Brains from neurologically normal adult individuals were provided from the Austro-German Brain Bank in Würzburg. The Ethics Committee of the University Clinics of Würzburg approved the use of post-mortem human brain tissue. NM was isolated from the substantia nigra, and a portion was treated with protease K as described previously (Double *et al.* 2000). DAM and Cys-DAM were prepared by oxidation of DA in the absence and presence of L-cysteine as reported (Ben-Schachar *et al.* 1991). Melanin was suspended in 10 % dimethyl sulfoxide (DMSO) to be 1 mg/ml, sonicated for 30 s and shaken gently for 3 days for the rehydration at the room temperature under protection from light.

Measurement of apoptosis by morphological observation, FACS, and with LIVE/DEATH assay kit

SH-SY5Y cells were cultured in a 24-well poly-L-lysine-coated tissue culture plate and treated with melanin for 16 h at 37°C. After stained with 50 μM Hoechst 33342 or propidium iodide (PI), the cells were observed with a fluorescence microscope, Olympus Bx60 (Tokyo, Japan) equipped with an epi-illuminator. In other experiments, the cells

were cultured in 6-well culture flasks with melanin (10 μg/ml of Cosmedium-001) for 16 h. After gathered and washed with phosphate-buffered saline (PBS), the cells were stained with Hoechst 33342 (5 μg/ml) at 37°C for 20 min, washed twice with PBS, applied on a glass slide and observed with the fluorescence microscope.

Apoptosis was quantitatively assessed by fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Benton Dickinson, San Jose, CA, USA). The cells cultured in a 6-well culture flask were treated with or without melanin (10-25 μg/ml in the final concentration) for 16 h. To determine apoptosis, the cells were stained with 75 μM PI solution in PBS containing 1% Triton X-100 at room temperature for 5 min in the dark, washed, suspended in PBS, then subjected to FACS analysis (Yi *et al.* 2006). The fluorescence intensity at 560-640 nm (FL-2 channel) was measured with excitation at 488 nm. Cells with a lower DNA content showing PI staining less than G_1 peak were defined to be apoptotic (subG_1 peak).

The cell viability was also measured using LIVE/DEAD viability/cytotoxicity kit according to the manufacture's instruction. The cells cultured in 6-well flasks, treated with melanin (5-25 μg/ml) for 16 h at 37°C, gathered by scraping, washed with PBS, and

suspended with Hanks' balanced salt solution. The cells were stained with 4 μM calcein AM to determine live cell number. Fluorescence intensity of calcein produced by esterase in live cells was measured at 485 nm with excitation at 530 nm in a Corona MTP-600F microplate fluorometer (Corona Electrics, Hitachinaka, Japan). The number of live cells were calculated by comparison of the fluorescence intensity of samples with that of control and expressed as % of the control. The protein content was measured according by Lowry method using DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Measurement of mitochondrial membrane potential, ΔΨm, by FACS

ΔΨm in isolated mitochondria was quantified by FACS using $DiOC_6(3)$. Mitochondria were prepared from the wild and Bcl-2 cells, suspended in DMEM and incubated with 10-25 μg/ml NM or DAM at 37°C for 3 h. After stained with 25 nM $DiOC_6(3)$ for 15 min, the mitochondria were washed, suspended in PBS, and subjected to FACS. The laser emission at 560-640 nm (FL-2) with excitation at 488 nm was used for the detection of ΔΨm.

Western blot analysis for released cytochrome c

To detect cytochrome c released from mitochondria, the wild cells were incubated with melanin (10 μg/ml) for 1 to 6 h, gathered, washed with PBS, and treated with the extraction buffer [50 mM PIPES-KOH buffer, pH 7.4, containing 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol (DTT) and a complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany)]. The supernatant of centrifugation at 11 000 g for 20 min was mixed with an equi-volume of the RIPA buffer (10 mM Tris-HCl buffer, pH 7.5, containing 1% NM-40, 0.1% sodium dodecylcholate, 0.1% SDS, 150 mM NaCl and 1 mM EDTA). The sample (5 μg protein) was subjected to SDS-PAGE with a 10-20% polyacrylamide gel (Bio-Rad, Hercules, CA, USA), and electroblotted onto PVDF membranes (Du Pont, Boston, MA, USA). After blocking with nonfat milk, cytochrome c was visualized by use of antibodies against cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were treated with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, WI, USA) and visualized with an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA).

Assay for activities of caspase 3

The wild and Bcl-2 cells were cultured in 6-well tissue culture flasks in the presence of melanin (10-25 μg/ml) for 16 h at 37°C. The cells were gathered, washed with PBS, then lyzed in the lysis buffer (20 mM HEPES-KOH, pH 7.0, containing 10 mM KCl, 15 mM $MgCl_2$, 1 mM EDTA 2Na, 1 mM EGTA, 1 mM DTT, 250 mM sucrose and the protease inhibitor cocktail). The enzymatic activity was measured in the reaction mixture (20 mM HEPES buffer, pH 7.5, containing 10% glycerol and 2 mM DTT) with a substrate, Ac-DEVD-MCA (10 μM in the final concentration). After incubation at 37°C for 1 h, the fluorescence at 460 nm was measured with excitation at 360 nm in an MTP-600F microplate fluorometer. The fluorescence intensity was compared with standard 7-amino-4-methyl-coumarin (AMC) solution.

Determination of SH residue, GSH and GSSG

SH contents in mitochondria and melanin were quantitatively measured using Measure-iT™ Thiol assay kit, according the manufacture's instruction. The

fluorescence at 520 nm was measured with excitation at 490 nm in the microplate fluorometer. To differentiate the free and pellet-bound SH residues, mitochondria were treated with melanin (2 h at 37°C), incubated with 10 mM sodium phosphate buffer, pH 7.4, in an ice bath for 30 min, centrifuged at 12 000 g for 15 min, and the sediment was washed twice with the phosphate buffer by centrifugation. SH contents in the combined supernatant fractions and the pellets were quantified using the Thiol assay kit. GSH and GSSG in cells and mitochondria were also quantitatively measured using the enzymatic recycling method according to Rahman *et al.* (2006). The cells and mitochondria were centrifuged at 800 g for 5 min, or 12 000 g for 20 min, respectively, and the sediment was dissolved in the extraction medium (0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM EDTA, 0.1% Triton X-100 and 0.6% sulfosalicylic acid). The supernatant of the centrifugation at 12 000 g for 20 min was subjected to the enzyme recycling assay. The cytosol and mitochondria fraction were prepared from the cells treated with 10 μg/ml NM, DAM or 100 μM dopamine for 2 h, according to Muyderman *et al.* (2004). HPLC with electrochemical detection was used to identify and quantify GSH in melanin-treated mitochondria, using conditions reported previously (Naoi *et al.*

1996). GSH was identified and quantified by comparison with GSH standard.

Detection of *S*-glutathionylated protein (PrS-SG) in mitochondria

S-Glutathionylate protein (PrS-SG) in mitochondria were detected by Western blot analysis after non-reducing SDS-PAGE, according to Brennan *et al.* (2004). Mitochondria were incubated with 10-25 μg/ml melanin for 2 h at 37°C, centrifuged at 12 000 x g for 10 min, and the pellets were washed twice with PBS. Mitochondria were suspended in the extraction buffer for PrS-SG (50 mM Tris-HCl buffer, pH 7.5, containing 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 0.05% digitonin and 100 mM maleimide), allowed to stand in ice bath for 10 min, then centrifuged at 12 000 x g for 10 min. The extracts was reconstituted in SDS sample buffer containing 100 mM maleimide instead of reducing agents to block unreacted thiol group and subjected to SDS-PAGE. PrS-SG was visualized with anti-GSH antibody (Virogen, Watertown, MA, USA). To examine the reversibility of glutathionylation, parts of the samples were treated with the RIPA buffer containing 5% β-mercaptoethanol and subjected to SDS-PAGE under reducing conditions using SDS sample buffer. To identify

S-glutathionylated protein in mitochondrial complex I and III, the polyclonal antibodies against complex I and III were used (Tanaka *et al.* 1988).

Statistics

Experiments were repeated at least 3 times, and triplicate or quadruplicate measurements were carried out. The data were expressed as mean ± SD and the difference was evaluated by analysis of variance (ANOVA) followed by Scheffe's F-test. A p value less than 0.05 was estimated to be statistically significant.

Results

Apoptosis induced by NM

After incubation of SH-SY5Y cells with NM, apoptosis was detected by morphological observation. NM and dopamine induced cell death in the cells, and Hoechst 33342 staining showed apoptotic features with condensed nuclei among dead cells detected with PI (Fig. 1 A, Fig. 2 B). DAM was much less cytotoxic than NM and only few cells were positively stained with Hoechst 33342 and PI. The cytotoxicity of NM was

quantitatively analyzed by FACS and apoptotic cells were detected as the subG$_1$ peak (Fig. 1 B). NM treatment increased the number of apoptotic cells significantly to 37.9% of the total cells from 2.23% in control, whereas DAM induced apoptosis in 11.6% cells. Fig. 1 C shows the effects of NM and DAM concentrations on the viability of the wild and Bcl-2 cells as measured quantitatively using FACS. NM at concentrations higher than 10 μg/ml induced apoptosis in the wild cells in a dose-dependent way. Transfection-enforced Bcl-2 overexpression did not prevent apoptosis caused by NM and DAM.

Effects of melanin species and protein component of NM on the cytotoxicity

NM contains protein component in addition to mixed melanin of black eumelanin and brown pheomelanin. SH residues were detected in NM as measured with the fluorometric Thiol assay kit, which can detect GSH, cysteine and related SH compounds in free and protein-bound form. SH content in NM was determined to be 2.42 ± 0.80 nmol/mg melanin, whereas SH was not detected in DAM (Table 1). Protease K treatment of NM reduced the SH contents significantly to 0.57 ± 0.16 nmol/mg melanin,

23% of the un-treated NM. In Cys-DAM synthesized from dopamine in the presence of L-cysteine, high SH content was determined, 3.77 ± 0.19 nmol/mg melanin.

The cytotoxicity of these melanin species was quantitatively measured by calcein staining for live cells (Fig. 2 A). Among 4 melanin classes, only NM reduced the number of live cells, and the protease-K treatment suppressed the NM cytotoxicity. DAM and Cys-DAM were much less cytotoxic than NM and the difference from control was not statistically significant. The cell death was confirmed by histopathological observation after staining with Hoechst 33342 (Fig. 2 B). NM and dopamine induced apoptosis with the typical condensation and fragmentation of nucleus in most of the cells, while P-K NM, DAM and Cys-DAM virtually did not induce cell death.

Apoptosis pathway activated by NM

Mitochondria were prepared from the wild and Bcl-2 cells, treated with NM and then subjected to FACS analyses after stained with $DiOC_6(3)$. Fig. 3 A shows that NM reduced $\Delta\Psi m$ of the wild cells in a dose-dependent way, which GSH did not prevent. In mitochondria prepared from Bcl-2 cells, the reduction of $\Delta\Psi m$ by NM was also observed

(Fig. 3 B).

After treated with NM, cytochrome c was released from mitochondria into cytosol in wild SH-SY5Y cells in a time-dependent way (Fig. 3 C). The cells were treated with NM, DAM and dopamine for 16 h, and the activity of caspase 3 was measured fluorometrically. Fig. 3 D shows the significant increase in caspase-3 activity in the wild cells treated with NM and dopamine. On the other hand, DAM treatment did not affect the activity. In Bcl-2 cells, increase in caspase 3 activities was much less than in the wild cells, but NM still increased the activity markedly. Bcl-2 overexpression completely prevented the increase in caspase 3 activity by dopamine.

Effects of NM and DAM on SH state in mitochondria

Effects of NM and DAM on mitochondrial SH levels were examined. Mitochondria prepared from the wild and Bcl-2 cells were incubated with NM (25 μg/ml) and SH contents were measured for 3 h, and NM significantly increased SH levels in mitochondria from 30 min to 2 h and reached to a plateau (Fig. 4 A). On the other hand, DAM (25 μg/ml) and dopamine (100 μM) significantly reduced SH contents in a

time-dependent way (Fig. 4 B). The effects of four melanin species on mitochondrial SH levels are shown in Fig. 4 C. P-K NM did not increase SH, while DAM reduced SH after 2 h incubation. The increased SH residues were identified to be GSH by use of HPLC, as shown in Fig. 4 D. NM significantly increased GSH levels in mitochondria, whereas P-K NM did not affect and DAM reduced GSH levels, as in the case of the total SH contents measured by the fluorescent assay.

To confirm the localization of NM-increased SH compounds in sub-mitochondrial fractions, mitochondria were treated with these melanins, and differentiated into the soluble fraction and the precipitate. After NM-treatment, the SH contents increased significantly in both the fractions, whereas DAM and especially dopamine reduced SH contents (Fig. 5 A). Cys-DAM markedly increased SH in the soluble fraction and pellets.

NM- and DAM-treated cells were fractionated into mitochondria and cytosol fraction, and GSH contents were quantified by the enzyme-recycling method (Fig. 5 B). NM significantly increased GSH in mitochondrial and cytosol fraction, but DAM did not affect GSH levels.

Table 2 summarizes the total SH, GSH and GSSG contents, and the GSSG/GSH ratio in the wild cells, the subcellular fractions and the isolated mitochondria after NM- and DAM-treatment. NM increased the total SH and GSH contents in the cells, the mitochondria fraction and the isolated mitochondria, significantly ($p < 0.05$), but not in the cytosol. GSSG levels were also increased in these samples after treated with NM. DAM reduced the total SH and GSH contents and increased GSSG levels in the mitochondrial fraction and the isolated mitochondria. NM increased significantly the GSSG/GSH ratio in the cell lysate, mitochondrial fraction and isolated mitochondria and DAM increased the ratio more markedly.

Effects of antioxidant on the cytotoxicity and SH reduction by NM

The involvement of ROS-RNS in NM-induced cell death and increase of mitochondrial SH levels was examined. After incubated with NM in the presence of antioxidants, the cell viability was quantitatively measured by calcein staining. Iron-chelating DFX, SOD and nitric oxide (NO)-scavenging EGCG protect cells from cell death induced by NM, but catalase did not (Fig. 6 A). At the same time, DFX, SOD and EGCG prevented

the NM-induced increase in mitochondrial SH contents, but catalase further increased SH levels (Fig. 6 B).

The effects of NADPH-dependent recycling system on the NM-increased SH levels were examined in mitochondria. NADPH enhanced NM-induced increase in SH levels, but did not affect the levels in control and DAM-treated mitochondria.

Effects of NM on *S*-glutathionylated protein (PrS-SG) in mitochondria

To find the origin of GSH increased in mitochondria by NM, mitochondria prepared from the wild cells were treated with NM and other melanin (10 μg/ml), and the protein was subjected to Western blot analysis for *S*-glutathionylated protein (PrS-SG). Under non-reducing conditions PrS-SG was detected in mitochondria without NM treatment (Fig. 7 A). NM reduced ProS-SG especially with high molecular mass, while other melanin increased PrS-SG. Under reducing conditions, reduction of PrS-SG was confirmed again in NM-treated mitochondria, where PrS-SG proteins with molecular mass higher than 50 kDa were reduced significantly (Fig. 7 B). Mitochondrial complex I proteins were visualized with anti-complex I antibody. Under non-reducing conditions,

NM treatment disaggregated the macromolecular structure of complex I. Complex I proteins with 100-150 kDa disappeared, and complex I subunits with less than 75 kDa increased significantly. On the other hand, other melanin did not affect the high structure of complex I. Under reducing condition the amount and electrophoresis-pattern of complex I subunits did not change by NM-treatment, as shown by Western blot analyses, indicating the reversibility of NM-induced dissociation of complex I subunits. Under non-reducing conditions, the reactivity against anti-complex III antibody was slightly reduced by NM treatment, but other melanin did not affect the amounts and pattern of complex III subunits. In mitochondria prepared from Bcl-2 cells, the same results were obtained.

Discussion

This paper presents a novel role of NM in the pathogenesis of PD. NM induces apoptosis in SH-SY5Y cells through activation of death cascade, which depends on the protein component, SH content and melanin species. In PD NM contents in the substantia nigra reduced to 1.2-1.5 mg/g wet weight from 2.3-3.5 mg/g wet weight (Zecca

et al. 2004), as indicated by loss of dark brown color in this region. However, it remains unclear whether the protein, lipid and inorganic components of NM change the nature in PD. Protein associated with NM granules from normal brains was subjected to the proteomic studies and about 70 kinds of protein were detected, but the accumulation of specified proteins was not confirmed (Tribl *et al.* 2005). In PD brain, α-synuclein is associated with NM (Fasano *et al.* 2003), and NM isolated from PD brains is composed mainly of highly cross-linked, protease resistant protein-like materials (Aime *et al.* 2000). Considering the increase of oxidative stress in parkinsonian brains, proteins in NM granules might be highly modified with ROS-RNS and tend to be more aggregated, which may be accelerated further by dysfunction of the ubiquitin-proteasome system (McNaught *et al.* 2001). However, the cytotoxicity of NM-associated protein itself has never been reported in NM prepared from either normal or parkinsonian brains. In A9 neurons of PD brains, the loss of cholesterol and the aggregation of α-synuclein to lipid in NM were reported by histopathological observation (Halliday *et al.* 2005). However, the lipid components may not be involved in the toxicity of NM observed in this paper, since lipid is washed out during the purification procedure (Double *et al.* 2000).

The role of protein component in the NM toxicity was clearly demonstrated in this paper. Protease-K treatment totally reduced NM cytotoxicity, and synthesized DAM and Cys-DAM did not induce the cell death in SH-SY5Y cells under used conditions. In NM, SH residues were detected as exposed on the surface, which protease K-treatment reduced to one fourth, suggesting that a major part of SH groups is derived from the protein and the rest from the pheomelanin component. This result may be comparable with the previous result that protease K-treatment reduced amino aid contents from 165 to 57 μg/mg melanin (Double *et al.* 2000). According to the reported amino acid composition, the cysteine content of NM is 10.6 ± 3.7 nmol/mg melanin (Double *et al.* 2000). SH level in NM is quantified to be 2.42 ± 0.80 nmol/mg melanin, suggesting that most of cysteine is sequenced in protein as intra- or inter-disulfide bond, or occurs as the mixed disulfide bond between protein SH and GSH, cysteine or related SH derivatives. In pheomelanin produced from 5-S-cysteinyl-dopamine a conjugate of cysteine with *o*-quinone, free SH residues were detected, even though pheomelanin has been considered to polymerize into a benzothiazine structure.

NM and DAM affected SH state in mitochondria in quite opposite ways. NM and

Cys-DAM increased SH contents in mitochondria, whereas DAM and dopamine reduced them markedly. These results might be comparable to the previous results that DAM increased ROS-RNS and induced cell death in neuronal SK-N-SH cells, whereas NM protected the cells from hydroxyl radicals produced by the Fenton reaction (Li *et al.* 2005). Melanosomes containing eumelanin and pheomelanin have oxidation potentials of - 0.2 and + 0.5 V, respectively (Samokhvalov *et al.* 2005). Pheomelanin in NM and Cys-DAM may reduce disulfide bonding and release GSH or cysteine from the mixed disulfide bonding and increases SH content in mitochondria, while eumelanin and dopamine oxidatively modify SH residues. These results are relevant with the fact that NM has the surface oxidation potential different from synthesized DAM (Bush *et al.* 2006).

The role of NM-associated protein was examined in concern to regulation of mitochondrial "redox state", which is related to many physiological and pathological phenomena of cells (Schafer and Buettner, 2001; Mahler 2006). Redox state depends on the reduction potential of redox pairs, GSH/GSSG, reduced/oxidized thioredoxin [$Trx(SH)_2$/TrxSS] and NADPH/$NADP^+$. GSH/GSSG pool is the largest in cells and a

major indicator of the redox state. Most glutathione is localized in the cytosol, but there is a GSH pool in mitochondria, which accounts for about 15% of the total (Meister 1995). GSH reduces thiol modifications of disulfide bonding and thioesters, and it is a substrate of protein *S*-glutathionylation. *S*-Glutathionylation is reversible and transitional in cytosol, where mM order of GSH and ascorbic acid are present, but in mitochondria with high oxidative environments (Table 2), *S*-thiolated proteins are more stabilized and detected in complex I, as reported here and by Taylor *et al.* (2003). Glutathionylated $NADP^+$-dependent isocitrate dehydrogenase was detected in brains from a PD model of MPTP-treated mice (Kil and Park, 2005). Under oxidative conditions, actin, glyceraldehyde-3-phosphate dehydrogenase, protein kinase, HSP27, protein-tyrosine phosphatase 1B, protein kinase Cα are the substrate of *S*-thiolation (Eaton *et al.* 2002). In physiological conditions or mild oxidative stress, thiols in protein (PrS-SG) or GSH are reversibly modified into active intermediates, such as thiolate, sulfenate and sulfenic acid, by NO, superoxide, hydrogen peroxide and peroxynitrite (Klatt and Lamas, 2000). The activated protein SH groups reacts with GSH, or *vice verse* activated GSH reacts with reduced protein SH to generate GSH-protein mixed disulfide (PrS-SG). Prolonged

or intense generation of ROS-RNS irreversibly oxidized thiols into protein cysteic acid by sulfonation, which was detected in the brains of patients with PD and Alzheimer' diseases (Choi *et al.* 2005). Glutathionylation is reversed by glutaredoxin (GRX), other thioredoxin (TRX) and protein disulfide isomerase [EC 5.3.4.1], and yields free SH in protein (Pr-SH) and GSH from PrS-SG. This reaction is recycled by TRX reductase [EC 1.6.4.5] or GSH reductase [EC 1.6.4.2] using NADPH as a cofactor.

NM significantly reduces *S*-glutathionylated proteins detected in mitochondria, especially in complex I (Fig. 7). In addition, NM dissociates high structure of complex I into the subunits, and the dissociation is reversed by reducing agents, suggesting that *S*-glutathionylation stabilizes the high structure of complex I under physiological condition. It may be reasonable to consider that SH group of NM (NM-SH) functions as that in GRX, reduces the disulfide bonding in mitochondrial protein, release free GSH or cysteine and exposes free protein SH. Only NM, but not Cys-DAM, reduces mitochondrial PrS-SG, indicating that NM-associated protein, not pheomelanin, affects the mixed disulfide bonding in mitochondria. The SH amounts in NM used for these experiments were less than 1% of the total SH content present in mitochondria,