Original research article

DOPA pheomelanin is increased in nigral neuromelanin of Parkinson’s disease

Waijiao Cai\textsuperscript{a, b, c, 1}, Kazumasa Wakamatsu\textsuperscript{d, 1}, Fabio A. Zucca\textsuperscript{e, 1}, Qing Wang\textsuperscript{a, f}, Kai Yang\textsuperscript{b, c}, Niyaz Mohamadzadeh-Deonarvar\textsuperscript{a, f}, Pranay Srivastava\textsuperscript{a, f}, Hitomi Tanaka\textsuperscript{a}, Gabriel Holly\textsuperscript{a}, Luigi Casella\textsuperscript{h}, Shosuke Ito\textsuperscript{d}, Luigi Zecca\textsuperscript{e}, Xiqun Chen\textsuperscript{a, f, 2}

\textsuperscript{a} Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, USA
\textsuperscript{b} Institutes of Integrative Medicine, Fudan University, Shanghai, China
\textsuperscript{c} Department of Integrative Medicine, Huashan Hospital, Shanghai, China
\textsuperscript{d} Institute for Melanin Chemistry, Fujita Health University, Toyoake, Japan
\textsuperscript{e} Institute of Biomedical Technologies, National Research Council of Italy, Segrate, Milan, Italy
\textsuperscript{f} Aligning Science Across Parkinson’s Collaborative Research Network, Chevy Chase, USA
\textsuperscript{g} Department of Medical Technology, School of Health Sciences, Gifu University of Medical Science, Seki, Japan
\textsuperscript{h} Department of Chemistry, University of Pavia, Pavia, Italy

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\textbf{ABSTRACT}

Neuromelanin (NM) in dopaminergic neurons of human substantia nigra (SN) has a melanic component that consists of pheomelanin and eumelanin moieties and has been proposed as a key factor contributing to dopaminergic neuron vulnerability in Parkinson’s disease (PD). While eumelanin is considered as an antioxidant, pheomelanin and related oxidative stress are associated with compromised drug and metal ion binding and melanoma risk. Using postmortem SN from patients with PD or Alzheimer’s disease (AD) and unaffected controls, we identified increased L-3,4-dihydroxyphenylalanine (DOPA) pheomelanin and increased ratios of dopamine (DA) pheomelanin markers to DA in PD SN compared to controls. Eumelanins derived from both DOPA and DA were reduced in PD group. In addition, we report an increase in DOPA pheomelanin relative to DA pheomelanin in PD SN. In AD SN, we observed unaltered melanin markers despite reduced DOPA compared to controls. Furthermore, synthetic DOPA pheomelanin induced neuronal cell death in vitro while synthetic DOPA eumelanin showed no significant effect on cell viability. Our findings provide insights into the different roles of pheomelanin and eumelanin in PD pathophysiology. We anticipate our study will lead to further investigations on pheomelanin and eumelanin individually as biomarkers and possibly therapeutic targets for PD.

1. Introduction

In substantia nigra (SN) and locus coeruleus (LC) of the human brain there are catecholaminergic neurons which accumulate particular autolysosomal organelles. These organelles contain neuromelanin (NM) pigment, lipids, and proteins (Zecca et al., 2008a; Zucca et al., 2018). With aging a continuous accumulation of these NM-containing organelles occurs in neurons of different brain regions, albeit in a lower amount compared to SN and LC, which remain the most heavily pigmented regions of the aged brain (Zecca et al., 2004, 2008a). Loss of pigmented dopaminergic neurons and consequently depigmentation of SN is a pathological hallmark of Parkinson’s disease (PD), a common neurodegenerative disorder manifested clinically by resting tremor, rigidity, bradykinesia, and gait instability (Surmeier, 2018). L-3,4-dihydroxyphenylalanine (L-DOPA or DOPA in this report) is the most commonly used symptomatic treatment for PD (Mueller et al., 2020).

Unlike the well-characterized biosynthesis of melanin in the periphery (i.e., skin), which begins with the oxidation of tyrosine by tyrosinase to DOPA and then dopaquinone, followed by the addition of cysteine (Cys) to form cysteinyldopa (Cys-DOPA) and eventually

\footnotesize{\textsuperscript{1} Corresponding author at: Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, USA.\textsuperscript{1} \textit{E-mail address: xchen17@mgh.harvard.edu (X. Chen).}\textsuperscript{1} Equal contribution.\textsuperscript{2} 114 16th Street, Room 3003, Charlestown, MA 02129–2000.\textsuperscript{2} \textbf{https://doi.org/10.1016/j.pneurobio.2023.102414}\textsuperscript{2}

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pheomelanin or eumelanin when cysteine (Cys) is absent (Fedorow et al., 2005; Zucca et al., 2017), NM in SN is considered mainly a non-enzymatic product from dopamine (DA) iron-mediated auto-oxidation (Fedorow et al., 2005; Sulzer et al., 2000; Zucca et al., 2018). NM has a complex structure with the following components: melanin portion, lipids among which dolichols are the most abundant species, proteins and other constituents including iron and other metals (Engelen et al., 2012; Zucca et al., 2017). The melanic moiety of NM pigment has been identified as composed of benzothiazine (BT), benzothiazole (BZ) and dihydroxyindole units (Wakamatsu et al., 2003), which means that melanic component of NM consists of pheomelanin and eumelanin moieties. Indeed, we have previously performed chemical analyses to elucidate the structure of NM in the SN (Wakamatsu et al., 2003, 2012; Zecca et al., 2008a), which suggested that the melanic part of NM pigment in the SN is mainly derived from DA and Cys in a molar ratio of 2:1 (Wakamatsu et al., 2012).

Our previous studies also indicated DOPA as a precursor for NM in brain regions, including SN, LC, putamen, premotor cortex, and cerebellum (Wakamatsu et al., 2014; Zecca et al., 2008a). It was recently suggested that various catecholic metabolites are incorporated into NM synthesis from the SN and the LC. These compounds are metabolites of DA and norepinephrine formed by oxidative deamination by monoamine oxidase followed by reduction/oxidation (Eisenhofer et al., 2004; Wakamatsu et al., 2015, 2014). The involvement of enzymes in NM synthesis has also been proposed, including tyrosinase and tyrosine hydroxylase (TH), which converts tyrosine to DOPA and is a marker for dopaminergic neurons. However, to date a neuronal specific enzymatic synthesis pathway of NM has not been unequivocally demonstrated (Zucca et al., 2017). The existence and function of tyrosinase in the human brain are a matter of debate. Studies have shown that tyrosinase is not present in human SN neurons (Ikemoto et al., 1998; Tribi et al., 2007, 2005; Zucca et al., 2018) or it is expressed at very low levels (Miranda et al., 1984; Xu et al., 1997; Greggio et al., 2005; Carballo-Carbalaj et al., 2019). Overexpression of human tyrosinase in rat SN has been reported to result in age-dependent production of melanin within dopaminergic neurons (Carballo-Carbalaj et al., 2019). NM interacts with several metals (Zecca et al., 2008a), quinones from DA oxidation, dopaminergic neurotoxins such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and other xenobiotics (Capucatti et al., 2021), indicating active roles of NM in aging and diseases, especially PD. However, the exact role of NM appears to be complex and can be neuroprotective or neurotoxic, depending on the specific cellular context (Zucca et al., 2017).

Structurally, the casing model of mixed melanogenesis in the periphery (Bush et al., 2006; Ito, 2006), previously applied to brain NM, has been reformulated based on more recent evidence (Monzani et al., 2019). Protein/peptide fibrils constitute the core of NM particles on which quinone molecules produced by oxidative stress react, producing melanin within dopaminergic neurons (Carballo-Carbalaj et al., 2019). NM interacts with several metals (Zecca et al., 2008a), quinones from DA oxidation, dopaminergic neurotoxins such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and other xenobiotics (Capucatti et al., 2021), indicating active roles of NM in aging and diseases, especially PD. However, the exact role of NM appears to be complex and can be neuroprotective or neurotoxic, depending on the specific cellular context (Zucca et al., 2017).

To test the pre-defined hypothesis that pheomelanin is neurotoxic and eumelanin is not, differentiated dopaminergic SH-SYSY cells and primary mouse cortical neurons were treated with synthetic DOPA pheomelanin and synthetic DOPA eumelanin or control vehicle. Each treatment condition was assigned to 3–5 wells at random. Cell viability and cell death were determined by established methods (Cai et al., 2022; Ye et al., 2022; Zheng et al., 2020) by the investigators who were blind to the sample group information. Experiments were independently performed at least 3 times.

2.2. Human postmortem SN samples

Human postmortem ventral midbrain blocks containing SN were provided by the Harvard Brain Tissue Resource Center through the National Institutes of Health (NIH) NeuroBioBank. The cohort includes PD, n = 12, age 70–74 years old, PMI 13.9–35.4 h; AD, n = 11, age 65–74 years old, PMI 6.5–26.5 h; and CON with no neurological disorder, n = 8, 68–73 years old, PMI 18.0–32.7 h. All subjects were Caucasian. The neuropathological assessment confirmed PD or AD diagnosis. The SNpc was dissected from midbrain blocks, and tissues were carefully and mechanically ground without buffer. Aliquots were weighed and frozen at −80 °C for the determination of NM concentration. The remaining aliquots of samples were weighed and freeze-
dried for chemical degradation analyses. All study protocols were approved by the institutional review board at the Massachusetts General Hospital and the institutions of the co-authors.

2.3. Determination of NM concentration

Multiple aliquots of ~5 mg obtained from homogenized SNpc wet tissues were processed to extract and measure NM concentration according to a previously published spectrophotometric method (Zecca et al., 2002). The method was markedly improved to remove interfering tissue components (Cassidy et al., 2019). Three samples from PD group had limited amount of SNpc tissue; we thus prioritized determination of pheomelanin and eumelanin markers and did not measure NM in these 3 PD samples. For each of the 9 PD, 11 AD, and 8 CON samples, NM value have been widely used as a model for dopaminergic neurons (Xicoy moieties of NM using our well-established chemical oxidation and reduction methods followed by high performance liquid chromatography (HPLC) detection of markers for pheomelanin and eumelanin (Ito et al., 2011; Mitra et al., 2012; Wakamatsu and Ito, 2002). Samples were homogenized in water with Ten-Broeck glass homogenizer at a concentration of 10 mg/mL (if samples were < 5 mg, we used 0.5 mL water) and 100 µL aliquots were subjected to the chemical reactions. Samples were oxidized with 1.5% H2O2/K2CO3. After the termination of reaction, the mixtures were left for 20 h at 25 °C to induce secondary production of pyrrole-2,3,5-tricarboxylic acid (PTCA), pyrrole-2,3-dicarboxylic acid (PDCA), and thiazole-2,3,5-tricarboxylic acid (TTCA), the oxidative products from DOPA eumelanin, DA eumelanin, and DA pheomelanin, respectively, followed by HPLC analysis with UV detection (UVD) (Ito et al., 2011). For the reductive reaction, samples were heated with 57% hydroiodic acid (HI) in the presence of H2PO4 at 130 °C for 20 h. Levels of 4-amino-3-hydroxyphenylalanine (4-AHP), the degradative product of DOPA pheomelanin, and 4-amino-3-hydroxyphenylethylamine (4-AHPEA), the degradative product of DA pheomelanin were analyzed by HPLC-electrochemical detection (ECD) (Del Bino et al., 2015; Wakamatsu et al., 2002; Wakamatsu and Ito, 2002). DA and DOPA were analyzed by HPLC-ECD under the same conditions as for 4-AHP and 4-AHPEA, respectively. Both oxidative and reductive reactions were performed on two separate occasions and the averages were reported. Contents of eumelanin and pheomelanin markers were calculated by the comparison with standard solution, and presented as ng/mg dry tissue (Wakamatsu and Ito, 2002). We also calculated all variables using wet tissue weight and found high correlations between dry tissue and wet tissue values.

2.4. Chemical degradation and determination of pheomelanin and eumelanin markers

Aliquots of the freeze-dry SN tissue (ca. 5–7 mg) prepared as previously described were used to determine pheomelanin and eumelanin moieties of NM using our well-established chemical oxidation and reduction methods followed by high performance liquid chromatography (HPLC) detection of markers for pheomelanin and eumelanin (Ito et al., 2011; Mitra et al., 2012; Wakamatsu and Ito, 2002). Samples were homogenized in water with Ten-Broeck glass homogenizer at a concentration of 10 mg/mL (if samples were < 5 mg, we used 0.5 mL water) and 100 µL aliquots were subjected to the chemical reactions. Samples were oxidized with 1.5% H2O2/K2CO3. After the termination of reaction, the mixtures were left for 20 h at 25 °C to induce secondary production of pyrrole-2,3,5-tricarboxylic acid (PTCA), pyrrole-2,3-dicarboxylic acid (PDCA), and thiazole-2,3,5-tricarboxylic acid (TTCA), the oxidative products from DOPA eumelanin, DA eumelanin, and DA pheomelanin, respectively, followed by HPLC analysis with UV detection (UVD) (Ito et al., 2011). For the reductive reaction, samples were heated with 57% hydroiodic acid (HI) in the presence of H2PO4 at 130 °C for 20 h. Levels of 4-amino-3-hydroxyphenylalanine (4-AHP), the degradative product of DOPA pheomelanin, and 4-amino-3-hydroxyphenylethylamine (4-AHPEA), the degradative product of DA pheomelanin were analyzed by HPLC-electrochemical detection (ECD) (Del Bino et al., 2015; Wakamatsu et al., 2002; Wakamatsu and Ito, 2002). DA and DOPA were analyzed by HPLC-ECD under the same conditions as for 4-AHP and 4-AHPEA, respectively. Both oxidative and reductive reactions were performed on two separate occasions and the averages were reported. Contents of eumelanin and pheomelanin markers were calculated by the comparison with standard solution, and presented as ng/mg dry tissue (Wakamatsu and Ito, 2002). We also calculated all variables using wet tissue weight and found high correlations between dry tissue and wet tissue values.

2.5. Synthesis of DOPA pheomelanin and eumelanin

DOPA eumelanin and DOPA pheomelanin were prepared using tyrosinase according to the methods described in detail elsewhere (d’Ischia et al., 2013). The melanin powder was dried by lyophilization and equilibrated with moisture in a desiccator with a saturated CaCl2 solution.

2.6. Human neuroblastoma cell line SH-SY5Y cells

Although not exclusively dopaminergic, differentiated SH-SY5Y cells have been widely used as a model for dopaminergic neurons (Xicoy et al., 2017). SH-SY5Y cells (ATCC, Manassas, VA, USA, Cat# CRL-2266, RRID:CVCL_0019) were seeded at 70–80% density, and the medium was changed to DMEM/F12 supplemented with 15% FBS, 1% penicillin-streptomycin and 10 µM retinoic acid (11320–033, Gibco) for differentiation for 4 days (Encinas et al., 2000).

2.7. Primary mouse cortical neuron cells

Primary cortical neurons were prepared from the cerebral cortex of embryonic day 16–17 mice as previously described (Cai et al., 2022; Ye et al., 2022; Hilgenberg et al., 2007). Cells were cultured in Neurobasal™ medium (21103049, Thermo Fisher Scientific) with 2% B-27™ plus supplement (A3582801, Thermo Fisher Scientific), 2 mM GlutaMAX™ supplement (35050061, Thermo Fisher Scientific). On day 7 in vitro (DIV) cells were treated with synthetic DOPA pheomelanin or synthetic DOPA eumelanin or vehicle PBS.

2.8. Synthetic DOPA melanin

Synthetic DOPA pheomelanin (DOPA:Cys = 1:1) and DOPA eumelanin (DOPA:Cys = 1:0) were suspended in sterile PBS and sonicated overnight.

2.9. Cell viability assessment

Cell viability was assessed using a MTS cell proliferation assay kit (ab197019, Abcam) (Su et al., 2021; Ye et al., 2022). Cells were seeded and differentiated for 4 days. The differentiated cells were treated with synthetic DOPA pheomelanin or synthetic DOPA eumelanin or vehicle PBS to determine the time and dose responses of the cells to the treatment. Culture medium was removed and a 110 µL solution containing 10 µL MTS and 100 µL medium was added to each well and incubated in humidified air containing 5% CO2 at 37 °C for 1 h. Absorbance at 490 nm was measured using a microplate reader.

2.10. Live and dead cell staining

Differentiated SH-SY5Y cells and primary cortical neurons were treated with 40 µg/mL eumelanin or pheomelanin or PBS for 24 h. Live and dead cells were stained using a LIVE/DEAD® cell imaging kit (Invitrogen™ R37601, Fisher Scientific). Cells were incubated for 15 min at 25 °C (Zheng et al., 2020) and then imaged using a confocal laser scanning microscope FV3000 under 10x (primary neurons) or 40x (SH-SY5Y cells) objective lens with FITC and Texas Red™ filter sets with the same camera gain, exposure time and pixel setting for all samples. Integrated optical density of green and red fluorescence generated by live and dead cells were captured randomly from each well using FluoView FV300 confocal microscope under a 60x objective lens. MAP2-positive cells and DAPI and live and dead cells were stained using a LIVE/DEAD® cell imaging kit (Invitrogen™ R37601, Fisher Scientific). Cells were incubated for 15 min at 25 °C (Zheng et al., 2020) and then imaged using a confocal laser scanning microscope FV3000 under 10x (primary neurons) or 40x (SH-SY5Y cells) objective lens with FITC and Texas Red™ filter sets with the same camera gain, exposure time and pixel setting for all samples. Integrated optical density of green and red fluorescence generated by live and dead cells in each random visual field of 1.619999 mm2 (primary neurons) or 0.025312 mm2 (SH-SY5Y cells) were quantified using ImageJ. A total of nine images from three replicate wells were analyzed.

2.11. Immunofluorescent staining for neuronal marker MAP2

Differentiated SH-SY5Y cells were treated with 40 µg/mL eumelanin or pheomelanin or PBS for 24 h. Cells were then processed using Cytofix/Cytoperm™ fixation/permeabilization solution (BD554714, Thermo Fisher Scientific) and blocked with 5% normal goat serum. Primary antibody against microtubule-associated protein 2 (MAP2) was added (30 µg/mL, OSM00036G, Thermo Fisher Scientific) and incubated at 4 °C overnight (Royo et al., 2008; Cai et al., 2022; Ye et al., 2022). Secondary antibody (1:1000, Alexa 594-conjugated, A11012, Thermo Fisher Scientific) was incubated for 2 h at room temperature. Nuclei were stained with DAPI. For MAP2-positive cell counting, three images were captured randomly from each well using FluoView FV300 confocal microscope under a 60x objective lens. MAP2 and DAPI channels were merged, and MAP2-positive cells in each random visual field of 0.045000 mm2 were counted using ImageJ. A total of nine images from three replicate wells were analyzed.
2.12. Statistical analysis

All values were presented as mean ± SEM. For results using the human postmortem samples, statistical analysis was performed using ANOVA to initially compare differences among groups and Tukey test for pairwise comparisons. Analysis of covariance (ANCOVA) was used to compare outcome variables among groups adjusting for gender or other confounding factors as indicated in Results. Linear correlations between analytes were estimated by Pearson correlation. For results from the cell experiments, differences among groups were analyzed using one-way or two-way ANOVA and Tukey post hoc test. \( p < 0.05 \) was considered statistically significant.

3. Results

3.1. Characteristics of study subjects and materials

We obtained postmortem SN samples from 12 patients with PD and 8 CON with no neurological conditions. We also included analysis of postmortem SN tissues from the same tissue bank from 11 patients with AD. Diagnosis of PD or AD was confirmed pathologically. There were no statistical differences in age and postmortem interval (PMI) among PD, AD, and CON subjects (Table 1). All subjects were Caucasian.

3.2. Reduced NM and DA in postmortem PD SN, not in AD SN

We dissected SN pars compacta (SNpc) and determined DA and its precursor DOPA in the postmortem SN tissues by HPLC coupled with ECD. Consistent with the pathological diagnosis, HPLC revealed markedly lower DA in SN of PD as compared with CON (\( p = 0.001 \), Fig. 1A). DOPA levels in PD were reduced by 30%, not significantly different from CON, which likely reflects exogenous DOPA from PD medications (Fig. 1B). DA level was still significantly lower in PD (\( p = 0.001 \)) when DOPA level was adjusted to control for the confounding effect of exogenous DOPA in PD group. Surprisingly, AD patients displayed significantly decreased DOPA (\( p = 0.019 \)) compared to CON. There were no significant differences in DA and DOPA levels between PD and AD (Fig. 1A&B). The conversion rate from DOPA to DA expressed as DA/DOPA in PD group was lower in PD compared with CON (\( p = 0.020 \)). The rate was unaltered in AD compared with CON (Fig. 1C). It is noteworthy that we calculated DOPA after HI hydrolysis, which affords total DOPA, including Cys-DOPA, DOPA conjugated with proteins, as well as free DOPA.

NM was isolated and its level was measured by spectrophotometry. Consistent with previous studies, PD patients showed decreased NM concentration in SN as compared with CON (\( p = 0.032 \), Fig. 1D). No significant difference in NM concentration was observed between AD and CON groups. Compared with AD, PD showed borderline reduced concentration in SN (\( p = 0.050 \)) (Fig. 1D). NM correlated with DA in CON (\( r = 0.833 \), \( p = 0.010 \)). This correlation is consistent with the current knowledge that NM is derived mainly from DA under normal conditions. Of note, NM and DA were determined independently in different laboratories of the co-authors’. The NM and DA correlation was lost in PD and AD groups (Fig. 1E). DOPA-adjusted NM was still significantly lower in PD group as compared with CON (\( p = 0.013 \)).

3.3. Increased DOPA pheomelanin and reduced DOPA eumelanin and DA eumelanin in postmortem PD SN, not in AD SN

DOPA pheomelanin and DA pheomelanin in the SN were determined based on the formation of 4-AHP and 4-AHPEA, respectively, from HI reductive hydrolysis (Wakamatsu and Ito, 2002) (Fig. S1). HPLC-ECD analysis revealed a significantly higher 4-AHP level (\( p = 0.025 \)) and a trend for the higher rate of DOPA to DOPA pheomelanin conversion (4-AHP/DOPA ratio) in PD SN as compared to CON (Fig. 2A). The difference in 4-AHP between PD and CON was stronger (\( p = 0.003 \)) when DOPA was adjusted to control for exogenous DOPA. There were no differences in 4-AHP and 4-AHP/DOPA ratio in AD group as compared to CON. Compared to AD, PD had a significantly higher 4-AHP level (\( p = 0.015 \), Fig. 2A).

Level of 4-AHPEA, a BT unit marker for DA pheomelanin from HI reductive hydrolysis, was 59% lower in PD SN as compared with CON (\( p = 0.005 \), Fig. 2B). However, PD patients showed a greater 4-AHPEA to DA ratio in SN than CON (\( p = 0.042 \), Fig. 2B). Level of 4-AHPEA in AD SN was not statistically different from CON, and AD group showed unchanged 4-AHPEA to DA ratio as compared to CON (Fig. 2B). PD displayed no significant difference in 4-AHPEA from CON when DA was adjusted in the analysis, suggesting that the unadjusted difference may be explained by lower DA in PD despite the higher conversion rate as reflected by 4-AHPEA to DA ratio.

Alkaline \( \mathrm{H}_2\mathrm{O}_2 \) oxidation (AHPO) was performed, and TTCA, a BZ unit marker for DA pheomelanin, was determined by HPLC-UVD. PD SN demonstrated a significantly lower level of TTCA as compared with CON (\( p = 0.039 \), Fig. 2C). However, a markedly higher TTCA/DA ratio was revealed in PD vs CON (\( p = 0.012 \)) and vs AD (\( p = 0.008 \)). TTCA and TTCA to DA ratio were not different in AD as compared with CON (Fig. 2C).

DOPA eumelanin marker PTCA and DA eumelanin marker PDCA from AHPO of the SN tissues were determined by HPLC-UVD. Reduced PTCA levels were revealed in PD SN as compared with CON (\( p = 0.049 \)). The borderline significance disappeared when DOPA was adjusted. PDCA was similarly lower in PD SN than CON with (\( p = 0.016 \)) or without adjusting for DA levels (\( p = 0.010 \)). PTCA and PDCA levels were not different in AD as compared with either CON or PD (Fig. 2D&E). PTCA to DOPA and PDCA to DA ratios among all three groups were not significantly different (Fig. 2D&E).

As a result of changes in DOPA pheomelanin and DA pheomelanin, DOPA incorporation into pheomelanin as reflected by the ratio of 4-AHP/4-AHPEA was significantly higher in PD SN as compared with CON or AD (\( p = 0.036 \) vs CON, \( p = 0.005 \) vs AD), whereas DOPA eumelanin to DA eumelanin ratio PTCA/PDCA in PD SN was not different from CON and AD (Fig. 2F).

The ratio of pheomelanin/eumelanin is not only an indicator of NM composition but also melanin-related oxidative stress. A significantly greater 4-AHP/PTCA, which represents DOPA pheomelanin to DOPA eumelanin ratio, was revealed in PD SN as compared with CON or AD (\( p = 0.011 \) vs CON, \( p = 0.045 \) vs AD, Fig. 2G). There was no difference in 4-AHP/PTCA between AD and CON. The 4-AHPEA to PDCA ratio, which reflects DA pheomelanin to DA eumelanin ratio from HI hydrolysis and AHPO, respectively, in PD SN and AD SN were not different from CON. Similarly, there was no difference among PD, AD, and CON groups in TTCA/PDCA, another DA pheomelanin to DA eumelanin ratio from AHPO (Fig. 2G).

Original p-values and gender adjusted p-values for all the comparisons are included in Table S1.

We additionally explored Cys, cystine, and oxidized glutathione (GSSG) in the remaining SN samples from PD, AD, and CON subjects by HPLC-UVD at 294 nm (Imai et al., 1987; Tanaka et al., 2018). Compared to CON, there was a trend for lower cystine to Cys ratio and higher Cys to cystine+cyt in PD (Fig. S2A). Although suggestive for lower Cys to cystine conversion and possibly more available Cys for pheomelanin production, these results need to be further validated due to limited...
remaining sample sizes. GSSG levels in PD SN were not different from CON or AD (Fig. S2B). Reduced glutathione (GSH) was not detectable in all samples.

3.4. Synthetic DOPA pheomelanin, not DOPA eumelanin, induces neuronal cell death in differentiated SH-SY5Y cells and in primary neurons

The above results from the human postmortem study show that DOPA pheomelanin is more concentrated in NM of PD subjects than that of CON. To explore the roles of DOPA pheomelanin and eumelanin in neuronal cell survival and death, we differentiated SH-SY5Y cells into neuronal cells and treated the cells with synthetic DOPA pheomelanin or DOPA eumelanin or vehicle PBS. At 20, 40, 60, and 80 μg/mL, pheomelanin treatment for 24 h induced a 12%, 21%, 23%, and 24% decrease in cell viability, respectively, which was statistically significant as compared to cells treated with vehicle or eumelanin at the same dose (Fig. 3A). To determine the time course of the melanin effect on cell viability, differentiated SH-SY5Y cells were treated with 40 μg/mL pheomelanin or eumelanin or control PBS. Cell viability was significantly reduced in pheomelanin-treated group by 14%, 21%, and 22% at 12, 24, and 48 h, respectively, as compared to vehicle-treated controls at the same time point (Fig. 3B). Eumelanin treatment did not show any effect on cell viability at any of the doses or time points tested (Fig. 3A,B).

Effects of synthetic pheomelanin and eumelanin on survival of differentiated SH-SY5Y cells were additionally tested by the neuronal marker MAP2 staining. Cells were treated with 40 μg/mL synthetic DOPA pheomelanin or DOPA eumelanin or vehicle PBS for 24 h. MAP2-positive cell counting revealed reduced number of MAP2-positive cells in pheomelanin-treated group as compared to vehicle control (p = 0.011) or eumelanin group (p = 0.023) (Fig. 3C).

We further employed dye staining for live cells, which produce green fluorescence, and dead cells, which produce red fluorescence. Following 40 μg/mL pheomelanin treatment for 24 h, cell death was evident by red fluorescence. Quantified analyses indicated a significant increase in red fluorescence integrated optical density (normalized by total fluorescence density) in cells treated with pheomelanin as compared to cells treated with vehicle (p = 0.046) or eumelanin (p = 0.039). The green fluorescence produced by live cells appeared to show cells that were smaller in size with shorter processes. Eumelanin-treated cells did not show any apparent differences from vehicle-treated cells (Fig. 3D).

Primary mouse cortical neurons were prepared and treated with 40 μg/mL synthetic pheomelanin or eumelanin or vehicle for 24 h. Live and dead cell staining was performed. Pheomelanin treatment similarly induced cell death in primary cortical neurons. Red fluorescence integrated optical density (normalized by total fluorescence density) in cells treated with pheomelanin was significantly higher than that in vehicle (p = 0.018) or eumelanin- (p = 0.006) treated cells (Fig. 3E).

4. Discussion

Pheomelanin and eumelanin possess distinct chemical and biological characteristics (Wakamatsu et al., 2021). Their metabolism and functions in the brain as the typical elements of NM are poorly understood, especially in the context of neurodegenerative disease. The present study identified in PD postmortem SN increased DOPA pheomelanin and increased conversion rates of DA to pheomelanin markers. Eumelanin derived from both DOPA and DA was decreased. In addition, we reported an increase in DOPA pheomelanin relative to DA eumelanin in PD SN. In AD SN, we observed unaltered melanin markers despite reduced levels of DOPA. Furthermore, we showed that synthetic DOPA pheomelanin, not DOPA eumelanin, induced neuronal cell death in vitro.

The reduced DA melanin, both pheomelanin and eumelanin, in PD SN can be attributed to the substantially reduced DA levels at the advanced stage of the disease as a result of dopaminergic neuron degeneration, altered conversion from DOPA (since DA/DOPA was lower in PD SN), and altered DA metabolism (Dorszewska et al., 2014; Masato et al., 2019). This reduction is particularly evident for DA pheomelanin given the unaltered high correlations between Cys-DA and DA in PD as compared to CON. However, the significantly higher ratios of TTCA to DA and 4-AHPEA to DA in PD SN still suggest enhanced DA oxidation and conversion to form prooxidant pheomelanic pigment. Accelerated DA oxidation in PD is supported by considerable literature evidence (Burbulla et al., 2017; Carballo-Carbaljai et al., 2019; Kulikovskaja and Seibler, 2018). Forsnstedt et al. reported that, while SN DA was lower, the Cys-DA/DA ratio was substantially higher in SN in patients with degenerated SN dopaminergic neurons, and that the ratio seemed to be correlated with the degree of SN depigmentation and neurodegeneration (Forsnstedt et al., 1989). Spencer et al. later detected significant increases in cysteinyl adducts of DA in PD SN (Spencer et al., 1998). Together with reduced DA eumelanin, these findings may indicate a shift of the NM production towards imbalanced pheomelanin and eumelanin and as results, oxidative stress despite the unchanged DA pheomelanin to eumelanin ratios.
Unlike DA, DOPA in PD SN was maintained at “normal” levels, likely by exogenous DOPA from DOPA therapies and reduced DOPA to DA conversion. The unaltered DOPA, however, is associated with a decrease in DOPA eumelanin and an increase in DOPA pheomelanin, suggesting further preferential production of pheomelanic over eumelanic DOPA pigment in PD SN in addition to the already favored pheomelanin production in NM synthesis based on casing model (Ito, 2006). DOPA treatment has been reported to lead to increased pheomelanogenesis and increased pheomelanin/eumelanin ratio in melanocytes in vitro (Sato et al., 1987). The abovementioned study from Spencer et al. also detected significant increases in cysteinyl adducts of DOPA in addition to cysteinyl adducts of DA in PD SN (Spencer et al., 1998). Furthermore, the availability of Cys may be another important factor that facilitates the enhanced preference for pheomelanogenesis in PD (Ito, 2006). Cys normally is present in low concentrations in the brain, and pheomelanin biosynthesis could deplete Cys and related antioxidants (Aoyama et al., 2006; Napolitano et al., 2014). However, increased Cys has been reported in neurodegenerative diseases (Paul et al., 2018), including PD likely due to the need to increase antioxidant protection.

Greater DOPA pheomelanin, together with reduced DA pheomelanin substantially increased DOPA pheomelanin accumulation in PD SN to ~90% of DA pheomelanin (4-AHP/4-AHPEA=0.88 in PD). Cause or effect of neurodegeneration in PD, or DOPA therapy-related, the higher levels of DOPA pheomelanin as well as reduced DOPA eumelanin and DA eumelanin in PD revealed dysregulated melanin production as well as redox homeostasis in PD SN (Wei et al., 2018). Although it remains to be determined how the changes in chemical composition in PD SN may affect the external structure of NM with its pheomelanic and eumelanic components on the surface, more pheomelanin and less eumelanin are ultimately associated with thinning protective surface (Bush et al., 2006; Ito, 2006). Depletion of eumelanin will invariably lead to release of bound iron that, together with the exposure of the more redox reactive pheomelanin component, will lead to more oxidative damage. The vicious circle between oxidative stress and neurodegenerative events in the SN likely plays a role in PD progression if not its initiation. Only ~50% of the binding capacity of NM for iron is saturated in normal subjects. It is unclear whether in PD this binding ability of NM for iron can be saturated and reactive iron can be available to generate toxic effect (Shima et al., 1997).

The relevance of the postmortem findings to PD pathophysiology is...
pheomelanin, may increase expression and aggregation of the key PD (Surmeier, 2018; Zecca et al., 2008a), notwithstanding eumelanin or protein normally occurs in human SN dopaminergic neurons during aging (Carballo-Carbajal et al., 2019; Zucca et al., 2018, 2017, 2014). Our findings in vitro provided evidence that the pheomelanin and eumelanin act differently in neuronal cells. Pheomelanin and eumelanin may differentially impact microglia and indirectly affect neurons will need to be elucidated, especially in in vivo settings. Future studies using other cellular and animal models as well as human samples will be necessary to better understand the roles of pheomelanin and eumelanin in PD and their complex interactions with lipids, proteins, metals, and other components of human NM and NM-containing organelles (Carballo-Carbajal et al., 2019; Zucca et al., 2018, 2017, 2014).

The reduced DOPA and DA levels in AD as compared to CON when DOPA was adjusted were unexpected. Despite certain shared epidemiological, clinical, and neuropathologic features, evidence for dopaminergic pathology in AD has not been consistent and conclusive (Han et al., 2018). A meta-analysis found lower levels of DA in patients with AD as compared to controls, however subanalysis showed that the difference was not significant in studies using brain tissues (Pan et al., 2019). Transgenic mice overexpressing a mutated human amyloid precursor protein (APP), a model of AD, displayed age-dependent loss of dopaminergic neurons in ventral tegmental area (VTA) but not SN (Nobili et al., 2017). While not as great as in PD patients, loss of dopaminergic neurons has been reported in AD patients (Zarow et al., 2003), especially loss of VTA volume appeared to correspond to hippocampal size and memory indices (De Marco and Venneri, 2018). No SN pathology was reported in the present study in all but one of the AD patients, who had mild SN neuron loss but not the lowest DA and DOPA values in the group. Except for a trend for reduced PTCA, we did not find changes in NM content or any of the eumelanin and pheomelanin markers in AD as compared with CON. Compared to PD, AD had lower 4-AHP/PTCA, lower 4-AHP level as well as lower ratio of 4-AHP/PTCA. It is unclear whether AD shares common pathological factors, which in the presence of massive release of bound iron from degraded NM, will cause further neurodegeneration with subsequent release of NM from degenerating neurons and microglia activation. This cascade produces a vicious cycle of neuroinflammation/neurodegeneration, which may contribute to progression of PD (Zecca et al., 2008b; Zhang et al., 2011). How pheomelanin and eumelanin may differentially impact microglia and indirectly affect neurons will need to be elucidated, especially in in vivo settings. Future studies using other cellular and animal models as well as human samples will be necessary to better understand the roles of pheomelanin and eumelanin in PD and their complex interactions with lipids, proteins, metals, and other components of human NM and NM-containing organelles (Carballo-Carbajal et al., 2019; Zucca et al., 2018, 2017, 2014).
samples were total DOPA, which consisted of not only free DOPA but also Cys-DOPA, protein conjugated DOPA, and other forms of DOPA. Due to the relatively small sample size, we did not stratify gender differences. Second, DOPA values from postmortem samples were total DOPA, which consisted of not only free DOPA but also Cys-DOPA, protein conjugated DOPA, and other forms of DOPA. Third, although our chemical methods for postmortem tissue assessments were well-validated, the values reported here were degradation markers for pheomelanin and eumelanin, not actual levels of pheomelanin and eumelanin. Lastly, although our previous studies did not identify differences in dopaminergic cell survival between gold suspension control and PBS, we cannot exclude in differentiated SH-SYSY cells and primary neurons possible particle effects from melanin suspension (Zecca et al., 2008b), especially since eumelanin is more insoluble than pheomelanin. In addition, more investigations are needed to elucidate mechanisms underlying the cytotoxic effects of pheomelanin in neurons.

Nevertheless, our findings provide insights into the possible distinct roles of pheomelanin and eumelanin in PD pathophysiology and they form a foundation for further investigation on possible intervention to the dysregulated NM pathways in PD. With advances in the development of NM detection methods (Bradberry, 2020; Cassidy et al., 2019; Sulzer et al., 2018), it may be possible and necessary to distinguish pheomelanin and eumelanin in the human brain as a biomarker for oxidative stress status, aging, and PD. The distinct functions of pheomelanin and eumelanin in the periphery have been extensively probed. High pheomelanin and related oxidative stress in redheaded people carrying loss-of-function variants of MCIR (melanocortin-1-receptor), which activates tyrosinase and facilitates eumelanin synthesis (Nasti and Timares, 2015), have been associated with compromised drug and metal ion binding, abnormal skin sun damage, UV-independent skin aging as well as melanoma risk (d’Ischia et al., 2015; Mitra et al., 2012; Roeder and Fisher, 2016). We have reported the compromised nigrostriatal dopaminergic system in redheaded mice and increased PD risk in red-haired people (Chen et al., 2017a, 2017b). Further investigations should be warranted to better understand whether/how systemic pheomelanin might relate to pheomelanin in the NM of CNS and how it may affect dopaminergic neuron survival.

5. Conclusions

Dopaminergic neurons in the human brain contain NM and are particularly vulnerable in PD. NM pigment consists of pheomelanin and eumelanin, which possess distinct chemical and biological characteristics. We found increased pheomelanin and reduced eumelanin in PD brain as compared to control subjects. Additionally, synthetic pheomelanin promoted neuronal cell death in vitro while synthetic eumelanin did not show neurotoxicity. Our study provides insights into the different roles of pheomelanin and eumelanin moieties in PD pathophysiology. It forms a foundation for further investigations on pheomelanin and eumelanin individually as biomarkers and therapeutic targets for PD.

CRediT authorship contribution statement

Waijiao Cai, Kazumasa Wakamatsu, Shosuke Ito, Luigi Zecca, Xiqun Chen: Conceptualization. Waijiao Cai, Kazumasa Wakamatsu, Fabio A. Zucca, Qing Wang, Kai Yang, Pranay Srivastava, Hitomi Tanaka, Gabriel Holly, Shosuke Ito, Luigi Zecca, Xiqun Chen: Methodology. Waijiao Cai, Kazumasa Wakamatsu, Fabio A. Zucca, Qing Wang, Kai Yang, Pranay Srivastava, Hitomi Tanaka, Gabriel Holly, Luigi Casella, Shosuke Ito, Luigi Zecca: Investigation. Waijiao Cai, Kazumasa Wakamatsu, Fabio A. Zucca, Qing Wang, Kai Yang, Niyaz Mohamadzadehkonvar, Pranay Srivastava, Shosuke Ito, Luigi Zecca, Xiqun Chen: Visualization. Waijiao Cai, Luigi Zecca, Xiqun Chen: Funding acquisition. Xiqun Chen: Project administration. Shosuke Ito, Luigi Zecca, Xiqun Chen: Supervision. Kazumasa Wakamatsu, Niyaz Mohamadzadehkonvar, Xiqun Chen: Writing – original draft. Waijiao Cai, Kazumasa Wakamatsu, Fabio A. Zucca, Kai Yang, Pranay Srivastava, Luigi Casella, Shosuke Ito, Luigi Zecca, Xiqun Chen: Writing – review & editing.

Competing interest statement

The authors declare there are no conflicts of interest.

Data availability

All the raw data have been deposited and can be found at open-access repository through zenodo.com. doi – 10.5281/zenodo.7067188.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pneurobio.2023.102414.

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