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The absolute concentration of nigral neuromelanin, assayed by a new sensitive method, increases throughout the life and is dramatically decreased in Parkinson's disease

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Abstract The concentration of neuromelanin (NM) in substantia nigra pars compacta (SNPC) has been measured in male and female normal subjects at different ages in the range 1–97 years old and in SNPC of parkinsonian patients. A very similar age trend of NM concentration was found in both sexes. In the first year of life NM was not detectable, between 10 and 20 years the NM levels were 0.3–0.8 μg/mg of SNPC, between 20 and 50 years were 0.8–2.3 μg/mg SNPC and between 50 and 90 were 2.3–3.7 μg/mg of SNPC. In parkinsonian subjects, the NM levels were 1.2–1.5 μg/mg of SNPC, which is less than 50% with respect to the age-matched controls. These data demonstrate a continuous NM accumulation in SNPC neurons during aging, the presence of large amounts of NM in SNPC and severe depletion of NM in Parkinson's disease. **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Neuromelanin; Parkinson's disease; Substantia nigra

1. Introduction

Neuromelanin (NM) is an insoluble pigment found in neurons of specific brain regions of several animal species including humans [1–6]. Neurons rich in NM are found especially in the substantia nigra (SN) and locus coeruleus (LC) [5,7,8]. The structure and role of NM have been only partially characterized [9–14] and are still a matter of investigation because of the possible involvement in brain aging and Parkinson's disease (PD). In fact, this pigment first appears in humans at 2–3 years of age and accumulates with aging [8]. NM is possibly associated with the vulnerability of dopaminergic neurons in the SN [15,16]. In PD the melanized neurons of SN are more vulnerable than the non-melanized ones. However, such vulnerability is not correlated with the amount of NM [17]. A recent study showed that NM synthesis is driven by excess cytosolic catechols which are not accumulated in synaptic vesicles [18]. A role of enzymatic activity of macrophage migration inhibitory factor was proposed in the NM synthesis, since it oxidizes catecholamines to dihydroxyindole derivatives and these are potential precursors of NM [19]. The NM of SN shares some characteristics of melanins such as redox activity and chelating ability for metals [2,20–23]. In NM isolated from SN and LC, a significant amount of iron was found [13]. Since iron can generate cytotoxic free radical, NM, due to its strong chelating ability for iron and other toxic metals (cadmium, mercury and manganese), could play an important protective role in neurons [2,24,25]. In the SN the presence of NM partially saturated with iron was indeed shown [25]. NM specifically adsorbs lipids at high molecular weight and probably plays a role in the metabolism of brain lipids [14]. There are different results about the loss of pigmented neurons in aging probably because of the different methodologies in the counting of SN sections [26–30]. The current methods for NM estimation are semiquantitative [17,31] and are affected by several limitations as detailed further in Section 4. In a recent study the concentration of NM in SN of a small group of normal male subjects was reported [32]. Despite the interest in the NM role in PD, to date no data have been reported about the variation of nigral NM with aging in normal male and female subjects, nor its variation in PD patients. With the present work we describe a precise and sensitive chemical method for NM determination and report the age-related trend of NM accumulation in SN pars compacta (SNPC) of normal subjects and parkinsonian patients.

2. Materials and methods

2.1. Collection of samples

Samples of midbrain were removed from people who had died at different ages without evidence of neurologic or psychiatric disorders. These tissues were collected during autopsies carried out at the Institute of Forensic Medicine, University of Milan, Milan, Italy, and Department of Pathology, IRCCS San Raphael Hospital, Milan, Italy. Autopsies were carried out within 48 h of death. The SNPC was carefully dissected and stored at −80°C until assay. For NM

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Abbreviations: NM, neuromelanin; SN, substantia nigra; SNPC, substantia nigra pars compacta; PD, Parkinson's disease; LC, locus coeruleus

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determinations, 19 samples of SNPC from normal male subjects and 18 from normal females were employed in the age range from 1 to 97 years old. Further samples were used to isolate the NM to prepare the calibration curves. The normal subjects included in this study at pathological examination did not show macroscopic alterations of neurologic and vascular type. At histological examination, no Lewy bodies or other pathological markers were observed. Only in one female subject, without story of PD symptoms, Lewy bodies and consistent neuronal loss were observed. Samples of SNPC were also taken from four male and three female PD patients with the same procedure as above. Diagnosis of PD was established by clinical records and histologically confirmed by the presence of neuronal loss and Lewy bodies in the SN.

This study was approved by the Ethical Committee of the C.N.R. Institute of Biomedical Technologies (Milan, Italy) and was carried out in agreement with the Policy of Italian National Research Council.

2.2. Isolation of NM from SNPC for preparing calibration standards

Pooled SNPC samples were weighed and processed for NM isolation as previously described [12–14]. The identity and purity of the obtained NM sample was confirmed by the following determinations. Elemental analysis (%): carbon = 58.80; hydrogen = 7.16; nitrogen = 6.51; sulfur = 2.56. The total amino acid content was 9.4%. The electron paramagnetic spectrum showed typical g4 and g2 signals. Purity was over 98%.

2.3. Procedure of NM determination

The SNPC of each subject was carefully ground in a plastic tube, then a 10 mg aliquot of ground tissue was weighed into a 5 ml glass tube. Then, 1.5 ml of pH 7.4 phosphate buffer (50 mM) was added, the tube shaken, centrifuged at $9000\times g$ for 30 min and the supernatant discarded. Washing with phosphate buffer was repeated one more time. The pellet was incubated with shaking for 2 h at 37°C with 1.5 ml of Tris buffer (50 mM, pH 7.4) solution containing sodium dodecyl sulfate (5 mg/ml) and 0.2 mg/ml proteinase K (EC 3.4.21.14; Sigma, St. Louis, MO, USA). The fine suspension of pigment was centrifuged at $9000\times g$ for 30 min. The pellet was washed with 1.5 ml of NaCl solution (9 mg/ml) and 1.5 ml of water, then centrifuged with the above given conditions. The NM residue was dissolved in 1 ml of 1 M NaOH at 80°C for 1 h. The obtained solution was centrifuged and the supernatant transferred into a quartz cuvette, and its absorbance at 350 nm was measured by spectrophotometer. Calibrations curves were run by dissolving known amounts (1, 5, 10, 20 and 30 μg) of NM in 1 ml of 1 M NaOH at 80°C for 1 h. To this purpose, NM isolated from SNPC as described above was suspended in water (0.5 mg/ml), then diluted to a lower concentration to obtain a suspension which was employed for the preparation of the calibration curve. The values presented in this work are the average content of NM from three to five determinations in the analyzed samples. The NM content in SN tissue was stable for not less than 96 h after death (data not shown).

Protein concentration was measured in each sample by using the Lowry method [33].

2.4. Recovery of NM from brain tissue

In order to evaluate the recovery of NM in the analysis of SN tissue, aliquots of white matter from normal human brain were employed, since this region is known not to contain NM. Standards of NM prepared as described above were added to white matter samples which were ground and processed for NM determination as described above.

2.5. Statistical analysis

The data were analyzed using paired Student's *t*-test and ANOVA. Values are given as mean ± S.E.M.

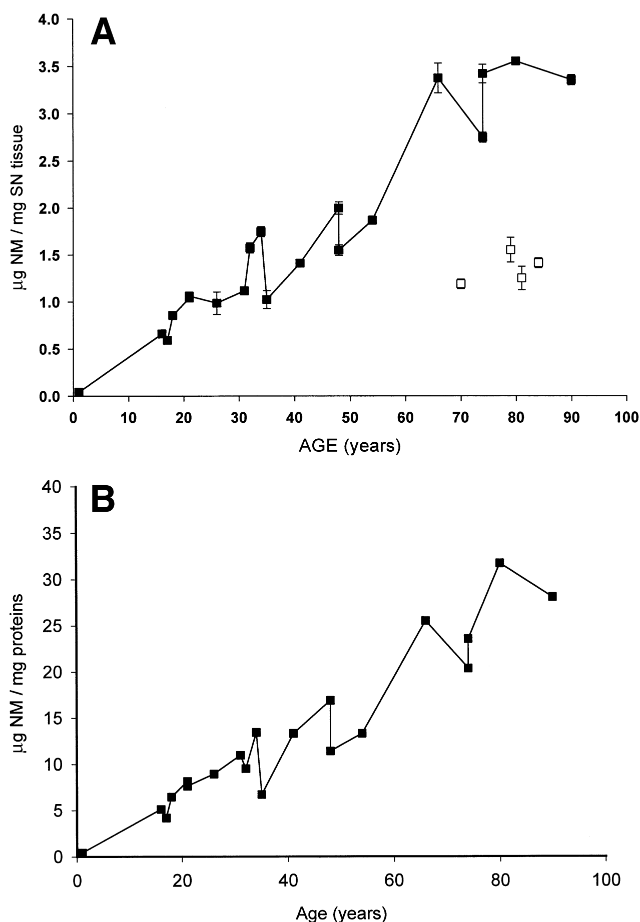

Fig. 1. A: NM content (μg/mg tissue) in SNPC of normal male subjects (■) at different ages and four parkinsonian patients (□). B: NM content (μg/mg protein) in SNPC of the same normal subjects. Values are given as mean ± S.E.M. of three to five determinations for each subject.

3. Results

The parameters of the assay method here reported are given in Table 1. It appears that this method is linear in the adopted concentration range, and has good sensitivity, reproducibility and recovery.

For both sexes there was a clear variation of NM concentrations according to the subjects age, with the levels steadily increasing with senescence. In males (Fig. 1A), the youngest subject aged 10 months, the concentration of NM was below the method's detection limit, i.e. less than 0.05 μg/mg SNPC, which is well in agreement with histological studies showing the appearance of the first granules of NM at the age of 2–3 years. Levels of NM in teenagers were found to be between 0.5 and 1.0 μg/mg SNPC. Then, an increase of NM concentration occurs throughout all the rest of the life span until the

Table 1
Main parameters of the new method for NM determination

Linearity	Interassay reproducibility (CV, %)	Detection limit	Recovery (%)
$Y = 0.0053X + 0.0083$ $R^2 = 0.9896$	4.96 ($n = 4$)	50 ng/mg tissue	83.2 ± 7.1 ($n = 3$)

latest phase of life when levels around 3.5 μg/mg are present. Individual variations occur over this increasing trend. A very similar time course of NM concentration is observed in normal females (Fig. 2A) with an analogous trend as to age. One outstanding exception was a female aged 69 whose NM value of 1.61 μg/mg was much lower than the average level at this age. This subject died of a cardiovascular condition and had no parkinsonian signs. However, the neuropathological examination revealed the presence of Lewy bodies and extensive loss of melanized neurons in SNPC, thus showing a condition of preclinical PD.

The four PD subjects in the male group had NM significantly lower concentrations than normal subjects ($P < 0.05$, t-test) in the corresponding age interval that is 75–85. In the female group the three PD subjects also had a significantly lower concentration ($P < 0.05$, t-test) of NM than corresponding normal subjects (age interval 75–85). In both male and female groups the PD patients had a 50–60% average decrease of NM concentration, which is in good agreement with that of neuronal loss of pigmented neurons histologically observed.

The concentration of proteins found in the SNPC samples was in the range 95–140 μg/mg wet tissue.

NM concentrations, expressed as values in μg/mg protein, of the same normal male subjects and female subjects are given in Figs. 1B and 2B, respectively. Due to the paucity of SN tissue obtained from parkinsonian subjects, the protein determination was not sufficiently accurate to calculate concentrations also as μg/mg protein; therefore values for parkinsonian subjects are shown only as μg/mg SN tissue.

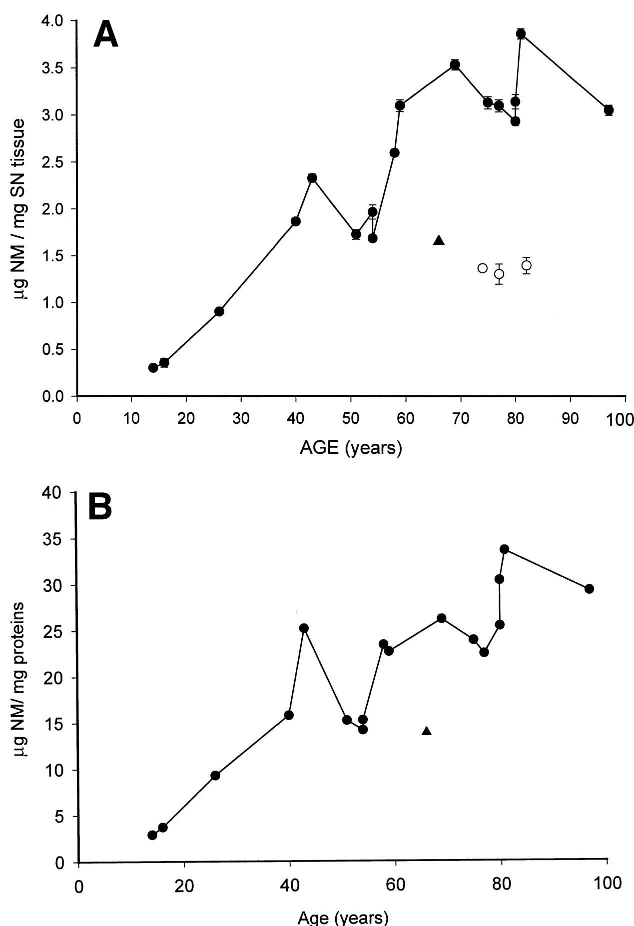

Fig. 2. A: NM content (μg/mg tissue) in SNPC of normal female subjects (●) at different ages, one subject with preclinical PD (▲), and three parkinsonian patients (○). B: NM content (μg/mg protein) in SNPC of the same normal subjects and the subject with preclinical PD (▲). Values are given as mean ± S.E.M. of three to five determinations for each subject.

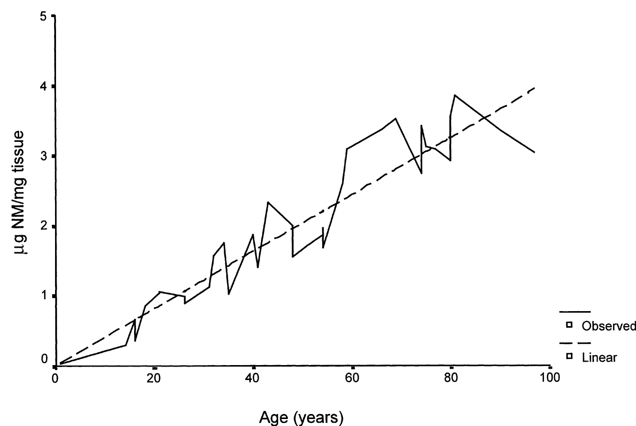

Fig. 3. Regression analysis of the relationship between NM content (μg/mg tissue) and age, of male and female subjects at different ages. The data from the two groups of subjects were pooled together. Data best fit to a linear regression model.

Since normal male and female subjects had very similar concentration of NM in the corresponding age ranges, all values were pooled and the fitting analysis performed by using several regression models. Among all tested models, the linear, logistic and quadratic ones resulted to better fit to the experimental data. By applying these approaches, and as shown in Fig. 3, the relationship between NM concentration and age was linear ($Y = 0.040645X + 0.0009231$).

4. Discussion

To our knowledge this is the first report concerning the absolute content of NM in the SN of normal male and female subjects at different ages, and PD patients. The NM assay here reported consists of a new, sensitive and specific method of quite acceptable reliability. Moreover, this method is simple, rapid and accurate and therefore suitable as a routine assay of NM in normal and pathological SN, in addition to the measurement of dopamine and other parameters.

Previous assessments of the NM content in SNPC were based only on histological methods and indirect determinations. Those studies reported qualitative [30] and semiquantitative [19,31] evaluation of NM neuronal content by histological methods employing stained and unstained sections of SNPC. The semiquantitative evaluation of NM content in stained and unstained sections [17,31] was performed using as a reference standard a synthetic dopamine-derived melanin, whose structure is quite different from that of authentic NM of SN [13,14]. Another potential source of error in histological studies is that the NM aggregates estimated within neurons contain large amounts of lipids and peptides associated with NM, as reported [14]. Furthermore, the use of a suspension of synthetic melanin of unknown concentration poses an additional limitation resulting in an unreliable estimate of NM. Histological studies [17,30,31] reported that lightly melanized

neurons are more vulnerable than heavily melanized ones in PD.

Other studies [26,28,29] on age trend of the number of pigmented neurons in SNPC reported either an age-dependent loss [26] or no variations [27–29]. However, different counting methods were employed in these studies. The absolute number of pigmented neurons in SNPC of normal subjects seems to be dependent on the ethnic group [28].

In the present work in both male and female subjects a progressive accumulation of NM has been documented with aging and the values are in the range 0–3.5 μg/mg of SNPC. The only exception was observed in one woman, aged 69, who had very low NM concentration at the level found in subjects aged less than 35 and she was found to be a case of preclinical PD. The age trend of total NM concentration in SNPC reported here is quite different from the age trend of the number of melanized neurons in SNPC reported in previous studies [26–29]. In fact, the previously mentioned studies described the distribution of pigmented neurons in specific layers of SN. Our method measures the total NM content in SNPC including the low amount of extraneuronal NM the magnitude of which should not significantly affect the total amount of neuronal NM.

In both male and female PD patients the percentage of decrease of NM concentration is similar to that of neuronal loss reported in neuropathological studies [26,27].

Previous studies showed a decreased level of pigmentation in the surviving neurons in PD patients [17,31]. This could be the result of a reduced NM synthesis, increased NM degradation, higher vulnerability of heavily pigmented neurons or a combination thereof. Since pigmented neurons may have different NM content, so an evaluation of the neuronal content of NM in the different cellular groups should be done in future studies on normal subjects and PD patients, by using the present method and neuronal counting on the same SNPC samples.

Because of the documented ability of NM to bind toxins such as redox active metals, cadmium, mercury, manganese, pesticides, etc. [23,34–36], NM could play, in a first phase, an efficient protective role in neurons. Moreover, the NM synthesis itself is a protective mechanism since it removes the excess cytosolic catechols which are not accumulated in synaptic vesicles [18]. However, when peroxidation processes prevail, the NM may be degraded, consequently releasing large amounts of toxins into the cytosol. If this is the case, then NM alone may not be a factor in increasing neuronal vulnerability but probably behaves as a sink collecting toxins which in some conditions can be broken releasing its content.

NM appears to be a good marker of damage to the SNPC in PD. As such, the development of NM in vivo imaging techniques may offer diagnostic and disease staging measurements matching the available tools used to monitor striatal dopamine depletion and offering the first direct approach to recognize and quantify nigral damage. The concentration values of NM here described could be used for correlation with in vivo images of SN in normal and PD subjects.

Further studies should expand the above observations in other extrapiramidal disorders to asses specificity of NM depletion and correlation with other established markers of nigrostriatal dysfunction.

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