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Neuromelanins in various regions of human brain are associated with native and oxidized isoprenoid lipids

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ABSTRACT

Neuromelanin (NM) isolated from seven regions of the human brain is found to contain series of natural and oxidized isoprenoid lipids. Specifically, dolichols (dol) and dolichoic acids (dol-CA) with 14-22 and 14-21 isoprene units are identified. Standards of nor-dolichol and nor-dolichoic acid were used to determine the relative amounts of dol and dol-CA compared to the total lipids present in NM for each region. The cerebellum, putamen, globus pallidus, and premotor cortex contained similar amounts of dol, comprising approximately 8–9.5% of the total lipid weight. Interestingly, the corpus callosum contains substantially lower quantities of both dol and dol-CA compared to the other regions—less than 4% dol relative to the total lipid weight. Oxidized and reduced dolichol-related species were identified and determined to be region-dependent.

Neuromelanin (NM)[1] is a pigment found in catecholaminergic neurons of select brain regions [1]. Proteins, lipids, and metals are intertwined within the catechol backbone [2–4], with lipids also present in droplets adjacent to pigment aggregates in enclosed organelles [5]. Dolichol (dol) has been reported to comprise ~12–14% of the total weight of NM in the substantia nigra (SN) [6,7]. In 2007, the first observation of naturally occurring dolichoic acid (dol-CA) was detected in NM isolated from the SN, in which ~3% of the total NM weight is dol-CA [6].

Until recently, only NMs from the SN and locus coeruleus (LC) had been characterized [8]. Zecca et al. recently identified neuronal pigments of the NM type in the cerebellum (CB), putamen (PU), premotor cortex (CX), globus pallidus (PA), and other brain regions [9]. Imaging and spectroscopy suggest that NM organelles from different regions are comparable [9].

In brains afflicted with Parkinson's disease (PD), NM organelles are degenerated in the SN pars compacta, releasing NM and toxins. Increased toxins and alterations in the organelle composition in PD brain regions result in oxidative stress, free radical production, and the consequential lipid peroxidation [10]. The present study examines the region dependence of isoprenoids, providing additional evidence to back the supposition that NM is comparable between the regions. Determining the lipids present in neurologically normal NM will describe important aspects of aging processes which involve accumulation of NMs and new lipid pathways.

Herein we demonstrate that the dol and dol-CA chain length distributions are similar between brain regions. However, the NM from the SN contains over twice the amount of dol and dol-CA present in any of the other regions. In addition, oxidized and reduced dolichol-related species are identified and their relative concentrations are found to be region-dependent. Reduction of dol involves hydrogenation of double bonds along the isoprenoid chain. Oxidized products reflect addition of oxygen(s) along the isoprenoid chain.

Experimental procedures

Isolation of NM and lipid extraction

This study was approved by the Institutional Review Board and performed in accordance with the Ethics Committee of Duke University. NM was isolated from the seven regions of human midbrain of neurologically normal adult individuals within 48 h after death and immediately frozen at −80 °C as described previously [11,12]. The dissection of each region from the frozen human brain tissue was performed on a cold plate at −10 °C, homogenized in distilled H_2O (0.03 g/mL) in a glass-Teflon homogenizer, followed by centrifugation at 12,000 *g* for 10 min. The pellets were washed twice with 30 mL of phosphate buffer (0.05 M, pH 7.4)/g of tissue. The pellets were next incubated at 37 °C for 3 h with 20 mL Tris buffer (0.050 M, pH 7.5) containing SDS (5 mg/mL)/g of tissue, as standard protocol for the isolation of NM granules. The suspension

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[1] *Abbreviations used:* dol, dolichol; dol-CA, dolichoic acid; NM, neuromelanin; SN, substantia nigra; CX, premotor cortex; CB, cerebellum; PA, globus pallidus; PU, putamen; CC, corpus callosum; LC, locus coeruleus.

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was centrifuged at 18,000g for 20 min at 20 °C. The supernatant was removed, and 20 mL/g of tissue of the previously described incubating solution containing 4 mg of proteinase K/g tissue was added. The sample was incubated for 3 h at 37 °C. The NM pigment was separated by centrifugation at 18,000g and washed/centrifuged (12,000g) twice with 5 mL of NaCl (9 mg/mL). The NM was then washed/centrifuged (12,000g) with 5 mL of water.

After dialysis against water (to remove low molecular weight compounds and remaining salts), the NM was suspended in 1 mL of methanol, sonicated for 5 min, and centrifuged (12,000g, 30 min, 20 °C). The supernatant containing the lipid component was aspirated. The precipitate was resuspended in 1 mL of hexane and centrifuged identically to the methanol fraction. The solvent extracts were combined and evaporated under nitrogen.

Using the above procedures, lipids were obtained from the following specimens: CB (one subject; 82 y.o.), CX (one subject; 92 y.o.), PA (pooled tissues from 6 subjects; age range 65–82 y.o.), PU (pooled tissues from 3 subjects; age range 82–91 y.o.), CC (pooled tissues from 3 subjects; age range 65–72 y.o.), LC (pooled tissues from 37 subjects; age range 52–89 y.o.), and SN (pooled tissues from 36 subjects, age range 63–99 y.o.).

The dried lipid fractions were dissolved to give a concentration of 1 mg lipid per 100 μL of chloroform. Then 25 μL of each stock solution was transferred to separate vials and diluted to 100 μL in chloroform:dimethyl sulfoxide (DMSO) (1:1, v/v). Nor-dol and nor-dol-CA internal standards, synthesized by Avanti Polar Lipids (Alabaster, AL) with $n = 13–21$, were added to give the concentrations listed in Table 1; 5 μL of the final mixture was injected onto a reversed-phase column for LC/MS analysis as described below. The levels of dol species in NM granules were estimated by comparing the monoisotopic ion abundance of the naturally occurring dol with those of the nor-dol standard [6]. A more rigorous quantitation was not pursued due to sample limitations and the lack of a stable isotope-labeled dol standard. Because there is no commercial standard, the concentration of dol-CA relative to dol in NM was estimated by measuring the signal response ratio of the nor-dol-CA to the nor-dol standards [6], When analyzed by LC/MS, equal weight quantities (50 ng) (about equal molar) of nor-dol and nor-dol-CA yielded $[M+Ac]^-$ to $[M-H]^-$ monoisotopic ion abundance ratio of 1.7. The Q-STAR mass spectrometer used for these measurements was calibrated to an accuracy of at least 0.01 amu, and the measurements were collected using an internal standard differing by a single methyl group. Thus, the reported concentrations are a close estimate. Because of the limited amount of sample obtained from a single subject, the organelles were pooled and, a detailed statistical analysis of the data in Table 2 could not be performed. This method of estimating the ng of isoprenoid per gram of tissue was previously used in the initial report of dolichoic acid (Ward et al.).

HPLC/MS experimental conditions

LC/MS analysis was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps, and a SCL-10A system controller) coupled to a QSTAR XL high-resolution quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). The negative ion TOF mode of Q-Star XL mass spectrometer was calibrated using PPG3000 (Applied Biosystems). HPLC was operated at a flow rate of 200 μL/min with a linear gradient as follows: 100% A was held isocratically for 2 min and then linearly increased to 100% B over 14 min and held at 100% B for 4 min. Mobile phase A consisted of methanol/acetonitrile/aqueous 1 mM ammonium acetate (60/20/20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. Five microliters of the lipid sample dissolved in 50/50 chloroform/DMSO was injected onto a Zorbax SB-C8 reversed-phase column (5 μm, 2.1 × 50 mm) obtained from Agilent (Palo Alto, CA). The post-column split diverted ~10% of the HPLC flow to the ESI source of the mass spectrometer.

Table 1
Amounts of nor-dol and nor-dol-CA standards injected for each region used for quantification.

Region	ng nor-dol injected	ng nor-dol-CA injected
CB	150	30
CX	200	30
PA	150	120
PU	150	120
CC	50	15
LC	100	20

Table 2
Quantification of dol and dol-CA content in NMs isolated from human brain regions.

Region	Dol ng/μg lipid	Dol-CA ng/μg lipid
SN	200	50
CB	84	7.5
PU	94	6.9
PA	81	8.5
CX	86	3.6
LC	2	0.6
CC	8.9	0.5

Results

Table 1 lists the ng of nor-dol and nor-dol-CA standards per μg of lipid of NM isolated for each region. These internally-spiked standards were used to quantify the ng of dol and dol-CA per μg of total lipid for each region, as listed in Table 2.

The structures of dol and dol-CA are illustrated in Fig. 1. Individual species within the class of the two isoprenoids are identified by the chain length, represented as the number of isoprenoid units (i.e. for dol-19 there are 19 isoprenoid units). Fig. 1 brackets the straight portion as n-4 units, illustrating the head and tail, containing unrepeated segments. Fig. 2 displays the chain length distribution of the dol (2A) and dol-CA (2B) present for each region. The

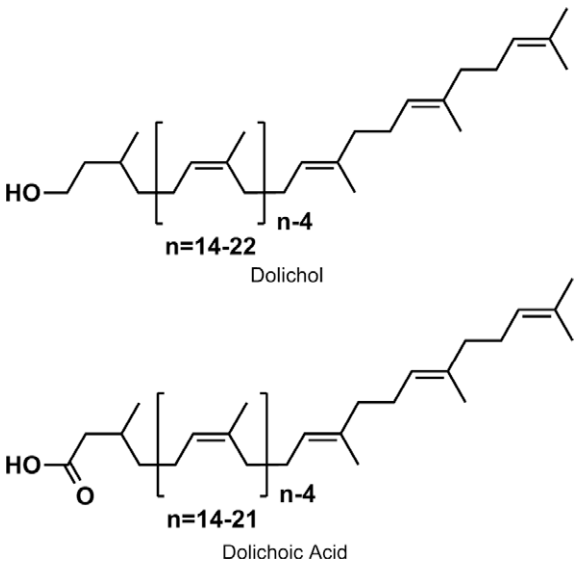

Fig. 1. Structures of dol and dol-CA.