) indicated that these two forms, although conformationally related, differed in their quaternary structure, with $PG14^{RML}$ forming larger, more tightly packed aggregates. We found that the majority of $PG14^{RML}$ aggregates have a sedimentation coefficient of >50S, with ~30% of them having a sedimentation coefficient of >120S. If composed exclusively of PrP, the latter aggregates would contain >200 molecules of the protein. In contrast, 15–20% of $PG14^{Spon}$ PrP was monomeric (3.2S), with the rest sedimenting at 16–20S (corresponding to oligomers containing ~20–30 molecules of PrP). These results suggest that while highly aggregated polymers of PrP are a prerequisite for prion infectivity, small, β-rich oligomers are the species that are toxic to nerve cells (Chiesa et al., 2003). This conclusion is strikingly reminiscent of recent work in Alzheimer's disease, which has demonstrated that small oligomers of the Aβ peptide

(ranging in size from 2–100 subunits), rather than large amyloid fibers, are the primary toxic species (Haass and Selkoe, 2007; Walsh and Selkoe, 2007).

In a more recent study (Biasini et al., 2008), we have subjected $PG14^{Spon}$ and $PG14^{RML}$ to a panel of assays commonly used to distinguish infectious PrP (PrP^{Sc}) from cellular PrP (PrP^{C}), including immobilized metal affinity chromatography, precipitation with sodium phosphotungstate, and immunoprecipitation with PrP^{C}- and PrP^{Sc}-specific antibodies. Surprisingly, we found that aggregates of $PG14^{Spon}$ and $PG14^{RML}$ behave identically to each other, and to authentic PrP^{Sc}, in each of these biochemical assays. $PG14^{Spon}$ however, in contrast to $PG14^{RML}$ and PrP^{Sc}, was unable to seed the misfolding of PrP^{C} in an *in vitro* protein misfolding cyclic amplification reaction. Collectively, these results suggest that infectious and non-infectious aggregates of PrP share common structural features accounting for their toxicity, and that self-propagation of PrP involves more subtle molecular differences that remain to be identified.

Gain vs. loss of function as a pathogenic mechanism

How do mutant PrP molecules induce neurodegeneration? For most dominantly inherited neurodegenerative disorders, a gain-of-function mechanism is invoked in which the mutant protein is presumed to acquire a toxic property unrelated to its normal physiological activity (Winklhofer et al., 2008). For example, aggregates of the mutant protein could disrupt important cellular processes and so impair neuronal viability.

Such a mechanism appears to play a role in the pathogenicity of the PG14 PrP, based on our studies of the localization of this mutant in neurons in the brains of transgenic mice. We generated transgenic mice expressing PG14-EGFP, a fluorescent fusion protein that can be directly visualized *in vivo* (Medrano et al., 2008). Tg(PG14-EGFP) mice develop an ataxic neurological illness characterized by astrogliosis, PrP aggregation, and accumulation of a partially protease-resistant form of the mutant PrP. Strikingly, PG14-EGFP forms numerous fluorescent aggregates in the neuropil and white matter of multiple brain regions. These aggregates are particularly prominent along axonal tracts in both brain and peripheral nerve, and similar intracellular deposits are visible along the processes of cultured neurons. Our results suggest that intra-axonal aggregates of mutant PrP could contribute to the pathogenesis of familial prion disease by disrupting axonal transport. A similar mechanism of axonal blockage has recently been proposed to explain the pathogenesis Alzheimer's disease (Stokin et al., 2005).

To investigate whether a loss-of-function mechanism could also play a role in the neurotoxicity of PG14 PrP, we crossed Tg(PG14) mice with Tg(PrPΔ32–134) mice (Li et al., 2007c). Tg(PrPΔ32–134) mice, which express an N-terminally truncated form of PrP, spontaneously develop a neurodegenerative phenotype that is stoichiometrically reversed by co-expression of wild-type PrP. We found that, at equivalent expression levels, PG14 PrP is significantly less efficient than wild-type PrP in suppressing the development of clinical symptoms and neuropathology in Tg(PrPΔ32–134) mice. Thus, our results suggest that some features of the neurological illness associated with inherited PrP mutations may be attributable to a loss of PrP neuroprotective function.

Since PrP-null mice do not display features of a prion disease (Büeler et al., 1992; Manson et al., 1994), it is unlikely that these disorders are due exclusively to the simple loss of an essential physiological function of PrP^{C}. A loss of function mechanism also appears to be incompatible with the dominant mode of inheritance of familial prion diseases. However, these facts do not rule out a contribution of PrP functional deficiency to prion-related neurodegeneration. For example, PrP^{Sc} or mutant PrP may sequester wild-type PrP^{C} into aggregates that lack functional activity, thereby producing a dominant-negative effect. Moreover a biological activity of PrP^{C} that is dispensable under normal conditions may become essential in the disease state due to cellular or organismal stress.

Taken together, our results also indicate that more than one pathogenic mechanism (both gain- and loss-of-function) may contribute to neurotoxicity in inherited prion diseases.

Topological variants of PrP

There is evidence that alterations in membrane topology can result in neurotoxic forms of PrP. While most PrP molecules are linked to the plasma membrane exclusively via a GPI anchor, three topological variants have been described: ^{Cyto}PrP, NtmPrP, and ^{Ctm}PrP (Chakrabarti et al., 2009). ^{Cyto}PrP, in which the polypeptide chain lies entirely in the cytoplasm, is produced at low levels as a result of inefficient translocation into the ER (Drisaldi et al., 2003). The amount of ^{Cyto}PrP can be regulated by cellular stress, a mechanism that has been termed “pre-emptive quality control” (Kang et al., 2006). Mice expressing a transgenically encoded form of ^{Cyto}PrP show a severe neurodegenerative phenotype, indicating that this molecule possesses neurotoxic activity (Ma et al., 2002). It has been claimed that prion infection increases production of ^{Cyto}PrP (Rane et al., 2008), thereby contributing to neurotoxicity, but this observation is not confirmed by out own data (Stewart and Harris, 2003). At present, the pathogenic role of ^{Cyto}PrP in prion diseases remains unclear.

Two transmembrane variants of PrP have also been described. ^{Ctm}PrP and ^{Ntm}PrP span the lipid bilayer once via a stretch of hydrophobic amino acids in the central region of the protein (residues 111–135), with the N- or C-terminus, respectively, in the cytoplasm. These molecules were first observed in cell-free translation/translocation systems, where it was shown that their amounts could be altered by mutations in the central hydrophobic domain, as well as in an adjacent “stop transfer effector” segment that contains several positively charge amino acids (Hay et al., 1987; Hegde et al., 1998; Yost et al., 1990). Subsequent work from our laboratory (Stewart and Harris, 2003) and others (Kim et al., 2001) demonstrated that non-conservative substitutions in the core of the N-terminal signal sequence also increased the proportion of ^{Ctm}PrP, and that combining these mutations with ones in the central domain resulted in almost exclusive synthesis of the ^{Ctm}PrP. Cell biological analysis of one such compound mutant (L9R/3AV) (Fig. 1) led to the conclusion that ^{Ctm}PrP contains an uncleaved signal peptide as well as a GPI anchor, and that it is retained intracellularly in either the ER or the Golgi (depending on the cell type) (Stewart et al., 2001; Stewart and Harris, 2005).

Transgenic mice expressing PrP molecules with ^{Ctm}PrP-promoting mutations display a neurodegenerative phenotype, implying that ^{Ctm}PrP has neurotoxic potential *in vivo* (Hegde et al., 1998; Stewart et al., 2005). PrP^{Sc} accumulation has been claimed to cause enhanced generation of ^{Ctm}PrP, and on this basis it has been suggested that ^{Ctm}PrP represents a key neurotoxic intermediate in prion disorders (Hegde et al., 1999). However, this hypothesis is called into question by our observations that scrapie infection does not significantly alter ^{Ctm}PrP levels in cultured cells or brain (Stewart and Harris, 2003), and that pathogenic mutations outside of the central, hydrophobic domain do not alter the membrane topology of PrP (Stewart and Harris, 2001).

Whether or not ^{Ctm}PrP itself plays a role in naturally occurring prion diseases, it is likely to be revealing PrP-related neurotoxic signaling mechanisms that could contribute to the pathological process. Our studies of Tg(L9R/3AV) mice exemplify this point. These animals develop a fatal neurological illness characterized by ataxia and neuronal loss in the cerebellum and hippocampus (Stewart et al., 2005). Importantly, this phenotype is strongly dependent on co-expression of endogenous, wild-type PrP, suggesting a synergistic interaction between transmembrane and exclusively GPI-anchored forms of PrP in transducing a toxic signal.

Neurotoxicity of PrP deletion mutants

One of the most surprising and intriguing observations to have emerged concerning the neurotoxic effects of PrP was first made over 10 years ago by Shmerling et al. (1998) as part of a study to examine which parts of the PrP molecule were necessary for sustaining prion infection. These authors discovered that mice expressing PrP harboring either of two large, N-terminal deletions (Δ32–121 and Δ32–134) (Fig. 1) developed a spontaneous neurodegenerative illness even without inoculation with scrapie prions. This illness was characterized by ataxia and massive degeneration of cerebellar granule neurons. Mice with shorter deletions (Δ32–80, Δ32–93, Δ32–106) were phenotypically normal, implying a critical role for amino acids distal to residue 106. What was even more surprising, the neurodegenerative phenotype of the Tg(Δ32–121) and Tg(32–134) mice was only observed on the *Prn-p*$^{0/0}$ background: co-expression of endogenous, wild-type PrP from even a single *Prn-p* allele completely abrogated clinical symptoms and neuropathology. To explain these puzzling observations, the authors proposed a loss-of-function model based on the existence of two hypothetical molecules, one of which (L_{prp}) is a ligand that binds to PrP and the other (π) a receptor that binds the ligand when PrP is absent. It was suggested that binding of L_{prp} to wild-type PrP delivered an essential trophic signal, while binding to N-terminally deleted PrP failed to elicit this signal and also prevented interaction with π.

To more precisely map the region of PrP responsible for the spontaneous neurodegenerative phenotype, we created Tg(ΔCR) mice expressing PrP with a much smaller deletion, comprising residues 105–125 within the Central Region of the molecule (Li et al., 2007b) (Fig. 1). The deleted segment lies within a highly conserved, unstructured region of the protein, and encompasses a cluster of three positively charged amino acids (residues 105, 109, and 110) followed by a stretch of 15 hydrophobic residues (residues 111–135). There were three motivations for focusing on the 105–125 region as a possible toxicity-

determining domain in PrP. First, the study by Shmerling et al. (1998) had pinpointed residues distal to position 106 as being critical determinants of neurotoxicity. Second, it was known that a synthetic peptide derived from this region (PrP106-126) was toxic when applied to cultured neurons from *Prn-p*$^{+/+}$ but not from *Prn-p*$^{0/0}$ mice (Brown et al., 1994a). Third, the 105–125 region is one of the most highly conserved portions of the sequence, being virtually identical in PrP molecules from zebrafish to humans (Rivera-Milla et al., 2006).

Tg(ΔCR) mice display a spontaneous neurodegenerative phenotype that is more severe than any we have ever encountered in other transgenic mice (Li et al., 2007b). This phenotype is observed at modest expression levels of the mutant protein (0.5–1X). On the *Prn-p*$^{0/0}$ background, Tg(ΔCR) mice become ill and die within one week of birth. This phenotype is reversed in a dose-dependent fashion by co-expression of wild-type PrP: one *Prn-p* allele (0.5X expression level) delays death until 25 days, and two *Prn-p* alleles (1X expression level) delays death until 48 days. The presence of one Tga20 allele (5X expression level of wild-type PrP) strongly rescues the neurodegenerative phenotype, with mice remaining alive for well over one year.

Tg(ΔCR) mice show two major kinds of neuropathology (Li et al., 2007b). First, there is massive degeneration of cerebellar granule cells (with no effect on Purkinje cells), resulting in severe atrophy of the cerebellum. Degenerating granule neurons display extensive DNA fragmentation, but there is no activation of caspases 3 or 8 (Christensen et al., manuscript in preparation). By electron microscopy, granule cells display a unique, non-apoptotic ultrastructural morphology (Christensen et al., manuscript in preparation). In conjunction with evidence that neurodegeneration in Tg(ΔCR) mice is Bax-independent (Li et al., 2007a), our ultrastructural and biochemical data indicate that PrPΔCR is activating a novel form of neuronal death that is not related to apoptosis, necrosis, or autophagy.

The second major neuropathological feature of Tg(ΔCR) mice is vacuolar degeneration of white matter areas of the brain and spinal cord, particularly in older animals. This abnormality, which was also seen Tg(Δ32–134) mice (Radovanovic et al., 2005), may be due either to axonal or myelin damage.

The biochemical and cell biological properties of PrPΔCR suggest that the neurotoxicity of this molecule results from an alteration of a normal activity of PrP^{C}, rather than from accumulation of misfolded protein aggregates or cellular mislocalization. PrPΔCR is not aggregated or protease-resistant like PrP^{Sc} (Li et al., 2007b). Moreover, the cellular localization of PrPΔCR is identical to that of wild-type PrP (Christensen and Harris, 2009). Like its wild-type counterpart, ΔCR PrP is present in lipid rafts on the plasma membrane, where it is attached by a GPI anchor, and it selectively localizes to the apical surface of polarized MDCK epithelial cells. Moreover, PrPΔCR is distributed in a punctate pattern on the surface of neuronal processes, similar to the wild-type protein.

Recently, Baumann et al. (Baumann et al., 2007) described lines of mice expressing PrP with a deletion of residues 94–134. These animals also display a neurodegenerative phenotype that is suppressible by co-expression of wild-type PrP. It is instructive to compare the

features of mice expressing the four different pathogenic deletions thus far published (Δ32–121, Δ32–134, Δ94–134, and Δ105–125) (Fig. 1). Each of these deletions encompasses the 105–125 region, demonstrating the importance of these residues in determining the neurotoxic activity of PrP. Interestingly, the four kinds of mice differ in the severity of the phenotypes they display (as indicated by age at symptom onset), and there is a correlation between the severity of the phenotype and the amount of wild-type PrP required for rescue (Table 1). Tg(ΔCR) mice, which have the strongest phenotype (illness onset at <1 week on the *Prn-p*$^{0/0}$ background), require >5X expression of wild-type PrP for complete rescue, while Tg(PrPΔ34–134) mice (illness onset at 3–5 weeks) are almost completely rescued by 0.5X co-expression of wild-type PrP. This quantitative relationship between neurotoxic potency and the ability to be suppressed by wild-type PrP provides important clues to the underlying molecular mechanisms (see below).

Doppel and shadoo

Studies of two proteins with structural similarities to PrP have provided additional insights into the neurotoxic activities of PrP. Doppel (Dpl) is a PrP paralog that resembles the C-terminal half of PrP, but lacks residues homologous to the flexible N-terminal domain (Mo et al., 2001) (Fig. 1). Dpl thus structurally resembles the deleted forms of PrP that produce neurodegeneration in transgenic mice. The Dpl gene, which is normally expressed primarily in testis, is expressed ectopically in the brain in certain lines of *Prn-p*$^{0/0}$ mice as a result of intergenic splicing events between the adjacent PrP and Dpl genes (Moore et al., 1999). These lines, as well as transgenic lines expressing elevated levels of Dpl in the brain, display a neurodegenerative phenotype that is stoichiometrically rescued by wild-type PrP (Moore et al., 2001; Rossi et al., 2001). Dpl-induced neuronal death involves Purkinje cells as well as granule cells in the cerebellum, and is partially Bax-independent (Dong et al., 2007; Heitz et al., 2007). Taken together, the available data make it very likely that the neurotoxicity of Dpl involves the same molecular pathways as those activated by deleted forms of PrP.

While Dpl is structurally related to the C-terminal half of PrP, the recently described protein, Shadoo (Sho), resembles a GPI-anchored version of the flexible, N-terminal tail of PrP, including the highly conserved central region (reviewed in Watts and Westaway, 2007) (Fig. 1). Sho is expressed in many of the same regions of the brain as PrP^C, although the cellular distribution of the two proteins is complementary (Watts et al., 2007). In the cerebellum, for example, Sho is most abundant in Purkinje cell bodies and dendrites, while PrP^C is present at highest levels in granule neurons and their axons in the molecular layer. In a neuronal culture assay, Sho had no toxic activity on its own, but was able to suppress the toxicity of co-expressed Dpl and PrPΔ32–121 (Watts et al., 2007). Thus, Sho displays a neuroprotective activity like that of wild-type PrP^C. The protective activity of PrP^C presumably resides in the N-terminal half of the protein, based on the observation that fusion of this region to Dpl prevents neurodegeneration in transgenic mice (Yoshikawa et al., 2008). Interestingly, Sho protein levels are dramatically down-regulated in scrapie-infected mice, raising the possibility that loss of Sho neuroprotection may play a role in prion diseases (Watts et al., 2007). It has been suggested that Sho is a candidate for π, the hypothetical receptor that has been postulated to deliver an essential neurotrophic signal in the absence of PrP (Watts et al., 2007).

A model for PrP neurotoxicity: subversion of function

Studies of the biological activities of ΔCR and other deleted forms of PrP, along with Dpl and Sho, provide powerful mechanistic insights into the normal function of PrP^{C}, and how it can be subverted to produce neurotoxic effects. The dose-dependent, opposing effects of truncated and wild-type PrP are most easily explained by supposing that both proteins compete for binding to a common molecular target, with binding of truncated PrP eliciting a neurotoxic signal, and binding of wild-type PrP silencing the signal (or restoring a non-toxic, physiological signal) (Fig. 2). The molecular target for PrP is presumably a receptor or other cell-surface complex capable of transducing a signal to the interior of the cell. We hypothesize that binding to the target involves at least two sites on PrP: the CR region (residues 105–125), as well as a more C-terminal region. In the absence of the CR region (as in PrPΔCR), binding of the C-terminal region alone would elicit a neurotoxic signal. Binding at both sites (wild-type PrP) would produce a physiological signal or no signal. We suggest that an identical mechanism is responsible for the neurotoxicity of all four deleted forms of PrP as well as Dpl, with variations in the toxic potency of these molecules being attributable to differences in their affinity for the putative binding target. The cytoprotective effect of Sho could be due to its ability to occupy the CR binding site on the putative transducer molecule.

Although ΔCR and other deleted forms of PrP are artificial molecules, working out how they kill neurons will likely provide crucial insights into neurodegeneration in naturally occurring prion diseases. Like PrPΔCR, PrP^{Sc} (or other toxic forms of PrP that accumulate during prion infection) appears to act by subverting a normal function of PrP^{C} (see above). It is therefore possible that PrP^{Sc}/PrP^{toxic} interacts with the same putative membrane target as PrPΔCR, resulting in generation of a similar neurotoxic signal (Fig. 2). Interestingly, there is evidence that PrP^{Sc} is conformationally altered in the CR region (Peretz *et al*, 1997), perhaps preventing binding interactions involving this site. Thus, PrP^{Sc}/PrP^{toxic}, like PrPΔCR, may be able to interact with its target only via C-terminal binding sites. The same may be true for PG14 PrP, which is aggregated and conformationally altered in the CR region (Biasini et al., 2008) (Fig. 2).

The model outlined here may also explain the toxicity of ^{Ctm}PrP, as well as the synthetic peptide PrP106–126. PrP106–126 has been reported to be toxic to neurons cultured from *Prn-p*$^{+/+}$ but not *Prn-p*$^{0/0}$ mice (Brown et al., 1994a; Fioriti et al., 2005; Forloni et al., 1993). This result suggests that the peptide alters interaction between PrP and the hypothetical transducer by competitively blocking binding within the 105–125 region of PrP. This would then produce a toxic signal equivalent to the one elicited by PrPΔCR which lacks the 105–125 domain. In the absence of PrP^{C}, no signal would be delivered and the peptide would have no effect. In ^{Ctm}PrP, the CR region is partially embedded in the lipid bilayer, and so would be unavailable for binding to the transducer (Fig. 2). In contrast to the situation for PrPΔCR, however, activation of the transducer by ^{Ctm}PrP is presumably facilitated by the presence of wild-type PrP, which potentiates the toxicity of ^{Ctm}PrP.

Conclusions and perspective

Table 1 summarizes the properties of the non-infectious, neurotoxic mutants of PrP discussed in this review, and compares them to the properties of PrP^{C} and infectious PrP^{Sc}. Studies of neurotoxic PrP mutants have provided a number of important insights into the pathogenic species and cellular pathways that may underlie prion-induced neurodegeneration. An important principle exemplified by all of these mutants is the distinction between prion infectivity and neurotoxicity. For some of the mutants associated with familial prion disorders, such as octapeptide insertions, small, β-rich aggregates are likely to represent a key neurotoxic intermediate. There is evidence that these aggregates act via both gain- and loss-of-function mechanisms. In contrast, transmembrane mutants as well as mutants harboring deletions within the central region display biochemical and cell biological properties indistinguishable from those of wild-type PrP. The neurotoxicity of these mutants is likely to depend on alteration or subversion of a neuroprotective function of PrP^{C}.

Further understanding prion neurotoxicity will require progress on several fronts. First, it will be necessary to identify the molecular components of the cellular signaling pathways activated by neurotoxic forms of PrP. This will involve searching for PrP-interacting proteins via biochemical methods, as well as via genetic means using model organisms. The availability of highly potent neurotoxic forms like PrPΔCR should facilitate this search, since these molecules should have an especially high affinity for putative cell surface transducers. Second, it will be important to develop cell culture systems that allow the neurotoxic effects of PrP to be studied *in vitro*. Unfortunately, the mutant PrP molecules discussed here, all of which are neurotoxic in transgenic mice, have only modest effects on cell viability when expressed in cultured cells (our unpublished data) (Ashok and Hegde, 2008; Drisaldi et al., 2004; Rambold et al., 2008; Watts et al., 2007). Presumably, some factor present in the brain milieu required for maximal toxicity of these molecules must be missing in cell culture systems. Finally, and perhaps most importantly, we will need to demonstrate a connection between the neurotoxic mechanisms activated by artificial mutants like PrPΔCR and those operative in “natural” prion diseases of humans and animals. The most direct way to achieve this goal will be to knock down expression of the relevant signaling components *in vivo*, and see if this mitigates the disease phenotype.

Most current approaches to treatment of prion diseases are based on inhibiting accumulation of PrP^{Sc} (Trevitt and Collinge, 2006). Identification of the cellular pathways activated by neurotoxic forms of PrP would allow development of an entirely new class of anti-prion therapeutics based on blocking these pathways. Assuming appropriate cell culture models can be developed, some of the mutant forms of PrP described in this review may provide particularly good substrates for a drug screening approach to identify inhibitors of neurotoxic pathways. Such compounds may have wide applicability, given some of the underlying similarities between prion diseases and other neurodegenerative disorders in terms of pathogenic mechanisms. Indeed, recent evidence that PrP^{C} is a cell-surface receptor for the Alzheimer’s Aβ peptide provides a direct link between neurotoxic mechanisms involved in these two diseases (Laurén et al., 2009).

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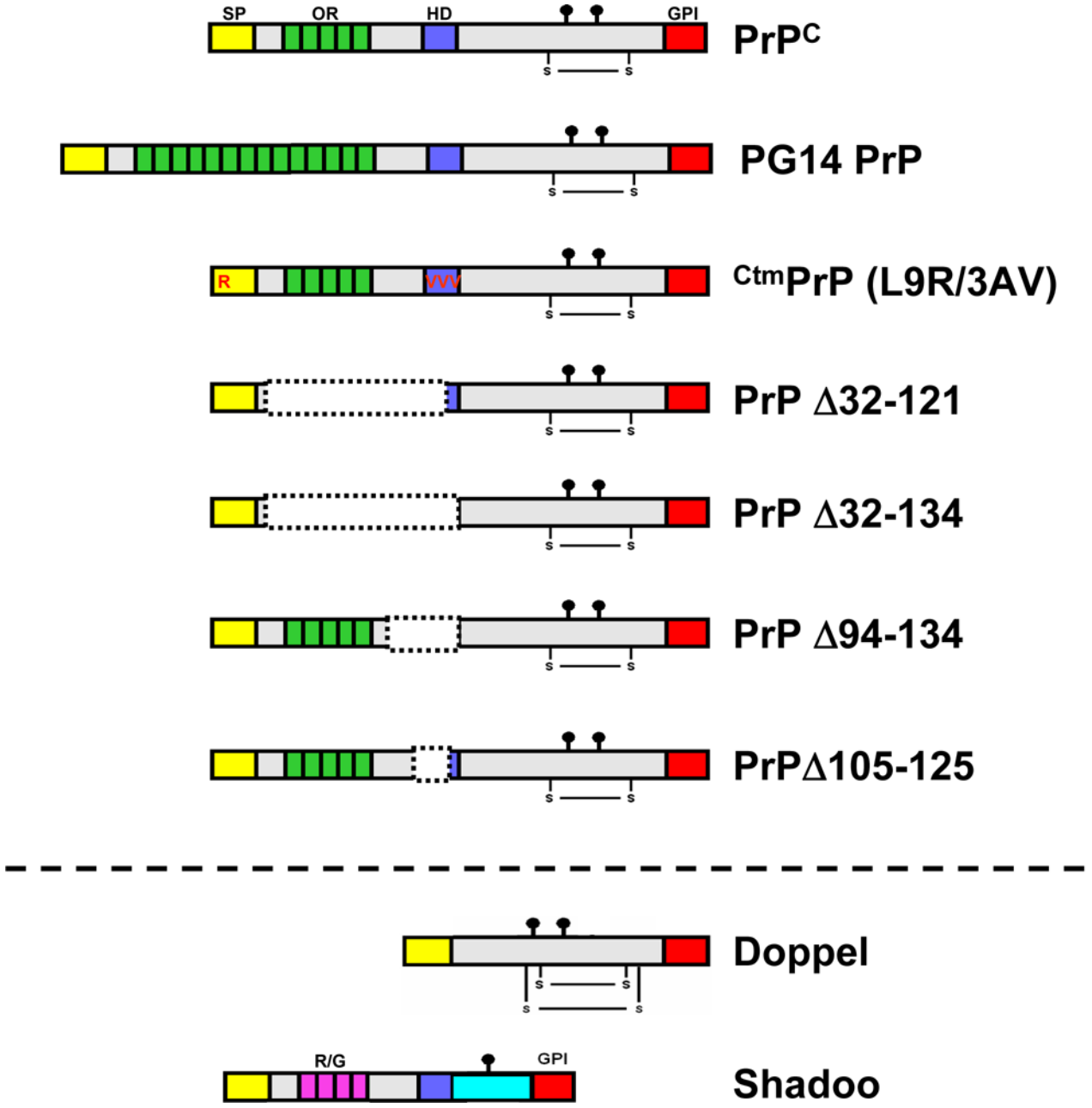

FIGURE 1. Schematic of wild-type and mutant PrP molecules, Doppel, and Shadoo

Structural domains are indicated by the colored blocks: SS (yellow), signal sequence; OR (green), octapeptide repeats; HD (blue), hydrophobic domain; GPI (red), glycosyl-phosphatidylinositol attachment signal; R/G (pink), arginine/glycine repeats of Sho. The lollipop symbols indicate sites of N-linked glycosylation, and the S—S symbols indicate disulfide linkages.

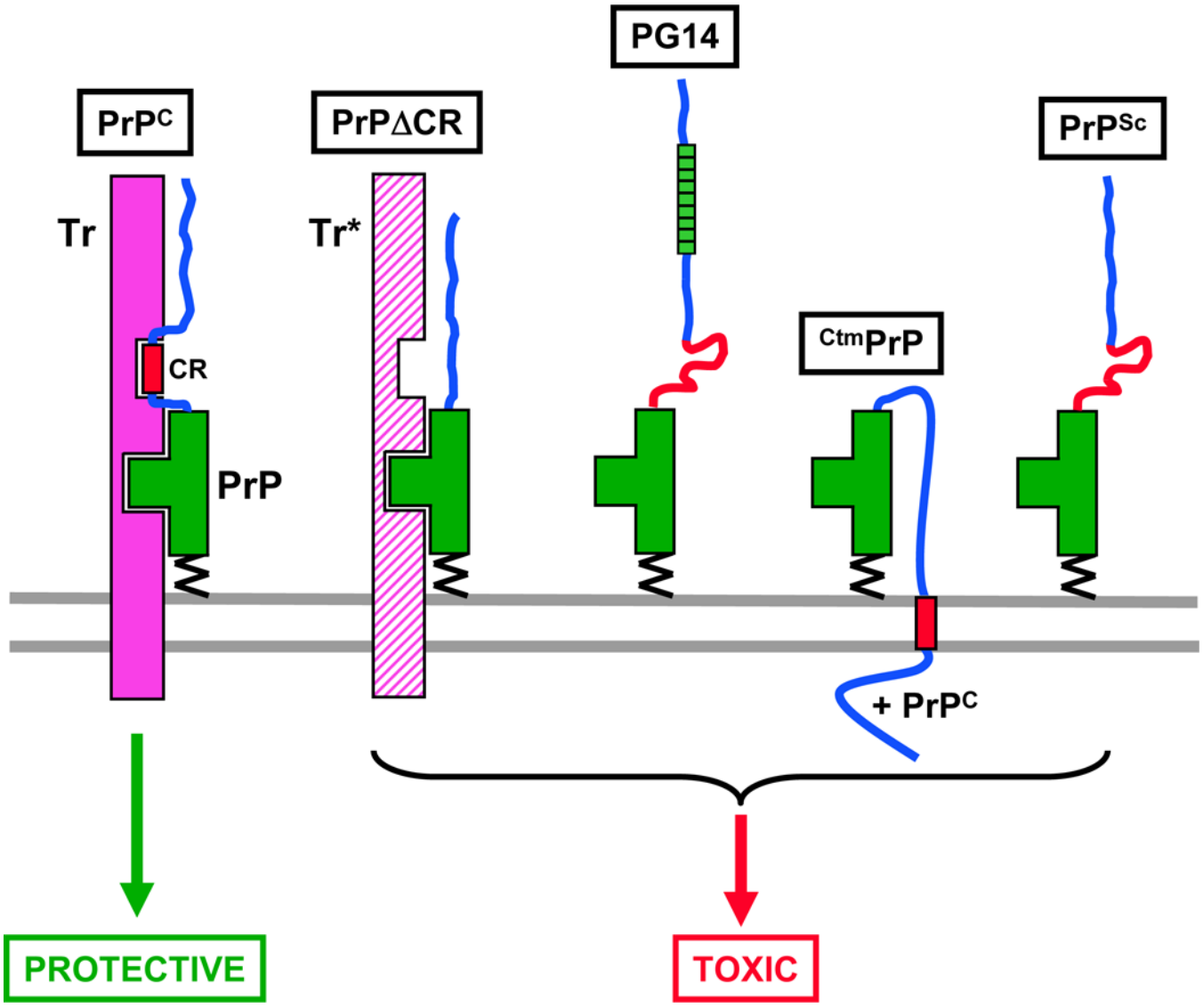

FIGURE 2. Model for the neuroprotective activity of PrP^C, and subversion of this activity by neurotoxic forms of PrP

The structured, C-terminal half of PrP is shown in green and the flexible, N-terminal tail as a blue line. The CR segment of PrP (residues 105–125) is shown as a red rectangle. Tr, hypothetical signal transducing protein that normally generates a neuroprotective signal (solid pink), but which can assume an altered conformation (crosshatched pink) that generates a neurotoxic signal. Two binding sites between PrP and Tr are shown, one involving the C-terminal half of PrP and the other CR segment of PrP. When both binding sites are occupied, Tr elicits a non-essential neuroprotective signal (PrP^C). When only the C-terminal site is occupied, as would be the case when the CR segment is absent (PrPΔCR), embedded in the lipid bilayer (^{Ctm}PrP), or conformationally altered (PG14 and PrP^{Sc}), the transducer delivers a neurotoxic signal. The toxicity of ^{Ctm}PrP requires the cooperation of wild-type PrP^C.

TABLE 1

Properties of PrP^{C}, PrP^{Sc}, and neurotoxic mutants of PrP

Molecule	Protease-resistance/aggregation	Cellular localization	Infectivity	Disease onset (days)[a]	Pathological targets	Effect of wild-type PrP (expression level)
PrP^{C}	−	PM/Rafts	−	N/A	None	N/A
PrP^{Sc}	+++	PM, endosomes	+	Depends on strain/host	Neurons, WM	Exacerbation
$PG14^{Spon}$ [1–3]	+	ER	−	240	CGN, dendrites, axons/myelin	No effect (1X)
$PG14^{RML}$ [1, 2]	+++	Unknown	+	240 (post-inoculation)	CGN	Not tested
^{Ctm}PrP (L9R/3AV) [4–6]	−	ER (CHO cells); Golgi (neurons)	−	85–172[b]	CGN, HPN	Exacerbation (0.5–1X)
PrPΔ32–121 [7]	−	Not tested	−	42–56[c]	CGN	Rescue (0.5X)
PrPΔ32–134 [7]	−	PM	−	21–35[c]	CGN, WM	Rescue (0.5X)
PrPΔ94–134 [8]	−	Rafts	−	14[c]	WM	Rescue (2–3X)
PrPΔ105–125 [9]	−	PM/Rafts	−	7[c]	CGN, WM	Rescue (5X)

Abbreviations: CHO, Chinese hamster ovary; CGN, cerebellar granule neurons; ER, endoplasmic reticulum; HPN, hippocampal neurons; N/A, not applicable; PM, plasma membrane; WM, white matter.

[a] Disease onset varies with transgene expression level. Data are for highest expressing lines (1–3X).

[b] For mice on the *Prn-p*$^{+/+}$ background.

[c] For mice on the *Prn-p*$^{0/0}$ background.

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[8] Baumann et al., 2007;

[9] Li et al., 2007b.