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Iron, copper and their proteins in substantia nigra of human brain during aging

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Scarce information is available on the content of metals and their molecules in the human brain. Iron, copper and other metals are involved in neurodegenerative disorders like Parkinson's Disease (PD), however, their behavior in physiological conditions is poorly understood. In this study we have measured iron, copper and their major proteins (ferritins and ceruloplasmin) in substantia nigra (SN) of normal subjects at different ages, since this is the main target region of PD. An increasing trend for iron and copper concentration was found in aging. Ferritins were also increasing in aging while ceruloplasmin did not vary. These data show that the accumulation of these metals requires an increased expression of storage molecules to prevent toxic effects of iron and copper.

Introduction

The crucial role of some metals, like copper, iron and other transition metals, has been studied for their involvement in neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer disease (AD), and amyotrophic lateral sclerosis (ALS). Multivalent transition metals are necessary for many biological reactions and also act as cofactors of various enzymes. Alterations in the homeostasis of these metals, resulting in oxidative stress and increased free radical production, can lead to molecular and cellular damage and finally to cell death. Oxidative stress, that is the imbalance between the processes resulting in the reactive oxygen species (ROS) production and the antioxidant mechanism, is the pre-existing condition clearly associated with the neurodegeneration. The hydroxyl radical, the most dangerous free radical produced in cells, results from the Fenton reaction between reduced transition metals [iron(II) and copper(I)] and H_2O_2. Among all transition metals, iron is the most responsible in inducing oxidative stress through the production of oxygen free radicals. Alteration in free iron levels and iron homeostasis are implicated in the pathogenesis of PD[1,2] and of AD.[3] The potential role of iron in inducing cellular damage is partially controlled by various iron binding proteins such as lactoferrin (Lf), with a protective role against inflammation, and iron regulatory proteins (IRP-1 and IRP-2), that control other iron binding proteins. IRP proteins are important in the regulation of cellular iron metabolism, thus inhibiting the Fe capacity in ROS production. Melanotransferrin (MTf), ceruloplasmin (CP) and ferritin (Ft) are also involved in the homeostasis of iron. In particular ferritin is the most important iron binding protein in the brain and is mostly concentrated in microglial cells and oligodendrocytes.[4] In neurons, neuromelanin (NM) is an excellent binder of metals, especially iron.[5–7] This is an insoluble granular pigment present in dopaminergic neurons of the substantia nigra (SN), that is the main target area of the degenerative process in PD. In PD and related syndromes the level of SN iron increases by 30–35%.[8,9] Iron accumulation seems to occur within NM granules, where iron concentration is higher than in NM granules of normal subjects.[10,11] The NM strong chelating ability for iron and other transition metals strengthens the hypothesis that NM could have a protective role by inactivating these metals that can induce oxidative stress.

Like in the case of iron, copper has a functional role in many enzymes but, on the other side, it could result in cytotoxicity by increasing the production of free radicals. For this reason it is necessary a strictly regulated homeostasis of copper including both transport and storage proteins, such as prion protein (PrP) and ceruloplasmin (CP). PrP is a constitutive transmembrane glycoprotein whose normal role is still unclear. Nevertheless, it was observed that PrP has a SOD activity[12,13] and specifically binds copper(II),[14,15] thus it may play a protective role against cellular oxidative damage. CP is an important metalloprotein containing copper. Many functions have been ascribed to CP including metabolism, transport and homeostasis of Cu, ferroxidase activity,[16–18] amino oxidase activity and antioxidant activity against lipid peroxidation.[19] Consequently, alterations of CP levels could have a significant relationship to disease in which free radical damage is considered part of the pathology as in the case of aging, PD and AD.

Here, we investigate the age trend of these metals and their related proteins in physiological conditions in order to understand their role in aging and in neurodegenerative diseases.

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Experimental

Samples

Human SN samples were obtained from autopsies of normal subjects who had died without neurological or psychiatric disorders. Autopsies were carried out within 48 hours of death. The SN tissues were carefully dissected in a clean area using titanium-carbide and plastic tools to avoid any contamination. After separation, tissue samples were stored at –80 °C until analysis.

Standards, reagents and blanks

For instrumental neutron activation analysis (INAA) and for electro-thermal atomic absorption spectroscopy (ET-AAS) the primary standards consisted in standard solutions for inductively coupled plasma mass spectroscopy (ICP-MS) obtained from BDH (U.K.). The irradiation vials used for INAA were of high purity quartz (specpure Suprasil, Hereaus, Germany) and of polyethylene (Kartell, Milan, Italy). All the vials were cleaned before irradiation by an ultrasonic bath with a 0.5M solution of high purity HNO_3 (Aristar BDH – U.K.) and Milli-Q-Plus water (Millipore, Milan, Italy). A strict blank control was carried out analyzing both the quartz and plastic irradiation vials used for short and long irradiation, respectively. Analytical quality control was carried out by analyzing the standard reference materials SRM 1515 Apple Leaves and SRM 1547 Peach Leaves. All reagents used for ELISA and immunonephelometric assay were of high purity grade (Sigma-Aldrich Co., St. Louis, Mo, USA; Carlo Erba S.p.a., Milano, Italy) and were prepared using Milli-Q-Plus water.

Analysis by INAA and ET-AAS

Neutron irradiations were carried out by the TRIGA Mark II research nuclear reactor of the University of Pavia following two different procedures.

(1) For Cu determinations, short irradiations of 5 minutes of plastic vials were carried out in the pneumatic irradiation facility at a neutron flux of $5 \cdot 10^{12}$ $n \cdot cm^{-2} \cdot s^{-1}$. After a decay ranging from 1 to 6 minutes, a 300–600-second gamma-counting was carried out.

(2) For Fe, long irradiations of 30 hours of quartz vials were carried out in the central thimble facility at a nominal flux of 10^{13} $n \cdot cm^{-2} \cdot s^{-1}$. After a decay of 10 days, a 30,000-second gamma-counting was performed. HPGe (gamma–X) detectors (Ortec, USA) coupled to a computerized multichannel analyzer (Ortec ADMAC, USA) were used for the gamma-spectra evaluation. The relative efficiencies of the detectors ranged between 40 and 50% with resolutions of 1.7–1.8 keV on the 1332.5 keV gamma-line of ^{60}Co.

ET-AAS analysis of Cu were carried out by a Shimadzu AA 660. Samples were dissolved in conc. HNO_3 in high pressure Teflon lined dissolution bombs for microwave ovens (Milestone 1200, USA). The resulting solutions were evaporated to near dryness (80 °C), reconstituted with 0.1M HNO_3 and brought to final volume for the ET-AAS measurements. The determinations of Cu by both INAA and ET-AAS were considered.

Determination of H- and L-ferritin concentration

The concentration of ferritins was determined by ELISA using monoclonal antibodies specific for H-ferritin (rH02) and L-ferritin (LF03).[20]

Determination of ceruloplasmin concentration

SN tissues were homogenized in lysis buffer containing Tris (20 mM, pH 7.4) and protease inhibitor cocktail (Sigma), and centrifuged at 5000 rpm for 10 minutes at 4 °C. The supernatants were collected and the content of ceruloplasmin was determined by immunonephelometric assay with an automatic analyzer BNA II (Behring), using rabbit polyclonal antibodies against ceruloplasmin.

Results

Iron concentration in SN of normal subjects is reported in Fig. 1. The levels of iron had a smooth increase within the age interval here considered (16–90 years) according to a linear model ($p = 0.040$), with a value range between 103.0±7.2 and 249.0±25.4 ng/mg wet tissue. H- and L-ferritin concentrations in human SN samples are reported in Fig. 2. H-ferritin was more abundant than L-ferritin, with a ratio H : L of 2–3 : 1. H-ferritin levels were in the range of 145.9±8.7 and 290.9±26.1 ng/mg wet tissue, and L-ferritin concentration was in the range of 34.3±1.3 and 122.6±6.7. Both types of ferritins showed a smooth increase during aging according to a linear model ($p = 0.002$ for H-ferritin; $p = 0.0003$ for L-ferritin). In Fig. 3 the age trend of copper in human SN samples is represented. Copper concentration was lower than that of iron and showed values between 5.6±1.1 and 47.5±4.7 ng/mg wet tissue, with an increasing trend during aging according to an S regression model ($p = 0.025$). The levels of ceruloplasmin in human SN tissues were in the range of 573.7±46.4 and 2581.1±292.8 ng/mg wet tissue (Fig. 4), and were higher than those of ferritins and did not show any specific trend.

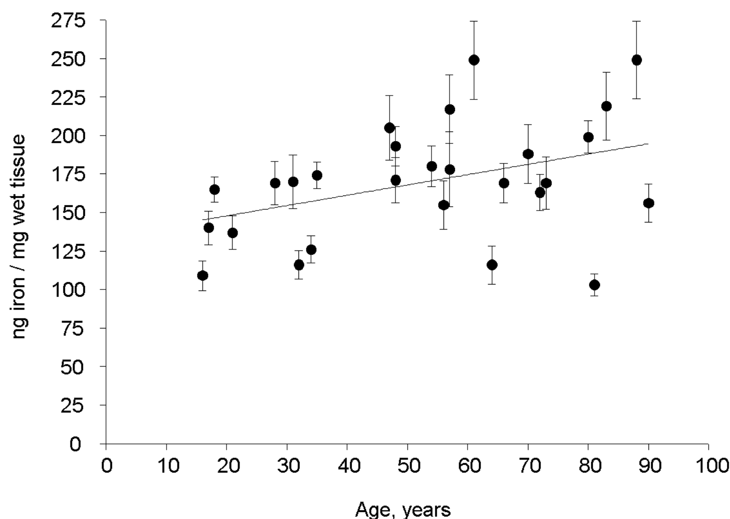

Fig. 1. Iron concentration in substantia nigra of human normal subjects during aging. Values are given as single determination ± total combined uncertainty

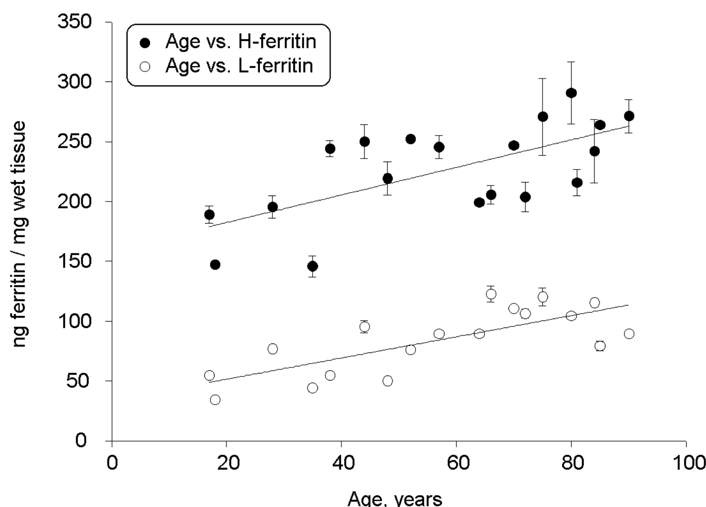

Fig. 2. Concentration of H- and L-ferritin in substantia nigra of human normal subjects during aging. Values are reported as mean ± S.E.M.

Discussion

A clear picture appears from the data presented here on iron and ferritin concentration in SN of normal subjects over the whole life span. It is known that iron levels in the brain are very low at birth and a large amount of iron is required for brain development and particularly for myelinogenesis.[21] We have previously observed that iron concentration which is attained by 20 years is maintained for the rest of the life span.[20] In this work, including a larger number of subjects, we have found a smooth increase of iron concentration during aging. To date, the quantitative distribution of the different iron pools (carrier, storage and enzyme molecules) in a normal human brain is unknown. In the phylogenetic analysis of iron distribution using histochemical methods in brain cells, a species-dependent situation was found.[22] However, the methodology used suffers the limitation of not detecting iron bound to NM. For the above-mentioned reasons, data from animal studies cannot be extrapolated to the human brain and more information is needed on the cellular and subcellular distribution of iron, considering its role in brain aging and neurodegenerative disorders.

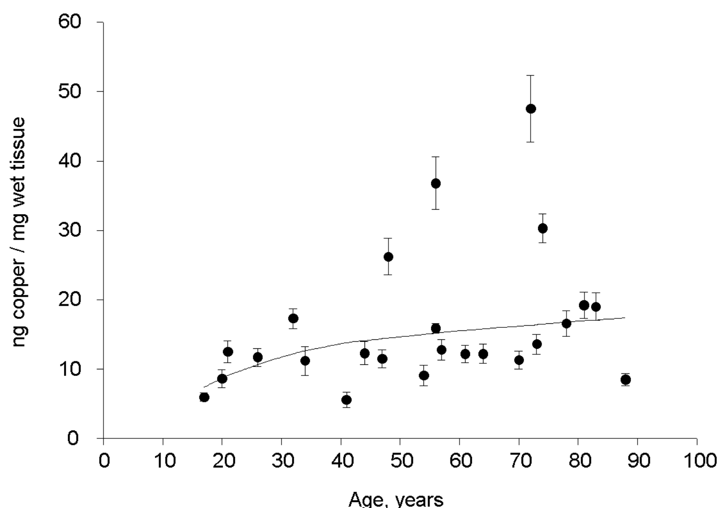

Fig. 3. Copper concentration in substantia nigra of human normal subjects during aging. Reported values are single determination ± total combined uncertainty

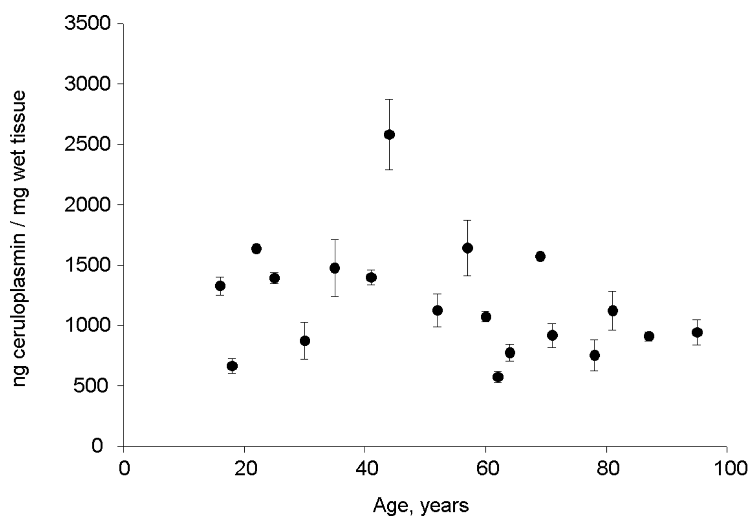

Fig. 4. Ceruloplasmin concentration in substantia nigra of human normal subjects. Values are expressed as mean ± S.E.M.

The increase in ferritin concentration in the elderly and the general pattern of ferritin accumulation is in reasonable agreement with previous studies,[23,24] except that the values we detected here for L-ferritin using the ELISA sandwich system were lower than those detected using a quantitative dot/blot assay.[24] This is probably related to the quaternary structure of the ferritin in which hybrid molecules are under-evaluated in sandwich-type assays.[25] Ferritin concentration was related to iron concentration, in keeping with its iron storage functions. To date, it has been shown that ferritin in human basal ganglia is present in oligodendrocytes and astrocytes as H- and L-ferritin.[4,26] In SN neurons, ferritin content is very low and a major storage molecule for iron is NM.[20] It was reported that ferritin content is very low in all neurons of the human brain.[26] This is notable with respect to the well documented increase of iron content in SN neurons during PD.[8]

Like in the case of iron, the concentration of copper had an increasing trend during aging. It was reported that total copper levels were reduced by 34–45% in the SN in the course of PD.[8] However, because copper levels were reduced in the Parkinsonian substantia nigra, copper cannot account for the increased lipid peroxidation observed in this brain region. The process by which copper is transported into the brain and distributed within brain proteins is not well understood. There are at least 10 copper-containing proteins in the brain[27] the most prominent of these are cytochrome oxidase, copper–zinc superoxide dismutase and neurocuprein. Ceruloplasmin seems to be not the major cuproprotein in the brain, unlike plasma. However,

immunocytochemical staining indicated that CP was present both intracellularly (in neurons and astrocytes) and extracellularly (AD neuropil and diffuse plaques).[28] Staining of neurons and astrocytes suggests that CP synthesis may occur in these cells. In this study we found that levels of CP remains constant during the aging of normal subjects. Since CP shows an age trend different than that of copper, the increase of copper content in SN during aging is likely due to the increase of other copper proteins like ZnCu SOD or cytochrome oxidase. In PD an increase of CP levels occurs in SN.[28] Oxidative stress is a possible mechanism for promoting brain CP synthesis. Increased regional CP in the brain may not necessarily confer increased anti-oxidant capacity, however, because CP may in some cases function as a pro-oxidant.[19]

Conclusions

For both iron and copper, an age increasing trend is observed and such an increase is a potential risk factor for neurodegenerative processes generated by these metals. Proteins and other buffering molecules like NM are expressed to prevent the toxicity of iron and copper. Ferritins, CP and NM are among the most important molecules playing this role but others need to be investigated for a better understanding of brain aging and neurodegenerative disorders.

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