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The prion-ZIP connection: From cousins to partners in iron uptake

Neena Singh*, Abhishek Asthana, Shounak Baksi, Vilok Desai, Swati Haldar, Sahi Hari, and Ajai K Tripathi

Department of Pathology; School of Medicine; Case Western Reserve University; Cleveland, OH USA

ABSTRACT. Converging observations from disparate lines of inquiry are beginning to clarify the cause of brain iron dyshomeostasis in sporadic Creutzfeldt-Jakob disease (sCJD), a neurodegenerative condition associated with the conversion of prion protein (PrP^C), a plasma membrane glycoprotein, from α-helical to a β-sheet rich PrP-scrapie (PrP^{Sc}) isoform. Biochemical evidence indicates that PrP^C facilitates cellular iron uptake by functioning as a membrane-bound ferrireductase (FR), an activity necessary for the transport of iron across biological membranes through metal transporters. An entirely different experimental approach reveals an evolutionary link between PrP^C and the Zrt, Irt-like protein (ZIP) family, a group of proteins involved in the transport of zinc, iron, and manganese across the plasma membrane. Close physical proximity of PrP^C with certain members of the ZIP family on the plasma membrane and increased uptake of extracellular iron by cells that co-express PrP^C and ZIP14 suggest that PrP^C functions as a FR partner for certain members of this family. The connection between PrP^C and ZIP proteins therefore extends beyond common ancestry to that of functional cooperation. Here, we summarize evidence supporting the facilitative role of PrP^C in cellular iron uptake, and implications of this activity on iron metabolism in sCJD brains.

KEYWORDS. Prion protein, iron, ferrireductase, sCJD, ZIP14, ZIP proteins

INTRODUCTION

Brain iron dyshomeostasis has been reported in several neurodegenerative conditions, including sporadic Creutzfeldt-Jakob disease (sCJD), Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). The underlying cause has been the subject of much debate.[1-3] Recent reports indicating a physiological role of protein(s) implicated in the

*Correspondence to: Neena Singh; Email: neena.singh@case.edu

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pathogenesis of these disorders in cellular iron metabolism provide a partial answer to this long-standing question.[4] Here, we review evidence supporting the role of PrP^C, the protein underlying sCJD pathogenesis, in cellular iron uptake by functioning as a ferrireductase (FR) partner for a member of the Zrt, Irt-like protein (ZIP) family of divalent metal transporters, especially zinc and iron.[1,2,5] It is likely that loss of this function combined with gain of abnormal activity by its β-sheet rich PrP-scrapie (PrP^{Sc}) isoform causes imbalance of iron homeostasis in sCJD brains, creating a potentially neurotoxic environment.[6-8] A complete understanding of this subject would pave the way for the development of disease-specific therapeutic strategies where none exist, and allow a critical assessment of the use of iron chelators as disease-modifying agents.[9]

Iron is essential for vital metabolic processes, but unbound iron is highly toxic because of its ability to shuttle between 2 oxidation states, ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron. Although ideal as a catalyst, uncontrolled oxidation/reduction of iron is likely to generate toxic hydroxyl radicals by Fenton chemistry. Iron homeostasis is therefore tightly regulated at the cellular and systemic levels by the coordinated action of iron uptake, efflux, and storage proteins that are themselves regulated by iron regulatory proteins and the peptide hormone hepcidin.[10] Although iron pools in the brain are separated from systemic circulation by the blood brain barrier (BBB), similar regulatory mechanisms operate within the brain with minor differences.[11] Nevertheless, iron accumulates in the aging brain, and the process is hastened by certain neurodegenerative conditions associated with protein aggregation.[4] Whether this is an active process mediated by loss or altered function of specific protein(s) or a consequence of neuronal death combined with inefficient clearance of iron-rich cell debris has been difficult to parse out. One of the reasons is the difficulty in developing appropriate experimental models that reproduce brain pathology and associated changes in iron homeostasis faithfully.

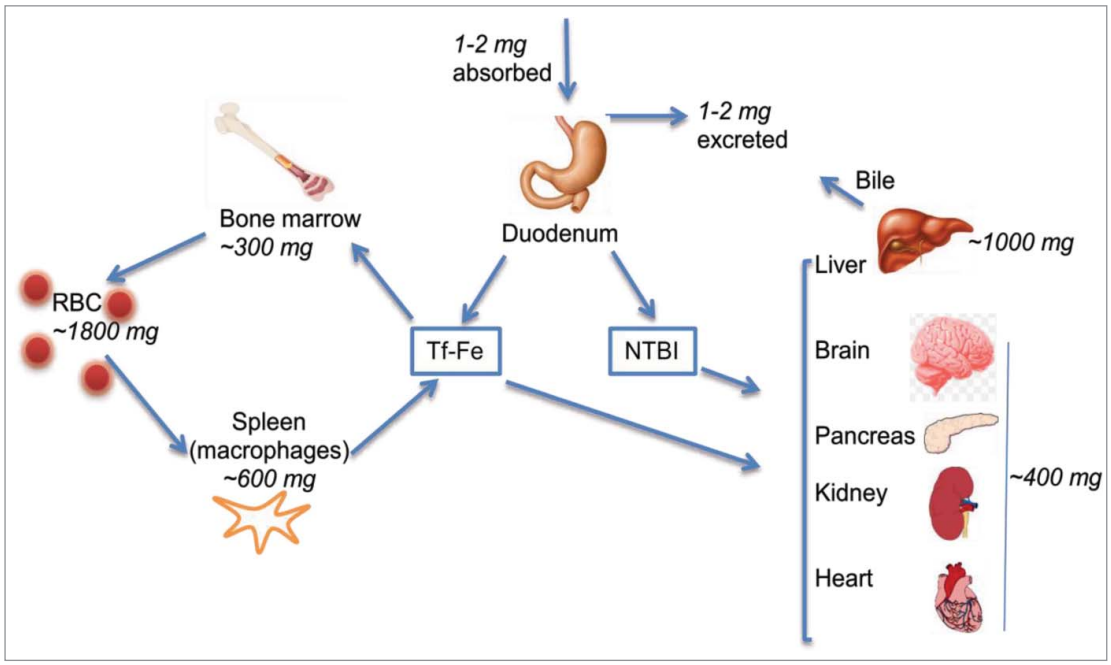
In this regard, models of sCJD and other prion disorders offer 2 specific advantages: 1) PrP^C is expressed ubiquitously on all cells, providing an opportunity to study its role in iron metabolism in systemic organs that are experimentally accessible and iron metabolism is better understood, and 2) animal models reproduce major pathological features of sCJD faithfully in a relatively short time, providing an opportunity to identify specific changes in brain iron metabolism with disease progression.[12,13] This approach has yielded useful information on the physiological function of PrP^C in iron metabolism, and possible causes of iron dyshomeostasis in sCJD brains.[8,13] Below, we summarize recent literature on the functional role of PrP^C in cellular iron uptake, and implications of this activity on brain iron homeostasis in healthy and sCJD affected brains.

IRON TRAFFICKING: A SHORT OVERVIEW

Iron trafficking in the systemic circulation: Of the total body iron, ~80% is utilized for hemoglobin synthesis, and the rest is distributed among systemic organs and the brain. Only ~2 mg of iron are lost daily, and an equivalent amount is absorbed from the intestinal lumen. At the cellular level, iron is taken up by 2 principal mechanisms; transferrin (Tf)-iron via the Tf-receptor (TfR) pathway, and non-Tf bound iron (NTBI) through divalent metal transporters. Heme-associated iron is transported and metabolized by distinct pathways, and will not be discussed here. Under physiological conditions plasma Tf is ~35% saturated with iron, leaving enough buffering capacity for a pathological increase in circulating iron. Nonetheless, circulating NTBI is detected under physiological and several pathological conditions, and is largely sequestered by the liver to prevent cytotoxicity (**Fig. 1**). For detailed explanation of systemic iron uptake and metabolism the reader is directed to recent reviews on this subject.[2,10,14]

Iron trafficking in the brain: Transport of iron from the peripheral circulation to the brain is mediated by the BBB via mechanisms that are still emerging.[11] Current understanding of this process is illustrated in **Figure 2**. It is known that plasma Tf is not transported across the BBB and vice versa. Brain Tf is secreted by the choroid plexus

FIGURE 1. Iron trafficking in the systemic circulation: Systemic iron homeostasis is maintained by regulating uptake from the duodenum. Only 1-2 mg of iron is absorbed daily from the duodenum, and an equivalent amount is lost through epithelial desquamation. Majority of circulating iron is utilized for erythropoiesis, and re-used after recovery from senescent RBCs by splenic macrophages. Iron in the systemic circulation binds plasma Tf or circulates as NTBI in conjugation with small molecular weight compounds. Most organs utilize Tf-Fe preferentially, though some take up significant amounts of NTBI as well. Liver sequesters excess circulating iron and stores in ferritin. Tf-Fe: transferrin iron, NTBI: non-transferrin-bound iron. Numbers indicate the amount of iron in each organ.

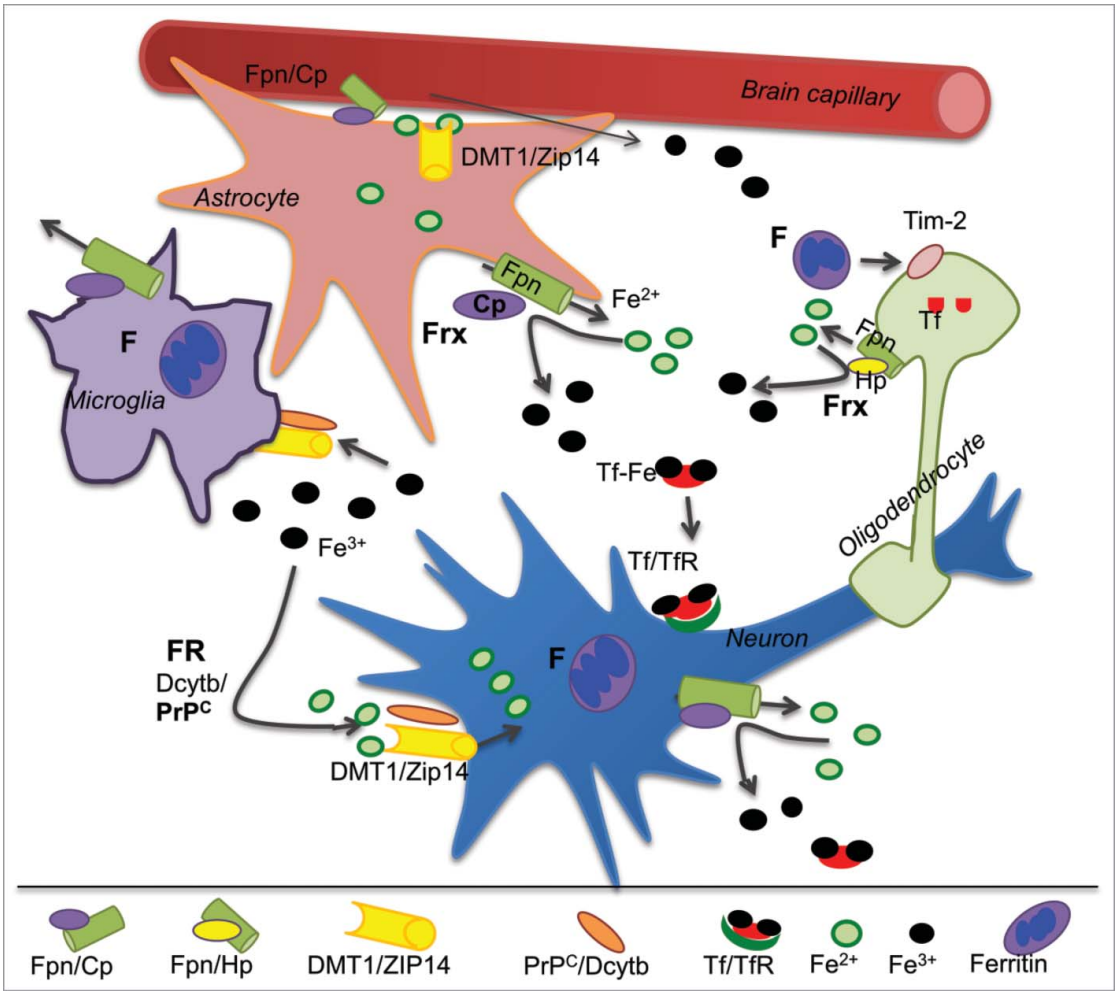


and perhaps oligodendrocytes.[15] Unlike serum Tf, brain Tf is ~99% saturated with iron, leaving little buffering capacity for excess iron. Within the brain, each cell type has preference for Tf-iron or NTBI, and express receptors and transporters that facilitate uptake of one or both forms. Neurons utilize both Tf-iron and NTBI, and express TfR for the uptake of Tf-iron and divalent metal transporter 1 (DMT1) and possibly members of the ZIP family for the uptake of NTBI. Evidence for the latter, however, is inconclusive. Astrocytes do not express TfR in vivo and utilize mainly NTBI. Excess intracellular iron is stored in cytosolic ferritin or exported from the cell through the combined action of ferroportin (Fpn) and a ferroxidase (Frx) such as ceruloplasmin (Cp) or hephaestin (Hp) (**Fig. 2**). Since iron is vital for cell survival, redundancies exist in iron transport pathways such that absence of one does not result in acute depletion or accumulation of iron.

REDOX-CYCLING FOR IRON TRANSPORT

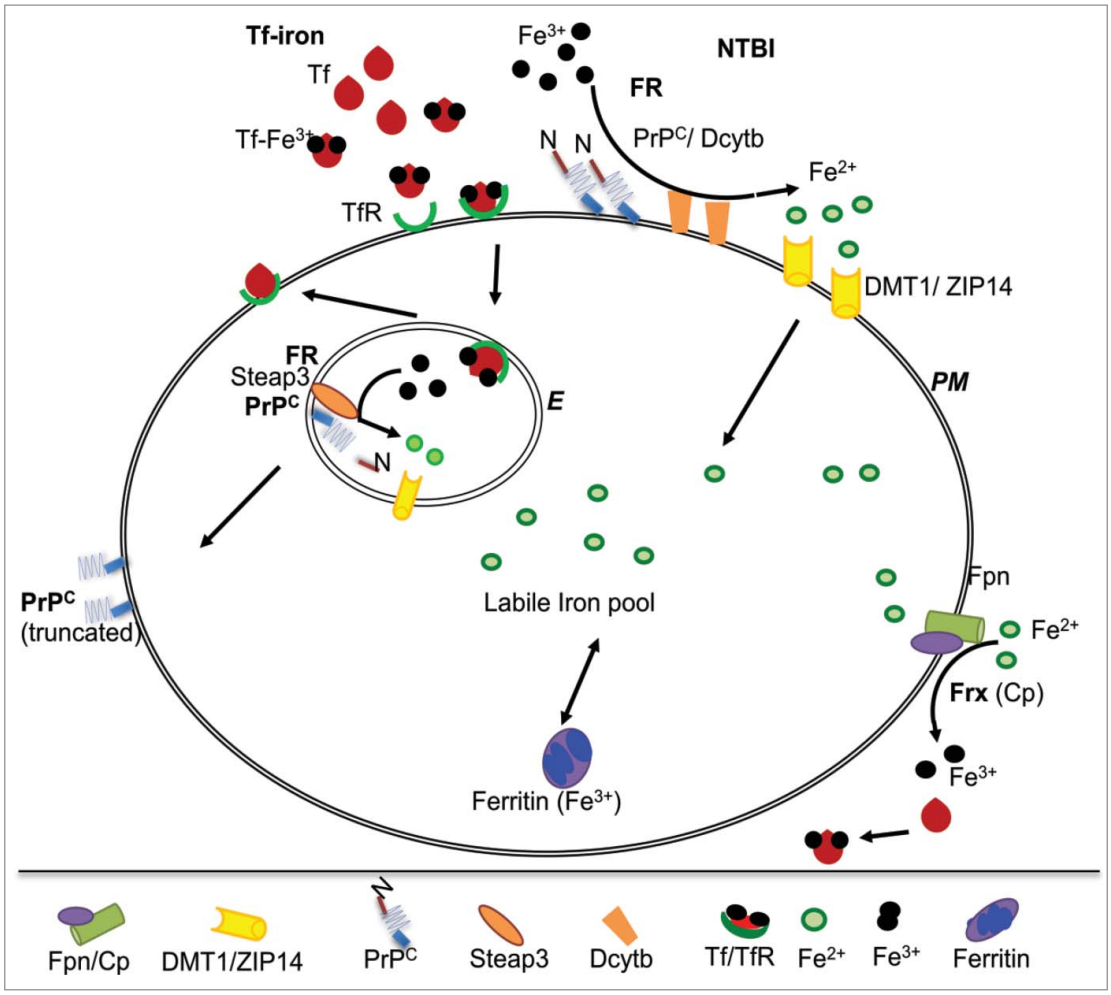
Iron exists in 2 oxidation states, the highly reactive ferrous (Fe^{2+}) form, and the oxidized and relatively stable ferric (Fe^{3+}) form. Both Tf-iron and NTBI are in the Fe^{3+} form, and require reduction to Fe^{2+} for transport across biological membranes through metal transporters such as divalent metal transporter 1 (DMT1) and the ZIP family of proteins. This reaction is mediated

FIGURE 2. Iron trafficking in the brain: Fe^{2+} in capillary endothelial cells is exported from the basolateral membrane through Fpn and oxidized to Fe^{3+} by the Frx Cp expressed on the plasma membrane of astrocytes. Majority of Fe^{3+} binds to Tf in the brain interstitial fluid. The rest binds to citrate and circulates as NTBI. Astrocytes utilize only NTBI, while neurons take up both Tf-Fe and NTBI. Tf-Fe is internalized through the TfR pathway, and NTBI is reduced to Fe^{2+} by Dcytb and/or PrP^C before transport through DMT1 or ZIP14. Microglia take up NTBI from the extracellular milieu and store iron released from phagocytosed cells in cytosolic ferritin. Oligodendrocytes synthesize and utilize Tf-iron and also take up ferritin iron by the Tim-2 receptor. Frx: ferroxidase, FR: ferrireductase, Cp: ceruloplasmin, Dcytb: duodenal cytochrome b, DMT1: divalent metal transporter 1.



by FR proteins on the plasma membrane and the endosomal membrane. A major plasma membrane FR is duodenal cytochrome b (Dcytb),[16] and the principal endosomal FR is Steap 3.[17] The cellular localization of Dcytb and Steap 3 is suited to their ability to function optimally at neutral and acidic pH respectively. Following transport across the membrane, Fe^{2+} is utilized for metabolic purposes, oxidized and stored in ferritin, or exported from the cell through the combined action of Fpn and Cp or Hp that oxidize Fe^{2+} to Fe^{3+} for binding to extracellular Tf. The rest associates with citrate or other small molecular weight compounds that comprise the NTBI pool of iron. Thus, redox-cycling is essential for iron

FIGURE 3. PrP^C functions as a FR partner for ZIP14 and DMT1: At the plasma membrane, FR proteins Dcytb and PrP^C reduce NTBI to Fe^{2+} for transport to the cytosol through DMT1 and ZIP14. Fe^{3+} released from Tf in acidic endosomes is reduced by Steap3 and PrP^C for transport to the cytosol through DMT1. PrP^C undergoes α-cleavage during this process, releasing its N-terminal FR domain. N-terminally truncated PrP^C is transported back to the plasma membrane. PM: plasma membrane, E: endosome.



transport, and all iron transporters are linked to an oxido-reductase mechanism (**Fig. 3**).[18]

EVIDENCE SUPPORTING FERRIREDUCTASE ACTIVITY OF PRP^C

Initial cues suggesting a functional role of PrP^C in iron uptake emerged from studies on PrP-knock-out transgenic mice ($PrP^{-/-}$) that showed impaired incorporation of supplemental iron administered by the oral or parenteral route in major systemic organs and the brain.[19] Reduced levels of iron in $PrP^{-/-}$ mouse brains were also noted using synchroton-based X-ray fluorescence imaging technology.[20] However, introduction of PrP^C in mice with $PrP^{-/-}$ background reversed their iron deficiency, supporting a non-redundant role of PrP^C in iron uptake and metabolism.[19] Observations on neuroblastoma cells suggested that PrP^C functions as a

plasma membrane FR, and the octa-peptide repeat region and linkage to the plasma membrane are essential for this activity.[21]

The FR activity of PrP^C was especially evident in hepatocyte and kidney proximal tubule (PT) cells that absorb significant amounts of NTBI and Tf-iron under physiological conditions, requiring FR activity for uptake and transport. Liver is the main organ that sequesters excess Tf-iron and NTBI from the circulation, and PT cells re-absorb ~99% of Tf-iron and NTBI filtered into the glomerular filtrate, providing excellent models for these studies. Expression of PrP^C on HepG2 cells, a hepatocyte cell line, increased uptake of Fe^{3+}, not Fe^{2+} iron from the extracellular medium, an effect that was reversed by deleting the octa-peptide repeat domain of PrP^C.[5,21] A similar effect of PrP^C was noted on PT cells that form polarized monolayers on filter supports. In these cells, PrP^C was localized to the apical plasma membrane and intracellular vesicles, and facilitated trans-cellular transport of NTBI from the apical to the basolateral domain. Again, this activity was abolished by deleting the octa-peptide repeat domain, re-affirming that the facilitative role of PrP^C in iron uptake is due to its FR activity.[22]

In vivo evaluation of FR activity of a specific protein is complicated by the fact that both Tf-iron and NTBI require reduction for transport across biological membranes, and the former pathway is likely to be influenced by factors that alter Tf/TfR recycling. This, experimental models that allow quantification of NTBI uptake in vivo have proved more useful in assessing FR activity of a specific protein in transgenic mouse models. In a typical experiment, circulating NTBI is radiolabeled preferentially with ^{59}Fe by prior saturation of plasma Tf with unlabeled ferric ammonium citrate (FAC), and uptake of ^{59}Fe-NTBI by different organs is monitored over time. Using this approach, it was determined that liver, kidney, heart, and pancreas take up significant amounts of ^{59}Fe-NTBI.[23] Evaluation of mouse models using a similar approach revealed significant reduction in the uptake of ^{59}Fe-NTBI by the liver, kidney, and pancreas of $PrP^{-/-}$ mice relative to wild-type controls, supporting a non-redundant role of PrP^C as a FR in vivo.[5,22]

FUNCTIONAL COOPERATION BETWEEN PRP^C AND ZIP TRANSPORTERS

Recent reports suggest an evolutionary link between PrP^C and the ZIP family of metal transporters that mediate zinc uptake and maintain cellular zinc homeostasis.[24] Two members of this family, ZIP8 and ZIP14, transport iron, cadmium, and manganese in addition to zinc, and are expressed on the plasma membrane and the membrane of endocytic vesicles. PrP^C and ZIP proteins are expressed on most cells, and certain members of the ZIP family co-immunoprecipitate with PrP^C, suggesting close physical proximity on the plasma membrane.[25]

Interestingly, expression of ZIP14 in PrP^C-expressing hepatoma cells increased physiological processing of PrP^C at the α-cleavage site, releasing the octa-peptide repeat region including the FR domain. Co-expression of PrP^C and ZIP14 increased levels of intracellular iron more than each protein individually, suggesting functional co-operation between the 2 and coupling of FR activity of PrP^C with its α-cleavage (**Fig. 3**).[5] Since ~99% of PrP^C in the urine is truncated, it is likely that similar processing events occur in PT cells that are known to express PrP^C and ZIP transporters on the apical plasma membrane that is bathed in iron-rich glomerular filtrate. It is therefore likely that in addition to mediating iron uptake, PrP^C regulates this process by shedding its FR domain. Further investigations are necessary to understand this phenomenon fully.

IMPLICATIONS FOR PRION DISORDERS

Brains of sCJD cases and prion infected mice show deficiency of iron,[8] zinc, and manganese.[26] The underlying cause of iron deficiency is accumulation in aggregated ferritin in a biologically unavailable form.[13] Although it is argued that these changes result from neuronal death, disease-specific alterations in the level(s) of iron modulating proteins in pre-mortem cerebrospinal fluid (CSF) of sCJD and

other rapidly progressing dementias suggest otherwise.[27] Considering the physical proximity and functional co-operation of PrP^{C} and ZIP proteins, it is likely that loss of function combined with gain of abnormal activity by PrP^{Sc}-ZIP aggregates alters homeostasis of iron, zinc, manganese, and other divalent cations in diseased brains. Altered expression of iron regulatory and storage proteins has been reported in the brains of mice inoculated with scrapie intra-cerebrally,[28] and spleens of mice where PrP^{Sc} was introduced intra-peritoneally.[29] Cell lines infected with PrP^{Sc} also show altered expression of iron regulatory and storage proteins, supporting the above hypothesis.[30] It is noteworthy that prion infectivity is transmitted from cell-to-cell via exosomes that are associated with PrP^{Sc}.[31] Exosomes also transport other aggregated proteins such as amyloid-β that bind metals with high affinity, and are likely to include PrP^{Sc}-ferritin aggregates that resist degradation by lysosomes. Fusion of such vesicles with neighboring cells is likely to alter their metal homeostasis, a phenomenon that is likely to contribute to iron dyshomeostasis and requires further exploration.

METAL CHELATION AS A POTENTIAL DISEASE-MODIFYING STRATEGY

Metal chelation therapy for the management of neurodegenerative conditions has been the focus of several recent reviews. Specific metals targeted by this approach include iron, copper, and zinc. The apparently critical involvement of metals, particularly iron, in both oxidative stress and protein aggregation renders chelation therapy a sensible strategy. An essential feature of such an approach would be the ability to scavenge the free redox-active metal to form a nontoxic metal complex which would be excreted, and secondly, to cap the metal at its labile binding site, preventing Fenton activity and secondary damage to neighboring cells.

Thus far, most trials have been conducted on iron chelators. Deferiprone, desferrioxamine, desferasirox, clioquinol, and certain other compounds have shown promise in cases of PD, AD, and Friedreich's ataxia.[32,33] PBT2 (5,7-dichloro-2-(dimethylamino)-methyl-8-hydroxyquinoline) that chelates zinc, copper, and possibly iron has shown cognitive improvement in cases of AD.[32] Other trials have shown mixed results, and are summarized in recent reviews.[34,35]

Limited studies have evaluated the therapeutic potential of metal chelation in sCJD and other variants of human prion disorders. Published reports have focused on reducing copper, zinc, and manganese in diseased brains. Although iron imbalance is clearly a feature of sCJD and experimental scrapie, iron chelation has not been tried, and we hope this review will be instrumental in triggering these studies. However, it is important to consider that chelation of one metal is likely to create imbalance of other metals, creating an equally neurotoxic microenvironment. This scenario is especially likely for metals such as iron, copper, and zinc that share transporters such as the ZIP family of proteins. It is therefore essential to devise strategies that restore metal homeostasis in the diseased brain rather than deplete one or the other metal. As new metal chelators emerge, it may be possible to achieve this goal. This remains a promising area for further research.

FUTURE DIRECTIONS

The functional connection between PrP^{C} and ZIP proteins has significant implications for metal homeostasis under physiological and pathological conditions. Altered function of either partner is likely to influence cellular uptake of iron and other cations transported by ZIP proteins. A better understanding of the functional cooperation between PrP^{C} and members of the ZIP family is likely to impact the therapeutic management of sCJD. Significant areas of future investigation include 1) further characterization of FR activity of PrP^{C} and whether its reductase function extends to copper, 2) a better understanding of the prion-ZIP connection and identification of specific members of the ZIP family that interact with PrP^{C}, 3) functional implications of proteolytic cleavage of PrP^{C} on metal transport, and 4) the

impact of conversion of PrP^{C} to PrP^{Sc} on PrP-metal interaction and metal homeostasis in neurons and other cell types in the brain. We hope that this review will stimulate research in these areas and improve our understanding of the prion-ZIP connection.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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