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MINI-SYMPOSIUM: PRION DISEASES

Prion neurotoxicity

Nhat T. T. Le*; Bei Wu*; David A. Harris

Department of Biochemistry, Boston University School of Medicine, Boston, MA.

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Corresponding author:
David A. Harris, Department of Biochemistry, Boston University School of Medicine, 72 E. Concord Street, K-225, Boston, MA 02118 (E-mail: *daharris@bu.edu*)

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*These authors contributed equally.

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Abstract

Although the mechanisms underlying prion propagation and infectivity are now well established, the processes accounting for prion toxicity and pathogenesis have remained mysterious. These processes are of enormous clinical relevance as they hold the key to identification of new molecular targets for therapeutic intervention. In this review, we will discuss two broad areas of investigation relevant to understanding prion neurotoxicity. The first is the use of *in vitro* experimental systems that model key events in prion pathogenesis. In this context, we will describe a hippocampal neuronal culture system we developed that reproduces the earliest pathological alterations in synaptic morphology and function in response to PrP^{Sc}. This system has allowed us to define a core synaptotoxic signaling pathway involving the activation of NMDA and AMPA receptors, stimulation of p38 MAPK phosphorylation and collapse of the actin cytoskeleton in dendritic spines. The second area concerns a striking and unexpected phenomenon in which certain structural manipulations of the PrP^{C} molecule itself, including introduction of N-terminal deletion mutations or binding of antibodies to C-terminal epitopes, unleash powerful toxic effects in cultured cells and transgenic mice. We will describe our studies of this phenomenon, which led to the recognition that it is related to the induction of large, abnormal ionic currents by the structurally altered PrP molecules. Our results suggest a model in which the flexible N-terminal domain of PrP^{C} serves as a toxic effector which is regulated by intramolecular interactions with the globular C-terminal domain. Taken together, these two areas of study have provided important clues to underlying cellular and molecular mechanisms of prion neurotoxicity. Nevertheless, much remains to be done on this next frontier of prion science.

INTRODUCTION

Prion diseases are a group of fatal, infectious neurodegenerative diseases affecting humans and animals. A defining feature of prion diseases is their association with a protein-only infectious agent, PrP^{Sc}, which is self-propagating and transmissible (87, 88). Once PrP^{Sc} is introduced into individuals from the environment or is generated endogenously, it converts the normal, cellular conformer, designated PrP^{C}, into additional molecules of PrP^{Sc}. Consistent with this model, PrP knockout mice, in which PrP^{C} expression is absent, are completely resistant to prion infection (14, 89). Probably the strongest evidence for the prion model is the fact that infectious molecules can be generated and amplified *in vitro* from purified or recombinant PrP^{C} in reactions seeded by small amounts of PrP^{Sc} (18, 32, 125). A prion-like mechanism may play a role in the spread of misfolded protein aggregates in other, more common neurodegenerative disorders (59), and in the maintenance of certain physiological states (81).

While a great deal is now known about the mechanisms of prion infectivity and propagation, we have a much more limited understanding of how misfolded PrP actually damages neurons and causes the neuropathological abnormalities characteristic of the disease. Previous studies have established several relevant points. First, there is considerable evidence that PrP^{C} plays an essential role in mediating prion neurotoxicity, beyond its function as a required precursor to PrP^{Sc} (12, 75). In this regard, it has been hypothesized that PrP^{C} may act as a cell surface receptor that binds PrP^{Sc} and transduces downstream neurotoxic signals, a process that could involve the subversion of a normal, physiological activity of PrP^{C} (7, 93). Second, analogous to the case of Aβ in Alzheimer's disease (123), it is likely that oligomeric, possibly noninfectious forms of misfolded PrP, rather than large, self-propagating polymers of PrP^{Sc}, are the most neurotoxic (23). Finally, there is strong evidence that, as in other neurodegenerative disorders, synapses are the earliest anatomical targets of prion neurotoxicity, and their degeneration and dysfunction are primary causes of the cognitive and motor symptoms of the disease (76).

Further investigation of the mechanisms of prion neurotoxicity has been hampered by two major obstacles. First

is the lack of suitable *in vitro* model systems that can be interrogated at the cellular and molecular levels. There are a limited number of cell lines capable of propagating prions in culture (16, 92), and none of these exhibit signs of cytotoxicity as a result of chronic prion infection. Moreover, most of the cells used to propagate prions are transformed cell lines (e.g., N2a neuroblastoma cells), and there is very little published literature on prion infection of cultured primary neurons (30, 47). Beyond their importance for understanding the biology of prion neurodegeneration, *in vitro* systems are necessary to test potential therapeutic compounds in a systematic manner.

A second major obstacle to understanding prion neurotoxicity has been the limited information available about the normal function of PrP^C. PrP^C is a GPI-anchored, cell surface glycoprotein that is widely expressed on neurons throughout the CNS beginning early in development (48, 77). A number of physiological functions have been proposed for PrP^C (128), but the absence of a strong phenotype in PrP knockout animals has precluded the definitive identification of a normal molecular or cellular activity for PrP^C (127, 132). Importantly, PrP knockout animals do not display symptoms of a prion disease (15), suggesting that the disease phenotype is due primarily to a gain-of-function attributable to PrP^{Sc} or a related toxic species, rather than to a loss of the normal function of PrP^C. However, there are now a large number of studies demonstrating that certain manipulations of the PrP^C molecule, including the introduction of particular mutations in the flexible N-terminal domain or the binding of antibodies to specific epitopes in the globular, C-terminal domain, can endow the protein with powerful neurotoxic activities (70, 104, 111). How these artificially induced toxic activities relate to the pathophysiology of prion diseases, and whether they are triggered by conversion of PrP^C to PrP^{Sc} is uncertain. Taken together, however, these studies raise the possibility that prion neurotoxicity could involve the subversion of the normal physiological activity of PrP^C as a result of disruption of structural interactions within the protein.

In this contribution, we will review work over the past several years from our laboratory and several others in two areas relevant to understanding the mechanisms of prion neurotoxicity. In the first section, we will discuss the use of simplified, *in vitro* experimental systems (brain slices, cultured neurons) that model key features of prion neurotoxicity. We will discuss in detail the results obtained from a novel neuronal culture system developed in our laboratory that we believe reproduces some of the earliest events in prion pathogenesis at the level of individual synapses. This system has allowed us to define what we think is a core signaling pathway activated by PrP^{Sc}. In the second section, we will discuss how artificial manipulations of the PrP molecule (deletion mutations, antibody binding) can, in the absence of PrP^{Sc}, trigger neurotoxic sequelae in mice, brain slices and cultured cells. We will review work from our own laboratory, involving a combination of electrophysiological, cellular and biophysical approaches, which leads to a new structural model to explain toxic as well as physiological activities of PrP^C.

We will begin by first reviewing several topics that are conceptually relevant to understanding the mechanisms of prion neurotoxicity.

INFECTIVITY VS. NEUROTOXICITY

There is evidence that infectivity (the ability to self-propagate) and neurotoxicity (the ability to produce neuropathology) may be distinct properties attributable to different molecular forms of misfolded PrP (7). Misfolded forms of PrP purified from infected brain are known to be heterogeneous in terms of aggregation state, protease resistance and possibly protein conformation (63, 74, 96, 97, 134). Although, historically, proteinase K (PK) resistance has been used to define PrP^{Sc} in biochemical analyses, it has been estimated that a large fraction of the PrP^{Sc} present in the brain after prion infection is actually sensitive to PK digestion (63, 96, 97, 134). There is debate about the relative infectivity of the PK-resistant and PK-sensitive forms, and it has been suggested that the latter species may represent small aggregates that are particularly neurotoxic without being infectious (6, 7, 23). Our own previous work demonstrates that noninfectious, weakly PK-resistant, oligomeric forms of PrP are responsible for neuropathology in a mouse transgenic model of a familial prion disease (6, 22, 23). Consistent with a distinction between infectivity and neurotoxicity, there is evidence that prion disease progression in mice is characterized by two, mechanistically discrete phases: rapid accumulation of PrP^{Sc} and infectivity, followed by slower development of neuropathology and clinical symptoms over a time course that is inversely related to the expression levels of PrP^C (99, 100). It was postulated that the second phase occurred because of the accumulation of toxic, protease-sensitive species, although these studies did not biochemically isolate or characterize these forms.

THE ROLE OF PRP^C IN PRION NEUROTOXICITY

An important clue to the mechanism underlying prion neurotoxicity is the observation that PrP^C knockout neurons are relatively resistant to the toxic effects of PrP^{Sc} that is supplied exogenously by wild-type astrocytes or by neighboring neurons (12, 75). This result suggests that a critical neurotoxic signal is generated as part of the process by which endogenous cell surface PrP^C is converted into PrP^{Sc}, and in the absence of PrP^C, this signal is not produced. As PrP^C is normally attached to the cell surface by a glycolipid anchor, one might predict that a signal-transducing function for PrP^C would require its membrane anchoring. Consistent with this prediction, scrapie-inoculated mice expressing an anchorless form of PrP^C show an altered neuropathological profile, suggesting that the neurotoxic signaling processes normally mediated by PrP^C require its attachment to the plasma membrane (20, 21). Although there are a

number of studies suggesting signal-transducing activities for cell surface PrP^{C} (72), the pathways by which its interaction with PrP^{Sc} produces neurotoxic signals remain mysterious.

THE IMPORTANCE OF THE SYNAPSE

The terminal neuropathology of prion diseases encompasses a number of features, including spongiform change, amyloid deposition, astrogliosis and neuronal loss (71). However, some of the earliest and potentially most critical changes occur at the level of the synapse (76). Neuropathological and *in vivo* imaging studies in prion-infected mice suggest that synaptic degeneration begins very early in the disease process, predating other pathological changes, and eventually contributing to the development of clinical symptoms (5, 17, 27, 31, 41, 58, 67, 76). Synaptic pathologies include morphological and functional abnormalities, leading eventually to complete elimination of synapses. Suggesting a causative role for PrP^{Sc} in synaptic pathology, PrP^{Sc} is often found in neuropil deposits that are referred to as "synaptic-like" because they appear to surround synaptic sites (5, 17, 27, 31, 58, 67). Two-photon imaging studies of living, prion-infected animals demonstrate that the swelling of dendritic shafts and the retraction of dendritic spines occur early during the disease course, well before symptoms appear (41). Taken together, these studies pinpoint synapses, in particular dendrites and dendritic spines, as important initial targets of prion neurotoxicity. Dendritic spines are protuberances on dendrites at which synaptic contacts (usually excitatory) occur (83). Changes in their morphology are now believed to underlay synaptic plasticity associated with learning and memory, as well as degenerative events that occur during aging and neurological disease (51, 98). Given these considerations, any experimental system designed to study prion neurotoxicity must be capable of reproducing acute degenerative effects on synapses and dendritic spines.

IN VITRO SYSTEMS FOR STUDYING PRION NEUROTOXICITY

A major roadblock in studying prion neurotoxicity has been the lack of experimentally tractable model systems in which pathological changes can be studied *in vitro*. As noted above, the transformed cell lines typically used for prion propagation show no cytopathology, and so are not useful for studying the toxic effects of prions (16, 92). In principle, one might model prion synaptotoxicity using cultures of dissociated neurons (primary or iPS-derived), three-dimensional neuronal cultures, acute or chronic brain slices or brain organoids. Studies of prion neurotoxicity have thus far been restricted to brain slices and dissociated neurons (Figure 1).

Aguzzi and colleagues recently described an organotypic brain slice system in which slices of cerebellum or hippocampus can be infected with scrapie prions (34–36) (Figure 1, top). The cytotoxic readout in this system is typically neuronal death, which is registered by staining the slices with a neuronal marker like NeuN or the DNA-binding dye propidium iodide. This system has been used to demonstrate a role for calpains, reactive oxygen species, metabotropic glutamate receptors and the unfolded protein response in prion neurotoxicity, and to analyze the transcriptional response to prion infection (36, 43, 52). A drawback of this system is that it requires chronic infection of the slices with scrapie prions to generate sufficient PrP^{Sc}, which takes place over several weeks. This makes it difficult to disentangle the effects of prion propagation from prion neurotoxicity, which *in vivo* are thought to represent distinct phases of the disease process (99, 100). In addition, the synaptic abnormalities (dendritic spine loss) observed in these slices (17) occur in the context of more general pathological changes secondary to neuronal death, making it challenging to dissect the acute effects of PrP^{Sc} on synaptic structure and function.

There have been very few published studies of prion infection of neurons in culture. In one early study, Cronier *et al* (30) showed that cultured cerebellar granule neurons and astrocytes from transgenic mice over-expressing ovine PrP could be infected by ovine prions, with a proportion of the infected neurons undergoing apoptosis after several weeks in culture as the amount of PrP^{Sc} increased. In a second study, Hannaoui *et al* (47) infected cultured cerebellar granule, striatal, or cortical neurons with three different strains of scrapie prions, and reported strain-specific effects on neuronal viability that were related temporally to the accumulation of PrP^{Sc} over a time period of 10–14 days. These experiments demonstrate that chronic infection of cultured neurons results in the gradual accumulation of PrP^{Sc}, and this correlates with decreases in neuronal viability. Again, however, these systems do not allow the assessment of prion toxicity independent of propagation. In addition, the use of neuronal viability as a readout is likely to miss earlier, more subtle effects on synaptic structure and function.

A NOVEL NEURONAL CULTURE SYSTEM TO STUDY PRION NEUROTOXICITY

We were interested in analyzing the acute synaptotoxic effects of prions over the course of 0–48 h, independent of the development of a chronic infected state. We therefore adopted the strategy of adding purified preparations of PrP^{Sc} directly to the extracellular medium bathing neuronal cultures. This allowed us to assess the earliest neuronal responses elicited by interaction of exogenous PrP^{Sc} with endogenous, cell surface PrP^{C}. There is evidence that this interaction results in an extremely rapid conversion reaction, with generation of new molecules of PrP^{Sc} within minutes of the initial contact of the two conformers (44, 45). We anticipated that this system would allow us to monitor the acute effects of PrP^{Sc} formation without

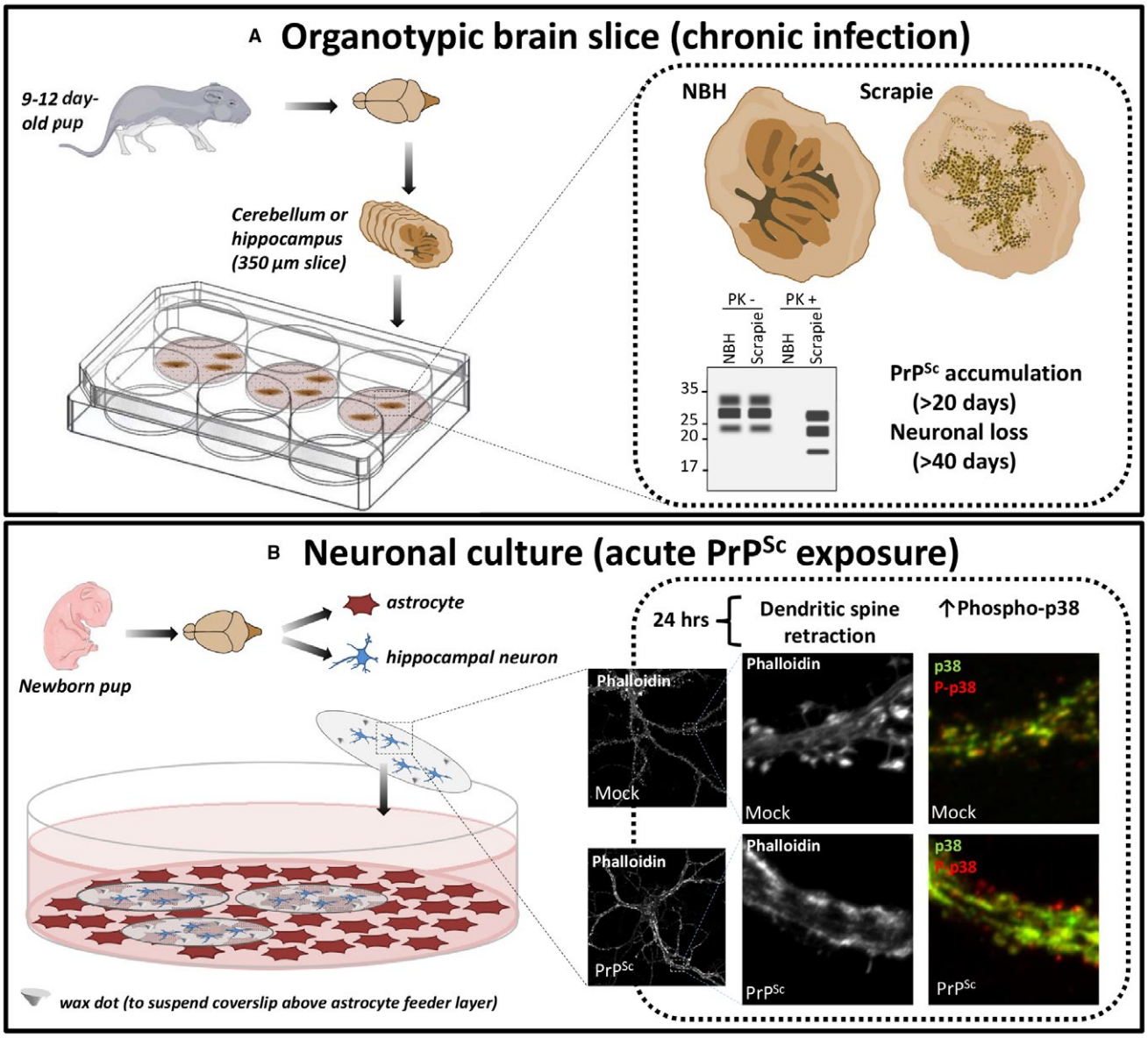

Figure 1. *In vitro systems to study prion neurotoxicity.* **A.** An organotypic brain slice system registers the effects of chronic prion infection, monitored by reductions in neuronal viability as PrP^{Sc} accumulates (34–36). **Left:** Cerebellar or hippocampal slices from WT or Tga20 (PrP-over-expressing) mice are prepared from neonatal pups and are treated with normal brain homogenate (NBH) or scrapie-infected brain homogenate. **Right:** The slices become chronically infected, accumulating PrP^{Sc} (detectable by Western blotting for PK-resistant PrP) and suffering neuronal loss over a matter of weeks. **B.** A hippocampal neuronal culture system allows analysis of the acute toxic effects of prions on synaptic structure and function (37, 38). **Left:** Neurons are isolated from hippocampi of neonatal pups and cultured at low density on coverslips that are suspended facedown, via wax dots, over a feeder layer of astrocytes. **Right:** Neurons in this system are susceptible to the synaptotoxic effects of prions within 24 hrs. Treatment with purified PrP^{Sc}, but not mock-purified material, leads to retraction of dendritic spines, revealed by staining with fluorescent phalloidin, as well as local increases in p38 MAPK phosphorylation, visualized by staining with antibodies to phospho-p38 (red) and total p38 (green). Electrophysiological abnormalities in synaptic transmission are also detectable using patch-clamping techniques (38). Images of mice were taken from Servier Medical Art (http://smart.servier.com).

the necessity for the establishment of a chronic infected state, which is thought to involve additional steps related to intracellular trafficking of PrP^{Sc} (121).

We have adopted a specialized kind of neuronal culture system (Figure 1, bottom) that has several advantages over conventional methods for co-culturing neurons and astrocytes on the same substrate (37, 60). In this system, neonatal mouse hippocampal neurons are cultured at low density on coverslips suspended face-down over a feeder layer of astrocytes. A key feature of this system is that the neurons are physically separated from the astrocyte feeder layer. This means that visualization of neuronal

morphology is greatly improved. In addition, the coverslip can be readily removed for extraction of proteins and RNA from the neurons, with minimal contamination from astrocytes, a crucial advantage for subsequent proteomic or genomic analyses. In this system, neurons can be maintained for as long as four weeks, during which time they elaborate highly differentiated axons and dendrites, and they develop functionally active synapses that can be analyzed by patch-clamping techniques. The dendrites are studded with large numbers of mature, mushroom spines, which are the postsynaptic sites of excitatory transmission. The spines can be readily visualized using fluorescent phalloidin, which stains F-actin in the spine cytoskeleton, or by filling them with cytoplasmic GFP. The cultures can be co-stained for additional markers to reveal the overall morphology of axons and dendrites, as well as the location of presynaptic and postsynaptic elements. This system is ideal for studying the effects of PrP^{Sc} on synaptic structure and function, and it provides a facile platform for genetic and pharmacological manipulation of the underlying processes.

Effect of PrP^{Sc} on synaptic morphology

Our initial validation of the system involved monitoring the effects of PrP^{Sc} on the morphology of dendritic spines (37). We found that the addition of purified PrP^{Sc} to the hippocampal cultures resulted in a rapid and dramatic retraction or collapse of dendritic spines on neurons throughout the culture, as monitored by staining with fluorescent phalloidin (Figure 1, bottom). This effect was dose-dependent, and could be detected at PrP^{Sc} concentrations as low as 2.2 μg/mL. Retraction of spines was detectable within 24 h of PrP^{Sc} exposure, and occurred prior to other changes in overall dendritic morphology or neuronal cell death, and well before chronic prion infection was established. The effect on spines was specific to PrP^{Sc}-containing samples, and was seen both with crude PrP^{Sc}-containing brain homogenates, as well as with highly purified preparations of PrP^{Sc}. Importantly, spine collapse caused by exposure to PrP^{Sc} requires the expression of full-length PrP^{C} by the target neurons, consistent with an essential signal transduction role for cell surface PrP^{C} (12, 75).

Taken together, these results demonstrated that our neuronal culture system was capable of reproducing the earliest changes in dendritic spine morphology that have been observed in the brains of living, scrapie-infected mice by two-photon imaging (41), as well as in postmortem brain sections (5, 27, 31, 58, 67). As detailed in the following sections, we have utilized this culture system to elucidate key cellular and molecular mechanisms underlying prion synaptotoxicity, to identify new, druggable therapeutic targets and to compare prion synaptotoxic pathways with the pathways operative in other neurodegenerative diseases.

Highly selective effects of PrP^{Sc} on synaptic function

We have found that PrP^{Sc} produces a strikingly selective synaptotoxic effect, specifically targeting the postsynaptic elements of excitatory synapses (38). Treatment of hippocampal neurons with purified PrP^{Sc} from infected brains caused a marked reduction in the frequency of miniature excitatory postsynaptic currents (mEPSCs), and a less pronounced but statistically significant decrease in mEPSC amplitude. These effects were not observed in neurons derived from PrP knockout mice, demonstrating that the functional as well as the morphological effects of PrP^{Sc} on synapses are entirely PrP^{C}-dependent. In contrast, PrP^{Sc} did not significantly affect the frequency or amplitude of miniature inhibitory postsynaptic currents (mIPSCs). In addition, PrP^{Sc} treatment did not change the number of inhibitory synapses, as determined by staining for gephyrin (a postsynaptic anchoring component for glycine and $GABA_A$ receptors). These results indicate that PrP^{Sc} targets primarily excitatory and not inhibitory synapses.

The reduction in mEPSC amplitude and frequency caused by PrP^{Sc} could be because of the effects on either presynaptic processes (e.g., synaptic release) or postsynaptic characteristics (e.g., number and distribution of active zones). When neurons were treated with purified PrP^{Sc}, there was a dramatic reduction in staining for GluR1, an AMPA receptor subunit, but not in staining for synaptophysin, a presynaptic marker. These data demonstrate that PrP^{Sc} exerts a highly selective effect on postsynaptic elements, with no detectable effect on presynaptic structures, even in the face of massive morphological changes in dendritic spines.

The requirement for PrP^{C}

PrP^{Sc}-induced synaptotoxicity in our neuronal culture system requires the expression of cell surface PrP^{C} by the target neurons (37). In contrast to wild-type neurons, PrP knockout neurons showed no significant change in spine number or area after treatment with PrP^{Sc}. Moreover, knockout neurons, unlike WT neurons, did not suffer any functional changes in synaptic transmission or alterations in intracellular calcium levels in response to PrP^{Sc}. These observations demonstrate that the toxicity of PrP^{Sc} is not attributable to nonspecific effects of the protein aggregates on neuronal integrity or viability, but rather require the mediation of a specific cell surface receptor (PrP^{C}). Moreover, they are consistent with numerous reports that neuronal expression of membrane-anchored PrP^{C} is necessary for prion-induced neurodegeneration *in vivo* (12, 20, 21, 75).

The PrP^{C} requirement in our assay system has allowed us to dissect which parts of the PrP^{C} molecule are essential for mediating synaptotoxic effects (37). Hippocampal neurons from two lines of transgenic mice expressing N-terminally deleted forms of PrP (Δ23-31 and Δ23-111) were completely resistant to the toxic effects of purified PrP^{Sc}. This result indicates that a small, polybasic region of PrP^{C} (residues 23-31; KKRPKPGGW) expressed on target neurons is essential for spine loss induced by exogenously applied PrP^{Sc}. This result could be attributable to the previously documented role of residues 23-31 in PrP^{C} binding to PrP^{Sc} (78, 106, 117). Alternatively, the N-terminal

domain may play a direct role in the ability of PrP^C or PrP^{Sc} to elicit downstream neurotoxic signals.

What is the precise mechanistic role of PrP^C in mediating prion neurotoxicity? One possible scenario is that synaptic degeneration results directly from binding of PrP^{Sc} to PrP^C on the cell surface. This idea is consistent with evidence that PrP^C binds very selectively to PrP^{Sc}, and that this interaction represents the first step of the conversion process by which more molecules of PrP^{Sc} are generated (32, 54, 125). In this scenario, PrP^C may be acting as a toxicity-transducing receptor, akin to other cell surface receptors that generate intracellular signals upon binding of extracellular ligands. In fact, there is evidence that PrP^C can participate in certain kinds of signal transduction phenomena (72). A related hypothesis is that PrP^C serves to trap and concentrate PrP^{Sc} molecules at the cell surface, and that the captured PrP^{Sc} molecules then cause toxic effects by virtue of their interaction with the lipid bilayer or with other cell surface proteins. This mechanism would be akin to the function of some GPI-anchored proteins that serve as non-signaling co-receptors for binding of extracellular ligands (119). A third possibility is that cell surface PrP^C is first converted to PrP^{Sc} (or some other misfolded form) which then elicits a toxic signal. This hypothesis is consistent with recent reports (45) that cell surface PrP^C is converted to PrP^{Sc} within minutes of contact with exogenously applied PrP^{Sc}. Future studies will be directed toward testing these three hypotheses. The astrocyte feeder layer in our culture system is required for trophic support of the adjacent neurons, but the contribution of astrocytes to PrP^{Sc} generation and synaptotoxicity remains to be investigated.

Assaying the neurotoxicity of different forms of PrP

What is the neurotoxic form of PrP? As outlined above, there are reasons to draw a distinction between infectious and neurotoxic forms of PrP. The neuronal culture system we have described allows us to assay neurotoxicity independent of infectivity. PrP^{Sc} purified from brain typically consists of both PK-resistant and PK-sensitive species. PK treatment results in the digestion of ~90% of the PrP^{Sc}, which represents PK-sensitive PrP^{Sc} (96, 100). We have found that there is no significant difference between the toxicity of equivalent amounts of undigested and PK-digested PrP^{Sc}, in terms of the ability of these preparations to induce dendritic spine retraction in our culture system (37). This result suggests that both PK-sensitive and PK-resistant forms of PrP^{Sc} contribute to dendritic spine loss. In addition, it demonstrates that residues 23 through ~90 of PrP^{Sc} are not essential for synaptotoxicity. In the future, our system can be used to assay the neurotoxicity of various PrP^{Sc}-like forms generated by *in vitro* conversion reactions. As some of these species are infectious, while others are not (84), the results obtained will allow analysis of the structural relationship between the toxic and infectious properties of misfolded PrP. Thus far, all of our toxicity studies have been carried out with the RML strain of scrapie prions. In the future, we can use our system to investigate whether different prion strains, which are associated with characteristic PrP^{Sc} conformations, produce strain-specific synaptotoxic effects.

The role of the neuronal cytoskeleton

Actin is abundant in dendritic spines and has been shown to regulate spine morphology (112). In addition, there is evidence that most signaling pathways linking synaptic activity to spine morphology influence local actin dynamics (53, 55). We found that PrP^{Sc}-induced spine retraction was prevented by SiR-actin (73), a fluorogenic, cell-permeable peptide derived from jasplakinolide that both stabilizes and labels F-actin (38). In contrast, the microtubule-stabilizing agent, taxol did not have a protective effect. SiR-actin also prevented the loss of the AMPA receptor subunit, GluR1, in response to PrP^{Sc}, as well as the decreases in mEPSC amplitude and frequency caused by PrP^{Sc} treatment. These data demonstrate that actin dynamics play an important role in the morphological changes in dendritic spines induced by PrP^{Sc}, and that stabilizing actin filaments can prevent these changes.

The role of glutamate receptors and calcium influx

We have found that NMDA receptor blockers, and to a lesser extent, AMPA receptor blockers, protect neurons from the synaptotoxic effects of PrP^{Sc} (38). In addition, we observed that purified PrP^{Sc} caused a rapid (within 30 minute) increase in intracellular Ca^{2+} as monitored with the calcium-sensitive dye Fluo-3 (38). The effect of PrP^{Sc} on Ca^{2+} levels was absent in neurons derived from $Prnp^{0/0}$ mice, and was completely blocked by the NMDA receptor antagonist, memantine. These results imply that one of the first steps in the PrP^{Sc} synaptotoxic signaling cascade is the activation of NMDA- and AMPA-type glutamate receptors.

This conclusion is consistent with several previous observations in the literature. First, there is evidence that PrP^C modulates the NMDA receptor's function (9) and post-translational modification (42), and also interacts physically with NMDA receptor subunits (62), processes that could be altered by binding of PrP^{Sc} to cell surface PrP^C. Second, NMDA receptors have been shown to regulate the actin cytoskeleton in dendritic spines via Ca^{2+}-induced effects on cytoplasmic actin-binding proteins (39). Finally, glutamate receptor-dependent excitotoxicity contributes to the pathogenesis of many neurodegenerative diseases, and is known to cause morphological changes in dendrites similar to those observed here in response to PrP^{Sc} (2, 56, 64, 69, 103).

A p38 MAPK synaptotoxic signaling pathway

Mitogen-activated protein kinases (MAPKs) are important signal transducers downstream of many kinds of intracellular and extracellular stimuli (79), including stressful stimuli

like excitotoxicity (28). In mammals, the MAPKs are grouped into three main families, ERKs (extracellular signal-regulated kinases), JNKs (Jun amino-terminal kinases) and p38/SAPKs (stress-activated protein kinases) (79). In the nervous system, p38 MAPK has been found to play a role in synaptic plasticity as well as in neuronal damage and survival, and it has been linked to a number of neurodegenerative diseases (28, 116). In this regard, there is evidence that p38 MAPK and its downstream substrates regulate AMPA receptor trafficking, dendritic spine morphology, cytoskeletal dynamics and synaptic transmission.

Several pieces of evidence demonstrate that p38 MAPK plays an essential role in mediating the toxic effects of PrP^{Sc} on synaptic structure and function in our neuronal culture system (38). First, pharmacological inhibitors of p38 MAPK, including those selective for the α isoform, blocked and even reversed dendritic spine collapse and defects in synaptic transmission induced by PrP^{Sc}. In contrast, pan-isoform inhibitors of ERK and JNK, the two other major classes of MAPK, were without effect. Second, genetic suppression of p38 MAPK signaling by a dominant-negative form of p38 MAPK prevented PrP^{Sc}-induced synaptic abnormalities. Finally, the amount of phosphorylated p38 in dendritic spines increased after 1 h of PrP^{Sc} treatment, and remained elevated after 24 h in the region of collapsed spines. Using selective pharmacological inhibitors, we have shown that the likely downstream targets phosphorylated by p38 MAPK include MAPK-activated protein kinase (MAPKAP or MK) isoforms 2 and 3.

Taken together, our results have allowed us to define a glutamate receptor/p38 MAPK-dependent signaling pathway underlying prion synaptotoxicity (38) (Figure 2). Importantly, this work identifies a new, druggable target that could be used to block synaptotoxic sequelae in prion diseases: p38 MAPK. Inhibitors of this kinase are already in clinical use for the treatment of inflammatory and neurodegenerative disorders (including Alzheimer's disease) (1, 10, 29, 80, 101, 133), and some of these compounds might be easily repurposed for therapy of prion diseases. The neuronal culture system described here could be readily adapted to screen additional therapeutic agents, as we have already done for some novel anti-PrP^{Sc} compounds (57).

PrP^{Sc} and Aβ active distinct synaptotoxic signaling pathways

Our neuronal culture system allows direct comparisons between pathogenic mechanisms involved in prion diseases and other neurodegenerative disorders. It has been proposed that PrP^{C} is a cell surface receptor for Alzheimer's Aβ oligomers, and that it mediates some of the neurotoxic effects of these assemblies (11, 19, 40, 68, 82). Binding of Aβ oligomers to PrP^{C} is thought to trigger a signaling pathway involving mGluR5 and Fyn kinase, and pharmacologically inhibiting this pathway has been shown to prevent Aβ neurotoxicity and ameliorate neurological symptoms in mice (46, 61, 118, 120).

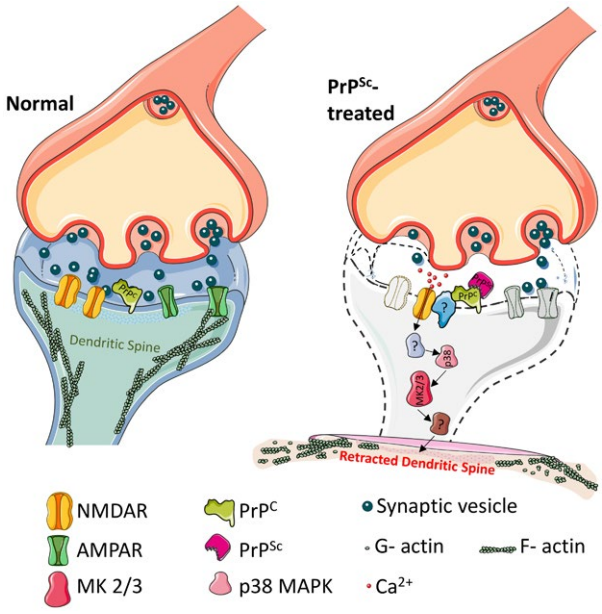

Figure 2. *Model for a prion synaptotoxic pathway*. The pathway is initiated by binding of PrP^{Sc} to endogenous PrP^{C} on the cell surface. This binding event itself, or the subsequent conversion of PrP^{C} to PrP^{Sc}, results in the activation of NMDA and AMPA receptors with influx of calcium ions. Calcium influx leads to subsequent activation of p38 MAPK and MK2/3, collapse of the dendritic spine cytoskeleton, spine retraction and decreases in synaptic transmission. Question marks indicate unknown components of the pathway. Figure was produced using Servier Medical Art (http://smart.servier.com).

We used specific pharmacological inhibitors to compare the synaptotoxic signaling pathways activated by PrP^{Sc} and Aβ oligomers (38). We found, consistent with a previous report (118), that the mGluR5 inhibitor, MPEP, completely blocked the ability of Aβ oligomers to cause dendritic spine retraction. In contrast, MPEP had no influence on PrP^{Sc}-induced retraction of dendritic spines. Moreover, a p38 MAPK inhibitor, which completely blocked PrP^{Sc} synaptotoxicity, had no significant effect on Aβ oligomer-induced dendritic spine loss. These data suggest that Aβ oligomers and PrP^{Sc} trigger different neurotoxic signaling pathways downstream of a common cell surface receptor, PrP^{C}.

MANIPIULATIONS OF THE PRP^{C} MOLECULE PRODUCE NEUROTOXIC EFFECTS

In this section of the review, we will discuss a body of work that began 20 years ago with the publication of a paper by Weissmann, Aguzzi and colleagues (104), in which the authors described a curious and striking phenomenon in some lines of transgenic mice expressing PrP molecules with deletions in the flexible, N-terminal domain. These mice displayed an ataxic neurodegenerative disorder with

specific cerebellar lesions, a phenotype that could be rescued by co-expression of wild-type PrP in the same animals. This phenomenon immediately attracted great interest because of the possibility that it could be a clue both to the normal physiological function of PrP^C, as well as to how PrP^C might generate neurotoxic signals during prion diseases. Our own laboratory began working on this subject approximately 10 years ago, and we continue to believe that it offers important insights about structural interactions within the PrP molecule, and how these influence the biological activities of the protein. The original work has now been extended to include a related phenomenon in which antibody binding to specific epitopes in the globular, C-terminal domain mimics some of the toxic effects of the deletions within the N-terminal domain (111, 131).

N-terminal deletion mutants of PrP cause neurodegeneration

As described in the original publication (104), transgenic mice expressing PrP molecules with deletions of residues 32-121 or 32-134 within the flexible, N-terminal domain developed ataxia and degeneration of the granular layer of the cerebellum at 2–3 months after birth. It was subsequently shown that smaller deletions within this region also produced a neurodegenerative phenotype (4, 13). Amazingly, we found that the smallest deletion to be examined (referred to as ΔCR), which removes only 21 amino acids from the Central Region of PrP (residues 105-125) produced the most severe phenotype, causing death of the mice within a week of birth (70). In all cases, co-expression of WT PrP in the same animals dose-dependently suppressed the neurodegenerative phenotype, with larger amounts of WT PrP required for rescue of ΔCR mice than Δ32-121/134 mice. This striking genetic interaction between mutant and WT PrP immediately suggested a molecular model in which the mutant and WT proteins physically interact with each as part of a molecular complex, in a dominant-positive fashion, or else they compete with each other for binding to a common molecular target. Consideration of these models suggests the concept that deletions in the PrP molecule somehow alter the normal biological activity of PrP^C in a way that produces neurotoxic effects.

N-terminally deleted forms of PrP induce abnormal ionic currents

In an attempt to understand why ΔCR and related deletion mutants are so toxic, we used whole-cell patch clamping techniques to record electrical activity from cells expressing these mutants. To our surprise, we observed large, spontaneous inward currents in a variety of transfected cell lines as well as primary neurons expressing the highly pathogenic ΔCR deletion mutant (8, 108–110). These currents, which were inward at negative holding potentials, were highly irregular, fluctuating randomly over a time course of seconds to minutes and reaching amplitudes of several thousand picoamps. They were present only at hyperpolarized holding potential (<−30 mV) (131). Ion substitution experiments demonstrated that the currents resulted from relatively nonselective, cation-permeable pathways in the membrane. The unusual kinetic properties of the currents suggested that they reflect the transient formation of membrane pores or localized disruptions of the lipid bilayer, rather than the presence of conventional ion channels. Several different deletion mutants spanning the central region, including Δ94-134, Δ94-110, Δ111-134 and Δ114-121, all of which produce neurodegeneration in transgenic mice, as well as three disease-associated point mutations, including P101L, G130V and G113V, also induced spontaneous currents (110). Importantly, WT PrP potently suppressed the currents induced by all of these mutants, in parallel with the ability of WT PrP to reverse the neurodegenerative phenotype of Tg(ΔCR) and Tg(Δ32-121/134) mice (70, 104). The antagonistic effects of WT and mutant PrP in both settings argue that the current-inducing activity of mutant PrPs observed *in vitro* is mechanistically related to their neurotoxicity *in vivo*.

The N-terminal domain of PrP possesses a toxic effector function

We have carried out a series of experiments designed to identify which parts of the PrP molecule are responsible for the toxic effects of the deletion mutants. These studies revealed that the flexible, N-terminal domain of PrP^C possesses a powerful, toxic effector activity, which, when unregulated, produces neurodegenerative changes in mice and abnormal ion currents in cells. In support of this conclusion, we found that the spontaneous currents induced by ΔCR and related mutants are inhibited by treatment with ligands that bind to the N-terminal domain of PrP^C, including sulfated glycosaminoglycans (pentosan polysulfate), Cu^{2+} ions and antibodies (131). We interpret these results to mean that ligands bound to the N-terminal domain prevent its interaction with the lipid bilayer and/or with other membrane-associated targets that are essential for ion channel activity and other toxic effects.

We also found that ion channel activity depends on the presence of a polybasic amino acid segment at the very beginning of the N-terminal domain of PrP (KKRPKPGGW, residues 23-31) (110, 131). ΔCR PrP carrying a deletion of the KKRPKPGGW sequence, or mutations that reverse the positive in this region, completely lacked channel activity. A requirement for the KKRPKPGGW sequence is also seen *in vivo*: removal of this segment abolishes the neurotoxicity of Δ32-134 PrP when expressed in transgenic mice (129).

The KKRPKPGGW region is similar in sequence to those of positively charged "cell penetrating peptides" or "protein transduction domains," originally derived from the HIV Tat protein, which can transiently permeabilize

the lipid bilayer by interacting with negatively charged membrane phospholipids (50, 122). An analogous mechanism may account for the ability of ΔCR PrP to create transient pores in the membrane, by acting as a "tethered protein transduction domain." Support for this model is provided by our demonstration that a C-terminally lipidated, recombinant form of ΔCR PrP incorporated into synthetic liposomes created membrane pores that were permeable to fluorescent dyes and ionic quenchers (25). An alternative model is that the N-terminal polybasic domain interacts with other membrane proteins, for example endogenous ion channels or channel-modulating proteins, to induce current activity.

In order to determine whether N-terminal domain can function as an autonomous effector, we constructed a series of chimeric proteins (collectively designated PrP(N)-EGFP-GPI) consisting of various lengths of the N-terminal domain of PrP^C (residues 23-109) fused to an EGFP molecule that was equipped with the GPI addition signal from PrP^C (131). We found that the PrP(N)-EGFP-GPI constructs induced spontaneous currents similar to those produced by ΔCR PrP, with the amount of current activity increasing as the length of the N-terminal segment became longer. These currents, like those associated with ΔCR PrP, were silenced by application of pentosan polysulfate, Cu^{2+} ions and N-terminal antibodies, and by the removal of residues 1-31.

Taken together, these results indicate that certain structural alterations (deletion of residues in the central region; absence of the C-terminal domain) allow the N-terminal domain of PrP^C to acquire a powerful, toxic effector function, which is manifested by abnormal ionic currents in cultured cells and neurodegeneration in transgenic mice.

Anti-prion antibodies cause neuronal toxicity

Several groups, including our own, have found that another kind of structural perturbation, caused by binding of antibodies to the C-terminal domain of PrP^C, also unmasks latent toxic activities. We have reported that three different antibodies (POM1, D18 and ICSM-18) targeting overlapping epitopes in helix 1 induce ionic current activity in cells expressing WT PrP^C (131). The properties of these currents are identical to those of spontaneous currents associated with ΔCR PrP, in terms of their sporadic nature, their blockage by N-terminal ligands (pentosan sulfate and antibodies targeting the octapeptide repeat domain) and their absolute dependence on the presence of the 23-31 region.

In addition to acutely inducing ionic currents, helix 1 antibodies (POM1, D18 and ICSM18) cause major degenerative changes in the dendrites of cultured hippocampal neurons when applied for 48 h (131). This antibody-induced dendritic degeneration is, like the currents induced by the same antibodies, entirely dependent on the N-terminal domain of PrP^C, and is blocked by N-terminal ligands and deletions of residues 23-31. The parallel characteristics of the ionic currents and the dendritic changes induced by C-terminal antibodies suggest a mechanistic connection between the two phenomena, perhaps via the activation of excitotoxic pathways. Dendritic varicosities similar to those caused by anti-PrP antibodies are a characteristic feature of glutamate excitotoxicity (49), and glutamate excitotoxicity has been implicated in neuronal degeneration induced by ΔCR PrP (8, 24).

Studies from two other laboratories have also reported that antibodies targeting specific epitopes in the structured domain of PrP^C cause neuronal death when administered *in vivo* or in brain slices (43, 95, 107, 111). Investigation of the underlying mechanisms revealed the involvement of several different pathways, including the generation of reactive oxygen species, calpain activation and stimulation of the PERK arm of the unfolded protein response (52, 111). It remains to be determined whether these pathways are operative in our neuronal culture system, in which we observe acute changes in dendritic morphology without loss of neuronal viability.

Aside from their relevance to understanding the mechanisms of PrP toxicity, these studies have important clinical implications, as anti-PrP antibodies have been proposed as therapeutic agents for the treatment of both prion and Alzheimer's diseases (26, 66, 130). However, if the antibodies themselves have significant neurotoxic potential, this could constitute an unacceptable side effect precluding their use. Unfortunately, there is now considerable controversy surrounding this subject. Although several studies, including our own (131), have reported neurotoxic effects of anti-PrP antibodies (52, 95, 107, 111), another study found that the same antibodies were nontoxic (65). It has been claimed that the neurotoxic effects observed are nonspecific, and related to the use of high antibody concentrations (90, 91), despite the fact that mice lacking PrP expression, or expressing N-terminally deleted forms of PrP (Δ23-31 or Δ23-111), are resistant to antibody toxicity (94, 131). In light of these uncertainties, it would seem prudent to exercise caution in administering anti-PrP antibodies to patients for therapeutic purposes.

Structural mechanisms regulating the toxic activity of PrP^C

The findings described thus far suggest a structural model in which the flexible, N-terminal domain of PrP^C functions as a powerful toxicity-transducing effector whose activity is tightly regulated in *cis* by the globular C-terminal domain (Figure 3). Deletions of the hinge region connecting the N- and C-terminal domains (as in ΔCR PrP), or complete elimination of the C-terminal domain (as in the PrP(N)-EGFP-GPI constructs), would be predicted to interfere with the regulatory interaction between these two domains, thereby freeing the N-terminal domain to produce spontaneous ionic currents and neurodegenerative changes. Antibodies targeting helix 1 in the C-terminal domain would have a similar effect by disrupting the regulatory

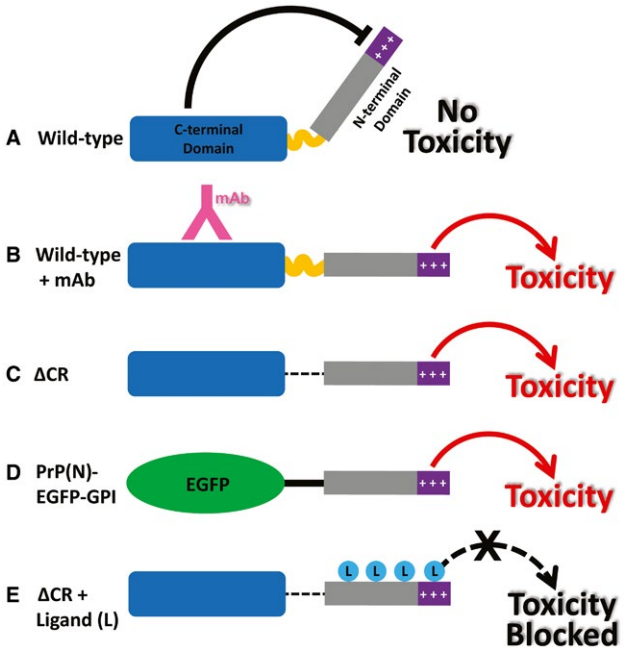

Figure 3. *Models for the neurotoxic effects of PrP.* **A.** The C-terminal domain of PrP^C negatively regulates the toxic effector function of the N-terminal domain. +++, basic residues within the 23-31 region at the extreme N-terminus, which are essential for the toxic action of PrP. **B.** Binding of monoclonal antibodies to the C-terminal domain disrupts this regulatory interaction, releasing the N-terminal domain to produce toxic effects. **C.** Deletion of the central region, as in ΔCR PrP, produces a similar loss of regulation, with toxic consequences. **D.** When EGFP is substituted for the C-terminal domain of PrP^C, regulation is also lost. **E.** Binding of ligands (PPS, antibodies, Cu^{2+}) to the N-terminal domain of ΔCR PrP blocks its ability to exert toxic effects. Reprinted from (131).

action of the C-terminal domain on the N-terminal domain. Ligands targeting the N-terminal domain, as well as deletion of residues 23-31, would abrogate these toxic effects. Aguzzi and colleagues have proposed a similar model to explain the effect of anti-PrP antibodies (111).

NMR studies we have carried out in collaboration with the laboratory of Glenn Millhauser provide direct support for this model. In these experiments, we performed ^1H-^{15}N HSQC NMR analysis of PrP in the presence and absence of Cu^{2+} ions. Cu^{2+} ions are physiological ligands that bind to histidine residues in the octapeptide repeat region within the N-terminal domain (124). Broadening of specific NMR peaks in the structured C-terminal domain as a result of Cu^{2+}-induced paramagnetic relaxation enhancement is then used as an indicator of the proximity of the corresponding residues to Cu^{2+} ions bound to the octapeptide repeats. Previous studies had shown that that both Cu^{2+} and Zn^{2+} ions drive the N-terminus of wild-type PrP^C to associate with the C-terminal domain (33, 113). We found that, compared to WT PrP, ΔCR PrP showed many fewer residues in the C-terminal domain that were affected by the presence of Cu^{2+} ions bound to the N-terminal domain. This indicated diminished interaction between the two domains in this mutant, consistent with the proposed release of regulatory inhibition (131). In subsequent experiments, we have used chemical cross-linking in combination with mass spectrometry to pinpoint specific interacting residues in the N- and C-terminal domains, and how these are affected by toxic mutations (manuscript submitted).

Implications for pathology and physiology

The toxic effects we have described above are all produced by artificial manipulations of the PrP^C molecule, including the introduction of specific deletion mutations, or binding of antibodies. How do these effects relate to neurotoxic processes that occur during the course of a naturally occurring prion disease, or other neurodegenerative disorders where PrP^C is thought to play a role? One hypothesis is that pathologic ligands, including PrP^{Sc} or Aβ, which bind to PrP^C, produce neurotoxic effects by disrupting the normal regulatory cis-interaction between the N- and C-terminal domains, thereby unleashing the toxic effector activity of the N-terminal domain. Consistent with this model, Aguzzi and colleagues have reported that prion infection and anti-PrP antibodies activate similar downstream neurotoxic pathways in brain slices (52). On the other hand, mice expressing N-terminally truncated PrP^C remain susceptible to prion diseases (114, 117), arguing against a primary neurotoxic role for the N-terminal domain. However, the disease course is considerably prolonged in these animals, which may reflect the recruitment of additional, less efficient toxic pathways, or else an effect of the deletions on the PrP^C-PrP^{Sc} conversion process. One test of the toxic effector hypothesis would be to determine whether the exposure of PrP^C-expressing cells to PrP^{Sc} or Aβ induces abnormal ionic currents similar to those produced by antibodies and deletion mutations. Thus far, we have not observed such currents (unpublished data). At this point, the disease relevance of the deletion- and antibody-induced toxic activities of PrP requires further investigation.

It is also possible that the effects of deletion mutations and antibodies reflect aberrations in the normal physiological activity of PrP^C. For example, natural ligands, including proteins, small molecules or metal ions, may exist, whose binding to PrP^C regulates a physiologically relevant effector activity of the N-terminal domain. Previous studies have implicated the N-terminal domain in several physiological activities of PrP^C (85, 86, 102, 105, 115, 126), some of which may be regulated by the interaction with the C-terminal domain. Copper ions are examples of natural ligands, binding of which to the octapeptide repeats promotes docking of the N- and C-terminal domains (33). This phenomenon may be important if PrP^C functions as a sensor or transporter of these divalent metal ions. Endogenous ligands for the globular domain may also exist, which either enhance or disrupt the N-C interaction.

SUMMARY AND PERSPECTIVE

Although the mechanisms underlying prion propagation and infectivity are now well established, the processes accounting for prion toxicity and pathogenesis have remained less clear. Beyond its role as a precursor to infectious PrP^{Sc}, the cellular form of the prion protein, PrP^{C}, functions as an essential mediator or transducer of PrP^{Sc}-initiated neurotoxicity. Progress in understanding the nature of prion neurotoxicity has been hampered by the dearth of suitable *in vitro* experimental systems that model the earliest neurotoxic events at the level of synapses, which are key pathological targets of prions. A hippocampal neuronal culture system we have developed has allowed us to define a core prion synaptotoxic pathway involving the activation of NMDA and AMPA receptors, and the stimulation of p38 MAPK phosphorylation. Another line of investigation into prion toxicity is based on the striking observation that certain structural manipulations of the PrP^{C} molecule itself (deletion mutations and antibody binding) endow the protein with powerful toxic activities, including induction of neurodegeneration in transgenic mice and abnormal ionic currents in cultured cells. These effects are caused because of altered intramolecular interactions between the globular C-terminal and flexible N-terminal domains of PrP^{C}, resulting in disinhibition of a toxic effector activity of the latter.

Much remains to be learned about prion neurotoxicity. We need to identify additional, missing steps in the synaptotoxic cascade shown in Figure 2, and understand the role of PrP^{C}-PrP^{Sc} conversion in this process. Discovery-based genomic and proteomic approaches may help in this regard. We also need to validate findings from neuronal cell culture in other systems, including brain slices or organoids, and mouse models. We will also want to exploit the therapeutic potential of our neuronal culture system, in terms of identifying novel drugs and drug targets that inhibit prion neurotoxicity. Such compounds could be combined in a dual therapy with drugs that block prion replication. The artificially induced toxic effects of N-terminal deletion mutants like ΔCR and anti-C-terminal PrP antibodies remain an intriguing puzzle requiring further investigation. We need to determine whether these effects relate to processes occurring during the disease state, and whether they reflect aberrations of normal, physiological states of the PrP^{C} molecule. Finally, we will want to investigate how the neurotoxic mechanisms uncovered in prion diseases relate to other neurodegenerative disorders, many which may display features of prion-like propagation (59), and some of which may even involve PrP^{C} as a cell surface receptor for protein oligomers (3, 68). Investigations of prion neurotoxicity hold great promise from both a biological and medical standpoint, and they constitute the next major frontier of prion research.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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