Cellular and subcellular localizations of nonheme ferric and ferrous iron in the rat brain: a light and electron microscopic study by the perfusion-Perls and -Turnbull methods

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Summary. Iron in the brain is utilized for cellular respiration, neurotransmitter synthesis/degradation, and myelin formation. Iron, especially its ferrous form, also has the potential for catalyzing the Fenton reaction to generate highly cytotoxic hydroxyl radicals. The amount of iron in the brain must therefore be strictly controlled. In this study, we focused on the cellular and subcellular localizations of nonheme ferric (Fe(III)) and ferrous (Fe(II)) iron in the adult female rat brain using light and electron microscopic histochemistry. Although Fe(II) deposition was much less dominant than Fe(III), the brain contained iron in both forms. Among the cellular elements of the brain, oligodendrocytes were numerically the most prominent and heavily iron-storing cells. Pericapillary astrocytes and sporadic microglial cells also showed dense iron accumulation. Large neurons involved in the motor system were relatively strongly iron-positive. Subcellularly, Fe(III) and Fe(II) were mainly localized in lysosomes, and occasionally in the cytosol and mitochondria.

Furthermore, capillary endothelial cells had Fe(III)-positive reactions in lysosomes and the cytosol, with Fe(II)-positive reactions on the luminal membrane. With advancing age, both Fe(III) and Fe(II) became more extensively distributed and accumulated more numerously in oligodendrocytes and astrocytes. These findings suggest that age-related increases in Fe(II) accumulation may raise the risk of tissue damage in the normal brain.

Introduction

Iron is present at the active site of the molecules that play crucial roles in biological functions such as oxygen transport, electron transfer, the neurotransmitter metabolism, and DNA synthesis. However, iron generates highly reactive and cytotoxic oxygen species such as superoxide (O₂⁻) and hydroxyl radical (OH⁻) through iron-catalyzed Haber-Weiss and Fenton reactions (reviewed by Halliwell and Gutteridge, 2007). OH⁻ damages biologically important macromolecules (e.g. membrane lipids, DNA, and proteins) at the site of its generation. Therefore, the localization and increased accumulation of iron species which can release catalytic iron are critical for the development of tissue injury.

Indeed, the iron-catalyzed OH⁻ generation and peroxidation of cellular components are believed to be the primary cause of neuronal cell death in various neurodegenerative diseases, e.g. Parkinson’s disease and Alzheimer’s disease (Schipper, 2004). Excessive nonheme iron accumulation and/or changes in the redox state of iron have been reported in such conditions (Kress et al.,...
Nonheme iron species such as iron-binding transport proteins (transferrin and lactoferrin), iron storage proteins (ferritin and hemosiderin), iron-sulfur proteins, and low molecular weight iron (nonprotein-bound iron) are prone to release catalytic iron (Symons and Gutteridge, 1998). Senescent or defective macromolecules and organelles are autophagocytosed by the cellular degrading system, and indigestible substances including nonheme iron progressively accumulate in long-lived post-mitotic cells such as neurons and cardiac muscle cells. Excretion routes for iron from the brain are still unknown. Therefore, to decrease the risk of iron-mediated cellular injury, the therapeutic control of excess iron accumulation in the brain is required. In this concern, a knowledge of the cellular and subcellular localization and redox state of nonheme iron would be highly useful for the development, assessment, and improvement of target-oriented neurotherapeutics.

Thus, in the present study, we histochemically investigated the brain nonheme ferric (Fe(III)) and ferrous (Fe(II)) iron depositions with a principal focus on the cellular and subcellular localizations because previous studies had never provided detailed data regarding the subcellular localization of nonheme Fe(III) and Fe(II) in various types of brain cells. In this study we took advantage of the highly sensitive perfusion-Perls and -Turnbull methods (Yu et al., 2001; Meguro et al., 2003, 2007) which enabled the production of insoluble nonheme Fe(III) and Fe(II) compounds (Prussian blue and Turnbull blue) in vivo, thereby preventing the inevitable loss of loosely bound nonheme Fe(III) and Fe(II) and the oxidation of Fe(II) during tissue treatments in the traditional Perls and Turnbull staining.

Materials and Methods

All animal experiments in this study were approved by the Animal Research Committee, Hirosaki University, and strictly adhered to the Guidelines for Animal Experimentation, Hirosaki University. Young-adult female Wistar rats (4–6 months old, 200–300 g) and middle-aged female Wistar rats (14–15 months old, 350–400 g) were supplied from the Institute of Animal Experimentation, Hirosaki University School of Medicine.

Animal treatments

The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). After thoracotomy, a thin plastic tube was inserted into the ascending aorta from an incision in the left ventricle, and the left auricle was cut. At a rate of 20 ml/min, each animal was perfused with a heparinized 0.005M phosphate buffer containing 0.9% NaCl (0.005 M PBS) (100 ml, pH 7.4) followed by a fixative (pre-fixative) containing 1% glutaraldehyde (GA) and 4% paraformaldehyde (PA) in 0.005 M PBS (100 ml, pH 7.4). The animals were then perfused with a conventional fixative (1% GA and 4% PA in 0.005 M PBS; 800 ml, pH 7.4), the Perls fixative (800 ml, pH 0.8–1.0) for the perfusion-Perls method, or the Turnbull fixative (800 ml, pH 0.8–1.0) for the perfusion-Turnbull method, as described below (Meguro et al., 2003, 2007). Before brain removal, the animals were perfused with saline (0.9% NaCl in distilled water) to flush out the excess Perls or Turnbull fixatives. Then the tissues were postfixed with 0.5% GA in 0.005 M PBS (pH 5.5–6.0), or 0.5% GA in saline.

The fixatives for the perfusion-Perls and -Turnbull methods to visualize Fe(III) and Fe(II)

The Perls fixative (pH 0.8–1.0) contained 1% potassium ferrocyanide and 4% PA in saline. The Turnbull fixative (pH 0.8–1.0) consisted of 1% potassium ferricyanide and 4% PA in saline. Immediately before perfusion, the desired pH of the fixative was obtained by dropping concentrated HCl.

The section-Perls method for the visualization of Fe(III)

The microslicer sections in the conventionally fixed brains were postfixed with 1% GA in 0.005 M PBS for 10 min and then treated with the Perls solution (pH 0.8–1.0, adjusted by dropping HCl) consisting of 1% potassium ferrocyanide and saline for 30 min. The sections were washed with saline.

Tissue treatments for light and electron microscopy

The brains fixed by the conventional, perfusion-Perls, or -Turnbull methods were sliced at a 40 μm thickness in 0.005 M PBS on a microslicer (DTK-1000, Dosaka, Japan). The sections were washed with saline. Then the sections with conventional fixation were treated by the section-Perls method as above.

The sections stained by the section-Perls, perfusion-Perls, or perfusion-Turnbull method were treated with 0.3% H₂O₂ and 0.065% sodium azide (NaN₃) in 0.005M PBS for 15 min, washed with 0.005 M PBS for 5 min, pretreated with 0.025% 3,3’-diaminobenzenidine-HCl (DAB, Sigma, USA) in a 0.01 M phosphate buffer.
containing 0.9% NaCl (0.01 M PBS) (DAB solution) or 0.04% nickel and 0.025% DAB in 0.01 M PBS (Ni-DAB solution) for 10 min, and then treated with DAB or Ni-DAB solution containing 0.005% H₂O₂ for 20 min. After washing with 0.01 M PBS, those sections were processed for light or electron microscopy as described below. For the DAB controls, the conventionally fixed sections were treated with saline (pH 0.8 - 1.0) for 30 min, 0.3% H₂O₂ and 0.065% NaN₃ in 0.005 M PBS, and then with DAB solution containing 0.005% H₂O₂.

The sections for light microscopy were dehydrated with graded ethanol, cleaned with xylene, and coverslipped. For electron microscopy, the sections were postfixed with 1% osmium-tetroxide in 0.1 M PB 60 min, washed with distilled water, dehydrated with a graded ethanol series, treated with propylene oxide and Epon mixture, and embedded in Epon mixture for more than 24 h at 60°C. Ultrathin sections (80-100 nm thickness) were made on an ultramicrotome (ULTRACUT S, Reichert-nissei, Austria) and observed under an electron microscope (H-600, Hitachi, Japan). All ultrathin sections were observed without electron staining.

Results

Neurons and each type of glia were morphologically identified by light and electron microscopy according to the standard criteria in the literature (Pannese, 1994; Kettenmann and Ransom, 1995). Both Fe(III)- and Fe(II)-positive reactions supplemented by DAB (Fig. 1) or Ni-DAB (Fig. 2, 8) intensification were observed as brown to black staining by light microscopy and as amorphous deposits of high electron density by electron microscopy (Fig. 3–7).

Regional distribution of Fe(III) and Fe(II) in the rat brain

The Fe(III)- and Fe(II)-positive reactions were observed in comparable brain regions (Fig. 1, Table 1). The Fe(II)-positive reaction was much less intense than the Fe(III)-positive reaction in the young-adult brain (Fig. 1a, b). The aged rat brain was more strongly stained for Fe(III) and Fe(II) (Fig. 1c, d). The iron positive reaction visualized by DAB intensification appeared brown, whereas the DAB control was scarcely stained except for some red blood cells (Fig. 1e, f).

As shown in Table 1, particularly strong Fe(III)- and Fe(II)-positive reactions were found in the olfactory bulb, basal ganglia, amygdaloid body, hypothalamus, cerebellar nuclei, and many brain stem structures.

Cellular and subcellular distribution of Fe (III) and Fe (II)

In both the Fe(III)- and Fe(II)-positive reactions, glial cells, especially oligodendrocytes, were most prominent in staining intensity and number. In contrast, most neurons were only palely stained—except for some moderately Fe(III)-positive neurons in certain brain regions as described below.

1) Neurons

Both the Fe(III) and Fe(II)-positive reactions were considerably weak in neurons compared to those of glial cells. Fe(II)-positive neurons were far fewer than Fe(III)-positive ones. By light microscopy, Fe(III)- and Fe(II)-positive neurons were observed in the cerebellar nuclei and cortex (Purkinje cells), globus pallidus, red nucleus, trigeminal motor nucleus, facial nucleus, vestibular nuclei, cochlear nuclei, and caudal pontine and medullary reticular formation. The Fe(III)- and Fe(II)-positive reactions were mainly observed in cytoplasmic small granules (Fig. 2b, i). A small number of neurons also exhibited Fe(III) in the cell nuclei. Purkinje cells and large neurons in the lateral vestibular nucleus and caudate pontine and medullary reticular formation showed homogenous Fe(III)- and Fe(II)-staining of the cytoplasm (Fig. 2a, h, i). In addition, thick axons of the white matter were occasionally positively stained for Fe(III).

By electron microscopy, Fe(III)-positive reaction was localized in lysosomes and mitochondria (Fig. 3a, b, e, f), which corresponded to Fe(III)-positive small granules by light microscopy. The electron density of those iron deposits was stronger than that in the DAB controls (Fig. 3d, h). The Fe(II)-positive reaction was also seen in lysosomes (Fig. 3c, g), strongly suggesting that they contain nonheme iron in both Fe(III) and Fe(II) forms. However, both Fe(III) and Fe(II)-positive reactions were scarcely observed in the neuronal cytosol. In axons and dendrites, a small number of mitochondria were positively stained for Fe(III) (Fig. 3i), but not for Fe(II).

2) Astrocytes

Fe(III)- and Fe(II)-positive protoplasmic astrocytes (Fig. 2c, j) were sparsely found in the gray matter, where pericapillary astrocytes were darkly stained. However, the other astrocytes did not stain for Fe(III) and Fe(II) even though they were in the close vicinity of the positive astrocytes or capillaries. Fibrous astrocytes (Fig. 2d, k) were the major cell type stained for Fe(III) and Fe(II) in the white matter.
By light microscopy, Fe(III)- and Fe(II)-positive reactions in pericapillary astrocytes were found homogeneously and/or in the small granules scattering in the cell body and projections—including the capillary end-feet (Fig. 2c, d, j, k). In the Fe(II)-positive reaction, fibrous astrocytes were more strongly stained than protoplasmic astrocytes (Fig. 2j, k).

By electron microscopy, protoplasmic astrocytes (Fig. 4a, b) showed the Fe(III)-positive reaction primarily in lysosomes (Fig. 4d - f) and occasionally along the mitochondrial membrane (Fig. 4d, e) and nucleus (Fig. 4f). Electron-dense Fe(III)-deposits were present in several astrocytic cytosols (Fig. 4e), the staining pattern of which apparently corresponding to light microscopically homogeneous Fe(III)-staining in the cytoplasm of astrocytes. However, the deposits were hardly seen in the cytosol of most astrocytes. This is probably because the reaction was too weak to be visualized by electron microscopy. The Fe(II)-positive reaction was also found in lysosomes, the nucleus, and the cytosol (Fig. 4g, j) of the protoplasmic astrocytes.

Fibrous astrocytes, characterized by the long, thin, and relatively straight processes, also showed the Fe(III)-positive reaction, but their electron density was stronger than that in protoplasmic astrocytes (Fig 4c, h). Fibrous astrocytes also displayed both Fe(III)- and Fe(II)-positive reactions in lysosomes, the nucleus, and the cytosol (Fig. 4c, k).

The astrocytic projections and end-feet surrounding the capillary wall were also Fe(III)- and Fe(II)-positive in lysosomes and the cytosol (Fig. 4j, 7b, 7f).

| Table 1. Regional distribution of Fe(III) and Fe(II) in the rat brain. The intensities are from very weak (+) to strongest labeling (++++). |
|----------------|----------------|
| **Forebrain** |
| Olfactory bulb, ventral hypothalamus, amygdaloid body | ++++ | +++ |
| Thalamus, medial and lateral geniculate nuclei | ++ | ++ |
| Cerebral cortex, hippocampus | ++~+ | + |
| Subcortical white matter, corpus callosum, internal capsule | ++ | + |
| Globus pallidus, ventral pallidum, substantia nigra pars reticulata | ++++ | ++ |
| Corpus striatum, substantia nigra pars compacta | ++ | + |
| **Midbrain** |
| Inferior colliculus, interpeduncular nucleus, dorsal tegmental nucleus | ++++ | +++ |
| Superior colliculus | +++ | ++ |
| Red nucleus | + | + |
| **Pons, medulla oblongata and cerebellum** |
| Nucleus of the lateral lemniscus, superior olivary nucleus, inferior olivary nucleus, cranial nerve nuclei (V, VII, VIII), cerebellar nuclei | ++++ | +++ |
| Trapezoid body, brain stem reticular formation, solitary, gracile and cuneate nuclei, cerebellar cortex, circumventricular organs, area postrema | +++ | ++ |
| Cerebellar white matter, pyramidal tract | ++~+ | + |
**Fig. 1.** The distribution of Fe(III) (a, c) and Fe(II) (b, d) visualized by the perfusion-Perls and perfusion-Turnbull methods with DAB intensification, respectively, in the rat brain (sagittal sections). An iron-positive reaction is shown by brown deposit. The brains were from 5-month-old (a, b) and 14-month-old (c–e) rats. The DAB control section was almost unstained with DAB; whole image (e) and vestibular nucleus (f). Acb: accumbens nucleus, ac: anterior commissure, CBC: cerebellar cortex, CBM: cerebellar medulla, CBN: cerebellar nucleus, CC: cerebral cortex, cc: corpus callosum, CPu: caudate putamen (striatum), GP: globus pallidus, HT: hypothalamus, IC: inferior colliculus, O: olfactory bulb, Pn: pontine nucleus, Rt: brain stem reticular formation, SC: superior colliculus, SNR: substantia nigra pars reticulata, SO: superior olivary nucleus, Sp5: spinal trigeminal nucleus, T: thalamus, VP: ventral pallidum, 7N: facial nucleus, 8N: vestibular nucleus. Bars = 5 mm (a–e), 100 μm (f)
Fig. 2. Different cell types of the rat brain treated by Fe(III) (a–g) and Fe(II) (h–n) histochemistry. An iron-positive reaction is identified as a brown or dark brown deposit. Thick arrows and asterisks indicate stained cells and capillaries, respectively; neuron in the pontine reticular nucleus (a, h), neuron in the facial nucleus (b, i), protoplasmic astrocyte in the inferior colliculus (c, j), fibrous astrocyte in the medial lemniscus (d, k), oligodendrocyte in the globus pallidus (e, l), microglia in the cerebellar cortex (f, m), capillary in the globus pallidus (g, n). Thin arrows indicate the glial projections. A: astrocyte, O: oligodendrocyte. Bar = 50 μm
Fig. 3. Subcellular localization of Fe(III) (a, b, e, f, i) and Fe(II) (c, g) in neurons of the rat brain. a, c, d: Neurons in the brain stem reticular formation; b: Purkinje cells in the cerebellum; i: myelinated axons in the white matter. e–h: Higher magnifications of a–d. Thick black arrows: iron-positive lysosomes. thin black arrows: lysosomes in DAN control, white arrows: iron-positive mitochondria. Bars = 1 μm (e–h), 2 μm (i), 5 μm (a–d)
Fig. 4. Legend on the opposite page.
Fig. 4. Subcellular localization of Fe(III) (a–f) and Fe(II) (g–i) in astrocytes of the rat brain. High magnifications d, e, i are from a, b, h. An iron-positive reaction is found in lysosomes (thick black arrows), mitochondria (white arrows), endoplasmic reticulum (thin black arrows), cytoplasm, perinuclear cytoplasm, and nuclei (N). f: Arrowheads indicate Fe(III)-positive fragments on the inner and outer surface of the myelin sheath. Bars = 2 μm (a–c, g, h), 1 μm (d–f, i)

Fig. 5. Subcellular localization of Fe(III) (a–f) and Fe(II) (g–i) in oligodendrocytes of the rat brain. High magnifications d, e, i are from a, b, h. An iron-positive reaction is found in lysosomes (thick black arrows), mitochondria (white arrows), endoplasmic reticulum (thin black arrows), cytoplasm, perinuclear cytoplasm, and nuclei (N). f: Arrowheads indicate Fe(III)-positive fragments on the inner and outer surface of the myelin sheath. Bars = 2 μm (a–c, g, h), 1 μm (d–f, i)
3) Oligodendrocytes
By light microscopy, numerous Fe(III)- and Fe(II)-positive oligodendrocytes were distributed in the gray matter. Oligodendrocytes showed homogeneous Fe(III)- and Fe(II)-positive reactions in their cytoplasm with a small number of Fe(III)- and Fe(II)-positive particles (Fig. 2e, l)—although the Fe(II)-positive reaction in oligodendrocytes was less intense than the Fe(III)-positive reaction. The processes of oligodendrocytes densely filled with the Fe(II)-positive reaction extended to the myelinated nerve fiber bundles in the corpus striatum, substantia nigra pars reticulata, and corpus callosum (Fig. 2e).

By electron microscopy, oligodendrocytes showed Fe(III)-positive reactions in lysosomes (Fig 5d, e), occasionally in mitochondria (Fig. 5e) and the endoplasmic reticulum (Fig. 5d, e), and in fine particles scattered throughout the cytosol (Fig 5a, b, d, e). Small to medium-sized oligodendrocytes densely filled with the Fe(II)-positive reaction extended to the myelinated nerve fiber bundles in the corpus striatum, substantia nigra pars reticulata, and corpus callosum (Fig. 2e).

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4) Microglia
By light microscopy, a small number of microglial cells were heavily stained for Fe(III) and Fe(II), and they were scattered solitarily and sporadically. The cell body and blanched processes of the se heavily-labeled ramified microglia were filled with reaction products (Fig. 2f, m), and the shapes of these cells were characteristic as shown in the normal, nonpathological brain (Kettenmann and Ransom, 1995). However, most of the other microglia showed light to moderate Fe(III)- and Fe(II)-reactions, and sometimes they were not labeled (not shown).

By electron microscopy, heavily Fe(III)- and Fe(II)-laden, ramified, and phagocytic microglial cells had highly electron-dense reaction deposits that filled the cytosol and cytoplasmic compartments (Fig 6a, b), except for the nucleus and engulfed tissue debris. Lightly to moderately stained microglia revealed by light microscopy contained only a few Fe(III)- and Fe(II)-positive lysosomes (Fig. 6c, d). Pericapillary microglia in the very close vicinity of the capillary wall occasionally had a Fe(III)-positive nucleus.

5) Capillary endothelial cells and pericytes
Light microscopy, revealed many capillary walls positively stained for Fe(III) and Fe(II) in various brain regions. The Fe(III)-positive reaction was observed as a thick and fuzzy line-shape (Fig. 2g), whereas the Fe(II)-positive reaction was a sharp and thin line-shape (Fig. 2n).

Electron microscopy showed iron-positive sources of
6) Specific cell types of circumscribed areas

Strongly Fe(III)- and Fe(II)-positive reactions were observed in Bergmann glia in the cerebellar cortex (Fig. 8a), round and swollen astrocytes in the mammillary body, arcuate nuclei and tuber cinereum of the ventral hypothalamus (Fig. 8c), subpial radial astrocytes in the supraoptic nucleus, optic chiasm, and glial limiting membrane of the ventral brain stem (Fig. 8d). Positive reactions were also found in radial glia-like cells which ventrodorsally gave off long, thick projections in the hypoglossal nucleus and the ventromedial central gray matter of the medulla oblongata (Fig. 8e), and tanyocytes which were found in the walls of the third and fourth ventricles, optic chiasm, area postrema and central canal and extended long, thin projections into the parenchyma (Fig. 8c, f, g). By electron microscopy, Fe(III)- and Fe(II)-deposits in those cells were found primarily in lysosomes and the cytosol and occasionally in the mitochondrial outer membrane and the endoplasmic...
reticulum (not shown). One exception was Bergmann glia in the caudal-ventral cerebellar cortex (10th lobule and copula pyramis of the cerebellar cortex; e: round- and swollen-shape glia (right-arrow) and tanycyte (down-arrow) in the ventral hypothalamus (lower portion); f: subpial radial astrocyte in the glial limiting membrane of the ventral brain stem; e: radial glia-like cell in the ventromedial central gray matter and hypoglossal nucleus; f: tanycytes in the area postrema; g: tanycytes (down-arrows) and ependymal cells (up-arrows) in the central canal of the medulla oblongata; h: ependymal cells in the fourth ventricle (4V); i: pial cell in the pia mater of the cerebellum. Gliofilaments characterizing astrocytes were observed in the above site-specific astrocytes by electron microscopy. Arrows indicate Fe(III)-positive cells and their projections. cc: central canal, gcl: granular cell layer of the cerebellar cortex, ml: molecular layer of the cerebellar cortex. Bar = 100 μm (a–i)

Fig. 8. Specific types of glial cells stained for Fe(III) histochemistry in the rat brain. a: Bergmann glia in the large area of cerebellar cortex; b: Bergmann glia in the lateral part of the tenth cerebellar lobule and copula pyramis of the cerebellar cortex; c: round- and swollen-shape glia (right-arrow) and tanycyte (down-arrow) in the ventral hypothalamus (lower portion); d: subpial radial astrocyte in the glial limiting membrane of the ventral brain stem; e: radial glia-like cell in the ventromedial central gray matter and hypoglossal nucleus; f: tanycytes in the area postrema; g: tanycytes (down-arrows) and ependymal cells (up-arrows) in the central canal of the medulla oblongata; h: ependymal cells in the fourth ventricle (4V); i: pial cell in the pia mater of the cerebellum. Gliofilaments characterizing astrocytes were observed in the above site-specific astrocytes by electron microscopy. Arrows indicate Fe(III)-positive cells and their projections. cc: central canal, gcl: granular cell layer of the cerebellar cortex, ml: molecular layer of the cerebellar cortex. Bar = 100 μm (a–i)

Fe(III)- and Fe(II)-positive ependymal cells (Fig. 8g, h) and pial cells (Fig. 8i) were distributed in a mosaic pattern throughout the ependymal layer and pia mater, and the subcellular iron-deposits were localized in lysosomes.
There were distinct differences between the young-adult brain (5-month-old) (Fig. 1a, b) and middle-aged brain (14-month-old) (Fig. 1c, d) in the extent and intensity of Fe(III)- and Fe(II)-positive reactions. Various brain regions in both of the gray and white matters notably showed increased reactions in the older brain (Fig. 1c, d). Fe(III)- and Fe(II)-positive glial cells were more numerous and more extensively distributed in the older brain than in the younger brain. Iron-positive oligodendrocytes were markedly increased in number in the older rats. The subcellular localization and intensity of Fe(III)- and Fe(II)-positive reactions in each type of glia were almost comparable in young and older brains, whereas neurons increased in positive lysosomes or lipofuscin granules.

Discussion

Fe(III) and Fe(II), are extensively distributed throughout the brain, are especially highly concentrated in the diencephalon, basal ganglia, brain stem, and cerebellum, similar to previous observations for Fe(III) in various mammalian and human brains (Hill and Switzer, 1984; Connor et al., 1990; Morris et al., 1992; Gilissen et al., 1998).

In the present study, we demonstrated the precise cellular and subcellular localizations of nonheme Fe(III) and Fe(II) in the rat brain by the perfusion-Perls, perfusion-Turnbull, and section-Perls methods. The results demonstrated the colocalization of nonheme Fe(II) and Fe(III) in lysosomes and the cytosol of various neurons and glial cells (Fig. 9).
Cellular and subcellular localization of nonheme Fe(III) and Fe(II)

1) Neurons
Because nonheme iron is acquired from the interstitial fluid or retrieved from degraded iron-binding substances, post-mitotic neurons ought to sequestrate excess Fe (III) and Fe (II) into lysosomes occasionally in association with lipofuscin granules to keep cytosolic labile iron at the minimum level. The present results showed that neurons successfully treated nonheme iron following this strategy, in that, contrary to glial cells, most neurons showed iron-deposits in lysosomes only. However, some large neurons involved in the motor system were intensely stained for Fe(III) and Fe(II) throughout the cytoplasm. Leibold et al. (2001) demonstrated that neurons of the motor nuclei highly expressed iron regulatory proteins that coordinate the cytosolic ferritin level. It appears that neurons with a large cell body or a long axon continually require iron to maintain their high metabolic activities and therefore contain a relatively large amount of iron in the cytosol that is histochemically detectable.

Rouault and Cooperman (2006) reported that ferritin is present in the axons of normal neurons in the brain, implying that there may be a trafficking mechanism that permits ferritin to carry iron from the neuronal cell body to the synapse. Rouault (2001) also suggested that ferritin could be degenerated within lysosomes and that Fe(II) is released in distal axons—although we rarely observed iron-positive lysosomes in axons. Instead, we found iron-positive mitochondria in axons as well as in neuronal cell bodies. The mitochondrion is the major site of heme synthesis and contains nonheme iron-protein complexes including ferritin, aconitase (iron-sulfur enzyme), and ferredoxin (iron sulfur protein) (Napier et al., 2005; Meguro et al., 2007). Therefore, mitochondria would be expected to have a putative function as an iron transporter to axon terminals. In addition, ferroportin (an iron exporter) is present on synaptic vesicles, suggesting that Fe(II) may be released into the synaptic cleft (Wu et al., 2004).

2) Astrocytes
Astrocytes, considered to be important regulators for iron import into the brain (Moos et al., 2006), have iron transporters such as ferroportin for iron excretion into the interstitial fluid (Zoller et al., 2002; Dringen et al., 2007). We observed pericapillary astrocytes containing nonheme Fe(III) and Fe(II) in lysosomes and the cytosol. The lysosomal iron is probably in the form of degraded nonheme iron, and the cytosolic iron is probably in the form of a redox-active low molecular weight iron pool. In some white matter regions, pericapillary fibrous astrocytes were more intensely stained for iron than oligodendrocytes. These astrocytes probably sequestrate iron to protect myelinated nerve fibers against oxidative stress, in addition to the regulation of iron uptake and transport into the parenchyma. Astrocytes are the major source of ceruloplasmin, a ferroxidase which effectively inhibits Fe(II)-mediated OH· formation and lipid peroxidation (Vassiliev et al., 2005; Oide et al., 2006).

3) Oligodendrocytes
Oligodendrocytes synthesize transferrin and ferritin and use iron as a cofactor for the synthesis of cholesterol and lipids, which are required for myelination and the maintenance of myelin (reviewed in Connor and Menzies, 1996). The present results demonstrated that oligodendrocytes stored nonheme Fe(III) and Fe(II) in lysosomes and the cytosol, and Fe(III) in the endoplasmic reticulum. Transferrin was found in the cytosol and bound to the endoplasmic reticulum in oligodendrocytes (Cho et al., 1997), and an iron regulatory protein was found on the membranes of the endoplasmic reticulum and Golgi apparatus (Patton et al., 2005).

Considering that the well known function of oligodendrocytes is myelin formation, it is interesting that iron-positive oligodendrocytes were more numerous and more intensely stained for both Fe(III) and Fe(II) in the gray matter than in the white matter. This suggested that the iron stored in oligodendrocytes in mature animals was significantly important not only for myelin synthesis and maintenance but also for the iron-requiring metabolism and functions of neurons and glia in the gray matter. Correspondingly, it is noteworthy that, in the peripheral nerves of adult rats, Schwann cells were not positively stained for iron while mantle cells in the sensory ganglion were (unpublished data).

In addition, we observed Fe(III) accumulation in the inner and outer collars of the myelin sheath, particularly in the region where oligodendrocytes were intensely stained for iron. Iron is probably mobilized from the cell body of oligodendrocytes to the inner portion of the myelin sheath. It is also possible that iron in the myelin sheath is concerned with delayed myelination or remyelination in the central nervous system. Quintana et al. (2006) observed ferritin accumulation in such myelin-associated processes of oligodendrocytes in the human brain with Alzheimer's disease.

4) Microglia
Microglial cells are immunopositive to ferritin and transferrin in the adult rat brain (Benkovic and Connor, 1993). In the developing brain, microglia provides ferritin...
as a trophic factor to oligodendrocytes for myelination (Zhang et al., 2006). In our results, in sporadic occurrence throughouts the brain, a small number of resting microglia and phagocytic microglia were heavily filled with Fe(III)- and Fe(II)-deposits throughout the cell body and processes where lysosomes and the cytosol were strongly stained. The reason why these microglial cells contain abundant iron is unclear, but they seem to be specialized for iron sequestration to prevent iron-toxicity (Benkovic and Connor, 1993) and possibly act as a source of iron supply for local iron shortage.

5) Capillary endothelial cells
Blood iron crosses the capillary endothelial cell membrane via the transferrin, lactoferrin, or possibly ferritin receptors (Fisher et al., 2006; Moos et al., 2007). Interestingly, we observed Fe(II)- but not Fe(III)-deposits along the luminal surface of endothelial cells. This suggested that Fe(III) in the blood was reduced to its ferrous form on the endothelial luminal surface, possibly under the expression of some ferric reductases located near the Fe(II)-transporter (e.g. DMT1), and then transported into the cytosol to be used for cellular metabolisms.

Alternatively, it seemed possible that ferritin Fe (III) in the cytoplasm of endothelial cells was reduced to Fe(II) by nitric oxide (NO), the endothelium-derived relaxing factor (Palmer et al., 1987), released from the endothelial cell—because NO can reductively bind to Fe(III). Moreover, the nonheme Fe(III)- or Fe(II)-NO complex may be present in the low molecular weight iron pool near the endothelial luminal membrane for the donation of NO (Cooper, 1999; Alencar et al., 2003; Vlasova et al., 2003).

6) Specific cell types
The bergmann glia probably expresses transferrin to regulate iron delivery to Purkinje cells (Cho 1998). Our results suggest that the Bergmann glia executes an active iron uptake and iron metabolism, similar to pericapillary astrocytes. Interestingly, however, the Bergmann glia in the cerebellar cortex facing the fourth ventricle accumulated iron almost exclusively in lysosomes. Thus, the Bergmann glia in that region might have a different iron metabolism or additional roles.

Tanyocytes in the lateral and third ventricular walls and radial glia-like cells in the fourth ventricular wall and area postrema may be morphologically and functionally comparable to immature radial glia in the developing brain (Spassky et al., 2005; Borrell et al., 2006; Pecchi et al., 2007). In the present study, a large number of radial glia-like cells were strongly iron positive in the caudal medulla oblongata. Although neurogenesis in the adult brain may be quite infrequent, the rich iron accumulation in the radial glia-like cells is required for a possible neurogenesis, in which the ribonucleotide reductase, a nonheme iron enzyme, is essential for DNA synthesis. Furthermore, their localization was closely associated with capillaries, suggesting that they regulate the uptake of iron and nutrients from blood. Tanyocytes were strongly stained for both Fe(III) and Fe(II), in agreement with previous findings that they contain ferritin and low-molecular weight iron (Benkovic and Connor 1993; Dickinson and Connor, 1995). It has been suggested that they participate in the transport of iron from the CSF into the parenchyma (Burdo JR 1999); the CSF contains iron in the form of transferrin, ascorbate, citrate, and an iron-albumin complex (Bradbury 1997).

Ependymal cells were Fe(III)- and Fe(II)-positive. Ferroportin is localized on the ventricular side of ependymal cells, suggesting that ferroportin exports iron from the brain parenchyma and circulation into the cerebro-spinal fluid (Wu et al., 2004).

Topography of nonheme Fe(III) and Fe(II) distribution in relation to those of neurotransmitter systems

The reason for the dense iron accumulation in the specific brain regions—including the globus pallidus, substantia nigra pars reticulata, and cerebellar dentate nucleus—is not known. However, these regions appear to be strongly correlated with massive GABAergic inhibitory input. The globus pallidus and substantia nigra pars reticulata receive GABAergic projections from the striatum. Similarly, the deep cerebellar nuclei receive GABAergic projections from Purkinje cells. Recent studies revealed that GABA transaminase has an iron-sulfur cluster at the center of the GABA transaminase dimer (Storici et al., 2004). This enzyme is synthesized in postsynaptic neurons and astrocytes (reviewed in Kugler, 1993) and plays an essential role in the degradation of GABA, which is released from GABAergic synaptic terminals. Moreover, the inherent high neural activity in the above brain regions may cause an increased turnover of iron-containing proteins, thereby creating a high demand for iron supply. We consider these the most likely reasons why oligodendrocytes, the major iron-storing and -donoring cells, show densely accumulated iron in the globus pallidus, substantia nigra pars reticulata, and dentate nucleus.

Besides the GABAergic system, nonheme iron has been known to be required by monoaminergic and glutamatergic systems for the production of the enzymes involved in the synthesis of norepinephrine, epinephrine, dopamine, and glutamate (Ramsey et al., 1996;
McGahan et al., 2005). However, the topography of the monoaminergic and glutamatergic neural systems did not seem to specifically match the topography of dense iron accumulation.

**Increased nonheme Fe(III) and Fe(II) accumulation with age**

Progressive iron accumulation with age in specific brain regions has long been known in humans and other mammals (Hill and Switzer, 1984; Morris et al., 1992). Recent magnetic resonance imaging (MRI) studies of the human brain also demonstrated such increased iron accumulation with age in these brain regions (Drayer et al., 1986; Xu et al., 2008).

The exact reasons for the above accumulation have not been determined. There are two predominant characteristics of iron metabolism in the brain: the first is that the brain may not have an essential system for discharging iron, and the second is that iron is an essential element for normal brain functions. Furthermore, endothelial cells appear to exhibit a decreased or impaired function of the blood-brain barrier with age-related increase of cholesterol and membrane lipid peroxidation, which would accelerate the age-related iron increase with aging (Ong et al., 2004).

Moreover, most iron accumulated into autophagosomes could not be easily reused for the cellular iron metabolism. Endogenously formed H$_2$O$_2$ easily diffuses into iron-laden autophagosomes to generate highly reactive OH$^-$ generation by the Fenton reaction and so damage the lysosomal membrane and iron-containing macromolecules, causing a further production of OH$^-$ and accumulation of iron-containing lipofuscin which deteriorates the autophagocytic capacity (Kurz et al. 2008). The increased numbers of iron-rich lysosomes and lipofuscin possibly cause increased neuronal damage with age. Bartzokis et al. (2007) showed that the increased iron accumulation level correlates with the onset of Parkinson's and Alzheimer's diseases in older humans.

Although the amount of Fe(II) should be maintained at a low level, our results unexpectedly demonstrated the parallel increase of Fe(II) with Fe(III) in the normal aged brain. This indicates that an increased risk of tissue damage is inevitable with increased amounts of iron in the brain.

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