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Copper-induced structural conversion templates prion protein oligomerization and neurotoxicity

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Prion protein (PrP) misfolding and oligomerization are key pathogenic events in prion disease. Copper exposure has been linked to prion pathogenesis; however, its mechanistic basis is unknown. We resolve, with single-molecule precision, the molecular mechanism of Cu2+-induced misfolding of PrP under physiological conditions. We also demonstrate that misfolded PrPs serve as seeds for templated formation of aggregates, which mediate inflammation and degeneration of neuronal tissue. Using a single-molecule fluorescence assay, we demonstrate that Cu2+ induces PrP monomers to misfold before oligomer assembly; the disordered amino-terminal region mediates this structural change. Single-molecule force spectroscopy measurements show that the misfolded monomers have a 900-fold higher binding affinity compared to the native isoform, which promotes their oligomerization. Real-time quaking-induced conversion demonstrates that misfolded PrPs serve as seeds that template amyloid formation. Finally, organotypic slice cultures show that misfolded PrPs mediate inflammation and degeneration of neuronal tissue. Our study establishes a direct link, at the molecular level, between copper exposure and PrP neurotoxicity.

INTRODUCTION

Protein misfolding and oligomeric protein accumulation are key pathogenic mechanisms in many major neurodegenerative disorders, including prion diseases, Parkinson’s disease, and Alzheimer’s disease (1). Propagation of protein misfolding in these disorders is believed to proceed through a common prion-like mechanism, which means that proteins misfold, impose their structures on natively folded proteins, and template their aggregation (2). Although the structure of natively folded prion protein (PrP) has been resolved (3–6), the mechanistic basis for its misfolding, aggregation, and neurotoxicity is unknown.

PrP is a metal-binding protein; the unstructured N-terminal tail of PrP contains four copies of an octarepeat sequence, which bind a number of divalent ions, including Cu2+, Ni2+, and Mn2+ (Fig. 1A) (7–9). Metal ions also bind to an additional site in the flexible region between the octarepeats and the C-terminal domain (10). It has been proposed that Cu2+ ions play a role in PrP structural conversion (11); however, direct molecular proof for the role of Cu2+ in PrP misfolding and aggregation is lacking. Biochemical experiments show that PrPs incubated with Cu2+ ions misfold from a native, a helix-rich structure to a predominantly β-sheet conformation that is resistant to proteolytic digestion (12, 13). Along the same lines, aggregation of PrP is enhanced either by the insertion of additional octapeptide repeats or by exposure to high concentrations of Cu2+ (14, 15). However, the mechanisms by which Cu2+ induces PrP misfolding, aggregation, and neurotoxicity are unknown.

Here, we resolve, with single-molecule precision, Cu2+-induced misfolding of PrP under physiological conditions. We also demonstrate that the misfolded PrPs serve as seeds for the templated formation of aggregates, which mediate inflammation and degeneration of neuronal tissue. We compare the biophysical properties of full-length, human PrP with globular protein lacking the N-terminal octapeptide repeats in the presence and absence of Cu2+, Ni2+, and Mn2+ (Fig. 1). Using a fluorescence-based single-molecule assay, we demonstrate that PrP monomers misfold to a protease-resistant conformation before oligomer assembly; the N-terminal region and Cu2+ ions mediate misfolding. This is a striking result because the conventional model is that protease resistance is acquired only by oligomers; in fact, protease resistance is often used as a readout for PrP oligomerization (16). Using single-molecule force measurements with an atomic force microscope (AFM), we show that misfolded PrP monomers have an almost 900-fold higher affinity (Kd) compared to the native isoform and rapidly form oligomers. Using real-time quaking-induced conversion (RT-QuIC) (17), which is a cell-free seeding assay designed for rapid clinical diagnosis of prion disease, we show that misfolded PrPs form active seeds that template aggregation. Finally, to investigate the effect of Cu2+-induced PrP misfolding on neuronal viability, we quantify protein markers for inflammation, apoptosis, and oxidative stress in brain explants exposed to native and misfolded PrPs. Analysis of these organotypic slice cultures provides direct molecular proof that the misfolded PrPs mediate neuroinflammation and neurodegeneration. Together, our results identify conditions for Cu2+-induced PrP misfolding, oligomerization, and neurotoxicity.

RESULTS

N-terminal region and Cu2+ ions mediate protease resistance in PrP monomers

A common characteristic of PrP misfolding is the conversion of native, protease K (PK)–sensitive PrP (PrPSEN) into an isoform that is prone to aggregation and resists PK digestion (PrPRes) (18–20). To identify misfolded PrPs and determine the role of different metal ligands and of the N- and C-terminal domains in PrP misfolding, we developed a fluorescence-based single-molecule PK-resistance assay. The single-molecule resolution...
of our assay allowed us to measure the misfolding of PrP monomers and avoid artifacts introduced by higher-order oligomerization. In our assay, the PrPs were first biotinylated and then covalently immobilized on a glass substrate decorated with maleimide-functionalized polyethylene glycol (PEG) tethers (Materials and Methods). Surface density measurements (Materials and Methods; figs. S1 and S2) showed that the PrPs bound to the surface were well-separated monomers (table S1). The immobilized PrP monomers were incubated overnight either in the presence or in the absence of 1 mM Ni\(^{2+}\), Mn\(^{2+}\), or Cu\(^{2+}\) ions; dissociation constants indicate that the PrP would be saturated with the divalent ions under these experimental conditions (11). The samples were then treated with PK for different time periods (0 to 13 hours) and subsequently labeled with fluorescent streptavidin (Fig. 2C). On the other hand, biotinylated PrP\(^{\text{sen}}\) was readily digested by PK, which resulted in a decreased binding to fluorescent streptavidin (Fig. 2C). On the other hand, biotinylated PrP\(^{\text{sen}}\) was readily digested by PK, which resulted in a decreased binding to fluorescent streptavidin (Fig. 2C).

In contrast, fluorescence signal from the negative control was completely eliminated after 10 hours of PK digestion (Fig. 2, D and E). The data in Fig. 2E were acquired from a total (summed across all time points) of 9634, 12,438, 10,680, and 8869 PrP\((90–231)\) molecules in the absence and in the presence of Cu\(^{2+}\), Mn\(^{2+}\), and Ni\(^{2+}\), respectively. These results show that monomeric PrP\(^{\text{res}}\) formation requires both the intrinsically disordered N-terminal region and the Cu\(^{2+}\) ions; elimination of either results predominantly in PrP\(^{\text{sen}}\) conformation.

**Full-length PrPs incubated in Cu\(^{2+}\) ions have a higher binding affinity**

Next, we proceeded to use single-molecule AFM force measurements (21, 22) to characterize the kinetics of the initial stage of PrP aggregation and to identify the role of different divalent metal ions and protein domains in this process. AFM–force spectroscopy (AFM–FS) has previously been used to study the misfolding and interactions of neurodegenerative proteins (23, 24). Single-molecule measurements with tethered PrP monomers enabled us to directly determine association and dissociation rates while avoiding artifacts due to the formation of higher-order oligomers. Relative dimerization rates (relative on-rates) were determined from the measured binding probabilities, whereas off-rates were measured using single-molecule dynamic FS. In both sets of experiments, the PrP constructs were covalently immobilized on an AFM tip and substrate (Fig. 3, A and B; Materials and Methods) and incubated in either the presence or the absence of 1 mM Cu\(^{2+}\), Mn\(^{2+}\), or Ni\(^{2+}\). The AFM tip and surface were first brought into contact, enabling opposing PrPs to dimerize. The tip was then withdrawn from the substrate and the force required to rupture the PrP dimer was recorded. If the PrPs did not interact, no unbinding forces were recorded. However, dimer formation resulted in unbinding events from the substrate and the force required to rupture the PrP dimer was recorded. If the PrPs did not interact, no unbinding forces were measured (Fig. 3C). However, dimer formation resulted in unbinding events characterized by the nonlinear stretching of the PEG tethers (Fig. 3D); the stretching of PEG served as a molecular fingerprint for PrP dimer formation because its extension under load has been extensively characterized (25). As described in Materials and Methods, the unbinding events were fit to an extended freely jointed chain (FJC) model (25) using a total least squares (TLS) fitting protocol; only unbinding events that occurred at a distance that corresponds to the contour length of two PEGs in series were used in further analysis (Materials and Methods).
Because each PrP can be tethered to the AFM tip or substrate via either one or two PEGs (covalently bound to Cys179 and/or Cys214), we fitted the unbinding events to alternate FJC models that assumed stretching of either a single tether or two parallel tethers (26). Our analysis showed that, in 78% of the selected events, PrP was immobilized on both the AFM tip and substrate through a single PEG tether. In contrast, 21% of unbinding corresponded to rupture events where one of the PrPs was tethered via two PEG linkers, whereas the other PrP was immobilized via a single PEG. Only 1% of the events corresponded to unbinding events where both PrPs were immobilized via two tethers. However, this heterogeneous tethering distribution did not affect the measured binding probability or unbinding force; identical results were obtained when events corresponding to the stretching of only single tethers were compared to measurements that correspond to the stretching of both single and parallel PEG tethers. The measurements were repeated several thousand times at 6 or 11 different pulling velocities and at different positions of the substrate. The surface density of PrP was estimated for every condition using single-molecule fluorescence microscopy (Materials and Methods; figs. S1 and S2); measured probabilities were normalized for direct comparison.

To identify the role of the unstructured N-terminal region in PrP interactions, we first compared the binding probability of full-length PrP(23–231) and globular PrP(90–231) in the absence of divalent metal ions. We established that our selected unbinding events had a nonspecific
binding probability of $0.15 \pm 0.04\%$ by eliminating PrP from either the tip or the substrate (Fig. 3E). A comparable binding probability of $0.05 \pm 0.02\%$ was measured for opposing PrP(90–231), suggesting that globular PrPs lacking the N-terminal domain do not form dimers (Fig. 3E). In contrast, PrP(23–231) dimerized with a fivefold higher probability ($0.81 \pm 0.04\%$), showing that the N-terminal tail plays a role in dimerization (Fig. 3E).

Next, we quantified the effect of divalent ions on the homotypic binding probabilities between opposing PrP(23–231) (Fig. 3A) and between opposing PrP(90–231) (Fig. 3B). Homotypic PrP(90–231) binding probabilities were comparable to nonspecific adhesion; interaction frequencies in the presence of Mn$^{2+}$, Ni$^{2+}$, and Cu$^{2+}$ and in the absence of divalent ions were $0.03 \pm 0.02\%$, $0.03 \pm 0.02\%$, $0.14 \pm 0.08\%$, and $0.05 \pm 0.02\%$, respectively (Fig. 3F). However, homotypic PrP(23–231) binding probability was significantly enhanced by the presence of divalent ions. Although a binding probability of $0.81 \pm 0.04\%$ was measured in the absence of divalent ions, the interaction frequencies increased to $1.85 \pm 0.18\%$, $3.92 \pm 0.34\%$, and $4.21 \pm 0.45\%$ when Mn$^{2+}$, Ni$^{2+}$, and Cu$^{2+}$, respectively, were added to solution (Fig. 3F).

On the basis of the probabilities of interaction, we calculated the relative on-rates for PrP(23–231) interactions (table S2) (27). Because the association rates depend on factors besides microscopic binding rates, such as protein surface density, the local geometry of the tip and substrate, and the location of the proteins, all rates were calculated relative to the association rate of homotypic PrP(23–231) interactions in the absence of divalent ions (Materials and Methods) (27). The on-rate of homotypic PrP(23–231) was increased 6.3 ± 0.9 times in 1 mM Cu$^{2+}$, 5.8 ± 0.7 times in 1 mM Ni$^{2+}$, and 2.6 ± 0.3 times in 1 mM Mn$^{2+}$ (Fig. 3F and table S2).

Next, using PrP(23–231), we calculated the dissociation rate by measuring the most probable rupture force at different rates of application of force; the data were fitted to the Bell-Evans equation (28) to extract the intrinsic off-rate ($k_{off}$) and the width of the potential energy barrier ($\Delta x_0$) that inhibits dimer dissociation (Fig. 4 and figs. S3 to S6; Materials and Methods). Our data show that PrP dimer off-rates in the absence of divalent ions and in the presence of Mn$^{2+}$ and Ni$^{2+}$ were comparable; the measured off-rates corresponded to $3.1 \times 10^{-2}$, $1.3 \times 10^{-2}$, and $1.0 \times 10^{-2}$ s$^{-1}$, respectively (Fig. 4, A to C, and table S2). In contrast, the measured off-rate in Cu$^{2+}$ was two orders of magnitude smaller with a value of $2.2 \times 10^{-4}$ s$^{-1}$ (Fig. 4D and table S2). This indicates that dimers of PrP in Cu$^{2+}$ have lifetimes that are around 100 times longer than those of PrP$^{\text{sen}}$ dimers.

Using the measured off-rates and the relative on-rates determined from the binding probabilities, we calculated the relative association
constant \((K_A)\) for PrP(23–231) in the presence of different metal ions (Materials and Methods). The \(K_A\) of PrP incubated in Cu\(^{2+}\), Ni\(^{2+}\), and Mn\(^{2+}\) was 863, 18, and 6 times higher, respectively, than that of PrP in the absence of divalent ions (table S2).

**PrPs incubated in Cu\(^{2+}\) serve as seeds for templated aggregation**

Next, we used RT-QuIC (17), an in vitro seeding assay, to test whether PrPs exposed to divalent metal ions form seeds that template aggregation. In a conventional RT-QuIC assay, trace amounts of scrapie seeds are added to a recombinant PrP substrate and repeatedly agitated (17); amyloid fiber formation is monitored through an increase in fluorescence intensity when thioflavin T (ThT) binds to the aggregate. If the seeds are active, a more rapid onset of aggregation is measured with increasing seed concentration. In our measurements, we generated seeds by incubating human recombinant PrP(23–231) in the absence of divalent ions, a more rapid onset of aggregation is measured with increasing seed concentration (one-tailed t-test) and energy barrier width (\(k_{off}\)). Error bars indicate the SD of forces. Ninety percent confidence interval was calculated using a bootstrap with replacement protocol. (B to D) Similar analysis for PrP(23–231) in (B) 1 mM Mn\(^{2+}\) (709 events), (C) 1 mM Ni\(^{2+}\) (1133 events), and (D) 1 mM Cu\(^{2+}\) (606 events).

![Figure 4](https://example.com/figure4.png)

Fig. 4. Dissociation rate of PrP(23–231) dimers measured using single-molecule dynamic FS. (A) Plots of the measured rupture forces and loading rates in the absence of divalent ions. Colored open squares correspond to individual unbinding events at different loading rates (556 events). Black circles representing the most probable rupture forces were fit to the Bell-Evans model (black dashed line) to determine the off-rates (\(k_{off}\)) and energy barrier width (\(k_{on}\)). Error bars indicate the SD of forces. Ninety percent confidence interval was calculated using a bootstrap with replacement protocol. (B to D) Similar analysis for PrP(23–231) in (B) 1 mM Mn\(^{2+}\) (709 events), (C) 1 mM Ni\(^{2+}\) (1133 events), and (D) 1 mM Cu\(^{2+}\) (606 events).
suggesting an inverse correlation as predicted by theory (Fig. 5D). Similarly, when PrP(23–230) seeds prepared in 10 μM Cu2+ ions were used in the experiments, a more rapid onset of aggregation with increasing seed concentration was measured (Fig. S12). In contrast, a decrease in \(T_{th}\) with increasing seed concentration was not observed when seeds were prepared in Mn2+ and Ni2+ ions (one-tailed t test; \(P = 1\) and 0.81, respectively) (Fig. 5, E and F). These results demonstrate that only PrP(23–230) exposed to Cu2+ ions serve as seeds for the formation of PrP aggregates.

**PrPs incubated in Cu2+ induce neuroinflammation and neurodegeneration in brain tissue**

Finally, we demonstrated the cytotoxic nature of Cu2+-induced PrP amyloids using a mouse organotypic slice culture assay. Slices of
brain tissue from young mice were incubated with either misfolded PrP(23–230) prepared by pre-exposure to Cu2+ ions (PrP-Cu2+) or PrP(23–230) monomers that were not pre-exposed to Cu2+ (PrP–no metal). PrP-Cu2+ and PrP–no metal were prepared using a protocol identical to that of the RT-QuIC assay but without adding ThT (Materials and Methods). At the end of the treatment period, whole-cell lysates were prepared from the brain slices, and three protein markers for neuroinflammation and neurodegeneration were assayed using Western blot analysis (Materials and Methods). We first characterized the levels of glial fibrillary acidic protein (GFAP), an important indicator of astrocytic activation during neuroinflammation. Western blot analysis indicated that PrP-Cu2+ results in higher GFAP immunoreactivity than PrP–no metal or control slices that were not exposed to PrP (Fig. 5, G and H). Next, because elevated gliosis enhances oxidative stress and downstream neuronal degeneration, we also analyzed the expression of protein kinase C-δ (PKC-δ), a key oxidative stress–sensitive kinase of the novel PKC family. Activation of PKC-δ has been shown to induce neuronal cell death, and its relevance to oxidative stress–mediated neurodegeneration is seen in many neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, and prion diseases (32–35). We observed significantly increased levels of PKC-δ when brain slices were incubated with PrP-Cu2+. Finally, because PKC-δ is a proapoptotic kinase, its activation results in a downstream apoptotic protein cascade and neuronal degeneration. We therefore investigated the activation of one such proapoptotic protein, Bax. Our Western blot analysis showed an increase in Bax activity in mouse brain slices incubated with PrP-Cu2+. We ruled out any role of the minuscule amounts of Cu2+ ions, which are introduced into the slice cultures upon addition of protein seeds, in enhancing these protein markers (fig. S13). Together, the notable increase in the levels of GFAP, PKC-δ, and Bax upon addition of PrP-Cu2+ suggests that Cu2+-induced misfolding contributes to neurotoxic signaling and mediates neuronal cell death (Fig. 5, G and H).

**DISCUSSION**

Because PK degrades accessible regions of proteins that have flexible secondary structures, proteolytic digestion is routinely used to identify PrP domains that have misfolded into a β sheet–rich conformation. We therefore developed a fluorescence assay with single-molecule sensitivity to measure the PK resistance and, consequently, the misfolding of PrP monomers. Our experiments demonstrate that the unstructured N-terminal region and Cu2+ ions mediate PrP monomer misfolding and PK resistance. It has been reported that Cu2+ binding reduces the folding stability of PrP, making a conformation transition more thermodynamically favorable (36). Ensemble biophysical experiments with PrP(23–231) show that exposure to Cu2+ ions results in a PK-resistant conformation and that PrPsen isoforms are observed in Zn2+, Mg2+, Ca2+, Mn2+, and Fe2+ (12). In contrast, PrP(90–231) forms oligomeric aggregates rich in β sheets only at acidic pH (37).

It has been suggested that the direct inhibition of PK by copper may be responsible for previous reports of Cu2+-induced PK resistance in ensemble biochemical experiments (38). However, in our experiments, the Cu2+ ions are washed out of solution before PK incubation. Thus, our fluorescence experiments demonstrate that Cu2+ promotes PK resistance through direct interaction with PrP(23–231) and not by inhibiting PK itself. Furthermore, we show that PrP(90–231) exposed to Cu2+ remains more PK-sensitive, confirming that the Cu2+ ions do not inhibit PK activity. Previous studies show that PK degrades the N-terminal region of scrapie PrP without compromising pathogenicity (39, 40). Experiments with transgenic mice also show that globular PrP is sufficient for propagating scrapie in vivo (41). In contrast, both our single-molecule and RT-QuIC data show that the N-terminal region is essential for Cu2+-induced misfolding and aggregation in vitro. One possible explanation for this inconsistency is that Cu2+ ions trigger PrP(23–231) monomers to misfold into a distinct, noninfective strain that resists PK digestion (42).

In solution, unmodified PrPsen and PrPsen tagged with biotin and PEG tethers are equally sensitive to digestion by PK. We demonstrated this by using PK to digest unlabeled PrP(23–230), biotin/PEG-labeled PrP(23–230), unlabeled PrP(90–230), and biotin/PEG-labeled PrP(90–230) in solution (fig. S14). However, because the PrPs are tethered to a surface in our single-molecule protease resistance assay, biotinylated fragments of the digested PrP that remain immobilized following PK treatment likely account for the 40% fluorescence background measured when PrPsen is digested with PK for 10 hours. A candidate biotinylated fragment is the region of PrP containing three biotinylation sites (Lys185, Lys194, and Lys204) that lies in between the two Cys tether points. However, because the tethered fragments are expected to contribute a similar fluorescence background signal when PrPsen is digested with PK, this isoform is at least 60% more PK-resistant than PrPsen. Our hypothesis on the origin of the background signal is supported by the complete elimination of fluorescence signal measured in the negative control (biotinylated BSA), which lacks tethered, biotinylated peptide fragments (Fig. 2, D and E).

By comparing the interaction probabilities of PrP(23–231) and PrP(90–231), we show that the unstructured N-terminal tail has a dominant influence on the first stage of oligomerization, that is, PrP dimerization. Our data suggest that in the absence of the N-terminal tail, PrP(90–231) does not dimerize within the 100-ms time window that the AFM tip and surface are held in contact. It has previously been suggested that one of the key functions of the unstructured N-terminal region is enhancing the efficiency of aggregation (43). This effect may be mediated by the polybasic region (residues 23 to 31) in the disordered N-terminal region, which is believed to be involved in PrP propagation (44). Our data also indicate that the relative association constant of full-length PrP incubated with Cu2+ ions was 863 times higher than the affinity in the absence of divalent ions. This increase in relative K_A could arise from a Cu2+-induced tertiary structural conversion of PrP(23–231) (45).

In our single-molecule force and fluorescence assay, PrPs were immobilized by reducing the intramolecular disulfide bond between Cys179 and Cys214 and functionalizing the Cys residues with PEG tethers. Because tagging the Cys residues with PEG tethers can potentially destabilize PrP and promote misfolding, we used nuclear magnetic resonance (NMR) spectroscopy, dynamic light scattering, circular dichroism (CD) spectroscopy, and thermal denaturation experiments to confirm that PrP molecules are in a stable, native, monomeric conformation upon functionalization. We confirmed that the PrPs remained in their native conformation following disulfide bond reduction and PEG labeling using CD spectroscopy (Supplementary Materials; fig. S15) and 1H-NMR (Supplementary Materials; figs. S16 to S23). Our CD data show that native, reduced, and PEG-functionalized PrP(23–230) and PrP(90–230) retain an α-helical content that is characteristic of native-
changed from 28 to 27%, whereas the α-helical content of PrP(90–230) changed from 43 to 35%, upon reduction of the disulfide bond. Similarly, the α-helical content of PrP(23–230) and PrP(90–230) bound to PEG is 21 and 32%. In contrast, previous CD measurements show that misfolded PrP is typically characterized by a low α-helical content of ~7% (47). We also used 1H-NMR to confirm that the native structure of PrP(23–230) and PrP(90–230) was preserved when the disulfide bond between Cys179 and Cys214 was reduced (figs. S20 and S21) and when PrP(23–230) and PrP(90–230) were functionalized with PEG linkers (figs. S22 and S23), the chemical shift dispersions of all these structures were similar to natively folded PrP.

Furthermore, by monitoring temperature-induced unfolding of PrP(23–230) and PrP(90–230) before and after attaching PEG tethers, we confirmed that PEG functionalization does not alter the stability of the folded PrP (fig. S24) (48). Identical CD thermal denaturation curves with melting temperatures of 61.4° ± 0.4°C and 62.9° ± 0.7°C were measured for PrP(23–230) before and after PEG functionalization (fig. S24A). Similarly, the melting temperatures (65.1° ± 0.4°C and 65.9° ± 0.8°C) of PrP(90–230) before and after attaching PEG tethers were similar (fig. S24B). Finally, light scattering data showed that although 100% of PrP(23–230) and 96% of PrP(90–230) with intact disulfide bonds existed as monomers, reducing this linkage and functionalizing the Cys residues with PEG linkers do not significantly decrease the fraction of monomers (Supplementary Materials; fig. S25). In agreement with our results, previous studies show that reducing the disulfide bond between Cys79 and Cys214 does not alter the pathway for PrP misfolding (49) and that misfolding occurs without disulfide exchange (50).

Because previous studies have shown that aging of PrP promotes oligomerization and copper-induced PK resistance (12), we used PrP(23–230) and PrP(90–230) within 1 month after purification. Furthermore, we used CD and dynamic light scattering to confirm that the proteins retained their native conformation and remained in a monomeric state, even after 2 months of storage (Supplementary Materials; figs. S26 and S27). After a 2-month storage period, 96% of PrP(23–230) and 95% of PrP(90–230) remained monomeric (fig. S26), with α-helical contents of 38 and 33%, respectively (fig. S27). Because the presence of even trace amounts of misfolded PrP will result in spontaneous aggregation in an RT-QuIc experiment, the absence of large deviations between replicates (figs. S9 to S11) confirms that the PrP substrate used in our experiments is in a native conformation.

We also confirmed that the presence of an N-terminal His tag does not promote PrP denaturation or aggregation by using CD and dynamic light scattering (Supplementary Materials; figs. S26 and S27). After His tag cleavage, 92% of PrP(23–230) and 97% of PrP(90–230) remained in a monomeric state (fig. S26) and had α-helical contents of 43% (fig. S27). Finally, it is important to note that the Cu2+ ion–induced structural conversion of full-length PrP is not an artifact arising from the presence of His tag because the globular PrP constructs, which also contain an identical His tag, do not show Cu2+–induced misfolding and aggregation.

Because the prion hypothesis proposes that misfolded seeds can template the aggregation of normal PrP into amyloid fibrils, we tested whether PrP exposed to different divalent ions can serve as seeds using RT-QuIc. Our data showed that a more rapid onset of aggregation was measured as the seed concentration increased. This correlation is predicted by theory and has previously been used to quantify the aggregation kinetics of two major forms of amyloid-β peptides (30, 51). Several studies have shown that different synthetic prion fragments promote neuroinflammatory and apoptotic responses in neuronal cells without evidence of being infectious (52–54). Therefore, to investigate the neurotoxic effect of Cu2+-induced misfolded PrP, we used a mouse organotypic slice culture assay, which had been previously adopted as an excellent ex vivo model of progressive neurodegenerative disorders (34, 55); a key advantage of this platform is that intact tissue explants preserve the basal cellular and molecular environment of local circuits similar to that of the whole brain. In the central nervous system, many neuropathological disorders are characteristic of being accompanied by activation and proliferation of glial cells that promote neuroinflammation, which is initiated relatively early in the disease process (56–59). Consistent with this, we observed an increase in the levels of the neuroinflammatory marker GFAP upon exposing brain slices to misfolded PrP. Furthermore, because oxidative stress is a critical initiator of neurotoxic insult (32–35), we analyzed the expression of the oxidative stress–sensitive kinase PKC-δ; notably, an increase in PKC-δ expression was measured when we exposed brain slice cultures to PrP amyloids. Finally, consistent with the finding that activated PKC-δ triggers the redistribution and activation of Bax, a Bcl-2 family protein that can directly induce cytochrome c release and downstream apoptotic cascade (60, 61), we observed markedly increased levels of Bax in brain slices exposed to misfolded PrP. Overall, our experiments demonstrate the neurotoxicity of amyloidogenic structures resulting from PrP interaction with Cu2+ ions. The neuronal cell death we observed in the mouse organotypic slice culture assay directly confirms the effect of abnormal PrP, ruling out any role for the minuscule amount of Cu2+ ions (100 nM) in the oligomeric protein solution (fig. S13). Previous studies show that copper–induced neurotoxicity normally requires concentrations ranging from 0.1 to 1 mM in ex vivo and in vivo experimental models (62, 63).

Although its physiological function is still uncertain, it has been proposed that cellular PrP plays a role in regulating Cu2+ homeostasis (64–66) and protecting neurons against apoptosis induced by Bax (67). Previous studies show that PrP is associated with Cu2+ ions in vivo (9, 68). The average Cu2+ concentration in the human brain is about 80 μM (65), but concentrations as high as 1.3 and 0.4 mM are found in the locus coeruleus and the substantia nigra, respectively (69). Consequently, our experimental conditions mimic these physiological Cu2+ concentrations. Our data suggest that an excess of Cu2+ ions contributes to the structural instability and oligomerization of cellular PrP and induces neuroinflammation and neurodegeneration.

**MATERIALS AND METHODS**

**PrP constructs used**

We used recombinant, full-length, human PrP that was either purified in-house [PrP(23–230)] or purchased commercially [PrP(23–231); Jena Bioscience]. The biophysical properties of full-length PrP were compared with homegrown [PrP(90–230)] or commercially obtained [PrP(90–231); Jena Bioscience] globular PrP. 1H-NMR spectra showed that the structure of PrP(23–231) and PrP(90–231) are similar to that of PrP(23–230) and PrP(90–230), respectively (Supplementary Materials; figs. S16 to S19). Plasmids for recombinant human PrP(23–230) and PrP(90–230) with an N-terminal histidine tail and an engineered histidine tag were a gift from K. Wüthrich (University of Zürich, Switzerland) (70). The PrP constructs were expressed and purified as previously described with minor modifications (71). Expression
of human PrP(23–230) and PrP(90–230) proteins was performed in *Escherichia coli* BL21(DE3) in a pRSET-A vector expression system. At an optical density at 600 nm (OD600) of 0.5, PrP expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM; after 8 hours of IPTG induction, bacterial cell pellets were harvested by centrifugation. Cell lysis was carried out in 25 ml of buffer A [6 M guanidinium chloride, 10 mM tris-HCl, 100 mM Na2HPO4, and 10 mM reduced glutathione (pH 8.0)]. The lysate was incubated with 5 ml of Ni–nitrilotriacetic acid agarose beads (Qiagen) on a rocking shaker at room temperature for 1 hour; 5 mM imidazole for PrP(90–230) or 10 mM imidazole for PrP(23–230) was added during the incubation to prevent the nonspecific binding of protein. The resin was transferred to a column, and the beads were washed with a 200-ml gradient of buffer A to buffer B [10 mM tris-HCl and 100 mM Na2HPO4 (pH 8.0)] to induce PrP refolding. The column was washed with 20 ml of buffer B plus 50 mM imidazole at 4°C, and PrP was eluted with buffer C [10 mM tris-HCl, 100 mM Na2HPO4, and 500 mM imidazole (pH 5.8)] and dialyzed against 10 mM sodium phosphate buffer (pH 5.8). Protein concentrations were determined by a Bradford protein assay using BSA as reference. Protein fractions collected after elution were assayed for the presence and purity of PrP using SDS-PAGE with Krypton fluorescent protein staining (Pierce) and Western blot analysis using either POM1 (Prionatis AG) or SAF32 (Cayman Chemical) (72, 73) (Fig. 1C). The purified PrP was stored at 4°C and used within 1 month.

**Surface and AFM tip functionalization and PrP immobilization**

The protocols for functionalization of AFM cantilevers and substrates for AFM-FS experiments have been described elsewhere (74, 75). Silicon nitride AFM cantilevers (Olympus) and glass substrates (Fisher) were sequentially cleaned in a solution of piranha [H2SO4/H2O2 in a 3:1 (v/v) ratio], deionized water, and acetone. The cantilevers and substrates were then silanized using a 2% (v/v) 3-aminopropyltriethoxysilane (Sigma) solution in acetone for 30 min and functionalized with a monolayer of maleimide PEG [maleimide–PEG–succinimidyl valerate (MW, 3400); Laysan Bio]. For PK digestion experiments, glass coverslips were functionalized with a mixture of 20% maleimide PEG and 80% mPEG succinimidyl valerate (MW, 3400); Laysan Bio]. For PK digestion experiments, glass coverslips were functionalized with a mixture of 20% maleimide PEG and 80% mPEG succinimidyl valerate (MW, 3400); Laysan Bio]. Before PrP immobilization, the disulfide bond was reduced with an equal volume of 10 mM tris(carboxyethyl)phosphine (TCEP) for 3 hours at 4°C. The reduced PrP (23–231) or PrP(90–231), at a concentration of 1 μg/ml, was incubated on the surface in reaction buffer [50 mM sodium phosphate, 50 mM NaCl, and 10 mM EDTA (pH 7.2)] for 1 or 4 hours, respectively, at room temperature. Unlinked maleimide groups were quenched using 1 mM cysteine and 10 mM TCEP in reaction buffer overnight at 4°C.

**PK digestion experiments**

Biotinylation of PrP for fluorescence measurements was performed by mixing 10 mM Sulfo-NHS-LC-Biotin (Thermo Scientific) with human recombinant PrP(23–231) or PrP(90–231) (10 μg/ml) in reaction buffer for 3 hours at 4°C. The reaction was quenched by adding 1-lysin (1 mg/10 μl) (Sigma) for 1 hour at room temperature. The biotinylated PrP was immobilized on PEG-functionalized glass coverslips and incubated overnight either with or without 1 mM Mn²⁺, 1 mM Ni²⁺, or 1 mM Cu²⁺. Following incubation, the divalent ions were washed away, and the immobilized PrP was treated with PK (100 μg/ml) (Sigma) in buffer [25 mM tris-HCl, 5 mM CaCl2, and 1 mM EDTA (pH 7.4)] for different time courses at 37°C. Following PK digestion, the PrP was labeled using Alexa Fluor 555–conjugated streptavidin (0.7 μg/ml) (Invitrogen) for 10 min at room temperature and rinsed with buffer [25 mM tris-HCl and 100 mM NaCl (pH 7.4)]. Before incubation with fluorescent streptavidin, the substrate was incubated with BSA (1 mg/ml) for 2 hours to minimize nonspecific fluorescence background. For every measurement, a blank control sample without PrP was prepared in parallel using an identical protocol. 3′-tetramethylrhodamine–modified DNA (ITD) was used as a positive control because of its resistance to PK digestion. The 5′ terminal of the DNA was modified by dithiol for surface immobilization. Biotin-conjugated BSA (Sigma), which is sensitive to PK digestion, was used as a negative control. A 50 nM solution of biotin-BSA was immobilized on maleimide PEG as described above.

For each time point, 8 to 20 fluorescence images were acquired for both the fluorescent samples and the blank control samples. We randomly selected one image each from the fluorescent and blank control data sets and recorded their difference in fluorescence intensity. This process was repeated 100,000 times. The mean and SD of these values were normalized to the intensity of an identically prepared sample without PK treatment.

**Single-molecule AFM-FS**

Force-ramp experiments were performed in a measurement buffer [25 mM tris-HCl and 100 mM NaCl (pH 7.4)] using an Agilent 5500 AFM system with a closed-loop scanner. The AFM tip and substrate were brought into contact for 0.1 s and then withdrawn at 6 or 11 different speeds ranging from 27 to 10,000 nm s⁻¹. Cantilever spring constants were measured using the thermal fluctuation method. Force measurements were performed continuously for 24 hours at room temperature; a LabVIEW script allowed us to operate the AFM in an automated mode to eliminate operator bias. For every experiment performed with divalent metal ions, we first acquired data for 24 hours in the measurement buffer and then acquired data for the next 24 hours in a pH 7.4 buffer with 1 mM divalent metal ions (25 mM tris-HCl, 97 mM NaCl, and 1 mM NiCl₂/MnCl₂/CuCl₂) to enable direct comparison. Before the experiment, the AFM cantilever and substrate functionalized with PrP were incubated in BSA (1 mg/ml) for 3 hours to minimize nonspecific protein adhesion. Each unbinding force trace was fitted to an extended FJC model (Eq. 1) (25), which describes the stretching of a polymer, L, under a stretching force, F.

\[
L = L_C \times \left( \frac{\coth \left( \frac{F L_K}{k_B T} \right)}{\frac{F L_C}{L_m K_s}} \right) + \frac{F L_C}{L_m K_s} \tag{1}
\]

The model contains only one fitting parameter—the contour length of PEG tethers, L_C. The values of all other parameters were obtained from the literature (25). We used a value of 0.7 nm for L_K (the Kuhn length of PEG), 0.2837 nm for L_m (the average length of a PEG monomer), and 150,000 pN/nm for K_s (the stiffness of a PEG monomer). Force curves were fit using a TLS fitting protocol; goodness of fit was estimated from the residuals. For each experimental condition, the distribution of L_C from selected events was fitted to a Gaussian distribution. Only events with fitted L_C within the range of Gaussian center ± 1 SD were used to calculate the probability of interaction and off-rate.
Calculation of relative on-rates
A detailed derivation of the formulas used for the calculation of relative on-rates can be found in the study by Chesla et al. (27). Briefly, the specific binding probability was calculated as $P_{\text{corrected}} = (P_m - P_{\text{control}})/(1 - P_{\text{control}})$, where $P_m$ is the measured binding probability when PrP is immobilized on both tip and substrate and $P_{\text{control}}$ is the measured binding probability when PrP is immobilized only on the substrate. The average number of bonds formed between opposing PrPs during each tip-substrate contact is a function of contact area between the AFM tip and substrate $A$, the protein density on opposing surfaces $\rho_m$ and $\rho_b$, the contact time $t$, and the on-rate $k_{\text{on}}$ and the off-rate $k_{\text{off}}$. Their mathematical relation is given by $\ln(1 - P_{\text{corrected}}) = A \rho_m \rho_b (1 - e^{-t k_{\text{on}}}) k_{\text{on}}/k_{\text{off}} = A \rho_m \rho_b t k_{\text{on}}$. Assuming that $A$, $\rho_m$, and $\rho_b$ are fixed, the on-rate $k_{\text{on}}$ is proportional to $-\ln(1 - P_{\text{corrected}})$.

RT-QuIC assay
PrP seeds for the RT-QuIC assay were created by incubating 6 µM PrP with 10 µM divalent metal ions (NiCl₂/MnCl₂/CuCl₂) in ThT assay buffer [20 mM tris-HCl, 150 mM NaCl, and 10 µM ThT (pH 7.4)]. The sample was incubated in a Cytation3 multimode microplate reader (BioTek) at 37°C for 64 to 95 hours with continuous cycles of 1-min shaking alternating with 1-min resting periods. The formation of prion seeds was detected as an increase in ThT fluorescence intensity, which was measured every 15 min. Seeds were created by three replicates for each condition. The PrP(23–230) seeds were serially diluted to a final amount of 1.5 ng, 150 pg, 15 pg, 1.5 pg, or 150 fg in 10 µl of buffer [20 mM tris-HCl and 150 mM NaCl (pH 7.4)]. Reactions were prepared in a black, 96-well, optical-bottomed plate (Nunc) with 90% of the seed in 1 ml of slice culture medium for 48 hours. The sample was incubated in a Cytation3 multimode microplate reader, incubated at 37°C, and shaken intermittently (1-min shake–1-min rest cycle) at 807 cpms in a double orbital configuration for 71 to 95 hours. ThT fluorescence was recorded every 15 min throughout the experiment. In each experiment, three to five replicates of blank samples [150 mM NaCl, 10 µM ThT, 1 mM EDTA, and 20 mM tris-HCl (pH 7.4)] were used. Experimental samples using seeds generated in either NiCl₂/MnCl₂ or CuCl₂ were examined in the RT-QuIC assay with four and five replicates, respectively. For the same seeding condition, fluorescence intensity was averaged across three to five replicates, followed by baseline subtraction and robust, locally weighted scatterplot smoothing. The duration of $T_{\text{th}}$ was determined from the point where the ThT fluorescence intensity first reached a threshold value, where the presence of amyloid can be detected (31). This threshold was defined as five times the SD of the fluorescence intensity from blank samples. The SEM of $T_{\text{th}}$ was calculated using a bootstrap with replacement protocol.

Organotypic slice culture assay and analysis
All procedures involving animal handling were approved by the Institutional Animal Care and Use Committee at Iowa State University and conducted in compliance with Association for Assessment and Accreditation of Laboratory Animal Care accreditation. Organotypic slice cultures were prepared as previously described with some modifications (34, 55). Briefly, 9- to 12-day-old C57BL/6 pups were anesthetized using isoflurane, and after decapitation, brains were quickly removed and brain blocks were prepared in 2% (w/v) low–melting point agarose (Invitrogen 15517–022) in Hanks’ balanced salt solution. Once the agarose was cooled on ice, 350-µm-thick cortical-striatal slices were prepared using a Compresstome VF300 microtome (Precisionary Instruments Inc.). Slices were transferred to Millicell CM Biopore PTFE membrane inserts (Millipore PIM03050) and maintained in slice culture medium (50% minimum essential medium, 25% Basal Eagle medium, 25% horse serum, and 0.65% glucose supplemented with penicillin/streptomycin and GlutaMAX) in a humidified 37°C incubator with 5% CO₂ and 95% air for 7 days. The PrP(23–230) seeds used in the organotypic assay were prepared similarly to seeds used in the RT-QuIC assay except that ThT was not used in the buffer. Seed materials were incubated and shaken using a thermomixer under the same conditions as the ThT assay either in the presence (PrP-Cu²⁺) or in the absence (PrP–no metal) of Cu²⁺ ions. A separate ThT assay was carried out in parallel to monitor seed formation. The formation of PrP–no metal and PrP-Cu²⁺ seeds was terminated upon observing the increase of ThT fluorescence in the ThT assay. After 1 week, organotypic slice cultures were exposed to 10 µl of the seed in 1 ml of slice culture medium for 48 hours, and 90% of the medium was exchanged every other day for another week. At the end of the treatment, slices were collected and washed with phosphate-buffered saline, and whole-cell tissue lysates were prepared using modified radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktail (Thermo Scientific), as previously described (35, 76–78). Western blot analysis of the lysates was performed using GFAP (1:2000; Millipore), Bax (1:1000; Cell Signaling), or PKC-8 (1:1000; Santa Cruz Biotechnology) antibodies. To confirm equal protein loading, blots were reprobed with β-actin (1:15,000; Sigma) antibody.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/7/e1600014/DC1

Supplementary Methods
Supplementary Results
fig. S1. Fluorescence images with different amounts of fluorescent streptavidin on the substrate.
fig. S2. Calibration plot of the number of streptavidin versus fluorescence intensity.
fig. S3. Histogram of unbinding force for PrP(23–231) measured in the absence of divalent ions.
fig. S4. Histogram of unbinding force for PrP(23–231) measured in 1 mM Mn²⁺.
fig. S5. Histogram of unbinding force for PrP(23–231) measured in 1 mM Ni²⁺.
fig. S6. Histogram of unbinding force for PrP(23–231) measured in 1 mM Cu²⁺.
fig. S7. Formation of PrP(23–230) seeds monitored in real time using ThT fluorescence intensity.
fig. S8. No seeds were formed when PrP(90–230) was incubated with divalent metal ions for 84 hours.
fig. S9. Seeding activity of PrP(23–230) seeds generated in 10 µM Mn²⁺ measured using RT-QuIC.
fig. S10. Seeding activity of PrP(23–230) seeds generated in 10 µM Ni²⁺ measured using RT-QuIC.
fig. S11. Seeding activity of PrP(23–230) seeds generated in 10 µM Cu²⁺ measured using RT-QuIC.
fig. S12. Formation of PrP(23–230) seeds using 1 µM Cu²⁺ and its corresponding seeding activity.
fig. S13. Upon addition of protein seeds to brain slice cultures, residual copper does not increase levels of PKC-8 and Bax.
fig. S14. Biotinylation of PrP and functionalization with PEG tethers do not alter sensitivity to PK digestion.
fig. S15. PrP(23–230) and PrP(90–230) remain in a native conformation after reduction of disulfide bond and functionalization with PEG tethers.
fig. S16. One-dimensional ¹H-NMR spectra of PrP(23–231) show that the protein is in a natively folded conformation.
fig. S17. One-dimensional ¹H-NMR spectra of PrP(90–231) show that the protein is in a natively folded conformation.
fig. S18. One-dimensional ¹H-NMR spectra of PrP(23–230) show that the protein is in a natively folded conformation.
fig. S19. One-dimensional ¹H-NMR spectra of PrP(90–230) show that the protein is in a natively folded conformation.
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