In patients with aceruloplasminemia, the serum hepcidin levels and hepatic hepcidin mRNA levels are lower than those in control subjects.[12,13] Ceruloplasmin knockout mice also showed decreased hepatic hepcidin mRNA levels in comparison to wild type and heterozygous mice.[14] The low serum hepcidin levels may induce increased iron absorption in the intestine, where the ceruloplasmin homolog protein, hephaestin, retains ferroxidase activity that is involved in basolateral intestinal iron transport. Therefore, the low hepcidin level in the serum and the loss of cell surface ferroportin due to mutant ceruloplasmin may enhance the cellular iron accumulation.

Clinical manifestations

Unique among iron-overload syndromes, aceruloplasminemia involves both the systemic and brain iron metabolism. In a major systemic iron-overload disorder, hereditary hemochromatosis, tissue iron accumulation arises from the increased serum ferrous iron, but the brain iron level is not increased despite the elevated serum ferrous iron. Aceruloplasminemia is classified as an inherited neurodegenerative disorder associated with systemic iron-overload syndrome. A marked accumulation of iron in the affected parenchymal tissues, such as the liver, pancreas, heart and thyroid, results in DM, cardiac failure and hypothyroidism.[3] The prevalence of aceruloplasminemia was estimated to be approximately one per 2 000 000 offspring in non-consanguineous marriages,[15] and subsequent studies have now identified more than 60 affected families around the world. Heterozygotes for the ceruloplasmin gene were estimated to account for 0.1% of individuals with diabetes in Japan.[16]

The clinical manifestations of aceruloplasminemia are a triad of retinal degeneration, DM and neurological signs/symptoms.[17] A summary of the clinical manifestations in the 71 patients is shown in Table 1. Ophthalmological examinations usually reveal evidence of peripheral retinal degeneration secondary to iron accumulation. The neurological manifestations (in order of frequency) include ataxia, involuntary movements, cognitive dysfunction and parkinsonism, corresponding to the specific regions of brain iron accumulation. These symptoms generally appear in the fourth or fifth decade of life. More than 40% of the involuntary movement is dystonia, and approximately 25% of cases exhibit chorea and choreoathetosis. The cognitive dysfunction includes forgetfulness, mental slowing and apathy. The phenotypic expression varies even within families.

The diagnosis of aceruloplasminemia in a symptomatic individual relies upon the demonstration of the complete absence of serum ceruloplasmin and abnormal laboratory findings, as well as neuroimages suggesting iron overload. The neuroimaging studies in aceruloplasminemia patients are strongly supported by the characteristic MRI findings of abnormal low intensities reflecting iron accumulation in the liver and brain (Fig. 2). A T2*-weighted MRI study can be used to distinguish these patients from those with other neurodegeneration with brain iron accumulation (NBIA).[18]

Table 1 The clinical characteristics of 71 patients with aceruloplasminemia

Clinical manifestations
• Anemia (80%)
• Retinal degeneration (76%)
• Diabetes mellitus (70%)
• Neurological symptoms (68%)
1) Ataxia (71%): dysarthria > gait ataxia > limb ataxia
2) Involuntary movement (64%): dystonia (blepharospasm, grimacing, neck dystonia) > chorea > tremors
3) Parkinsonism (20%): rigidity > akinesia
4) Cognitive dysfunction (60%): apathy > forgetfulness
Onset of clinical manifestations
• Diabetes mellitus: under 30 years old, 18%; 30–49 years old, 66%; over 50 years old, 16%
• Neurological symptoms: under 40 years old, 7%; 40–59 years old, 80%; over 60 years old, 13%

Recently, a method to map iron distribution in the human brain has been developed based on the measurement of the apparent transverse relaxation rate and water content in a high-field MRI at 4.7 Tesla.[19] In almost all regions in the aceruloplasminemia patient's brain, the non-heme iron concentration exceeded 20 mg/100 g fresh weight, while such a high level of iron was seen only in the globus pallidus in the healthy brain (Fig. 3).[20] The neuropathological process in these patients extended beyond the basal ganglia to the cerebral cortex. Some patients have been recognized prior to the onset of neurological symptoms due to biochemical abnormalities indicating iron metabolism.[21] These laboratory findings include microcytic anemia, a decreased serum iron content and an increased serum ferritin concentration, usually greater than 1000 ng/mL. Genetic testing can thereafter confirm the diagnosis. The genetic analyses of aceruloplasminemia patients worldwide have identified 49 mutations in the ceruloplasmin gene.[6] The symptoms, onset and prognosis in single cases have demonstrated that there is no genotype-phenotype association.[22]

Disease mechanisms

Pathological studies in patients with aceruloplasminemia showed severe iron deposition in the astrocytes and nerve cells in the basal ganglia, thalamus and cerebellum, and neuronal loss in the same regions associated with the highest iron accumulation.[23] Neuronal and glial cell bodies were also found to display increased levels of redox-active

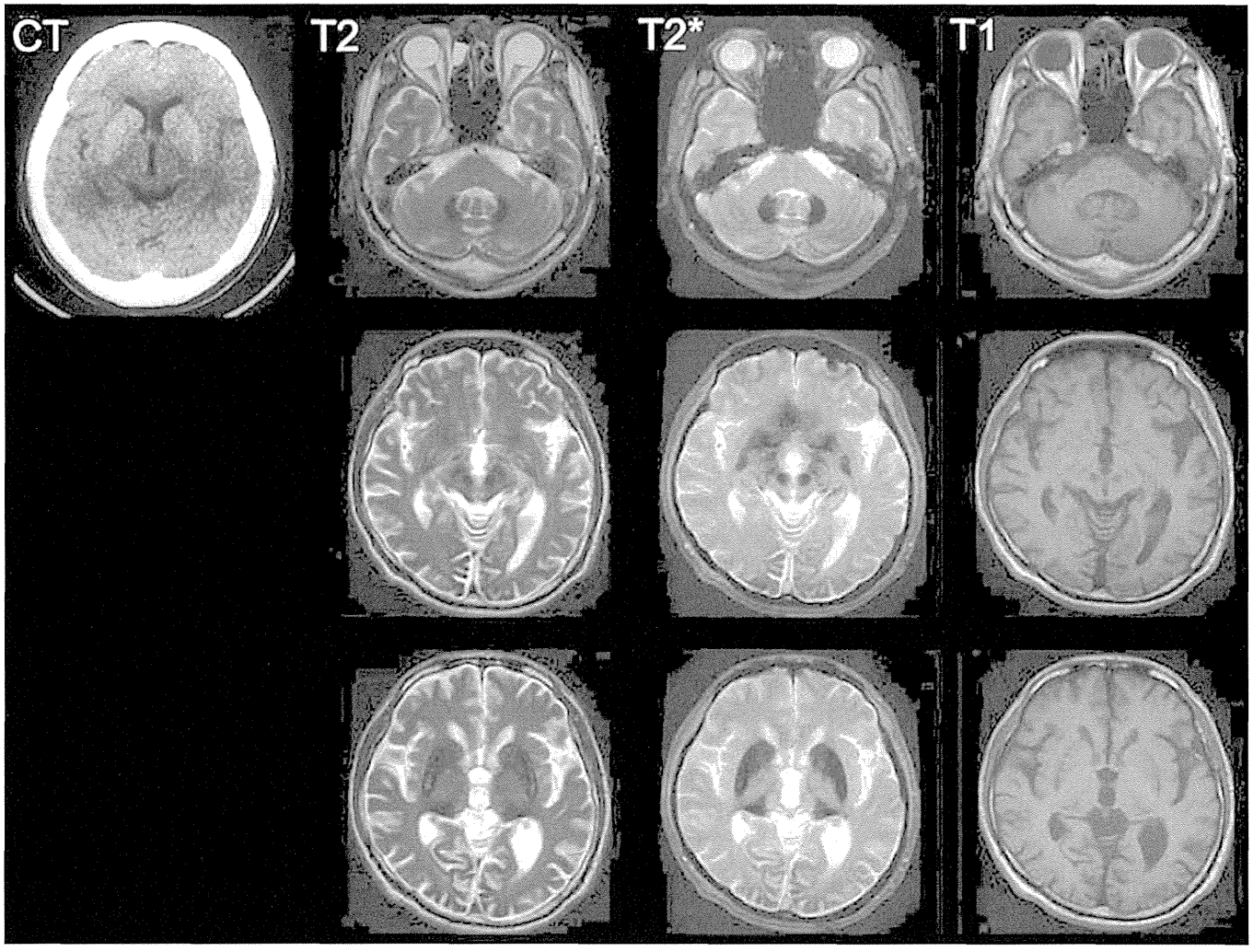

Fig. 2 The brain CT and MRI findings of a patient with aceruloplasminemia. Brain CT showed abnormal high density areas in the basal ganglia. T1-, T2- and T2*-weighted axial images of the brain showed signal attenuation of the dentate nucleus of the cerebellum, globus pallidum, putamen, caudate nucleus and thalamus.

iron (Fig. 4a,b).[24] Intense redox-active iron deposition was demonstrated in the terminal astrocytic processes and the globular structures.[25] The Fenton catalysis of iron plays an important role in cellular redox chemistry by reducing H_2O_2 to the highly cytotoxic hydroxyl (OH•) radical, which may be injurious to astrocytes and neurons. The most characteristic histopathological findings observed in patients with aceruloplasminemia were abnormal/deformed astrocytes and globular structures.[26] In the basal ganglia, many astrocytes were enlarged (up to 50 μm in size), with abundant cytoplasm and prominent nuclei associated with massive iron accumulation (Fig. 4c).[16,26] Some giant astrocytes with large, multiple or lobulated nuclei have been observed in the caudate nucleus and putamen.

These astrocytes were also positive for 4-hydroxynoneal (HNE) staining, a marker of lipid peroxidation (Fig. 4d).[16,26] A number of globular structures have also been identified in the superficial layers of the frontal cortex, as well as in the basal ganglia. The globular structures vary in size from 10 to 60 μm in diameter. Many structures show a grumose internal structure and iron deposition (Fig. 4e,f).[26] Immunohistochemically, many globular structures were positive for GFAP and S-100 but not for neurofilament or synaptophysin, suggesting that they were ballooned foot processes of astrocytes.[16] These structures were stained by anti-HNE and anti-ubiquitin antibodies.[26]

These findings may be related to iron-induced tissue damage, since these abnormal astrocytes were more frequently observed in the basal ganglia, where marked iron accumulation was seen. The morphological deformities in astrocytes are linked to subsequent oxidative stress induced by iron accumulation, and astrocyte dysfunction may contribute to neuronal cell loss, in addition to the direct effects of free radicals on neurons.[27] Ceruloplasmin

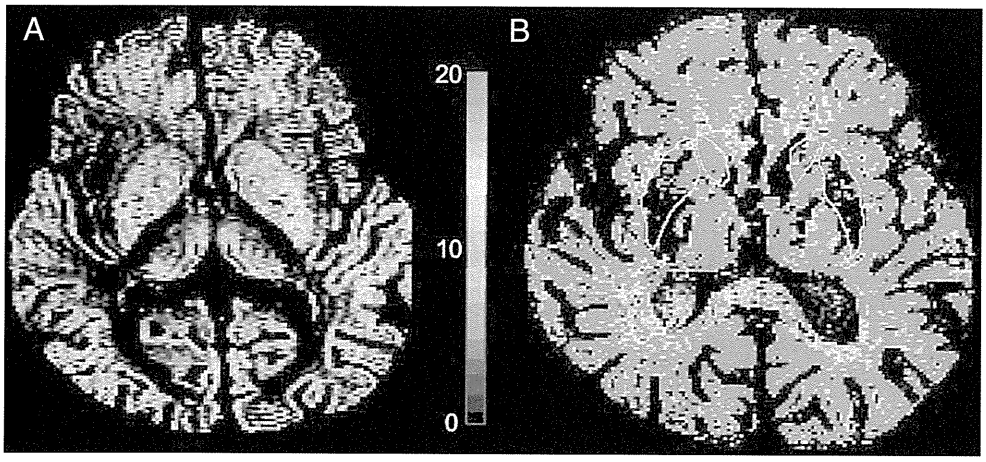

Fig. 3 Iron mapping of the brain using a 4.7 Tesla MRI $T_2^{\dagger}$ scan. The colored bar indicates the iron content in mg/100 g fresh weight of the brain. In the healthy brain, a high level of iron was seen only in the globus pallidus. In almost all regions in the patient's brain, the iron level exceeded 20 mg/100 g fresh weight. In the basal ganglia, voids were seen due to too short $T_2^{\dagger}$ caused by the very high iron accumulation, over 60 mg/100 g fresh weight. (a) A 60-year-old healthy male; (b) a 60-year-old male suffering from anemia, retinal degeneration and diabetes with insulin treatment, who had experienced gait ataxia for 5 years.

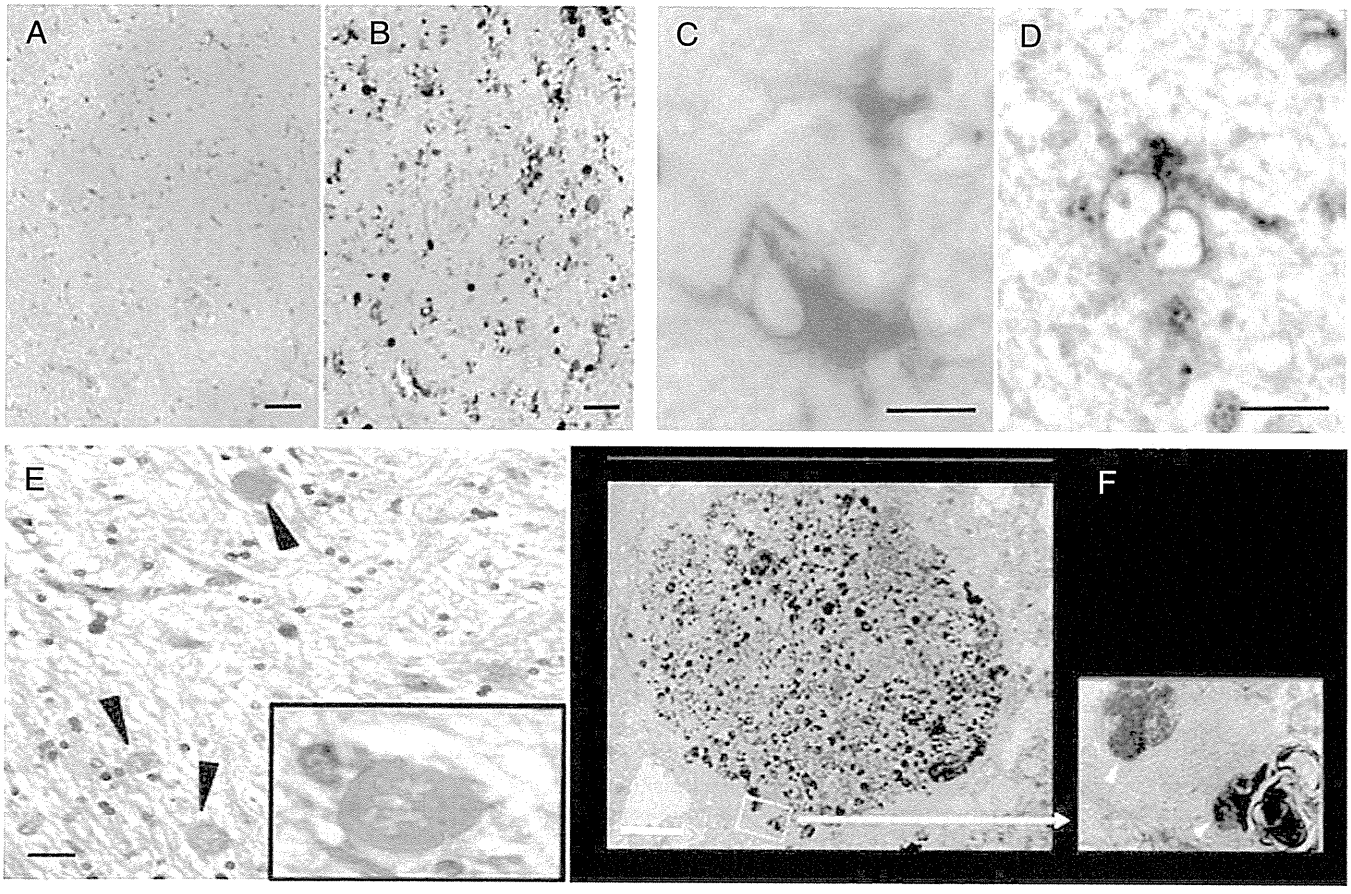

Fig. 4 The histopathological findings in the brains of patients with aceruloplasminemia. Marked accumulation of redox-active iron was shown in the globus pallidus of an aceruloplasminemic brain (b) compared with that from a control subject (a) (redox-active iron staining: a modified Perl's technique). In the basal ganglia, deformed astrocytes showed marked iron deposition (c: Berlin blue stain) and reacted positively to anti-4-hydroxynonenal antibody (d). Globular structures, indicated by arrowheads, were seen in the putamen (e: HE stain). The electron microscopic findings of the globular structures indicated that they contained many electron-dense bodies, indicated by the arrowhead (f). Scale bars: a, b = 100 μm; c–e = 50 μm; f (left panel) = 5 μm. (c–f from Kaneko *et al.*[26]).

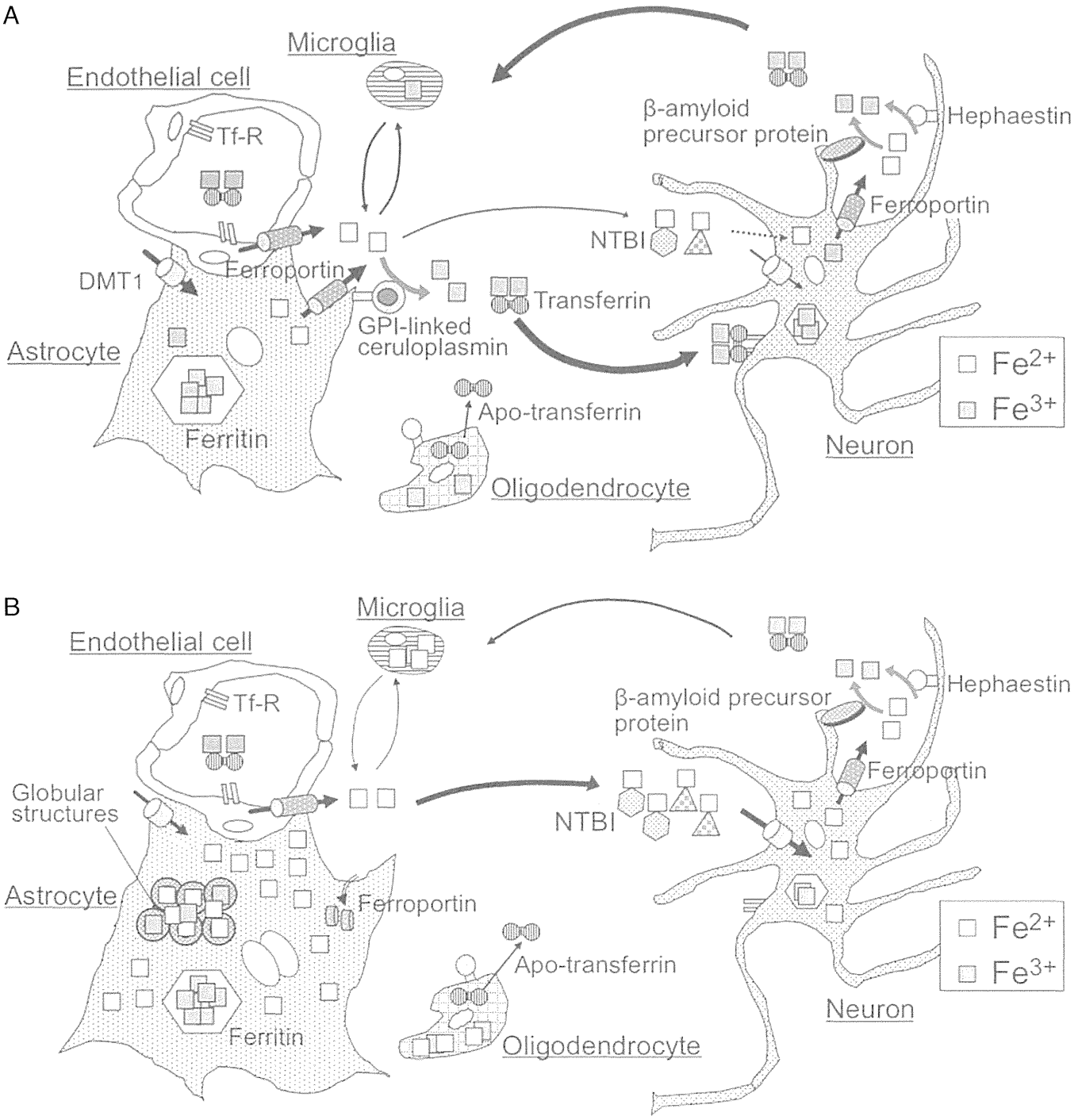

Fig. 5 A model of the iron metabolic cycle in the brain. In the normal brain (a), iron may be recycled between astrocytes and neurons. Transferrin acts as a shuttle to deliver iron from astrocytes to neurons. The glycosylphosphatidylinositol (GPI)-ceruloplasmin on astrocytes is a ferroxidase that mediates the oxidation of ferrous iron and its subsequent transfer to transferrin. Neurons take up the transfferin-bound iron and also take up iron from alternative sources (non-transferrin-bound iron; NTBI). β-amyloid precursor protein and hephaestin also play roles as a ferroxidase and interact with neuronal ferroportin. In the brain of a patient with aceruloplasminemia (b), neurons take up the iron from alternative sources (NTBI), where the iron is complexed to molecules such as citrate and ascorbate, because astrocytes without GPI-linked ceruloplasmin cannot transport iron to transferrin. Mutant ceruloplasmin cannot prevent hepcidin-mediated ferroportin internalization. Tf-R, transferrin receptor 1; DMT1, divalent ion transporter.

knockout mice developed deficits in motor coordination, and showed increased iron deposition in several regions of the CNS.[28] Increased lipid peroxidation due to iron-mediated cellular radical injury was also seen in these regions. These results indicate that ceruloplasmin plays an important role in protecting the brain from iron-mediated free radical injury.

In the brain, serum transferrin-bound iron is endocytosed by brain endothelial cells in a manner dependent on the transferrin receptor 1, and iron is released into the brain interstitial fluid through ferroportin.[6] Extracellular iron is oxidized by GPI-ceruloplasmin, which is located in the foot processes of astrocytes, and then the iron binds to the transferrin synthesized by oligodendrocytes, and is transported into neurons. GPI-linked ceruloplasmin also plays an important role in iron efflux from astrocytes.[6] Neurons take up the iron mainly from transferrin and from the alternative sources of non-transferrin-bound iron (NTBI), including citrate and ascorbate. β-amyloid precursor protein was found to possess ferroxidase activity like ceruloplasmin, and to interact with neuronal ferroportin.[29] The ceruloplasmin homologue, hephaestin, is also expressed on the neurons and functions as a ferroxidase. The brain needs several times the concentration of iron obtained from the blood to maintain its normal function.[30] Taken together, the known functions of the iron metabolic molecules suggest the presence of a cycle of iron storage and reutilization within the brain (Fig. 5a).

In aceruloplasminemia, the brain ferroportin expressed on the astrocytes is markedly reduced, probably due to degradation caused by the absence of ceruloplasmin.[12] Murine models of aceruloplasminemia showed that the neuronal cell loss might result from iron starvation in regions where the iron in astrocytes was not able to be mobilized for uptake into neurons, and later, the iron accumulation was observed in neurons, thus suggesting that the neurons take up significant amounts of iron from alternative sources of NTBI.[31] Neuronal cell injury may therefore result from iron deficiency in the early stage and from iron-mediated oxidation in the late stage. The excess iron in astrocytes could result in oxidative damage to these cells, with the subsequent disruption of neuronal cell protection by astrocytes. GPI-linked ceruloplasmin may be associated with normal iron homeostasis and neuronal survival in the CNS (Fig. 5b).

Treatment

Aceruloplasminemia is a slowly progressive neurodegenerative disorder, and its early diagnosis and the early treatment of patients are important issues. Iron-mediated lipid peroxidation and oxidative stress are considered to be the main causes of the neuronal degeneration in patients with aceruloplasminemia. The prognosis may also involve heart failure due to cardiac iron overload. To reduce the iron accumulation, systemic iron chelation therapy has been introduced in some patients. The intravenous administration of deferoxamine or an oral iron chelating agent, deferasirox, led to a mild improvement in clinical symptoms.[32,33] Treatment with oral zinc sulfate therapy (200 mg/day) was effective for extrapyramidal and cerebellar symptoms.[34] Combination therapy with an iron chelator and zinc sulfate may ameliorate the neurological symptoms.

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CHAPTER

45

Aceruloplasminemia

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CLINICAL FEATURES

In 1987, we described the first case of aceruloplasminemia as a form of familial apoceruloplasmin deficiency. The patient was a 52-year-old Japanese female suffering from blepharospasm, retinal degeneration, and diabetes mellitus.[1] Subsequent evaluations revealed the complete absence of serum ceruloplasmin, the presence of mild anemia, a low plasma iron concentration, an elevated plasma ferritin level, and significant iron accumulation in the basal ganglia and liver on T2-weighted magnetic resonance imaging (MRI). Ferrokinetics and a histochemical study showed accumulation of iron, but not copper, in the liver and brain, although ceruloplasmin is a multicopper oxidase harboring six copper ions. Careful family studies in this original case showed that the lack of serum ceruloplasmin was inherited although in an autosomal recessive fashion. A genetic analysis of the ceruloplasmin gene revealed that this patient was homozygous, with a five-base insertion in exon 7, resulting in a frameshift mutation and a truncated open reading frame.[2] The clinical findings and identification of a mutation in the ceruloplasmin gene confirmed that the disorder was a novel disorder of iron metabolism resulting from a lack of ceruloplasmin in the serum. The disorder was termed aceruloplasminemia (MIM 604290). Treatment with the iron chelator desferrioxamine decreased the brain iron stores, prevented the progression of neurological symptoms and reduced the level of plasma lipid peroxidation.[3] The identification of aceruloplasminemia implies that there is a direct connection between iron accumulation in the brain and liver and that ceruloplasmin plays an essential role in the transport and/or metabolism of iron, but not copper, despite its need for copper to complete its functions. An epidemiological study in Japan demonstrated that the prevalence of aceruloplasminemia was estimated to be approximately 1 per 2,000,000 in nonconsanguineous marriages, and subsequent studies have now identified more than 35 affected families from around the world.[4]

The clinical diagnosis should be made based on the complete absence of serum ceruloplasmin and the identification of a mutation in the ceruloplasmin gene. There are two variants associated with mutations in the ceruloplasmin gene. The first variant involves a low amount of ceruloplasmin detected in the serum, as reported in one case.[5] In that case, the patient presented with the typical symptoms of aceruloplasminemia, including asymptomatic hepatic iron overload, retinal degeneration, and diabetes mellitus. MRI of the liver and basal ganglia showed T2-hypointensity signals associated with parenchymal iron accumulation. A gene analysis disclosed a G969S homozygous mutation in the ceruloplasmin gene. An immunoblot analysis of serum ceruloplasmin revealed only apo-form of ceruloplasmin without ferroxidase activity. Another patient with compound heterozygous mutations of R882X and H978Q also presented with typical symptoms, including hepatic and brain iron accumulation.[6] The serum ceruloplasmin level was half the normal value, and apo- and holo-ceruloplasmin were detected in an immunoblot analysis. However, no serum ferroxidase activity was detected in the patient. The patient's mother was heterozygous for the H978Q mutation, and her serum ceruloplasmin level was normal with a level of ferroxidase activity that was half the normal value. Therefore, the serum ceruloplasmin present in H978Q mutation carriers is speculated to be devoid of ferroxidase activity, resulting in the development of aceruloplasminemia in spite of the detection of ceruloplasmin in the serum.

Rosenberg's Molecular and Genetic Basis of Neurological and Psychiatric Disease
http://dx.doi.org/10.1016/10.1016/B978-0-12-410529-4.00045-0

The second variant is symptomatic heterozygous disease. Aceruloplasminemia is an autosomal recessive inherited disease, and heterozygous individuals with a partial ceruloplasmin deficiency may have normal iron metabolism and no clinical symptoms. The first report of symptomatic heterozygous disease showed three Japanese patients from two families with half the normal ceruloplasmin levels in the serum, and who developed cerebellar ataxia from the fourth decade of life.[7] They were all heterozygous for a W858X mutation, and their serum iron concentrations and transferrin saturation levels were normal. At autopsy, pathological and biochemical examinations showed marked loss of Purkinje cells, a large amount of iron deposition in the cerebellum, and small deposits in the basal ganglia, thalamus, and liver. The W858X mutation is frequently detected in Japanese patients; however, most of the carriers heterozygous for the W858X mutation are asymptomatic.[8,9] The second report of a symptomatic heterozygous patient was a young patient who presented with subacute progressive extrapyramidal movement disorders.[10] Although her brain MRI showed no iron accumulation, the iron content in a liver biopsy specimen exceeded the normal expected range. A genetic analysis of her ceruloplasmin gene revealed that she was heterozygous for a R701W mutation. However, her father, who was also heterozygous for the R701W mutation, was asymptomatic and the pathological effects of the mutation on the neurological symptoms are unclear.

These findings have important diagnostic implications, indicating that the presence of ceruloplasmin in the serum of patients with the typical clinical features of aceruloplasminemia requires ceruloplasmin gene analysis before the diagnosis of aceruloplasminemia can be conclusively ruled out.

LABORATORY TESTING

Aceruloplasminemia patients present in the fourth or fifth decade of life with neurological symptoms.[11,12] These neurological features are usually progressive at the time of diagnosis, and are associated with the iron accumulation in the basal ganglia and cerebellum as detected on T2-weighted MRI (Figure 45.1). Ophthalmological examinations usually reveal evidence of peripheral retinal degeneration secondary to iron accumulation and photoreceptor cell loss (Figure 45.2A). Although the neurological features dominate the clinical features in most patients, all individuals have evidence of systemic iron accumulation at the time of diagnosis. The laboratory findings demonstrated microcytic anemia, decreased serum iron content and an increased serum ferritin concentration, usually greater than 1000 ng per ml. T2-weighted MRI of the liver shows low intensity signals associated with the iron accumulation (Figure 45.2B). Liver biopsy samples reveal normal hepatic architecture and histology without cirrhosis or fibrosis; however, they do demonstrate excess iron accumulation (>1200 μg per g dry weight) within hepatocytes and reticuloendothelial cells (Figure 45.2C).

Aceruloplasminemia patients also present with diabetes or evidence of abnormal glucose tolerance. Autopsy studies have revealed significant iron accumulation within the endocrine portion of the pancreas, with marked diminution in the β cell population within the islets of Langerhans.[13-15] Thus, the diagnosis of aceruloplasminemia in a symptomatic individual relies upon the demonstration of the complete absence of serum ceruloplasmin and abnormal laboratory findings, as well as MRI findings suggesting iron overload in both the liver and brain. The neuroimaging studies in aceruloplasminemia patients are strongly supported by the characteristic MRI findings of abnormal low intensities reflecting iron accumulation in the liver and brain, including the basal ganglia, thalamus and dentate nucleus on both T1- and T2-weighted images. Functional neuroimaging studies using fluorodeoxyglucose (FDG)-positron emission tomography (PET) demonstrated hypometabolism in the basal ganglia.[16-18] The clinical characteristics of the patients are summarized in Table 45.1. In general, aceruloplasminemia patients present neurological symptoms, including extrapyramidal signs, in the fourth or fifth decade of life. Although the neurological findings dominate the clinical features in most patients, some patients have been recognized prior to the onset of neurological symptoms due to biochemical abnormalities indicating changes in iron metabolism, the presence of diabetes or evidence of abnormal glucose tolerance and abnormal MRI findings of the liver and the brain.[19-22]

The molecular diagnosis of aceruloplasminemia is usually made based on a sequence analysis of the ceruloplasmin gene using genomic DNA derived from leukocytes. The presence of a processed pseudogene on chromosome 8 encoding the carboxyl-terminal 563 amino acids of this protein must be taken into account when designing polymerase chain reaction (PCR) primers for molecular diagnostic testing.[23] When an intronic mutation that can affect the acceptor or donor splice site is found, a sequence analysis of mRNA using reverse transcriptase PCR should be performed to confirm the production of an aberrant transcript. The use of reverse transcriptase PCR is preferable to conducting assessments of liver samples, the predominant source of the secreted form of ceruloplasmin, because few ceruloplasmin mRNAs are expressed in leukocytes.

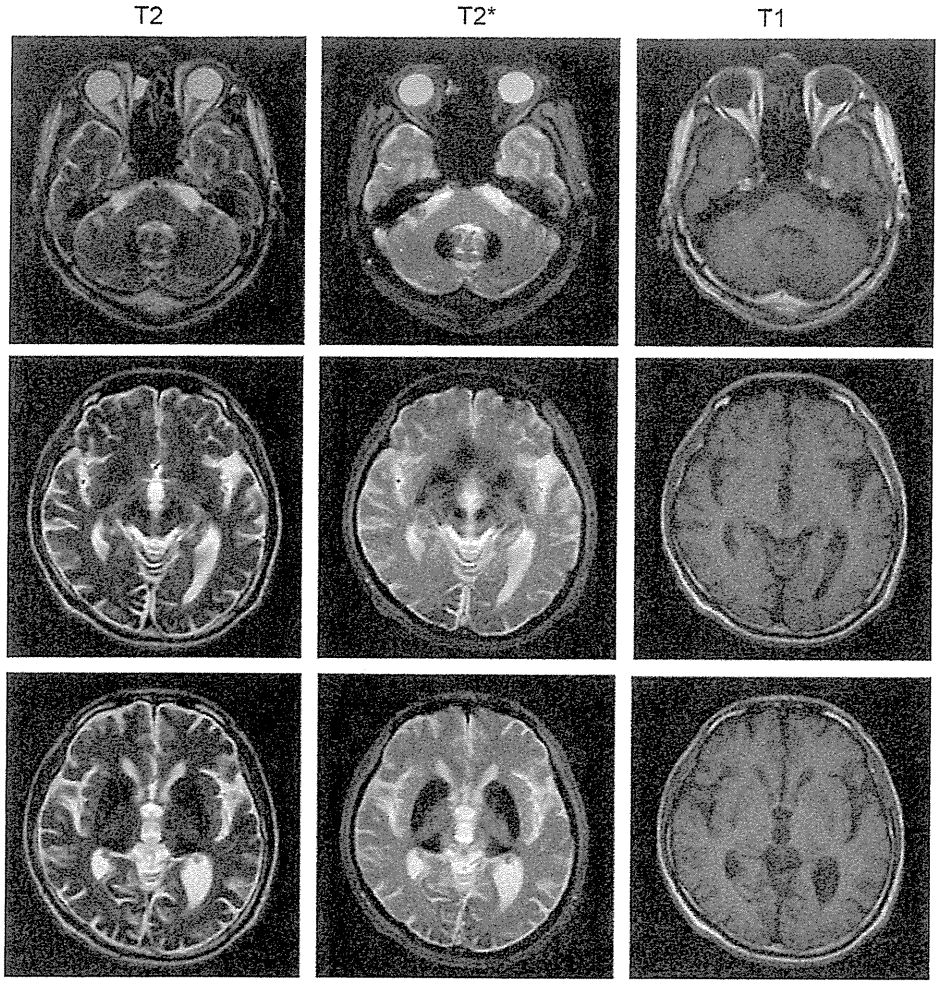

FIGURE 45.1 **Magnetic resonance images (MRI) of an aceruloplasminemia patient.** T1, T2 and T2*-weighted axial images of the brain showed signal attenuation of the dentate nucleus of the cerebellum, globus pallidum, putamen, caudate nucleus, and thalamus.

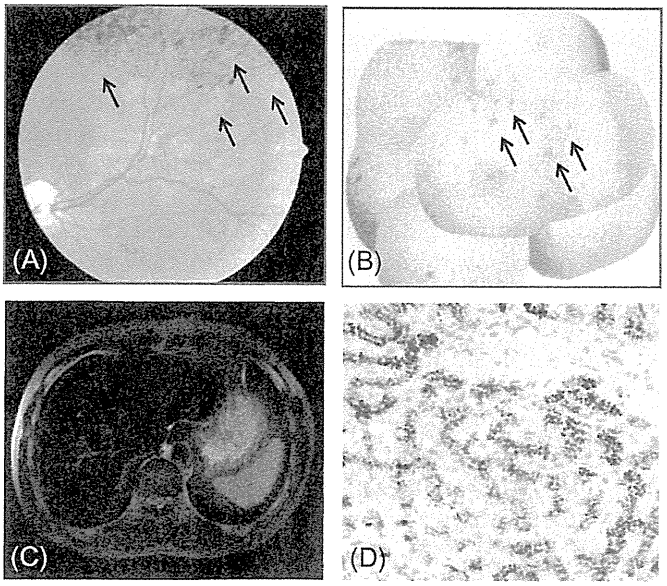

FIGURE 45.2 Ophthalmoscopic findings show several small, yellowish areas of opacity (arrows) scattered over grayish areas of atrophy in the retinal pigment epithelium (A). Fluorescein angiography demonstrates window defects (arrows) corresponding to yellowish areas of opacity (B). T2-weighted axial images of the liver also showed signal attenuation (C). A liver biopsy specimen stained with Perl stain showed iron in the hepatocytes (original magnification ×200) (D).

TABLE 45.1 The Clinical Characteristics of Patients with Aceruloplasminemia

Clinical manifestations in 71 patients with aceruloplasminemia:
- Anemia (80%)
- Retinal degeneration (76%)
- Diabetes mellitus (70%)
- Neurological symptoms (68%)
  1. Ataxia (71%): dysarthria > gait ataxia > limb ataxia
  2. Involuntary movement (64%): dystonia (blepharospasm, grimacing, neck dystonia) > chorea > tremors
  3. Parkinsonism (20%): rigidity > akinesia
  4. Cognitive dysfunction (60%): apathy > forgetfulness

Onset of clinical manifestations:
- Diabetes mellitus: under 30 years old, 18%; 30–39 years old, 35%; 40–49 years old, 31%; over 50 years old, 16%
- Neurological symptoms: under 40 years old, 7%; 40–49 years old, 38%; 50–59 years old, 42%; over 60 years old, 13%

Laboratory findings:
- Undetectable serum ceruloplasmin
- Elevated serum ferritin
- Decreased serum iron, iron-refractory microcytic anemia
- Low serum copper and normal urinary copper levels

MRI (magnetic resonance imaging) findings:
- Low intensity on both T1- and T2-weighted MRI in the liver and the basal ganglia, including the caudate nucleus, putamen and pallidum, and the thalamus

Liver biopsy results:
- Excess iron accumulation (>1000 μg/g dry weight) within hepatocytes and reticuloendothelial cells
- Normal hepatic architecture and histology without cirrhosis or fibrosis
- Normal copper accumulation

MOLECULAR GENETICS

Ceruloplasmin is a single-copy gene on chromosome 3 in the human genome. The human ceruloplasmin gene contains 20 exons with total length of about 65 kb.[24] The ceruloplasmin secreted into the plasma is considered to be involved in iron homeostasis. Although the liver is the predominant source of serum ceruloplasmin, the extrahepatic expression of ceruloplasmin has been shown in several tissues, including the central nervous system (CNS).[25] In the brain, ceruloplasmin is expressed in the astrocytes lining the brain microvasculature located in the basal ganglia, where a distinct form of ceruloplasmin is expressed as a glycosylphosphatidylinositol (GPI)-linked form by the alternative splicing of exons 19 and 20.[24,26] Earlier studies showed that the GPI-linked ceruloplasmin was located in leptomeningeal cells, the Müller glial cells in the retina, the Sertoli cells in the testes, and the Schwann cells in peripheral nerves;[27,28] however, recent studies reported the expression of GPI-linked ceruloplasmin in various tissues.[29,30] Although the precise function of GPI-linked ceruloplasmin remains unknown, the GPI-linked ceruloplasmin likely plays an important role in the mobilization of iron and the antioxidant effects in the CNS.[31,32] GPI-linked ceruloplasmin may be associated with iron homeostasis and antioxidant defense by protecting the CNS from iron-mediated free radical injury. The ferroxidase activity of GPI-linked ceruloplasmin is also essential for the stability of cell surface ferroportin.[29,33] The requirement for a ferroxidase to maintain iron transport activity represents a novel mechanism of regulating cellular iron export.

The genetic analyses of aceruloplasminemia patients have identified more than 40 distinct mutations in the ceruloplasmin gene (Figure 45.3).[19,5,6,8–10,20,21,34–51] Most of the mutations detected are unique to specific families, where there is often a history of consanguinity. The majority of mutations are truncated mutations leading to the formation of a premature stop codon. The ferroxidase activity of ceruloplasmin is dependent upon the trinuclear copper cluster, the ligands for which are encoded by exon 18.[52] The truncated mutations identified are predicted to result in the formation of a protein lacking the copper cluster sites presumed to be critical for enzymatic function. The symptoms, onset, and prognosis in single cases have demonstrated that there is no genotype–phenotype association.[12] This finding suggests that unknown genetic or environmental factors may regulate iron accumulation in the brain.

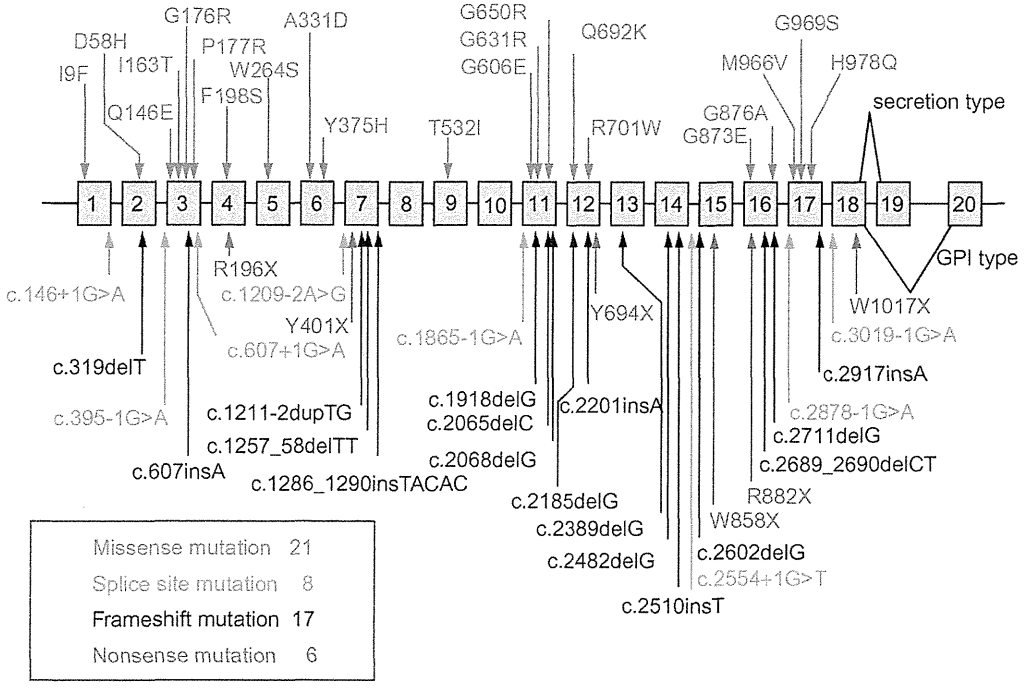

FIGURE 45.3 **Genetic mutations characterized in patients with aceruloplasminemia and their family members.** The indicated mutations are referenced in the text and some mutations are included in our unpublished data.

Biosynthesis studies of mutant ceruloplasmin in mammalian cell culture systems without endogenous ceruloplasmin were performed to investigate the molecular pathogenesis of aceruloplasminemia.[29,37,40,42,49,53] The biosynthesis studies of missense mutants revealed three distinct pathological mechanisms (Figure 45.4). A first group comprising the I9F, G176R, P177R, D58H, F198S, W264S, A331D, G606E and G873E mutants were retained in the endoplasmic reticulum (ER).[29,40,49] The mutants presumably result from the misfolding of ceruloplasmin in the ER. The amino acid sequence of a G(FLI) (LI)GP repeat motif is believed to affect folding during the early secretory pathway.[49] The G873E, G176R, and P177R mutants affect the conserved repeated G(FLI) (LI)GP motif, which is consistent with this hypothesis, whereas the G876A mutant was not retained in the ER.[29] The mutants located beside this motif are speculated to have other molecular mechanisms involved in the cellular trafficking of ceruloplasmin. A second group of mutants, including the G631R, Q692K, M966V and G969S mutants, was synthesized and all were found to be secreted with normal kinetics, but failed to incorporate copper during the late secretory pathway, resulting in apo-ceruloplasmin.[5,29,37] The G631R and G969S mutations are located in the nearby type I copper-binding His637 and His975 sites, respectively. The Q692K and M966V mutations are also located near the type I copper-binding sites of M690 and His975, respectively. A site-directed mutagenesis analysis of the type I copper-binding site indicated that these mutants failed to incorporate copper into the apo-ceruloplasmin.[37] These biochemical studies demonstrated that the type I copper-binding site did not affect either the protein folding for intracellular trafficking from the ER to the Golgi body or the subsequent protein secretion from the cell. However, the copper-binding site may play an essential role in the protein structure for copper incorporation into the apo-ceruloplasmin. The third group containing the Y356H, R701W, and G876A mutants reconstituted both the apo- and holo-proteins, and were secreted extracellularly.[29] However, the mutants had impaired ferroxidase activity, which is required for ferroportin stability. These mutants may have altered iron-binding sites or changes in the trinuclear copper cluster, which are essential for the oxidase activity of the protein. It will be necessary to analyze the crystal structure of the mutant ceruloplasmin proteins in order to obtain insight into the mechanism of ferroxidase activity.

A biogenesis study of nonsense mutations, including Y694X, W858X and R882X, demonstrated that the Y694X and W858X mutants were retained in the ER, while the R882X mutant was secreted.[42] Subsequent site-directed mutagenesis analyses revealed that the truncated mutant containing the cysteine residue at amino acid 881(Cys-881) was able to pass through the ER and was secreted, while the truncated mutant protein without Cys-881 appeared to accumulate in the ER, leading to ER stress, and eventually resulting in cell death. Thus, Cys-881 is necessary for the secretion of almost all of the truncated ceruloplasmin, although a recent biogenesis study of the W1017X mutant with Cys-881 showed that the mutant was exclusively retained in the ER.[54]

The observations made in the cases of symptomatic heterozygous patients indicate that the specific mutations of W858X and R701W may cause a dominant-negative effect of the mutations overriding the influence of ceruloplasmin function in iron metabolism. *In vitro* biogenesis studies showed a potential function of the dominant-negative effect of mutant ceruloplasmin occurring via silencing of the wild-type ceruloplasmin function. The W858X mutant accumulated in the ER, leading to the ER stress, which resulted in cell death.[40] The R701W mutant induced the subcellular relocalization of the copper-transporting ATPase ATP7B in the Golgi complex and fragmentation of the Golgi complex, resulting in a failure of copper loading in wild-type ceruloplasmin.[53] The Arg701 site is located in one of the repeat CX(R/K) motifs consisting of large exposed loops connecting domains. A mutagenesis study of the motifs revealed that the external loops play an important role in copper incorporation.

The clinical phenotype in most patients shows little variation, regardless of the specific mutation.[12] While almost all patients have a complete absence of serum ceruloplasmin, H978Q and G969S mutations were reported in patients who had detectable levels of serum ceruloplasmin, but who presented with the clinical features of aceruloplasminemia. The H978Q mutation, which is located at one of the type I copper-binding sites and constitutes holo-ceruloplasmin, was speculated to be devoid of ferroxidase activity.[6] The G969S mutation that led to apo-ceruloplasmin in the serum was suggested to be less fragile than the wild-type apo-ceruloplasmin, allowing it to remain in circulation for a longer period of time.[5]

DISEASE MECHANISMS

The abnormalities of iron homeostasis observed in patients with aceruloplasminemia can be understood by considering the cellular physiology of systemic iron metabolism. A small amount of total iron is delivered from absorption in enterocytes while a large amount of the iron arising from recycling of the heme iron from aging red blood cells is turned over within the reticuloendothelial system. The recycled iron is released from endothelial cells in the liver and the spleen, and binds to the transferrin in the plasma, resulting in its return to the bone marrow for erythropoiesis. Ceruloplasmin functions as an important factor in the iron cycle by performing iron oxidation, which is required to sustain the iron release and uptake by transferrin (Figure 45.4). The lack of plasma ferroxidase activity in ceruloplasmin results in increased extracellular ferrous iron, which is rapidly taken up into cells. Pathological studies in patients with aceruloplasminemia showed that iron accumulates within hepatocytes, pancreatic endocrine cells, and astrocytes.[13,14,55] Although the mechanism underlying the neurodegeneration in aceruloplasminemia has not been clarified, its pathogenesis is presumably secondary to consistent accumulation of iron within neurons and astrocytes. The electronic properties of iron enable the metal to take part in chemical reactions because the Fenton catalysis of iron plays an important role in cellular redox chemistry by reducing H_2O_2 to the highly cytotoxic hydroxyl (OH•) radical, which may be injurious to neural and other cellular substrates. The antioxidant activity of ceruloplasmin can be mainly ascribed to its ferroxidase activity, which effectively inhibits ferrous ion-stimulated lipid peroxidation and ferrous ion-dependent formation of hydroxyl radicals in the Fenton reaction. A direct role for iron in oxidant-mediated neuronal injury is supported by findings of increased lipid peroxidation and subsequent mitochondrial dysfunction in the brain tissues, cerebral spinal fluid, and erythrocytes of aceruloplasminemia patients.[56-61]

The pathological findings in the brain showed severe iron deposition in both the astrocytes and neurons, and neuronal loss in the same regions associated with the highest iron accumulation and necrosis.[13,14] The neurodegenerative changes were observed in the cerebral cortex, as well as in the basal ganglia, dentate nuclei, and cerebellar cortices. The distribution in order of the iron level is the globus pallidus > putamen > cerebellar cortex and cerebral cortex.[11] The characteristic histopathological findings of the patients were deformed astrocytes and globular structures, which were observed more frequently in the striatum than in the cerebral cortex, which occurred in parallel with significant iron deposition and neuronal loss (Figure 45.5).[55,62] The globular structures were immunologically reactive for a glial marker protein, suggesting that they were ballooned foot processes of astrocytes. The deformed astrocytes were more frequently observed in the basal ganglia in which marked iron deposition was observed. Glial fibrillary acidic protein (GFAP) is one of the proteins most severely modified by oxidative stress in the brains of aceruloplasminemia patients.[63] Intense ferrous iron deposition was demonstrated in the terminal astrocytic processes and the globular structures.[64] The morphological changes of astrocytes may be related to iron-induced tissue damage.

The iron accumulation was observed in neurons as well as astrocytes. This finding indicates that the neurons take up significant amounts of iron due to alternative sources of non-transferrin-bound iron complexed to molecules, such as citrate and ascorbate, because astrocytes without any expression of ceruloplasmin are not able to transport iron to transferrin that binds to transferrin receptor 1 on neurons. A recent pathological study of a murine model of aceruloplasminemia showed that the neuronal cell loss may result from iron deficiency in regions where the iron in

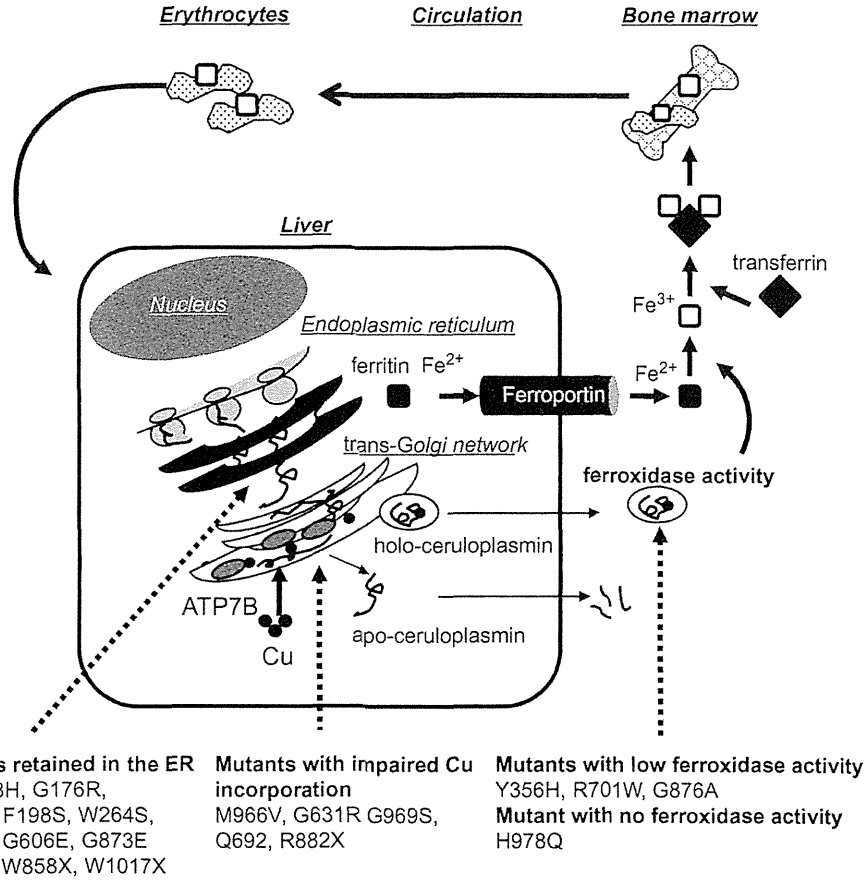

FIGURE 45.4 **A model for the interaction between iron and copper homeostasis in normal subjects and in the aceruloplasminemia patients.** In normal subjects, iron is continuously recycled between the bone marrow and hepatocytes, with serum transferrin acting as a shuttle to deliver iron from hepatocytes to the bone marrow. The role of ceruloplasmin, formed as holo-ceruloplasmin, is as a ferroxidase mediating ferrous iron oxidation and subsequent transfer to transferrin. In normal subjects, copper enters the cell and binds the copper chaperones, which deliver the copper to ATP-7B. The ATP-7B pumps the copper into the trans-Golgi network. Ceruloplasmin is initially synthesized as apo-ceruloplasmin and incorporates the copper into the apo-protein in the Golgi body, resulting in the formation of holo-ceruloplasmin prior to extracellular secretion. Mutant ceruloplasmin biosyntheses were investigated using a cell culture system without endogenous ceruloplasmin. The mutant proteins revealed three distinct pathological mechanisms, including mutants associated with mistrafficking, resulting in the retention of the protein in the ER, mutants altering the intrinsic protein structure, resulting in abrogation of copper incorporation into apo-ceruloplasmin, and mutants with impaired ferroxidase activity.

astrocytes is not able to be mobilized for uptake into neurons, and the excess iron accumulation in astrocytes could also result in oxidative damage to these cells, with subsequent loss of the glial-derived growth factors critical for neurons.[65]

The generation of murine models of aceruloplasminemia provided a critical clue to study the role of ceruloplasmin in iron homeostasis. Three distinct research groups generated ceruloplasmin knockout mice that develop hepatic and reticuloendothelial iron overload. The first knockout mice generated were reported by Harris et al.[66] The mice have an increased iron content, with lipid peroxidation in the brain.[67] However, there is no evidence of neurological symptoms in these mice. Double knockout mice lacking both ceruloplasmin and hephaestin were generated by crossing the ceruloplasmin knockout mice with *sla* mice, which are hephaestin knockout, sex-linked anemic mice. Hephaestin is a ceruloplasmin homolog, and is also a multicopper oxidase with ferroxidase activity, which is abundantly expressed in the neurons in the murine brain, as well as in the enterocytes in the duodenum. The knockout mice lacking both ceruloplasmin and hephaestin exhibited a neurodegenerative phenotype and retinal degeneration consistent with the aceruloplasminemia patients.[67–69] In the mice, hephaestin expression may play a more important role to maintain the cellular redox environment than it does in the CNS in the humans. The iron homeostasis associated with ceruloplasmin was investigated using the ceruloplasmin knockout mice. When the mice were injected with damaged red blood cells in order to induce an increased reticuloendothelial iron overload, or when the mice received a phlebotomy to accelerate reticuloendothelial iron transport to the bone marrow, the mice failed to show

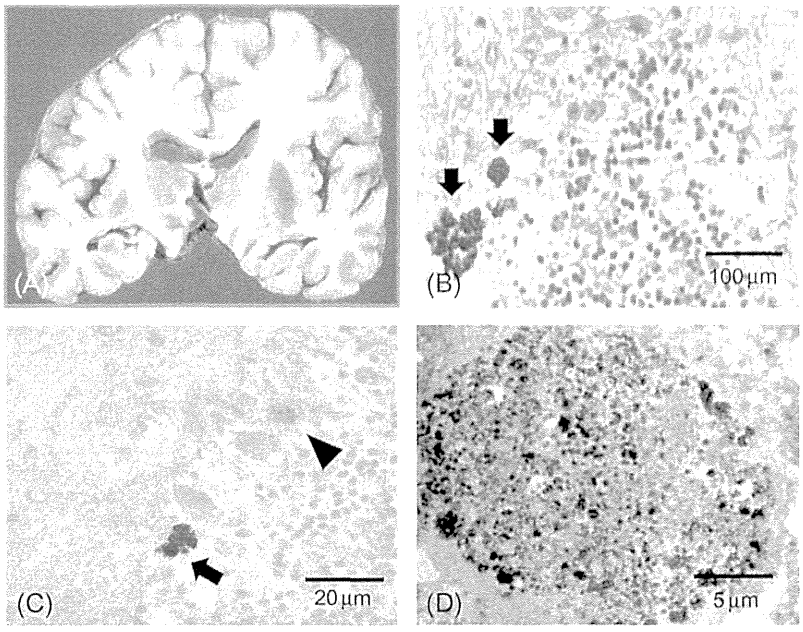

FIGURE 45.5 **The histopathological findings of cerebellar cortices in the brains of patients with aceruloplasminemia.** A coronal section of the brain of the affected patient shows brown pigmentation of the basal ganglia (A). Globular structures, indicated by arrows, were seen in the Purkinje cell layer. Many of the globular structures contained brown materials (B; hematoxylin and eosin stain). At the cellular layer, iron deposits in Purkinje cells (indicated by an arrowhead) with a decreased number of cells were seen (C; Prussian blue stain). The globular structures (arrow) exhibited siderous features. The electron microscopic findings of the globular structures indicated that they contain many electron-dense bodies (D).

an increase in their serum iron levels. Only upon injection of holo-ceruloplasmin were the mice able to release iron from a storage compartment for delivery to a synthetic compartment, the bone marrow, thus confirming the essential role for ceruloplasmin in regulating efficient iron efflux. Of interest, the copper metabolism is normal in ceruloplasmin knockout mice, and none of the clinical pathology associated with aceruloplasminemia is secondary to copper deficiency or toxicity.

The second groups of knockout mice were reported by Patel et al.[31] The mice developed a neurodegenerative phenotype and showed increased iron deposition in several regions of the CNS, such as the cerebellum and brainstem. Increased lipid peroxidation due to iron-mediated cellular radical injury was also seen in some regions. Cerebellar neuronal cells from neonatal mice were also more susceptible to oxidative stress *in vitro*. These mice showed deficits in motor coordination that were associated with a loss of brainstem dopaminergic neurons. These results indicate that ceruloplasmin plays an important role in maintaining iron homeostasis in the brain, and in protecting the brain from iron-mediated free radical injury.

The third group of knockout mice were reported by Yamamoto et al.[70] Although the mice showed hepatic iron overload, there was no evidence of iron accumulation in the brain, even after treatment with rotenone, a mitochondrial complex 1 inhibitor that enhances oxidative stress.[71]

Ceruloplasmin plays an essential role in cellular iron efflux by oxidizing the ferrous iron exported from ferroportin. Ferroportin is posttranslationally regulated through internalization triggered by hepcidin binding.[72] Previous studies showed that the ferroxidase activity of GPI-linked ceruloplasmin was essential for the stability of cell surface ferroportin in rat glioma cells lines.[33] The *in vitro* biological analyses suggested that the ceruloplasmin mutants had impaired ferroportin stability on the cell surface, resulting in exacerbated iron accumulation.[29,53] The hepatic expression of ferroportin proteins and the mRNA levels were analyzed to evaluate the involvement of ferroportin in the pathogenesis of aceruloplasminemia.[29] The hepatic ferroportin protein levels were decreased despite the presence of high ferroportin mRNA levels in two aceruloplasminemia patients. Decreased ferroportin protein levels in the liver may be due to degradation due to the absence of ceruloplasmin rather than due to a decreased synthesis of ferroportin at the transcriptional level. Clinical analyses of the hepcidin level in patients with aceruloplasminemia revealed that the serum hepcidin levels and hepatic hepcidin mRNA levels are lower than in control subjects.[29,73] An analysis of ceruloplasmin knockout mice also showed the hepatic hepcidin mRNA levels to decrease in comparison to wild-type and heterozygous mice.[74] The low serum hepcidin levels may induce increased iron absorption in the intestine, where the ceruloplasmin homolog hephaestin retains ferroxidase activity that is involved in basolateral intestinal iron transport. Therefore, the low hepcidin level in the serum and the loss of cell surface

ferroportin due to mutant ceruloplasmin may enhance the cellular iron accumulation, contributing to the pathology of aceruloplasminemia.

DIFFERENTIAL DIAGNOSIS

Inherited neurodegenerative disorders termed "neurodegeneration with brain iron accumulation" (NBIA) should be considered in the differential diagnosis of aceruloplasminemia, as the characteristic syndrome of NBIA presents with progressive extrapyramidal symptoms and excessive iron deposition in the brain, particularly affecting the basal ganglia. The main causes of the syndromes are mutations in neuroaxonal dystrophies: panthothenate kinase-associated neurodegeneration (PKAN, formerly known as Hallervorden–Spatz disease, NBIA1) and PLA2G6-associated neurodegeneration (PLAN, NBIA2). Intensive genetic approaches have identified additional genes that cause other NBIA syndromes, including Kufor–Rakeb disease (NBIA3, PARK9), fatty acid hydroxylase-associated neurodegeneration (FAHM), neuroferritinopathy, mitochondrial membrane protein-associated neurodegeneration (MPAN), Woodhouse–Sakati syndrome and beta-propeller protein-associated neurodegeneration.[75,76] Neuroimaging studies using T2* MRI have shown that a wide range of hypointensity regions, including the basal ganglia, caudate nucleus, putamen, pallidum, and thalamus, are typically observed in aceruloplasminemia patients and can be used to distinguish these patients from those with other NBIA syndromes.[77] Among patients with NBIA syndrome, only those with aceruloplasminemia have abnormal serum ceruloplasmin levels. The level of serum ceruloplasmin is usually found to be decreased in Wilson disease patients with progressive extrapyramidal symptoms. In patients with Wilson disease, the inability to transfer copper into the ceruloplasmin precursor protein apo-ceruloplasmin and a decrease in biliary copper excretion results in serum ceruloplasmin deficiency and excess copper accumulation.[78] The presence of Kayser–Fleischer rings on slit-lamp examinations in conjunction with massive accumulation of hepatic copper on liver biopsies is helpful for diagnosing Wilson disease. Increases in the brain level of copper are detectable on MRI relatively early in the course of the disease. T2-weighted images show increased signal intensity with a central core of decreased intensity in the basal ganglia. Neuroimaging may also prove useful for confirming the diagnosis.

MANAGEMENT

Aceruloplasminemia is a fatal disease, and its early diagnosis and early treatment of patients are issues of paramount importance. Iron-mediated lipid peroxidation and oxidative stress are considered to be the main cause of the neuronal degeneration in aceruloplasminemia patients. To reduce the iron accumulation, systemic iron chelation therapy has been introduced in some patients. Desferrioxamine (deferoxamine) is a high-affinity iron chelator that combines with ferric iron. It has been shown to cross the blood–brain barrier and to promote the excretion of excess iron in patients with inherited and acquired forms of iron overload.[79] The administration of desferrioxamine was effective for reducing the hepatic iron overload and leading to a partial improvement of the neurological symptoms and brain iron accumulation, as reported in a single case report.[3] However, subsequent studies showed little effect of desferrioxamine on the central nervous symptoms, despite normalization of the serum ferritin and hepatic iron concentrations and improvement in the insulin requirement and the regional brain iron levels in T2*-weighted MRI.[21,38,80] Desferrioxamine therapy was often discontinued because of a concomitant decrease in hemoglobin and the serum iron level was observed after several months of therapy, suggesting that desferrioxamine sequestered the iron available for erythropoiesis. Combination therapy with fresh frozen plasma for 6 weeks to replenish the blood ceruloplasmin levels and, thereafter, administration of deferoxamine for an additional 6 weeks to deplete ferric iron stores showed unprecedented improvement in neurological symptoms.[81] Deferiprone, which has a lower molecular weight and more lipophilic properties, had no beneficial effects in a patient.[21] Deferasirox, an oral iron-chelating agent, did not lead to any improvement in the neurological symptoms or brain iron accumulation quantified by MRI,[82,83] while deferasirox therapy has been reported to lead to mild improvement in neurological symptoms, including cognitive performance, gait, and balance in an aceruloplasminemia patient who had no response to both desferrioxamine and fresh–frozen plasma therapy.[84] Short-term iron chelation therapy is therefore effective for reducing the hepatic iron overload and improving the diabetic mellitus, but is ineffective for the treatment of neurological symptoms due to brain iron accumulation. In many reports of single cases, the side effects of the iron chelation therapy prohibited the long-term treatment that may be required to mobilize iron from the brain. However, it seems rational to suggest that the therapy should be initiated early in the course of aceruloplasminemia in order to remove the iron before it induces neurodegeneration.

In comparison with iron chelation therapy, oral zinc sulfate therapy (administered for 1.5 years) led to dramatic neurological improvement in a patient with extrapyramidal and cerebellar-mediated movement disorder caused by a heterozygous mutation in the ceruloplasmin gene.[85] Although the patient was bedridden before the zinc treatment, she was able to stand for a short time and walk a few steps after undergoing this treatment. The antioxidant properties of zinc, as well as its effects on iron absorption, are well established.[86,87] While the mechanisms of antioxidation are not fully understood, the induction of metallothionein synthesis is considered to be one relevant aspect. The zinc therapy could be used as an alternative treatment when iron-chelation therapy is discontinued due to side effects or progression of the symptoms, because the zinc therapy shows no side effects and may ameliorate the neurological symptoms in aceruloplasminemia patients.

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Chapter 28
IP_3 Receptors in Neurodegenerative Disorders: Spinocerebellar Ataxias and Huntington's and Alzheimer's Diseases

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Abstract Modulation of intracellular calcium concentration is a ubiquitous signaling system involved in numerous biological processes in diverse cell types. Alterations of intracellular calcium homeostasis have been implicated in age-related neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and spinocerebellar ataxias (SCAs). Inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs), calcium release channels in the ER membrane, play a key role in regulating intracellular calcium concentration. IP_3R type 1 (IP_3R1), a major neuronal type of IR_3R, is expressed ubiquitously and is involved in diverse biological processes. Cerebellar Purkinje cells are mainly affected by alterations in IP_3R1. Heterozygous deletion or missense mutations in *ITPR1,* the *IP3R1* gene, result in autosomal dominantly inherited ataxias, including SCA type 15 or 29. In addition, mutations in carbonic anhydrase-related protein VIII, which suppresses the binding ability of IP_3 to IP_3R1, cause recessively, inherited ataxia. These results indicate that IP_3R1-mediated calcium signaling has an important role in maintaining the function of Purkinje cells. Moreover, cytosolic calcium overload with excessive IP_3R1 activity has been implicated in pathogenesis of other neurodegenerative diseases, including SCA type 2, SCA type 3, Huntington's disease, and Alzheimer's disease, where dysregulation of IP_3R1-mediated calcium signaling may link to the pathogenesis.

Abbreviations (alphabetical)

AD	Alzheimer's disease
Aβ	Amyloid β

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AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATXN2	Ataxin-2
ATXN3	Ataxin-3
CAMRQ3	Cerebellar ataxia and mental retardation with or without quadrupedal locomotion 3
CARP	Carbonic anhydrase-related protein VIII
ER	Endoplasmic reticulum
HAP1	Htt-associated protein1
HD	Huntington's disease
Htt	Huntingtin
mHtt	Mutant huntingtin
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	IP_3 receptor
IP_3R1	Inositol 1,4,5-trisphosphate receptor type 1
LTD	Long-term depression
mGluR	Metabotropic glutamate receptors
MSN	Medium spiny neuron
nAChR	Nicotinic acetylcholine receptor
NCX	Sodium-calcium exchanger
NMDA	*N*-methyl-D-aspartic acid
PMCA	Plasma membrane calcium ATPase
PS	Presenilin
RyR	Ryanodine receptor
SCA	Spinocerebellar ataxia
SERCA	Sarco-/endoplasmic reticulum calcium ATPase
SUMF1	Sulfatase modifying factor 1
VGCC	Voltage-gated calcium channel

28.1 Introduction

Modulation of cytoplasmic free calcium (Ca^{2+}) concentration is a universal intracellular signaling system involved in numerous biological processes, including learning and memory, membrane transport, cell excitability, synaptic transmission, axonal transport, cell division, apoptosis, and cell development, in diverse cell types (Foskett et al. 2007; Bezprozvanny 2010; Finch et al. 2012; Stutzmann and Mattson 2011; Goto and Mikoshiba 2011). Inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) form a group of Ca^{2+} release channels localized in the endoplasmic reticulum (ER) membrane (Foskett et al. 2007; Bezprozvanny 2005). They function to release Ca^{2+} from ER, the major Ca^{2+} storage organelle, into the cytoplasm in response to IP_3, an intracellular second messenger, which is