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Prion Protein Regulates Iron Transport by Functioning as a Ferrireductase

Ajay Singh^a, Swati Haldar^a, Katharine Horback^c, Cynthia Tom^c, Lan Zhou^a, Howard Meyerson^a, and Neena Singh^{a,b,*}

^aDepartment of Pathology, Case Western Reserve University, Cleveland, OH, USA

^bDepartment of Neurology, Case Western Reserve University, Cleveland, OH, USA

^cCase Medical School, Case Western Reserve University, Cleveland, OH, USA

Abstract

Prion protein (PrP^C) is implicated in the pathogenesis of prion disorders, but its normal function is unclear. We demonstrate that PrP^C is a ferrireductase (FR), and its absence causes systemic iron deficiency in PrP knock-out mice (PrP^{-/-}). When exposed to non-transferrin-bound (NTB) radioactive-iron (⁵⁹FeCl₃) by gastric-gavage, PrP^{-/-} mice absorb significantly more ⁵⁹Fe from the intestinal lumen relative to controls, indicating appropriate systemic response to the iron deficiency. Chronic exposure to excess dietary iron corrects this deficiency, but unlike wild-type (PrP^{+/+}) controls that remain iron over-loaded, PrP^{-/-} mice revert back to the iron deficient phenotype after 5 months of chase on normal diet. Bone marrow (BM) preparations of PrP^{-/-} mice on normal diet show relatively less stainable iron, and this phenotype is only partially corrected by intraperitoneal administration of excess iron-dextran. Cultured PrP^{-/-} BM-macrophages incorporate significantly less NTB-⁵⁹Fe in the absence or presence of excess extracellular iron, indicating reduced uptake and/or storage of available iron in the absence of PrP^C. When expressed in neuroblastoma cells, PrP^C exhibits NAD(P)H-dependent cell-surface and intracellular FR activity that requires the copper-binding octa-peptide-repeat region and linkage to the plasma membrane for optimal function. Incorporation of NTB-⁵⁹Fe by neuroblastoma cells correlates with FR activity of PrP^C, implicating PrP^C in cellular iron uptake and metabolism. These observations explain the correlation between PrP^C expression and cellular iron levels, and the cause of iron imbalance in sporadic-Creutzfeldt-Jakob-disease brains where PrP^C accumulates as insoluble aggregates.

Keywords

Brain; ferrireductase; iron; neurotoxicity; prion; sporadic Creutzfeldt-Jakob disease

*Correspondence to: Neena Singh, Department of Pathology, Case Western Reserve University, 2103 Cornell Road, Cleveland, Ohio 44106, USA. neena.singh@case.edu.

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INTRODUCTION

Cellular prion protein (PrP^C) is a ubiquitously expressed, evolutionarily conserved cell surface glycoprotein that serves as the obligatory substrate of PrP-scrapie (PrP^{Sc}), a β -sheet rich isoform implicated in the pathogenesis of prion disorders including sporadic Creutzfeldt-Jakob disease (sCJD) in humans and scrapie in sheep and goats [1, 2]. Although investigations on the mechanisms underlying PrP^{Sc}-induced neurotoxicity have provided important insight, the physiological function of PrP^C has remained ambiguous. Diverse functions have been ascribed, in part reflecting the models and experimental paradigms used for the analyses [2–4]. Lack of consensus on this subject is partly due to the absence of an overt phenotype in transgenic mice lacking PrP expression (PrP^{-/-}) [5]. Reported abnormalities in these mice include disturbance of circadian rhythm and sleep pattern [6, 7], synaptic dysfunction [7], peripheral demyelination with ageing [8], hippocampal dysfunction [9], increased susceptibility to neuronal damage by oxidative stress and cerebral ischemia [10], impaired ability of hematopoietic progenitor cells to colonize in irradiated wild-type mice [11], and slow recovery from experimentally induced anemia [12]. PrP^{-/-} mice also show a phenotype of iron deficiency [13], suggesting a functional role of PrP^C in iron uptake. Among the various proposed functions, involvement of PrP^C in systemic iron metabolism is of particular interest since sCJD and scrapie infected brains show imbalance of iron homeostasis that increases with disease progression and is directly related to PrP^{Sc} load [14]. A likely cause of this phenotype is sequestration of iron in PrP^{Sc}-protein complexes that include the iron storage protein ferritin, creating insufficiency of bioavailable iron [15]. These complexes are redox-active [16] and likely to create a potentially neurotoxic environment due to the propensity of iron to participate in Fenton and Haber Weiss reactions, generating highly reactive hydroxyl radicals [17, 18]. A complete understanding of this phenomenon is possible only by delineating the role of PrP^C in iron metabolism.

Investigations on the functional role of PrP^C in brain iron homeostasis have been challenging due to limited understanding of iron transport and utilization by different cell types within the complex environment of the brain [19, 20]. On the other hand, mechanisms regulating systemic iron acquisition and utilization are relatively better known [21–30], and provide experimentally accessible models that can be utilized to understand the role of ubiquitously expressed proteins such as PrP^C in iron homeostasis. Available information indicates that uptake of iron from ingested material is regulated at the duodenum by systemic iron levels [22, 23]. To allow efficient uptake by the divalent metal transporter 1 (DMT1) expressed on the apical plasma membrane of enterocytes, ingested iron requires reduction to the ferrous (Fe²⁺) form by an ascorbate-dependent b-type cytochrome (Dcytb) ferrireductase (FR) [24–26]. Absorbed Fe²⁺ iron is delivered to the blood stream by the coupled action of ferroportin and hephaestin on the basolateral membrane of enterocytes that transport and oxidize Fe²⁺ iron respectively for loading to plasma transferrin (Tf) [22]. Non-Tf bound iron circulates in association with low molecular weight chelates such as citrate, and gains access to cells through DMT1 after reduction by membrane bound FR proteins [27]. Major known ferrireductases are Dcytb and the Steap family of proteins, and ferroxidases include ceruloplasmin and hephaestin [28, 29]. Most of the absorbed iron is utilized for erythropoiesis, and excess is stored within ferritin in the relatively inert Fe³⁺ form in

hepatocytes and reticuloendothelial cells of the liver and bone marrow (BM). Minimal iron is absorbed or excreted daily. Most of the iron is recycled from senescent red blood cells (RBCs) by BM and splenic macrophages and re-utilized for erythropoiesis [21, 28]. Majority of cell types acquire iron from Tf by the Tf-transferrin receptor (Tf/TfR) pathway, wherein Tf-bound Fe^{3+} iron released in the acidic endosomes is reduced to the Fe^{2+} form by Steap 3 for transport through DMT1 to the cytosol. Other cells such as enterocytes and reticuloendothelial cells utilize the DMT1 pathway of iron uptake, though concomitant uptake by the Tf/TfR pathway also occurs [24, 25, 30].

Published reports indicate that PrP^C facilitates iron transport across duodenal enterocytes and uptake by parenchymal and hematopoietic cells of various organs [13]. In neuroblastoma cells, PrP^C increases the labile iron pool and iron stores in cytosolic ferritin [31], indicating that the facilitative role of PrP^C in iron uptake and utilization is common among cells of systemic organs and the brain. Though suggestive, these observations fall short of identifying the function of PrP^C in systemic or brain iron metabolism. In this study, we use a combination of wild type (PrP^{+/+}) and PrP^{-/-} mouse models and human neuroblastoma cells transfected to express PrP^C and mutant PrP forms to identify the function of PrP^C in iron metabolism. We demonstrate that PrP^C is a ferrireductase, and the copper binding octa-peptide repeat region in its N-terminal domain and expression on the plasma membrane are necessary for this function.

MATERIALS AND METHODS

Ethics statement

Animal protocols were approved by the Animal Resource Center and the IACUC committee at Case Western Reserve University (CWRU) (protocol number 2011-0070), and procedures were performed in accordance with guidelines established by the IACUC committee at CWRU.

Materials, cells, and culture conditions

Human neuroblastoma (M17) cells were obtained from the American Type Culture Collection and transfected with PrP (PrP^C) and mutant PrP forms including the pathogenic mutation at codon 102 (PrP^{102L}) [31], deletion within the octa-peptide repeat region (PrP⁵¹⁻⁸⁹), deletion of N-terminal 90 amino acids (PrP²³⁻⁸⁹), secreted PrP lacking the glycosylphosphatidyl inositol (GPI) anchor signal-peptide by introducing a stop codon at residue 231 (PrP^{231stop}), and cytosolic PrP lacking both N- and C-terminal signal peptides (PrP²³⁻²³¹) as described in previous reports [16, 31]. Non-transfected M17 cells were cultured in Opti-MEM supplemented with 5% donor bovine serum and penicillin, streptomycin, and fungizone (1% of each). Transfected cells were cultured in the presence of 500 µg/ml of hygromycin to maintain constant selection pressure. Bulk selected cells were used for all experiments to avoid artifacts due to cloning. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Culture media and supplements were obtained from Invitrogen (Carlsbad, CA). Primary antibody to PrP (3F4) was from Signet Laboratories (Dedham, MA), and antibodies to

ferritin and β -actin were obtained from Sigma-Aldrich (St. Louis, MO) and Millipore (Bedford, MA) respectively. Horseradish peroxidase-conjugated secondary antibodies and ECL Detection Kit were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Radiolabeled $^{59}\text{FeCl}_3$ was obtained from Perkin-Elmer. Iron-deficient diet (TD 80396) was purchased from Harlan Teklad (Madison, WI). Iron supplementation was done by gavage feeding 200 μg of carbonyl iron in 200 μl of water per mouse every day for 2 months. Iron estimation kit was from Stanbio laboratory (Boerne, TX). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Transgenic mice

Wild type ($\text{PrP}^{+/+}$) and transgenic FVB/NJ mice lacking PrP ($\text{PrP}^{-/-}$) were originally obtained from George Carlson (McLaughlin Research Institute). Colonies of $\text{PrP}^{+/+}$ and $\text{PrP}^{-/-}$ mice were bred and maintained in our facility. Age-matched females between the ages of 2 to 6 months were used for all experiments.

Evaluation of iron uptake and transport

Two experimental paradigms were tested: 1) Two-month old age- and sex-matched $\text{PrP}^{+/+}$ and $\text{PrP}^{-/-}$ mice were provided normal diet (C), normal diet supplemented with carbonyl iron for 2 months (I+) [32], or iron-deficient diet (I-) for 2 months. One group was sacrificed and evaluated at the end of 2 months (0 M). Remaining animals were chased on normal diet for 5 months (5 M) before evaluation. At the indicated chase times, animals were prepared and evaluated as described below and in a previous report [13]. 2) Three-month old matched $\text{PrP}^{+/+}$ and $\text{PrP}^{-/-}$ mice maintained on normal diet were inoculated with iron-dextran (5 mg/25 g body weight) intraperitoneally [33] and chased for 24 h before evaluation.

At each chase time point, control and experimental mice were starved overnight and 20 μCi of $^{59}\text{FeCl}_3$ diluted in 0.2 ml of sterile normal saline was introduced orally with a gavage needle. Following a chase of 4 h, animals were euthanized and blood was collected by cardiac puncture in heparinized vials. Plasma and red cells were separated by centrifugation at $100\times g$ for 5 min and red cells were washed three times with normal saline before spotting 100 μl on a PVDF membrane for autoradiography. The first 10 cm of duodenum were rinsed with cold PBS, vacuum dried on filter paper, and autoradiographed. Subsequently, dried duodenum (10 cm) was cut out of the filter paper and absolute ^{59}Fe counts were quantified in a γ -counter. Brain, liver, spleen, and femur were harvested and snap frozen on dry ice. All organs were weighed and radioactivity was counted in a γ -counter (Beckman Gamma 5500).

Bone marrow evaluation and culture of macrophages

Long bones of control and iron-dextran injected mice were crushed and marrow was smeared on glass slides. Air-dried smears were stained for iron by Perl's reaction and examined. Additionally, cells collected from long bones were fractionated by SDS-PAGE and transferred proteins were probed for ferritin.

For culturing bone marrow macrophages, $\text{PrP}^{+/+}$ and $\text{PrP}^{-/-}$ mice were euthanized and marrow cells from the tibia and femur were flushed out with DMEM under sterile

conditions. Isolated cells were washed with DMEM, passed through a 40 μ m cell strainer (BD biosciences), and cultured in DMEM supplemented with 10% fetal bovine serum, 1% antibiotic cocktail, and 25 ng/ml of mouse macrophage colony stimulating factor (R&D systems) for 6 days. Cells were re-plated and cultured overnight before evaluation.

For evaluation of iron uptake, BM macrophages were cultured in the absence (control) or presence of 30 μ M ferric ammonium citrate (FAC) spiked with $^{59}\text{FeCl}_3$ for 16 h. Cells were either harvested at 0 h or chased in serum containing medium for 24 h before evaluation. At each time point cells were washed with cold PBS and incorporation of ^{59}Fe was quantified in a γ -counter and normalized to total protein in each sample.

Sequence alignment of PrP

Sequence alignment of mouse and human PrP^C, STEAP-2, STEAP-3, STEAP-4, and the *Methanothermobacter thermautotrophicus* F420H2:NADP-oxidoreductase (FNO) was performed using MultAlin [34].

Ferrireductase assay

FR assay was performed essentially as described by Riedel et al. [35]. Freshly prepared Fe^{3+} /NTA (Fe^{3+} /nitrilotriacetic acid) solution in a molar ratio of 1 : 4 was titrated to neutral pH before use. A standard 1 ml assay mix consisted of 50 μ M Fe^{3+} /NTA, 200 μ M ferrozine, and 100 μ M NADH in assay buffer A (140 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 5 mM NaH_2PO_4 , and 5 mM Hepes, pH 7.4). Cells were lysed by adding PBS containing 1% Triton X-100 directly to the culture dish and lysates were collected. For estimation of total FR activity in PrP^C and mutant cell lines, 10 μ g of protein from cell lysates was added to the reaction mix and incubated at 37°C for 90 min away from light. Subsequently, samples were centrifuged at 10,000 $\times g$ for 2 min, and change in OD of the supernatant relative to the control assay mix (without cells) was measured at 562 nm. Background arising from buffers without the addition of lysates was subtracted before calculating FR activity from a standard curve established from graded concentrations of ferrous ammonium sulfate, and expressed as nmoles of Fe^{2+} /min/mg protein. To determine the effect of heat on FR activity, samples were heated at 100°C for 10 min and assayed as above. For measuring FR activity on the cell surface, PrP^C and mutant cell lines cultured overnight in complete medium were washed with buffer A, and exposed to 1 ml of assay mix (described above) at 37°C for 2 h in a cell culture incubator away from light. Control samples consisted of assay mix incubated in the same dish without cells. Cell viability was checked by trypan blue exclusion, and the assay mix was collected and centrifuged to pellet any cells. Change in OD of cell-free supernatant was quantified at 562 nm as above and compared with controls. Subsequently, cells were lysed and total protein estimated with the D_c Protein Assay Kit (BioRad). FR activity was normalized to total protein in each sample. Cell viability was >95% before and after the completion of this assay. All assays were performed from triplicate cultures of each cell line, and the experiment was repeated at least three times.

SDS-PAGE and western blotting

Samples prepared in lysis buffer were resolved by SDS-PAGE, transblotted to a PVDF membrane, and probed for specific proteins as described in previous reports [13–16].

Statistical analysis

All results are expressed as mean $\pm$ standard error of mean (SEM). Comparison of two groups was performed by unpaired Student's *t*-test, and of multiple groups by one way ANOVA followed by Bonferroni multiple comparison *post hoc* test using GraphPad Prism software (Version 4.03, GraphPad Inc., San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

RESULTS

Excess dietary iron corrects the iron deficiency in PrP^{-/-} mice temporarily

Systemic iron deficiency reported in PrP^{-/-} mice [13] could be attributed to 1) decreased uptake or transport of dietary iron from the duodenum, 2) inefficient uptake, storage, and re-utilization of absorbed iron by parenchymal and hematopoietic cells, or 3) a combination of the above. To distinguish between these possibilities, uptake of radioactive iron (⁵⁹FeCl₃) introduced orally (Figs. 1–2) or intraperitoneally (Fig. 3A, B) to age and sex-matched PrP^{+/+} and PrP^{-/-} mice was compared under different experimental conditions.

The response of PrP^{-/-} mice to dietary iron supplementation or depletion was evaluated in age and sex-matched PrP^{-/-} and PrP^{+/+} mice maintained on normal diet (C), normal diet supplemented with carbonyl iron (I+), or custom formulated iron deficient (I-) diet for 2 months. One set of mice were evaluated at the end of 2 months (0 M), and the other chased on normal diet for 5 months (5 M) before evaluation. At the indicated times, equal counts of ⁵⁹FeCl₃ were introduced by gastric gavage, and following a chase of 4 h, incorporation of ⁵⁹Fe in the duodenum, RBCs, femur, liver, and other peripheral organs was quantified. Duodenum samples were dried and exposed to X-ray film, subsequent to which ⁵⁹Fe in the first 10 cm was quantified (Figs. 1–2).

Comparison of duodenum samples from 0 M controls revealed significantly more ⁵⁹Fe in PrP^{-/-} relative to matched PrP^{+/+} controls, indicating increased uptake by duodenal epithelial cells or impaired transport to the blood stream as reported previously (Fig. 1A, lanes 1 & 4, Fig. 1B) [13]. Chronic exposure to excess dietary iron resulted in significant inhibition of ⁵⁹Fe uptake in 0 M PrP^{-/-} (I+) and PrP^{+/+} (I+) mice relative to controls (C), reflecting excess systemic iron and appropriate inhibition of ⁵⁹Fe uptake from the intestinal lumen in both sets of mice (Fig. 1A, lanes 2 & 5 versus 1 & 4, Fig. 1B). Iron deficient diet (I-) increased ⁵⁹Fe uptake significantly in both mouse lines, indicating appropriate response to systemic iron deficiency by the duodenal epithelium (Fig. 1A, lanes 3 & 6 versus 1 & 4, Fig. 1B).

However, significant differences surfaced between PrP^{+/+} (I+) and PrP^{-/-} (I+) mice after a chase of 5 M. In contrast to PrP^{+/+} (I+) mice that continued to absorb significantly less ⁵⁹Fe relative to matched controls (Fig. 1A, lanes 7 & 8, Fig. 1B), PrP^{-/-} (I+) mice incorporated

equivalent amounts of ^{59}Fe as $\text{PrP}^{-/-}$ (C) mice (Fig. 1A, lanes 9 & 10, Fig. 1B), and significantly more than comparable $\text{PrP}^{+/+}$ (I+) mice (Fig. 1A, lanes 8 & 10, open arrow-head, Fig. 1B), indicating reversion to the iron deficient phenotype. Chase of 0 M (I-) mice on normal diet restored systemic iron to control levels in both mouse lines, and these animals were not evaluated further (data not shown).

Utilization of absorbed ^{59}Fe for hematopoiesis was assessed by spotting washed RBCs from equal volume of blood on a PVDF membrane followed by autoradiography (Fig. 2A). Uptake of ^{59}Fe by systemic organs was quantified in a γ -counter (Fig. 2B–D). As in Fig. 1, untreated $\text{PrP}^{-/-}$ (C) mice showed significantly more ^{59}Fe relative to matched $\text{PrP}^{+/+}$ (C) controls in the RBCs, femur, and liver in both 0 M and 5 M groups (Fig. 2A, lane 1 versus 4 & 7 versus 9, Fig. 2B–D). $\text{PrP}^{+/+}$ (I+) and $\text{PrP}^{-/-}$ (I+) samples showed negligible incorporation of ^{59}Fe in these organs at the 0 M time point (Fig. 2A–D). However, while $\text{PrP}^{+/+}$ (I+) samples continued to show minimal uptake of ^{59}Fe after a chase of 5 M, $\text{PrP}^{-/-}$ (I+) mice reverted back to control levels when the source of iron was removed, and incorporated significantly more ^{59}Fe relative to matched $\text{PrP}^{+/+}$ (I+) samples (Fig. 2A–D, open arrowhead). Iron deficient (I-) samples from both groups incorporated significantly more ^{59}Fe relative to matched controls (C) as expected (Fig. 2A–D). Notably, RBCs from the 0 M $\text{PrP}^{-/-}$ (I-) group incorporated significantly more ^{59}Fe than matched $\text{PrP}^{+/+}$ (I-) samples, reflecting amplification of the iron deficiency in this group (Fig. 2A, B). Uptake of ^{59}Fe by the spleen and brain revealed a similar pattern, indicating that the observed differences are not restricted to specific organs (data not shown). Surprisingly, RBCs, femur, and liver from $\text{PrP}^{-/-}$ (I+) mice incorporated significantly more ^{59}Fe than matched controls in the 5 M group, indicating rebound iron deficiency after a chase on normal diet (Fig. 2A–D).

Together, these results demonstrate appropriate response of duodenal epithelial cells and other organs of $\text{PrP}^{-/-}$ mice to systemic iron deficiency. However, persistence of the iron deficiency in these mice at steady state [13] despite increased uptake, correction of the iron deficient phenotype by excess dietary iron, and re-appearance of iron deficiency when weaned off of excess iron indicates inefficient uptake, storage, and/or recycling of absorbed iron by systemic organs in the absence of PrP^{C} . Subsequent studies were therefore directed at evaluating these possibilities.

Bone marrow macrophages of $\text{PrP}^{-/-}$ mice show impaired iron uptake and storage

Since ~80% of the absorbed iron is utilized for erythropoiesis, we evaluated the uptake, storage, and utilization of iron by BM macrophages of $\text{PrP}^{-/-}$ mice. Accordingly, iron-dextran was introduced intraperitoneally to achieve iron overload, and BM smears were prepared after 24 h. This approach allowed flux of iron between organ systems during 24 h of chase, and simulated the normal and excess dietary iron paradigms in Figs. 1 and 2 after eliminating regulation of iron uptake at the duodenum.

Examination of crushed BM smears from control and iron-dextran inoculated mice showed significantly less stainable iron in $\text{PrP}^{-/-}$ macrophages relative to matched $\text{PrP}^{+/+}$ controls (Fig. 3A, panels 1–4). Immunoblotting of BM extracts revealed significantly less reactivity for ferritin in $\text{PrP}^{-/-}$ samples (Supplemental Figure 1A, B; available online: <http://www.j->

alz.com/issues/35/vol35-3.html#supplementarydata03), partly explaining the decreased reactivity for stainable iron in these samples.

To differentiate between iron uptake, storage, or release as the primary cause of decreased iron in PrP^{-/-} BM macrophages, cultures of primary BM macrophages from PrP^{+/+} and PrP^{-/-} mice were exposed to ⁵⁹FeCl₃ in the absence or presence of 30 μM FAC for 16 h. One set of samples was analyzed after 16 h (0 h), and the other set chased for 24 h (24 h) in complete medium at 37°C before evaluation. At the indicated times the cultures were checked for viability, and incorporation of ⁵⁹Fe was quantified in a γ-counter. At the 0 h time point, PrP^{-/-} macrophages incorporated 0.4 fold less ⁵⁹Fe relative to PrP^{+/+} samples (Fig. 3B, 0 h). Chase of 24 h did not alter ⁵⁹Fe counts in PrP^{+/+} or PrP^{-/-} macrophages significantly, indicating minimal efflux to the extracellular medium under these conditions (Fig. 3B, 0 & 24 h). Presence of FAC in the extracellular medium increased ⁵⁹Fe incorporation in both PrP^{-/-} and PrP^{+/+} macrophages significantly. However, the increase in PrP^{-/-} macrophages was 0.5 fold less than matched PrP^{+/+} samples even in the presence of FAC (Fig. 3B, +FAC 0h). Notably, FAC treated PrP^{+/+} macrophages retained most of the absorbed ⁵⁹Fe after a chase of 24 h, while PrP^{-/-} samples lost most of the acquired ⁵⁹Fe and reverted back to levels similar to the 24 h untreated samples (Fig. 3B, -/+FAC 24 h).

Together, these results indicate that absence of PrP^C impairs iron uptake and storage in ferritin by BM macrophages of PrP^{-/-} mice.

PrP^C functions as a ferrireductase

Since PrP^{-/-} mice show altered uptake and storage of iron at anatomic sites that involve distinct proteins and pathways [13], investigations were focused on shared mechanisms of iron utilization such as ferroxidase and FR activities necessary for oxidizing and reducing iron respectively. Predominant ferroxidases involved in systemic iron metabolism include ceruloplasmin and hephaestin [28], and major known ferrireductases are Dcytb [24, 25] and the Steap family of proteins (Steap 2–4) [29]. Sequence alignment of mouse and human PrP with known FR proteins revealed NAD(P)H binding domain in the N-terminal region that shares sequence homology with the Steap family of reductases (Steap 2, 3, & 4) and flavin FNO from *Methanothermobacter thermautotrophicus* (Fig. 4), suggesting a functional role as a FR.

To determine whether PrP^C possesses FR activity, human neuroblastoma (M17) cells expressing barely detectable levels of PrP^C or transfected to express 6–9 fold higher levels of PrP^C (PrP^C-cells) were used as models (Fig. 5). Since PrP^C is expressed on the plasma membrane, FR activity was checked on the cell surface and in cell lysates. Minimal difference was noted in ferroxidase activity between the two cell lines (Supplementary Figure 2). However, FR activity was ~0.3 fold lower in M17 cells relative to matched PrP^C-cells at the plasma membrane and in lysates (Fig. 5A, B). Activity in lysates was 9-fold higher than cell surface in both cell lines probably due to contribution from other intracellular FR proteins (Fig. 5B). Cell surface FR activity was undetectable in the absence of NADH (data not shown) and was sensitive to heat (Supplementary Figure 3), supporting protein-associated origin.

To identify the region of PrP^C critical for this function, M17 cells expressing mutant PrP forms with a deletion in the octa-peptide repeat region (PrP^{51–89}), deletion of N-terminal 90 amino acids (PrP^{23–89}), lacking the C-terminal GPI-signal peptide (secreted PrP, PrP^{231stop}), lacking both N- and C-terminal signal peptides (cytosolic PrP, PrP^{23–231}), or carrying the pathogenic mutation 102L (PrP^{102L}) were tested (Fig. 5C & D and Supplementary Figure 4A). Comparison of FR activity of PrP^C-cells with mutant cell lines showed a significant reduction in cell surface and intracellular activity in all cell lines except PrP^{102L} that, surprisingly, showed a significant increase in activity (Fig. 5C, D). The difference in FR activity between cell lines did not correlate with PrP expression level (Supplementary Figure 4B, C), suggesting that specific region(s) of PrP are responsible for this function.

DISCUSSION

Despite the ubiquitous expression of PrP^C on all cell types, little significance has been attributed to its presence except on neuronal cells. This study demonstrates that PrP^C is a FR, and its absence in PrP^{-/-} mice impairs iron uptake, storage, and utilization in most organ systems including the hematopoietic system. The FR activity of PrP^C is NADH dependent, and requires the copper binding octa-peptide repeat region and linkage to the plasma membrane for optimal function. These observations identify PrP^C as a novel FR that influences iron uptake and storage at distinct anatomical sites. Since PrP^C is most abundant on neuronal cells, these observations have significant implications for sCJD and other prion disorders where brain iron dyshomeostasis is a prominent feature [14, 36, 37].

To understand the implications of FR activity of PrP^C on systemic iron homeostasis, it is pertinent to identify anatomic and cellular sites and specific iron metabolic pathways where dysfunction or absence of PrP^C is likely to have the most impact. Since only reduced (Fe²⁺) iron is transported through DMT1, FR activity of PrP^C is likely to have the most impact on this pathway. Increased uptake of orally introduced ⁵⁹Fe by duodenal enterocytes, liver, femur, and RBCs of PrP^{-/-} mice indicates that absence of PrP induces systemic iron deficiency, and duodenal epithelial cells respond appropriately by increasing uptake of iron from the intestinal lumen. Although this phenotype is corrected by oral supplementation of excess inorganic iron, it resurfaces when the iron source is removed while similarly treated PrP^{+/+} mice remain iron overloaded as expected. These observations can be explained by 1) inefficient uptake and/or storage of excess iron by the liver in PrP^{-/-} mice, or 2) impaired release of stored iron to meet systemic iron demands. Since the amount of iron in PrP^{-/-} livers is significantly less than matched controls as revealed by increased incorporation of orally introduced ⁵⁹Fe and decreased total iron at steady state (Supplementary Figure 5), the latter possibility is unlikely [13, 27]. Moreover, the increase in liver iron is significantly less in PrP^{-/-} mice relative to matched PrP^{+/+} controls even after intraperitoneally inoculation of excess iron-dextran (Supplementary Figure 5), indicating reduced uptake or storage as the underlying cause. Surprisingly, alternate mechanisms of iron uptake do not compensate for this deficiency, suggesting that PrP^C participates in a non-redundant pathway of iron uptake and storage by hepatocytes and reticuloendothelial cells of the liver.

The iron deficient phenotype of PrP^{-/-} RBCs is more surprising since red cell precursors acquire most of their iron by the Tf/TfR pathway, and the predominant FR in these cells is Steap 3 [29, 38, 39]. Increased uptake of ⁵⁹Fe by the femur and RBCs of PrP^{-/-} mice within 4 h of oral administration suggests enhanced erythropoiesis and premature release of reticulocytes to the peripheral circulation, perhaps in response to the iron deficiency. The relatively quick incorporation of ⁵⁹Fe by PrP^{-/-} RBCs suggests direct uptake by erythrocyte precursors from the peripheral circulation by the Tf/TfR pathway, and minimal role of PrP^C in this process. However, more than 80% of iron for erythropoiesis is recovered from phagocytosed senescent RBCs by the BM and splenic macrophages and reutilized [40–42]. Relatively low stainable iron in PrP^{-/-} BM macrophages at steady state suggests ineffective erythrophagocytosis and/or storage of released iron in ferritin. Since PrP^{-/-} RBCs show minimal increase in ⁵⁹Fe levels after the initial spike while PrP^{+/+} RBCs show a linear increase in ⁵⁹Fe content for upto 11 days [13], it is likely that PrP^{-/-} macrophages do not store the iron released from senescent RBCs effectively. However, inability to increase stainable iron in PrP^{-/-} BM macrophages even after intraperitoneally inoculation of iron dextran points to an additional deficiency in uptake from the peripheral circulation. Since iron uptake by both the DMT1 and Tf/TfR pathway require the activity of a FR [26], it is likely that PrP^C is the principal FR in these cells, and inhibits uptake of iron for subsequent storage in ferritin. Reduced uptake of NTB-iron by cultured PrP^{-/-} macrophages in the absence or presence of excess extracellular iron supports this assumption. PrP^{-/-} macrophages have also been shown to exhibit reduced phagocytic activity [43, 44], suggesting that a combination of inadequate uptake from peripheral circulation and inefficient recovery from senescent RBCs are responsible for the reduced iron content of PrP^{-/-} macrophages. These observations also explain the slow recovery of PrP^{-/-} mice from experimentally induced hemolysis, a condition that requires efficient recovery of iron from damaged RBCs for re-utilization [12]. Interestingly, PrP^C is expressed on the plasma membrane of erythroid precursors and decreases as the cells mature and accumulate hemoglobin, suggesting a role in their development [45].

The FR activity of PrP^C is demonstrated clearly in transfected neuroblastoma cells. PrP^C-associated FR activity is dependent on NADH, the copper binding octa-peptide repeat region in the N-terminus, and linkage to the plasma membrane. Interestingly, PrP possesses a NADPH binding motif in the N-terminus that is similar to the Steap family of ferrireductases [29, 38]. Proximity of this site to the copper binding octa-peptide repeat region equips PrP^C to reduce Fe³⁺ iron at the plasma membrane, facilitating the transport of iron and perhaps other divalent cations across the lipid bilayer through DMT1. Deletion of the copper binding region abolishes this activity regardless of the NADPH binding region, emphasizing the requirement of available copper for this function. Cytosolic and secreted forms of PrP show minimal FR activity, indicating that linkage to the plasma membrane is necessary for active FR function. A direct correlation between ⁵⁹Fe uptake and FR in neuroblastoma cells expressing PrP^C and mutant PrP forms [31] provides further evidence that PrP^C facilitates iron uptake through this process. A cupric-reductase activity of the octa-peptide repeat domain of PrP^C has been described [46], and in this regard mimics the Dcytb and Steap family of ferrireductases that possess both ferri- and cupric reductase activities [29, 47]. Surprisingly, the FR activity and iron uptake are increased in neuroblastoma cells expressing

PrP^{102L}, a pathogenic mutation that segregates with an inherited form of human prion disease. Although PrP^{102L} undergoes aberrant recycling and cleavage in neuroblastoma cells [48], the influence of this abnormality on FR activity and iron uptake by these cells is unclear. Further investigations are necessary to resolve this question.

In conclusion, the relatively mild but persistent iron deficiency of PrP^{-/-} mice underscores the function of PrP^C in systemic iron homeostasis. A rather serious consequence of this function can be envisioned in sCJD brains where loss of function combined with toxic gain of function by aggregated PrP^{Sc} is likely to induce brain iron dyshomeostasis. This report provides important insight into the role of PrP^C in iron metabolism, and may help in the development of therapeutic strategies that reduce iron-induced neurotoxicity in sCJD brains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Excess dietary iron compensates the iron deficiency in $\text{PrP}^{-/-}$ mice temporarily. A) Uptake of orally introduced ^{59}Fe was checked in $\text{PrP}^{+/+}$ and $\text{PrP}^{-/-}$ mice maintained on normal diet (C), iron supplemented diet (I+), or iron deficient diet (I-) for 2 months. Evaluation was performed at the end of 2 months (0 M) (lanes 1–6) or after a chase of 5 months (5 M) on normal diet (lanes 7–10). Autoradiographs of duodenum samples show increased uptake by C $\text{PrP}^{-/-}$ samples at 0 M and 5 M time points (lanes 1 versus 4 & lanes 7 versus 9). This difference is abolished in I+ samples at 0 M (lanes 2 & 5), but enhanced after 5 M of chase (lanes 2 versus 5 & lanes 8 versus 10, open arrowhead). Samples from I- $\text{PrP}^{+/+}$ and $\text{PrP}^{-/-}$ group show similar uptake of ^{59}Fe at 0 M (lanes 3 & 6). B) Quantification of ^{59}Fe in the first 10 cm of duodenum shows a significant decrease in I+ $\text{PrP}^{+/+}$ and increase in I- $\text{PrP}^{+/+}$ and C $\text{PrP}^{-/-}$ samples relative to C $\text{PrP}^{+/+}$ samples in the 0 M and 5 M groups (** $p < 0.01$, *** $p < 0.001$). There is a significant decrease in I+ $\text{PrP}^{-/-}$ relative to matched C $\text{PrP}^{-/-}$ samples at the 0 M time point (### $p < 0.001$). However, this difference disappears in at the 5 M time point (open arrowhead). On the other hand, the insignificant difference between I+ $\text{PrP}^{+/+}$ and I+ $\text{PrP}^{-/-}$ samples at 0 M becomes highly significant in the 5 M samples (@ @ @ $p < 0.001$). Values are mean $\pm$ SEM; ns: non-significant.

Fig. 3.

Bone marrow (B) macrophages of PrP^{-/-} mice show impaired iron uptake and storage. A) Perl's reaction of BM smears shows more stainable iron in control and iron dextran inoculated PrP^{+/+} mice relative to matched PrP^{-/-} samples after 24 h (panels 1–4). B) Cultured primary BM macrophages from PrP^{-/-} mice take up significantly less ⁵⁹Fe from the extracellular medium in the absence or presence of excess iron relative to matched PrP^{+/+} samples (0 h time point, -/+FAC. ****p* < 0.001; ###*p* < 0.001). PrP^{+/+} cells incorporate 2.1 fold more ⁵⁹Fe in the presence of excess extracellular iron relative to a modest increase of 0.8 fold in PrP^{-/-} samples (0 h time point, -/+FAC; ****p* < 0.001; ###*p* < 0.001). There is minimal efflux of absorbed iron from PrP^{+/+} and PrP^{-/-} macrophages in both sets of samples after 24 h of chase in normal medium (0 versus 24 h, -/+FAC). However, ⁵⁹Fe content of +FAC PrP^{+/+} samples is significantly higher than -FAC samples after 24 h (@ @ *p* < 0.01), whereas +FAC PrP^{-/-} samples lose the absorbed iron after 24 h and show minimal difference from 24 h -FAC PrP^{-/-} samples. Values are mean ± SEM; ns: non-significant.

HumanPrP	1	MANLGC	WHLVLFVATW	SDLGLCKRRP	KPGGWNTGGS	RYFGQGSPPG	NRYPPQGGGG	WGQPHGGGWW	QPHGGGWGQP	HGGGWGQPHG	GGWGQGGGTH	SQU--NKPSK	130
MousePrP		MANLGY	WLLALFVIMW	TDVGLCKRRP	KPGGWNTGGS	RYFGQGSPPG	NRYPPQGGT-	WGQPHGGGWW	QPHGGGWGQP	HGGGWGQPHG	GGWGQGGGTH	NQU--NKPSK	
STEAP2		MESISMHGSP	KSLSETFLPN	GINGIKDARK	VTGVIGSGD	FAKSLTIRLI	RCGGYHVIGS	RNPKRTARLF	PHVVDVTHHE	DALTKTNIIF	VAIHREHYTS	LWDLRLHLLG	KILIDVENNM
STEAP3		NPEEMDKP	LISLHLVDS	SSLAKVPDEA	PKVGILGSGD	FARSLATIRLV	GGGFKVVIGS	RNPKRTARLF	PSAAQVTFQE	EAVSSPEVIF	VAVFREHYTS	LCSLSDQLAG	KILVDVENMT
STEAP4			MEKTCIDAL	PLTMSSEKQ	ETVCIFGTGD	FGRSLGLKML	CCGYSVVIGS	RNPKRTT-LL	PSGAEVLSYS	EAAKSGIIL	IATHREHYDF	LTELTEVLNG	KILVDISNNL
FNO				MIKVMVM	KIAVIGGTGD	QGLGLALRFA	VAGEEVIIGS	RDAEKASKAA	SKVLEIAGRD	DISVEGATNP	DAAAS---AD	VVVLTVPLQA	QMTLASIRD
Consensus					gtgd	g..f..	g..v..	GS	R..p..	s..	g..	g..	a..
												l..l..g	s..
													#.p.k
HumanPrP	131	PKTNHKKHAG	AAAAGAVVGG	LOGYMLGSAM	SRPIHFGSD	YEDRYVRENH	HRYPNQVYVR	PNDVYSNQNN	FVHDGVNITI	K-QHTVITTT	KGENFTETDV	KHNEVVEQOM	CITQYERESQ
MousePrP		PKTNLKHVAG	AAAAGAVVGG	LOGYMLGSAM	SRPIHFGSD	WEDRYVRENH	HRYPNQVYVR	PNDVYSNQNN	FVHDGVNITI	K-QHTVITTT	KGENFTETDV	KHNEVVEQOM	CVTQYKESQ
STEAP2		NAEYLASLFP	DSLIVKGFNV	VSAVALQGP	RDASRQVVIC	SNNIQARQGV	IELARQLNFI	PIDLGLSLSA	DEIENLPLRL	FTLURGPPVV	ATSLATFFFL	YSFVBDVTHP	YA--RWQNSD
STEAP3		NAEYLASLFP	TCTVVKAFNV	ISAVTLQAGP	RDGNRQVVIC	GDOPEAKRAV	SEMALANGFM	PVDHGLSLASA	WEVEAMPLRL	LPAWKVPVTL	ALGLFVCVYA	YNFVBDVLOP	YV--QESONK
STEAP4		NAEYLASLFP	GAHVVKAFNV	ISAVTLQAGP	LDASRQVVIC	GNDSEAKQV	NDIVRNLGLT	PVDHGLSLASA	WEVEAMPLRL	FPNWRFPVYL	SAVLGVFLFF	YCFVBDVTHP	YV--YERKDN
FNO		VLIDATVPID	SCIGSSAURY	IDLWEGSALE	RAARFLREQG	TRVAAAFNNI	SASALLEVSE	PVDCDCLVAS	DHRDALEVA	ELAEEKIDGVR	AIECGGLENA	RIEEKITPLL	IN--LMIRNR
Consensus			g..v..	w.l..a.	a..	ae#n	a..q..	PvD..sl..	#..	a..e..	e..v..	..s..	y..
													iri.n
HumanPrP	261	MVLFSSPPVI	LLISFLIFLI	VG									390
MousePrP		TVLFSSPPVI	LLISFLIFLI	VG									
STEAP2		KTLPIVAITL	LSLVYLAGLL	AAAYQLYVGT	KYRRFPFWLE	TWLQCRKQLG	LLSFFFAVMH	VATSLCLPMR	RSERYFLNM	AYQQVHANIE	NSWNEEEVVR	IENYISFGIM	SLGLLSLLAV
STEAP3		TTLPCVAVYL	LSLVYLPVGL	AAALQLRRGT	KYQRFDDWLD	HVLQHRKQIG	LLSFFCAALH	ALYSFCLPLR	RAHRYDLVNL	AVKQVLANKS	HLWVEEEVVR	NEIYLSLGVL	ALGTLSLLAV
STEAP4		RIFPITALTL	LALVYLPVGI	AAILQLYRGT	KYRRFPDDWLD	HVHLCKRQLG	LVALGFALFH	VLYTLVIPIR	YVVRVRLGNL	TVTQAILKKE	NPFSTSSAUL	SDSYVALGIL	GFLLFVLLGI
FNO		LPEQE											
Consensus			l..l..l..l										
HumanPrP	391	NWREFSFIQI	FCSFADTQTE	LELEFVFLIT	LLL								492
MousePrP		NWREFSFIQI	FCSFADTQTE	LELEFVFLIT	LLL								
STEAP2		NWREFSFIQI	FCSFADTQTE	LELEFVFLIT	LLL								
STEAP3		NWREFSFIQI	FCSFADTQTE	LELEFVFLIT	LLL								
STEAP4		NWREFSFIQI	FCSFADTQTE	LELEFVFLIT	LLL								
FNO		NWREFSFIQI	FCSFADTQTE	LELEFVFLIT	LLL								
Consensus													

Fig. 4.

PrPC shares sequence homology with known NAD(P)H reductases. Alignment of mouse and human PrPC with Steap 2, 3, 4, and the F420H2:NADP+oxidoreductase (FNO) of bacterium *Methanothermobacter thermautotrophicus* by multiple sequence alignment with MultAlign shows the predicted NAD(P)H binding motif GSR in the N-terminus of PrPC (underlined). High and low consensus residues are shown in red and blue respectively.

Fig. 5.

PrP^C is a ferriredutase. A, B) FR activity is significantly increased in PrP^C cells relative to non-transfected M17 controls on the cell surface and in lysates. C, D) Cells expressing mutant PrP forms show significantly less FR activity on the cell surface and in lysates relative to PrP^C cells except PrP^{102L}-cells which show a significant increase in activity. Values represent mean $\pm$ SEM calculated from 5–8 separate experiments carried out in triplicate or quadruplet. (***) $p < 0.001$, (**) $p < 0.01$.