A. 研究目的

神経変性疾患の治療を考えた場合、有用なアプローチの一つは、神経細胞の死のメカニズムを解明することである。本年度の研究の目的は、アポトーシス時のミトコンドリア外膜透過性亢進メカニズムを解析するとともに、アポトーシスとネクローシスに関与することが示唆されているMPTの分子メカニズムの解明と疾患治療のための標的分子の同定に向け、

（1）Cyclophilin Dに結合する因子の同定と、（2）Cyclophilin D欠損マウスの詳細な解析を行った。

B. 研究方法

（1）Baxのアポトーシス誘導機能発現に対するVDACの必要性を検討するために、VDAC2欠損細胞を用いた解析を行った。

（2）Cyclophilin Dに結合する分子を同定するために、免疫沈降法を用いた。Cyclophilin Dに対する良質な抗体が確立されていなかったため、Cyclophilin Dにタグを付けた遺伝子を哺乳動物細胞に導入し、タグに対する抗体を利用し免疫沈降を行った。不安定な相互作用分子の同定も考慮し、数種類のたんぱくクロスリンカーも利用した。Cyclophilin Dと免疫共沈するものを電気泳動により分離し、アミノ酸配列解析により結合因子を同定した。

（3）Cyclophilin D欠損マウスが示す記憶異常と情動行動異常の原因を探るために、マイクロダイアリシス法を用いニューロトランスミッターの遊離をCyclophilin D欠損マウスとコントロールマウスの海馬で比較検討した。

　動物を用いる実験は、全て大阪大学医学部の動物実験安全委員会の許可を得ている。動物愛護上の配慮からの審査基準は以下の通りである。（1）代替え手段がないこと（特定遺伝子Cyclophilin Dの生体内での機能解析のために利用できるのはマウスに限られている）、（2）苦痛を回避する手段を講じている。

C. 研究結果

（1）Baxのアポトーシス誘導機能発現に対するVDACの必要性

　我々は、すでに抗VDAC抗体などを用いた手法により、ミトコンドリアを介したアポトーシスにVDACが重要な役割を演じることを報告してきた。しかし、VDACのアポトーシスへの関与には否定的な報告もあることを考慮し、この問題をより厳密に検討するために、VDACを欠損した細胞株を利用した解析を行った。また、系をより単純化するために、siRNAによるsilencingを利用し、ミトコンドリア膜透過性亢進に関わる機能的に重複した因子BaxとBakを別々に検討する方法を取った。その結果、VDAC2を

欠損したマウス胚繊維芽細胞株において、種々の刺激で誘導されるBax依存的なアポトーシスが強く抑制されることを見出した。一方、Bak依存的なアポトーシスはむしろ亢進した：これは、VDAC2がBakに結合し、その機能を抑制するという既報の結果と一致した。VDAC2欠損細胞に、VDAC2遺伝子を再導入するとBax依存的なアポトーシスへの感受性が回復したことから、「VDAC2はBax依存的アポトーシスに重要な役割を演じる」と結論した。

VDAC2欠損細胞と対照細胞株を比較することで、VDAC2は、Baxの活性化（ミトコンドリア膜への挿入とオリゴマー形成）に必須であることが明らかになった。

（2）Cyclophlin D結合因子の同定
Cyclophilin DはPPIase (Peptidyl prolyl cis-trans isomerase)の活性を有しており、MPTにその活性が必要と考えられているため、その機能発現に結合ターゲット分子が想定される。Cyclophlin Dに結合し、MPTに関与する因子の候補を得るために、Cyclophilin DのC末にFLAGタグを付加したコンストラクトを作製し、293T細胞株に安定導入した。クロスリンカーの存在下あるいは非存在下で、また細胞死誘導刺激前後の細胞株からライセートを作製し、FLAGタグに対する抗体を用いてCyclophlin Dを免疫沈降させ

た。その沈降物をSDS/PAGEで展開し、Cyclophlin Dに結合する分子を複数種類（ゲル上のバンドとして）得た。これらの分子をMASS解析により同定した。現在、得られた分子のMPTへの関与の可能性を解析中である。

（3）CyclophilinD欠損マウスの解析
CyclophilinD欠損マウスが行動異常（記憶障害と情動障害）を呈することを見出したが（論文投稿中）、この分子基盤を明らかにする目的で、海馬におけるニューロトランスミッター（アセチルコリンとグルタメイト）の遊離をマイクロダイアリシス法により検討した。その結果、高カリウムにより誘導されるアセチルコリンとグルタメイトの量が、CyclophilinD欠損マウスでは有意に低下していことが判明した。行動異常の原因の一つになっているものと思われる。

D. 考察
アポトーシス制御の主過程の一つであるミトコンドリア外膜透過性亢進のメカニズムを解明する研究の一環として、そのプロセスに関わるBcl-2ファミリーたんぱくの機能ターゲットの一つとして我々が提唱してきたVDACについて解析を行い、特にVDAC2欠損細胞を利用することにより、Bax依存的なアポトーシスが強く抑制されることを示し、我々のモデルに対するその遺伝学的証拠を得た。アポトーシス時のミトコンドリア外膜透

過性亢進の詳細なメカニズムの理解に役立つ知見である。

今回、我々はCyclophilin Dと相互作用する分子を複数確認したが、これらの分子がCyclophilin Dの機能ターゲットである可能性があり、その詳細な解析はMPTのメカニズム解明に向け重要な情報を与えるものと考えている。さらに、これらの分子は、MPTが関与する疾患の治療薬ターゲットになる可能性もあり、今後の解析が急務である。

MPTやCyclophilin Dの生理的な役割を明らかにするために、Cyclophilin D欠損マウスは有用な材料であり、このマウスの解析から、Cyclophilin D（あるいはCyclophilin D依存的MPT）が記憶や情動性など幾つかの行動に関与することを明らかにしてきた（論文投稿中）。その詳細なメカニズムの理解に向けた解析から、今回、マウス海馬において、高カリウム刺激によるニューロトランスミッターの遊離がCyclophilin D欠損マウスでは有意に低下していることを見出した。行動異常とCyclophilin Dの機能をつなぐための重要な知見であり、Cyclophilin D機能の生化学的基盤を解く上でも重要な発見であると考えている。

E.結論

アポトーシス制御の主過程の一つであるミトコンドリア外膜透過性亢進のメカニズムを解明する研究の一環として、Bcl-2ファミリーたんぱくの機能ターゲットの一つとして我々が提唱したきたVDACについて、遺伝子欠損細胞を利用した詳細な解析を行い、特にVDAC2がミトコンドリア外膜透過性亢進において重要な役割を演じていることを明らかにした。

MPTの分子メカニズムの解明と細胞死における役割を解明するために、MPTに必須分子であるCyclophilin Dの結合分子の探索を免疫沈降法を用いて行い、Cyclophilin Dと相互作用する分子の存在を確認した。

Cyclophilin Dの機能を明らかにする目的で、Cyclophilin D欠損マウスの解析を行い、Cyp D欠損マウスは、記憶障害や情動反応に異常を呈することを示したが、その分子基盤の一つとして、海馬におけるニューロトランスミッターの遊離がCyclophilin D欠損マウスでは有意に低下していることを見出した。行動異常とCyclophilin Dの機能をつなぐための重要な知見を提示した。

F.健康危険情報

　特になし

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III. 研究成果の刊行に関する一覧表

西田友哉、荒川聡子、水田健、藤谷健司、
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**ストレス応答性オートファジーの分子
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神戸
平成２０年１２月１２日

H.知的財産権の出願・登録状況
　　特になし

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III. 研究成果の刊行に関する一覧表

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精神・神経変性疾患治療薬と精神疾患関連遺伝子の発見

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医療薬学分野

准教授　新田　淳美　（にった　あつみ）

一言アピール

神経栄養因子の研究をライフワークに選び、創薬に結びつく研究を行うことを研究者人生の目標としてきました。名古屋大学医学部附属病院教員への着任を機に神経変性・精神疾患治療薬の開発につながる研究を目指すようになりました。神経栄養因子の産生を誘導する化合物が、薬物依存・脊髄損傷・アルツハイマー病・パーキンソン病などに有効であることを示唆する結果を基礎研究で得ています。私達の研究グループでは、shati という新しい遺伝子（名古屋大学にて発見したことから、名古屋城の金の鯱にちなんで命名しました）と脳での機能について全く研究されていなかった piccolo という2つの遺伝子が精神疾患に関連していることを報告しました。これらの遺伝子の機能を調べて、神経・精神系の疾患の原因解明や治療法に結びつくことを目指しています。まずは、これら遺伝子が精神疾患の早期発見のマーカーとならないか調べ、産学連携研究を行いたく思っています。

研究のキーワード

薬物依存、行動薬理、神経再生、神経栄養因子、培養神経細胞、アルツハイマー病、パーキンソン病、酵素免疫測定法、遺伝子導入、抗体アレイ

プロフィール

平成　2 年　岐阜薬科大学薬学部製造薬学科卒業
平成　4 年　岐阜薬科大学大学院薬学研究科
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平成　7 年　名古屋大学大学院医学研究科生理系
　　　　　　医療薬学専攻修了
平成　7 年　岐阜薬科大学薬学部分子生物学教室　助手
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　　　　　　大学院医学系研究科・医療薬学・助教授
　　　　　　（19 年4月より呼称変更にて准教授）

保有技術・機器（医療薬学講座　山田清文教授の研究室として）

【保有技術】・行動薬理学実験（薬物嗜好性・学習記憶・情動性等）・培養神経細胞への遺伝子導入
　　　　　　・神経栄養因子の高感度酵素免疫測定法　・in vivo マイクロダイアリシス　・cDNA サブトラクション
【主な機器】神経細胞へのエレクトロポレーション装置、蛍光透過型顕微鏡、蛍光顕微鏡、行動実験装置、吸光および
　　　　　　蛍光プレートリーダー、リアルタイム RT-PCR、in vivo マイクロダイアリシス装置、小型冷却超遠心機

主な特許・論文・著書

【主な特許】（海外特許への移行状況については本学知財部にお問い合わせください）
・特開 2002-300247　神経栄養因子誘導剤　・特開 2003-279070　抗薬物依存形成剤　・特願 2007-504648　Akt 刺激薬
・特願 2007-505883　薬物依存関連遺伝子　・特願 2006-287639　脳内酸化抑制剤およびその使用

【主な論文】・Identification of piccolo as a regulator of behavioral plasticity and dopamine transporter internalization.
　　　　　　　Mol. Psychiatry, In press
・A novel molecule 'shati' is involved in methamphetamine-induced hyperlocomotion, sensitization, and
　conditioned place preference.　J. Neurosci., 27, 7604-7615 (2007)
・An inducer for glial cell line-derived neurotrophic factor and tumor necrosis factor-α protects against
　methamphetamine-induced rewarding effects and sensitization.　Biol. Psychiatry, 61, 890-901 (2007)
・Tumor necrosis factor-α and its inducer inhibit morphine-induced rewarding effects and sensitization.
　Biol. Psychiatry, 62, 658-668 (2007)
・An analogue of dipeptide-like structure of FK506 increases GDNF expression through CREB activated
　by Hsp90/Akt signaling pathway.　J. Neurosci., 26, 3335-3344 (2006)
・Hydrophobic dipeptide, Leu-Ile, protects against neuronal death by inducing brain-derived neurotrophic
　factor and glial cell line-derived neurotrophic synthesis.　J. Neurosci. Res., 78, 250-258 (2004)

研究 01　精神疾患関連遺伝子

概要

覚せい剤精神病モデルマウスの脳で過剰に発現している遺伝子を検索したところ、新規遺伝子 shati（名古屋大学発という意味を込めて、命名しました）と脳・神経系での研究がほとんどなされていない piccolo という遺伝子を見つけました。これらの遺伝子が精神疾患と関係していることが強く予想されるため、神経・精神疾患との関連について研究を進めている最中です。

この研究の新規性・独創性

shati と piccolo は、情動や運動機能、さらに人間の三大欲にも関係が深いドパミンという神経伝達物質の遊離を調節することを見出しています。ドパミンは統合失調症などの精神疾患やパーキンソン病のような神経変性疾患の key 物質でもあることから、本研究を進めていけば、多くの精神・神経疾患の治療薬になる可能性があります。私たちは両タンパクの研究に必要な多くのツールをすでに作成・現有しています。

産学連携を目指した応用研究

覚せい剤精神病モデルマウスから見出した遺伝子であることから、精神疾患病の早期発見マーカーとしての可能性を考えています。将来的には、脳疾患の遺伝子治療薬への応用を目指したいです。

ピッコロ　　shati
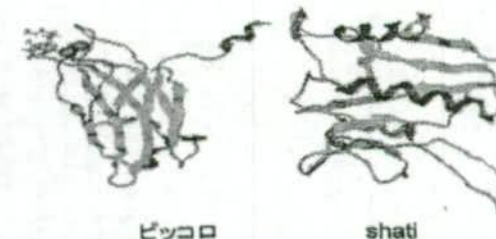
研究 02　多くの種類の神経疾患に対して効果のあるジペプチド

概要

私たち神経化学の研究者ですら神経幹細胞の概念が浸透するまでは、神経細胞は一旦形成されると2度と再生されることはなく、人間の寿命とともに神経細胞数は減少し、神経機能は衰えるのみと考えていました。神経栄養因子とよばれるタンパクは、神経細胞の死滅を防ぐ因子として大変注目されています。しかしながら、それらタンパクの多くは、大きな分子のため、血液中で分解されやすく、血液・脳関門の通過が難しいため、脳疾患の治療薬として用いることは不可能です。私たちは、神経栄養因子の産生を促進する低分子化合物 Leu-Ile が、神経変性疾患の治療薬として用いる可能性を示す研究成果を得ています。

この研究の新規性・独創性

Leu-Ile は、パーキンソン病、アルツハイマー病、薬物依存、脊髄データーを得ています。おもしろいことに、疾病の種類、すなわち生体側の状況によって異なる作用メカニズムが発揮されているようデーターを得ます。おもしろいことに、疾病の種類、すなわち生体側の状況によって異なる作用メカニズムが発揮されているようです。

産学連携を目指した応用研究

Leu-Ile は化合物としてはシンプルなため、用途特許の出願しか難しいようです。多くの作用を持ったジペプチドを世の中に出すお手伝いをしていただけませんでしょうか？

Leu-Ile
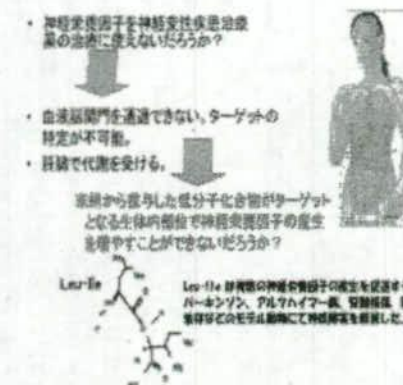
これまでの研究テーマ

・神経栄養因子の高感度酵素免疫測定法の確立
・神経栄養因子産生誘導物質のスクリーニング
・脊髄損傷治療法の確立への基礎研究
・薬物依存形成メカニズムの解明および治療薬の開発研究
・アルツハイマーおよびパーキンソン病治療薬の開発に向けての基礎研究
・ニコチンによる抗うつ作用
・アルツハイマー病モデルの開発および神経機能障害メカニズムの解明
・薬物依存および精神異常に関係する新規遺伝子クローニング
・ドパミントランスポーター過剰発現細胞のクローン化
・うつ病治療薬の開発

The State of Drug Dependence

Associate Professor Atsumi Nitta / Neuropsychopharmacology and Hospital Pharmacy, Graduate School of Medicine

## Seeds 1. A Novel Therapeutic Agent against Drug Dependence

Abstract

Leu-Ile, which induces neurotrophic factors, could be a novel therapeutic agent against not only neurodegenerative diseases such as Parkinson's disease and damage of the spinal cord, but also drug dependence by inhibiting drug-induced place preference and behavioral sensitization. Moreover, we have found that Leu-Ile inhibits neurodegenerative diseases and drug dependence by activating Akt, since Leu-Ile activates Akt signaling.

Novelty and Originality

There are few efficacious medications for drug dependence. Since we have found that Leu-Ile inhibits methamphetamine and morphine-induced dependence, we will investigate the effects of Leu-Ile on nicotine, alcohol, and other drugs of abuse-induced dependence in subsequent studies.

Applied Research Collaboration

Leu-Ile, a hydrophobic dipeptide, is safe, since mice show no toxicity for receiving 1g/mouse of Leu-Ile. Leu-Ile is contained in milk, salmon, red wine, etc. Leu-Ile would also be useful as a functional food as well as a medical drug, if Leu-Ile inhibits nicotine and alcohol-induced dependence.

Inhibitory effects of Leu-Ile on drug-induced dependence
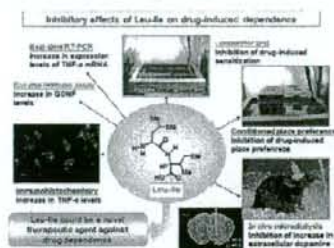
Seeds 2. Involvement of Novel Molecules in Drug Dependence

Abstract

We have identified novel molecules from the nucleus accumbens of mice treated with methamphetamine. We have named one of the novel molecules "shati". The other has been identified as a diabetes-related protein, although there is no report of the physiological role it plays in the brain.

Novelty and Originality

We have found important molecules related to drug dependence. These molecules reduce dopamine release, which blocks drug dependence. We will investigate the relationship between these molecules and neuropsychological disorders.

Applied Research Collaboration

We are considering the application of diagnostic kits for several psychiatric diseases, based on the concentrations of the new proteins in blood.

Roles of shati in METH-induced hyperlocomotion and sensitization
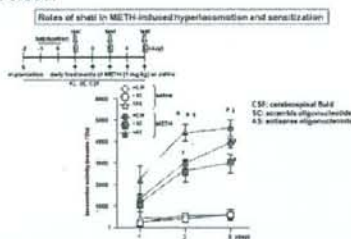
Repeated METH administration leads to a progressive augmentation of many behavioral effects of the drug (behavioral sensitization). Sensitization is of interest as a model for drug-induced neuroplasticity in neuronal circuits important for addiction. It is well established that the induction of sensitization involves complex neuronal circuitry. Repeated METH treatment for 5 days produced locomotor sensitization. The AS treatment potentiated the development of locomotor sensitization to METH compared with -SC or CSF-treated mice.

Research Performance

Neuromelanin selectively induces apoptosis in dopaminergic SH-SY5Y cells by deglutathionylation in mitochondria: involvement of the protein and melanin component

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Abstract

Parkinson's disease (PD) is characterized by selective depletion of nigral dopamine (DA) neurons containing neuromelanin (NM), suggesting the involvement of NM in the pathogenesis. This study reports induction of apoptosis by NM in SH-SY5Y cells, whereas protease-K-treated NM, synthesized DA- and cysteinyl dopamine melanin showed much less cytotoxicity. Cell death was mediated by mitochondria-mediated apoptotic pathway, namely collapse of mitochondrial membrane potential, release of cytochrome c, and activation of caspase 3, but Bcl-2 over-expression did not suppress apoptosis. NM increased sulfhydryl content in mitochondria, and a major part of it was identified as GSH, whereas dopamine melanin significantly reduced sulfhydryl levels. Western

blot analysis for protein-bound GSH demonstrated that only NM reduced S-glutathionylated proteins in mitochondria and dissociated macromolecular structure of complex I. Reactive oxygen and nitrogen species were required for the deglutathionylation by NM, which antioxidants reduced significantly with prevention of apoptosis. These results suggest that NM may be related to cell death of DA neurons in PD and aging through regulation of mitochondrial redox state and S-glutathionylation, for which NM-associated protein is absolutely required. The novel function of NM is discussed in relation to the pathogenesis of PD.

Keywords: apoptosis, mitochondria, neuromelanin, Parkinson's disease, Redox status, S-glutathionylation.

J. Neurochem. (2008) 105, 2489–2500.

Neuromelanin (NM) is a pigment localized in the catecholaminergic neurons in the substantia nigra, locus coeruleus, and other brainstem nuclei. In Parkinson's disease (PD), dopaminergic and noradrenergic neurons are preferentially affected (Hirsch et al. 1988), and the presence of NM in most of these neurons suggests that NM may be involved in the cell vulnerability (Kastner et al. 1992). NM is known to appear after 2–3 years of life and increase with age to a level of 2.3–3.7 mg/g wet weight of the substantia nigra pars

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Abbreviations used: ΔΨm, mitochondrial membrane potential; Cys-DAM, cysteinyl dopamine melanin; DAM, dopamine melanin; DFX, deferoxamine mesylate; EGCG, (−)-epigallocatechin gallate; FACS, fluorescence-augmented flow cytometry; GRX, glutaredoxin; mPT, mitochondrial permeability transition; NM, neuromelanin; NO, nitric oxide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PD, Parkinson's disease; P-K NM, protease-K-treated neuromelanin; PrS-SG, S-glutathionylated protein; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SH, sulfhydryl; SOD, superoxide dismutase; TRX, thioredoxin.

compacta at 50–90 years of age (Zecca et al. 2001). A major NM composition is melanin, which is synthesized from quinones and semiquinones, products of autooxidation of dopamine (DA), noradrenaline, and L-DOPA, and accounts for about 11–13% of NM (Wakamatsu et al. 2003). The melanin composition is a mixture of two melanin classes, eumelanin and pheomelanin in the ratio of 1–3 : 1 (Odh et al. 1994). Eumelanin is black, insoluble, and composed of indole monomers derived from oxidized catechol derivatives. Pheomelanin contains about 10% sulfur and is brownish red, alkaline soluble, and produced from 5-S-cysteinyl-DA and -DOPA derived by conjugation of DA quinone with GSH or cysteine (Odh et al. 1994). Recently, studies on the surface oxidation potential of NM suggest a spherical architecture of the pheomelanin core with eumelanin surface (Bush et al. 2006). In addition, protein, lipids, and trace metals are detected in NM, but it has never been clarified whether these components are integrated in a complex structure of NM or only associated to melanin in NM granules. NM contains protein components of about 15% of NM mass (Gerlach et al. 1995; Zecca et al. 2000). Proteomics of NM granules identified about 70 proteins occurring commonly in human brain tissues, mainly lysosomal proteins, suggesting non-selective protein binding to DA quinone (Tribl et al. 2005). In parkinsonian brains, α-synuclein, a component of Lewy body was reported to bind to NM or NM granules, but not in control brain (Fasano et al. 2003; Halliday et al. 2005). Solid-state NMR studies indicate the presence of glycidic and aliphatic components attributed to lipid materials, which corresponds to 20% of NM weight and identified as C_{14}–C_{18} fatty acids and dolichol (Zecca et al. 2000; Fedorow et al. 2005). NM also contains inorganic components, iron, copper, and zinc, as being about 1.5% of NM weight (Zecca et al. 1994). The high iron content in NM increases vulnerability of NM-containing DA neurons through the production of reactive oxygen and nitrogen species (ROS and RNS) (Ben-Shachar et al. 1991) and of cytokines and nitric oxide (NO) in microglia (Wilms et al. 2003). Recently, NM was found to inhibit the ubiquitin-proteasome system through increased ROS–RNS production, suggesting the involvement of NM in the accumulation of modified protein in the DA neurons (Shamoto-Nagai et al. 2004, 2006). However, NM may be also protective within dopaminergic cells by scavenging free radical species and binding toxic compounds. Human NM, but not synthesized melanin, scavenges hydroxyl radicals produced by the Fenton reaction (Li et al. 2005). NM binds and sequesters redox-active trace metals, dopaminergic neurotoxins, such as MPP^+ (D'Amato et al. 1986) and 1,2(N)-dimethyl-6,7-dihydroxy-isoquinolinium ion (Naoi et al. 1994), and cytotoxic DA quinine (Youdim et al. 1994).

Human NM and synthetic melanin from DA (dopamine melanin; DAM) or L-DOPA, induced cell death in rat pheochromocytoma PC12 cells (Offen et al. 1997) and primary mesencephalic culture cells (Nguyen et al. 2002). NM and DAM are phagocytosed into neuronal SK-N-SH, but not to glial U373 cells, and melanin is localized in the cytoplasm (Li et al. 2005). DAM-induced cell death in PC12 and SK-N-SH cells was reported to be apoptosis-like (Offen et al 1997; Li et al 2005), but the detailed mechanism behind the cytotoxicity remains to be elucidated. In addition, the involvement of the NM protein and melanin species in the cytotoxicity has been scarcely studied.

In PD, the reduction of GSH with corresponding increase in GSSG was confirmed in the substantia nigra (Riederer et al. 1989). GSH plays a major role in the cellular defense against oxidative stress by direct scavenging ROS–RNS, and GSH depletion initiates cell death by reduced compensation of oxidative stress. On the contrary, depletion of GSH protected cells against Fas-mediated cell death (Musallam et al. 2002), and N-acetyl-L-cysteine and GSH monoester enhanced hypoxia-induced apoptosis (Qanungo et al. 2004). The cytotoxic effects of GSH might be because of requirement for reducing conditions to activate caspase 3, an apoptosis executor, by caspase 8 (Hentze et al. 2000), and to suppress nuclear factor-κB transactivation (Qanungo et al. 2004). Recently, modification of protein thiols by ROS–RNS is gathering attention for its role in cellular dysfunction via reversible formation of mixed disulfides between the protein thiol and sulfhydryl (SH) residue of GSH (S-glutathionylated protein; PrS-SG) (Maher 2006). This post-translational modification of protein, referred as S-glutathionylation, regulates the essential cellular functions, such as energy synthesis, signal transduction pathway, and transcriptional activation (Schafer and Buettner 2001). Considering that NM contains SH groups, it should be clarified whether NM can affect S-glutathionylation in mitochondria and regulate the redox status, which might be involved in the selective death cascade of NM-containing DA neurons.

In this study, the cytotoxicity of NM was examined in human dopaminergic neuroblastoma SH-SY5Y cells using NM prepared from human substantia nigra. To examine the roles of the protein and melanin composition in the cytotoxicity, NM was treated with protease K (P-K NM), and eumelanin and pheomelanin were synthesized by oxidation of DA in the absence (DAM) or presence of L-cysteine (cysteinyl-dopamine melanin; Cys-DAM). Wild and transfection-enforced Bcl-2 over-expressed SH-SY5Y cells (Bcl-2 cells) (Akao et al. 2002) and mitochondria prepared from them were used to clarify the apoptotic process. The effects of NM on intracellular redox and SH states were studied to clarify the relation of S-glutathionylation to cell death process. The involvement of NM in the pathogenesis of PD is discussed, where increased oxidative stress, mitochondrial dysfunction and induction of apoptosis are proposed to induce selective degeneration of DA neurons containing NM.

Materials and methods

Materials
3,3'-Dihexyloxcarbocyanide iodide [DiOC$_6$(3)], LIVE/DEAD viability/cytotoxicity assay kit for mammalian cells, and Measure-iT^{TM} thiol assay kit were purchased from Molecular Probes (Eugene, OR, USA); superoxide dismutase (SOD), deferoxamine mesylate (DFX), and (−)-epigallocatechin gallate (EGCG) were from Sigma (St Louis, MO, USA). A substrate for caspase 3, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid α-(4-methyl-coumaryl-7-amide), was obtained from Peptide Institute (Osaka, Japan). Catalase from bovine liver, Dulbecco's minimum essential medium and other reagents were from Wako (Kyoto, Japan). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in 95% air and 5% CO$_2$. Bcl-2 was over-expressed in SH-SY5Y cells (Bcl-2 cells) by transfection as reported previously (Akao et al. 2002).

Preparation of neuromelanin and dopamine melanin
Brains from neurologically normal adult individuals were provided from the Austro-German Brain Bank in Würzburg, Germany. The Ethics Committee of the University Clinics of Würzburg (Würzburg, Germany) approved the use of postmortem human brain tissue. NM was isolated from the substantia nigra, and a portion was treated with P-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-Shachar et al. 1991). Melanin was suspended in 10% dimethyl sulfoxide to be 1 mg/mL, sonicated for 30 s, and shaken gently for 3 days for the rehydration at 20°C under protection from light.

Measurement of apoptosis by morphological observation, FACS, and with LIVE/DEAD 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 six-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 six-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 20°C for 5 min in the dark, washed, suspended in PBS, and then subjected to FACS analysis (Yi et al. 2006). The fluorescence intensity at 560–640 nm (fluorescene detector #2, 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 manufacturer's instruction. The cells cultured in six-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 percentage 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 by fluorescence-augmented flow cytometry
Mitochondrial membrane potential (ΔΨm) in isolated mitochondria was quantified by FACS using DiOC$_6$(3). Mitochondria were prepared from the wild and Bcl-2 cells, suspended in Dulbecco's minimum essential medium, and incubated with 10–25 μg/mL NM 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 (fluorescene detector, FL-2 channel) 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 1–6 h, gathered, washed with PBS, and treated with the extraction buffer [50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES)–KOH buffer, pH 7.4, containing 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 2 mM MgCl$_2$, 5 mM EGTA, and 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 sample buffer [10 mM Tris–HCl buffer, pH 7.5, containing 1% Nonidet P-40 (Wako, Osaka, Japan), 0.1% sodium dodecyl cholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, and 1 mM EDTA]. The sample (5 μg protein) was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) with a 10–20% polyacrylamide gel (Bio-Rad), and electroblotted onto polyvinylidene difluoride membranes (Du Pont, Boston, MA, USA). After blocking with non-fat 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 six-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, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid α-(4-methyl-coumaryl-7-amide) (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 solution.

Determination of SH residue, GSH, and GSSG

Sulfhydryl contents in mitochondria and melanin were quantitatively measured using Measure-iT™ Thiol assay kit, according the manufacturer'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 at 12 000 g for 20 min. 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 DA for 2 h, according to Musderman *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 in mitochondria

S-Glutathionylate protein 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 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 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 SDS sample buffer containing 5% β-mercaptoethanol and subjected to SDS–PAGE. To identify PrS-SG 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 three times, and triplicate or quadruplicate measurements were carried out. The data were expressed as mean ± SD and the difference was evaluated by ANOVA followed by Scheffe's F-test. A value of $p < 0.05$ was estimated to be statistically significant.

Results

Apoptosis induced by neuromelanin

After incubation of SH-SY5Y cells with NM, apoptosis was detected by morphological observation. NM and DA induced cell death in the cells, and Hoechst 33342 staining showed apoptotic features with condensed nuclei among dead cells detected with PI (Figs. 1a and 2b). 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. 1b). 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. Figure 1c 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

Neuromelanin 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). P-K NM reduced the SH contents significantly to 0.57 ± 0.16 nmol/mg melanin, 23% of the untreated NM. In Cys-DAM synthesized from DA 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. 2a). Among four melanin classes, only NM reduced the number of live cells, and the P-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. 2b). NM and DA 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.
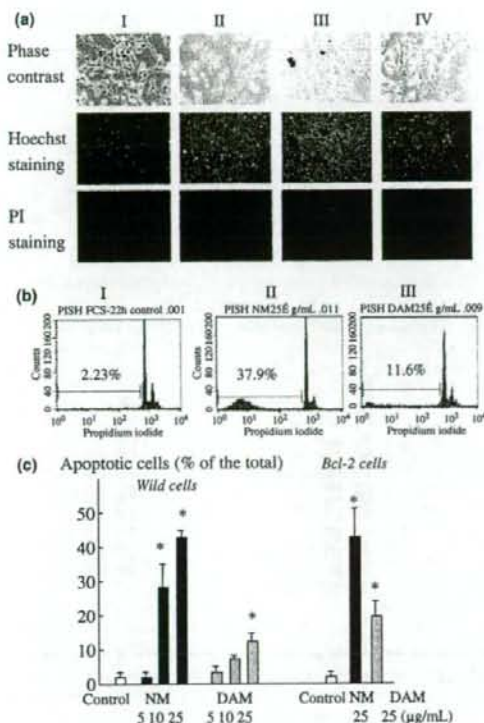
Fig. 1 Apoptosis induced by neuromelanin. (a) Morphological observation of NM cytotoxicity. SH-SY5Y cells were treated without (I, control) or with 10 μg/mL of NM (II), DAM (III), and 100 μM dopamine (IV) for 16 h. The cells were observed by phase contrast, or after staining with Hoechst 33342 for apoptotic cells and with PI for dead cells. (b) FACS analyses of apoptosis. The wild ells were incubated without (I, control) or with 25 μg/mL NM (II) or DAM (III) for 16 h, gathered, stained with PI with 1% Triton X-100, and subjected to FACS. The cells with a lower DNA content showing less PI staining than G₁ peak were defined to be apoptotic. (c) Quantitative analyses of apoptotic cell death by FACS. The wild and Bcl-2 cells were treated with 5, 10, or 25 μg/mL of NM or DAM for 16 h and were subjected to FACS. The column and bar represent the mean and SD of five experiments. *$p < 0.01$ from control.
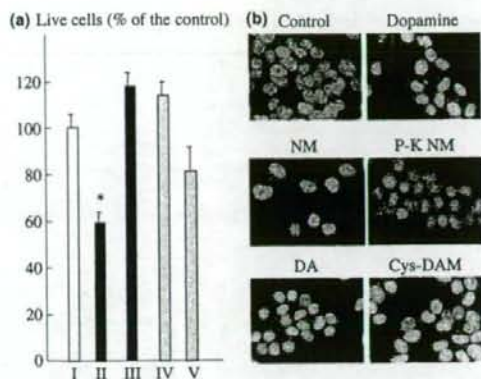

Fig. 2 Effects of protein component and melanin classes on cell viability. SH-SY5Y cells were cultured in a six-well tissue culture flask and treated without (I) or with 10 μg/mL NM (II), P-K NM (III), DAM (IV), or Cys-DAM (V) for 16 h at 37°C. (a) The number of live cells. The cells were gathered, washed with PBS, and the live cells were quantitatively measured after staining with calcein. The number of live cells was expressed as percent of control, and the column and bar represent the mean and SD of quadruplicate measurements of three experiments. *Difference from control, $p < 0.01$. (b) Morphological observation of cells. The cells were observed by fluoromicroscopy after staining with Hoechst 33342. The cells were also treated with 100 μM dopamine (Dopamine).

Table 1 SH contents in NM, P-K-treated NM, DAM, and cysteinyl-DAM

Melanin	SH content (nmol/mg melanin)
NM	2.42 ± 0.80
P-K-treated NM	0.57 ± 0.16
Dopamine melanin	Not detected
Cysteinyl dopamine melanin	3.77 ± 0.19

SH contents in three NM and two P-K NM samples from different brains were measured fluorometrically by use of Measure-iT™ assay kit. The values are mean ± SD of quadruplicate measurements of each sample. NM, neuromelanin; SH, sulfhydryl; P-K NM, protease-K-treated neuromelanin; DAM, dopamine melanin.

Apoptosis pathway activated by neuromelanin

Mitochondria were prepared from the wild and Bcl-2 cells, treated with NM, and then subjected to FACS analyses after stained with DiOC₆(3). Figure 3a shows that NM reduced ΔΨm of the wild cells in a dose-dependent way, which GSH did not prevent. In mitochondria prepared from Bcl-2 cells, the reduction of ΔΨm by NM was also observed (Fig. 3b).

After treated with NM, cytochrome *c* was released from mitochondria into cytosol in wild SH-SY5Y cells in a time-dependent way (Fig. 3c). The cells were treated with NM,

DAM, and DA for 16 h, and the activity of caspase 3 was measured fluorometrically. Figure 3d shows the significant increase in caspase 3 activity in the wild cells treated with NM and DA. 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 over-expression completely prevented the increase in caspase 3 activity by DA.
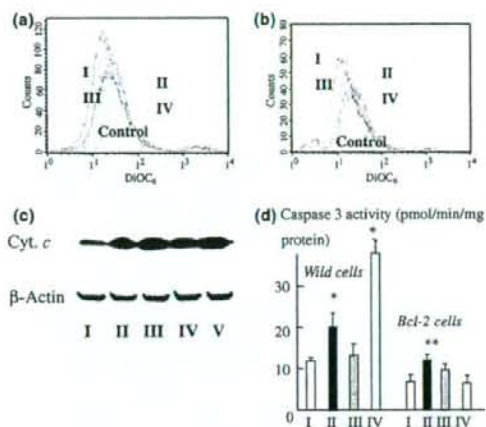
Fig. 3 Activation of apoptotic cascade by NM. (a and b) Reduction of ΔΨm by NM in mitochondria isolated from the wild (a) and Bcl-2 cells (b). I and II, NM (25 and 10 µg/mL) was incubated with mitochondria without or with 1 mM GSH (III and IV), respectively, at 37°C for 3 h. ΔΨm was measured by FACS after staining with DiOC₆(3). (c) Release of cytochrome *c* into cytosol. After treated with NM (25 µg/mL) for 6 h, cytosol fraction was prepared from SH-SY5Y cells and subjected to western blot analysis for cytochrome *c* (Cyt. *c*). I, Control and II, III, IV, and V, cells were treated with NM for 1, 2, 4, and 6 h. β-Actin was used as control. (d) Caspase 3 activity in the wild and Bcl-2 cells after treated for 16 h at 37°C. I, Control and II, III, and IV, cells treated with 10 µg/mL NM and DAM, and 100 µM dopamine, respectively. Caspase 3 activity was measured fluorometrically using acetyl-ʟ-aspartyl-ʟ-glutamyl-ʟ-valyl-ʟ-aspartic acid α-(4-methyl-coumaryl-7-amide) as a substrate. The column and bar represent the mean and SD of triplicate measurements of 4 experiments. *Difference from control, $p < 0.01$.

Effects of neuromelanin and dopamine melanin 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. 4a). On the other hand, DAM (25 µg/mL) and DA (100 µM) significantly reduced SH contents in a time-dependent way (Fig. 4b). The effects of four melanin species on mitochondrial SH levels are shown in Fig. 4c. 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. 4d. 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 submitochondrial fractions, mitochondria were
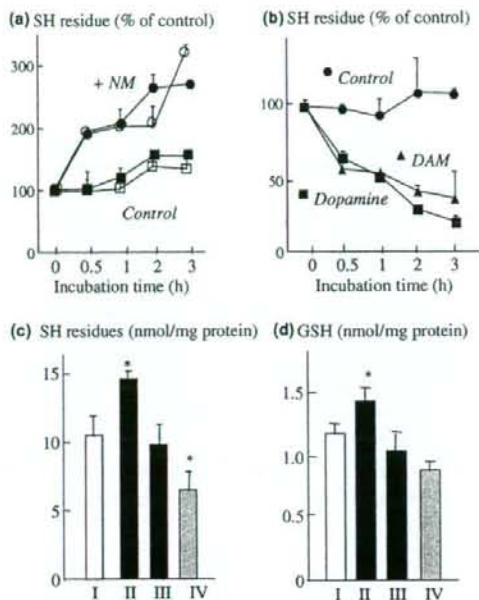

Fig. 4 Effects of melanin on SH levels in mitochondria. (a) Effects of NM on the mitochondrial SH contents. Mitochondria prepared from the wild (filled circle) and Bcl-2 cells (hollow circle) were incubated with NM (25 µg/mL) at 37°C, for 0.5, 1, 2, and 3 h. Mitochondria were treated without NM also in a similar way (filled and hollow square for mitochondria from wild and Bcl-2 cells, respectively). SH levels were quantified fluorometrically with the Thiol Assay Kit. The values were represented as percentage of SH levels at 0 time. The point and bar represent the mean and SD of quadruplicate measurements. (b) Effects of DAM and dopamine on SH levels in mitochondria prepared from the wild cells. Mitochondria were incubated with DAM (25 µg/mL) (triangle) and dopamine (100 µM) (square). Control, circle. (c) Effects of melanin species on mitochondrial SH levels. Mitochondria were treated without (I, control) or with 25 µg/mL NM (II), P-K NM (III), and DAM (IV) for 2 h at 37°C. SH residues were quantified by the fluorometric assay with Thiol Assay Kit. The column and bar represent the mean and SD of quadruplicate measurements of two experiments. *Difference from control, $p < 0.05$. (d) GSH was quantitatively measured by HPLC. Mitochondria were treated without (I, control) or with 25 µg/mL NM (II), P-K NM (III), and DAM (IV) for 2 h at 37°C. The column and bar represent the mean and SD of triplicate measurements of two experiments. *Difference from control, $p < 0.05$.

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 DA reduced SH contents (Fig. 5a). Cys-DAM markedly increased SH in the soluble fraction and pellets.
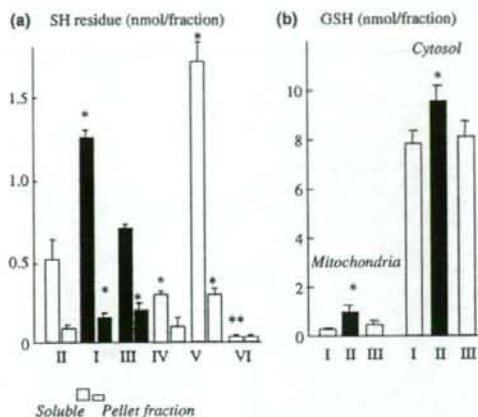
(a) SH residue (nmol/fraction)   **(b)** GSH (nmol/fraction)

Soluble □ Pellet fraction

Fig. 5 Effects of NM and DAM on SH levels in mitochondria and subcellular fractions. (a) Effects of melanin species on SH levels in the soluble and precipitate fraction of mitochondria. Mitochondria prepared from SH-SY5Y cells were incubated without (I) or with 25 µg/mL NM (II), P-K NM (III), DAM (IV), Cys-DAM (V), and 250 µM dopamine (VI) for 2 h at 37°C, then subjected to fractionation into the supernatant fraction and the sediment. SH levels were measured with Thiol Assay Kit and expressed as nmol/fraction. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *Difference from control, $p < 0.01$. (b) Effects of melanin on SH levels in cytosol and mitochondria fractions. The wild cells were treated with 25 µg/mL of NM and DAM at 37°C for 2 h and subjected to subcellular fractionation. Mitochondrial and cytosol fraction were prepared for GSH analysis by GSH reductase-dependent recycle method. I, Control. II and III, cells treated with NM and DAM. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *Difference from control, $p < 0.01$ from control.

Neuromelanin- and DAM-treated cells were fractionated into mitochondria and cytosol fraction, and GSH contents were quantified by the enzyme recycling method (Fig. 5b). 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 NO-scavenging EGCG protect cells from cell death induced by NM, but catalase did not (Fig. 6a). At the same time, DFX, SOD, and EGCG prevented the NM-induced increase in mitochondrial SH contents, but catalase further increased SH levels (Fig. 6b).

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 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 PrS-SG. Under non-reducing conditions, PrS-SG was detected in mitochondria without NM treatment (Fig. 7a). NM reduced PrS-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. 7b). 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 study 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

Table 2 Total SH, GSH, and GSSG contents, and GSSG/GSH ratio in the cells, subcellular fractions, and isolated mitochondria after treated with NM and DAM

Fraction	Total SH (nmol/mg protein)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSSG/GSH × 100
Cells				
Control	11.9 ± 0.2	6.90 ± 0.54	0.36 ± 0.02	5.22
Treated with NM	14.2 ± 0.7*	9.61 ± 0.36*	0.88 ± 0.02*	9.16
Treated with DAM	10.2 ± 0.4	6.77 ± 0.21	0.99 ± 0.02*	14.62
Cytosol				
Control	13.9 ± 1.6	10.2 ± 1.5	0.018 ± 0.003	0.18
Treated with NM	15.1 ± 1.6	12.9 ± 1.9	0.019 ± 0.001	0.15
Treated with DAM	13.4 ± 1.7	12.0 ± 1.1	0.015 ± 0.006	0.13
Mitochondria				
Control	9.1 ± 0.1	11.6 ± 0.2	0.55 ± 0.19	4.74
Treated with NM	11.1 ± 0.3*	19.9 ± 1.2*	1.66 ± 0.18*	8.34
Treated with DAM	7.9 ± 0.5*	13.3 ± 0.54	1.54 ± 0.11*	11.58
Isolated mitochondria				
Control	11.7 ± 1.3	6.13 ± 0.32	0.36 ± 0.13	5.87
Treated with NM	17.1 ± 1.7*	8.54 ± 0.19*	0.88 ± 0.07*	10.30
Treated with DAM	6.9 ± 0.2*	5.78 ± 0.59	0.99 ± 0.12*	17.13

The total SH contents were measured fluorometrically by use of Measure-iT™ Thiol Assay kit. GSH and GSSG were quantified using the enzymatic recycling method. The values are mean and SD of quadruplicate measurements of three experiments. *Difference from control, $p < 0.05$. SH-SY5Y cells were treated without or with 10 µg/mL NM or DAM for 2 h at 37°C, then subjected to subcellular fractionation according to Musderman et al. (2004). The precipitated mitochondrial fraction was treated with the extraction medium for GSH/GSSG and analyzed for GSH and GSSG. Isolated mitochondria were treated with 10 µg/mL NM or DAM for 2 h at 37°C, and the total SH was measured fluorometrically, then the rest was precipitated by centrifugation at 12 000 g for 20 min, treated as above for GSH-GSSG assay. NM, neuromelanin; SH, sulfhydryl; DAM, dopamine melanin.

1.2–1.5 mg/g wet weight from 2.3 to 3.5 mg/g wet weight (Zecca et al. 2001), 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 study, as 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 study. P-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 P-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 P-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-DA a conjugate of cysteine with o-quinone, free SH residues were detected, even though pheomelanin has been considered to polymerize into a benzothiazine structure.

Neuromelanin and DAM affected SH state in mitochondria in quite opposite ways. NM and Cys-DAM increased SH contents in mitochondria, whereas DAM and DA reduced them markedly. These results might be comparable to the