Meister A. (1995) Mitochondrial changes associated with glutathione deficiency. *Biochim. Biophys. Acta* **1271**, 5-12.

Musallam L., Ethier C., Haddad P. S., Denizear F. and Bilodeau M. (2002) Resistance to Fas-induced apoptosis in hepatocytes; role of GSH depletion by cell isolation and culture. *Am. J. Physiol. Gastrointest. Liver Physiol.* **283**, 709-718.

Musderman H., Nilsson M. and Sims N. R. (2004) Highly selective and prolonged depletion of mitochondrial glutathione in astrocytes markedly increases sensitivity to peroxynitrite. *J. Neurosci.* **24**, 8019-8028.

Naoi M., Maruyama W. and Dostert P. (1994) Binding of 1,2(N)-dimethyl-6,7-dihydroxy-isoquinolinium ion to melanin: effects of ferrous and ferric ion on the binding. *Neurosci. Lett.* **171**, 9-12.

Naoi M., Maruyama W., Dostert P., Kohda K. and Kaiya T. (1996) A novel enzyme enantio-selectively synthesizes (*R*)salsolinol, a precursor of a dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol. *Neurosci. Lett.* **212**, 183-186.

Nguyen A., Gille G., Moldino R., Hung S.-T. and Rausch W.-D. (2002) Synthetic neuromelanin is toxic to dopaminergic cell cultures. *J. Neural Transm.* **109**,

651-661.

Odh G., Garstam R., Paulsin J., Wittbjer A., Rosengren E. and Rorsman H. (1994) Neuromelanin of the human substantia nigra: A mixed-type melanin. *J. Neurochem.* **62**, 2030-2036.

Offen D., Ziv I., Barzilai A., Gorodin S., Glater E., Hochman A. and Melamed E. (1997) Dopamine-melanin induces apoptosis in PC12 cells; possible implications for the etiology of Parkinson's disease. *Neurochem. Int.* **31**, 207-216.

Qanungo S., Wang M. and Nieminen A.-L. (2004) N-Acetyl-L-cysteine enhances apoptosis through inhibition of nuclear factor-κB in hypoxic murine embryonic fibroblasts. *J. Biol. Chem.* **279**, 50455-50464.

Rahman I., Kode A and Biswas S. K. (2006) Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature Protocols* **1**, 3159-3165.

Riederer P., Sofic E., Raush W.-D., Schmidt B., Reynolds G. P., Jellinger K. and Youdim M. B. H. (1989) Transition metals, ferritin, glutathione and ascorbic acid in Parkinsonian brains. *J. Neurochem.* **52**, 515-520.

Samokhvalov A., Hong L., Liu Y., Garguilo J., Nemanich R. J., Edwards G. S. and Simon J. D. (2005) Oxidation potentials of human eumelanosomes and pheomelanosomes. *Photochem. Photobiol.* **81**, 145-148.

Schafer F. Q. and Buettner G. R. (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Rad. Biol. Med.* **30**, 1191-1212.

Shamoto-Nagai M., Maruyama W., Akao Y., Osawa T., Tribl F., Gerlach M., Zucca F. A., Zecca L., Riederer P. and Naoi M. (2004) Neuromelanin inhibits enzymatic activity of 26S proteasome in human dopaminergic SH-SY5Y cells. *J. Neural Transm.* **111**, 1253-1265.

Shamoto-Nagai M., Maruyama W., Yi H., Akao Y., Trible F., Gerlach M., Osawa T., Riederer P. and Naoi M. (2006) Neuromelanin induces oxidative stress in mitochondria through release of iron: mechanism behind the inhibition of 26S proteasome. *J. Neural Transm.* **113**, 633-644.

Tanaka M., Miyabayashi S., Nishikimi M., Suzuki H., Shimomura Y., Ito Y., Narisawa K., Tada K. and Ozawa T. (1988) Extensive defects of mitochondrial electron-transfer

chain in muscular cytochrome c oxidase deficiency. *Pediatr. Res.* **24**, 447-454.

Taylor E. R., Hurrell F., Shannon R. J., Lin T.-K., Hirst J. and Murphy M. P. (2003) Reversible glutathionylation of complex I increases mitochondrial superoxide formation. *J. Biol. Chem.* **278**, 19603-19610.

Tribl F., Gerlach M., Marcus K., Asan E., Tatschner T., Arzberger T., Meyer H. E., Bringmann G. and Riederer P. (2005) "Subcellular Proteomics" of neuromelanin granules isolated from the human brain. *Mol. Cell Proteomics* **4**, 945-957.

Wakamatsu K., Fujisawa K., Zucca F. A., Zecca L. and Ito S. (2003) The structure of neuromelanin as studied by chemical degradative methods. *J. Neurochem.* **86**, 1015-1023.

Wilms H., Rosenstiel P., Sievers J., Deuschl G., Zecca L. and Lucius R. (2003) Activation of microglia by human neuromelanin is NF-kB-dependent and involves p38 mitogen-activated protein kinase: implications for Parkinson's disease. *FASEB J.* **10**, 17:500-502.

Yi H., Akao Y., Maruyama W., Chen K., Shih J. and Naoi M. (2006) Type A monoamine oxidase is the target of an endogenous dopaminergic neurotoxin,

N-methyl(*R*)salsolinol, leading to apoptosis in SH-SY5Y cells. *J. Neurochem.* **96**, 541-549.

Youdim M. B., Ben-Shachar D. and Riederer P. (1994) The enigma of neuromelanin in Parkinson's disease substantia nigra. *J. Neural Transm. (Suppl.)* **43**, 113-122.

Zecca L., Pietra R., Goj C., Mecacci C., Radice D., and Sabbioni E. (1994) Iron and other metals in neuromelanin, substantia nigra and putamen of human brain. *J. Neurochem.* **62**, 1097-1100.

Zecca L., Costi P., Meccaci C., Ito S., Terreni T. and Sonnino S. (2000) The interaction of human substantia nigra neuromelanin with lipids and peptides. *J. Neurochem.* **74**, 1758-1765.

Zecca L., Tampellini D., Gerlach M., Riederer P., Fariello R. G. and Sulzer D. (2001) Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. *J. Clin. Pathol. Mol. Pathol.* **54**, 414-418.

Table 1. SH contents in NM, Protease K treated NM, DAM and Cysteinyl- DAM

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

SH contents in 3 NM and 2 P-K NM samples from different brains were measured fluorometrically by use of Measure-iT™ assay kit. The values are mean ± S.D. of quadruplicate measurements of each sample.

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 x 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
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
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
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
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 Muyderman *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, treated as above for GSH-GSSG assay.

Legends for Figures

Figure 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) 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 G_1 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.

Figure 2. Effects of protein component and melanin classes on cell viability.

SH-SY5Y cells were cultured in a 6-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**).

Figure 3. Activation of apoptotic cascade by NM.

A and **B**: Reduction of $\Delta\Psi$m 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**, **IV**), respectively at 37C for 3 h. $\Delta\Psi$m was measured by FACS after staining with $DiOC_6(3)$.

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. **II**, **III**, **IV** and **V**, cells were treated with NM for 1, 2, 4 and 6 h. β-Action 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. **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 Ac-DEVD-MCA as a substrate. The column and bar represent the mean and SD of triplicate measurements of 4 experiments. *, Difference from control, $p < 0.01$.

Figure 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 % 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 2 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 2 experiments. *, Difference from control, $p < 0.05$.

Figure 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.

Figure 6. Effects of anti-oxidants and NADPH on the NM-induced apoptosis and SH increase.

A: Effects of antioxidants on cell death induced by NM. The wild cells were treated without (**I**, control) or with NM alone (**II**) or NM in the presence of DFX (**III**, 1 μM), SOD (**IV**, 100 unit/ml), catalase (**V**, 300 u/ml) and EGCG (**VI**, 10 μM) for 16 h at 37°C. Live cells were quantified using calcein staining. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *, Difference from control, $P < 0.05$ from control. #, Difference from NM-treated cells, $p < 0.05$.

B: Effects of antioxidants on SH levels in mitochondria. Mitochondria were treated without or with NM in the absence (**I**, control) or presence of DFX (**II**, 1 μM), SOD (**III**, 100 unit/ml), catalase (**V**, 300 unit/ml) and EGCG (**VI**, 10 μM) for 2 h at 37°C. SH contents were measured fluorometrically using the Thiol Assay Kit. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *, Difference from control, $P < 0.05$. #, Difference from NM-treated cells, $p < 0.05$.

C: Effects of NADPH on SH contents in melanin-treated mitochondria. Mitochondria were treated without (Control) or with 25 μg/ml NM or DAM at 37°C for 2 h, in the

absence (-) or presence (+) of 1 mM NADPH. SH was quantified with the Thiol Assay Kit. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *, Difference from control, $P < 0.01$. #, Difference from NM-treated cells, $p < 0.05$.

Figure 7. Effects of NM on *S*-glutathionylated protein in mitochondria. Mitochondria were prepared from the wild cells and treated without (**I**, control), or with 10 μg/ml NM (**II**), P-K NM (**III**), DAM (**IV**), Cys-DAM (V) or 100 μM dopamine (**VI**), at 37°C for 2 h. The samples were washed with PBS, and subjected to SDS-PAGE under non-reducing (**A**) and reducing conditions (**B**). *S*-Glutathionylated protein (PrS-SG) was visualized by use of polyclonal anti-GSH antibody. Complex I and III were detected with the antibody against complex I and III, respectively. The left line of each gel represents the protein markers with molecular mass with 250, 150, 100, 75, 50, 37, 20, 15 and 10 kDa from the top.

Figure 1.

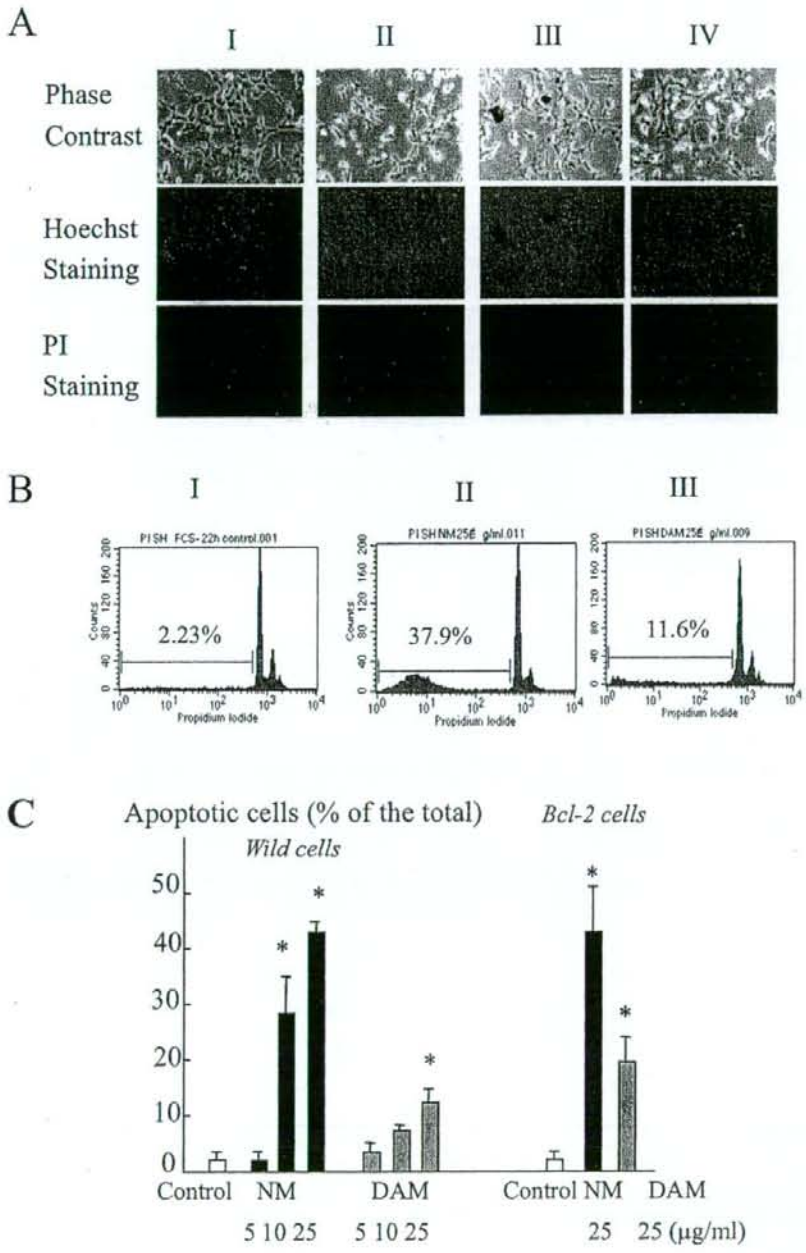

A
I
II
III
IV
Phase Contrast
Hoechst Staining
PI Staining
B
I
II
III
2.23%
37.9%
11.6%
Propidium Iodide
C
Apoptotic cells (% of the total)
Wild cells
Bcl-2 cells
50
40
30
20
10
0
Control
NM
DAM
5 10 25
5 10 25
Control NM DAM
25
25 (μg/ml)

Figure 2.

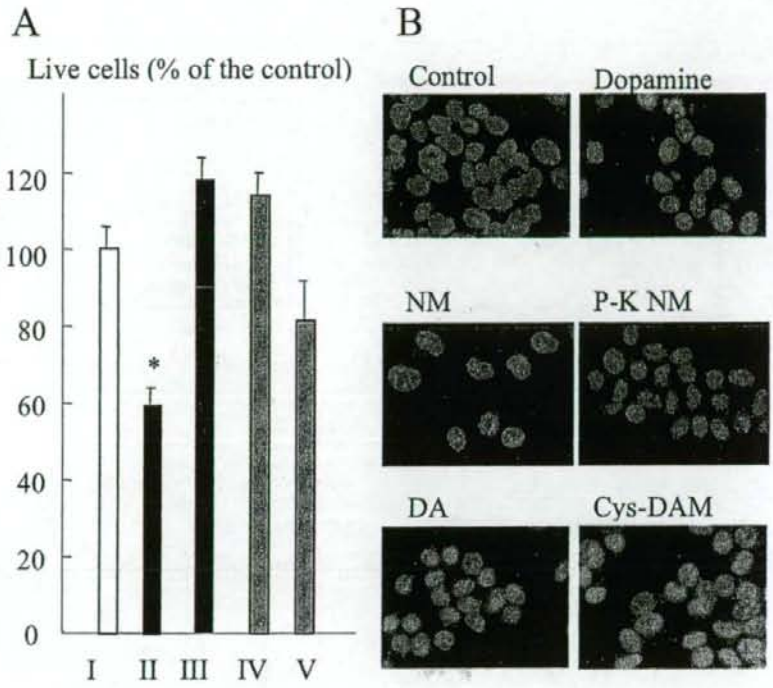

A
Live cells (% of the control)
120
100
80
60
40
20
0
*
I
II
III
IV
V
B
Control
Dopamine
NM
P-K NM
DA
Cys-DAM

Figure 3.

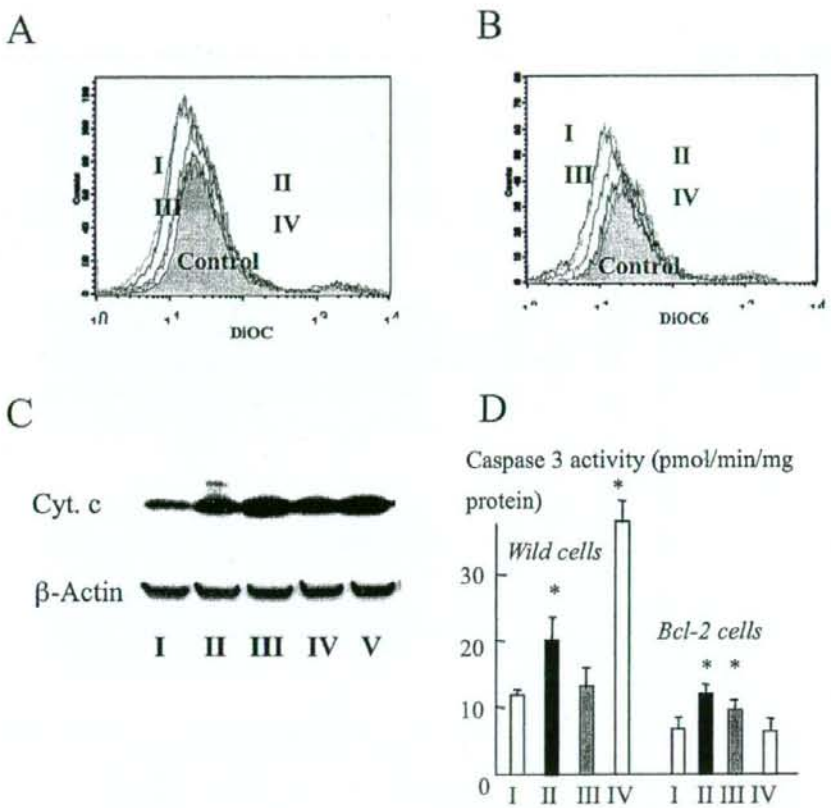

A
I
III
II
IV
Control
DiOC
B
I
III
II
IV
Control
DiOC6
C
Cyt. c
β-Actin
I II III IV V
D
Caspase 3 activity (pmol/min/mg protein)
Wild cells
Bcl-2 cells
30
20
10
0
I II III IV I II III IV

Figure 4

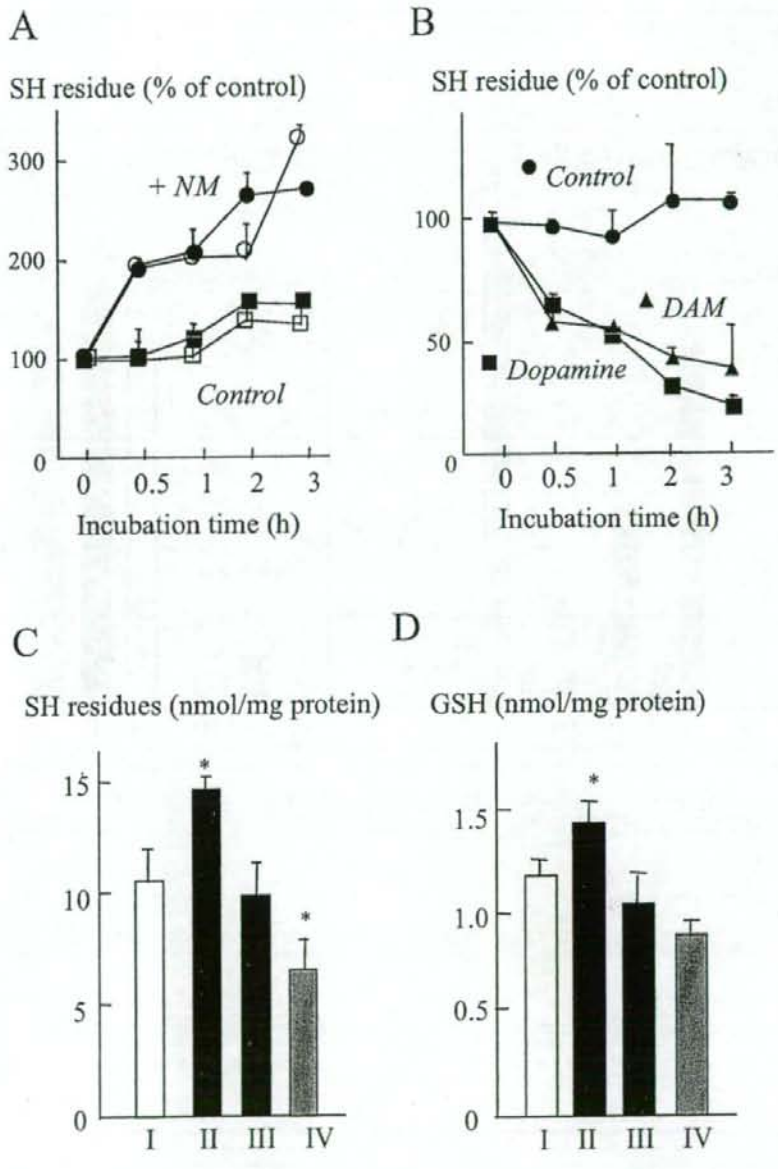
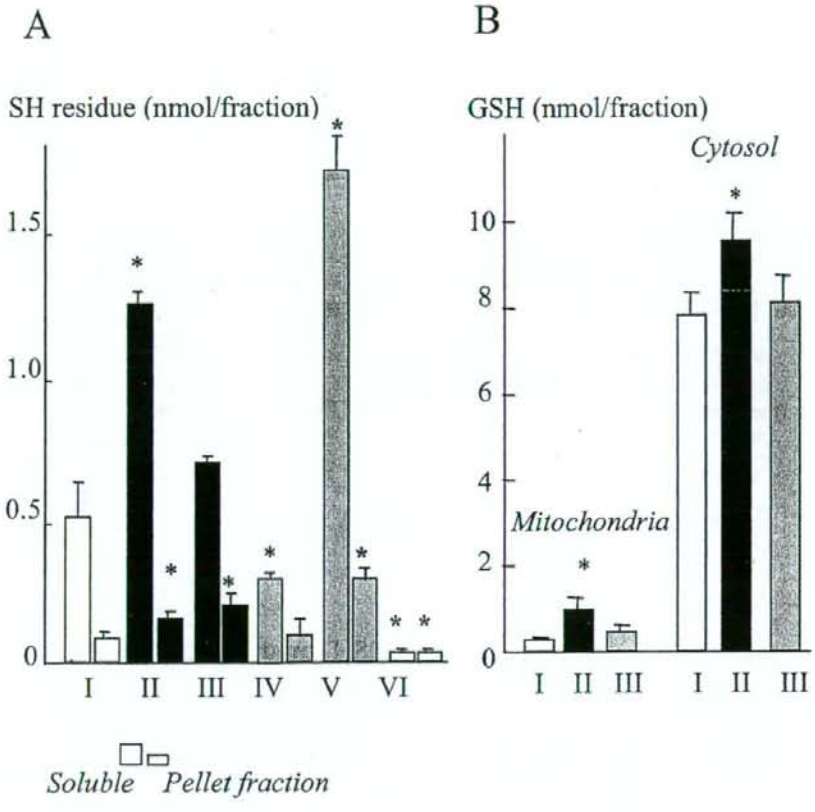

A
SH residue (% of control)
300
200
100
0
+ NM
Control
0 0.5 1 2 3
Incubation time (h)
B
SH residue (% of control)
100
50
0
Control
DAM
Dopamine
0 0.5 1 2 3
Incubation time (h)
C
SH residues (nmol/mg protein)
15
10
5
0
I II III IV
D
GSH (nmol/mg protein)
1.5
1.0
0.5
0
I II III IV

Figure 5.


A
SH residue (nmol/fraction)
1.5
1.0
0.5
0
I II III IV V VI
B
GSH (nmol/fraction)
Cytosol
10
8
6
4
2
0
Mitochondria
I II III I II III
Soluble Pellet fraction