

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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Abstract Iron is essential for a variety of cellular functions. However iron, especially ferrous form, has the potential for catalyzing the Fenton reaction to generate highly cytotoxic hydroxyl radicals. Therefore the amount of iron in the brain is 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 by 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 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. Fe(II)-specific deposit was shown on the luminal membrane of endothelium. With advancing age, both Fe(III) and Fe(II) became distributed more extensively, and accumulated more numerously into oligodendrocytes.

Hirosaki Med. J. 61, Supplement : S245—S251, 2010

Key words: nonheme iron; perfusion-Perls method; perfusion-Turnbull method

Introduction

Iron is present at the active site of the molecules that play crucial roles in biological functions such as oxygen transport, electron transfer, neurotransmitter metabolism, and DNA synthesis. However, iron generates highly reactive and cytotoxic oxygen species such as superoxide and hydroxyl radical (OH•) through iron-catalyzed Haber-Weiss and Fenton reactions¹⁾. Localization and increased accumulation of iron species which can release catalytic iron is critical for the development of tissue injury.

Indeed, iron-catalyzed OH• generation and peroxidation of cellular components is believed to be the primary cause of neuronal cell death in various neurodegenerative diseases, e.g. Parkinson's disease and Alzheimer's disease²⁾.

Excessive nonheme iron accumulation and/or change of the redox state of iron have been reported in these brains^{3,4)}. To decrease the risk of iron-mediated cellular injury, the therapeutic control of excess iron accumulation in the brain is required. In this concern, the knowledge of the cellular and subcellular localization and redox state of nonheme iron would be essential for the development, assessment and improvement of target-oriented neurotherapeutics. In the present study, we histochemically investigated the brain nonheme ferric (Fe(III)) and ferrous (Fe(II)) iron depositions with the principal focus on the cellular and subcellular localizations by the perfusion-Perls and perfusion-Turnbull methods.

Materials and methods

Animal treatments

Young-adult (5 months old) and aged (14–24 months old) Wistar rats were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Rats were perfused with a heparinized PBS, 4% paraformaldehyde (PA) and 1% glutaraldehyde (GA) in PBS, and then perfused with Perls fixative or Turnbull fixative of 800 ml, respectively^{5,6}.

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

The Perls fixative (pH 0.8) contained 1% potassium ferrocyanide and 4% PA in saline. The Turnbull fixative (pH 0.8) 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.

Tissue treatments for light and electron microscopy

The brains were sliced at 40 μ m thickness on a microslicer. The sections were washed with PBS, treated with 0.3% H₂O₂ and 0.065% NaN₃ in PBS, pretreated with 0.025% 3,3'-diaminobenzidine-HCl in PBS (DAB solution), and then treated with 0.005% H₂O₂-DAB. For electron microscopy, the sections were postfixed with 1% OsO₄ in 0.1 M PB 60 min and embedded in epon for more than 24 h at 60 °C. Ultrathin sections (80-100 nm thickness) were observed without electron staining.

Results

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

The Fe(III)- and Fe(II)-positive reactions were observed in the comparable brain regions. The Fe(II)-positive reaction was much less intense than the Fe(III)-positive reaction (Fig.1 a, b). The

aged rat brain was more strongly stained for Fe(III) and Fe(II) (Fig.1 c, d). 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. By contrast, most neurons were only palely stained except some moderately Fe(III)-positive neurons in certain brain regions as described below. The results demonstrated the colocalization of nonheme Fe(II) and Fe(III) in lysosomes and cytosol of various neurons and glial cells (Fig. 1g)

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 much less than Fe(III)-positive ones. 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. In those neurons, Fe(III)- and Fe(II)-positive reactions were localized in lysosomes and mitochondria. In axons and dendrites, a small number of mitochondria were positively stained for Fe(III), but not for Fe(II).

2) Astrocytes

Pericapillary astrocytes were darkly stained for Fe(III) and Fe(II) in the cell bodies and projections including the capillary end-feet. However, the other astrocytes did not stained for Fe(III) and Fe(II) even though they were in the close vicinity of the positive astrocytes or

capillaries. Fe(III)- and Fe(II)-positive reactions in pericapillary astrocytes were primarily found in lysosomes, and occasionally along the mitochondrial membranes (Fig. 1g). Electron-dense iron-deposits were homogeneously present in the several astrocytic cytosol. Fe(III)- and Fe(II)-positive neurons were observed in the brain stem reticular formation, cerebellar cortex and nuclei, corpus callosum, inferior and superior colliculi, interpeduncular nuclei, trapezoid body, and ventral hypothalamus.

3) Oligodendrocytes

Numerous Fe(III)- and Fe(II)-positive oligodendrocytes were distributed in the gray matter. The Fe(II)-positive reaction was less intense than the Fe(III)-positive reaction. Oligodendrocytes showed Fe(III) and Fe(II) reactions in lysosomes, occasionally in mitochondria and the endoplasmic reticulum, and in fine particles scattered throughout the cytosol (Fig. 1g). Small to medium sized oligodendrocytes showed Fe

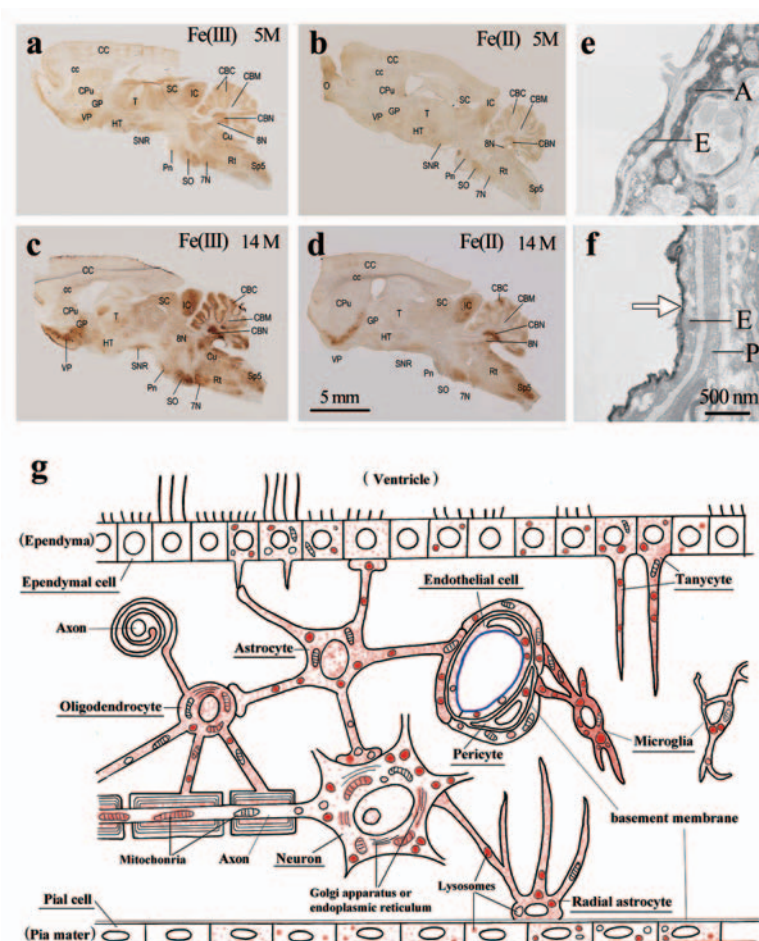


Figure 1 The distribution of nonheme Fe(III) and Fe(II) visualized by the perfusion-Perls and perfusion-Turnbull methods with DAB intensification in the rat brain. **a:** Fe(III)-positive reaction in 5-month-old brain. **b:** Fe(II)-positive reaction in 5-month-old brain. **c:** Fe(III)-positive reaction in 14-month-old brain. **d:** Fe(II)-positive reaction in 14-month-old brain. **e:** Subcellular localization of Fe(III) in capillary endothelial cell (E) and astrocyte (A). **f:** subcellular localization of Fe(II) is found on the capillary luminal membranes (white arrow). **g:** illustration of the subcellular distribution of Fe(III) and Fe(II) in the brain. Red indicates nonheme iron, mainly Fe(III), positive reaction. The red dot density is roughly proportional to the density of iron positive reaction subcellularly. Blue indicates Fe(II)-positive reaction. CBC: cerebellar cortex, CBM: cerebellar medulla, CBN: cerebellar nucleus, CC: cerebral cortex, cc: corpus callosum, CPu: caudate putamen, 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. Adapted from reference (7) with permission.

(III) and Fe(II) on the nuclear heterochromatin and the perinuclear cytoplasm. The processes of oligodendrocytes densely filled with the Fe(III) extended to the myelinated nerve fiber bundles in the corpus striatum, substantia nigra pars reticulata and corpus callosum. The myelinated nerve fiber bundles contained Fe(III)-positive cytoplasmic fragments on the inner and outer surfaces (i.e., inner and outer collars) of the myelin sheath. Dominant brain regions of Fe(III) - and Fe(II)-positive oligodendrocytes were the amygdaloid nuclear complex, brain stem reticular formation, cerebellar cortex and nuclei, caudate putamen, dorsal tegmental nucleus, globus pallidus, inferior and superior colliculi, inferior olivary nucleus, interpeduncular nuclei, olfactory bulb, pyramidal tract, substantia nigra pars reticulata, superior olivary nucleus, thalamus, ventral hypothalamus, and cranial nerve nuclei.

4) Microglia

A small number of ramified microglial cells and phagocytic cells were heavily stained for Fe(III) and Fe(II), and they were scattered solitarily and sporadically. These cells had highly

electron-dense reaction deposits that filled the cytosol and cytoplasmic compartments, except for the nucleus and engulfed tissue debris. Most of the other microglial cells were lightly stained for Fe(III) and Fe(II), containing only a few Fe(III)- and Fe(II)-positive lysosomes (Fig. 1g).

5) Capillary endothelial cells and pericytes

Many capillary walls were positively stained for Fe(III) and Fe(II) in various brain regions. Electron microscopy showed iron-positive sources of the capillary wall were endothelial cells, pericytes and/or astrocytic end-feet (Fig. 1g). Fe(III)-deposits were mainly localized in lysosomes and cytosol in the endothelial cells (Fig. 1e). Dense Fe(II)-deposits were observed along the luminal surface of endothelial cells including the cell membrane and subsurface cytosol (Fig. 1f).

Comparison of iron distribution between young-adult and middle-aged rat brains

Various brain regions in both of the gray and white matters notably showed increased the reactions in the older brain (Fig. 1a-d). Iron-

Table 1 Regional distribution of Fe(III) and Fe(II) in the rat brain. The intensities are from very weak (+) to strongest labeling (++++). Adapted from reference (7) with permission.

	Fe(III)	Fe(II)
<i>Forebrain</i>		
amygdaloid body, olfactory bulb, globus pallidus, ventral hypothalamus	++++	+++~++
thalamus, medial and lateral geniculate nuclei	+++	++
cerebral cortex, hippocampus, corpus callosum, corpus striatum	++~+	+
<i>Midbrain</i>		
inferior colliculus, interpeduncular nucleus, dorsal tegmental nucleus, substantia nigra pars reticulata	++++	+++
superior colliculus	+++	++
red nucleus, substantia nigra pars compacta	+	+
<i>Pons, medulla oblongata and cerebellum</i>		
cranial nerve nuclei, cerebellar nuclei, inferior olivary nucleus, nucleus of the lateral lemniscus, superior olivary nucleus	++++	+++
area postrema, brain stem reticular formation, cerebellar cortex, gracile and cuneate nuclei, trapezoid body	+++	++
cerebellar white matter, pyramidal tract	++~+	+

positive oligodendrocytes were prominently increased in number in the older brain. Subcellular localization and intensity of Fe(III) and Fe(II) in glia were almost comparable in young and older brains, whereas neurons increased positive lysosomes or lipofuscin granules.

Discussion

Cellular and subcellular localization of nonheme Fe(III) and Fe(II)

Post-mitotic neurons ought to sequester excess iron into lysosomes and lipofuscin granules to keep cytosolic labile iron at the minimum level. The present results showed that neurons successfully treated nonheme iron along this strategy, in that, contrast to glial cells, most neurons showed iron-deposits in lysosomes only. However, it appears that some large neurons involved in the motor system continually require iron to maintain their high metabolic activities and therefore contain a relatively large amount of iron in the cytosol. Mitochondrion is the major site of heme synthesis and contains nonheme iron-protein complexes including ferritin, aconitase and ferredoxin⁸. 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 vesicles⁹.

Astrocytes are considered to be important regulators for iron import into the brain and have iron transporters such as ferroportin for iron excretion into the interstitial fluid¹⁰⁻¹². Astrocytes are the major source of ceruloplasmin, a ferroxidase, which effectively inhibits Fe(II)-mediated OH• formation and lipid peroxidation^{13, 14}.

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 myelin¹⁵. 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. Considering that the well known function of oligodendrocytes is myelin formation, it is interesting that iron-rich oligodendrocytes were more numerous in the gray matter than in the white matter. In mature animals, oligodendrocytic iron would be significantly important not only for myelination but also for the iron-requiring metabolism and functions of neurons and glia in the gray matter.

Microglial cells are immunopositive to ferritin and transferrin in the adult rat brain¹⁶. In the developing brain, microglia provides ferritin as a trophic factor to oligodendrocytes for myelination¹⁷. Sporadic microglia with heavy-iron seems to be specialized for iron sequestration to prevent iron-toxicity, and possibly act as a source of iron supply for local iron shortage.

Blood iron crosses the capillary endothelial cell membrane via the transferrin or lactoferrin receptors^{18,19}. Interestingly, we observed Fe(II)-deposits along the luminal surface of endothelial cells. This suggested that Fe(III) in blood was reduced to the ferrous form on the endothelial luminal surface, possibly under the expression of some ferric reductases. Alternatively, it seemed possible that cytosolic Fe(III) was reduced to Fe(II) by nitric oxide (NO) released from the endothelial cell. Moreover Fe(II)-NO complex may be present for the NO donation, because NO can reductively bind to Fe(III)²⁰⁻²².

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

inputs. 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 iron-sulfur cluster at the center of GABA transaminase dimer²³. This enzyme is synthesized in postsynaptic neurons and astrocytes²⁴ and plays an essential role for the degradation of GABA which is released from GABAergic synaptic terminals. Moreover, the inherent high neural activity in the above brain regions may cause increased turnover of iron-containing proteins, thereby creating a high demand for iron supply. We think these are the most likely reasons why oligodendrocytes, the major iron-storing and -donating 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^{25,26}. 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 Fe(III) accumulation with age in specific brain regions has long been known in humans and other mammals^{27,28}. Endogenously formed H₂O₂ 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 further production of OH• and accumulation of iron-containing lipofuscin which deteriorates the autophagocytic capacity²⁹. Moreover, endothelial

cells appear to undergo decreased or impaired function of the blood-brain barrier with age-related increase of cholesterol and membrane lipid peroxidation, which would accelerate the iron increase with aging³⁰.

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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