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Copper Chelation Delays the Onset of Prion Disease*

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Einar M. Sigurdsson‡§¶, David R. Brown‖, Muhammad A. Alim‡, Henrieta Scholtzova, Richard Carp‡‡, Harry C. Meeker‡‡, Frances Prelli§, Blas Frangione‡§, and Thomas Wisniewski‡§**§§**

*From the Departments of ‡Psychiatry, §Pathology, and **Neurology, New York University School of Medicine, New York, New York, the ‖Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, United Kingdom, and the ‡‡Department of Virology, Institute for Basic Research in Developmental Disabilities, Staten Island, New York 10314*

The prion protein (PrP) binds copper and under some conditions copper can facilitate its folding into a more protease resistant form. Hence, copper levels may influence the infectivity of the scrapie form of prion protein (PrP^{Sc}). To determine the feasibility of copper-targeted therapy for prion disease, we treated mice with a copper chelator, D-(−)-penicillamine (D-PEN), starting immediately following intraperitoneal scrapie inoculation. D-PEN delayed the onset of prion disease in the mice by about 11 days ($p = 0.002$), and reduced copper levels in brain by 29% ($p < 0.01$) and in blood by 22% ($p = 0.03$) compared with control animals. Levels of other metals were not significantly altered in the blood or brain. Modest correlation was observed between incubation period and levels of copper in brain ($p = 0.08$) or blood ($p = 0.04$), indicating that copper levels are only one of many factors that influence the rate of progression of prion disease. *In vitro*, copper dose-dependently enhanced the proteinase K resistance of the prion protein, and this effect was counteracted in a dose-dependent manner by co-incubation with D-PEN. Overall, these findings indicate that copper levels can influence the conformational state of PrP, thereby enhancing its infectivity, and this effect can be attenuated by chelator-based therapy.

Transition metal ions, such as copper, manganese, iron, and zinc, are thought to be involved in the pathological events of various neurodegenerative diseases (1). Although these ions are unlikely to have a causative effect, disease-induced alterations in their homeostasis may contribute to the progression of the pathology, possibly by altering the conformation of proteins and/or by enhancing their aggregation. Additionally, these ions can promote oxidation and generation of free radicals.

Although copper has been implicated in the pathogenesis of prion disease, it is unclear if this ion may promote or attenuate disease progression. Prior to the discovery of the prion protein, some similarities were observed in the neuropathology associated with the toxicity of the copper chelator cuprizone and the neuropathology of scrapie (2, 3). However, copper supplementation does not prevent the development of cuprizone-induced spongy degeneration, indicating that these toxic effects may not be mediated by copper chelation. Conversely, one of Creutzfeldt's original patients later turned out to have had Wilson's disease (4), a copper storage disorder characterized by accumulation of copper in various organs (5). A biochemical link between the prion protein and copper was established when copper affinity chromatography allowed purification of the protein (6), and copper was subsequently shown to alter the secondary structure of prion peptides (7, 8). Further characterization has indicated that the octapeptide repeat region of the prion protein binds several copper ions at least in the micromolar range (9–13) and likely with a femtomolar affinity (14). The biological significance of this interaction is not clear but the normal cellular form of the prion protein, PrP^{C},[1] may be involved in copper uptake into cells (15, 16), and PrP^{C} has a copper-dependent superoxide dismutase activity (17).

To the best of our knowledge, only one study has determined whether there is an association between copper and prion infectivity (18). Those findings indicate that copper enhances reversibility of scrapie inactivation *in vitro* by facilitating refolding of denatured PrP^{Sc} into a protease-resistant and infectious form. Hence, increased copper may enhance the infectivity of PrP^{Sc}.

To determine the feasibility of copper-targeted therapy for prion disease, we treated mice with a copper chelator, D-penicillamine (D-PEN). This compound is a derivative of penicillin and is the chelator most commonly used to treat Wilson's disease. Additional uses are in treating cystinuria, heavy metal poisoning, rheumatoid arthritis, and certain collagen disorders. We report here that D-PEN delays the onset of prion disease in mice, indicating that copper promotes prionosis.

EXPERIMENTAL PROCEDURES

Animals—Two- to three-month-old CD-1 mice received 100 mg/kg of D-PEN (Sigma) dissolved in distilled water and delivered as a 100-μl intraperitoneal dose. The solution was prepared immediately before use to avoid disulphide formation. Control mice received water injections. The dose of D-PEN chosen for this experiment was based on a previous report showing that mice force-fed with 100 mg/kg body weight of D-PEN had a delay in the onset of amyotrophic lateral sclerosis (19). Mice fed 0.5 g of D-PEN per kg of diet for up to 48 weeks have been reported not to have any abnormalities (20). A mouse consumes on average around 150 g of chow per month, which corresponds to a dosage of about 75 mg of drug ingested per month. The injected dose used in the current study is equivalent to 43 mg of D-PEN per month for a mouse

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¶ To whom correspondence may be addressed: Depts. Psychiatry and Pathology, Millhauser Labs, Rm. HN418, 560 First Ave., New York, NY 10016. Tel.: 212-263-3913; Fax: 212-263-6751; E-mail: einar.sigurdsson@med.nyu.edu.

§§ To whom correspondence may be addressed: Depts. Psychiatry, Pathology, and Neurology, Millhauser Labs, Rm. HN419, 560 First Ave., New York, NY 10016. Tel.: 212-263-7993; Fax: 212-263-7528; E-mail: thomas.wisniewski@med.nyu.edu.

[1] The abbreviations used are: PrP^{C}, cellular form of PrP; PrP, prion protein; PrP^{Sc}, scrapie form of PrP; D-PEN, D-(−)-penicillamine; PBS, phosphate-buffered saline; PK, proteinase K; ANOVA, analysis of variance.

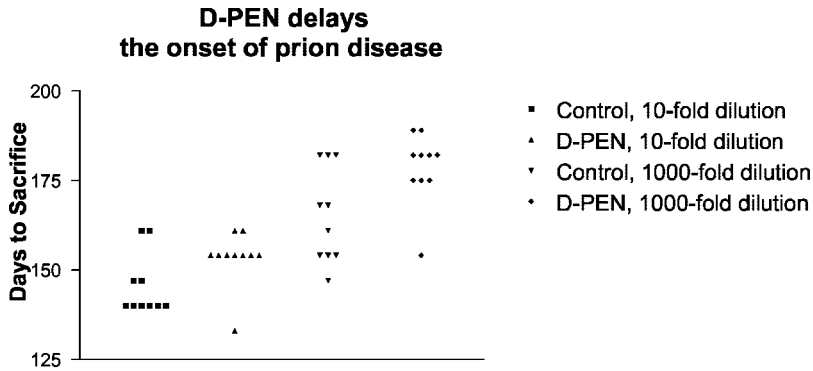

FIG. 1. **Copper chelator-mediated prolongation of the incubation period of prion disease.** The graph depicts the number of days from scrapie inoculation to the day of sacrifice in four groups of mice ($n = 10$ per group), infected with a 10- or 1000-fold dilution of scrapie agent and that subsequently received either D-PEN or were controls. Treatment with D-PEN significantly delays the onset of clinical signs of prion disease in mice ($p = 0.002$ by ANOVA). The therapeutic effect was more pronounced in the group receiving the 1000-fold dilution of the brain homogenate ($p = 0.006$, by Duncan's *post hoc* test) than within the group receiving the 10-fold dilution ($p = 0.1$, by Duncan's *post hoc* test).

weighing 20 g. The mice had access to food and water *ad libitum* and were fed a standard diet, Purina No. 5015. The first injection was delivered immediately following scrapie inoculation, and subsequent injections were performed daily, 5 days a week. The mice ($n = 10$ per group) were inoculated intraperitoneally with a brain homogenate of the mouse-adapted scrapie strain 139A at a 10-fold and a 1000-fold dilution. The mice were monitored for clinical signs of scrapie, starting a few weeks before the expected onset of symptoms. The activity level and competency of the mice were assessed on an apparatus that contained a series of parallel bars (3 mm in diameter) placed 7 mm apart, as per an established protocol (21–24). Mice with signs of prion disease have reduced activity and/or diminished ability to traverse the bars. The mice were euthanized when they rated as positive 3 weeks in a row by two observers blinded to the experimental status of the animals. This clinical end point is known to be associated with extensive scrapie pathology in the central nervous system of these mice (21–24).

Histology—At the clinical end point, the brains were removed from mice deeply anesthetized with ketamine/xylazine. The right hemisphere was immersion-fixed overnight in periodate-lysine-paraformaldehyde solution, and the left hemisphere was snap-frozen for measurements of copper levels. The periodate-lysine-paraformaldehyde solution-fixed brain hemisphere was transferred to 0.1 M sodium phosphate buffer containing 20% glycerol and 2% dimethyl sulfoxide and stored at 4 °C until sectioned. Coronal sections of 40 μm were cut, and a series of sections at 0.2-mm intervals were stained with cresyl violet and anti-PrP 78295 (courtesy of R. Kascsak, Institute for Basic Research in Developmental Disabilities, Staten Island, NY) (25) to confirm the diagnosis of prion disease.

Metal Analysis—Trace element content of brain was determined using inductively coupled plasma mass spectrometry as reported previously (26, 27). Briefly, all labware used was acid-leached overnight under pressure to reduce metal contaminants. Brain hemispheres were hydrolyzed in nitric acid (1 ml of SpA grade per 100-μl sample) at room temperature overnight. The samples were then sealed, pressurized to 120 p.s.i., and heated to at least 140 °C for 10 min. The hydrolyzed samples were diluted to 4% nitric acid before analyzing for metal contents. Standards were prepared from certified single elements (1000-ppm solutions). To further monitor the analysis, a 10-ppb standard solution was analyzed after each set of samples. Each sample was analyzed at least three times, and the instrument was washed with 2% nitric acid for 110 s between samples.

In Vitro Assay—Brains from mice at the terminal stage of infection with scrapie strain 139A were homogenized (10% w/v, pH 7.4) in various buffers containing 0.3 mM sucrose, and a mixture of protease inhibitors (Complete, Roche Applied Science). Brains from non-infected mice were used as controls. The buffers were: 1) phosphate-buffered saline (pH 7.4, PBS tablets, Sigma); 2) 10 mM Tris-HCl, 150 mM NaCl (pH 7.5); and 3) 25 mM *N*-ethylmorpholine, 100 mM potassium chloride (pH 7.4). Subsequently, the homogenate was centrifuged at 22,000 × *g*, and the supernatant was then incubated with 0.1, 1, 5, or 10 mM $CuCl_2$ or $CuSO_4$ and different amounts (0, 25, 50, and 100 mM) of D-PEN at 37 °C, for 72 h. In pilot studies, incubation with copper sulfate or copper chloride resulted in a similar dose-dependent (0.1–10 mM) enhanced resistance of the prion protein to proteinase K digestion, and copper chloride was used in all subsequent incubations. Initial studies also indicated that incubation of the samples for 72 h in PBS resulted in a

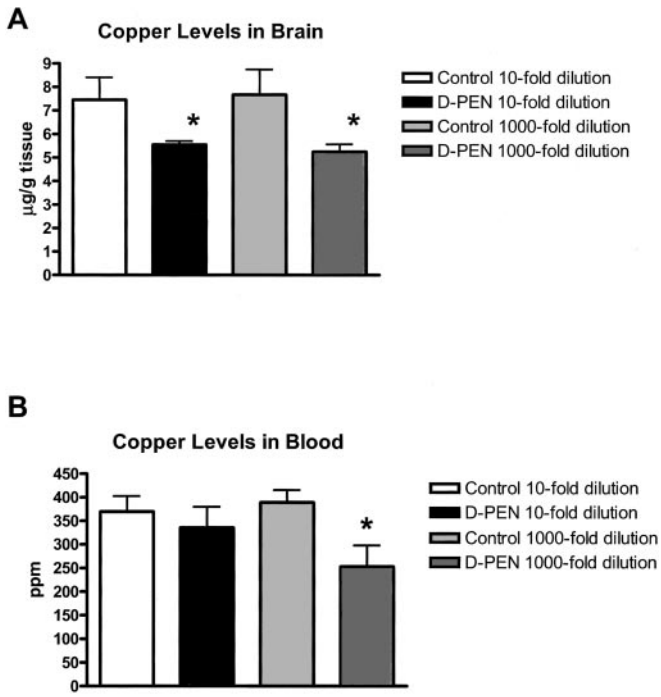

FIG. 2. **Selective chelator-mediated reduction in copper levels in brain and blood.** D-PEN reduced brain copper levels ($p < 0.01$ by ANOVA) by 26% (10-fold dilution; $p = 0.04$, by Duncan's *post hoc* test) and 32% (1000-fold dilution; $p = 0.02$, by Duncan's *post hoc* test) in the treated mice compared with control animals. Copper levels in blood were decreased in the treated mice ($p = 0.03$) by 9% (10-fold dilution) and 35% (1000-fold dilution: $p = 0.01$), respectively. Brain and blood levels of manganese, iron, zinc, nickel, selenium, and molybdenum were not altered (data not shown). Results are presented as means + S.E.

partial precipitation of the brain supernatant. No biological precipitate formed in the *N*-ethylmorpholine or Tris buffer, but at high concentrations copper was only partially soluble in *N*-ethylmorpholine or PBS. Therefore, Tris buffer was used for all subsequent incubations, and the data presented are from samples incubated in that buffer. One-half of each reaction mixture was transferred into a microcentrifuge tube and digested with 25 μg/ml proteinase K (PK; Sigma) at 37 °C, for 30 min. Brain supernatant incubated with 100 mM D-PEN as well as samples without copper and D-PEN were included as controls. Following the incubation, Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol (Sigma) was added, and the samples were boiled for 5 min and then were electrophoresed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel containing 10% glycerol. Western blots were performed with the 8H4 antibody (1:10,000 dilution; courtesy of Man-Sun Sy, Case Western Reserve University) that recognizes residues 175–185 of the prion protein (24, 28, 29). For confirmation, blots were incubated with 8F9 antibody, which binds to PrP residues 205–231 (24, 28, 29).

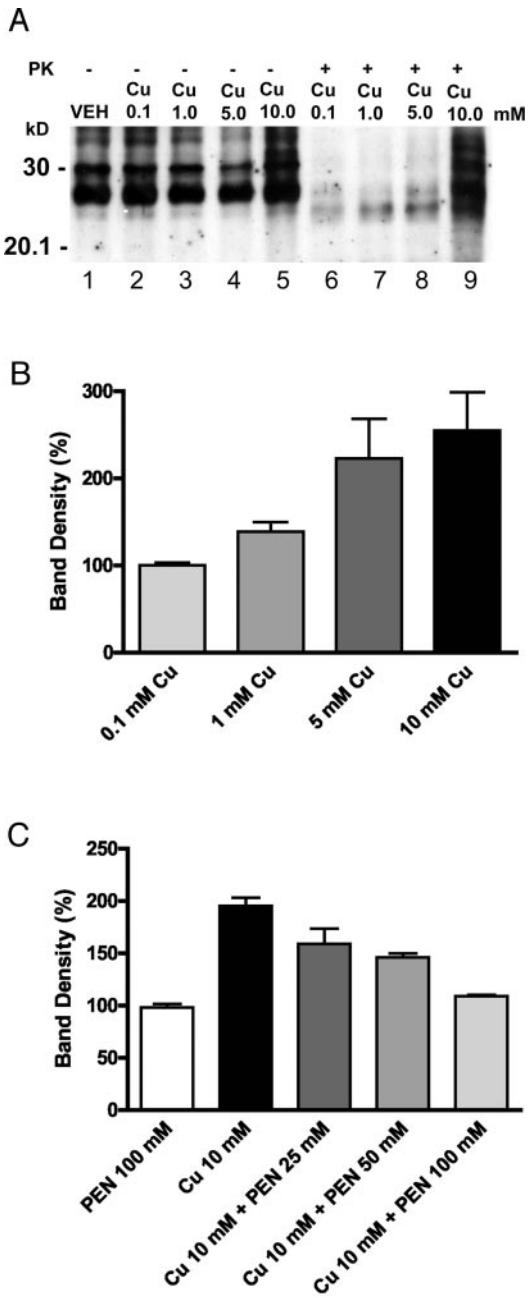

FIG. 3. ***In vitro*, D-PEN prevents copper-induced PK resistance of the prion protein.** Brains from terminally ill scrapie-infected mice were homogenized in Tris buffer, and the samples were incubated for 72 h at 37 °C prior to electrophoresis. *A* shows that copper chloride dose-dependently promotes PK resistance of the prion protein. Equivalent amounts of scrapie brain homogenate, without PK digesting in *lanes 1–5* and following PK digestion in *lanes 6–9*, was electrophoresed and transferred, followed by immunoprobing with monoclonal 8H4 antibody (anti-PrP residues 175–185). The − and + symbols above the lanes indicates whether PK digestion was not done or done, respectively. Also above each lane, it is indicated whether the samples were incubated with copper chloride and its concentration (mM). *Lane 1* shows scrapie brain, with no PK digestion and no copper added (*VEH*, vehicle). *Lanes 2–5* show scrapie brain incubated with increasing copper content (0.1–10 mM) without PK digestion. The bands in *lanes 1–5* represent PrP^{C} and PrP^{Sc}, with the band intensity being approximately the same in each sample as expected. *Lanes 6–9* are of scrapie brain homogenates following incubation with copper at difference concentrations and PK digestion. The bands shown in *lanes 6–9* are of PrP^{Sc} and are more intense in the samples incubated with higher concentrations of copper, prior to PK digestion. *B*, quantitation of the copper induced PK resistance of the prion protein. The pixel density of a PK-resistant band (25 kDa) was measured and normalized against the band observed following digestion of PrP^{Sc}-rich brain homogenate incubated without copper (rated as 100, not shown). Each *bar* depicts the mean band density + S.E. of four samples. ANOVA revealed significant group differences in the intensity of the PK resistant band ($p = 0.018$, $n = 4$ per group). The groups that received 5 and 10 mM copper chloride differed significantly from the 0.1 mM group (Duncan's *post hoc* test, $p = 0.02$ and 0.008, respectively). *C*, this effect is blocked by D-PEN, which by itself has no effect on PK-mediated protein degradation. Quantitation was performed in the same as manner as in *B*. D-PEN by itself had no effect on PK resistance, but dose-dependently reversed copper induced PK resistance of the prion protein. Each *bar* depicts the mean band density + S.E. of three samples. ANOVA revealed significant group differences ($p = 0.0017$, $n = 3$ per group) in the intensity of the PK-resistant band. The highest intensity was observed in the group that received 10 mM copper chloride compared with all the other groups (Duncan's *post hoc* test, $p = 0.02–0.0008$).

The secondary antibody (1:2,000 dilution) was peroxidase-linked anti-mouse IgG (Amersham Biosciences), and the immunoreactive material was visualized with the ECL detection kit (Amersham Biosciences). Quantitation was performed by measuring the pixel density of a PK-resistant band (~25 kDa) from individual lanes (SigmaGel, Jandel Scientific), compared with the density of the PK-resistant band from the same blot of a control PrP^{Sc} brain incubated without copper (given a value of 100).

Statistical Analysis—Data were analyzed with two-way (treatment and dilution effect; Figs. 1 and 2) or one way (*in vitro* assay; Fig. 3) analysis of variance (ANOVA), followed by the Duncan's *post hoc* test (Statistica software, Version 6.1, StatSoft Inc.). Correlation was determined by calculating the Pearson *r* correlation coefficient (Statistica software).

RESULTS

D-PEN delayed the onset of prion disease in the mice (Fig. 1). Two-way ANOVA revealed a treatment effect ($p = 0.002$) and a dilution effect ($p < 0.001$) but no significant interaction between the two factors. Duncan's *post hoc* analysis showed a significant delay in disease onset in the group receiving the 1000-fold dilution of the brain homogenate (D-PEN = 179 ± 3 days, vehicle = 165 ± 4, $p = 0.006$) and a trend for a delay within the group receiving the 10-fold dilution (D-PEN = 153 ± 2 days, vehicle = 146 ± 3, $p = 0.1$). As expected, D-PEN reduced brain copper levels ($p < 0.01$; two-way ANOVA) by 26% (10-fold dilution; Duncan's *post hoc* test: $p = 0.04$, one-tailed) and 32% (1000-fold dilution; $p = 0.02$, one-tailed) in the treated mice compared with control animals (Fig. 2). Brain levels of manganese, iron, zinc, nickel, selenium, and molybdenum were not altered (data not shown). The copper levels we report in untreated mice are similar to prior studies (26, 27). Blood levels of metals revealed a similar specificity in the copper-chelating effect of D-PEN at this dose. Copper levels were decreased in the treated mice ($p = 0.03$; two-way ANOVA) by 9% (10-fold dilution) and 35% (1000-fold dilution; Duncan's *post hoc* test: $p = 0.01$, one-tailed), but the two D-PEN-treated groups did not differ significantly from each other. The levels of manganese, iron, zinc, nickel, selenium, and molybdenum in the blood were not altered (data not shown).

To further support the notion that the therapeutic effect of D-PEN was mediated through its copper chelating properties, brain homogenates from terminally ill 139A-infected mice were incubated with copper with or without D-PEN. Incubation with copper chloride resulted in a dose-dependent (0.1–10 mM) enhanced resistance of the prion protein to proteinase K digestion ($p = 0.018$; Fig. 3, *A* and *B*). The increased PK resistance associated with the presence of copper was counteracted in a dose-dependent manner by co-incubation with D-PEN ($p = 0.0017$; Fig. 3*C*). A similar but less pronounced effect of copper-mediated enhancement in PK resistance of PrP, and its reversal by D-PEN was observed in control samples, in which the brain homogenate was from non-infected mice (data not shown), suggesting that copper may affect the conformation of both PrP^{C} and PrP^{Sc}. Overall, these findings support the pro-

posed *in vivo* effect of D-PEN in delaying the onset of prion disease in the animals.

DISCUSSION

To clarify the involvement of copper in the development of clinical symptoms of prion disease, we injected mice with the copper chelator D-PEN using a dose (100 mg/kg body weight) that has been shown to be without toxic effects in mice (20). Although D-PEN may chelate other ions at high doses, oral administration of a dose about 16-fold higher than we used in our study has been shown not to chelate other metals (30). Furthermore, our measurements of brain and plasma levels of copper, and other metal ions support the selectivity of D-PEN for chelating copper ions at the dose we injected. The mice in our study that received the chelator developed clinical symptoms significantly later than the control group indicating that lower copper levels may delay disease progression. A past *in vitro* study has shown that copper may enhance the refold of partially denatured PrP^{Sc} (18), while another study has documented that the protease cleavage pattern of PrP^{Sc} can be altered by copper and zinc ions as well as by metal chelators (31), suggesting that metal ions may be involved in maintaining certain conformations of the prion protein and thereby facilitate its conversion to the PrP^{Sc} form. Enhanced clearance of copper may, therefore, reduce PrP^{Sc} formation and/or its stability. The therapeutic effect of the chelator may also be indirect because copper has immunomodulatory effect (32), and the immune system is involved in propagation of prionosis (33).

The relatively modest correlation between incubation period and copper levels in brain ($p = 0.08$) and blood ($p = 0.04$) suggests that the progression of prion disease is influenced by multiple factors, only one of which may be copper levels. Prior studies indicate that copper levels are reduced by up to 50% in the brains of individuals that succumbed to Creutzfeldt-Jakob's disease (34). We have observed a similar reduction in scrapie-infected mice, in which brain copper levels are maximally reduced at the onset of clinical symptoms, while the amount of copper is increased in the liver (26, 27). Following clinical symptoms, blood copper levels are slightly elevated in these mice. Copper measurements in the previous studies are in a similar range our values. Also, prion knock-out mice have been shown to have lower copper levels but unchanged amounts of iron and zinc in synaptosomes (9), although brain copper content has also been reported not to be affected by prion expression levels (35). The findings of lower brain copper levels in prion-infected patients and animals do not provide information on the type of role copper has on prion infectivity. Since copper has been shown to bind with higher avidity to PrP^{C} than to the scrapie form, PrP^{Sc} (27, 36, 37), decreased copper levels observed in the brains of infected patients and animals may reflect enhanced clearance of unbound central nervous system copper ions. Our *in vitro* observation that copper enhances PK resistance of both PrP^{Sc} and PrP^{C} and that this effect can be reversed by D-PEN support a direct role of copper on the conformational state of PrP. Measurements of PrP^{Sc} and copper levels need to be performed over the whole course of the disease to thoroughly characterize the role of copper in progression of prion disease.

Overall, our findings suggest that chelator-based therapy can prolong the incubation phase of prion disease. Future studies will determine whether higher doses with acceptable side effects may prevent disease onset, and the usefulness of this approach in multitargeted combinational therapy. Our studies highlight the importance of PrP/copper interactions for prion infectivity.

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