Brain iron transport and neurodegeneration

Zhong Ming Qian and Xun Shen

Despite years of investigation, it is still not known why iron levels are abnormally high in some regions of the brain in neurodegenerative disorders. Also, it is not clear whether iron accumulation in the brain is an initial event that causes neuronal death or is a consequence of the disease process. Here, we propose that iron and iron-induced oxidative stress constitute a common mechanism that is involved in the development of neurodegeneration. Also, we suggest that, at least in some neurodegenerative disorders, brain iron misregulation is an initial cause of neuronal death and that this misregulation might be the result of either genetic or non-genetic factors.

Abnormally high levels of iron in the brain have been demonstrated in a number of neurodegenerative disorders (NDs), including Hallervorden–Spatz syndrome, Parkinson's disease (PD) and Alzheimer's disease (AD)[1–4]. Oxidative stress resulting from increased iron levels in the brain and possibly also from defects in antioxidant defense mechanisms is widely believed to be associated with neuronal death in these disorders[3,5,6]. However, a key question – why do iron levels increase abnormally in some regions of the brain? – has not been answered. During the past decade, a considerable research effort has been devoted to addressing this important issue. Although some relevant points are yet to be clarified, new findings provide important insights into understanding this key question. This article discusses recent advances in studies of iron transport mechanisms in the brain and the relationships among the disregulation of brain iron transport proteins, excess amounts of brain iron and neurodegeneration.

Mechanisms of iron transport in the brain

Transferrin-bound and non-transferrin-bound iron in the brain

The mechanisms of iron transport across the blood–brain barrier (BBB) have not yet been completely clarified. The accumulated evidence suggests that the transferrin–transferrin receptor (Tf–TfR) pathway might be the major route of iron transport across the luminal membrane of the capillary endothelium[7–9], and that iron, possibly in the form of Fe^{2+}, crosses the abluminal membrane and enters the interstitial fluid (IF). However, the molecular events of this process are not known[7,8] (Fig. 1). Evidence shows that the uptake of Tf-bound iron (Tf–Fe) by TfR-mediated endocytosis from the blood into the cerebral endothelial cells is no different in its nature from the uptake into other cell types[7]. This process includes several steps: binding, endocytosis, acidification and dissociation, then translocation of the iron across the endosomal membrane, probably by a process mediated by divalent metal transporter 1 (DMT1; previously referred to as Nramp2 or DCT1)[10,11]. Most of the Tf will return to the luminal membrane with TfR, whereas the iron crosses the abluminal membrane by an undetermined mechanism[7,8] (Fig. 1). Recent studies have shown that ferroportin 1–hephaestin (FP1–Hp) and/or Hp-independent iron export systems might play a key role in Fe^{2+} transport across the basal membrane of enterocytes in the gut[12–14], but it is not known if these two systems have the same role in Fe^{2+} transport across the abluminal membrane of the BBB; this is worth investigating.

Another proposed mechanism of Fe^{2+} transport across the abluminal membrane involves astrocytes. Astrocytes probably have the ability to take up Fe^{2+} from endothelial cells through their end-foot processes on the capillary endothelium[9,15]. In addition to the Tf–TfR pathway, it has been suggested that the lactoferrin receptor–lactoferrin (LfR–Lf) and glycosylphosphatidylinositol (GPI)-anchored p97–secreted p97 pathways might also play a role in iron transport across the BBB (Refs 3,9,16). It is also possible that a small amount of iron might cross the BBB in the form of intact Tf–Fe complex by receptor-mediated transcytosis[8] (Fig. 1).

After the iron has been transported across the BBB, it is likely to bind quickly to the Tf that is secreted from the oligodendrocytes and epithelial cells of the choroid plexus[7,8] (Fig. 2). Data from several experiments indicate that the iron concentration exceeds that of the binding capacity of Tf in the cerebrospinal fluid (CSF) and IF. Because the affinity of Tf with iron is higher than that of the other iron transporters (the equilibrium constant, log*K*, for the formation of diferric Tf is more than 10^{10} times that for ferric citrate), Fe^{3+} in CSF and IF will bind to Tf first. Unlike Tf found in blood, Tf in CSF and IF is fully saturated with iron; excess iron will bind to other transporters. Hence, it is possible that there are two transport forms of iron in the CSF and IF in the brain: Tf–Fe and non-Tf-bound iron (NTBI)[7,8]. The latter probably includes citrate–Fe^{3+} (or Fe^{2+}), ascorbate–Fe^{2+} and albumin–Fe (2+ or 3+), as well as Lf–Fe^{3+} and secreted p97–Fe^{3+}. If the remaining NTBI in the CSF is in oxidized form (Fe^{3+}), it will be bound almost completely to citrate, because the citrate–Fe^{3+} complex has a log*K* of ~11.4; if it is present as citrate–Fe^{2+}, it will be bound rather more loosely (log*K*~4.5). Low concentrations might be present in complex with ascorbate (log*K*~2) and a finite concentration (10^{-8}–10^{-7} M) as free Fe^{2+} (Refs 7,8). Tf–Fe, or probably Lf–Fe, and secreted p97–Fe will be taken up by brain cells via TfR- or LfR- and GPI-anchored p97-mediated processes, respectively[9]. NTBI will be acquired by neuronal

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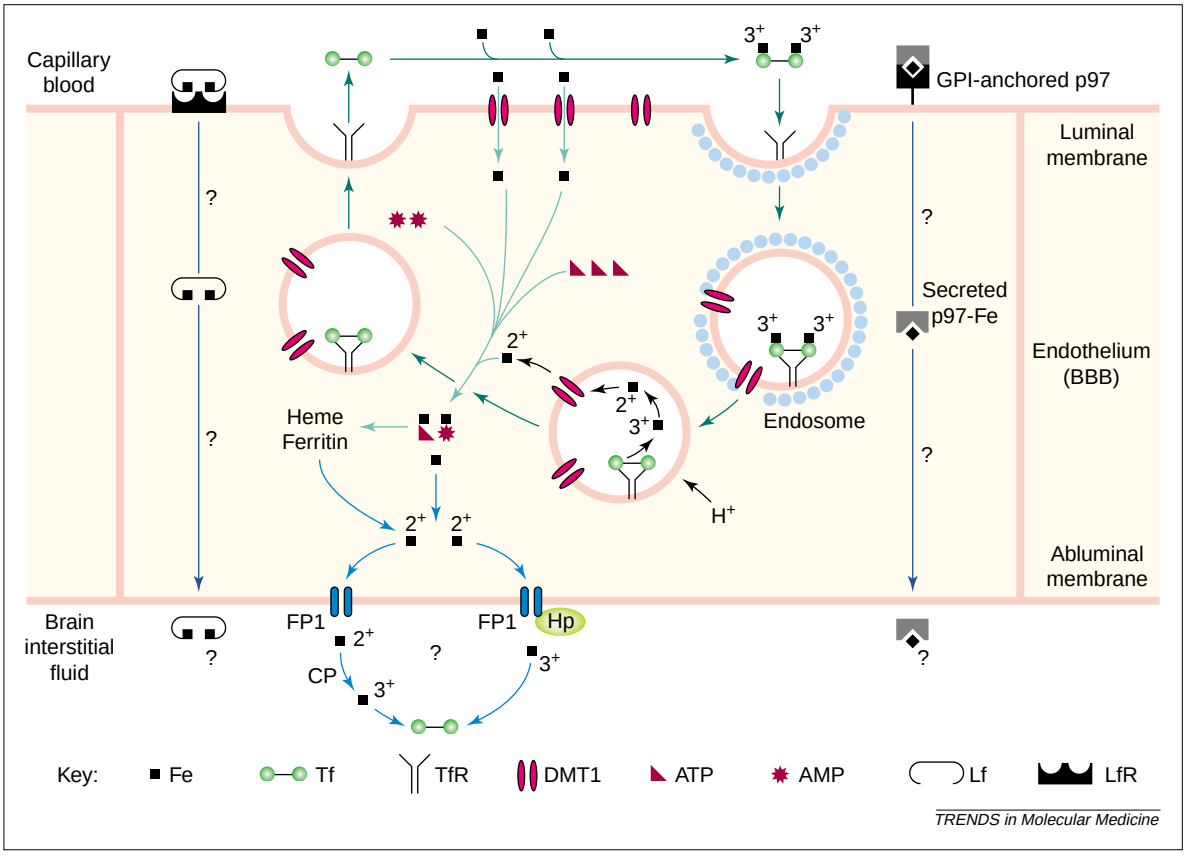

Fig 1. Proposed scheme for iron transport across the BBB. The Tf–TfR pathway might be the major route of iron transport across the luminal membrane of the BBB. Tf–Fe uptake by endothelial cells is similar in nature to the uptake into other cell types (green arrows). DMT1 might play a role in translocation of iron from endosome to cytosol. Iron (Fe^{2+}) probably crosses the abluminal membrane via FP1–Hp and/or Hp-independent export systems (light-blue arrows). LfR–Lf and GPI-anchored p97–secreted-p97 pathways might also be involved in iron transport across the BBB (dark-blue arrows); however, further study is needed. Abbreviations: BBB, brain–blood barrier; CP, ceruloplasmin; DMT1, divalent metal transporter; FP1, ferroportin 1; GPI-p97, GPI-anchored p97; Hp, hephaestin; Lf, lactoferrin; LfR, lactoferrin receptor; NTBI, non-transferrin-bound iron; Tf–Fe, transferrin-bound iron; Tf, transferrin; TfR, transferrin receptor.

cells or other brain cells, probably via DMT1- or trivalent cation-specific transporter[17] (TCT)-mediated mechanisms (Fig. 2). These mechanisms have not been elucidated.

Ceruloplasmin and the cause of excessive intracellular iron in the brain

For more than 30 years, ceruloplasmin (CP) has been postulated as the critical ferroxidase in the plasma of all vertebrate species. This protein is synthesized mainly in hepatocytes. Recent studies show that CP is also expressed in the mammalian central nervous system (CNS)[3]. A role for CP in iron efflux was first suggested in the 1960s, based on the observation that the ferroxidase activity of CP promoted iron incorporation into Tf (Ref. 18). This suggestion is supported by a recent study using an animal model of aceruloplasminemia[19], a disorder of iron metabolism resulting from inherited mutations in the *CP* gene[20–23]. Clinical data on aceruloplasminemia reveal the possible role of CP in release of iron from brain cells. Despite years of investigation, however, the functions of CP in brain iron metabolism are not well understood. Although it has been widely accepted that, on the basis of clinical studies of aceruloplasminemia[20], CP has an important role in iron release from brain neuronal cells, more recent findings show that this traditional viewpoint might need to be reconsidered.

It is highly likely that, through its ferroxidase activity, CP plays a role not only in iron efflux from brain cells but also in iron influx into these cells. Also, it is possible that the physiological role of CP is more important in iron uptake than in iron efflux. Several pieces of evidence support this possibility. First is the location of CP in the brain; CP expression in the brain is not observed in all astrocytes but rather identifies a unique subpopulation of these glial cells that predominantly surrounds the microvasculature. The CP located on these astrocytes is ideally positioned to effectively oxidize the highly toxic Fe^{2+} to Fe^{3+} (Refs 24,25). This unique location implies that CP is necessary for Fe^{2+} to be oxidized to Fe^{3+} after it crosses the abluminal membrane. Fe^{3+} can then bind to transport carriers Tf (or Lf and p97) in the CSF and IF, and is acquired by neurons or other brain cells. The second piece of evidence is provided by data obtained from *in vitro* studies on the effect of CP on iron transport across cell membranes. These studies show that addition of CP to cells results in enhanced uptake rather than release of NTBI (Refs 17,26). Although different results have been reported[21,27,28], our preliminary study shows that CP plays a role in iron influx into brain glioma cells. Iron uptake by these cells was observed after the addition of low (30 μg/ml) or high (300 μg/ml, physiological concentration in serum) (unpublished).

The similarities between CP and Fet3p (a membrane protein in *Saccharomyces cerevisiae*)[29] provide another indication. Studies in yeast demonstrate that iron uptake is dependent on what has been viewed as a CP homologue[30,31]. The similarities between Fet3p and CP suggest a possible

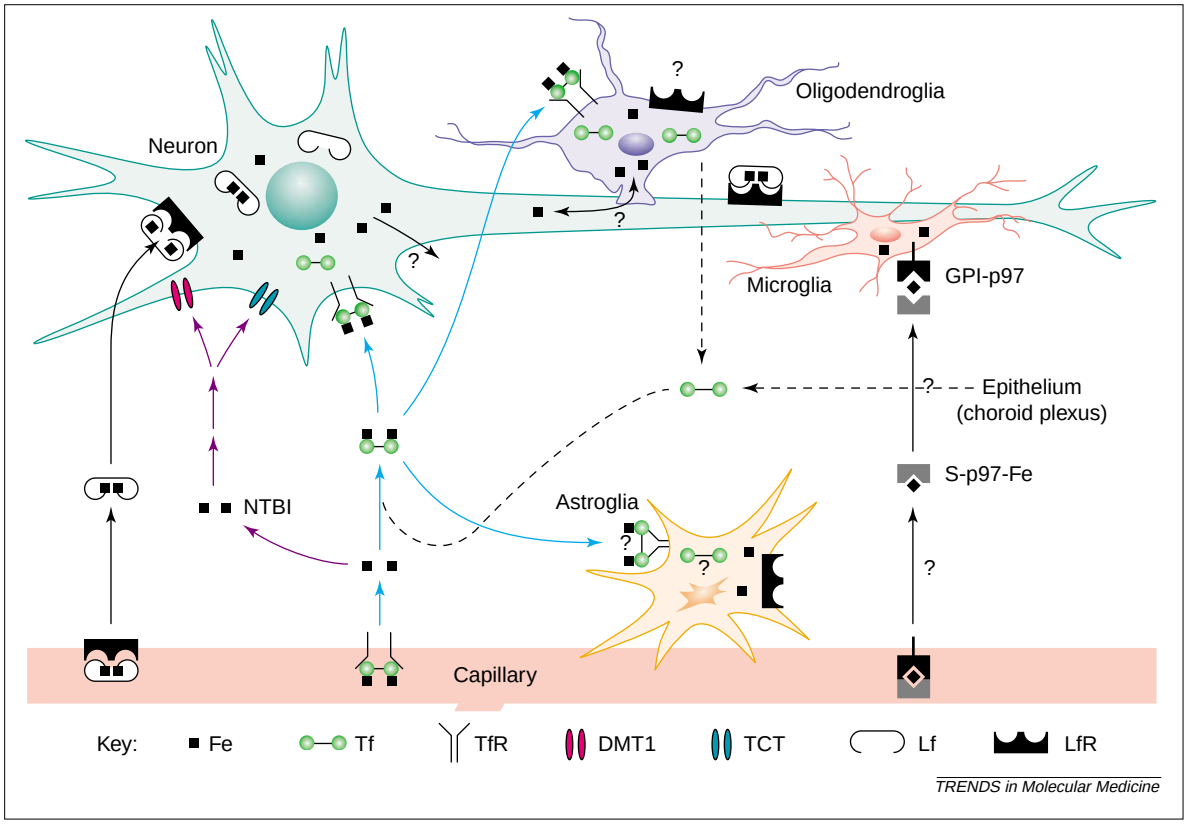

Fig. 2. Proposed scheme for iron transport in the brain. There are two transport forms of iron in the brain: Tf–Fe and NTBI. Brain Tf is synthesized in either oligodendrocytes or epithelial cells of the choroid plexus. Tf–Fe is taken up by neurons via a TfR-mediated process (blue arrows). NTBI, including Lf–Fe^{3+} and S-p97–Fe^{3+}, is acquired by neurons probably via DMT1- and TCT-mediated (purple arrows) processes or LfR- and GPI-p97-mediated processes (black arrows), respectively. The molecular mechanisms underlying these processes are unknown. Abbrevs: DMT1, divalent metal transporter; GPI-p97, GPI-anchored p97; Lf–LfR, lactoferrin–lactoferrin receptor; NTBI, non-transferrin-bound iron; S-p97, secreted p97; TCT, trivalent cation-specific transporter; Tf–Fe, transferrin-bound iron; Tf–TfR, transferrin–transferrin receptor.

role for the latter in iron uptake by mammalian cells[17]. The final piece of evidence is the existence of spontaneous oxidation activity in the brain. It has been suggested that the rate of spontaneous oxidation (Fe^{2+} to Fe^{3+}) is sufficient for the corresponding slow iron release rate. Only at higher release rates is exogenous ferroxidase activity, such as that provided by CP, required[27]. In other words, under physiological conditions, the role of CP in iron release in the brain might not be important, or only a small amount of CP is sufficient to maintain normal iron levels in brain cells.

Indeed, the role of CP in iron uptake by brain cells is consistent with the function of CP as a ferroxidase. It is probably more reasonable to conclude that the physiological function of CP is through its ferroxidase activity, which is involved in both iron efflux and influx. If it is true that CP has a role in iron uptake, how does the lack of CP production induce the excessive iron accumulation in neurons and other brain cells that is found in patients with aceruloplasminemia[20]? A possible explanation might be that excessive intracellular iron is due mainly to increased NTBI uptake and partly to decreased iron release. It is highly likely that 'uptake' is the major role of CP in brain cell iron balance and 'release' plays a minor role. Under physiological conditions, brain cells obtain iron mainly from Tf (Tf–Fe^{3+}). Most iron (Fe^{2+}), after crossing the BBB, is oxidized to Fe^{3+} by the ferroxidase activity of CP and then binds to Tf and is acquired by the brain cells. However, under pathological conditions, the loss of CP (and thus ferroxidase activity) makes it impossible for most of the Fe^{2+} to be oxidized to Fe^{3+}. Accordingly, the amount of Fe^{3+} and Tf–Fe decreases, and the amount of NTBI (e.g. citrate–Fe^{2+}, ascorbate–Fe^{2+}) and free Fe^{2+} increases. As a result, NTBI uptake (mainly as Fe^{2+}) by neurons increases abnormally. Because of the lack of CP within the cells, Fe^{2+} is unable to load into ferritin because only Fe^{3+} can be incorporated into ferritin and the ferroxidase activity of CP is necessary for the oxidation of Fe^{2+} to Fe^{3+} (Ref. 32). In addition, although intracellular Fe^{2+} increases, it might not be able to be released because extracellular Fe^{2+} also increases. The combined result will be 'excessive intracellular iron accumulation'. This induces oxidative stress and the formation of reactive oxygen species (ROS), triggering a cascade of pathological events leading to neuronal death.

The possible role of LfR, GPI-anchored p97 and DMT1

LfR, a monomeric 105-kDa glycoprotein that was originally identified only in monocytes and intestinal cells, is produced in the brain[16]. Immunohistochemical staining shows that LfR is localized on neurons, the cerebral microvasculature and, in some cases, glial cells[16]. In the brains of PD patients, LfR immunoreactivity on neurons and microvessels is increased and more pronounced in those regions of the mesencephalon where the loss of dopaminergic neurons is severe. In the substantia nigra, the intensity of immunoreactivity on neurons and microvessels is higher for patients with greater nigral dopaminergic loss[16]. Analysis of the distribution of Lf (an 80-kDa iron-binding glycoprotein) demonstrates that it is present in a

large population of neurons in the substantia nigra of neurologically normal individuals. Lf-positive neurons are severely affected by the neurodegenerative process in PD patients, as indicated by a significant decrease in the number of immunolabeled neurons[3]. These clinical investigations suggest that the Lf–LfR might play a role similar to that of Tf–TfR in brain iron transport under normal circumstances. If LfR and Lf levels are increased, intraneuronal iron might be able to rise to pathological levels and could contribute to the degeneration of nigral dopaminergic neurons in PD, and possibly in AD and other NDs (Refs 3,16). However, there is not much morphological evidence for LfR on the luminal membrane of the capillary endothelium, or physiological data on the role of Lf–LfR in brain iron transport; thus further studies are required.

p97, also called melanotransferrin (human melanoma tumor-associated antigen), was first identified on the surface of melanoma cells, but it can also be expressed by a wide range of cultured cell types, including liver and intestinal cells[3]. This protein has only one iron-binding site and exists in two distinct molecular forms: a plasma membrane-associated GPI-anchored form and a soluble form in the serum or CSF. In all cases, whenever cells have expressed GPI-anchored p97, they have also expressed a soluble form. Studies using Chinese hamster ovary cell lines[33] demonstrated that GPI-anchored p97 can bind and internalize iron into cells from citrate–Fe, but not from Tf–Fe. The internalization is temperature-sensitive, time-dependent and saturated at a media concentration of 2.5 μg/ml. These results imply the existence of a novel route for cellular iron uptake that is independent of Tf–TfR. In an immunohistochemical study, p97 was found to be highly localized in the capillary endothelium in all brain tissues examined. The distributions of p97 and TfR are remarkably similar, but quite different from that of Tf. Clinical investigation[34–36] shows that p97 is selectively expressed on reactive microglial cells in AD brains, and that expression is associated with amyloid plaques in postmortem brain tissue. Also, the p97 level is significantly elevated in the CSF of AD patients compared with that in the CSF of subjects suffering from other neuropathologies[35,36]. There is a significant correlation between the increase in the serum concentration of p97 and the progression of AD. These findings suggest that GPI-anchored p97 might deliver iron across the BBB by a mechanism analogous to that mediated by the TfR, as well as play a role in the iron uptake by microglial cells[3,9]. The dysregulation of p97 might be one of the causes of excess iron deposition in AD brain tissue. However, information on the cellular distribution and functional characterization of this protein in the brain is very limited. The mechanism of homeostatic control of p97 expression in the brain and the cause of p97 overexpression in AD are unclear.

DMT1 (Nramp2) was first identified in 1995 on the basis of its homology to Nramp1 (Ref. 37). In 1997, two groups[10,11] independently identified DMT1 as the first mammalian transmembrane iron transporter. This protein is widely expressed[10,11,38,39]; DMT1 mRNA was detected in all tissues tested, although levels were generally quite low[11]. DMT1 has an unusually broad substrate range, including Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} (Ref. 11). Fe^{2+} transport mediated by DMT1 is active and H^{+}-dependent[11,38]. In addition to expression on the plasma membrane, DMT1 can be expressed on the endosomal membrane and acts to export iron from the endosome into the cytoplasm of the cell[40,41]. There are at least two different splice forms of DMT1 (Ref. 42). One form, containing the iron-responsive element (IRE) sequence, encodes a 561-amino-acid protein. Another form does not contain a recognizable IRE and encodes a 568-amino-acid protein. Brain tissue appears to express the highest ratio of IRE to no-IRE forms. The regulation of DMT1 expression is most likely controlled by a feedback mechanism sensing body iron stores[11,43]. In the brain, DMT1 mRNA is consistently found in neurons and epithelial cells of the choroid plexus and is present at moderate levels in the substantia nigra[11,42]. The cellular localization of DMT1 and its functional characterization suggest that DMT1 might play a role in physiological iron transport in the brain. It has also been proposed that defects in DMT1 are likely to contribute to the etiology of certain NDs (Refs 11,42). It has been reported that the affected neurons of the substantia nigra in PD patients have a moderately high expression of DMT1 (Ref. 42). This might be the cause of the increased iron content in this region and might thereby contribute to neuronal death by inducing the production of harmful ROS (Ref. 42). However, further exploration of this possibility is required.

Excessive brain iron and neurodegeneration

Initial cause or secondary consequence

Despite considerable investigation, it is still not clear whether excessive iron accumulation in the brain is an initial event that causes neuronal death or is a consequence of the disease process[2,3]. On the basis of current knowledge, however, it seems reasonable to propose that brain iron misregulation is an initial cause of neuron death in some, but not all, NDs. The evidence suggests that the abnormally increased iron levels in the brain could result from disruption in the production of brain iron transport proteins. The cause might be genetic[20], as found in patients with aceruloplasminemia, or caused by non-genetic factors that can disrupt the normal control mechanisms of iron transport protein production[3,16,34,35]. These factors, however, have not been identified.

Intracellular iron balance depends on the amount of iron taken up as well as on the amount of iron released by the cell[2,3]. The excessive accumulation of iron in the brain that is found in NDs might result from increased uptake as well as decreased release[3]. In most cells of the

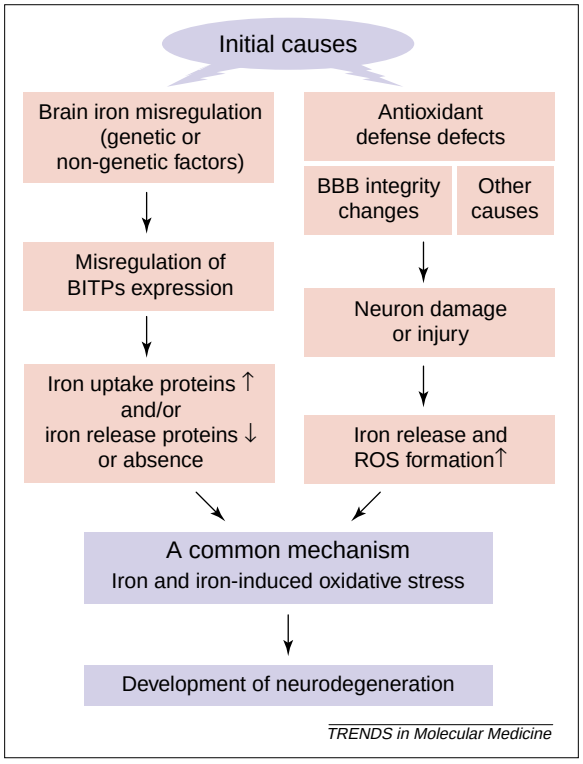

Fig. 3. Iron and iron-induced oxidative stress might be a common mechanism in the development of NDs. Brain iron misregulation, resulting from either genetic (as in aceruloplasminemia) or non-genetic (unknown) factors, is an initial cause of neuronal death in some NDs. Other NDs might be initiated by defects in antioxidant defense, changes in the integrity of the BBB or other (unknown) causes. Neuronal death, by any initial cause, could lead to large amounts of iron release and increased ROS formation, even though the initial cause(s) of the neuronal injury might be unrelated to the disregulation of brain iron metabolism. Therefore, iron and iron-induced oxidative stress might be a common mechanism involved in the development of neurodegeneration. Abbreviations: BBB, brain–blood barrier; BITPs, brain iron transport proteins; NDs, neurodegenerative diseases; ROS, reactive oxygen species.

body, the amount of iron uptake is mainly dependent upon the amount of TfR on the membrane[44]. TfR expression is controlled by cellular iron, occurs at the post-transcriptional level and is mediated by iron regulatory proteins[45]. However, the mechanism of iron uptake by brain cells might be more complicated. In addition to TfR, there are probably additional iron 'uptake' proteins, including LfR, DMT1 and p97 (Ref. 3), although their physiological importance in brain cell iron balance needs to be determined. Clinical data have demonstrated that there is increased production of some of these iron 'uptake' proteins in some NDs, as discussed above. These clinical findings imply that excessive iron accumulation could be induced by the abnormally increased expression of iron 'uptake' proteins. On the other hand, the increased intracellular iron in the brain can also result from decreased iron release from cells, as in the patients with aceruloplasminemia, because of the absence of CP expression, currently the only known iron 'release' protein. Decreased CP expression has also been considered as a possible cause for excessive iron accumulation in some other NDs, although no direct evidence has yet been obtained[3].

Disruption of the production of brain iron transport proteins provides a reasonable explanation as to how iron is deposited in high quantities in the brain cells in some NDs. However, it is necessary to investigate whether alterations in iron transport protein levels precede changes in brain iron levels and iron-dependent processes before the conclusion that dysfunction of iron metabolism is a primary event in the development of NDs can be reached. Also, it is important to identify the non-genetic factors that can disrupt the normal cycle of iron transport protein production as well as other genetic causes in addition to mutations in the *CP* gene.

A common mechanism

In some NDs, the disregulation of iron metabolism in the brain might not be the initial cause. There is a possibility that some NDs are initially induced by defects in antioxidant defense mechanisms, such as decreased expression of the Atox1 (a copper transport protein) gene[46] or reduced availability of glutathione (an important neuroprotectant for midbrain neurons) and other antioxidant substances in the brain (Fig. 3). Changes in the integrity of the BBB due to altered vascularization of tissue or inflammatory events could be another initial cause. It is also possible for some disorders to be initiated by multiple factors or other unknown causes. However, it should be noted that neuronal death, from any initial cause, could lead to large amounts of iron release and increased ROS formation, even though the initial cause(s) of the neuronal injury might be completely unrelated to the disregulation of brain iron transport proteins[2]. Therefore, it would be reasonable to propose that iron and iron-induced oxidative stress constitute a common mechanism that is involved in the development of neurodegeneration. Several pieces of evidence support this hypothesis. These include: an increase of iron in the brain; an increase in lipid peroxidation products

Outstanding questions

- How many types of iron transport proteins are there in the brain, what is their cellular and regional distribution and what is their physiological importance in brain iron homeostasis?
- How is the production of iron transport proteins controlled in the brain under physiological circumstances?
- What are the genetic or non-genetic causes that might lead to misregulation of the production of brain iron transport proteins?
- What is the transport form of iron across the abluminal part of the capillary endothelium (Fe^{2+} or other forms)?
- Do alterations in the production of iron transport proteins precede changes in brain iron levels and iron-dependent processes?

and ROS; and a reduction in the progression of neurological symptoms induced by treatment with the iron chelator desferrioxamine[2,22,23]. Based on this hypothesis, therefore, therapeutic efforts should be directed to reducing brain iron levels and inhibiting the generation of ROS.

Conclusion

In this article, we suggest that, at least in some NDs, brain iron misregulation is an initial cause of neuronal death and that this misregulation might be led by either genetic or non-genetic factors. Also, we propose that iron and iron-induced oxidative stress constitute a common mechanism involved in the development of neurodegeneration. However, it should be pointed out that many relevant questions need to be clarified. The precise roles and mechanisms of iron transport proteins in brain iron homeostasis are not completely understood. The uneven distribution of iron in the brain[6] implies that the distribution of the iron transport proteins is different in different types of brain cells and in different regions of the brain. A detailed analysis of the cellular and regional distribution of these proteins is therefore necessary. Although we propose that FP1 and Hp might play a role in Fe^{2+} transport across the abluminal membrane of the BBB, there is no evidence for the presence of FP1 and Hp (Refs 12–14) in the brain. How the expression of brain iron transport proteins is controlled in the brain under physiological circumstances and what the genetic and non-genetic causes are that might lead to misregulation of brain iron metabolism are two other key questions that need to be addressed. An understanding of these important aspects will greatly improve our knowledge of brain iron metabolism, as well as of the role of the disruption of brain iron metabolism in the development of NDs.

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