Disruption of copper homeostasis due to a mutation of *Atp7a* delays the onset of prion disease

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Copper influences the pathogenesis of prion disease, but whether it is beneficial or detrimental remains controversial. Copper homeostasis is also essential for normal physiology, as highlighted by the spectrum of diseases caused by disruption of the copper transporting enzymes ATP7A and ATP7B. Here, by using a forward genetics approach in mice, we describe the isolation of three alleles of *Atp7a*, each with different phenotypic consequences. The mildest of the three, *Atp7a*^{brown}, was insufficient to cause lethality in hemizygotes or mottling of the coat in heterozygotes, but did lead to coat hypopigmentation and reduced copper content in the brains of hemizygous males. When challenged with Rocky Mountain Laboratory scrapie, the onset of prion disease was delayed in *Atp7a*^{brown} mice, and significantly less proteinase-resistant prion protein was found in the brains of moribund *Atp7a*^{brown} mice compared with WT littermates. Our results establish that ATP7A-mediated copper homeostasis is important for the formation of pathogenic proteinase-resistant prion protein.

neurodegeneration | *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis | Menkes disease | ATPase | pigmentation

P rion diseases are fatal infectious neurodegenerative diseases caused by the misfolding and aggregation of the cellular prion protein, PrP, into the β -sheet-rich, proteinase-resistant, disease-associated form, PrP^{res}. Diseases caused by prions include Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy in cattle, scrapie in sheep, and chronic wasting disease in cervids, yet the normal function of cellular prion protein remains a mystery. PrP is known to bind copper, and copper can induce expression of the PrP-encoding gene *Pmp* (1–4). Histidine residues within the highly conserved octarepeat of PrP bind copper (5–8), and additional copies of the octarepeat within PrP are associated with early-onset prion disease. Octarepeat expansion can increase the copper-binding affinity of PrP by as much as 10-fold, suggesting that copper plays a significant role in prion pathogenesis (8). Copper also binds to histidines within region 95–115 of PrP.

The role of copper in prion disease pathogenesis is complex, and interpretations to date have been contradictory. Binding of copper to PrP destabilizes the native α -helical PrP and leads to an increase in β -sheet structure and conversion to the proteinase-resistant form (9–13), yet it has been reported that copper inhibits PrP^{res} amplification (14). Copper is known to promote prion infectivity (15), yet it is also important in limiting neuropathology caused by reactive oxygen species (16). Although copper chelation delays disease onset in mice (17), similar delays are observed in mice and hamsters maintained on a high-copper diet (18, 19). Mice fed a copper-depleted diet are also reported to be more sensitive to prion pathogenesis following i.p. inoculation (18).

The physiological consequences of copper deficiency were first recognized among a group of sheep in western Australia, which were noticed for their “stringy” coats, anemia, and the fact that they consistently gave birth to ataxic lambs (20). The affected ewes were subsequently found to be copper-deficient, and drenching them with copper sulfate solution would reverse anemia, improve their coat condition, and prevent ataxia in their lambs (21).

A similar link was established many years later in man, when patients with Menkes disease, an X-linked neurodegenerative condition, were discovered to be copper-deficient (22).

Menkes disease is caused by mutation of *ATP7A*, which encodes the P1B-type ATPase ATP7A (23). Along with the closely related ATPase ATP7B, ATP7A is a critical transporter of copper. Mutation of *ATP7A* is known to cause at least three distinct disorders (24), including Menkes disease (25–27), occipital horn syndrome (considered to be a milder form of Menkes disease) (28), and isolated distal motor neuropathy (29). Conversely, mutation of *ATP7B* causes Wilson disease (30, 31), which is characterized by neuronal and hepatic pathologic processes associated with copper accumulation.

Copper homeostasis is essential for two reasons. First, copper is an essential cofactor for enzymes such as superoxide dismutase, dopamine hydroxylase, tyrosinase, and lysyl oxidase, accounting for the characteristic defects in neurological function, pigmentation, and connective tissue formation seen in Menkes disease (32). Conversely, copper can promote the production of reactive oxygen species, and is therefore highly toxic in excess.

Here we describe the isolation of an *Atp7a* mutant mouse strain with an inherited copper deficiency. In hemizygous males, a 60% reduction in cerebral copper content was associated with delayed PrP^{res} conversion and prion pathogenesis.

Results

Positional Cloning of *brown* Phenotype. During the course of a C57BL/6J mouse mutagenesis program, we identified a male G3 mouse with a brown coat. This phenotype, designated simply as *brown*, was not seen in either parent, suggesting that it was inherited as an autosomal or X-linked recessive trait (ref. 33; http://mutagenetix.utsouthwestern.edu/phenotypic/phenotypic_rec.cfm?pk=469). To determine the chromosomal location of the causative mutation, the index *brown* male was outcrossed to C3H/HeN females, and then backcrossed to his F1 daughters. Among a total of 17 offspring, six were phenotypically normal (one female, five male) and 11 shared the coat color phenotype of their sire (seven female, four male). Each mouse was genotyped at 128 polymorphic markers across the genome, and individual genotypes were tabulated to calculate logarithm (base 10) of odds scores at each marker. This revealed a single peak score of 3.86 at DXMit172, flanked by the distal marker DXMit114 and proximal

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Fig. 1. A hemizygous viable allele of *Atp7a*. (A) Linkage mapping of the *brown* mutation to chromosome X. (B) Haplotype groups of affected and unaffected F2 progeny, isolating the *brown* mutation to distal chromosome X. (C) DNA sequence chromatogram indicates the mutated nucleotide in *Atp7a*. (D) Sequence chromatograms of two independent alleles of *Atp7a*: *Tigrou* and *Tigrou-like*. (E) Comparison of the coat color phenotypes of three *Atp7a* mutants. (F) Predicted relationship between ATP7A activity and tyrosinase-mediated pigmentation. (G) Predicted ATP7A topology and domain structure, indicating the relative positions of each mutation. MBD1 to MBD6 represent the N-terminal metal binding domains, with the copper-binding cysteines indicated. Critical residues thought to bind copper in transmembrane domains 6, 7, and 8 are shown. The canonical actuator (marked as "A") domain and phosphorylation ("P") domain residues are also indicated. The dileucine (LL) and PDZ (PSD-95/Discs-large/ZO-1) binding motif (PBM) in the C-terminal tail are necessary for subcellular localization of ATP7A. Conversion of ATP to ADP by the nucleotide-binding ("N") domain leads to phosphorylation of the P-domain and copper transport. The A-domain dephosphorylates the P-domain. Numbers 1, 2, and 3 indicate the order of copper binding and transport.

marker DXMit121 (Fig. 1 A and B). Within this 62.5-Mb interval lay 358 protein-coding and 22 miRNA genes, three of which had previously been described to affect coat pigmentation when mutated in the mouse (*Atp7a*, *Eda*, and *Htr2c*). As a result of an absence of ectodermal dysplasia phenotypes [commonly associated with *Eda* mutants (34)] or postnatal growth retardation [as seen in an *Htr2c* mutant (35)], all coding exons and splice junctions of *Atp7a* were sequenced. A single T-to-C missense

transition was detected at X:103283830, corresponding to position 1570 of the *Atp7a* transcript, in exon 5 of 23 total exons (Fig. 1C). The mutation is predicted to result in an isoleucine to threonine substitution at amino acid 483 of the ATP7A protein.

Allelic Series of *Atp7a* Mutations. Although not the case for *Atp7a*^{brown}, heterozygous *Atp7a* mutants typically present with variegated coat color, a consequence of random X-inactivation

in melanocyte precursors (36). Examples of this phenotype were previously observed in our mutagenesis program, and given their similarity to the classic *mottled* mutants (37, 38), were sequenced at the *Atp7a* locus. The mutants *Tigrou* and *Tigrou-like* were both found to harbor *Atp7a* mutations (Fig. 1D), respectively creating a premature termination codon at position 469, and an alanine-to-valine substitution at codon 998. Hemizygous *Tigrou* males were occasionally observed, lacked pigmentation (except at their extremities), and died within 3 wk of birth (Fig. 1E). *Tigrou-like* appeared to be entirely lethal in utero, as hemizygous males were not observed postpartum. Similar to *Tigrou-like* and *Tigrou*, the vast majority of *Atp7a* alleles described in mouse are hemizygous lethal in utero or shortly after birth (39). *Atp7a^{Blotchy}* and the *Pewter* series of alleles are all hemizygous viable, yet, unlike *brown*, cause variegated pigmentation in heterozygous form, making *brown* a uniquely mild allele in the *Atp7a* allelic series.

Based on the pigmentation phenotype of heterozygotes and lethality in hemizygotes, the three alleles could be arranged in a decreasing order of functional severity, with *Tigrou-like* predicted to cause the most severe copper deficiency and *brown* the least (Fig. 1F; protein schematic shown in Fig. 1G).

Although one might expect the *Tigrou* nonsense allele to be more detrimental than the A998V missense mutation of *Tigrou-like*, skipping of a nonsense mutation-harboring exon has been observed in a patient with Menkes disease (40). In the case of *Tigrou*, such a splice product would remove exon 5 yet remain in frame, but would do so at the expense of the fifth metal binding domain. Nevertheless, the effects of each missense mutation were consistent with PolyPhen-2 predictions, with respective scores of 1 and 0.476 for *Tigrou-like* and *brown*.

Reduced Quantities of Copper and Delayed Onset of Prion Disease.

The brains of *Atp7a^{brown/Y}* and *Atp7a^{+Y}* littermates were analyzed for copper content. The amount of copper in the brains of *Atp7a^{brown/Y}* mice was reduced by 60% compared with *Atp7a^{+Y}* littermates [$1.6 \pm 0.2 \mu\text{g Cu}^{2+}/\text{g}$ of brain (mean $\pm$ SEM) vs. $3.9 \pm 0.4 \mu\text{g Cu}^{2+}/\text{g}$; $P < 0.0001$; Fig. 2A].

Atp7a^{brown/Y} and *Atp7a^{+Y}* littermates were inoculated intracranially with the Rocky Mountain Laboratory (RML) strain of mouse scrapie. All 10 *Atp7a^{+Y}* mice became ill with clinical signs of scrapie, including altered gait, kyphosis, ataxia, disorientation, and lethargy within 161 d after infection. In contrast, only one of 10 *Atp7a^{brown/Y}* mice developed disease within the same period. The remaining *Atp7a^{brown/Y}* mice became terminally ill by 180 d after infection ($P < 0.0001$; Fig. 2B). Moribund mice from both groups were killed, and their brains were harvested for detection of abnormally folded PrP^{res} protein (Fig. 2C). Proteinase K-resistant PrP was detected by Western blot in the brains of clinically ill *Atp7a^{+Y}* (Fig. 2C, lanes 6, 8, and 10) and *Atp7a^{brown/Y}* mice (Fig. 2C, lanes 12, 14, and 16), confirming prion disease. No proteinase K-resistant PrP was detected in the brains of uninfected *Atp7a^{+Y}* or *Atp7a^{brown/Y}* mice (Fig. 2C, lanes 2 and 4).

Histological analysis showed that the brains of clinically ill *Atp7a^{brown/Y}* and *Atp7a^{+Y}* mice displayed spongiosis typical of transmissible spongiform encephalopathies, particularly in the cerebellum (Fig. 3, Top). Brain sections from both RML scrapie-infected *Atp7a^{+Y}* and *Atp7a^{brown/Y}* mice displayed considerable loss of MAP2-immunoreactive dendrites in the neocortex and hippocampus (Fig. 3, Middle), most apparent in the CA1–CA3 regions, suggestive of selective damage to pyramidal neurons. Analysis of reactive astrocytes by GFAP staining showed that both groups of RML scrapie-infected mice also showed extensive astrogliosis (Fig. 3, Bottom). Consistent with the widespread neurodegenerative alterations observed by MAP2 and GFAP staining, analysis of neuronal cells with NeuN showed rarefaction and loss of neurons in the CA1–CA3 regions in both RML scrapie-infected groups, and was accompanied by microgliosis as determined by Iba1 staining (not shown).

Fig. 2. Reduced copper content in *Atp7a^{brown}* mutant brains is associated with prolonged survival after intracranial RML scrapie infection. (A) The amount of copper in the brains of uninfected *Atp7a^{+Y}* and *Atp7a^{brown/Y}* littermates was determined by ICP-MS ($n = 5$; error bars represent SEM). (B) *Atp7a^{+Y}* and *Atp7a^{brown/Y}* mice were inoculated intracranially with RML scrapie and killed when clinical signs of prion disease were observed ($n = 10$). (C) The presence of PrP^{res} in the brains of clinically ill *Atp7a^{+Y}* and *Atp7a^{brown/Y}* mice was confirmed by Western blot ($n = 3$). Similar results to those shown were obtained for all other samples analyzed in each experiment.

However, substantially less PrP^{res} was detected by immunohistochemistry in the brains of clinically ill *Atp7a^{brown/Y}* mice compared with those of *Atp7a^{+Y}* (Fig. 4A). Upon further analysis, when equal amounts of protein from the brains of clinically ill mice were analyzed by Western blot (Fig. 4B), 41% less total PrP ($P < 0.01$; Fig. 4C) and 50% less PrP^{res} ($P < 0.01$; Fig. 4D) was detected in the brains of infected *Atp7a^{brown/Y}* mice compared with those of *Atp7a^{+Y}*. No significant difference was seen between the amount of PrP in the brains of uninfected *Atp7a^{brown/Y}* and *Atp7a^{+Y}* mice (Fig. 4B and C).

In summary, we observed widespread degeneration in the cortex, hippocampus, basal ganglia, and cerebellum of RML scrapie-infected *Atp7a^{+Y}* and *Atp7a^{brown/Y}* mice. The degree of neuronal loss, astrogliosis, and microgliosis in infected *Atp7a^{brown/Y}* mice was equivalent to that observed in infected *Atp7a^{+Y}* mice, yet the amount of PrP^{res} in the brains of *Atp7a^{+Y}* mice was significantly higher than in their *Atp7a^{brown/Y}* littermates, as measured by Western blotting and immunohistochemistry.

Discussion

This report describes the characterization of a hypomorphic allele of the Menkes disease gene orthologue, *Atp7a*, characterized by coat hypopigmentation and an insufficiency of copper in the

Fig. 4. Reduced PrP and PrP^{res} in the brains of RML scrapie-infected *Atp7a*^{brown} mutants. (A) Brain sections from clinically ill RML scrapie-infected *Atp7a*^{+Y} and *Atp7a*^{brown/Y} mice were stained for proteinase K-resistant PrP (i.e., PrP^{res}; $n = 3$). Low-magnification (Upper) and high-magnification (Lower) images are shown. (B) Representative Western blots of PrP and PrP^{res} levels in the brains of uninfected and RML scrapie-infected *Atp7a*^{+Y} (lanes 1–4) and *Atp7a*^{brown/Y} (lanes 5–8) mice. (C and D) Quantification of total PrP (C) and PrP^{res} (D) in the brains of uninfected and RML scrapie-infected *Atp7a*^{+Y} and *Atp7a*^{brown/Y} mice as determined by Western blot by using GE ImageQuant software ($n = 5$; error bars represent SEM). Similar results to those shown were obtained for all other samples analyzed in each experiment.

Triton X-100 solution for 1 h at 37 °C. For quantitative purposes, total protein concentration of each sample was determined by bicinchoninic acid assay (Pierce), and 20 μ g of total protein from each sample was untreated or treated with 50 μ g/mL of proteinase K. Reactions were stopped by adding an equal volume of 2 $\times$ SDS loading buffer (Invitrogen) and heating to 95 °C for 5 min. The samples were then run on a 16% Tris-glycine gel (Invitrogen) and transferred to Invitrolon PVDF membrane (Invitrogen). The membrane was blocked and all antibodies were diluted in 5% nonfat milk. Human anti-PrP antibody D13 was used at 3 μ g/mL to detect PrP, followed by peroxidase-conjugated goat anti-human antibody

(Jackson ImmunoResearch) used at a 1:10,000 dilution. For quantitative Western blots, a mouse anti- α -tubulin antibody was used at 1:10,000, followed by peroxidase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch) used at 1:5,000. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to X-ray film or imaged by using a GE ImageQuant LAS 4000. Signal intensities were analyzed with GE ImageQuantTL software.

Analysis of Brain Copper Content. Brains were analyzed individually for copper content using inductively coupled plasma mass spectroscopy (ICP-MS). Whole brain was homogenized in ddH₂O at 100 mg of tissue/mL. The tissue homogenate was cleared by ultrasonication and centrifugation at 500 $\times$ g for 15 min. One milliliter of cleared brain homogenate was digested with 3 mL of 70% nitric acid by using a microwave acid digestion vessel (Parr). Each sample was microwaved for 30 s at full power in a 1,200-W household microwave oven and allowed to cool for 30 min before opening. Separate microwave digestion vessels were used for mutant and WT samples. The digested sample was then diluted 1:2 with ddH₂O before analysis. Standard concentrations of Cu used were 0, 10, 25, 50, 75, 100, and 200 parts per billion. Each standard and sample contained 100 parts per billion yttrium as an internal standard. Each sample was analyzed three times, with 2% (vol/vol) nitric acid run for 2 min between each sample. The ICP-MS was recalibrated, and standards were rerun after every 15 samples.

Histology and PrP^{res} Immunohistochemistry. Brain hemispheres were fixed in 4% paraformaldehyde containing zinc for a minimum of 24 h before being dehydrated, embedded in paraffin wax, cut into 6- μ m sections, and placed on glass slides. Sections were deparaffinized with xylene and rehydrated with ethanol and water. For H&E staining, sections were stained with Shandon hematoxylin (Thermo Scientific) and differentiated in 1% HCl (in 70% isopropanol). Hematoxylin-stained sections were blued with Scott tap water substitute before being stained with Shandon eosin (Thermo Scientific), dehydrated with ethanol, cleared with xylene, and mounted with Permount. For PrP^{res} immunohistochemistry, antigen retrieval was performed by autoclaving in PBS solution for 12 min at 250 °F. Proteinase-sensitive PrP was removed by treatment with 50 μ g/mL of proteinase K for 20 min at room temperature. The sections were then blocked with 10% normal goat serum overnight, avidin-biotin blocked (Vector Laboratories), and incubated in 3% hydrogen peroxide for 20 min. PrP was detected with 3 μ g/mL antibody D13, biotinylated anti-human IgG (Jackson ImmunoResearch), and avidin-conjugated peroxidase (Jackson ImmunoResearch). After PrP staining, sections were counterstained with Shandon hematoxylin and Scott tap water substitute. The sections were dehydrated with ethanol and xylene and mounted using Permount (Fisher Scientific).

MAP2, GFAP, NeuN, and Iba1 Immunohistochemistry. Vibratome sections (40 μ m) were immunolabeled overnight with antibodies against the neuronal marker NeuN (1:1,000; Millipore), the astroglial marker GFAP (1:500; Millipore), the dendritic marker MAP2 (1:200; Millipore), and the microglial cell marker Iba1 (1:500; Wako), followed by incubation with species-appropriate secondary antibodies conjugated to HRP or FITC (1:2,000; Vector Laboratories). Sections labeled with HRP (NeuN, Iba1, and GFAP) were incubated with DAB and analyzed with a bright-field digital Olympus microscope. Signal intensities were assessed by optical density measurements by using the Image Quant 1.43 program (National Institutes of Health) and corrected against background. FITC-labeled sections (i.e., MAP2) were analyzed with an MRC1024 laser scanning confocal microscope (Bio-Rad).

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