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Glutathione depletion switches nitric oxide neurotrophic effects to cell death in midbrain cultures: implications for Parkinson's disease

S. Canals, M. J. Casarejos, S. de Bernardo, E. Rodríguez-Martín and M. A. Mena

Departamento de Investigación, Servicio de Neurobiología, Hospital 'Ramón y Cajal', Madrid, Spain

Abstract

Nitric oxide (NO) exerts neurotrophic and neurotoxic effects on dopamine (DA) function in primary midbrain cultures. We investigate herein the role of glutathione (GSH) homeostasis in the neurotrophic effects of NO. Fetal midbrain cultures were pretreated with GSH synthesis inhibitor, L-buthionine-(*S,R*)-sulfoximine (BSO), 24 h before the addition of NO donors (diethylamine/nitric oxide-complexed sodium and *S*-nitroso-*N*-acetylpenicillamine) at doses tested previously as neurotrophic. Under these conditions, the neurotrophic effects of NO disappeared and turned on highly toxic. Reduction of GSH levels to 50% of baseline induced cell death in response to neurotrophic doses of NO. Soluble guanylate cyclase (sGC) and cyclic GMP-dependent protein kinase (PKG) inhibitors protected from cell death for up to 10 h after NO addition; the antioxidant ascorbic acid also protected from cell death but its efficacy decreased when it was added after NO treatment (40% protection 2 h after NO addition). The pattern of cell death was characterized by an increase in chromatin condensed cells with no DNA fragmentation and with breakdown of plasmatic membrane. The inhibition of RNA and protein synthesis and of caspase activity also protected from cell death. This study shows that alterations in GSH levels change the neurotrophic effects of NO in midbrain cultures into neurotoxic. Under these conditions, NO triggers a programmed cell death with markers of both apoptosis and necrosis characterized by an early step of free radicals production followed by a late requirement for signalling on the sGC/cGMP/PKG pathway.

Keywords: ascorbic acid, cGMP-dependent protein kinase, dopamine neurones, glutathione, guanylate cyclase, nitric oxide.

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Glutathione (GSH) depletion occurs in several forms of cell death and is associated with Parkinson's disease (PD). GSH has been reported to be markedly reduced in PD, particularly in patients with advanced disease (Perry *et al*. 1982; Di Monte *et al*. 1992). Furthermore, the GSH decrease seems to appear before neurodegeneration in presymptomatic PD (Sian *et al*. 1994; Merad-Boudia *et al*. 1998) and is not a consequence thereof. This suggests that a link may exist between these two events although it remains to be established whether or not the loss of GSH can induce neurodegeneration. Nitric oxide (NO) has been also implicated in neurodegenerative diseases. Several authors have reported markers that suggest a NO overproduction in PD brains, i.e. NO radicals detected in PD substantia nigra (Shergill *et al*. 1996), as well as increased nitrosilated proteins such as α-synuclein (Giasson *et al*. 2000) and increased nitrite concentration in cerebrospinal fluid (Qureshi *et al*. 1995). Finally, the core of Lewy bodies in PD are immunoreactive for nitrotyrosine (Good *et al*. 1998).

Decreased GSH may predispose cells to the toxicity of other insults that are selective targets for dopaminergic neurones. GSH depletion synergistically increases the selective toxicity of MPP^+ in dopamine (DA) cell cultures (Nakamura *et al*. 1997), and the toxicity of 6-OHDA and MPTP *in vivo* (Pileblad *et al*. 1989; Wullner *et al*. 1996). GSH peroxidase (GPx)-knockout mice show increased

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Address correspondence and reprint requests to Dr M. A. Mena, Dpto Investigación, Hospital Ramón y Cajal, Ctra. de Colmenar, Km. 9, Madrid 28034, Spain. E-mail: maria.a.mena@hrc.es

Abbreviations used: AA, ascorbic acid; BSO, L-buthionine-(*S,R*)-sulfoximine; cGMP, cyclic GMP; DA, dopamine; DEA/NO, diethylamine/nitric oxide-complexed sodium; DMSO, dimethyl sulfoxide; GSH, glutathione; LDH, lactate dehydrogenase; NO, nitric oxide; PKG, cGMP-dependent protein kinase; PBS, phosphate-buffered saline; PD, Parkinson's disease; sGC, soluble guanylate cyclase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TH, tyrosine hydroxylase.

vulnerability to MPTP (Klivenyi *et al*. 2000). There is evidence that NO may play an important role in DA cell death and functionality (Przedborski *et al*. 1996; LaVoie and Hastings 1999; Liberatore *et al*. 1999; Rodríguez-Martín *et al*. 2000; Canals *et al*. 2001). A redox-based mechanism for the neuroprotective and neurodestructive effects of NO and related nitroso-compounds has been postulated (Lipton *et al*. 1993). In this regard, GSH is an endogenous thiol that reacts with NO to form *S*-nitrosoglutathione and which protects DA neurones from oxidative stress (Rauhala *et al*. 1998; Chiueh and Rauhala 1999).

We show that the NO donor diethylamine/nitric oxide complexed sodium (DEA/NO) at doses of 25 and 50 μM exert neurotrophic effects on DA cells, by increasing the number of tyrosine hydroxylase positive (TH^+) cells, TH^+ neurite processes, DA levels, [^{3}H]DA uptake and by elevating intracellular and extracelular GSH concentration (Canals *et al*. 2001). When we tried to block the NO neurotrophic effect by GSH synthesis inhibition with the γ-glutamylcysteine synthetase inhibitor L-buthionine-(*S*,*R*)-sulfoximine (BSO), NO effects switched from neurotrophic to induce cell death.

In this work, we study the combined effect of nontoxic GSH down-regulation and neurotrophic doses of NO for midbrain cultures and DA neurones. Cell viability in the culture was analysed, the nature of cell death, the cell type susceptibly, the time course of cell death were characterized, and the mechanism of cell death induction and neuroprotection were addressed.

Materials and methods

Materials

Culture media

Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L), Ham's F12 nutrient mixture, Eagle's minimal essential medium (EMEM) with Earl's salts and Leibovitz's L-15 medium, all of which were supplemented with L-glutamine, fetal calf serum (FCS), sodium pyruvate and L-glutamine, were purchased from Gibco BRL (Paisley, Scotland, UK). Glucose 45%, insulin, putrescine, progesterone and sodium selenite were from Sigma (Madrid, Spain) and human transferrin, 30% iron-saturated, from Boehringer-Mannheim (Barcelona, Spain).

Antibodies

Rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody was from Chemicon International, Inc. (CA, USA), anti-microtubule-associated protein 2a + 2b (MAP-2) antibody and anti-rabbit IgG conjugated with tetramethylrhodamine (TRITC) were purchased from Sigma (Madrid, Spain) and anti-mouse Ig fluorescein was from Jackson (West grove, PA, USA).

Chemicals

Trypan blue, bovine serum albumin, poly D-lysine, p-phenylenediamine, bis-benzimide, BSO, pargyline, *N*-(1-naphthyl)ethylenediamine, sulfanilamide, dimethyl sulfoxide (DMSO), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), reduced and oxidized forms of glutathione, and diethylamine/nitric oxide complexed sodium (DEA/NO) were from Sigma (Madrid, Spain), denitrosylated DEA/NO (– NO) was obtained by incubating a 5-mM stock solution of DEA/NO for 2 h at room temperature (22–24°C) under illumination. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was from Tocris (Bristol, UK); NADPH, lactate dehydrogenase standard (LDH), the cytotoxicity detection kit (LDH), cell proliferation kit I (MTT) and GSH reductase (GR) were from Boehringer-Mannheim (Barcelona, Spain); methylene blue and ascorbic acid were from Merck (Darmstadt, Germany); LY-83583 was from Biomol (Plymouth, PA, USA) and KT5823, caspase inhibitor III (Boc-D-FMK), cycloheximide and actinomycin D were from Calbiochem (Darmstadt, Germany). The radiochemicals [^{3}H]DA (70 Ci/mmol) and [^{3}H]GABA (90 Ci/mmol) were obtained from Dupont NEN (Boston, MA, USA). The apoptosis TUNEL detection kit was from Promega (Madison, WI, USA) and the Live/Dead Viability/Cytotoxicity kit from Molecular Probes (Eugene, OR, USA). The BCA protein assay kit was from Pierce (Rockford, IL, USA). All other reagents were of the highest purity commercially available from Merck or Sigma.

Neuronal culture

Neuronal-enriched cultures from embryonic Sprague–Dawley rat midbrain E-14 (crown-rump length 10–12 mm) were obtained and prepared as previously described (Mena *et al*. 1993; Pardo *et al*. 1997). The cells were seeded in DMEM with 15% fetal calf serum (DMEM–FCS) at a density of 10^5 cells/cm^2 in multiwells or glass cover slides previously coated with poly-D-lysine, 4.5 μg/cm^2, in 0.1 M borate buffer, pH 8.4. The cultures were kept in a humidified chamber at 37°C in a 5% CO_2 atmosphere. Twenty-four hours after plating, the cells were changed to serum-free defined medium (EF12) as reported elsewhere (Mena *et al*. 1993; Pardo *et al*. 1997). EF12 consisted of a 1 : 1 (v/v) EMEM and nutrient mixture of Ham's F-12, supplemented with D-glucose (6 mg/mL), insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), progesterone (20 nM) and sodium selenite (30 nM).

Immunocytochemistry

To study cell type susceptibility to BSO + DEA/NO treatment in the fetal midbrain cultures, we performed immunostaining techniques. Rabbit polyclonal anti-TH antibody (1 : 500) was employed to identify DA neurones and mouse monoclonal anti-MAP-2 antibody (1 : 250) to detect all neurones in the culture. In brief, cultures were fixed with 4% paraformaldehyde, washed in 0.1 M phosphate-buffered saline (PBS), pH 7.4, permeabilized with ethanol-acetic acid (19 : 1) and incubated at 4°C for 24 h with primary antibodies diluted in PBS containing 10% FCS. Fluorescein- and rhodamine-conjugated secondary antibodies were employed to visualise positive cells under fluorescent microscopy. The number of immunoreactive cells was counted in 1/7 of the total area of the cover slides. The cells were counted in predefined parallel strips using a counting reticule inserted in the ocular.

Cell viability measurements

Mitochondrial activity was measured with the MTT assay. Cells were grown on 24-well culture plates with 500 μL defined medium and treated with various reagents according to the experimental design. The MTT assay measures the ability of cells to metabolize 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). At

the end of the treatment period, 300 μL of culture medium were removed from each well and 20 μL of MTT solution (5 mg/mL) were added and incubated for 1 h. At this time, 200 μL of solubilization solution (10% SDS in HCl 0.01 M) were then added to the wells and after 24 h of incubation at 37°C, 100 μL were transferred into 96-well microtitre plates, and the absorption value at 540 nm was measured in an automatic microtitre reader (Spectra Fluor; Tecan, Mannedorf, Switzerland).

In some experiments, calcein acetoxymethyl ester and ethidium homodimer staining (live/dead viability/cytotoxicity assay) was employed to check viability under fluorescence microscopy (Mena *et al*. 1997). Calcein acetoxymethyl ester is a membrane-permanent dye that labels cells with esterase activity and an intact membrane is required to retain the esterase products (viable cells). Ethidium homodimer is a membrane-impermeable DNA dye that identifies cells in which plasma membrane integrity has been disrupted (dead cells). Cultures were washed and then incubated with 0.5 μM calcein acetoxymethyl ester and 1 μM ethidium homodimer for 30 min at room temperature before examination.

Apoptosis was measured by light microscopy features, DNA staining and the TUNEL assay. Cells growing on cover slides were fixed in 4% paraformaldehyde, nuclei were stained with bis-benzimide (Hoechst 33342) added in the antifading solution (3×10^{-6} M final concentration) (Hilwig and Gropp 1975; Pardo *et al*. 1997) and counted in 1/14 of the cover slide area; apoptotic cells were identified by chromatin condensation. TUNEL detection system for apoptosis measures the fragmented DNA of cells by incorporating fluorescein-12-dUTP* at the 3′-OH ends of the DNA by using the enzyme terminal deoxynucleotidyl transferase (TdT) (Kerr *et al*. 1972; Gavrieli *et al*. 1992). For this assay, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The fluorescein-12-dUTP-labelled DNA of apoptotic cells was visualized by fluorescence microscopy (positive cells with green fluorescence). The number of $TUNEL^+$ cells was counted in 1/14 of the cover slide area. Cells were counted in predefined parallel strips by using a counting reticule in the ocular. Cells incubated with buffer in the absence of TdT enzyme were used as negative controls.

For necrotic cell death determination, trypan blue dye exclusion assay was performed (Pardo *et al*. 1997), and lactate dehydrogenase (LDH) activity was measured in the culture medium by using a cytotoxicity detection kit (Decker and Lohmann-Matthes 1988), and expressed as a percentage versus detergent-extracted controls (100% cytotoxicity).

Nitrite measurement

NO production was routinely quantified by measuring nitrite, a stable oxidation end product of NO (Green *et al*. 1982). Briefly, 400 μL of culture medium were mixed with 800 μL of Griess reagent [1.5% sulfanilamide in 1 N HCl plus 0.15% *N*-(1-naphthyl]ethylenediamine dihydrochloride in distilled water, v/v). After 10 min of incubation at room temperature, the absorbance at 540 nm was determined in an automatic microtitre reader, by means of sodium nitrite as standard.

Uptake studies

[^{3}H]DA uptake was measured after incubation of the cells with 10^{-8} M [^{3}H]DA (70 Ci/mmol), in the presence of pargyline 10^{-5} M, and ascorbic acid 10^{-3} M, at 37°C for 30 min. Non-specific uptake/binding was calculated in the presence of 10^{-5} M mazindol and represented ≤ 5% (Beart and McDonald 1980). [^{3}H]GABA uptake was performed in the presence of 10^{-5} M aminooxyacetic acid and 10^{-3} M ascorbic acid and incubated for 4 min with 10 nM [^{3}H]GABA (90 Ci/mmol). Non-specific uptake/binding was calculated by incubating cultures at 0°C and represented ≤ 7% of the total (Michel and Hefti 1990). Proteins were measured by the BCA protein assay kit.

Glutathione measurements

Total glutathione levels were measured by the method of Tietze (1969). Briefly, 1×10^5 cells were washed with PBS, lysed in 100 μL of 3% perchloric acid (PCA) for 30 min at 4°C, centrifuged, and the supernatants were neutralized with 4 volumes of 0.1 M NaH_2PO_4, 5 mM EDTA, pH 7.5. Fifty microlitres of resulting supernatants were mixed with DTNB (0.6 mM), NADPH (0.2 mM) and glutathione reductase (1 U) and the reaction monitored in a P96 automatic microtiter reader at 412 nm during 6 min. Oxidized glutathione (GSSG) was measured in the cells by the method of Griffith (1980). Briefly, after PCA extraction and pH neutralization, GSH was derivatized with 2-vinylpyridine at room temperature for 1 h and the reaction carried out as above. GSH was obtained by subtracting GSSG levels from total glutathione levels.

Statistical analysis

The results were statistically evaluated for significance with one-way analysis of variance followed by the Newman–Keuls multiple comparison test as a *post-hoc* evaluation. Differences were considered statistically significant when $p < 0.05$.

Results

GSH depletion switches NO-induced neurotrophic effect on DA function to neurotoxic

Low doses of NO released by the NO-donor DEA/NO (25–50 μM) induce a neurotrophic effect on fetal midbrain cultures, characterized by increased TH^+ cell number and arborization, DA levels, [^{3}H]DA uptake, TH protein by western blot and GSH levels (Canals *et al*. 2001). Figure 1 shows how 50 μM DEA/NO-induced up-regulation of [^{3}H]DA uptake in midbrain cultures disappeared with 24 h of 20 μM BSO pretreatment and turned on down-regulation of neurotransmitter uptake. Cultures treated with 20 μM BSO alone without DEA/NO addition, did not show changes in neurotransmitter uptake. NO-induced neurotrophic effect is DA function-specific since [^{3}H]GABA uptake was not up-regulated, but in combination with GSH depletion, NO caused greater decrease in [^{3}H]GABA uptake than in [^{3}H]DA uptake (Fig. 1).

Neurotrophic levels of NO cause a loss of viability when applied to GSH down-regulated fetal midbrain cultures

NO treatment on GSH-down-regulated cultures not only decreased neurotransmitter uptake but also resulted in a loss of cell viability. Several doses of the NO donors DEA/NO (Figs 2a and b) and SNAP (Figs 2c and d) were applied to

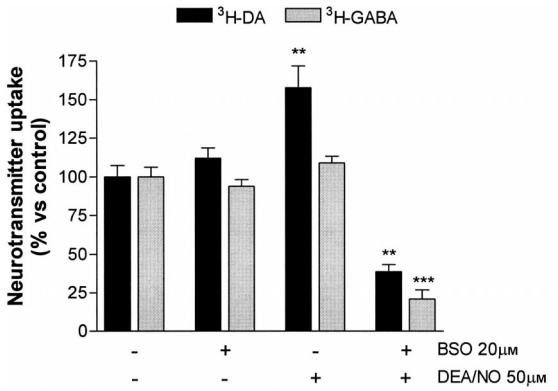

Fig. 1 GSH depletion switches NO-induced up-regulation of DA function to neurotoxic effect in midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for additional 24 h. The figure shows high-affinity [^{3}H]DA and [^{3}H]GABA uptakes expressed as a percentage versus controls. Values are the mean ± SEM from $n = 6$. Control values were 6.6×10^5 cpm/mg protein for [^{3}H]DA and 4.0×10^5 cpm/mg protein for [^{3}H]GABA. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $^{**}p < 0.01$; $^{***}p < 0.001$ versus their respective controls.

cultures pretreated for 24 h either with BSO 20 μM or vehicle. BSO 20 μM treatment without DEA/NO addition did not show any changes in cell viability. Neurotrophic doses of NO that caused no alterations in cell viability parameters (50 μM DEA/NO or 100 μM SNAP), turned on highly toxic in GSH-down-regulated cultures, increasing LDH release by 20–30% (Figs 2a and c) and decreasing MTT viability assay values by 75–85% (Figs 2b and d). A minimal amount of NO was required to switch on the cell death cascade, since 25 μM DEA/NO or 50 μM SNAP did not alter cell viability in the cultures. Both NO donors had similar effects on BSO-pretreated cultures and the addition of denitrosylated DEA/NO 50 μM (2 h after having been dissolved) exerted no effects on cell viability (Fig. 2, last bar to the right of graphs in (a) and (b), indicated as –NO), suggesting that NO is essential to initiate the cell death cascade.

GSH depletion after BSO treatment was dose- and time-dependent (Figs 3a and b). Treatment of cell cultures for 24 h with 1, 3, 10 and 20 μM BSO decreased intracellular GSH to 60%, 50%, 30% and 20% versus controls, respectively. GSH content in cultures treated with 20 μM BSO decreased from 20% at 24 h to 10% at 48 h of treatment, respectively. No signs of toxicity were seen with

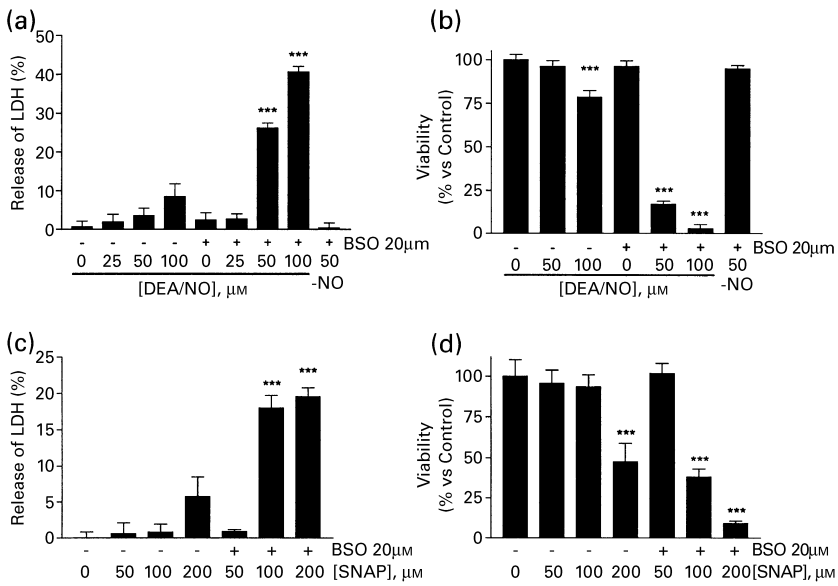

Fig. 2 Effects of NO donors and GSH depletion on cell viability. After 4 days *in vitro*, the cells were treated with BSO 20 μM or vehicle, and then on the fifth day, pre-established groups were treated with either the NO donors DEA/NO or SNAP in concentrations ranging from 25 to 200 μM, for 24 h. Cell viability was measured by MTT assay and is presented as a percentage versus controls. Cell cytotoxicity was measured by LDH activity in the culture medium and is expressed as a percentage versus detergent-extracted controls (100% cytotoxicity). (a) and (b) represent release of LDH and viability, respectively, in midbrain cultures treated with BSO 20 μM and DEA/NO in concentrations ranging from 25 to 100 μM. The last graph-bar on the right (– NO) corresponds to groups that were treated with denitrosylated DEA/NO 50 μM (added to the culture 2 h after DEA/NO reconstitution). (c) and (d) represent LDH cytotoxicity and MTT viability, respectively, in midbrain cultures treated with BSO 20 μM and SNAP in concentrations ranging from 50 to 200 μM. Values are the mean ± SEM from $n = 4$–6. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $^{***}p < 0.001$ versus controls.

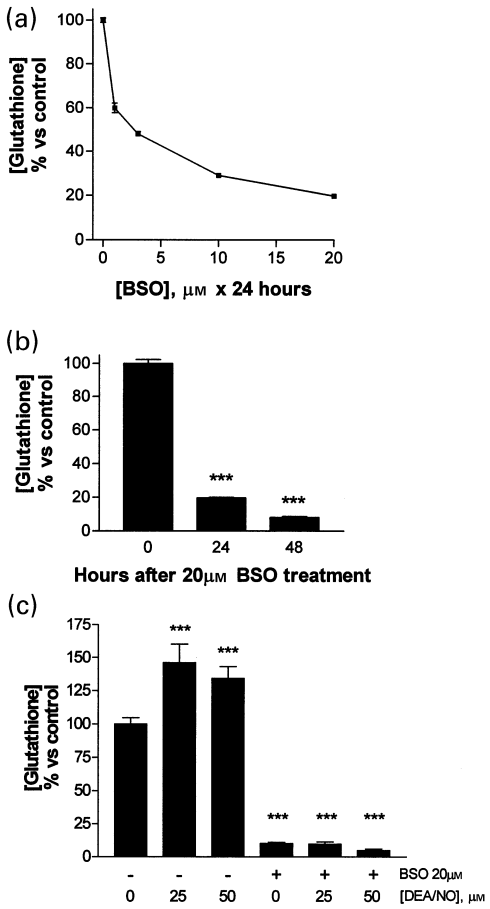

Fig. 3 Intracellular GSH levels in midbrain cultures treated with BSO and DEA/NO separately and in combination. GSH concentration is normalized by protein content and expressed as a percentage versus controls. (a) Dose–response curve of BSO (1, 3, 10 and 20 μM) treated for 24 h; (b) time-response curve of 20 μM BSO treatment; and (c) effect of pretreatment with 20 μM BSO for 24 h on DEA/NO-induced up-regulation of GSH synthesis. Basal levels of GSH were 17.2 ± 0.3 μg/mg protein in (a), 16.9 ± 0.4 μg/mg protein in (b), and 18.8 ± 0.9 μg/mg protein in (c). Values are the mean ± SEM from $n = 4$–8. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. ***$p < 0.001$ versus control.

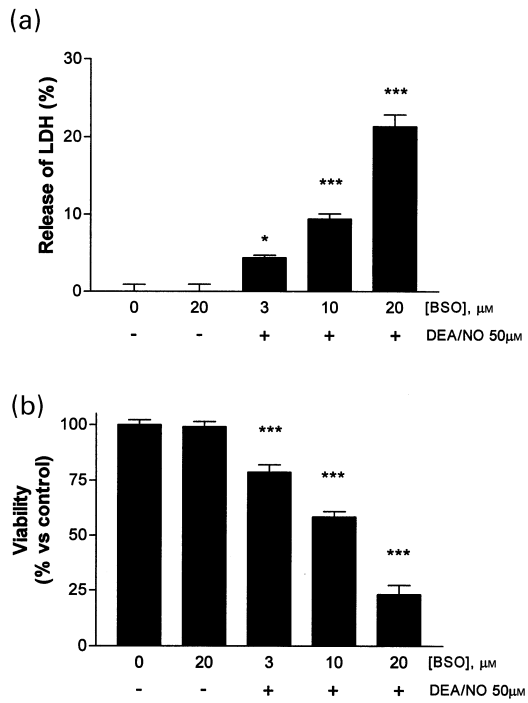

Fig. 4 Effect of pretreatment with different doses of BSO followed by DEA/NO 50 μM on cell viability. After 4 days *in vitro*, the cultures were treated with increasing concentrations of BSO (3, 10 and 20 μM) or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for an additional 24 h. (a) Cell cytotoxicity measured in the culture medium by LDH activity. (b) Cell viability measured by MTT assay and presented as a percentage versus controls. Values are the mean ± SEM from $n = 4$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. *$p < 0.05$; ***$p < 0.001$ versus controls.

BSO 20 μM at 48 h (BSO-pretreated DEA/NO-untreated groups) and at 72 h (data not shown) in midbrain cultures. When used in combination with 25 and 50 μM DEA/NO, 20 μM BSO prevented NO-induced GSH up-regulation in the cultures and depleted intracellular GSH to the same extent that when applied alone (Fig. 3c).

To investigate which level of GSH depletion is sufficient to trigger NO-induced cell death cascade, midbrain cultures were pretreated for 24 h with the same doses of BSO than in Fig. 3, and then 50 μM DEA/NO was added for additional 24 h. Results, as shown in Fig. 4, indicate that a neurotrophic dose of NO turns on neurotoxic from 3 μM BSO pretreatment, that is 50% depletion of intracelular GSH, and the toxicity increases in a dose-dependent manner in parallel with BSO concentration. We have used a pretreatment with 20 μM BSO for the cell death characterization and the cell death protection studies because such dose was not toxic by itself but it produced a high degree of toxicity when combined with 50 μM DEA/NO.

Soluble guanylate cyclase (sGC) and cyclic GMP-dependent protein kinase (PKG) are involved in the cell death cascade

GSH depletion has been shown to increase cyclic GMP (cGMP) synthesis in rat brain and primary culture neurones (Heales et al. 1996). Also, it is well known that NO is an endogenous activator for sGC (Bredt and Snyder 1989). However, we have previously shown that neurotrophism induced by 50 μM DEA/NO and cell death induced by 400 μM DEA/NO in fetal midbrain cultures are not mediated by cGMP (Canals et al. 2001). To study the implication of cGMP in the toxicity induced by low doses of NO in GSH down-regulated cultures, two structurally differentiated sGC inhibitors, LY83583 and methylene blue (MB), were used.

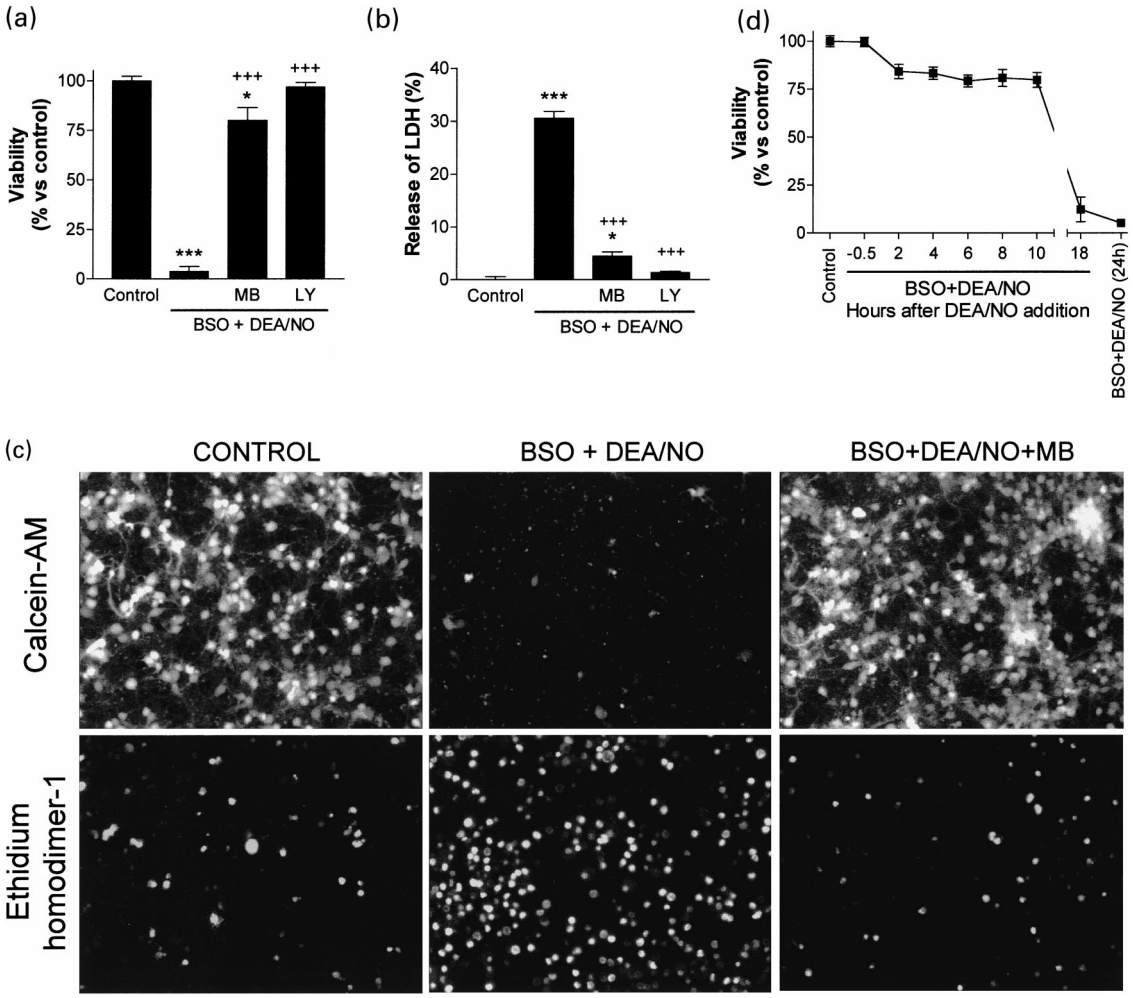

Fig. 5 Guanylate cyclase inhibitors protect from NO-induced toxicity in GSH-down-regulated midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM, then on the fifth day, pre-established groups were treated with methylene blue 0.1 μM (MB), 0.2 μM LY83583 (LY) or vehicle and 30 min later with DEA/NO 50 μM for 24 h. (a) Cell viability measured by MTT assay and presented as a percentage versus controls. (b) Cell cytotoxicity measured by LDH activity in the culture medium. (c) Photomicrographs of cells stained with calcein-AM and ethidium homodimer-1. (d) LY83583 was added to the cultures 30 min before DEA/NO addition or up to 10 h later, in 2 h-intervals. At 24 h of DEA/NO addition, cell viability was measured by MTT assay and is expressed as a percentage versus controls. Values are expressed as the mean ± SEM from $n = 4–6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $^{*}p < 0.05$, $^{***}p < 0.001$ versus controls; $^{+++}p < 0.001$ versus BSO 20 μM + DEA/NO 50 μM.

Addition of 0.2 μM LY83583 or 0.1 μM MB to the cultures 30 min before DEA/NO treatment (Figs 5a and b) or up to 10 h after (Fig. 5d), rescued the cultures from cell death as seen by MTT viability assay (Figs 5a and d), LDH activity assay (Figs 5b and 7), and calcein-AM/ethidium homodimer-1 staining (Fig. 5c). Protection was 100% when inhibitors were applied before NO treatment and by about 80% when applied 2–10 h after DEA/NO treatment (Fig. 5d). sGC inhibitors protected from cell death but not from GSH depletion (data not shown).

The PKG inhibitor KT5823 was used to investigate the way in which cGMP participates in the induced cell death. At concentrations of 0.5 and 1 μM, this inhibitor added to GSH-depleted cultures 30 min before DEA/NO treatment and up to 10 h after, strongly prevented the loss of viability as measured by MTT (Figs 6a and c) and LDH assays (Figs 6b and 7), indicating that cGMP-induced cell death occurs through a PKG-dependent mechanism. Since dimethyl sulfoxide (DMSO), used as solvent for KT5823, interfered with our model in a dose-dependent manner (Figs 6d and e), we reduced DMSO concentration as much as allowed by KT5823 solubility and treated the cultures with the usual dose of DEA/NO and a higher one. PKG inhibitor protected from cell death but not from GSH depletion (data not shown).

Free radicals are also involved in cell death

The antioxidant ascorbic acid (AA) was supplied to BSO-pretreated cultures 30 min before DEA/NO addition or up to 10 h after (Fig. 7). AA protected from cell death when was present in the culture before NO addition. The protection was progressively decreasing from 2 h (40% protection) to 10 h (no protection) after NO treatment, suggesting that free radical production occurs soon in the cell death cascade and that free radical levels correlate with cell death in the culture. When the ability of AA, sGC and PKG inhibitors to prevent cell death was compared in parallel, the protective effect of AA was lost sooner after NO treatment than the inhibitors protective effects (Fig. 7). This suggests that free radical production precedes sGC and PKG activation in the cell death cascade.

Involvement of free radicals in the BSO + DEA/NO-induced cell death is also suggested by GSH/GSSG ratios. Cultures treated with 50 μM DEA/NO alone doubled its GSH/GSSG ratio, 20 μM BSO treatment decreased it and BSO + DEA/NO combined treatment, although not statistically significant versus BSO alone, further decreased the GSH/GSSG ratio. Inhibition of PKG protected from cell death but did not restore GSH/GSSG ratio, indicating that free radical production precedes PKG activation (Table 1).

Cell type susceptibility to NO-induced toxicity in GSH-down-regulated cultures

Immunocytochemical characterization of cell death in cultures pretreated for 24 h with 20 μM BSO and treated for additional 24 h with 50 μM DEA/NO, reveals that all neurones in the cultures (TH^+ and MAP-2^+ cells) are strongly affected by the treatment (Fig. 8b), but TH^+ cells remain more preserved. Untreated groups showed that TH^+ cells represent a minor proportion of the total neurones in the cultures (Fig. 8a, top panels), but when treated with

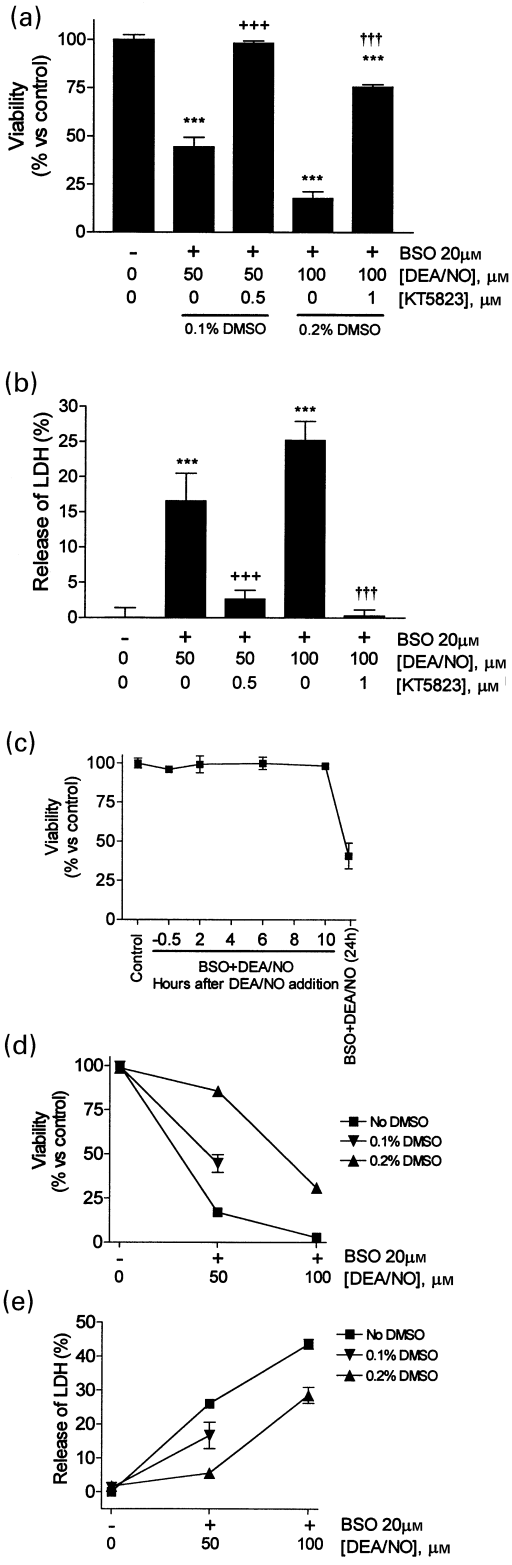

Fig. 6 The cGMP-dependent kinase inhibitor KT5823 protects from NO-induced toxicity in GSH-down-regulated midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM, then on the 5th day, preestablished groups were treated with DEA/NO 50–100 μM for 24 h. Cell viability was measured by MTT assay and is presented as a percentage versus controls. Cell cytotoxicity was measured by LDH activity in the culture medium. (a) and (b) represent viability and release of LDH, respectively, in cultures treated with 0.5 μM KT5823 in 0.1% DMSO or 1 μM KT5823 in 0.2% DMSO, 30 min before DEA/NO addition. Control and BSO + DEA/NO groups received the appropriate dose of DMSO under each condition. $^{***}p < 0.001$ versus controls; $^{+++}p < 0.001$ versus BSO 20 μM + DEA/NO 50 μM in 0.1% DMSO; †††$p < 0.001$ versus BSO 20 μM + DEA/NO 100 μM in 0.2% DMSO. (c) 0.5 μM KT5823 was added to the cultures 30 min before and 2, 6 and 10 h after 50 μM DEA/NO treatment. At 24 h of DEA/NO addition, cell viability was measured by MTT assay. Values are expressed as a percentage versus controls. (d) and (e) show viability and release of LDH, respectively, in BSO + DEA/NO-treated midbrain cultures in the absence or presence of 0.1% or 0.2% DMSO. $^{***}p < 0.001$ versus DMSO untreated group. Values are the mean ± SEM from $n = 4–6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test.

Table 1 Effects of treatments with BSO, DEA/NO and PKG inhibitor on GSH/GSSG ratios

Control	29.6 ± 4.9
DEA/NO 50 μM	60.4 ± 5.5***
BSO 20 μM	6.1 ± 1.2***
BSO + DEA/NO 50 μM	3.6 ± 0.6***
BSO + DEA/NO + KT5823 0.5 μM	3.4 ± 0.5***
KT5823 0.5 μM	28.3 ± 3.1

After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then, on the fifth day, pre-established groups were pretreated with 0.5 μM KT5823 or vehicle and 30 min later with DEA/NO 50 μM for additional 24 h. Control values for GSH and GSSG are 22.2 ± 1.5 and 0.8 ± 0.1 ng/μg of protein, respectively. Values are expressed as the mean ± SEM for $n = 4$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. ***$p < 0.001$ versus control.

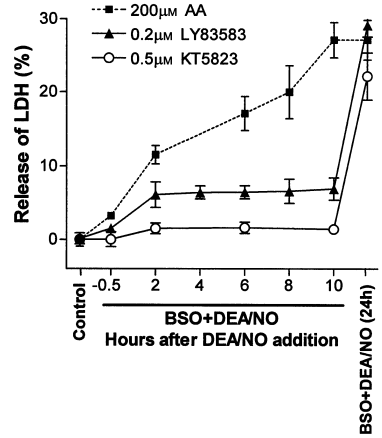

Fig. 7 Protection time pattern of the antioxidant ascorbic acid (AA), the sGC inhibitor LY83583 and the PKG inhibitor KT5823 on cell death induced by NO in GSH-down-regulated midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for an additional 24 h. At different time points after DEA/NO treatment, 200 μM AA, 0.2 μM LY83583 or 0.5 μM KT5823 were added to the culture. Cell death was measured at 24 h by LDH activity. Values are the mean ± SEM from $n = 4$.

BSO + DEA/NO, TH^+ cells reached the main population of surviving neurones (Fig. 8a, middle panels). These results are in agreement with those shown in Fig. 1 referred to [^{3}H]DA and [^{3}H]GABA uptakes, and indicate that DA neurones, although very affected, are the most resistant neurones to NO-induced toxicity in GSH-down-regulated midbrain cultures.

The sGC inhibitor MB, added to the culture 30 min before DEA/NO treatment in GSH-down-regulated cultures, totally protected MAP-2^+ and TH^+ cells from NO-induced toxicity, showing intact neuronal processes (Fig. 8a, bottom panels and 8b).

NO induces on GSH-down-regulated cultures a form of programmed cell death with characteristics of apoptosis and necrosis

Cell death induced by DEA/NO 50 μM in cultures pretreated with BSO 20 μM for 24 h is characterized by shrinkage, rounded cells with chromatin condensation without DNA fragmentation. Chromatin-condensed cells in the cultures increased above 70% with no increase in $TUNEL^+$ cells (Table 2). Chromatin condensed peripherally in the nucleus, as seen by bis-benzimide staining and phase contrast (inset in bis-benzimide photomicrograph and arrows in Fig. 9). TUNEL staining in the same fields showed that peripherally condensed chromatin did not comark as $TUNEL^+$ (insets in Fig. 9). Neither BSO 20 μM nor DEA/NO 50 μM alone caused changes in cell or nuclear morphology by phase contrast, or bis-benzimide or TUNEL staining. On the other hand, the combined treatment of BSO and DEA/NO gave rise to breakdown of plasmatic membrane, since LDH released to the culture medium increased to 30%, suggesting necrotic cell death. Furthermore, the membrane-impermeable DNA dye ethidium homodimer (Fig. 5c) and trypan

Table 2 Effects of BSO and DEA/NO treatment on chromatin condensation and DNA fragmentation

	TUNEL cells ($\times 10^3$)	Condensed nuclei ($\times 10^3$)	Condensed nuclei (% versus total nuclei)
Control	2.3 ± 0.3	2.6 ± 0.3	9.7 ± 0.9
DEA/NO 25 μM	2.3 ± 0.2	2.4 ± 0.1	9.0 ± 0.9
DEA/NO 50 μM	2.6 ± 0.2	2.9 ± 0.2	10.2 ± 1.1
BSO 20 μM	2.2 ± 0.2	2.5 ± 0.3	9.3 ± 0.6
BSO + DEA/NO 25 μM	2.2 ± 0.3	2.6 ± 0.3	9.8 ± 0.7
BSO + DEA/NO 50 μM	2.7 ± 0.2	19.3 ± 0.6***	72.6 ± 2.3***

After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then, on the fifth day, pre-established groups were treated with DEA/NO 25 or 50 μM for an additional 24 h. Values are expressed as the mean ± SEM for $n = 4$–6. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. ***$p < 0.001$ versus control.

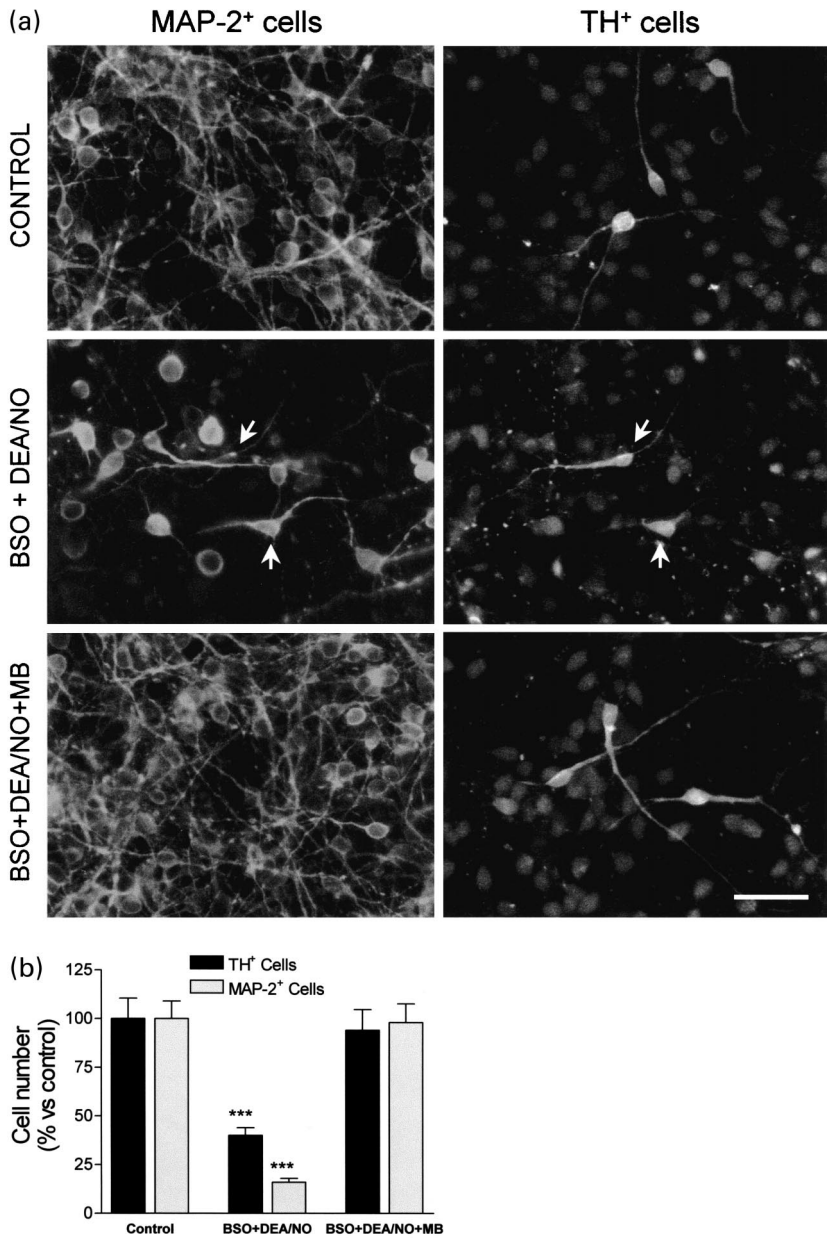

Fig. 8 Cell type selectivity of BSO + DEA/NO-induced neurotoxicity and cell protection by the sGC inhibitor methylene blue (MB). After 4 days *in vitro*, the cultures were treated with BSO 20 μM, then on the fifth day, pre-established groups were treated with MB 0.1 μM or vehicle and 30 min later with DEA/NO 50 μM for 24 h additional. (a) Photomicrographs show total neurones and DA neurones, corresponding to the same field, after cell treatments. Arrows indicate TH- and MAP-2-costained cells in the same field. Scale bar = 50 μm. (b) Number of total neurones (MAP-2$^+$) and DA neurones (TH$^+$) expressed as a percentage versus controls. Values are expressed as the mean ± SEM for $n = 4–6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. ***$p < 0.001$ versus their respective controls.

blue dye exclusion assay (data not shown), stained almost all the chromatin-condensed cells, indicating that both chromatin condensation and breakdown of plasmatic membrane occur simultaneously in the same cell. The definitive nature of programmed cell death is suggested because the inhibition of RNA and protein synthesis and of caspase activity did revert or attenuate cell death in the cultures (Fig. 10). The protein synthesis inhibitor, cycloheximide, at 0.01 μg/mL, added to the culture 30 min before DEA/NO 50 μM treatment, prevents the loss of viability as measured by MTT assay (Fig. 10a) and the increased LDH released to the culture medium (Fig. 10b). Similar results were obtained with the transcriptional inhibitor, actinomycin D, but the inhibitor by itself at 0.1 μg/mL caused more toxicity than did cycloheximide (Figs 10a and b), and lower concentrations failed to protect midbrain cultures from BSO + DEA/NO toxicity (data not shown). Furthermore, the broad spectrum inhibitor of caspases Boc-D-FMK also reverted the loss of viability induced by BSO- and DEA/NO-cotreatment (Fig. 10c).

Discussion

We have previously shown that the NO donor DEA/NO at low doses (25 and 50 μM) not only protects DA cells from apoptosis but also induces *de novo* TH synthesis and exerts

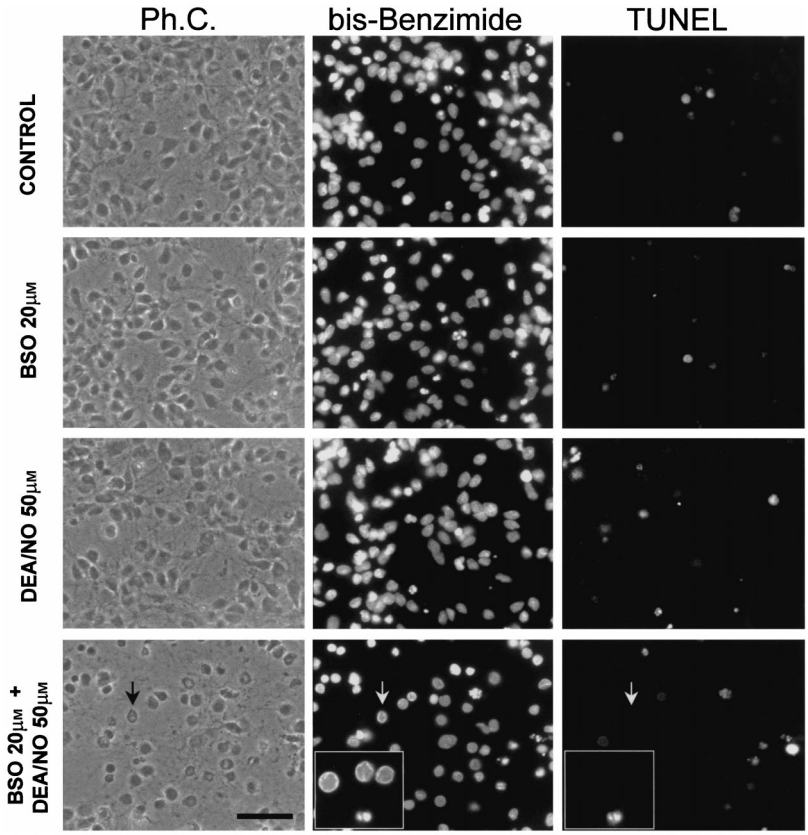

Fig. 9 Effect of GSH-down-regulation and NO treatment in apoptotic cell death of fetal midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for additional 24 h. The figure shows the phase-contrast microscopy of midbrain cultures and of total nuclei stained with bis-benzimide and nuclear fragmented cells stained by the TUNEL assay, corresponding to the same field. Arrows mark a constant position in the photomicrographs of BSO + DEA/NO group. Inset shows that chromatin-condensed nuclei are not comarked by TUNEL assay. Scale bar = 50 μm.

neurotrophic effects on DA function. In addition, it increases intracellular and extracellular GSH (Canals *et al*. 2001). To investigate the importance of correct GSH homeostasis for the neurotrophic capacity of NO, we pretreated cell cultures with different doses of the γ-glutamylcysteine synthetase inhibitor BSO. With this model, we demonstrated that a GSH depletion of only 50% is sufficient to transform the neurotrophic effect on DA function exerted by low doses of NO into neurotoxic effects. Under these conditions, NO triggers a programmed cell death with markers of both apoptosis and necrosis that is characterized by an early step of free radicals production followed by a late requirement for signalling on the sGC/cGMP/PKG pathway.

Several authors have reported that experimental depletion of GSH potentiates the toxicity of 6OH-DA and MPTP *in vivo* (Pileblad *et al*. 1989; Wullner *et al*. 1996; Klivenyi *et al*. 2000), sulfite plus $ONOO^-$ in CSM14.1.4 cell line (Marshall *et al*. 1999) and $ONOO^-$ in astrocytes (Barker *et al*. 1996). Furthermore, cellular GPx-deficient mice show increased vulnerability to MPTP (Klivenyi *et al*. 2000). A very important aspect of our data is that a 50% depletion of GSH, similar to that observed in PD (Sian *et al*. 1994; Merad-Boudia *et al*. 1998), is enough to enable NO, at low doses, to trigger the cell death cascade. Although under these conditions, cell death induced by NO is less significant than that induced with higher GSH depletions, it may become more outstanding over longer time periods, like in neurodegenerative diseases with development over decades.

Soluble guanylate cyclase inhibitors, LY83583 and MB, protect from cell death induced by BSO 20 μM pretreatment and DEA/NO 50 μM treatment up to 10 h after NO addition, suggesting that sGC is directly linked to neuronal death. Also, it suggests that NO released from DEA/NO is not the major source of sGC activation in our model because DEA/NO totally decomposes in less than 15 min (half-life decomposition of 3.5 ± 0.2 min) and sGC activation occurs up to 10 h after NO donor addition. Only a 20% decrease in cell viability was seen when sGC was inhibited 2 h or more after NO treatment, which may be explained by direct activation of sGC by NO. In addition to NO, other compounds like hydroxyl radicals and lipid peroxides activate sGC (Weber 1999; Snider *et al*. 1984; Reiser 1990) and have been involved in this way, with cell death (Li *et al*. 1997a). These compounds might participate in the cell death cascade observed in our model. This hypothesis is further supported by the experiments showing AA protection from BSO + DEA/NO-induced cell death. Because AA protects at earlier stages of the cell death cascade than sGC and PKG inhibitors, free radical species may initiate the death signalling. Furthermore, GSH/GSSG ratios indicate an increase in intracellular oxidative stress after the treatments that is not reverted by PKG inhibition. These results corroborate the

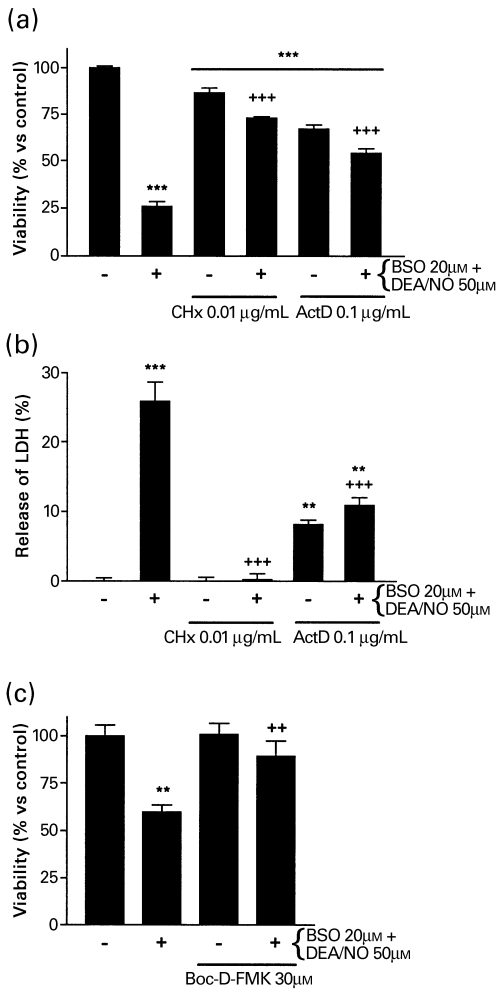

Fig. 10 Inhibition of macromolecular synthesis and caspase activity prevent cell death. After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for an additional 24 h. The transcriptional inhibitor actinomycin D (ActD), the protein synthesis inhibitor cycloheximide (CHx), the broad spectrum caspase inhibitor Boc-D-FMK or its correspond solvents, were added 30 min before DEA/NO treatment. (a) and (c) show cell viability measured by MTT assay and presented as a percentage versus controls. (b) Cell cytotoxicity measured by LDH activity in the culture medium. Values are expressed as the mean ± SEM for $n = 4–6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$ versus untreated groups. $^{++}p < 0.01$, $^{+++}p < 0.001$ versus BSO 20 μM + DEA/NO 50 μM.

involvement of oxidative stress as the major mechanism in this process and the neuroprotective role of AA in PD.

The PKG inhibitor KT5823 totally protects midbrain cultures from BSO + DEA/NO-induced cell death for up to 10 h after NO addition. PKG has been identified in neurones of the basal ganglia (Walaas *et al*. 1989), where its major substrate, the protein phosphatase I inhibitor DARPP-32, is highly represented (Tsou *et al*. 1993; Wang and Robinson 1997). The NO/cGMP/PKG pathway has been implicated in protein phosphatase regulation, calcium signalling, cytoskeletal dynamics, neurotransmitter release (Wang *et al*. 1997), the regulation of catecholamine synthesis, and secretion in CNS (Rodríguez-Pascual *et al*. 1999), but no data is available about its participation in cell death processes in CNS. Since we noted that macromolecular synthesis inhibitors also protects from cell death induced by BSO + DEA/NO treatment, and PKG has been implicated in *fos* promoter activation (Gudi *et al*. 1996; Gudi *et al*. 1999), the sGC/cGMP/PKG pathway may activate genes implicated in the cell death induced by NO in GSH down-regulated midbrain cultures.

NO is essential to initiate the cell death cascade. This is supported because two structurally differentiated donors exerted the same results and denitrosylated DEA/NO did not cause loss of viability. Also, in our experimental model, BSO by itself at any of doses used and for up to 3 days in culture (data not shown), did not result in any signs of toxicity. This is in agreement with other data in midbrain cultures that show no toxicity after 3 days of 50 μM BSO treatment (Mytilineou *et al*. 1999) and in rat mesencephalic cell line CSM14.1.4, in which 100 μM BSO for up to 60 h does not compromise cell viability detected by trypan blue exclusion or MTT reduction (Marshall *et al*. 1999). However, in other models with embryonic cortical primary neurones and HT22 hippocampal nerve cell line, GSH depletion above 80%, induced with glutamate or BSO treatment, causes cell death by sGC activation and extracellular Ca^{2+} influx, without PKG participation (Li *et al*. 1997a,b).

Here we show that 50 μM DEA/NO induces a type of cell death in the GSH down-regulated cultures that integrates simultaneously in the same cell morphological characteristics of apoptosis and necrosis. The biochemical study indicates that this type of cell death constitutes an active process with cell participation because inhibitors of macromolecule synthesis, such as cycloheximide and actinomycin D, and caspase inhibitors attenuate cell death, like in many other cases of apoptosis (Oppenheim *et al*. 1990; Koh *et al*. 1995; Ahn *et al*. 2000). A type of cell death with characteristics of both apoptosis and necrosis has been previously reported by using a mouse hippocampal cell line (HT-22) treated with either 5 mM glutamate or 250 μM BSO (Tan *et al*. 1998). Intracellular ATP depletion switches the mode of cell death from apoptosis to necrosis (Leist *et al*. 1999). NO donors caused necrosis in the absence of glucose due to inhibition of respiration and subsequent ATP depletion, but in the presence of glucose, to maintain ATP level via glycolysis, NO donors caused apoptosis. GSH depletion inhibits mitochondrial complex I activity (Jha *et al*. 2000) leading to mitochondrial respiration failure and ATP depletion. Finally, ATP is essential for the morphological changes in the nuclei typical of apoptosis (Kass *et al*. 1996).

These experiments may contribute to understand the type of programmed cell death observed in GSH-depleted cultures.

We found that DA neurones are more resistant than GABA neurones to GSH depletion plus NO treatment. Nakamura *et al*. (2000) reported that the preferential resistance of DA neurones to the toxicity of GSH depletion was independent of cellular GPx and was mediated by tetrahydrobiopterin (BH4). The resistance of dopaminergic neurones to oxidative stress may be critical to their survival and disturbances in their capacity to produce BH4 or other antioxidants from genetic mutations or exposure to exogenous toxins could underlie their demise in PD. Such a multihit hypothesis for DA cell death is consistent with the current view that PD is a heterogeneous disease that can arise from combinations of genetic susceptibilities and environmental insults (Langston 1998).

If our results can be extended to *in vivo* situations, then intervention of the sGC/cGMP/PKG pathway could be beneficial to individuals suffering from PD or other pathologies associated with NO and GSH disregulation. Experiments to elucidate the molecular and cellular mechanisms, to identify of cell death genes and the role played by mitochondria, leading to cell death initiated by NO when GSH synthesis is compromised in neuronal models, could lead to novel preventive or therapeutic strategies for PD.

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References

Ahn Y.-H., Koh J.-Y. and Hong S. H. (2000) Protein synthesis-dependent but Bcl-2-independent cytochrome c release in zinc depletion-induced neuronal apoptosis. *J. Neurosci. Res.* **61**, 508–514.

Barker J. E., Bolaños J. P., Land J. M., Clark J. B. and Heales S. J. R. (1996) Glutathione protects astrocytes from peroxynitrite-mediated mitochondrial damage: implications for neuronal/astrocyte trafficking and neurodegeneration. *Dev. Neurosci.* **18**, 391–396.

Beart P. M. and McDonald D. (1980) Neurochemical studies of the mesolimbic dopaminergic pathway: somatodendritic mechanisms and GABAergic neurons in rat ventral tegmentum. *J. Neurochem.* **34**, 1622–1629.

Bredt D. S. and Snyder S. H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA* **86**, 9030–9033.

Canals S., Casarejos M. J., Rodríguez-Martín E., de Bernardo S. and Mena M. A. (2001) Neurotrophic and neurotoxic effects of nitric oxide on fetal midbrain culturess. *J. Neurochem.* **76**, 56–68.

Chiueh C. C. and Rauhala P. (1999) The redox pathway of *S*-nitrosoglutathione, glutathione and nitric oxide in cell to neuron communications. *Free Rad. Res.* **31**, 641–650.

Decker T. and Lohmann-Matthes M. L. (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumour necrosis factor (TNF) activity. *J. Immunol. Methods* **115**, 61–69.

Di Monte D. A., Chan P. and Sandy M. S. (1992) Glutathione in Parkinson's disease: a link between oxidative stress and mitochondrial damage? *Ann. Neurol.* **32**, 111–113.

Gavrieli Y., Sherman Y. and Ben-Sasson S. A. (1992) Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493–501.

Giasson B. I., Duda J. E., Murray I. V. J., Chen Q., Souza J. M., Hurtig H. I., Ischiropoulos H., Trojanowski J. Q. and Lee V. M.-Y. (2000) Oxidative damage linked to neurodegeneration by selective α-synuclein nitration in synucleinopathy lesions. *Science* **290**, 985–989.

Good P. F., Hsu A., Werner P., Perl D. P. and Olanow C. W. (1998) Protein nitration in Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **57**, 338–342.

Green L. C., Wagner D. A., Glogowski J., Skipper P. L., Wishnok J. S. and Tannenbaum S. R. (1982) Analysis of nitrate, nitrite and ^{15}N-nitrate in biological fluids. *Anal. Biochem.* **126**, 131–138.

Griffith O. W. (1980) Determination of glutathione and glutathione disulphide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **106**, 207–212.

Gudi T., Huvar I., Meinecke M., Lohmann S. M., Boss G. R. and Pilz R. P. (1996) Regulation of gene expression by cGMP-dependent protein kinase. *J. Biol. Chem.* **271**, 4597–4600.

Gudi T., Hong G. K. P., Vaandrager A. B., Lohmann S. M. and Pilz R. B. (1999) Nitric oxide and cGMP regulate gene expression in neuronal and glial cells by activating II cGMP-dependent protein kinase. *FASEB J.* **13**, 2143–2152.

Heales S. J. R., Bolaños J. P. and Clark J. B. (1996) Glutathione depletion is accompanied by increased neuronal nitric oxide synthase activity. *Neurochem. Int.* **21**, 35–39.

Hilwig I. and Gropp A. (1975) pH dependent fluorescence of DNA and RNA in cytological staining with '33342 Hoechst'. *Exp. Cell Res.* **91**, 457–460.

Jha N., Jurma O., Lalli G., Liu Y., Pettus E. H., Greenamyre J. T., Liu R.-M., Forman H. J. and Andersen J. K. (2000) Glutathione depletion in PC12 results in selective inhibition of mitochondrial complex I activity. *J. Biol. Chem.* **275**, 26096–26101.

Kass G. E. N., Eriksson J. E., Weis M., Orrenius S. and Chow S. C. (1996) Chromatin condensation during apoptosis requires ATP. *Biochem. J.* **318**, 752.

Kerr J. F., Wyllie A. H. and Currie A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239–257.

Klivenyi P., Andreassen O. A., Ferrante R. J., Dedeoglu A., Mueller G., Lancelot E., Bogdanov M., Andersen J. K., Jiang D. and Beal M. F. (2000) Mice deficient in cellular glutathione peroxidase show increased vulnerability to malonate, 3-nitropropionic acid, and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *J. Neurosci.* **20**, 1–7.

Koh J.-Y., Wie M. B., Gwag B. J., Sensi S. L., Canzoniero L. M., Demaro J., Csernansky C. and Choi D. W. (1995) Staurosporine-induced neuronal apoptosis. *Exp. Neurol.* **135**, 153–159.

Langston J. W. (1998) Epidemiology versus genetics in Parkinson's disease: progress in resolving an age-old debate. *Ann. Neurol.* **44**, S45–S52.

LaVoie M. J. and Hastings T. G. (1999) Peroxynitrite- and nitrite-induced oxidation of dopamine: implications for nitric oxide in dopaminergic cell loss. *J. Neurochem.* **73**, 2546–2554.

Leist M., Single B., Naumann H., Fava E., Simon B., Kühnle S. and Nicotera P. (1999) Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis to necrosis. *Exp. Cell Res.* **249**, 396–403.

Li Y., Maher P. and Schubert D. (1997a) A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. *Neuron* **19**, 453–463.

Li Y., Maher P. and Schubert D. (1997b) Requirement for cGMP in nerve cell death caused by glutathione depletion. *J. Cell Biol.* **139**, 1317–1324.

Liberatore G. T., Jackson-Lewis V., Vukosavic S., Mandir A. S., Vila M., McAuliffe W. G., Dawson V. L., Dawson T. M. and Przedborski S. (1999) Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. *Nature Med.* **5**, 1403–1409.

Lipton S. A., Choi Y. B., Pan Z. H., Lei S. Z., Chen H. S., Sucher N. J., Loscalzo J., Singel D. J. and Stamler J. S. (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364**, 626–632.

Marshall K.-A., Reist M., Jenner P. and Haliwell B. (1999) The neuronal toxicity of sulphite plus peroxynitrite is enhanced by glutathione depletion: implications for Parkinson's disease. *Free Rad. Biol. Med.* **27**, 515–520.

Mena M. A., Pardo B., Paíno C. L. and de Yébenes J. G. (1993) Levodopa toxicity in foetal rat midbrain neurons in culture: modulation by ascorbic acid. *Neuroreport* **4**, 438–440.

Mena M. A., Khan U., Togasaki D. M., Sulzer D., Epstein C. J. and Przedborski S. (1997) Effects of wild-type and mutated copper/zinc superoxide dismutase on neuronal survival and L-DOPA-induced toxicity in postnatal midbrain culture. *J. Neurochem.* **69**, 21–33.

Merad-Boudia M., Nicole A., antiard-Baron D., Saille C. and Ceballos-Picot I. (1998) Mitochondrial impairment as an early event in the process of apoptosis induced by glutathione depletion in neuronal cells: relevance to Parkinson's disease. *Biochem. Pharmacol.* **56**, 645–655.

Michel P. P. and Hefti F. (1990) Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture. *J. Neurosci. Res.* **26**, 428–435.

Mytilineou C., Kokotos-Leonardi E. T., Kramer B. C., Jamindar T. and Olanow C. W. (1999) Glial cells mediate toxicity in glutathione-depleted mesencephalic cultures. *J. Neurochem.* **73**, 112–119.

Nakamura K., Wang W. and Kang U. J. (1997) The role of glutathione in dopaminergic neuronal survival. *J. Neurochem.* **69**, 1850–1858.

Nakamura K., Wright D. A., Wiatr T., Kowlessur D., Milstien S., Lei X. G. and Kang U. J. (2000) Preferential resistance of dopaminergic neurons to the toxicity of glutathione depletion is independent of cellular glutathione peroxidase and is mediated by tetrahydrobiopterin. *J. Neurochem.* **74**, 2305–2314.

Oppenheim R. W., Prevette D., Tytell M. and Homma S. (1990) Naturally occurring and induced neuronal death in the chick embryo *in vivo* requires protein and RNA synthesis: evidence for the role of cell death genes. *Dev. Biol.* **138**, 104–113.

Pardo B., Paíno C. L., Casarejos M. J. and Mena M. A. (1997) Neuronal-enriched cultures from embryonic rat ventral mesencephalon for pharmacological studies of dopamine neurons. *Brain Res. Protocols* **1**, 127–132.

Perry T. L., Godin D. V. and Hansen S. (1982) Parkinson's disease: a disorder due to nigral glutathione deficiency? *Neurosci. Lett.* **33**, 305–310.

Pileblad E., Magnusson T. and Fornstedt B. (1989) Reduction of brain glutathione by 1-buthionine sulfoximine potentiates the dopamine-depleting action of 6-hydroxydopamine in rat striatum. *J. Neurochem.* **52**, 978–984.

Przedborski S., Jackson-Lewis V., Yokoyama T., Shibata T., Dawson V. L. and Dawson T. M. (1996) Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. *Proc. Natl. Acad. Sci. USA* **93**, 4565–4571.

Qureshi G. A., Baig S., Bednar I., Sodersten P., Forsberg G. and Siden A. (1995) Increased cerebrospinal fluid concentration of nitrite in Parkinson's disease. *Neuroreport* **6**, 1642–1644.

Rauhala P., Lin A. M.-Y. and Chiueh C. C. (1998) Neuroprotection by S-nitrosoglutathione of brain dopamine neurons from oxidative stress. *FASEB J.* **12**, 165–173.

Reiser G. (1990) Mechanism of stimulation of cyclic-GMP level in a neuronal cell line mediated by serotonin (5-HT_3) receptors. *Eur. J. Biochem.* **189**, 547–552.

Rodríguez-Pascual F., Ferrero R., Miras-Portugal M. T. and Torres M. (1999) Phosphorylation of tyrosine hydroxylase by cGMP-dependent protein kinase in intact bovine cromaffin cells. *Arch. Biochem. Biophys.* **366**, 207–214.

Rodríguez-Martín E., Casarejos M. J., Bazán E., Canals S., Herranz A. S. and Mena M. A. (2000) Nitric oxide induces differentiation in the NB69 human catecholamine-rich cell line. *Neuropharmacology.* **39**, 2090–2100.

Shergill J. K., Cammack R., Cooper C. E., Cooper J. M., Mann V. M. and Schapira A. H. (1996) Detection of nitrosyl complexes in human substantia nigra, in relation to Parkinson's disease. *Biochem. Biophys. Res. Commun.* **228**, 298–305.

Sian J., Dexter D. T., Lees A. J., Daniel S. E., Agid Y., Javoy-Agid F., Jenner P. and Marsden C. D. (1994) Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann. Neurol.* **36**, 348–355.

Snider R. M., McKinney M., Forray C. and Rechelson E. (1984) Neurotransmitter receptors mediate cyclic GMP formation by involvement of arachidonic acid and lipoxygenase. *Proc. Natl. Acad. Sci. USA* **81**, 3905–3909.

Tan S., Wood M. and Maher P. (1998) Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. *J. Neurochem.* **71**, 95–105.

Tietze F. (1969) Enzymatic method for quantitative determination of nanogram amounts of total and oxidised glutathione: application to mammalian blood and other tissue. *Anal. Biochem.* **27**, 502–522.

Tsou K., Snyder G. L. and Greengard P. (1993) Nitric oxide/cGMP pathway stimulates phosphorylation of DARPP-32, a dopamine- and cAMP-regulated phosphoprotein, in the substantia nigra. *Proc. Natl. Acad. Sci. USA* **90**, 3462–3465.

Walaas S. I., Girault J. A. and Greengard P. (1989) Localisation of cyclic GMP-dependent protein kinase in rat basal ganglia neurons. *J. Mol. Neurosci.* **1**, 243–250.

Wang X. and Robinson J. (1997) Cyclic GMP-dependent protein kinase and cellular signalling in the nervous system. *J. Neurochem.* **68**, 443–456.

Weber G. F. (1999) Final common pathways in neurodegenerative diseases: regulatory role of the glutathione cycle. *Neurosci. Biobehav. Rev.* **23**, 1079–1086.

Wullner U., Loschmann P. A., Schulz J. B., Schmid A., Dringen R., Eblen F., Turski L. and Klockgether T. (1996) Glutathione depletion potentiates MPTP and MPP+ toxicity in nigral dopaminergic neurones. *Neuroreport* **7**, 921–923.