New melanic pigments in the human brain that accumulate in aging and block environmental toxic metals

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Neuronal pigments of melanic type were identified in the putamen, cortex, cerebellum, and other major regions of human brain. These pigments consist of granules 30 nm in size, contained in organelles together with lipid droplets, and they accumulate in aging, reaching concentrations as high as 1.5–2.6 μg/mg tissue in major brain regions. These pigments, which we term neuromelanins, contain melanic, lipid, and peptide components. The melanic component is aromatic in structure, contains a stable free radical, and is synthesized from the precursor molecule cysteinyl-3,4-dihydroxyphenylalanine. This contrasts with neuromelanin of the substantia nigra, where the melanic precursor is cysteinyl-dopamine. These neuronal pigments have some structural similarities to the melanin found in skin. The precursors of lipid components of the neuromelanins are the polyunsaturated lipids present in the surrounding organelles. The synthesis of neuromelanins in the various regions of the human brain is an important protective process because the melanic component is generated through the removal of reactive/toxic quinones that would otherwise cause neurotoxicity. Furthermore, the resulting melanic component serves an additional protective role through its ability to chelate and accumulate metals, including environmentally toxic metals such as mercury and lead.

lipids | neuromelanin | brain aging | neurodegenerative

Neuromelanins occur as dark brown granules with variable distribution in catecholamine neurons of the substantia nigra and locus coeruleus in the brains of humans and different animal species (1). In the human brain, neuromelanin has been found, isolated, and characterized only in the dopamine (DA) neurons contained in the substantia nigra and in the norepinephrine neurons in the locus coeruleus, and these two neuromelanins share some common structural features (2). In both of these regions of the human brain, the neuromelanin concentration normally increases linearly with age. In Parkinson's disease, the tissue concentration of neuromelanin decreases dramatically because DA pigmented neurons are preferentially lost compared with nonpigmented ones (3, 4). Reduced neuronal content of neuromelanin in substantia nigra has been reported in patients with Parkinson's disease (3), Alzheimer's disease (5), and Rett syndrome (6). In neuronal cultures and in the human brain, neuromelanin synthesis is driven by excess cytosolic catechols not accumulated in synaptic vesicles, is inversely related to the expression of vesicular monoamine transporter 2, and is directly related to the vulnerability of neurons during aging. It has been suggested that neuromelanin synthesis protects the neurons in the substantia nigra because it removes neurotoxic quinones (7, 8). Furthermore, the neuromelanin in the substantia nigra can trap endogenous and environmental toxins and immobilize them as stable adducts, thus protecting against toxicity (9–11).

Neuromelanin in the substantial nigra and locus coeruleus is an electron-dense brown/black pigment composed of aggregates of ≈30-nm diameter spheres with a pheomelanin core and eumelanin surface and a pheomelanin/eumelanin ratio of 3:1 (12, 13). Eumelanin is characterized by dihydroxyindole groups formed by DA oxidation and is responsible for the metal-chelating ability of neuromelanin (14). Pheomelanin is generated by oxidation of cysteinyl-dopamine (Cys-DA) and contains benzothiazine groups. In addition to the mentioned melanic component of neuromelanin, there are also poorly-characterized peptide and aliphatic components (15).

Before this work, it was not known whether pigments with structure and function similar to neuromelanin are present in noncatecholaminergic neurons and in brain regions other than the substantia nigra and locus coeruleus. In this work, we establish the presence of a type of aging pigment in neurons of the putamen, premotor cortex, and cerebellum and compare them with the neuromelanin of the substantia nigra. We have also examined their structure, behavior in aging, and interaction with metal ions and lipids.

Results

An Electron-Dense Pigment Is Contained in Organelles in the Neurons of the Putamen, Premotor Cortex, Cerebellum, and Substantia Nigra of the Human Brain. Transmission electron microscopy was used to investigate the existence of organelles containing melanic pigments in neurons of the putamen, premotor cortex, and cerebellum. Slices of these regions from human brains aged 70–80 years showed pigmented organelles similar to those in the substantia nigra (Fig. 1 *A–D*). Each organelle exhibited a typical double membrane, a large amount of dark pigment, lipid deposits, and a protein matrix. The ratios of these components varied with the brain region. Organelle shape also varied from round to oblong, with sizes ranging from 0.5 to 3.0 μm. The number of neurons containing organelles decreased in the order: substantia nigra > putamen ≅ premotor cortex > cerebellum. To characterize these organelles

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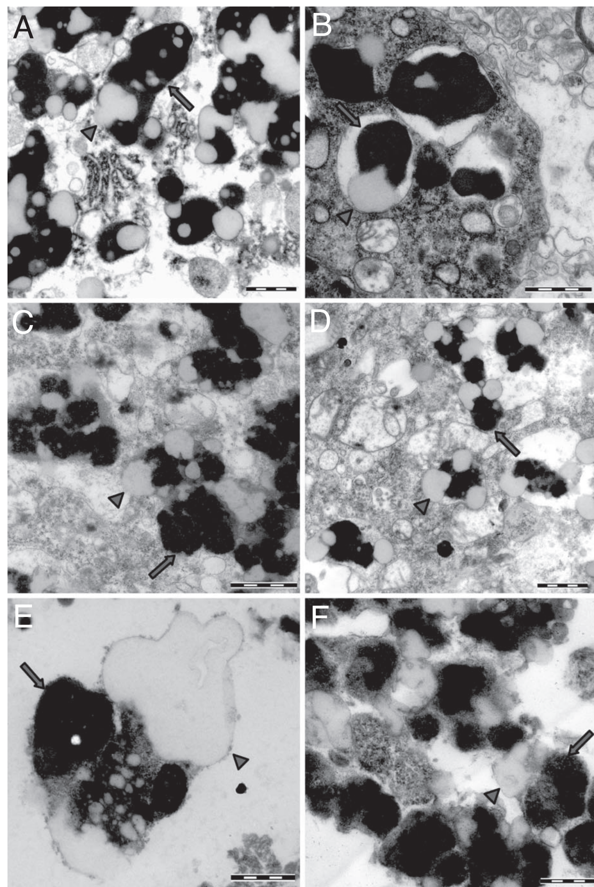

Fig. 1. Transmission electron microscopic images of pigmented organelles. (*A–D*) In putamen (*A*), premotor cortex (*B*), cerebellum (*C*), and substantia nigra (*D*) of the human brain, intraneuronal organelles containing dark pigment (arrow) and lipid droplets (arrowhead) are observed. (Scale bars, 1 μm.) (*E* and *F*) In aggregates of pigmented organelles isolated from cerebellum (*E*) and from substantia nigra (*F*), the same morphology with dark pigment (arrow) and lipid droplets (arrowhead) is present. [Scale bars, 1 μm (*E*) and 0.5 μm (*F*).]

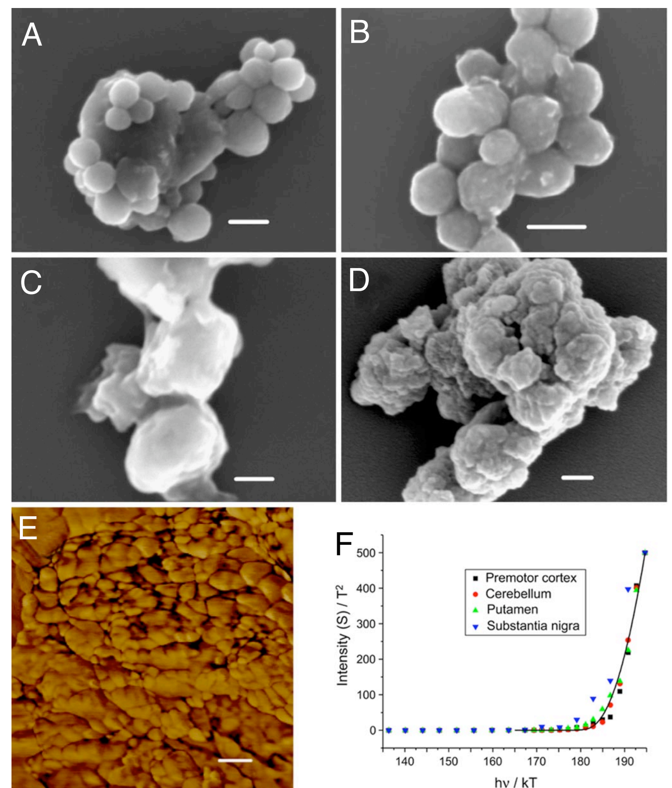

Fig. 2. Microscopic studies of isolated pigments. (*A–D*) Scanning electron microscopic images of pigment granules from different regions of the human brain: putamen (*A*), premotor cortex (*B*), cerebellum (*C*), and substantia nigra (*D*). (Scale bars, 200 nm.) (*E*) Atomic force microscopic image (phase) of pigment isolated from premotor cortex. (Scale bar, 100 nm.) (*F*) Wavelength-dependent UV free electron laser photoelectron emission microscopic data plotted for pigments isolated from different regions of the human brain, establishing that all have a common surface oxidation potential.

and their contents, they were isolated by a multistep gradient centrifugation and visualized by electron microscopy (Fig. 1 *E* and *F*). The morphology of the isolated organelles confirms the presence of both dark pigment and lipid droplets.

UV-Visible Spectra Confirm the Presence of Melanic Pigments in the Neuronal Organelles of Different Regions of the Human Brain. To assess the presence of melanic components in the dark pigment of organelles prepared from the putamen, premotor cortex, cerebellum, and substantia nigra, the organelles were washed with phosphate buffer and treated with SDS to remove proteins and then with methanol/hexane to eliminate lipids. The residual material was solubilized in NaOH and its UV-visible spectrum measured. The pigments from all regions exhibited the typical absorption profile of melanins: a broad shoulder extending from $\approx$250 nm to $>$300 nm on the tail of the intense absorption at higher energy, the latter arising from electronic transitions of the conjugated aromatic systems.

The UV-visible spectra of the pigments isolated from putamen, premotor cortex, cerebellum, and substantia nigra were also measured in DMSO, in which the pigments are appreciably soluble [supporting information (SI) Fig. S1]. In most cases, these spectra reveal poorly-defined, but detectable shoulders on the tail of the intense absorption band, the most interesting of which occurs $\approx$400 nm and could be assigned to the quinone residues of the melanic/peptidic component of the pigments.

Isolation of the Pigments from Human Brain Regions for Structural Investigations. The pigments from organelles of the same brain regions were isolated and purified in amounts sufficient for accurate structural determinations and for comparison with neuromelanin from substantia nigra (2, 15). For isolation of the pigments, tissues from brain areas were washed with phosphate buffer and SDS and successively treated with proteinase to remove proteins and methanol/hexane to remove lipids. The dark brown pigments are sparingly soluble in alkaline solution and polar organic solvents.

Imaging of Pigment Granules. Imaging with scanning electron microscopy showed that all pigments are aggregates of 200- to 300-nm-diameter spherical constituents (Fig. 2 *A–D*). Atomic force microscopy further reveals a substructure of 30-nm particles for all pigments (Fig. 2*E*). Spatial microscopy images on broken organelles confirm the existence of these small spherical substructures.

Pigments Accumulate in Human Brain Regions During Aging. To investigate whether these pigments accumulate during life, their concentrations were measured in putamen, premotor cortex, cerebellum, and substantia nigra samples from subjects of different ages. Pigment concentrations increase linearly with age for all regions. The concentrations of pigments in putamen, premotor cortex, and cerebellum are similar, reaching values as high as 24

Table 1. Products of chemical degradation and free and protein-bound catechols from pigments isolated from different brain regions

Method	Product	Putamen	Cortex	Cerebellum	Substantia nigra
H_2O_2 oxidation	PTCA	16 ± 3	24 ± 1	32 ± 5	62 ± 13
	PDCA	<5	<5	<5	106 ± 21
	TTCA	248 ± 48	100 ± 15	216 ± 82	717 ± 177
	TDCA	76 ± 3	39 ± 12	78 ± 41	449 ± 191
HI hydrolysis	4-AHP	30 ± 1	16 ± 4	38 ± 7	18 ± 3
	4-AHPEA	3.6 ± 1.4	3.9 ± 1.6	2.8 ± 0.6	454 ± 29
Free catechol	DOPA	2.2 ± 0.6	2.4 ± 0.6	2.4 ± 0.4	5.5 ± 1.2
	DA	<0.2	<0.2	<0.2	6.4 ± 2.7
	Cys-DOPA	1.5 ± 0.8	1.3 ± 0.6	1.3 ± 0.7	2.8 ± 1.3
	Cys-DA	<0.5	<0.5	<0.5	3.7 ± 1.3
Protein-bound catechol	DOPA	19 ± 6	53 ± 11	63 ± 9	265 ± 20
	DA	3.4 ± 0.4	1.2 ± 0.6	1.2 ± 0.4	948 ± 382
	Cys-DOPA	48 ± 1	21 ± 2	28 ± 9	60 ± 25
	Cys-DA	<1.0	<1.0	<1.0	47 ± 12

PTCA and PDCA are typical degradation products from DOPA–melanin and DA–melanin, respectively (13). Both TTCA and TDCA are degradation products from Cys-DOPA–melanin or Cys-DA–melanin. 4-AHP and 4-AHPEA are degradation products from Cys-DOPA–melanin and Cys-DA–melanin, respectively. Values are mean ± SEM (n = 2), each value being obtained in duplicate, and are shown in nanograms per milligram pigment.

μg/mg protein by the 9th decade of life (Fig. S2 *A–D*). However, these values are lower than the pigment concentration for the substantia nigra (2). The substantia nigra contains more pigmented neurons, and each neuron has more pigmented organelles than the neurons of the putamen, premotor cortex, and cerebellum. Pigments were also identified and measured in caudate nucleus, globus pallidus, and the occipital, parietal, and temporal cortexes (Fig. S2*E*).

A Stable Free Radical Is Present in All Pigments. EPR spectroscopy showed the presence of a stable free radical within the pigments and further revealed that this free radical interacts with iron. The spectra measured for these purified pigments are similar to that reported for pigment isolated from the substantia nigra. In all cases, a stable free radical signal at g~2, typical of melanins, was found together with a g~4 signal corresponding to a high-spin iron(III) complex in rhombic configuration (Fig. S3). The ratios of g~4/g~2 signal intensities were different among the pigments, showing that different amounts of iron were bound to the pigments from different regions of the brain. The intensity of the g~4 iron signal is 6–9 times larger in pigment of substantia nigra than pigments of putamen, premotor cortex, and cerebellum, indicating that the largest concentration of iron is found in neuromelanin from substantia nigra. This finding is in agreement with the concentrations of iron in these pigments, which are quantified below in analysis of metals.

Polyunsaturated Aliphatic Molecules Are Components of the Pigments. The ^{1}H NMR spectra of all pigments in deuterated DMSO exhibit a similar pattern of signals (Fig. S4), suggesting that the major components responsible for the spectra are common. The signals with highest intensity are observed between 1 and 2 ppm. Specifically, the five groups of signals occurring near 0.8, 1.0, 1.2, 1.6, and 2.0 ppm are prominent in all spectra and can be associated with saturated aliphatic CH_3 and CH_2 groups, which likely emanate from covalently bound lipids. Other important signals present in all spectra occur between 5.0 and 5.5 ppm, and near 7.2 ppm. The signal of lower intensity at 5.1 ppm corresponds to a proton on a C═C bond and is found in a nearly constant ratio with the aliphatic C—H signals. Therefore, together, these signals can be associated with molecules containing the dolichol skeleton (16). The group of weak signals between 5.2 and 5.5 ppm are also attributable to protons on unsaturated carbon atoms; they maintain the same characteristic intensity ratio in all of the spectra but occur with variable intensity ratio with respect to the 5.1-ppm signal. These signals could be caused by a different component of the pigments; a likely candidate is the unsaturated network of the melanic component.

Elemental analyses confirm the presence of large portion of aliphatic components in the pigments. In fact, the molar H/C ratio was found to be 1.58, 1.56, 1.61, and 1.30 for pigments of putamen, premotor cortex, cerebellum, and substantia nigra, respectively. These high ratios are indicative of aliphatic C—H groups, likely deriving from lipids, in addition to unsaturated C═C and ═C—H groups of the melanic component.

Pigmented Organelles Contain Lipids Similar to Those of Isolated Pigments. The attribution of aliphatic signals in the NMR spectra of pigments and the presence of lipid droplets in electron microscopy images prompted a lipid analysis of organelles to determine the droplet composition, quantify the major lipids present, and establish whether these lipids could participate in the pigment synthesis. Lipids from putamen, premotor cortex, cerebellum, and substantia nigra organelles were extracted by using a modified Bligh–Dyer procedure and prepared for liquid chromatography/mass spectrometry (LC/MS) analysis as described in ref. 16. The dominant lipid components are dolichol and dolichoic acid (Fig. S5), and the distribution of chain lengths for both lipids is similar for all of the organelles studied. These findings also confirm that the unsaturated aliphatic component of these pigments revealed by NMR derive from the dolichols present in organelles. A considerable number of glycerophospholipids, glycerolipids, and sphingolipids are also found by LC/MS but were not quantified.

Chemical Degradation Analysis Shows That the Melanin Component of Pigments Is Generated by Oxidation of Cysteinyl-DOPA (Cys-DOPA) and Cys-DA. The structure of the melanic component of these pigments was characterized by analyzing the products of oxidative (H_2O_2) and reductive (HI) chemical degradation (13, 17). As shown in Table 1, these pigments are mixed melanins, containing both eumelanin and pheomelanin moieties. Neuromelanin from subtantia nigra gave a pattern of degradation products similar to that reported (13). Notably, the high ratios of pyrrole-2,3-dicarboxylic acid (PDCA)/pyrrole-2,3,5-tricarboxylic acid (PTCA) and 4-amino-3-hydroxyphenylethylamine (4-AHPEA)/4-amino-3-hydroxyphenylalanine (4-AHP) are characteristic of DA-melanin with a partial incorporation of Cys-DA units (13). The yield of degradation products shows a lower melanic component in neuromelanins of

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putamen, premotor cortex, and cerebellum than in substantia nigra. The high ratios of PTCA/PDCA and 4-AHP/4-AHPEA in pigments from putamen, premotor cortex, and cerebellum indicate that they derive mostly from DOPA and Cys in the form of Cys-DOPA, but not from DA and Cys. The presence of large amounts of thiazole-2,3,5-tricarboxylic acid (TTCA), thiazole-4,5-dicarboxylic acid (TDCA), and 4-AHPEA or 4-AHP in the degradation products of pigments of putamen, premotor cortex, cerebellum, and substantia nigra suggests that Cys-catechols (Cys-DA or Cys-DOPA) are present in all of these regions and are oxidized to form the melanic component of pigments. Cys-catechols are produced by interaction of reactive quinones and Cys, and through this reaction Cys blocks the quinones, which otherwise would react with key proteins causing cellular damage. This suggests that the synthesis of pigments in these regions is a neuroprotective process removing cytotoxic quinones by converting them to Cys-catechols and finally to melanic pigments. In fact, small, but detectable amounts of free Cys-DOPA or Cys-DA are present in pigments from putamen, premotor cortex, and cerebellum, besides substantia nigra. In addition, large amounts of Cys-DOPA or Cys-DA are detected in the HCl hydrolysate of the pigments, indicating that quinones of DOPA or DA are trapped by Cys residues of proteins. Furthermore, DOPA and DA are also detected at very high levels in HCl hydrolysate of substantia nigra pigment. The pigments from putamen, premotor cortex, and cerebellum appear to be derived from DOPA, but not from DA, with interaction of Cys, as judged from the high ratios of PTCA/PDCA, 4-AHP/4-AHPEA, and free and protein-bound Cys-DOPA/Cys-DA.

The amino acid analysis reveals a relatively low peptide component, accounting for ≈15% of the pigment (Table S1), with a relative amino acid distribution that is similar for all pigments in the brain regions studied.

Pigments Have Low Oxidation Potential and Cannot Induce Redox Processes. Photoemission electron microscopy measurements of the surface photoionization threshold find that all pigments have a threshold potential of 4.6 ± 0.2 eV, which is reflective of eumelanin (Fig. 2*F*). Based on the melanic compositions given by the above chemical degradation and the data obtained by spatial imaging, these results establish that all pigments studied have a spherical structure consisting of a pheomelanin core encased by eumelanin (12). The threshold potential corresponds to an electrochemical potential of −0.2 V vs. normal hydrogen electrode, and so the surface of intact pigments is not thermodynamically positioned to trigger oxidative damage in neurons.

All Pigments Have the Same Spacing in Their Stacking of Structure Components. When wide-angle X-ray scattering was used to examine the noncrystalline samples of pigments, the result was striking: all of the spectra showed a single peak corresponding to a stacking spacing of 4.67, 4.65, 4.65, and 4.72 Å for pigments from putamen, premotor cortex, cerebellum, and substantia nigra, respectively. The similarity of these values shows that all pigments belong to the same class of substances, having the same skeleton with at most minor differences in substituent groups. The spacing values are very different from those of other melanins, which are typically close to 3.45 Å (Table S2). The coincidence between the X-ray diffraction pattern of pigments and that of the cross-β sheet structure of amyloid fibrils (18) is intriguing. Indeed, protein amyloid fibers appear to act as templates for melanin synthesis in melanosomes (19), although the pathway of melanic pigment synthesis in the brain may be different.

An attempt to measure the molecular mass of the pigments by using MALDI-TOF did not produce any molecular ion signals in the range 2,000–100,000 Da.

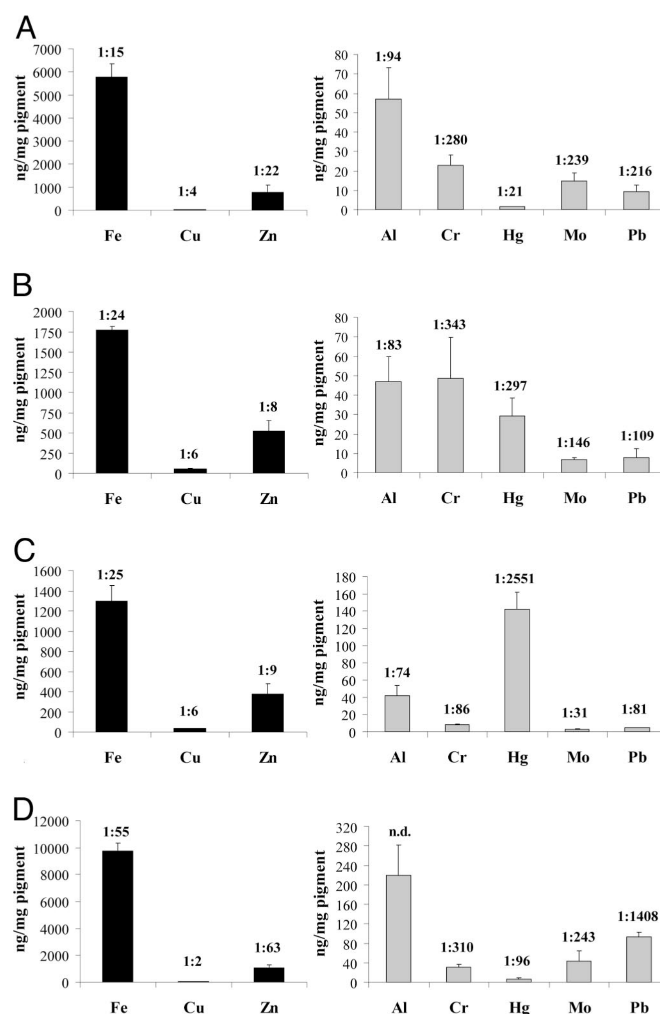

Fig. 3. Concentration of metals in pigments from putamen (*A*), premotor cortex (*B*), cerebellum (*C*), and substantia nigra (*D*). Values are nanograms per milligram pigment (mean ± SEM; $n \geq 2$), and relative ratios of accumulation are metal concentration in pigments per metal concentration in the brain region.

Pigments Accumulate Large Amounts of Environmental Toxic Metals. The ability of melanic pigments to accumulate metal ions was studied to evaluate their role in metal pathways and homeostasis for physiological metals (iron, copper, and zinc) and in their ability to sequester toxic metals that can arise from environmental exposure (aluminum, lead, chromium, molybdenum, and mercury). Metal content for the isolated pigments was analyzed by neutron activation and atomic absorption analysis. The corresponding concentrations in the tissues were also determined. It is striking that the pigments from all of the brain regions studied were able to accumulate large concentrations of metals selectively compared with tissue concentrations in each brain region (Fig. 3). In pigments of putamen, premotor cortex, cerebellum, and substantia nigra there was a low to moderate accumulation of iron (15- to 55-fold) and zinc (8- to 63-fold) compared with tissue. In pigments from all regions, there were high accumulations of aluminum (74- to 94-fold) and chromium (86- to 343-fold). A high accumulation of molybdenum (146- to 243-fold) was found in all pigments except that from the cerebellum (only 31-fold). Impressive accumulations were observed for lead (1,408-fold) in the substantia nigra pigment and for mercury (2,551-fold) in the cerebellum pigment. The large difference in metal uptake by the various pigments is probably dependent on availability of reactive metals because the metal fraction binding to pigments is the reactive form, which is the

potentially neurotoxic part of metal pool. These binding data clearly establish that pigments play a protective role by sequestering toxic metals through the formation of stable-insoluble complexes.

Discussion

The data presented above demonstrate that pigments present in putamen, premotor cortex, cerebellum, and other regions of the brain are substances that bear similar structure and partially resemble the neuromelanin present in the substantia nigra. For the sake of simplicity and classification, in the ensuing discussion all of these pigments from the different brain regions studied will be termed "neuromelanins," to embrace a family of pigments, including both those described here and those we characterized in substantia nigra and locus coeruleus (2, 15). Neuromelanins appear to be ubiquitous in the human brain and are contained in high concentrations, ranging from 0.2 to 3.5 mg/g tissue in the largest areas like putamen, premotor cortex, and cerebellum, in addition to substantia nigra.

Imaging data indicate that neuromelanins are found together with abundant lipids within organelles. In tissues, the number of neuromelanin organelles increases with the concentration of neuromelanin in the corresponding region in the order cerebellum $<$ premotor cortex $\cong$ putamen $<$ substantia nigra.

It is interesting to note that pigments of all of the brain areas have granules of the same size (200–500 nm), and these granules comprised substructures of the same size (30 nm). There is also striking analogy of the surface oxidation potential of these pigments (4.6 $\pm$ 0.2 eV), indicating the presence of eumelanins on the surface, and therefore establishing that the pheomelanin component is in the core of granular substructures. Such a low surface oxidation potential shows that neuromelanins cannot be the initiators of redox processes, with neurotoxic effects.

Both EPR and UV spectroscopy show the presence of an extended aromatic system typical of melanins; however, the sum of intensities corresponding to $g{\sim}4 + g{\sim}2$ signals in the EPR experiments established that the content of this aromatic/melanic component is much higher in neuromelanin of substantia nigra than in neuromelanins of putamen, cortex, and cerebellum. This difference in the content of the melanic component of the pigments is also reflected in the chemical degradation analyses, which show that the concentrations of the degradation products are higher in neuromelanin of the substantia nigra than the neuromelanins isolated from other regions.

Chemical degradation analysis establishes that the melanic component of all neuromelanins contains dihydroxyindole and benzothiazine groups, suggestive of mixed melanins containing both eumelanin and pheomelanin. These neuromelanins are strong metal chelators because of the presence of dihydroxyindole groups. Neuromelanins from putamen, premotor cortex, and cerebellum all have a melanic component partially similar to that of substantia nigra. An important difference, however, is found in the precursor of the pigments. For substantia nigra, the precursor is DA, whereas in other regions the precursor is DOPA.

In fact, the pigments of putamen, cortex, and cerebellum are generated by oxidation of DOPA and Cys-DOPA, whereas that of substantia nigra is generated by oxidation of DA and Cys-DA. The presence of different Cys-catechols as precursors of neuromelanin synthesis in these brain regions is in agreement with the different types of neurons present in the regions. In fact, the substantia nigra has many neurons containing DA, and its neuromelanin is generated by Cys-DA. In putamen, neuromelanin is likely synthesized within a particular type of neurons expressing tyrosine hydroxylase, the key enzyme for DOPA synthesis. These neurons are present in human and non-human primates and contain a high amount of DOPA; here, the precursor of neuromelanin synthesis is indeed Cys-DOPA (20, 21). In brain cortex, the presence of neurons containing tyrosine hydroxylase able to synthesize DOPA was also reported, and this is in agreement with our finding that DOPA is the precursor of neuromelanin identified in this region (22). In cerebellum, no description of neurons expressing tyrosine hydroxylase is available; however, the synthesis of neuromelanins could take place in neurons having this enzyme, similar to those of putamen and cortex.

The greatest functional importance lies in the fact that the synthesis of neuromelanins in each region removes cytotoxic quinones, DA-quinone and DOPA-quinone, as demonstrated for neuromelanin of substantia nigra (7, 13). The significance of free Cys-DA and Cys-DOPA as products of detoxification in the brain and the formation of protein-bound Cys-DA conjugates in neurons damaged by DA exposure has been suggested (23, 24). However, protein-bound Cys-DA and Cys-DOPA have never been detected in neuromelanin, possibly because of the low level of the metabolites and technical difficulties in their detection. Here, detection of protein-bound Cys-catechols was successfully achieved by using isolated neuromelanin granules that trapped DA-quinone and DOPA-quinone.

In all neuromelanins, an important aliphatic component is present, as shown by NMR spectra and elemental analysis. The relative abundance of aliphatic H vs. aromatic H demonstrates that unsaturated aliphatic chains are a major component of the pigments. This is also confirmed by the high elemental ratio of H/C ($>$1.5), considering that most of the melanic carbons bear no hydrogen atoms. The ratios of saturated H vs. unsaturated H of the lipid components observed in NMR spectra correspond to that of the polyisoprenic system of dolichols. In fact, polyisoprenic molecules like dolichols and dolichoic acids have been extracted and identified during isolation of the pigments from brain tissues. Moreover, the analysis of lipid deposits present in pigmented organelles separated from brain regions showed the presence of dolichols.

The organelles are hypothesized to contain the enzyme(s) required for dolichoic acid synthesis, although the function and turnover of this lipid remain to be determined (16). Based on the considerably high concentration of isoprenoids separated during isolation of neuromelanins and the presence of polyunsaturated aliphatic chains present in the NMR spectra of neuromelanins, we propose that these compounds participate in neuromelanin synthesis. Dolichols may bind to the melanic moiety through metal-induced radical reactions involving C—H groups adjacent to double bonds.

One more striking similarity in the structure of these pigments is that of stacking distances measured by X-ray scattering. These distances are much higher than those measured in natural and synthetic melanins. This shows once more that neuromelanins are a special class of melanins, with features recalling the peculiar amyloid cross-β sheet structure. Whether the stacking organization of neuromelanins involves protein components is currently not known. Amyloid fibril proteins vary greatly in amino acid sequence and size and often require participation of additional components and/or seeding (25). There is the possibility that peptide linkage to the growing melanic structure drives its structural organization, as in melanosomes, but clarification of this point must await the identification of the peptide composition of neuromelanin. Regarding melanosomal melanin, it should be recalled that the synthesis of melanins in skin and various organs requires tyrosinase, whereas the presence of this enzyme in the brain is currently excluded (26). Here, the synthesis of neuromelanins likely requires more than a single enzyme because different types of precursors are involved, including catechols, lipids, and peptides. Neuromelanins bear special characteristics that have not been reported in other classes of compounds involved in aging, such as lipofuscins, as discussed above. The localization of neuromelanins in the subcellular organelles is another special feature of these compounds.

The origin of the peptide component in neuromelanin is not clear. The presence of DOPA- and DA-peptide conjugates could suggest that in the first step of neuromelanin synthesis, DA or DOPA reacts with Cys residues of proteins. The protein adducts

thereby formed are cleaved by proteases present in organelles to generate small peptides that would further react with DA- or DOPA-quinones to form oligomers of melanic component of neuromelanins. The aliphatic component of neuromelanin could be inserted by the addition of dolichol residues to the melanic/protein components.

Quantification of the metal ions bound to different neuromelanins confirms that these pigments are strong metal chelators. This functional property of the pigments is caused by the presence of dihydroxyindole groups. There is an impressive selectivity in the accumulation of metals by the pigments from different brain regions, most likely because of differing availability of reactive metals in each brain region. Iron and zinc are found at highest concentrations in neuromelanins. However, metals with highest accumulation ratios (neuromelanin/tissue) are chromium, molybdenum, lead, and mercury. These environmental metals can enter the body and reach the brain by inhaling polluted air and eating food with a high content of metals. Neuromelanin in the cerebellum accumulates an enormous amount of mercury, showing that a large amount of this metal enters cerebellum neurons. This selective entry could explain the high susceptibility of cerebellum neurons to mercury toxicity. Indeed, mercury exposure induces motor deficits, targeting cerebellar cells (27, 28). Neuromelanin in the substantia nigra also accumulates an extremely high amount of lead. Such a high influx of lead into dopaminergic neurons could underlie the reported increase of risk for Parkinson's disease in occupational exposure to lead (29). In the past, people were exposed to organic lead by leaded gasoline, and that lead could cross the blood–brain barrier. However, upon entering neurons, the lead is efficiently bound by neuromelanin, forming a stable complex still detectable several years after the end of human exposure to leaded gasoline. The high accumulation of environmental metals into neuromelanin, with respect to the content in brain tissues, shows that neuromelanins continuously accumulate metals during aging without turnover. Thus, neuromelanins appear to be the most effective system for scavenging and long-term immobilization of toxic metals that invade neurons.

The neuromelanins recognized here are present throughout the human brain and accumulate over a lifetime. Their production and accumulation in putamen, premotor cortex, cerebellum, and substantia nigra during aging appear to be an important physiological process that serves both to remove toxic quinones and to bind and immobilize toxic metals. Because they have no turnover, neuromelanins constitute an effective and stable system for immobilizing/detoxifying the reactive/toxic metals in human body over its life span. Because substantial amounts of neuromelanins are found in all major brain regions, their detoxifying role is relevant throughout the human brain, and because neuromelanin accumulation takes place over the entire life span, the neuromelanin pigments contains the story of one's life exposure to several endogenous and environmental metals.

Materials and Methods

Because many different methods have been used in this work, we describe here the general scheme of methodology; for detailed procedures, see *SI Methods*.

Specific regions (putamen, premotor cortex, cerebellum, and substantia nigra) were dissected from normal human brains. Slices of these brain regions were used for electron microscopic investigations of organelles containing melanic pigments in neurons. From each brain region, the organelles containing the pigment were isolated by gradient centrifugation. UV-visible spectra were recorded on the pigments isolated from these isolated organelles.

The concentration of pigments in brain regions of subjects at different ages was measured. Large amounts of pigments were isolated from each brain region and used for the following structure determinations.

Imaging of size and shape pigments granules with scanning electron microscopy. EPR spectroscopy was used to detect stable free radical structure and interaction with iron.

Proton NMR spectra were recorded on all pigments in deuterated DMSO. The lipids adsorbed to the pigments were extracted with solvent and analyzed by LC/MS.

Chemical degradation of the pigments with alkaline H_2O_2 and HI was carried out, and the degradation molecules were analyzed to characterize the structure of melanic components of pigments. Catechols in both free and protein-bound forms were analyzed and correlated with the results of chemical degradation. Photoelectron emission microscopy was used to measure surface photoionization potential of pigments.

X-ray scattering was used for determination of stacking distance. Amino acid analysis was used to measure the peptide content.

Neutron activation and atomic absorption analysis were used to measure the content of metals in pigments.

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