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ELEVATION IN GLUTATHIONE IN RESPONSE TO AN OXIDATIVE STRESS: SIGNIFICANCE FOR NEUROPROTECTION FROM OXIDATIVE DAMAGE.

by

Shan-Kuo D. Han

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree Doctor of Philosophy, The City University of New York.

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ABSTRACT

ELEVATION IN GLUTATHIONE IN RESPONSE TO AN OXIDATIVE STRESS: SIGNIFICANCE FOR NEUROPROTECTION FROM OXIDATIVE DAMAGE.

by

Shan-Kuo D. Han

Advisor: Professor Gerald Cohen

Glutathione (L-gamma-glutamyl-L-cysteinyl-glycine; GSH) plays an important role in protecting cells from oxidative stress. My studies focus on upregulation of GSH induced by L-DOPA or dopamine, which results in protection against damage by a toxic agent, t-butylhydroperoxide. Upregulation of GSH was found in mesencephalic cell cultures, as well as cultured glia, neuroblastoma cells, and pig kidney epithelium. Not only L-DOPA and dopamine, but other autoxidizable compounds such as hydroquinone, catechol, alpha-methyl-DOPA and apomorphine also evoked an increase in GSH. However, structural analogs that do not autoxidize failed to elevate GSH (3-O-methyl-DOPA, 2,4-dihydroxyphenylalanine, tyrosine, resorcinol). The rise in GSH was preceded by a rise in GSSG and could be blocked by ascorbate, which is an antioxidant. Inhibitors of DOPA decarboxylase or monoamine oxidase had no effect; added superoxide dismutase or catalase had no effect. Dopamine-receptor blocking agents failed to affect the rise in GSH evoked by dopamine. However, the disulfide form of dithiothreitol, which was used as a model disulfide, also induced an elevation in GSH. The results indicate that the rise in GSSG is a response to an oxidative stress due to autoxidation, and that formation of disulfides may play a role. Upregulation of GSH was linked to an increase in gamma-glutamylcysteine synthetase activity, the rate-limiting step in GSH biosynthesis. Inhibitors of protein kinase C (staurosporine and H-7) blocked the rise in GSH, showing that protein kinase C was part of signaling pathway. In comparison with mesencephalic cell cultures, pure neurons (no glia) failed

to upregulate GSH and were badly damaged.

When mesencephalic cell cultures were treated with t-butylhydroperoxide, a progressive loss in viability was seen. Cells previously exposed to autoxidizing agents (L-DOPA or hydroquinone) were protected against the toxicity of t-butylhydroperoxide. However, when the rise in GSH was blocked with ascorbate, cell protection was no longer seen. These results show that upregulation of GSH provides protection in cell culture. Upregulation of GSH appears to be a response to a mild oxidative stress (autoxidation) and requires protein kinase C activity. The ability to upregulate GSH in brain may be an important factor in cell viability in neurodegenerative diseases, such as Parkinson's disease.

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ABBREVIATIONS

Carbidopa,	alpha-methyldopahydrazine (MK486)
CYS,	cysteine
2,4-DOPA,	DL-2,4-dihydroxyphenylalanine
DA,	dopamine
gamma-GCS,	gamma-glutamylcysteine synthetase
GSH,	glutathione (gamma-glutamylcysteinylglycine)
GSH-Px,	glutathione peroxidase
GSSG,	glutathione disulfide (oxidized form of GSH)
HQ,	hydroquinone
L-BSO,	L-buthionine sulfoximine
L-DOPA,	L-3,4-dihydroxyphenylalanine
LDH,	lactate dehydrogenase
MAO,	monoamine oxidase
MEM,	minimum essential medium
MPTP,	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+,	1-methyl-4-phenylpyridinium
MTT,	3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide
NSD-1055,	brocresine
NSE,	neuron-specific enolase
$\cdot OH$,	hydroxyl radical
$O_2^{\cdot -}$,	superoxide anion radical
6-OH-DOPA,	6-hydroxydopa (2,4,5-trihydroxyphenylalanine)
6-OHDA,	6-hydroxydopamine, (2,4,5-trihydroxyphenylethylamine)
SOD,	superoxide dismutase
t-BuOOH,	t-butylhydroperoxide
TH,	tyrosine hydroxylase

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A. BACKGROUND and SIGNIFICANCE

A1. GLUTATHIONE:

Glutathione (L-gamma-glutamyl-L-cysteinyl-glycine; **GSH**), is an essential tripeptide that is ubiquitously found in eukaryotic cells, It was first discovered by de Rey-Paihade (1888) and later named by Hopkins (1921), who initially thought it to be a dipeptide, containing glutamate and cysteine. GSH is synthesized intracellularly and is exported from cells. Its breakdown is by gamma-glutamyltranspeptidase, an enzyme attached to the external surface of membranes. GSH is the most prevalent cellular thiol and the most abundant low molecular-weight peptide presents in cells.

GSH is used for reduction of ribonucleotides to form the deoxyribonucleotide triphosphate precursors of DNA, and also serves as a storage and a transport form of cysteine moieties, protecting the thiols of cysteine from rapid metabolic utilization. It is also well known that glutathione is an important component of a pathway that uses NADPH to provide cells with their reducing environment. Such reducing power is used for maintenance of thiol groups of intracellular proteins and other molecules, such as cysteine, dihydrolipoate, and coenzyme A, as well as antioxidant molecules, such as ascorbate and alpha-tocopherol. Through its actions, GSH works as a "scavenger" for free radicals and it protects cells against oxidative stress (see below). It is also a cofactor for glutathione peroxidase, which detoxifies hydrogen peroxide and organic peroxides. Increased cellular GSH levels are normally associated with protection of cells against oxidative damage, toxic compounds, and radiation.

A2. WHAT IS AN "OXIDANT STRESS" ?

Damage to biological systems caused by the generation of active oxygen species (oxygen-free radicals) and peroxides is referred to as "oxidant stress" or "oxidative stress" (Cohen, 1985a). A free radical is defined as any molecule or atom with an unpaired electron in its outermost orbital. The presence of an unpaired electron in free radicals usually makes them chemically reactive. Some stable free radicals, such as the monodehydroascorbate radical, are also known. Conventionally, we use a dot · to represent an unpaired electron in the free radical. Two oxy-radicals (oxygen-centered radicals) that are important in biology are the superoxide anion radical ($O_2^{\cdot-}$) and the hydroxyl radical (·OH). Under acidic conditions, $O_2^{\cdot-}$ is easily protonated to $HO_2^{\cdot}$ (pK 4.9), which is a strongly oxidizing radical. The ·OH radical is very reactive and toxic to cells. Hydrogen peroxide (H_2O_2), while not a free radical, has the potential to generate ·OH radicals in reactions with $O_2^{\cdot-}$, catalyzed by iron. This is called the Haber-Weiss reaction. Ferrous ions (Fe^{2+}) can donate electrons to H_2O_2 to form ·OH. That is the well-known Fenton reaction (reviewed by Cohen, 1985b). Both reactions are shown in equations as follows:

$$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe^{2+}/Fe^{3+}} O_2 + OH^- + {\cdot}OH \quad \text{(Haber-Weiss reaction)}$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + {\cdot}OH \quad \text{(Fenton reaction)}$$

Free radicals are also produced in cells by the autoxidation of various substances (e.g., catecholamines) and by the microsomal cytochrome P-450 system. Radicals are also by-products of normal metabolism (e.g., synthesis of prostaglandins) or pathological functions of enzymes (e.g., xanthine oxidase in hypoxia-ischemia conditions). Because they are oxidizing agents and because they form new radicals that

can initiate particularly damaging chain reactions, radicals can damage proteins, nucleic acids, lipids, and extracellular matrix glycosaminoglycans (carbohydrates). They damage lipids by the peroxidation of fatty acids, resulting in alterations in membrane fluidity and permeability. Polyunsaturated fatty acids are particularly vulnerable. Radicals change protein functions by oxidation of sulfhydryl (SH) groups, resulting in, for example, activation of latent enzymes, such as collagenase, or inactivation of working enzymes, such as alpha-1-antitrypsin and alpha-1-protease inhibitor, allowing neutrophils to use elastase to destroy lung tissues. Superoxide radicals and other oxidants can also create DNA strand breaks. Damaged DNA will then activate the enzyme poly-ADP-ribose polymerase, which consumes NAD, a cofactor needed for ATP production, and impair ATP synthesis. Cerutti (1985) had proposed that the above mechanism might contribute to cancer development. Thus, when cells are under overwhelming "oxidant stress", a series of detrimentally toxic mechanisms are activated. They include a state of redox unbalance, thiol depletion, and disruption of intracellular calcium homeostasis. These changes can result in lethal cell injury by involving the activation of degradative enzymes such as phospholipases, proteases, and endonucleases.

A3. WHAT ARE THE DEFENSE MECHANISMS THAT PREVENT OXIDATIVE DAMAGE ?

A number of antioxidants prevent oxidative damage by free radicals. They are glutathione, ascorbate, alpha-tocopherol, glutathione peroxidase (GSH-Px), GSSG reductase, catalase, and superoxide dismutase (SOD) (Cohen et al, 1963; Cohen, 1983). Superoxide dismutase, which was discovered by McCord and Fridovich (1968), catalyzes the decomposition of two superoxide anion radicals into hydrogen peroxide and oxygen. Because H_2O_2 is a potential source of ·OH radicals (Haber-Weiss and

Fenton reactions), two protective enzymes, catalase and GSH-Px, can prevent formation of ·OH. Catalase converts H_2O_2 into O_2 and H_2O. Glutathione peroxidase catalyzes the oxidation of reduced glutathione to form glutathione disulfide (GSSG) at the same time reducing H_2O_2 or organic peroxides (such as lipid hydroperoxides). Glutathione reductase then will regenerate reduced glutathione (GSH) from GSSG. Two vitamins, vitamin C (ascorbate) in the cytosol and vitamin E (alpha-tocopherol) in lipid membranes are also major cellular antioxidants. Alpha-tocopherol (Tappel,1962) converts lipid peroxyl radicals to less active forms by donating a hydrogen atom to the radicals, stopping the chain process of lipid peroxidation (for example, of cell membranes). Alpha-tocopherol plays a significant role in defense mechanisms because it is lipid soluble. Other antioxidants, such as ascorbate, are active in the aqueous phase.

A4. FREE RADICALS IN THE PATHOGENESIS OF PARKINSON'S DISEASE AND BRAIN HYPOXIA-ISCHEMIA INJURY

The human brain is very vulnerable to "oxidant stress" for the following reasons:

a. It contains high concentrations of polyunsaturated lipids, which are substrates for lipid peroxidation.

b. The brain utilizes a high portion of total oxygen.

c. The brain is relatively deficient in GSH-Px and catalase, compared to organs such as liver

d. Iron (a catalyst for ·OH formation) is found in high concentrations in specific brain regions (such as the globus pallidus and the substantia nigra).

There are reports that patients with Parkinson's disease have increased iron in the zona compacta of the substantia nigra, accompanied by decreased GSH and increased

lipid peroxidation (Dexter et al., 1987, 1989, 1991; Cohen, 1994; Sian et al., 1994). The decreased GSH and consequent decrease in GSH-Px activity would reduce the capability to handle H_2O_2 and augment the risk of free radical damage via lipid peroxidation.

Much evidence indicates that oxy-radicals are generated during and after hypoxia-ischemia (Butterfield et al, 1978; Siesjo et al, 1981; McCord et al, 1985), by several mechanisms: During partial ischemia, a small amount of oxygen is still available to the tissue and can give rise to free radicals. When previously ischemic tissue is reoxygenated, a burst of radical production can occur. Neuronal membranes are rich in polyunsaturated fatty acids, which are particularly liable to free radical attack.

Energy impairment is also frequently seen during brain hypoxia-ischemia. For example, during hypoxia-ischemia, the adenine nucleotides, ATP, ADP, and AMP are depleted with the formation of adenosine, inosine, and hypoxanthine. Under the pathological condition of hypoxia-ischemia, xanthine dehydrogenase is converted to xanthine oxidase through the activation of a specific protease (calpain) by calcium. Xanthine oxidase uses molecular oxygen rather than NAD^+ and, on reperfusion of the tissue with oxygenated blood, it produces an abrupt surge of oxygen-free radicals (Halliwell and Gutteridge, 1989).

A5. WHY WE WANT TO STUDY L-DOPA

It is a controversial issue whether or not L-DOPA provokes toxic or damaging events in the CNS. We know that L-DOPA is toxic because: (1) the autoxidation of L-DOPA will give a variety of toxic species, including H_2O_2, oxygen-free radicals,

quinones, & semiquinones (Graham, 1978; Cohen, 1985a; Chieuh et al., 1992). (2) Our lab reported that oxidant stress is observed as a consequence of accelerated DA turnover in vivo (Spina & Cohen, 1989; Cohen & Spina, 1989) or upon exposure of striatal synaptosomes to L-DOPA in vitro (Spina & Cohen, 1988), as indicated by an increase in the formation of glutathione disulfide (GSSG).

On the other hand, there is evidence that L-DOPA has beneficial effects: (1) L-DOPA prolongs the lifespan of rodents (Cotzias et al., 1977) and it increases the survival rate of Parkinson patients. (2) Rodents do not show signs of dopaminergic toxicity after chronic exposure (Hefti et al., 1981; Perry et al., 1984), and L-DOPA does not influence the recovery of motor function after transplantation of fetal mesencephalic neurons into the striatum of 6-OHDA lesioned rats (Blunt et al., 1991).

In our study, we found that L-DOPA showed both toxic and protective effects on mesencephalic cell cultures. After 48 hr exposure of L-DOPA, the tyrosine hydroxylase-positive (TH^+) neurons (DA neurons) was reduced to 69.7 % of control values (Mytilineou et al., 1993). At the same time, the level of GSH, a major cellular antioxidant, rose to 125.2% of control values. My thesis will focus on studying both the mechanism of upregulation of GSH and the biological role of elevated GSH in mesencephalic cell cultures (see ahead).

A6. PROTECTIVE ROLES OF GSH

Glutathione plays a well known role in protecting cells from oxidative stress. It is a major reducing agent, present at levels of 1-2 mM in the CNS. It reacts directly with oxidizing agents, and as substrate for GSH peroxidase or for the peroxidatic activity of GSH transferase, it plays the predominant role in detoxifying cellular H_2O_2

or organic peroxides (such as lipid peroxides) (reviewed by Cohen, 1983; Meister & Anderson, 1983). Removal of H_2O_2 or lipid peroxides (ROOH) is catalyzed by GSH peroxidase and proceeds as shown below:

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH peroxidase}} GSSG + 2\,H_2O \qquad \text{(Eqn. 1)}$$

$$ROOH + 2GSH \xrightarrow{\text{GSH peroxidase}} GSSG + H_2O + ROH \qquad \text{(Eqn. 2)}$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} 2\,GSH + NADP^+ \qquad \text{(Eqn. 3)}$$

Generally, the accumulation of GSSG can have deleterious results. GSSG will promote formation of mixed disulfides with enzymes and structural proteins, alter calcium homeostasis, and enhance proteolytic degradation. GSSG is normally reduced by GSSG reductase (Eqn. 3) to maintain a cycle for removal of peroxides and to prevent GSSG effects on cellular metabolism.

Brain GSH can be depleted by inhibiting its synthesis with L-BSO (Slivka & Cohen, 1988). Depletion of brain GSH sensitizes DA neurons to the toxicity of 6-OHDA (Pileblad, Magnusson & Fornstedt, 1989) and produces other changes, such as accumulation of lipofuscin, reduced DA content, and axonal dilations in DA neurons, that mimic changes seen in mice during aging or after N-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP) administration (McNeill et al., 1988).

It has been reported that GSH levels are reduced in the substantia nigra, but not in other brain regions, of the parkinsonian brain (Riederer et al., 1989; Sian et al., 1994), and that the reduction in GSH correlates with the severity of Parkinson's disease

(Riederer et al., 1989). Therefore, the study of GSH regulation is important.

A7. GLUTATHIONE AND NEUROTOXICOLOGY IN MESENCEPHALIC CELL CULTURES

Up-regulation of GSH was consistently observed after treating mesencephalic cultures with L-DOPA, as described in this doctoral thesis. This observation (Mytilineou et al., 1993; Han et al., in press) was further pursued and the following main observations were made:

1. The elevation of GSH was commonly observed in different cell types and defined cell lines.

2. Pure neuronal cell cultures (lacking glial cells) behaved differently from mesencephalic cell cultures (which contain glia); therefore, we further dissected the differences between pure neuronal cell cultures and mesencephalic cell cultures.

3. The rise in GSH becomes strikingly greater after washout of the L-DOPA.

4. The results showed that redox-cycling compounds via autoxidation, but not via dopamine receptors, is the main mechanism for the upregulation of glutathione content.

5. Protein kinase C is involved in the modulation of the glutathione content.

6. An oxidant signal indeed will further trigger the upregulation of glutathione, while ascorbic acid (an antioxidant) can block the L-DOPA-induced elevation of GSH in mesencephalic cell culture.

7. Gamma-glutamylcysteine synthetase (γ-GCS) activity, the rate-limiting step in GSH biosynthesis, is increased after treatment with L-DOPA (or dopamine) in the mesencephalic cell culture.

8. Elevations in GSH evoked by L-DOPA will protect cells from t-butylhydroperoxide, a severe oxidative stress challenge.

9. Ascorbic acid can block the L-DOPA-induced upregulation of GSH and

prevent the protective effect of L-DOPA by reversing the elevation of GSH content.

10. L-DOPA and dopamine both inhibited catalase activity. Ascorbic acid can block the L-DOPA inhibition effect, but not the dopamine effect.

In summary, these studies showed that oxidative stress can up-regulate critical antioxidant defenses (e.g., GSH) and protect cells from further oxidative stress (e.g., that induced by t-butylhydroperoxide). We demonstrated that L-DOPA-induced elevated glutathione preserved biological function in mesencephalic cell cultures.

B. METHODS:

B1. Materials

Timed-pregnant Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY). Fetal calf serum, horse serum, minimum essential medium (MEM), and DMEM/F12 medium were purchased from GIBCO (Grand Island, N.Y.). Chemicals were obtained from Sigma (St. Louis, MO) or Research Biochemicals International (Natick, MA). DL-2,4-dihydroxyphenylalanine (2,4-DOPA) was a gift from Dr. A. Manian and Dr. J.S. Kennedy (Neurosciences Research Branch, National Institute of Mental Health, Bethesda, MD). Brocresine was a gift from Lederle Laboratories (Pearl River, NY)

B2. Cell cultures

B2.1 *Preparation of rat embryonic mesencephalic cell culture*

Cultures of embryonic rat mesencephalon (Mytilineou et al., 1993) were prepared on the 14.5 ± 0.5 day of gestation. The cells were dissociated mechanically and plated at a density of about 100,000 cells/cm^2 on plastic (Falcon) 35 mm dishes. The dishes had been pre-coated with polyornithine (0.1 mg/mL in 0.15 M sodium borate buffer, pH 8.5) for one hour, followed by three rinses with sterile distilled water and one rinse with BSS (Balanced Salt Solution). The feeding medium was MEM supplemented with glucose (33 mM), sodium bicarbonate (44.6 mM), 10% fetal calf serum, and 10% horse serum.

B2.2 *Preparation of glial cell cultures*

For the preparation of glial cell cultures (McCarthy and De Vellis, 1978), the cerebral cortex from newborn rats was rinsed in calcium-free and magnesium-free BSS, chopped with a razor blade, and incubated in balanced salt solution containing 0.25% trypsin and 1% DNase I (10 μg/mL) at 37°C for 15 min with shaking. The trypsin was inactivated with an equal volume of horse serum and the tissue was lightly centrifuged and rinsed once. The tissue was then triturated in MEM containing 10% fetal calf serum, passed through a 130 μm mesh, and plated in the same medium at a density of 1-2 x 10^6 cells in 25 cm^2 flasks. The medium was replaced after one hour. Cells were used after one week. Additional glial cultures were prepared by trypsinization and replating.

B2.3 *Preparation of pure neuronal cell cultures*

For pure neuronal cultures of fetal rat mesencephalon, the chemically-defined medium (N2) described by Bottenstein and Sato (1979) was used. The N2 medium consists of DMEM/F-12 medium supplemented with HEPES (15 mM), glucose (33 mM), glutamine (2 mM), sodium bicarbonate (44.6 mM), apotransferrin (100 ug/mL), insulin (25 ug/mL), putrescine (60 nM), sodium selenite (30 nM), and progesterone (20 nM).

B2.4 *Growth of the cell lines*

LLC-PK1 (porcine kidney epithelial cell) was obtained from Dr. Healy; C6 (rodent glioma), SK-N-MC (human neuroblastoma cell) and Neuro2A (mouse neuroblastoma cell) were gifts from Dr. Wilk; PC-12 (pheochromocytoma cell) was obtained from Dr. Salton. All cells were grown under the recommendations of American Type Culture Collection (Rockville, MD).

B3. Treatment with L-DOPA and other chemicals

After 4 or 5 days in vitro, the feeding medium was aspirated and replaced with 1.5 mL of fresh medium containing L-DOPA or the other test compounds. This treatment was repeated a second time after 24 hours. The stock solutions (20 mM) of L-DOPA and most of the other test compounds were prepared in 0.01M HCl, tyrosine was dissolved in 0.01N NaOH, ascorbic acid in water, and apomorphine in ethanol; control cultures received the appropriate vehicle. In experiments with ascorbate plus L-DOPA or hydroquinone, the test compounds were added to medium containing ascorbate, and then the mixture was added to the cell cultures. Experiments with other compounds were carried out with similar concentrations.

B4. Assays

Assays for glutathione, cell viability, lactic acid dehydrogenase, and proteins were conducted on a plate reader (Model 340 ATTC, SLT Laboratory Instruments, Hillsborough, NC).

B4.1 *Reduced glutathione (GSH) and oxidized glutathione (GSSG)*

The cell culture plates were placed on ice and the medium was aspirated. The remaining steps were carried out in the cold. Following three rinses with 1.5 mL sterile, phosphate-buffered saline, the cells were dislodged into 1.2 mL buffered saline with a cell scraper (Falcon) and transferred to plastic microcentrifuge tubes. Samples were centrifuged at 3,700 x g for 15 min. and the supernatant was discarded. Addition of 250 uL of 0.4M perchloric acid was followed by sonication for 10 sec. (setting 4,

Vibra-Cell Model V1A, Sonics and Materials, Inc., Danbury, CT) and centrifugation at 18,000 x g for 15 min. The pellet was reserved for protein assay. The supernatant (40 uL) was assayed for glutathione with a modification (Slivka et al., 1987b) of the enzymatic recycling assay of Tietze (1969). The conversion of 5,5'-dithio-bis-(2-nitro-benzoic acid) (DTNB) to the yellow chromophore thionitrobenzoate (TNB) is followed kinetically at 412 nm. To measure GSSG without interference from GSH, the GSH is first removed by reaction with N-ethylmaleimide (NEM), and excess NEM is subsequently removed by SepPak chromatography (Cf. Slivka et al., 1987b). Cysteine does not interfere in the assays for GSH or GSSG in the Tietze assay. Minor modifications were made to adapt the assays for a plate reader (Model 340 ATTC, SLT Laboratory Instruments, Hillsborough, NC). Results are reported in terms of the protein content of the samples.

B4.2 *Cell viability: measurement of MTT*

Cell viability was measured with a modification of the method of Mossman (1983). 3-(4,5-Dimethylthiazol-2-yl)- 2,5-di-phenyltetrazolium bromide (MTT, 5 mg/mL stock solution) was dissolved in phenol red-free MEM (Sigma), containing 44.6 mM sodium bicarbonate and 30 mM glucose, and filtered for sterilization and removal of any residue. Each well was incubated with MTT at a dilution of 0.5 mg/mL at 37°C for 3 h and then the supernatant fluid was aspirated. Then, 1.5 mL of acidic iso-propanol (0.04 N HCl in isopropanol) was added, gently mixed, and allowed to stand at room temperature for several minutes to dissolve the dye. The solution was centrifuged at 15,000 x g for 10 min and then the supernatant fluid was read at 550 nm and 630 nm on the plate reader. Data were expressed as the difference between the absorptions at the two wave lengths.

B4.3 *Lactic acid dehydrogenase (LDH)*

A modification of the method of Bergmeyer et al. (1963) was used. Cells were collected in 1.0 mL of 50 mM phosphate buffer at pH 7.5, and sonicated in the cold for 10 seconds. To 600 μL of buffer was added 200 μL of supernatant and 100 μL NADH (1.2 mg/mL in 120 mM sodium bicarbonate, stock) and the samples were vortexed. Reactions were initiated by the addition of 100 μL sodium pyruvate (1 mg/mL phosphate buffer, stock). Then, 250 μL aliquots (triplicates) were placed into 96-well plates at room temperature and kinetic analyses at 340 nm were initiated after 2 min to measure the rate of disappearance of NADH. Measurements were taken at 30-second intervals for 15 min with agitation for 15 seconds within each cycle.

B4.4 *Catalase*

The catalase measurement was modified from our previous established method (Cohen et al., submitted). The cells were rinsed three times with cold sterile phosphate-buffered solution (PBS) and harvested. We combined three 18 mm diameter wells of either treated or control cells for each assay sample. Following centrifugation of the cells at 4^0C and 3700 g for 10 min, the supernatant was discarded. The pellet was redissolved in 300 μl of ice-cold phosphate buffer and sonicated at setting 2 in an ice bath for 10 seconds (Vibra-Cell Model V1A, Sonics and Materials, Inc., Danbury, CT). After recentrifugation at 18,000 g for 15 min, a 50 μl supernatant was removed for catalase assay and another 50 μl supernatant was used for protein determination by the method of Lowry (1951). All reagents and buffer were maintained at 0^0C in an ice water bath, except for 0.6 N H_2SO_4 which is at room temperature. The final reaction mixtures included 800 μl phosphate buffer (10mM, pH 7.0) and 100 μl after-sonicated cell supernatant or 100 μl phosphate buffer as a non-cell blank. The reaction is

initiated by adding 100 μl H_2O_2 stock (60 mM), followed by gentle mixing (low speed vortex). At fixed time intervals (2 min and 10 min, respectively), triplicate 100 μl aliquots were removed and quenched by addition to a mixture of 4.0 ml of 0.6 N H_2SO_4 plus 1.0 ml of 10 mM $FeSO_4$ at room temperature. After all samples have been collected, 400 μl of 2.5 M KSCN was added to each tube. The red color of ferrithiocyanate develops immediately. 250 μL aliquots (triplicates) were placed into 96-well plates at room temperature and the color intensity was measured with the plate reader (Model 340 ATTC, SLT Laboratory Instruments, Hillsborough, NC) with a 492 nm filter. Results are averaged from triplicate specimens from a single tube. The catalase activity is expressed in terms of the first order reaction rate constant (k) and corrected by protein content, as follows:

$$\text{Catalase activity} = k \text{ /mg protein}$$
$$= [\ln (OD1/OD2)/t] \text{ /mg protein}$$

where ln is the natural log, OD1 and OD2 are the mean optical densities at the two selected time points (e.g., 2 min and 10 min), and t is the time difference between the two points (e.g., 8 min in our assay).

B4.5 *Gamma-glutamylcysteine synthetase (gamma-GCS)*

The gamma-glutamylcysteine synthetase assay was modified from the method of Fernandez-Checa and Kaplowitz, (1990). The cell culture plates were placed on ice and the medium was aspirated. All remaining steps were carried out in the cold. Following three rinses with 1.5 mL sterile, phosphate-buffered solution (PBS), the cells were harvested with 1.2 mL buffer A, which is composed of 100 mM Tris-Hcl, 150 mM KCl, 20 mM $MgCl_2$ and 2 mM sodium EDTA (pH 7.3), and transferred to plastic

micro-centrifuge tubes. Samples were centrifuged at 4°C and 3700 g for 10 min, and the supernatant was discarded. The pellet was redissolved in 1.0 ml of ice-cold buffer A and sonicated at setting 4 in an ice bath for 10 seconds (Vibra-Cell Model V1A, Sonics and Materials, Inc., Danbury, CT). After recentrifugation at 18,000 g for 15 min, a 100 μl supernatant is removed for τ-GCS assay and another 50 μl supernatant is used for protein determination by the method of Lowry (1951). The 2.0 ml final reaction mixtures should be added in sequence at 37°C as described below: buffer A (see above), glutamate (10 mM), glycine (10 mM), ATP (3 mM), pre-mixed 0.3mM L-cysteine + 0.2 mM DTT, and 100 μM monochlorobimane (mBCL), glutathione-S-transferase (GST) (0.1 unit/ ml) and cell supernatant (100 μl). The assay should immediately start just after the addition of cell supernatant. The glutathione-S-transferase was used to catalyze the formation of the GSH-bimane (GSH-mBCL) adduct. The formation of the fluorescent adduct was measured at 1 minute time intervals with a Perkin-Elmer LS-3B fluorescence spectrometer with an excitation wavelength of 385 nm and an emission wavelength of 478 nm.

The gamma glutamyl-cysteine synthetase (τ-GCS) activity is expressed in terms of the reaction rate after the subtraction of blank reading (BL, 100 μl buffer A, no cell) and corrected by the protein content, as follows:

$$\tau\text{-GCS activity} = [((OD2\text{-}BL) - (OD1\text{-}BL)) / t] / \text{mg protein}$$
$$= [(OD2 - OD1) / t] / \text{mg protein}$$

where OD1 and OD2 are the mean optical densities at the two selected time points and t is the time difference between the two points.

B4.6 *Protein*

Protein content was measured by the Lowry assay (Lowry et al, 1951). Protein was measured in the perchloric acid pellet obtained during the workup for GSH. It was shown that entrapment of trace L-DOPA in the protein pellet did not affect the assay (Mytilineou et al., 1993).

B5. Statistical assessment

For multiple comparisons, statistical analyses were performed by ANOVA followed by Tukey's HSD test. Where appropriate, a 2-tailed Student t-test was used, as indicated in the text.

C. RESULTS

PART I: UP-REGULATION OF GLUTATHIONE (GSH) IN CELL CULTURES

C1. Effect of L-DOPA on GSH levels in mesencephalic cell cultures, glia, and defined cell lines.

C1.1 *Study of Mesencephalic cell cultures*

C1.1.1 *L-DOPA dose-response*

The mesencephalon is a major site for dopamine cell bodies. We measured glutathione levels in rat fetal mesencephalic cell cultures exposed to L-DOPA to determine its effect on cellular GSH. The expectation was that L-DOPA oxidation or metabolism would lower the GSH level. In particular, the formation of adducts with DOPA quinone would be expected to irreversibly remove GSH. Contrary to expectation, a consistent effect in the opposite direction was observed. After 48 hr of exposure to 200 μM L-DOPA, the GSH content rose from 20.20 $\pm$ 1.24 nmole/mg protein to 25.28 $\pm$ 0.75 nmole/mg protein, an increase of 25.2% ($P<0.001$).

Dose-response experiments are shown in Table 1. The GSH rise was concentration-dependent and greater for higher concentrations of L-DOPA. A significant rise was seen with the lowest concentration tested (50 μM L-DOPA). In the same experiment, Lactic acid dehydrogenase (LDH) was assayed in the medium to monitor cell death induced by L-DOPA. Release of LDH was only significant for 400 μM L-DOPA ($p=0.005$ vs control), but not at the lower doses (data not shown).

TABLE 1. GSH levels for mesencephalic cultures after exposure to different concentrations of L-DOPA.

Group	GSH (nmole/mg protein)	GSH as (% control)	N
Control	14.18 ± 0.88	100.0 ± 6.2	(4)
50 μM L-DOPA	17.68 ± 0.61*	124.5 ± 4.4	(4)
100 μM L-DOPA	18.63 ± 0.31*	131.4 ± 2.3	(4)
200 μM L-DOPA	21.34 ± 0.97**	150.5 ± 6.9	(4)
400 μM L-DOPA	22.72 ± 0.70***	160.2 ± 4.8	(4)

Mesencephalic cultures were treated on the 5th day in vitro with the various concentrations of L-DOPA for 48 hours, with a change of medium after 24 hours. The values are the means ± SEM.

Compared to control: *$p<0.05$, **$p<0.01$, ***$p<0.001$

C1.1.2 *Effect of L-DOPA on glutathione disulfide (GSSG)*

The enzymatic recycling method of measuring glutathione levels detects both GSH and GSSG. In separate experiments, GSSG was measured: On exposure to 200 μM L-DOPA for 48 hr, the total glutathione level rose from 19.9 $\pm$ 0.59 to 26.1 $\pm$ 1.4 nmole/mg protein (n=6; +31.0%), and the GSSG level rose simultaneously from 0.94 $\pm$ 0.03 to 1.37 $\pm$ 0.10 nmole/mg protein (n=6; +44.8%). The relatively small increase in GSSG compared with the total glutathione shows that the increased total glutathione mainly is derived from the rise in GSH. The relatively low amounts of GSSG (approximately 5% or less of the total) means that the assay for total glutathione can be accepted as an index for GSH.

C1.1.3 *Time course for the GSH rise*

The time course for the rise in GSH content was examined. Initially cells were treated with 200 μM L-DOPA for 4, 24, and 48 hr and compared with corresponding controls (n=4 per group). At 4 hr, small changes in GSH (+3.4%) or GSSG (+7.2%) were not significantly different from control. However, there were significant changes at both 24 and 48 hr: Total glutathione expressed as a percentage of corresponding control was 116.9 $\pm$ 3.7% ($p<0.05$) and 123.6 $\pm$ 2.8% ($p<0.001$), respectively. GSSG was 135.8 $\pm$ 2.6% ($p<0.005$) of control and 147.1 $\pm$ 2.8% ($p<0.001$) of control, respectively, at 24 and 48 hrs.

The time course up to 24 hr was examined in a different design: all samples were incubated for 24 hr, and L-DOPA was added at staggered time points (Table 2); in this design, cells were compared at an identical age in vitro. Changes in total GSH were not significant until 24 hr (21.6%), whereas GSSG levels were significantly elevated

TABLE 2. Changes in total glutathione, GSSG, and % GSSG during exposure of mesencephalic cultures to L-DOPA (200 μM)

Exposure Time (hrs)	Total Glutathione (nmole/mg protein)	GSSG (nmole/mg protein)	% GSSG
0	14.77 $\pm$ 0.33	0.39 $\pm$ 0.03	2.65 $\pm$ 0.20
3	15.78 $\pm$ 0.33	0.52 $\pm$ 0.06	3.29 $\pm$ 0.38
6	15.29 $\pm$ 0.23	0.52 $\pm$ 0.03	3.50 $\pm$ 0.19
12	16.37 $\pm$ 0.20	0.65 $\pm$ 0.02[a]	3.97 $\pm$ 0.12[b]
18	15.00 $\pm$ 0.33	0.78 $\pm$ 0.03[a]	5.20 $\pm$ 0.22[a]
24	17.96 $\pm$ 0.72[a]	1.07 $\pm$ 0.03[a]	5.96 $\pm$ 0.18[a]

All cultures were incubated for 24 hrs. L-DOPA (200 μM) was added at staggered time intervals to yield the exposure times listed in the table. Values are the means $\pm$ SEM for N=4 per group. Total glutathione is GSH + GSSG, which is comprised predominantly of GSH; %GSSG is 100 x (GSSG/total glutathione).

Significantly different from the control without L-DOPA (0 hrs), [a]$p < 0.001$; [b]$p < 0.025$.

as early as 12 hr (+66.7%; $p<0.001$) and 18 hr (+ 100%; $p<0.001$), as well as at 24 hrs (+175%; $p<0.001$). The percentage of GSSG (i.e., GSSG expressed as a percent of the total glutathione) also rose significantly at the same time points. The percent change in GSSG from basal levels was greater than that of GSH, but the total amount of GSSG was still low.

C1.2 *Study of defined cell lines and various cell types*

Rat fetal mesencephalic cell cultures are heterogeneous and contain a variety of different kinds of neurons, as well as glial cells. DA neurons within the cultures comprise only a very small fraction of the total cells (less than 5%). The rise in GSH within the culture reflects the sum of changes in different cellular compartments. Some cells may increase their GSH a lot, others may show decreased GSH or no change at all. Additional experiments were performed with defined cell lines in order to better assess the effects on GSH.

Table 3 shows that the changes in cellular glutathione after exposure of several different types of cells in culture to 200 μM L-DOPA. The Neuro-2A (mouse neuroblastoma cell line), LLC-PK_1 (pig kidney epithelium cell line), and primary cultures of rat cerebral cortical glia, all showed elevated GSH: +38.0%, +33.0%, and +22%, respectively, compared to corresponding controls. GSH was also increased in two experiments with human SK-N-MC neuroblastoma (data not shown). An exception was the C6-glioma cell line, which did not show a significant rise in GSH, and also had the highest mean endogenous GSH in control (untreated) cultures. In other experiments, pure neuronal cultures from rat fetal mesencephalon were also tested. These data will be presented separately (see ahead) and further elaborated on when the differences between glia and neurons are discussed.

TABLE 3. Effect of L-DOPA (200 μM x 48 h) on the level of GSH in several cell types and defined cell lines.

Cells		GSH (nmole/mg protein, mean ± SEM) Control	L-DOPA	%Increase
Neuro-2A	(N=22)	5.55 ± 0.32	7.66 ± 0.28[a]	38.0%
LLC-PK$_1$	(N=35)	18.43 ± 0.93	24.51 ± 1.19[a]	33.0%
Mesencephalon	(N=24)	20.20 ± 1.24	25.28 ± 0.75[a]	25.2%
Glia	(N=14)	17.92 ± 2.84	21.87 ± 3.09[b]	22.0%
C6-Glioma	(N=14)	25.91 ± 1.50	27.80 ± 2.36	7.3%

Neuro-2A neuroblastoma, LLC-PK$_1$ pig kidney epithelium, glia from newborn rat brain, and C6-glioma were treated after they had achieved confluence. Cultures of rat fetal mesencephalon were treated on day 5 in vitro. The medium was removed after 24 h and fresh medium containing L-DOPA was added for an additional 24 h. Results are from 7 experiments with mesencephalic cultures and 4-5 experiments for the other cell types.

[a] $p < 0.001$ compared to corresponding control, Student t-test.

[b] $p < 0.002$ compared to control, paired t-test ($N = 14$). The control levels of GSH in 4 experiments with glial cultures were 6.8, 13.9, 21.5, and 34.5 nmole/mg protein, giving rise to the relatively broad SEM for the pooled data; GSH was increased by exposure to L-DOPA in each experiment.

C2. Effects of ascorbate, MAO inhibitors, DOPA-decarboxylase inhibitors, SOD and catalase.

Oxidizing species, such as hydrogen peroxide and associated oxy-radicals (namely, superoxide and hydroxyl radicals) can be generated during the autoxidation of L-DOPA; semiquinone radicals and reactive quinones are also generated during autoxidation. In addition, L-DOPA can be decarboxylated to form dopamine, which can then also undergo autoxidation; dopamine can also be metabolized by MAO, which forms H_2O_2.

We conducted experiments to evaluate the role of reactive oxygen species that might be derived from L-DOPA or dopamine. Ascorbic acid was used as a general antioxidant, pargyline as an MAO inhibitor, and catalase and superoxide dismutase (SOD) as scavengers of H_2O_2 and superoxide, respectively. In addition, inhibitors of L-DOPA decarboxylation (carbidopa and NSD-1055) were tested. Results are shown in Table 4 and Figures 1A and 1B.

In Table 4, GSH levels in mesencephalic cultures were increased to 125.2% of control ($p < 0.001$) after exposure to 200 μM L-DOPA for 48 hours. This effect was completely prevented by 200 μM ascorbic acid. On the other hand, it was not affected by pargyline, catalase, or SOD, indicating that neither monoamine oxidase activity nor the presence in the medium (extracellular) of H_2O_2 or superoxide radicals play an important role in the L-DOPA-induced increased in GSH levels.

The effect of ascorbate (Table 4) is very interesting because it means that the rise in GSH may need an "oxidant signal". Previously, in Table 2, we found that a rise in GSSG occurred before the rise in GSH. GSSG may behave as an oxidant signal to

TABLE 4. Effect of L-DOPA on the level of GSH in mesencephalic cultures.

Treatment	GSH as (% Control)	(N)
Control	100.0 ± 2.0	(24)
L-DOPA (200 μM)	125.2 ± 3.7[a]	(24)
Ascorbic Acid (200 μM)	100.7 ± 3.8	(10)
Ascorbic Acid + L-DOPA	97.0 ± 2.7	(11)
Pargyline (10 μM)	92.0 ± 3.7	(7)
Pargyline + L-DOPA	123.0 ± 4.0[ab]	(7)
Catalase (10 μg/mL)	103.9 ± 3.0	(10)
Catalase + L-DOPA	120.6 ± 3.7[a]	(11)
SOD (5 μg/mL)	93.7 ± 4.1	(9)
SOD + L-DOPA	120.6 ± 5.4[ab]	(10)

Mesencephalic cultures were treated on the 5th day in vitro with the various compounds for 48 hours, with a change of medium after 24 hours (pooled results of 2-7 experiments). The values are the means ± SEM; GSH levels of control cultures were 20.21± 1.24 nmole/mg protein. Although the method detects both GSH and GSSG, the relatively low levels of GSSG (see text) means that the values presented here reflect GSH predominantly.

By ANOVA followed by Tukey HSD test for multiple comparisons.

[a]: Differs from untreated control values, $p < 0.001$.

[b]: Differs from corresponding group without L-DOPA, $p < 0.001$.

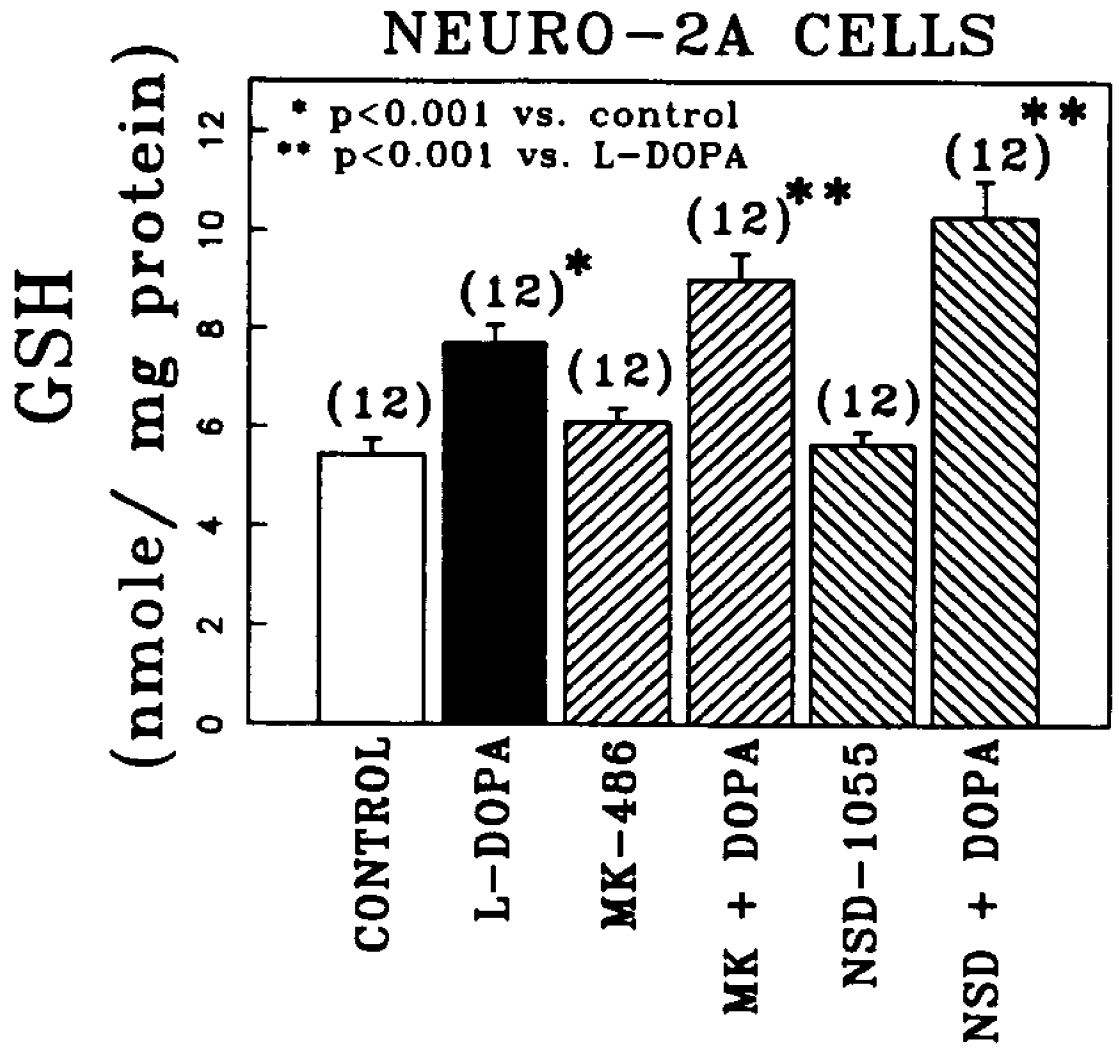

NEURO-2A CELLS
* p<0.001 vs. control
** p<0.001 vs. L-DOPA
GSH
(nmole/ mg protein)
0
2
4
6
8
10
12
(12)
(12)*
(12)
(12)**
(12)
(12)**
CONTROL
L-DOPA
MK-486
MK + DOPA
NSD-1055
NSD + DOPA

Fig. 1A

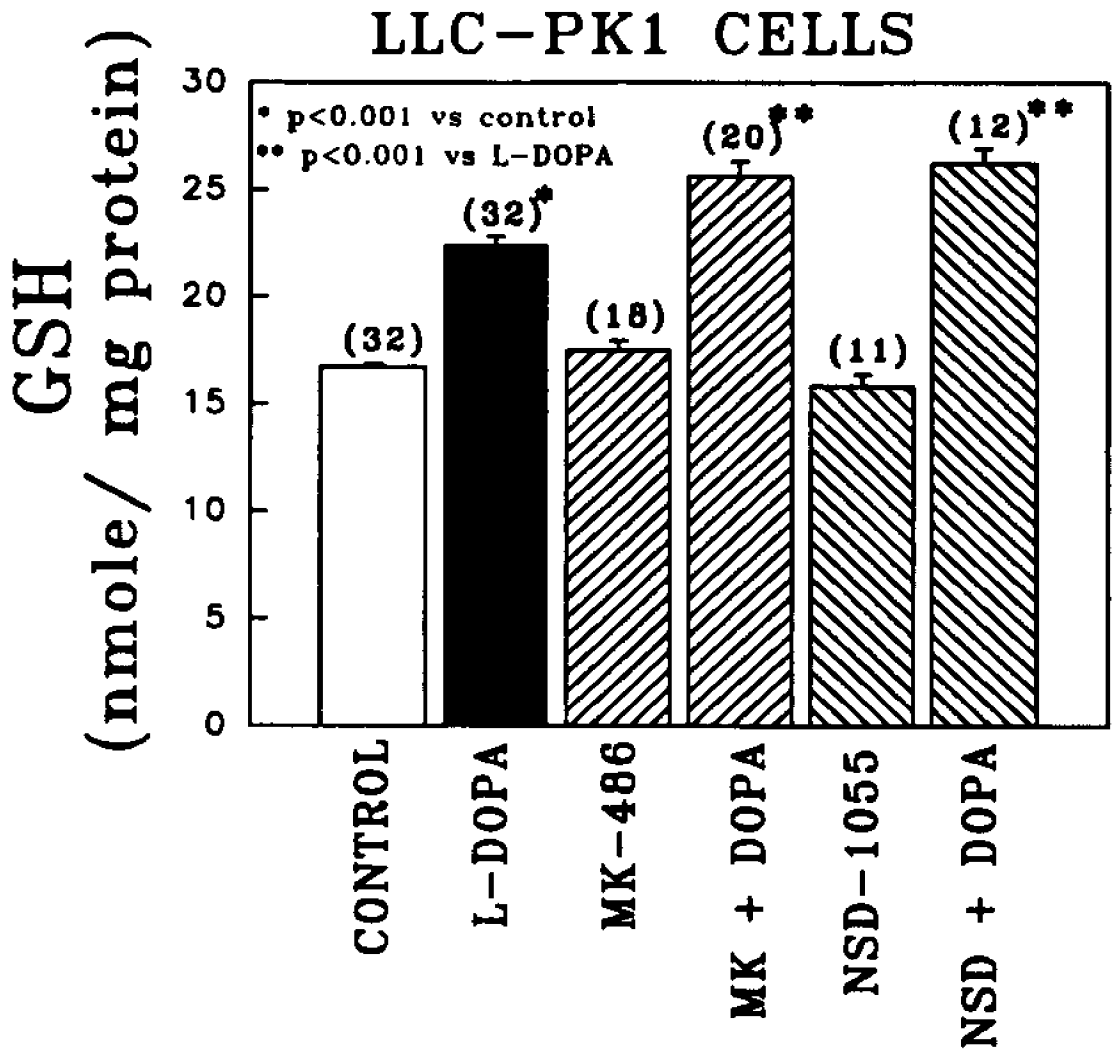

LLC-PK1 CELLS
GSH
(nmole/ mg protein)
30
25
20
15
10
5
0
* p<0.001 vs control
** p<0.001 vs L-DOPA
(32)
(32)*
(18)
(20)**
(11)
(12)**
CONTROL
L-DOPA
MK-486
MK + DOPA
NSD-1055
NSD + DOPA

Fig. 1B

trigger the upregulation of GSH content. For example, this may occur through the reaction of GSSG with protein sulfhydryl groups (PrSH) to form protein mixed-disulfides (Pr-SSG) as observed in other experiments in our laboratory by Werner & Cohen (unpublished observation).

It is conceivable that some of the effects of L-DOPA were mediated by dopamine formed via decarboxylation.

$$\text{L-DOPA} \xrightarrow{\text{DOPA decarboxylase}} \text{dopamine} \qquad \text{(Eqn. 4)}$$

Therefore, the effect of inhibiting DOPA decarboxylase was tested. The results shown in Figs 1A and 1B illustrate the lack of effect of two DOPA decarboxylase inhibitors on two different cell lines (Neuro-2A and LLC-PK_1). It is noteworthy that the LLC-PK_1 cells contain high amounts of the aromatic amino acid decarboxylase (L-DOPA decarboxylase; Grenader and Healy, 1991). Two inhibitors, namely carbidopa (MK-486) and brocresine (NSD-1055) failed to block the rise in tissue GSH. Indeed the inhibitors amplified the rise in GSH ($P < 0.001$). In additional experiments (Table 5), LLC-PK_1 cells showed time-dependent increases in GSH levels, and carbidopa magnified the rise in GSH. However, C6-glioma cells failed to respond at all (Table 6). Table 5 shows that production of dopamine from L-DOPA is not required.

C3. Effect of <u>washout</u> of L-DOPA on cellular level of GSH

It has been shown that exposure of mesencephalic cultures to L-DOPA is associated with toxic effects, such as loss of tyrosine hydroxylase-positive cells and decreased neurite length of surviving neurons (Olney et al., 1990; Steece-Collier et al., 1990; Mytilineou et al, 1993). In addition, autoxidation of L-DOPA would be

TABLE 5. Time course of the effect of 200 μM L-DOPA with or without 50 μM carbidopa (MK-486), DOPA decarboxylase inhibitor, on the level of GSH in LLC-PK1 cell cultures.

Treatment	Exposure Time (hrs)	GSH (nmole/mg protein)	% increase of control
Control		17.69 $\pm$ 0.62	
L-DOPA	24	18.91 $\pm$ 0.09 ^{a}	6.9
L-DOPA	48	22.66 $\pm$ 0.74 ^{a*}	28.1
L-DOPA	72	23.23 $\pm$ 0.97 ^{a*}	31.3
L-DOPA	96	27.53 $\pm$ 1.39 ^{a***}	55.7
L-DOPA + MK-486	24	22.87 $\pm$ 0.49 ^{a*,b}	28.9
L-DOPA + MK-486	48	29.00 $\pm$ 1.63 ^{a***,c**}	64.0
L-DOPA + MK-486	72	30.54 $\pm$ 1.30 ^{a***,d***}	72.7
L-DOPA + MK-486	96	35.44 $\pm$ 0.84 ^{a***,e***}	100.4

LLC-PK1 cells are exposed to 200 μM L-DOPA with or without 50 μM carbidopa (MK486) for 24, 48, 72, and 96 hours, with a change of medium every 24 hours (n=4, per group).

By ANOVA followed by Tukey HSD test for multiple comparisons.

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

a: compared to control

b: compared to L-DOPA 24 hr

c: compared to L-DOPA 48 hr

d: compared to L-DOPA 72 hr

e: compared to L-DOPA 96 hr

TABLE 6. Time course of the effect of 200 μM L-DOPA with or without 50 μM carbidopa (MK-486), DOPA decarboxylase inhibitor, on the level of GSH in C6 glioma cell cultures.

Treatment	Exposure Time (hrs)	GSH (nmole/mg protein)	% increase of control
Control		28.29 $\pm$ 0.32	
L-DOPA	24	27.04 $\pm$ 0.60	-4.4
L-DOPA	48	29.28 $\pm$ 0.68	3.5
L-DOPA	72	29.74 $\pm$ 1.09	5.1
L-DOPA	96	30.76 $\pm$ 0.49	8.7
L-DOPA + MK-486	24	27.57 $\pm$ 0.97	-2.6
L-DOPA + MK-486	48	29.85 $\pm$ 0.43	5.5
L-DOPA + MK-486	72	29.48 $\pm$ 0.50	4.2
L-DOPA + MK-486	96	29.30 $\pm$ 0.28	3.6

C6 cells are exposed to 200 μM L-DOPA with or without 50 μM carbidopa (MK-486) for 24, 48, 72, and 96 hours, with a change of medium every 24 hours (n=4, per group).

There are no difference among untreated control values and no difference among the corresponding time course group treated with L-DOPA. By ANOVA followed by Tukey HSD test for multiple comparisons.

expected to place a stress on the GSH content by generating hydrogen peroxide and reactive free radicals, which can oxidize GSH. Also, quinones, such as L-DOPA-quinone can remove GSH irreversibly by making a covalent bond (e.g., formation of 5-S-glutathionyl-DOPA; Ito, 1993). Such reactions would be expected to decrease the GSH content, so that the observed rise in GSH may not reflect the full potential to elevate cellular GSH. Therefore, the effect of washing out the L-DOPA was investigated. In addition, we wished to determine if the rise in GSH was transitory or could persist for several days in the absence of further stimulation.

The results in Table 7 show that the rise in GSH persisted for at least 48 h after removal of L-DOPA. Indeed GSH was strikingly elevated by removal of L-DOPA. The increase in GSH (compared to corresponding timed control) rose from 19.7% to 73.6% after 24 h. It then declined at 48 h, but was still significantly elevated compared to the corresponding control. During the extended washout procedure, the level of GSH in control cultures also fell from 10.6 nmole/mg protein to 7.0 nmole/mg, which can be attributed, in part, to a natural aging of the cells ex vivo. In addition, it was noted that protein levels in control cultures fell from 346 $\pm$ 6 to 244 $\pm$ 6 μg/culture ($p<0.01$) during the 48 h washout phase; protein levels in the L-DOPA-exposed cultures (344 $\pm$ 34 μg/culture) were not significantly altered by the washout procedure (control, 376 $\pm$ 6 μg/ culture; $p>0.05$).

C4. The toxic effects of L-DOPA on pure neuronal cell cultures from rat fetal mesencephalon.

When mesencephalic cell cultures are incubated with L-DOPA a relatively selective destruction of dopamine neurons takes place (Mytilineou et al., 1993): Tyrosine hydroxylase-positive neurons are lost (69.7% of control), while the total

TABLE 7. Effect of washout of L-DOPA on the level of GSH in mesencephalic cell cultures.

Washout hrs	GSH (nmole/mg protein) Control	L-DOPA-treated	% Increase
0	10.58 ± 0.46 (n=22)	12.66 ± 0.39[a] (n=20)	+19.7%
24	11.78 ± 0.49 (n=21)	20.47 ± 0.72[a,c] (n=18)	+73.6%
48	7.03 ± 0.81 (n=9)	9.92 ± 0.52[b] (n=9)	+41.2%

Mesencephalic cultures were exposed to 200 μM L-DOPA for 48 h with a change of medium at 24 h; control cultures did not receive L-DOPA. At 48 h, the medium was aspirated and the cells were rinsed twice with serum-free medium. Cultures were either assayed immediately (0 h washout) or subsequently incubated with fresh medium (containing serum, but without L-DOPA) for an additional 24 h or 48 h. The groups incubated for 48 h received fresh medium after the initial 24 h. Results are pooled from two experiments.

[a] $p < 0.005$ compared to corresponding control at 0 or 24 h

[b] $p < 0.05$ compared to control

[c] $p < 0.001$ compared to no washout

neurons, characterized by the presence of neuron-specific enolase, appear unaffected. In the studies described in this thesis, it was observed that pure neurons without glia were extremely sensitive to toxic effects of added L-DOPA. Toxicity in pure neuronal cultures was evident from disruption and disintegration of neuronal processes and cell bodies, observed by phase contrast microscopy, and by the appearance of LDH in the culture medium (Table 8). Neuronal destruction was accompanied by loss of GSH (Table 8). However, addition of ascorbate to the culture medium protected the neuronal cells (LDH release) and also partially preserved the GSH. Therefore, effects of L-DOPA and ascorbate in pure neuronal cultures are, in a sense, a mirror-image of what was observed with mesencephalic cultures containing both neurons and glia. Superoxide dismutase (SOD) added to the medium partially protected against both LDH release and loss of GSH.

In additional experiments, neuronal cultrures were exposed to lower levels of L-DOPA in order to avoid direct toxicity. Decreasing the concentration of L-DOPA diminished or prevented overt toxicity as viewed under the microscope and, as shown in Table 9, and also preserved the GSH content of the cultures as well as their protein content. The loss of protein can be taken as an index of toxicity. However, it is probable that some protein from lysed cells is associated with cytoskeletal elements that remain affixed to the cell culture wells and are harvested along with remaining intact cells. Such an effect would tend to amplify the decrease in GSH when expressed per mg. of protein. However, the general patter ı remains when GSH levels are assessed without correction for protein content (data not shown). Although the toxicity of L-DOPA is lessened and GSH is preserved with lower concentrations of L-DOPA, no statistically significant evidence for a rise in GSH was observed.

TABLE 8. GSH levels and leakage of lactic acid dehydrogenase (LDH) during exposure of pure neuronal cultures to 200 uM L-DOPA.

Group	GSH (% Control)	LDH (% Increase over Control)
Control	100.0 ± 4.0	0.0 ± 3.9
L-DOPA	23.2 ± 3.9[a]	181.2 ± 15.9[a]
Ascorbate	81.7 ± 5.2	11.4 ± 3.6
Ascorbate/DOPA	72.2 ± 1.9[ab]	9.9 ± 3.2[b]
SOD	107.1 ± 5.1	- 6.5 ± 6.2
SOD/DOPA	64.2 ± 3.7[abc]	69.3 ± 7.4[abc]

Pure neuronal cultures were exposed to 200 μM L-DOPA for 48 h with a change of medium at 24 h; control cultures did not receive L-DOPA. Ascorbic acid (200 μM) or superoxide dismutase (5 μg/mL) were added where indicated. At 48 h, the medium was removed and assayed for LDH. The wells were rinsed once and assayed for GSH and protein. Results are from one (ascorbate) or two (SOD) experiments with n=4/group in each experiment. Data are the mean ± SEM. Control levels of GSH were 3.01 ± 0.12 nmoles/mg protein (n=8); control LDH activity was 0.217 ± 0.010 μmoles NADH/min/mL (n=8).

[a] $p < 0.01$ compared to control

[b] $p < 0.01$ compared to L-DOPA alone

[c] $p < 0.01$ compared to SOD alone

TABLE 9. GSH levels and protein content of pure neuronal cultures after exposure to different concentrations of L-DOPA.

Group	GSH (% Control)	Protein (% Control)
Control	100.0 ± 6.1	100.0 ± 3.9
10 μM L-DOPA	107.6 ± 10.0[bd]	94.3 ± 6.3[c]
50 μM L-DOPA	48.2 ± 7.9[ad]	81.4 ± 5.8
200 μM L-DOPA	10.2 ± 3.5[a]	66.2 ± 5.9[a]

Cultures were treated as described in the legend to Table 8. Results are from 3 experiments with 3-4 samples per group (n=11). Data are the mean ± SEM. Control levels of GSH were 5.82 ± 0.25 nmoles/mg protein and protein was 152.6 ± 7.5 μg/culture (n=11).

[a] $p < 0.01$ compared to control

[b] $p < 0.01$ compared to 50 μM L-DOPA

[c] $p < 0.05$ compared to 200 μM L-DOPA

[d] $p < 0.01$ compared to 200 μM L-DOPA

Part II. MECHANISMS FOR THE RISE IN GSH

C5. Structure-activity relationships

An obvious property of L-DOPA is its ability to react with oxygen (autoxidation), a reaction that forms an insoluble black polymer known as melanin. After 48 hr incubation of mesencephalic cell cultures with L-DOPA, melanization was observed visually. Addition of ascorbate (200 μM, an antioxidant) blocked the formation of melanin at the same time that it blocked the rise in GSH. Therefore it seemed important to study structure-activity relationships to determine whether the ability to elevate GSH would be correlated with autoxidation.

Structure-activity studies were carried out with two defined homogeneous cell lines (Neuro-2A and LLC-PK_1), as well as with mesencephalic cultures. This was done because mesencephalic cultures are heterogeneous and there could be complications arising from different responses between the various cell types in the culture.

The results in Fig. 2 show that whereas L-DOPA evoked a 42.2% rise in GSH in Neuro-2A cells (Fig. 2A) and a 34.0% rise in LLC-PK_1 cells (Fig.2B), alterations in structure that eliminated the ability to autoxidize also eliminated the effect on cellular GSH. The compounds studied included those with methylation of one hydroxyl group (3-O-methyl-DOPA), rearrangement of the two hydroxyl groups in the benzene ring (2,4-DOPA), or removal of one hydroxyl group (tyrosine). These changes resulted in complete loss of the effect on cellular GSH. Whereas L-DOPA (and other catechol compounds) react spontaneously with oxygen (auto-oxidation), each of the structural changes (see Fig. 3) resulted in loss of a free catechol structure and the formation of a

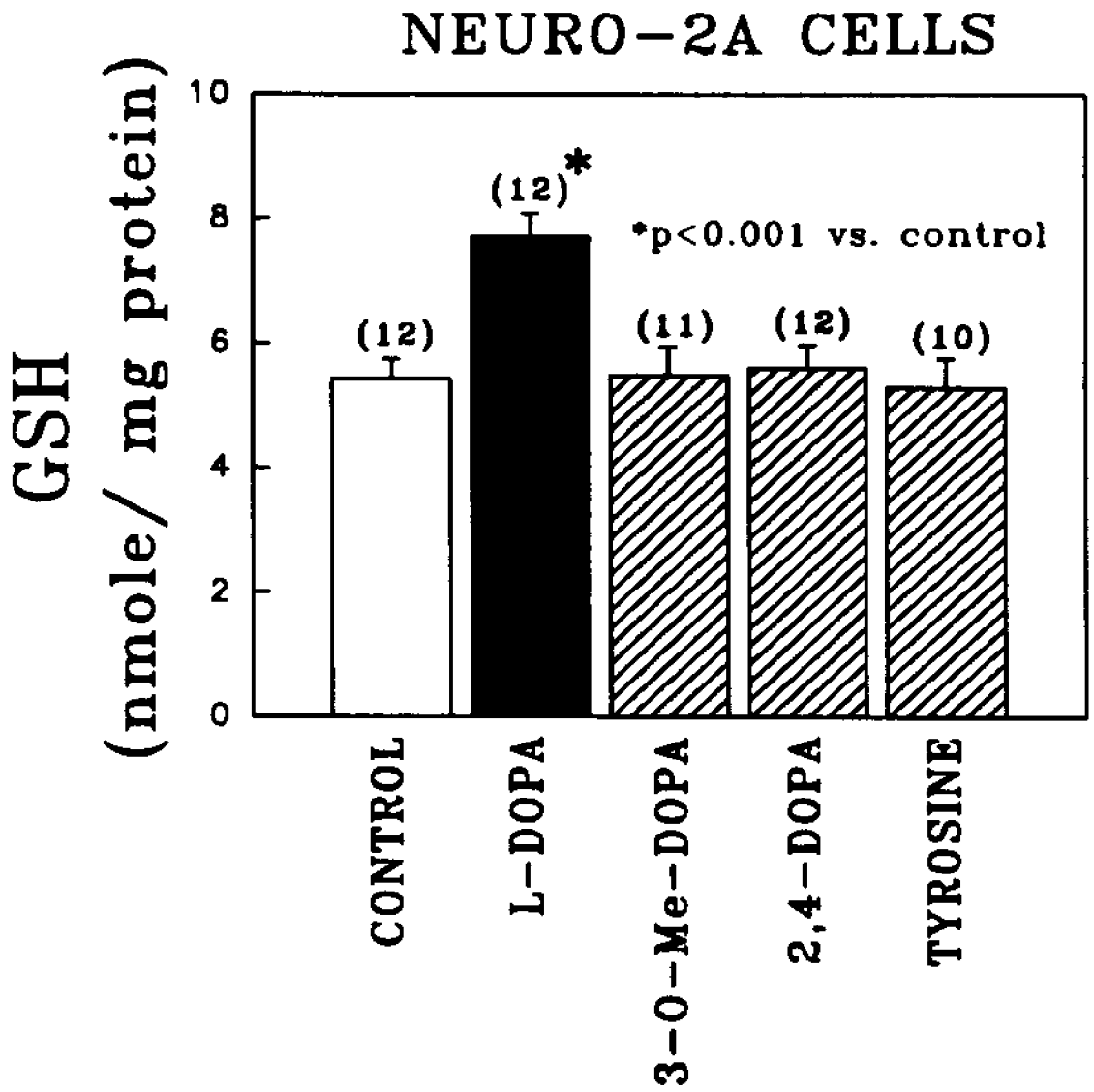

NEURO-2A CELLS
GSH
(nmole/ mg protein)
10
8
6
4
2
0
(12)*
*p<0.001 vs. control
(12)
(11)
(12)
(10)
CONTROL
L-DOPA
3-O-Me-DOPA
2,4-DOPA
TYROSINE

Fig. 2A

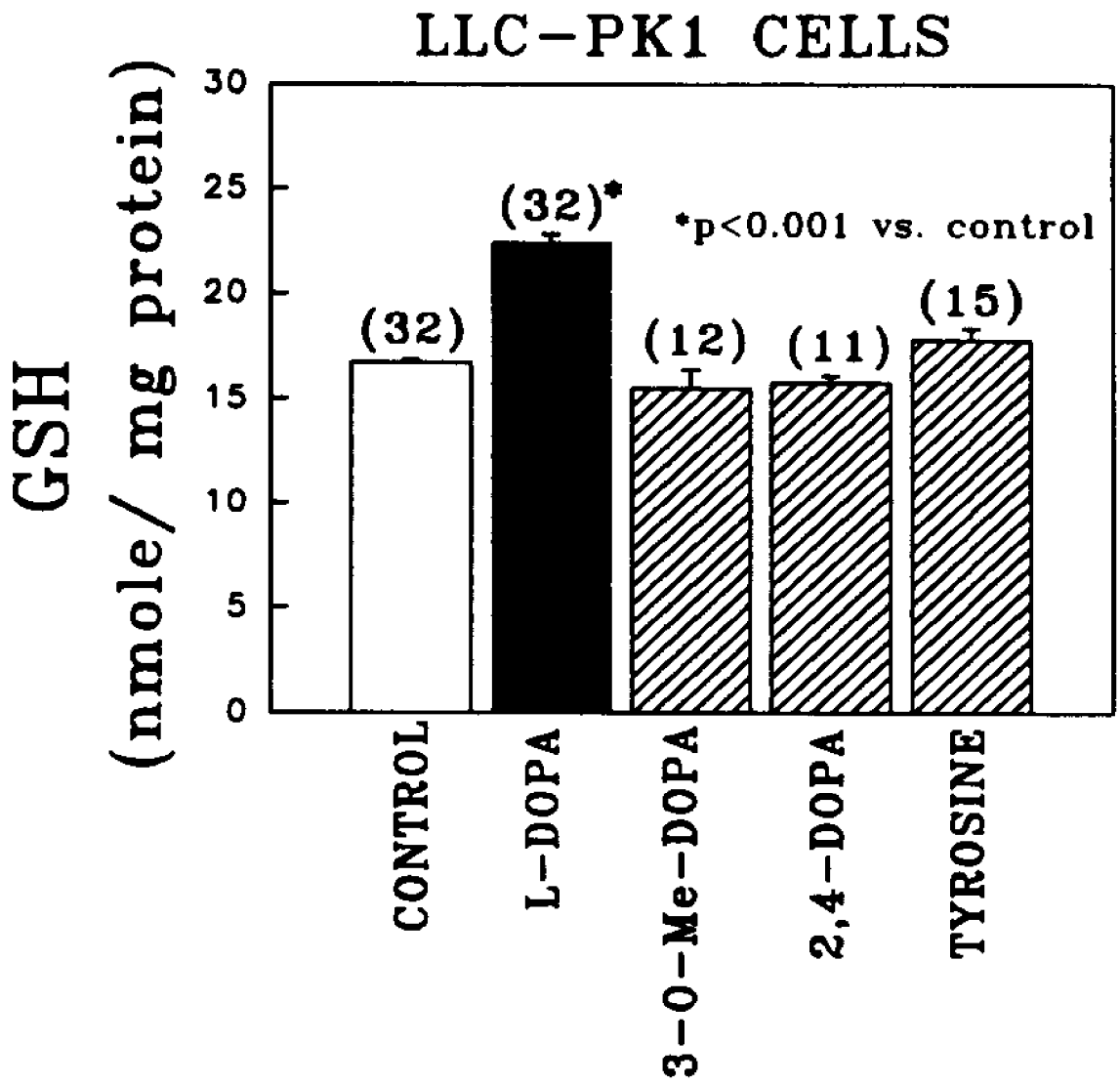
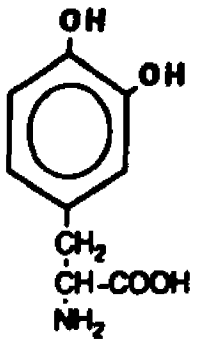

LLC-PK1 CELLS
GSH
(nmole/ mg protein)
30
25
20
15
10
5
0
(32)
(32)*
(12)
(11)
(15)
*p<0.001 vs. control
CONTROL
L-DOPA
3-O-Me-DOPA
2,4-DOPA
TYROSINE

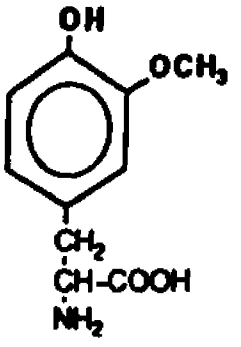
Fig. 2B

Fig. 3 ● = ACTIVE ○ = INACTIVE



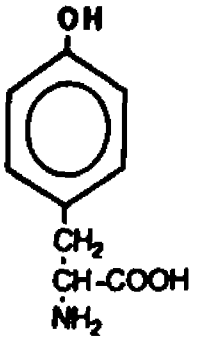
3,4-DOPA

●



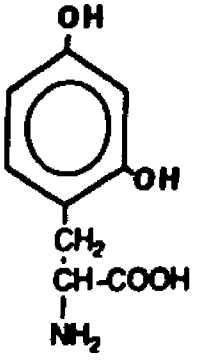
3-O-Methyl-DOPA

○



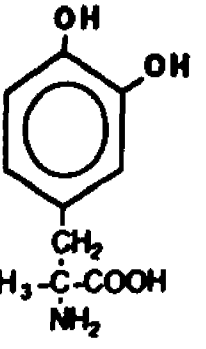
Tyrosine

○



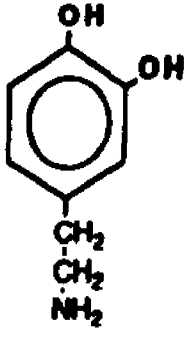
2,4-DOPA

○



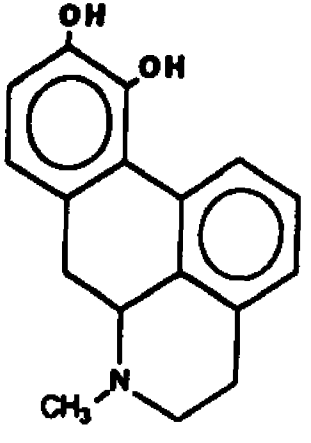
α-Methyl-DOPA

●



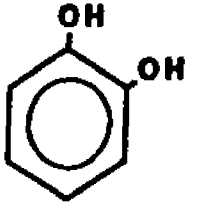
Dopamine

●



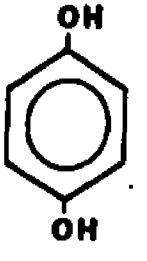
Apomorphine

●



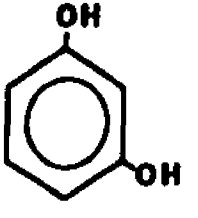
Catechol

●



Hydroquinone

●



Resorcinol

○

compound that cannot undergo autoxidation. Therefore, the results shown in Fig. 2 point to autoxidation as a determinant of the effect on cellular GSH.

In separate experiments alpha-methyl-DOPA was also tested as a positive amino acid control in experiments with mesencephalic cultures (Table 10). Alpha-methyl-DOPA contains a catechol structure (Fig.3) and therefore can undergo autoxidation. Alpha-methyl-DOPA (100 μM x 48 h) elevated GSH from 3.13 $\pm$ 0.06 to 5.47 $\pm$ 0.35 nmole/mg protein ($n=17$ per group), an increase of 74.8% ($p<0.001$). By way of comparison, dopamine (100 μM, also a catechol) in a parallel experiment elevated GSH to 6.59 $\pm$ 0.38 nmole/mg ($n=18$), an increase of 110.5% ($p<0.001$). Therefore, the ability to elevate GSH correlates with the presence of an autoxidizable catechol group in the amino acid series.

Primary cultures of fetal rat mesencephalon were also tested. They behaved similarly to the Neuro-2A and LLC-PK_1 cells. They failed to respond to 3-O-methyl-DOPA, tyrosine, or 2,4-DOPA in 3-4 experiments conducted in replicate (4-6 wells per compound; data not shown) although they did respond to L-DOPA (Table 1). Structure-activity relationships were then extended to other compounds that were not amino acids (see Fig. 3). Dopamine and apomorphine (100 μM), both of which are catechols, each elevated the mean cellular GSH level in mesencephalic cultures. Model compounds in which the entire side-chain was removed were also studied. The compounds were catechol itself (ortho-dihydroxybenzene) and two analogs based on rearrangement of the two phenolic hydroxyl groups on the benzene ring, namely, hydroquinone (para-dihydroxybenzene) and resorcinol (meta-dihydroxybenzene). Catechol and hydroquinone undergo auto-oxidation, while resorcinol does not. Catechol (100 μM) was active in elevating cellular GSH, whereas resorcinol at the same concentration was not (see Fig. 4). Hydroquinone, which undergoes vigorous

TABLE 10. GSH levels in mesencephalic cultures after 48 hr exposure to 100 μM alpha-methyl-DOPA or 100 μM DA.

Group	GSH (nmole/mg protein)	% increased over control	N
Control	3.13 ± 0.06		(17)
100 μM DA	6.59 ± 0.38[a]	110.4	(18)
100 μM alpha-methyl-DOPA	5.47 ± 0.35[a]	74.8	(17)

Mesencephalic cultures were treated on the 5th day in vitro for 48 hours, with a change of medium after 24 hours. The values are the means ± SEM.

[a] $p < 0.001$ compared to control

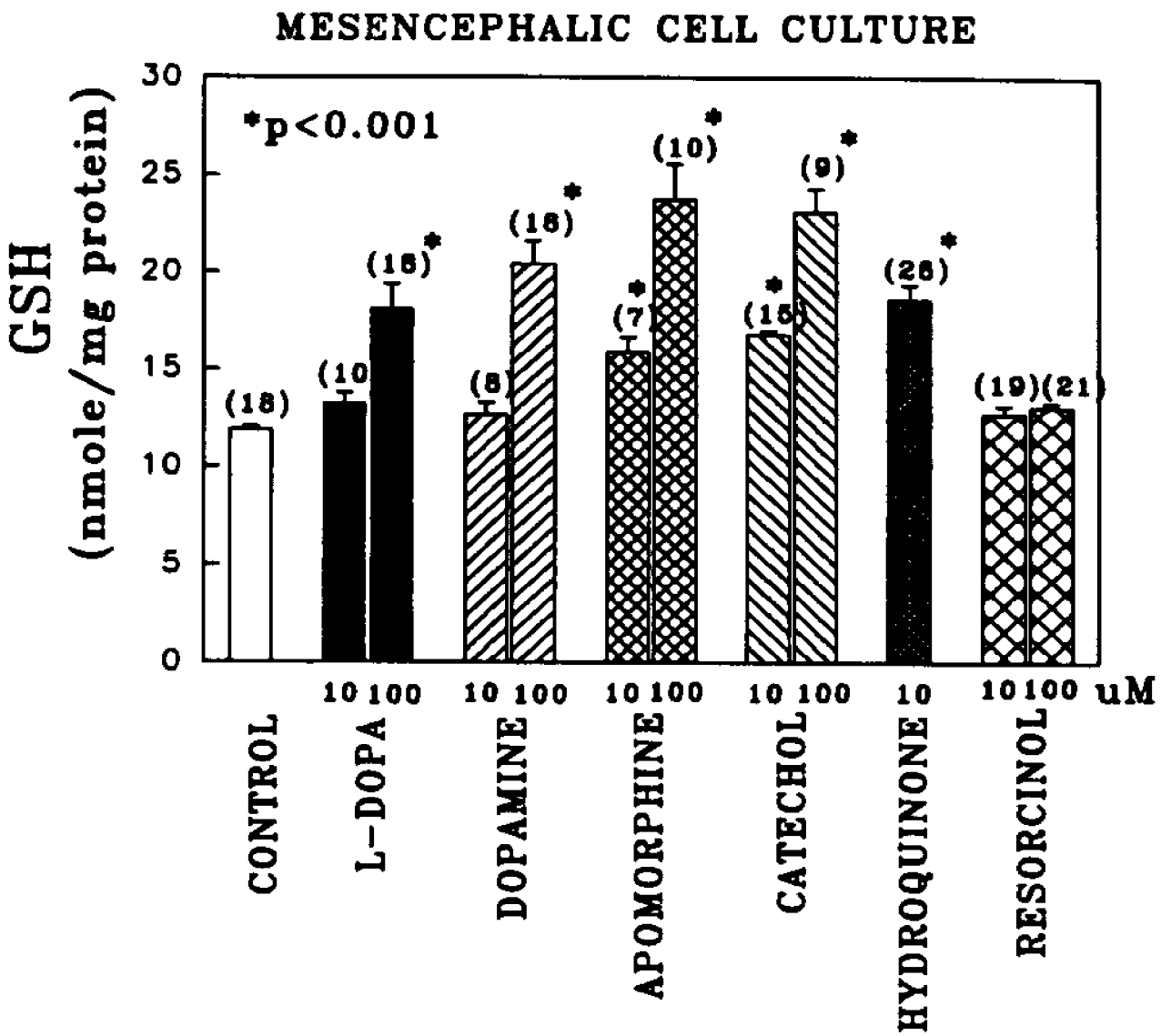

MESENCEPHALIC CELL CULTURE
GSH
(nmole/mg protein)
*p<0.001
30
25
20
15
10
5
0
(18)
(10)
(18)*
(8)
(18)*
(7)*
(10)*
(15)*
(9)*
(26)*
(19)
(21)
10 100
10 100
10 100
10 100
10
10 100
uM
CONTROL
L-DOPA
DOPAMINE
APOMORPHINE
CATECHOL
HYDROQUINONE
RESORCINOL

Fig. 4

autoxidation, proved to be very toxic to the cultures (disintegration and loss of cells). Therefore, a much lower concentration of 10 μM was used in order to avoid toxicity. Hydroquinone (10 μM) was effective in elevating cellular GSH. At a concentration of 10 μM, catechol and apomorphine, but not dopamine or L-DOPA, also induced a significant elevation of GSH.

These data, as a whole, show that the ability of a given compound to upregulate GSH in cell cultures is correlated with its ability to undergo autoxidation.

C6. DA receptors: Do they have a role?

C6.1 *Study of DA-receptor antagonists*

From the above structure-activity relationship study, we know that two DA receptor agonists, DA and apomorphine, can both elevate GSH. This raises the possibility that GSH may be regulated by DA receptors, in addition to effects brought on by autoxidation. To test this possibility we added DA-receptor antagonists to determine whether they would affect the rise in GSH (Fig. 5). Several DA receptor antagonists were tested, namely SCH 23390 (D1-receptor antagonist), spiperone (D2-receptor antagonist), and sulpiride (D2-receptor antagonist). After 48 hr of exposure to 100 μM dopamine in mesencephalic cell culture, the GSH content rose from 4.39 $\pm$ 0.22 nmole/mg protein to 9.24 $\pm$ 0.54 nmole/mg protein, an increase of 110.3% over control values ($p < 0.001$). All of the antagonists failed to suppress the DA-induced rise in GSH: 1 μM SCH23390 + DA, 92.8 $\pm$ 11.6% increase compared to control ($p < 0.001$); 1 μM spiperone + DA, 103.9 $\pm$ 9.3% increase ($p < 0.001$); and 1 μM sulpiride + DA, 75.2 $\pm$ 17.2% increase ($p < 0.001$). The final GSH level after exposure to DA plus DA receptor antagonists was not significantly different from

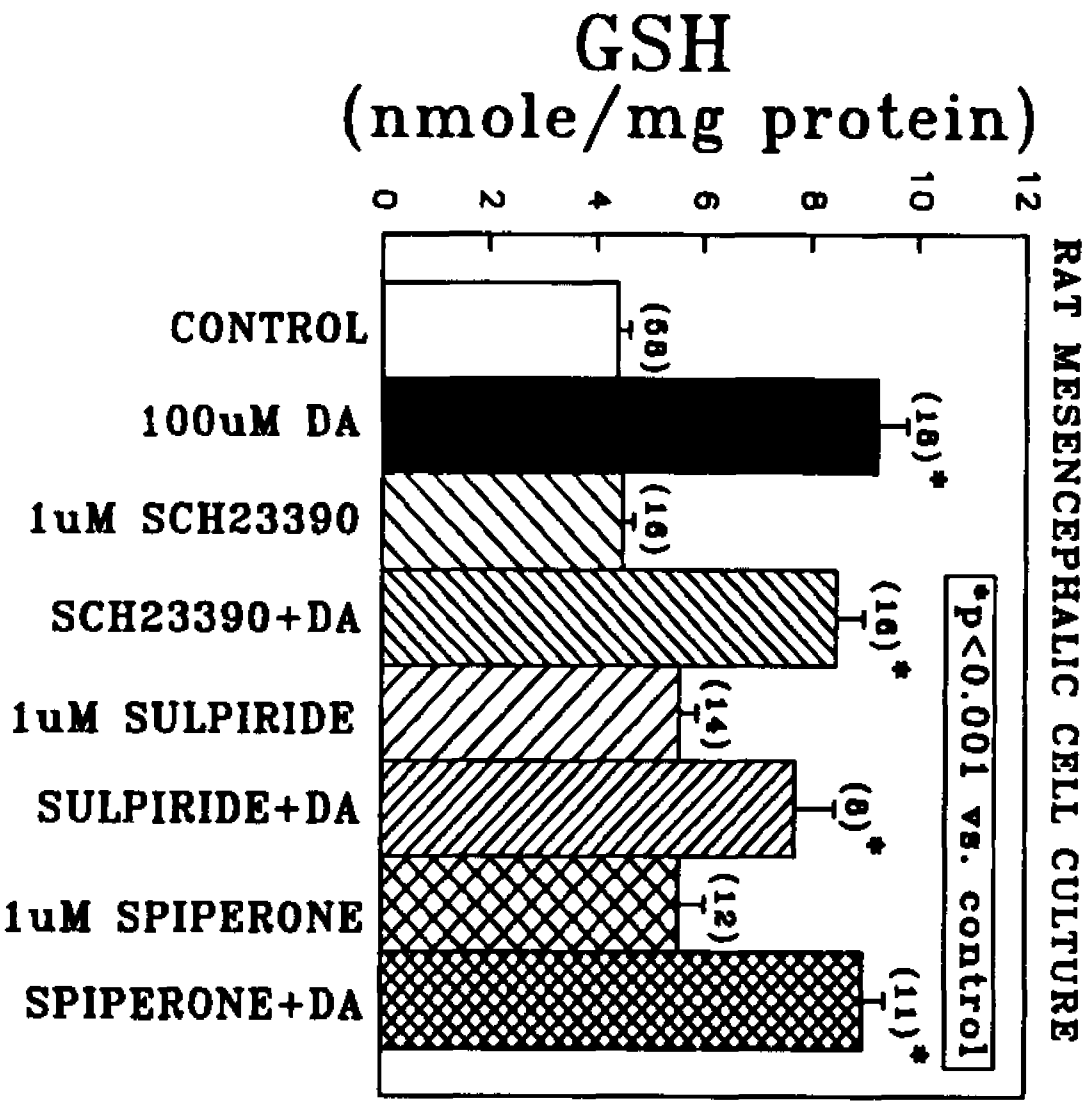

GSH
(nmole/mg protein)
0
2
4
6
8
10
12
CONTROL
100uM DA
1uM SCH23390
SCH23390+DA
1uM SULPIRIDE
SULPIRIDE+DA
1uM SPIPERONE
SPIPERONE+DA
(68)
(18)*
(16)
(16)*
(14)
(9)*
(12)
(11)*
*p<0.001 vs. control
RAT MESENCEPHALIC CELL CULTURE

Fig. 5

level observed with DA alone.

In additional experiments (Fig. 6), the concentrations of antagonist were increased from 1 μM to 25 μM or to 100 μM. Despite these high concentrations of receptor antagonists, no significant suppression in the GSH level was seen compared to DA alone. Dopamine (100 μM x 48 h) elevated GSH from 7.7 $\pm$ 0.19 to 10.1 $\pm$ 0.24 nmole/mg protein, an increase of 31.3% ($p<0.001$, $n=18$). All the antagonists plus DA groups such as SCH 23390 (100 μM, 10.0 $\pm$0.24 nmole/mg protein, $n=12$), sulpiride (100 μM, 10.8 $\pm$ 0.34 nmole/mg protein, $n=18$), and spiperone (25 μM, 12.0 $\pm$ 0.53 nmole/mg protein, $n=18$), showed an increase over control values: 30.2% ($p<0.001$), 40.5% ($p<0.001$), and 56.0% ($p<0.001$), respectively. The conclusion from the receptor antagonist study is that DA-receptors do not appear to be involved in up-regulation of glutathione in mesencephalic cell cultures.

C6.2 *Bypass of the DA receptor: The effects of Rp-cAMP, Sp-cAMP, forskolin and 1,9-dideoxy-forskolin on GSH levels.*

Dopamine receptors are well-known as regulators of adenylate cyclase: D1-receptor (stimulation of adenyl cyclase) and D2-receptor (inhibition of adenyl cyclase). Experiments were designed to bypass adenylate cyclase activity to determine the impact of such treatment on GSH levels. Two membrane-permeable cyclic nucleotide derivatives of adenosine-3',5'-cyclic monophosphothioate (cAMP) diastereomers were used, namely, Rp-cAMP (a specific inhibitor cAMP-dependent protein kinase I and II) and Sp-cAMP (a potent and specific activator of cAMP-dependent protein kinase I and II). These derivatives are also resistant to metabolism by phosphodiesterase. Neither Rp-cAMP nor Sp-cAMP altered the level of GSH in the mesencephalic cell cultures (Table 11). These data showed no change in GSH when cAMP-dependent kinases

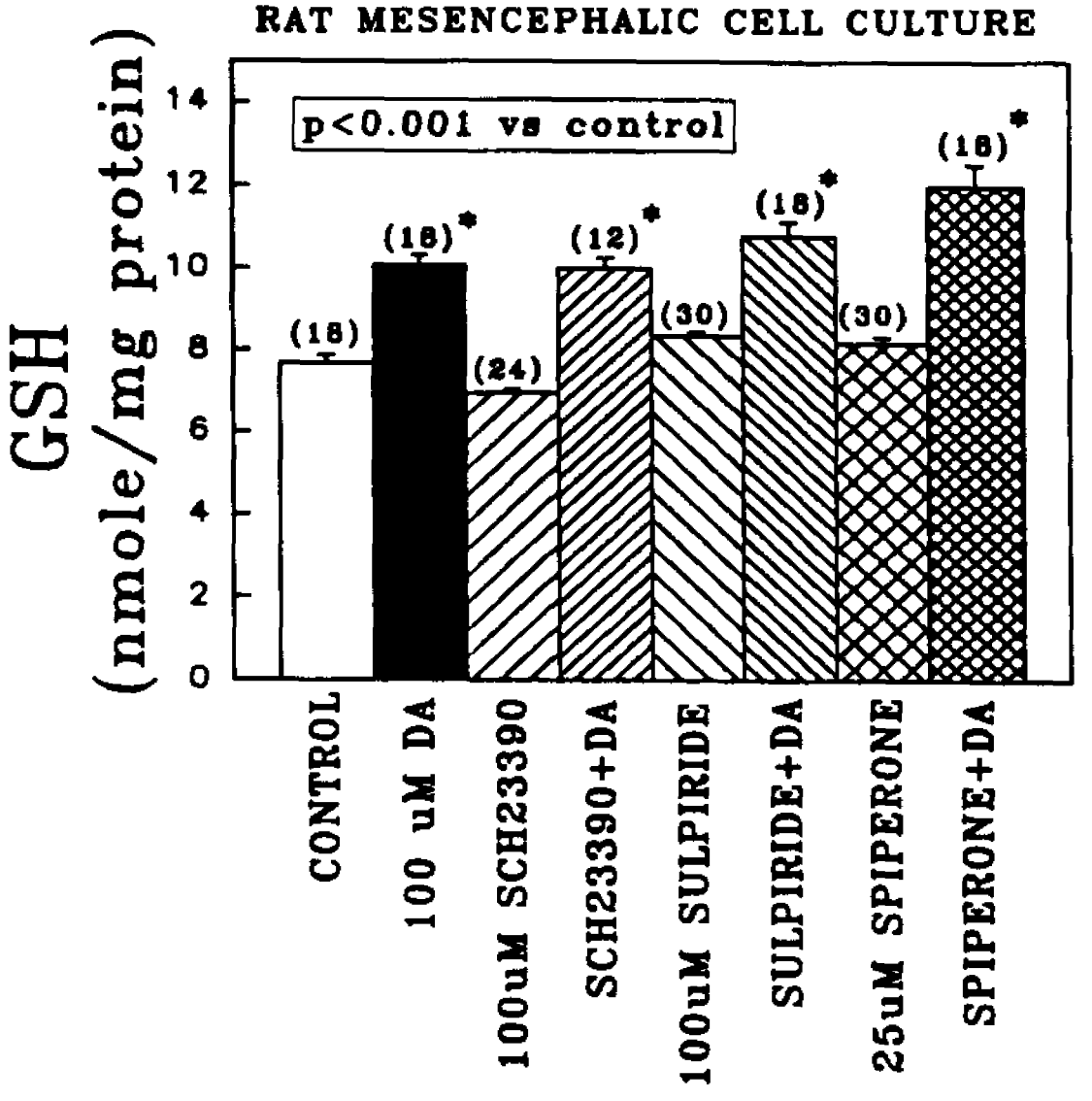

RAT MESENCEPHALIC CELL CULTURE
GSH
(nmole/mg protein)
p<0.001 vs control
0
2
4
6
8
10
12
14
(16)
(16)*
(24)
(12)*
(30)
(16)*
(30)
(16)*
CONTROL
100 uM DA
100uM SCH23390
SCH23390+DA
100uM SULPIRIDE
SULPIRIDE+DA
25uM SPIPERONE
SPIPERONE+DA

Fig. 6

Table 11. Lack of effect of 50 μM Sp- and Rp- diastereomers of adenosine 3',5'-cyclic monophosphothioate on levels of GSH in mesencephalic cultures.

Group	N	GSH (nmole/mg protein) Mean ± SEM
Control	16	7.35 ± 0.29
Sp-cAMP	16	6.90 ± 0.23
Control	4	6.05 ± 0.26
Rp-cAMP	4	6.09 ± 0.29

The cultures were incubated for 48 hrs. Results are from 3 independent experiments for Sp-cAMP and a single experiment for Rp-cAMP. Neither Sp-cAMP nor Rp-cAMP treatment resulted in GSH levels that were significantly diffferent from control ($p > 0.05$)

were either stimulated or inhibited directly, without involvement of DA receptors.

In separate experiments, forskolin, an activator of adenylate cyclase, was tested. Forskolin (50 μM) by itself failed to elevate GSH (Fig. 7). However, studies by Moullet et al. (1994) showed that forskolin can block the inhibition of adenylate cyclase by a quinone (p-quinone, the oxidized form of hydroquinone) in HepG2 cells. Therefore, the effect of forskolin on 100 μM dopamine- and 10μM hydroquinone-induced elevation of GSH was tested. Forskolin suppressed the rise in GSH (Fig. 7). On the other hand, 1,9-dideoxy-forskolin (50 μM), an inactive analog, which was used as a negative control, did not block the action of either dopamine or hydroquinone. These experiments imply that under the appropriate circumstances cAMP formation can block the upregulation of GSH by hydroquinone.

C6.3 *Study of R-(-)apomorphine and S-(+)apomorphine*

Apomorphine, which actively elevates GSH, preserves features that can be need for additional testing of the role of DA receptors. Apomorphine exists in two diastereomeric forms, R- and S-apomorphine. R-Apomorphine is a DA receptor agonist, while S-apomorphine is not an agonist and behaves as a DA receptor antagonist. Treatment with both R-apomorphine (10 μM x 48 h) and S-apomorphine (10 μM x 48 h) resulted in elevated GSH- from 5.3 $\pm$ 0.5 to 9.6 $\pm$ 0.5 and 8.9 $\pm$ 0.5 nmole/mg protein, respectively,- increases of 82.1% ($p<0.001$) and 69.2% ($p<0.001$) (Fig. 8). The results obtained with S-apomorphine, which can autoxidize but cannot activate dopamine receptor function, clearly showed that there is no need of activation of DA receptors to elevate GSH level.

These studies showed that autoxidation, without involvement of DA receptors, is

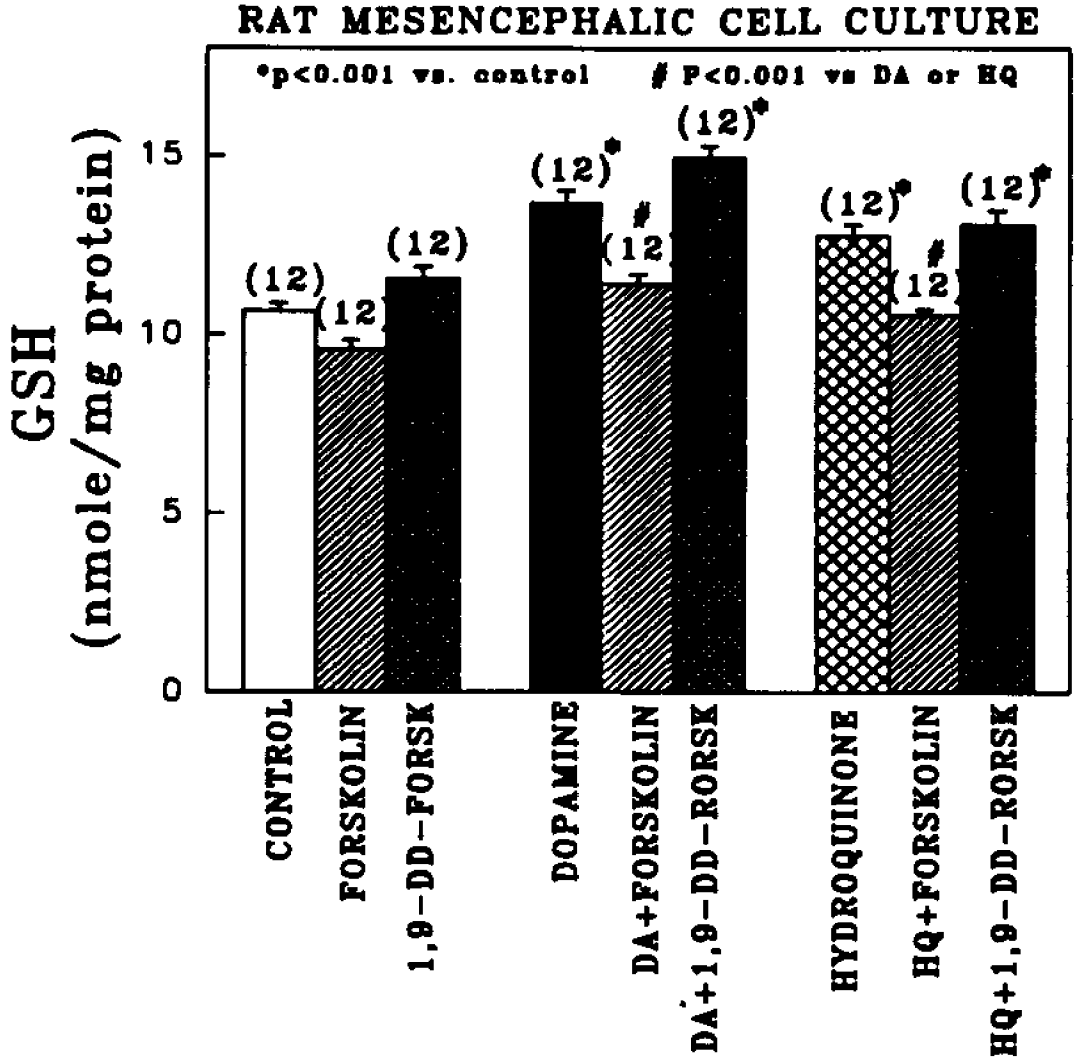

RAT MESENCEPHALIC CELL CULTURE
*p<0.001 vs. control
# P<0.001 vs DA or HQ
GSH
(nmole/mg protein)
0
5
10
15
(12)
(12)
(12)
(12)*
(12)
#
(12)
(12)*
(12)*
(12)
#
(12)
(12)*
CONTROL
FORSKOLIN
1,9-DD-FORSK
DOPAMINE
DA+FORSKOLIN
DA+1,9-DD-RORSK
HYDROQUINONE
HQ+FORSKOLIN
HQ+1,9-DD-RORSK

Fig. 7

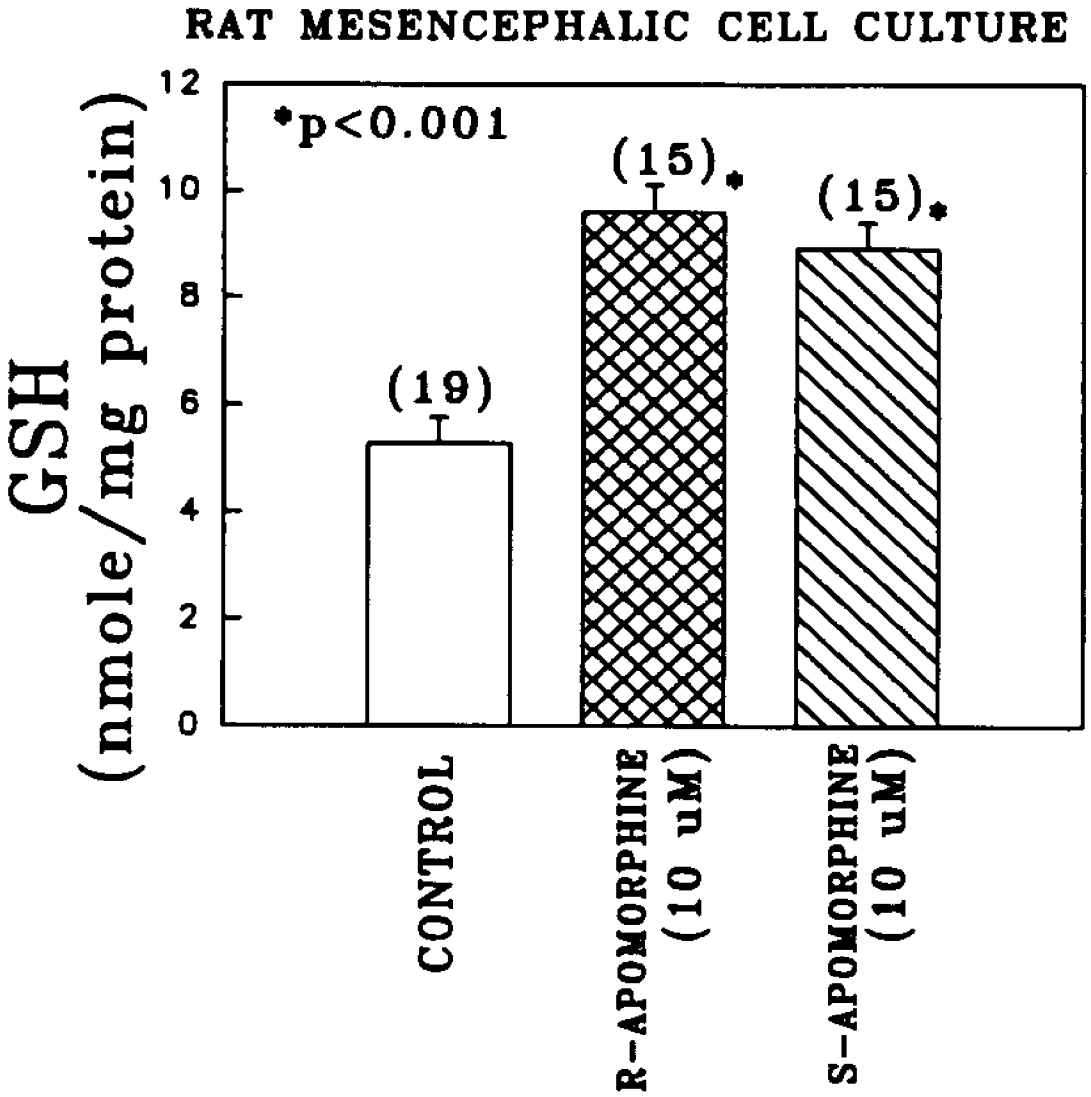

RAT MESENCEPHALIC CELL CULTURE
GSH
(nmole/mg protein)
*p<0.001
(19)
(15)*
(15)*
0
2
4
6
8
10
12
CONTROL
R-APOMORPHINE (10 uM)
S-APOMORPHINE (10 uM)

Fig. 8

the primary mechanism for the elevation of GSH level by redox cycling compounds in the mesencephalic cell culture.

C6.4 *An exception: Quinpirole*

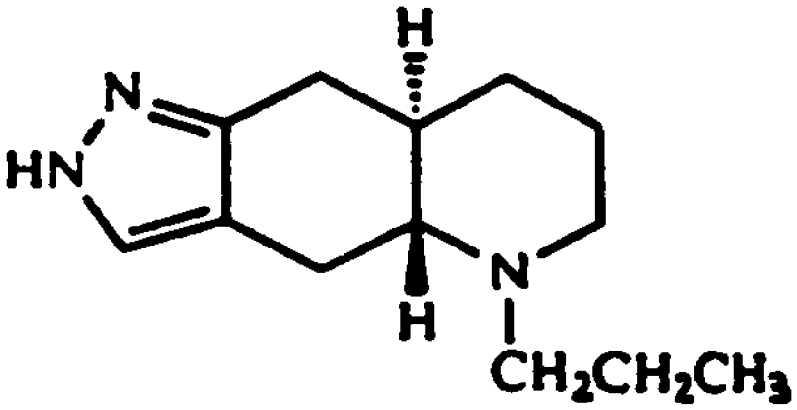
In the course of these experiments, we also tested quinpirole, which is a DA receptor agonist (major effector of D2 receptors and a minor effector of D3 receptors). Quinpirole (Fig. 9) does not undergo autoxidation. Nonetheless, quinpirole was active in elevating GSH in mesencephalic cultures (Table 12) at concentrations of 100 or 200 μM. Quinpirole was inactive at 10 μM (data not shown). Addition of sulpiride or spiperone (D2 receptor antagonists, 1 μM) did not affect the action of quinpirole. The effect of quinpirole does not appear to be mediated by either autoxidation or DA receptors. Other data with quinpirole will be presented later in this thesis when the role of protein kinase C is discussed.

C7. Disulfides as an oxidant signal: Experiments with glutathione disulfide (GSSG) and dithiothreitol disulfide.

We had previously observed that GSSG levels rose before an elevation in GSH could be detected (Table 2). For example, at 18 hrs, the level of GSSG had doubled ($p<0.001$), while GSH had not yet begun to rise (Table 2). Disulfides, like GSSG, are reactive and can form mixed disulfides with protein SH-groups:

$$\text{Protein-SH} + \text{GSSG} \text{ -----> } \text{Protein-SSG} + \text{GSH} \qquad \text{(Eqn. 5)}$$

Such reactions can modify the biological function of proteins. Therefore, we considered the possibility that the elevation in GSSG could serve as an oxidant signal to



THE STRUCTURE OF QUINPIROLE

Fig. 9

TABLE 12. GSH levels in mesencephalic cultures after 48 hr exposure to 100 μM or 200 μM quinpirole.

Group	GSH (nmole/mg protein)	% increased over control	N
Control	3.95 ± 0.06		(36)
100 μM Quinpirole	6.86 ± 0.35[a]	73.7	(31)
200 μM Quinpirole	6.86 ± 0.71[a]	73.7	(13)

Mesencephalic cultures were treated on the 5th day in vitro for 48 hours, with a change of medium after 24 hours. The values are the means ± SEM.

[a] $p < 0.001$ compared to control

trigger the upregulation of the GSH content.

Two kind of experiments were performed to address this question. First, GSSG was directly added to mesencephalic cell cultures as an oxidant signal. As seen in Fig. 10, incubation of cultures with 100 μM GSSG induced a significant increase of GSH from 9.2 $\pm$ 0.2 nmole/mg protein to 11.2 $\pm$ 0.3 nmole/mg protein (+21.7%, $p<0.001$, $n=24$ per group). But it is arguable that the elevation of GSH occurs via an oxidant signal because GSSG itself can be reduced to GSH by GSSG reductase. Therefore, a second disulfide was selected that cannot be directly reduced to GSH. For this experiment, the disulfide form of dithiothreitol (DTT) was used. Oxidized DTT (200 μM, Fig. 10) also provoked a significantly elevation of GSH to 11.5 $\pm$ 0.3 nmole/mg protein (+25.0%, $p<0.001$, $n=24$). In control experiments, the reduced form of DTT did not produce an effect on the level of GSH (data not shown). Therefore, the data with DTT-disulfide support the idea that the formation of GSSG during the autoxidation of L-DOPA (or other reactive diphenols) can be part of a biological signaling mechanism.

C8. Signal transduction at the level of protein kinase C (PKC).

Kass et al. (1989) reported that activation of hepatocyte cytosolic protein kinase C (PKC) by redox-cycling quinones, such as menadione or benzoquinone, is due to a change in the thiol/disulphide status (-SH/S-S status) of PKC. Activation of PKC was prevented by adding relatively high concentrations (10 mM) of beta-mercaptoethanol, GSH, or DTT. Direct addition of GSSG also activated PKC.

As noted above, hydroquinone (10 μM) can elevate the level of GSH (see Fig. 4) through a mechanism involving autoxidation. It was therefore of interest to know

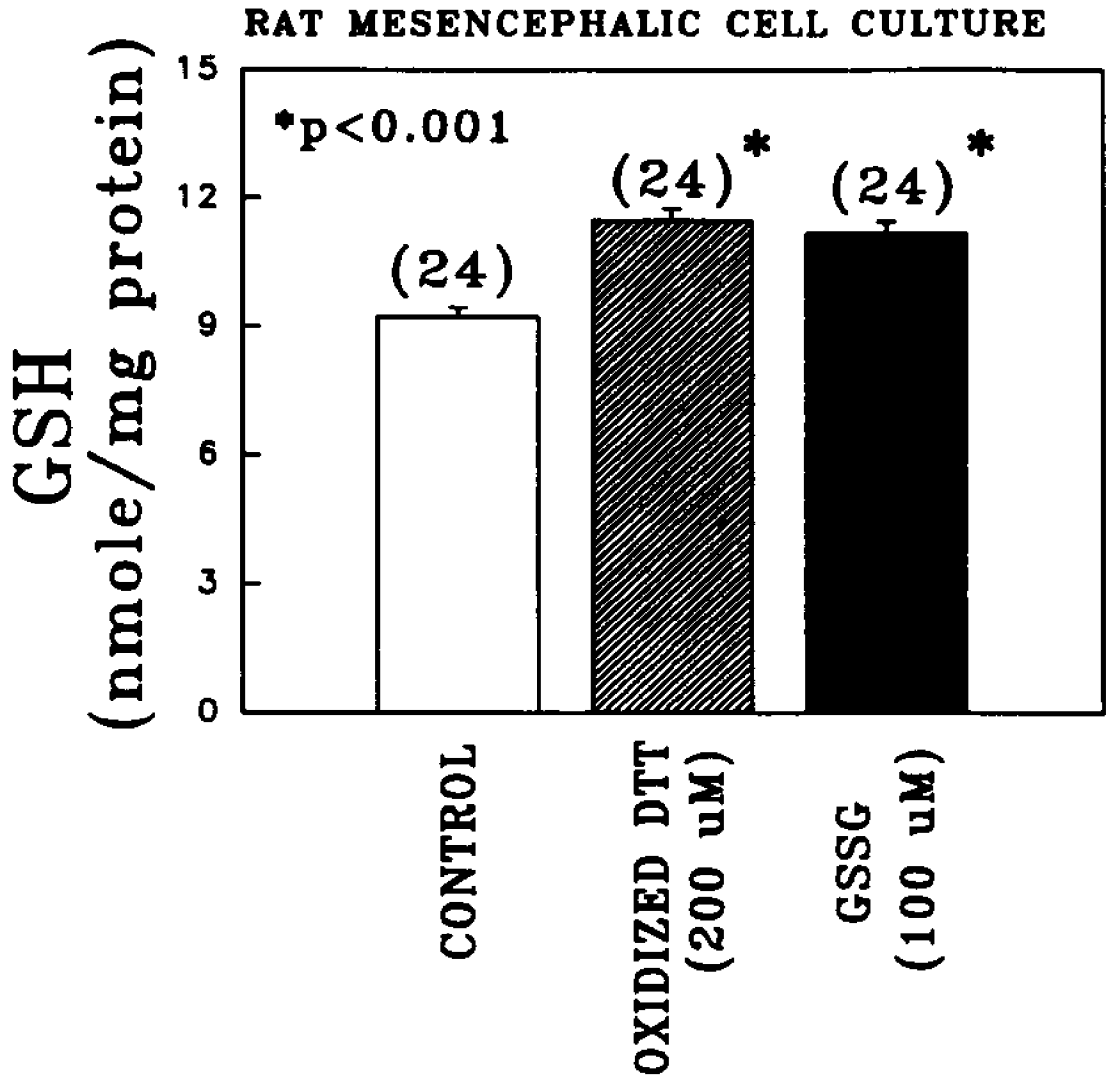

RAT MESENCEPHALIC CELL CULTURE
GSH
(nmole/mg protein)
15
12
9
6
3
0
*p<0.001
(24)
(24)*
(24)*
CONTROL
OXIDIZED DTT
(200 uM)
GSSG
(100 uM)

Fig. 10

whether up-regulation of GSH by L-DOPA or dopamine involved PKC activity. Two inhibitors of PKC were studied, namely, staurosporine and H-7. H-7 also exhibits weak activity as a PKA inhibitor, but staurosporin is relatively selective for PKC. H-7 and staurosporine efficiently block the elevation of GSH elicited by dopamine or L-DOPA. Fig. 11 shows experimental results with 100 μM DA added to mesencephalic cultures; DA elevated GSH by 262% ($p<0.001$, $n=16$). H-7 (200 μM) greatly suppressed the rise in GSH while staurosporine (0.2 μM) totally blocked the rise in GSH.

Table 13 shows similar experiments with L-DOPA. Both H-7 (100 μM) and staurosporin (0.2 μM) completely suppressed the rise in GSH induced by L-DOPA.

As also noted above, quinpirole, a DA-receptor agonist, elevates GSH (see Table 12), even though quinpirole does not have an autoxidizable structure. It was of interest to determine whether the rise in GSH induced by quinpirole also required PKC activity. The data in Fig. 12 show that the rise in GSH from 3.95 $\pm$ 0.06 nmole/mg protein to 6.86 $\pm$ 0.35 nmole/mg protein ($p<0.001$) induced by 100 μM quinpirole is greatly blocked by H-7 and completely blocked by staurosporine.

C9. Gamma-glutamylcysteine synthetase (gamma-GCS) activity.

Gamma-GCS catalyzes the first and rate-limiting step of glutathione synthesis. Shi et al. (1994) reported that both GSH and gamma-GCS activity were increased after incubation of bovine pulmonary artery endothelial cells with redox-cycling quinones. In the current study with mesencephalic cultures, L-DOPA (100 μM), DA (100 μM) and hydroquinone (10 μM) elevated the activity of gamma-GCS by 75.9% $\pm$ 4.9% ($p<0.001$, $n=7$), 35.1% $\pm$ 10.3% ($p<0.01$, $n=7$), and 31.5% $\pm$ 7.2% ($p<0.05$,

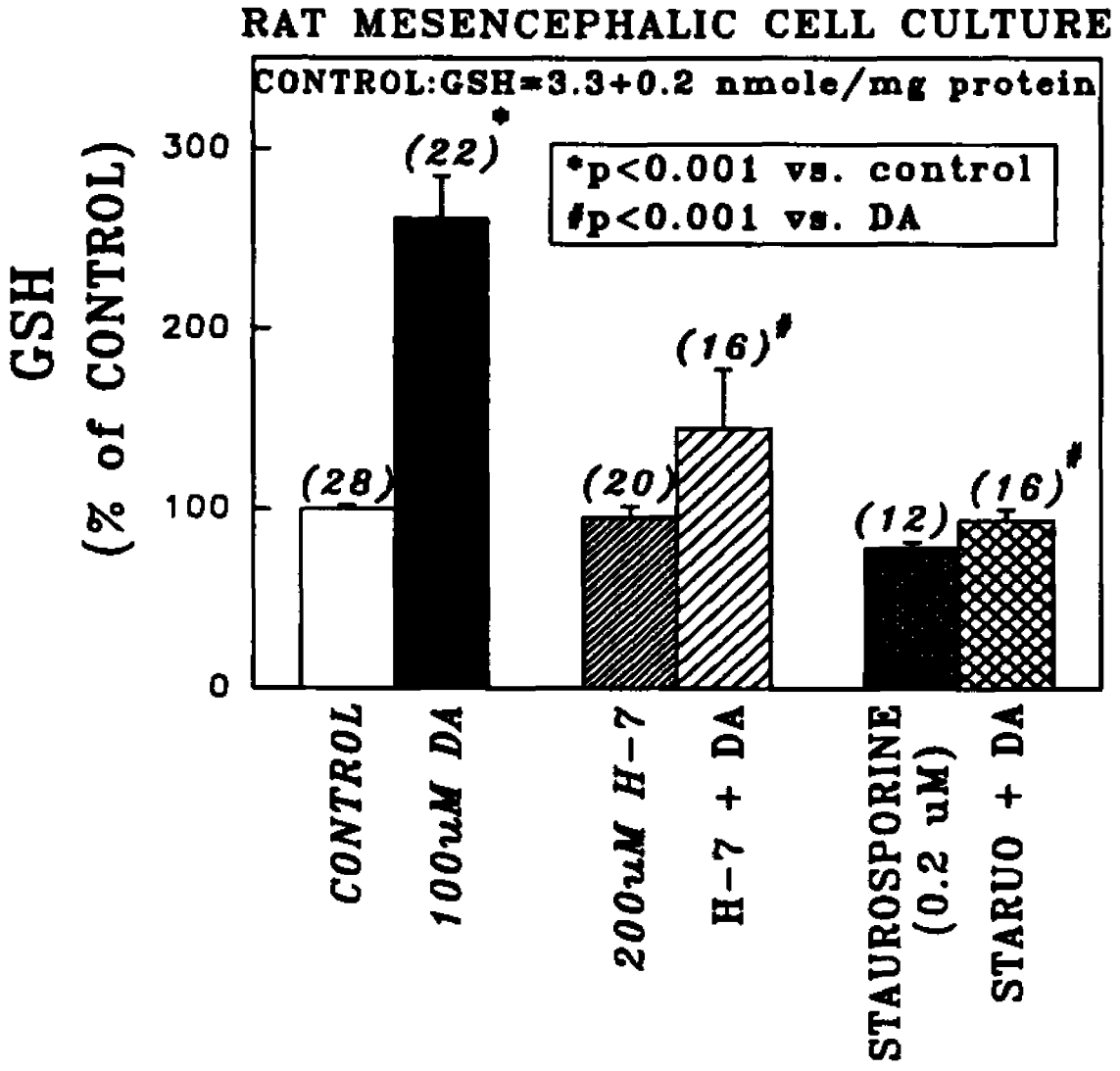

RAT MESENCEPHALIC CELL CULTURE
CONTROL:GSH=3.3+0.2 nmole/mg protein
*p<0.001 vs. control
#p<0.001 vs. DA
GSH
(% of CONTROL)
300
200
100
0
(28)
(22)*
(20)
(16)#
(12)
(16)#
CONTROL
100uM DA
200uM H-7
H-7 + DA
STAUROSPORINE (0.2 uM)
STARUO + DA

Fig. 11

TABLE 13. Effect of protein kinase C (PKC) inhibitors on the L-DOPA-induced increase in upregulation of GSH content in mesencephalic cultures.

Treatment	GSH as (% Control)	(N)
Control	100.0 ± 2.0	(28)
L-DOPA (100 μM)	161.3 ± 4.9[a]	(23)
L-DOPA (200 μM)	166.9 ± 7.7[a]	(15)
H-7 (100 μM)	86.4 ± 7.9	(8)
H-7 (100 μM) + L-DOPA (200 μM)	61.4 ± 1.4[ac]	(7)
Staurosporine (0.2 μM)	78.4 ± 3.5	(12)
Staur (0.2 μM) + L-DOPA (100 μM)	90.8 ± 4.6[b]	(14)
Staur (0.2 μM) + L-DOPA (200 μM)	85.6 ± 4.1[c]	(6)

Mesencephalic cultures were treated on the 5th day in vitro with the various compounds for 48 hours, with a change of medium after 24 hours. The values are the means ± SEM; GSH levels of control cultures were 3.38± 0.22 nmole/mg protein.

By ANOVA followed by Tukey HSD test for multiple comparisons.

[a] $p < 0.001$ compared to control

[b] $p < 0.001$ compared to 100 μM L-DOPA

[c] $p < 0.001$ compared to 200 μM L-DOPA

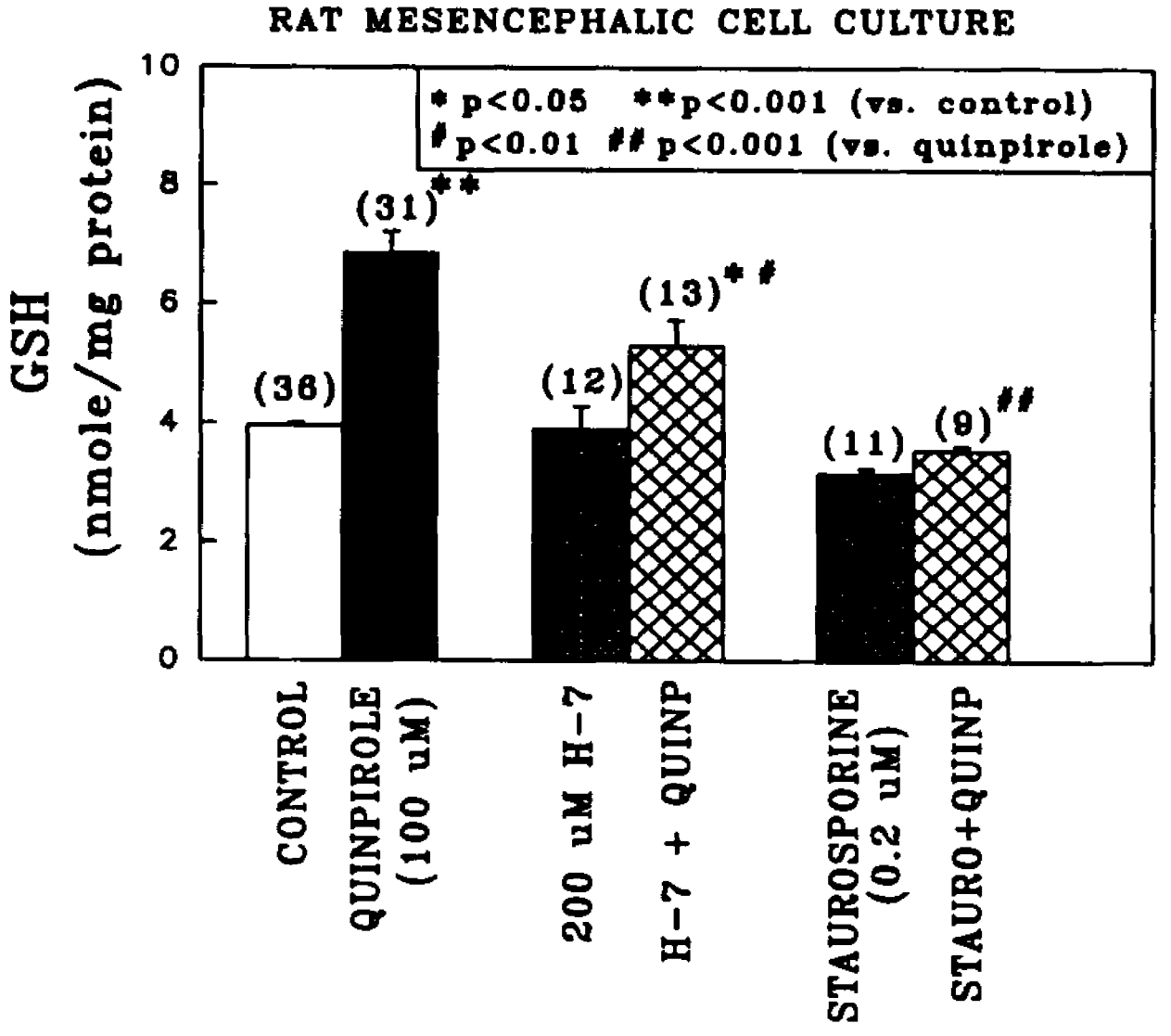

RAT MESENCEPHALIC CELL CULTURE
* p<0.05 **p<0.001 (vs. control)
# p<0.01 ## p<0.001 (vs. quinpirole)
GSH
(nmole/mg protein)
10
8
6
4
2
0
(36)
(31)**
(12)
(13)*#
(11)
(9)##
CONTROL
QUINPIROLE (100 uM)
200 uM H-7
H-7 + QUINP
STAUROSPORINE (0.2 uM)
STAURO+QUINP

Fig. 12

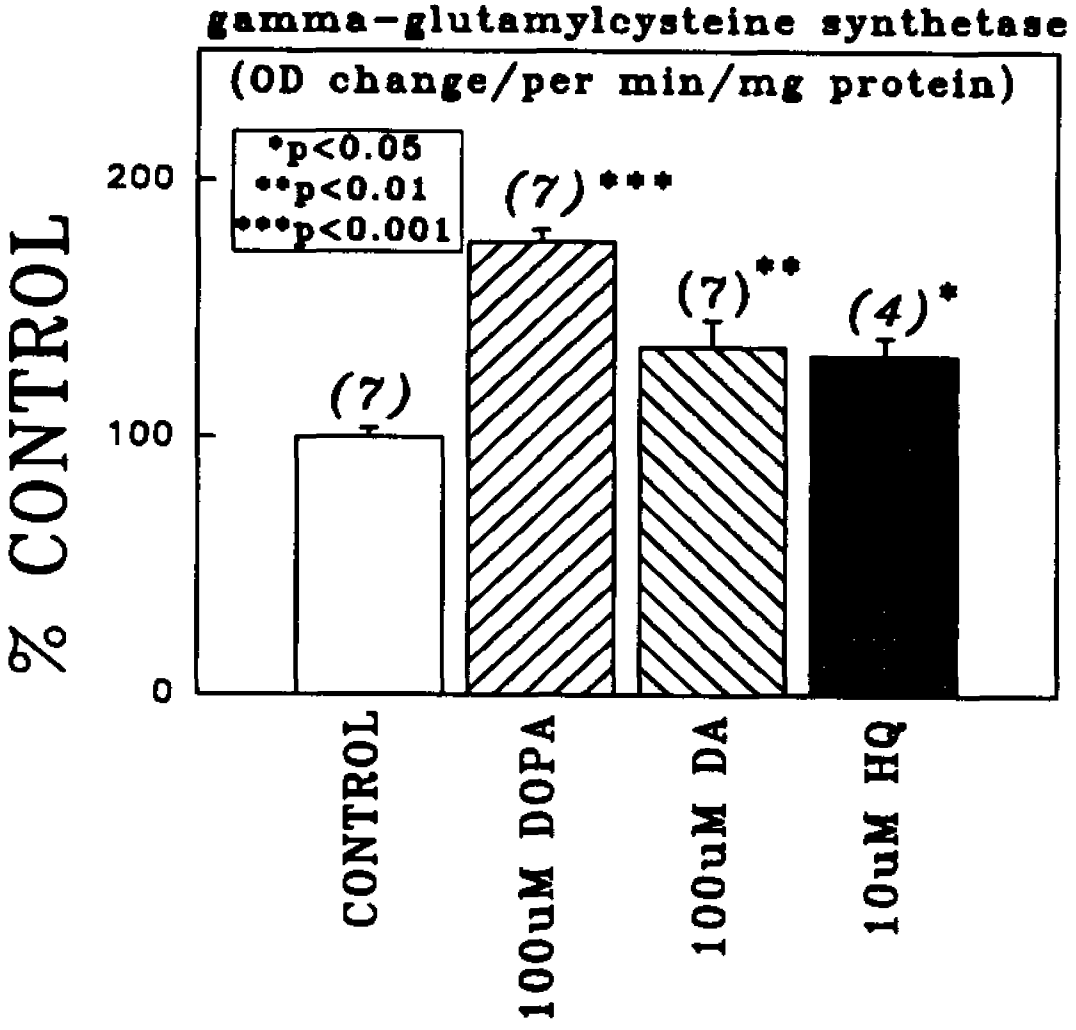

gamma-glutamylcysteine synthetase
(OD change/per min/mg protein)
*p<0.05
**p<0.01
***p<0.001
% CONTROL
200
100
0
(7)
(7) ***
(7) **
(4) *
CONTROL
100uM DOPA
100uM DA
10uM HQ

Fig. 13

n=4), respectively, compared to control values (Fig. 13).

C10. Catalase activity.

GSH is the required cofactor for GSH peroxidase, which removes or detoxifies peroxides. Catalase is another enzyme that metabolizes H_2O_2. Catalase activity in cell cultures exposed to L-DOPA, dopamine, and hydroquinone was also studied.

After 48 hr treatment of mesencephalic cell cultures with L-DOPA, dopamine, or hydroquinone, catalase activity was decreased (Fig. 14). The activity was reduced from 100.0 ± 3.6% (control) to 56.6 ± 3.4% of control by 100 μM L-DOPA ($p<0.001$), to 47.9 ± 2.9% of control by 100 μM DA ($p<0.001$), and to 79.2% ± 3.1% by 10 μM hydroquinone ($p<0.01$). Ascorbic acid blocked the effect of L-DOPA, bringing the catalase activity back to 90.8 ± 4.1 % of control (not significantly different from control). In contrast , ascorbic acid was ineffective in stopping the inhibitory effect of DA on catalase activity (52.9 ± 4.1% of control; $p<0.001$) in the same experiments. Monoamine oxidase (MAO) does not contribute to the effect of either L-DOPA or DA on catalase activity since treatment with the combination of 20 μM clorgyline (MAO-A inhibitor) and 20 μM pargyline (MAO-B inhibitor) resulted in catalase activity of 52.9 ± 3.7% of control (L-DOPA) and 47.6 ± 3.6% of control (dopamine), respectively, compared to 56.6% of control and 47.9% of control, respectively, for L-DOPA or dopamine alone. These results show that catalase activity is decreased by autoxidizable compounds.

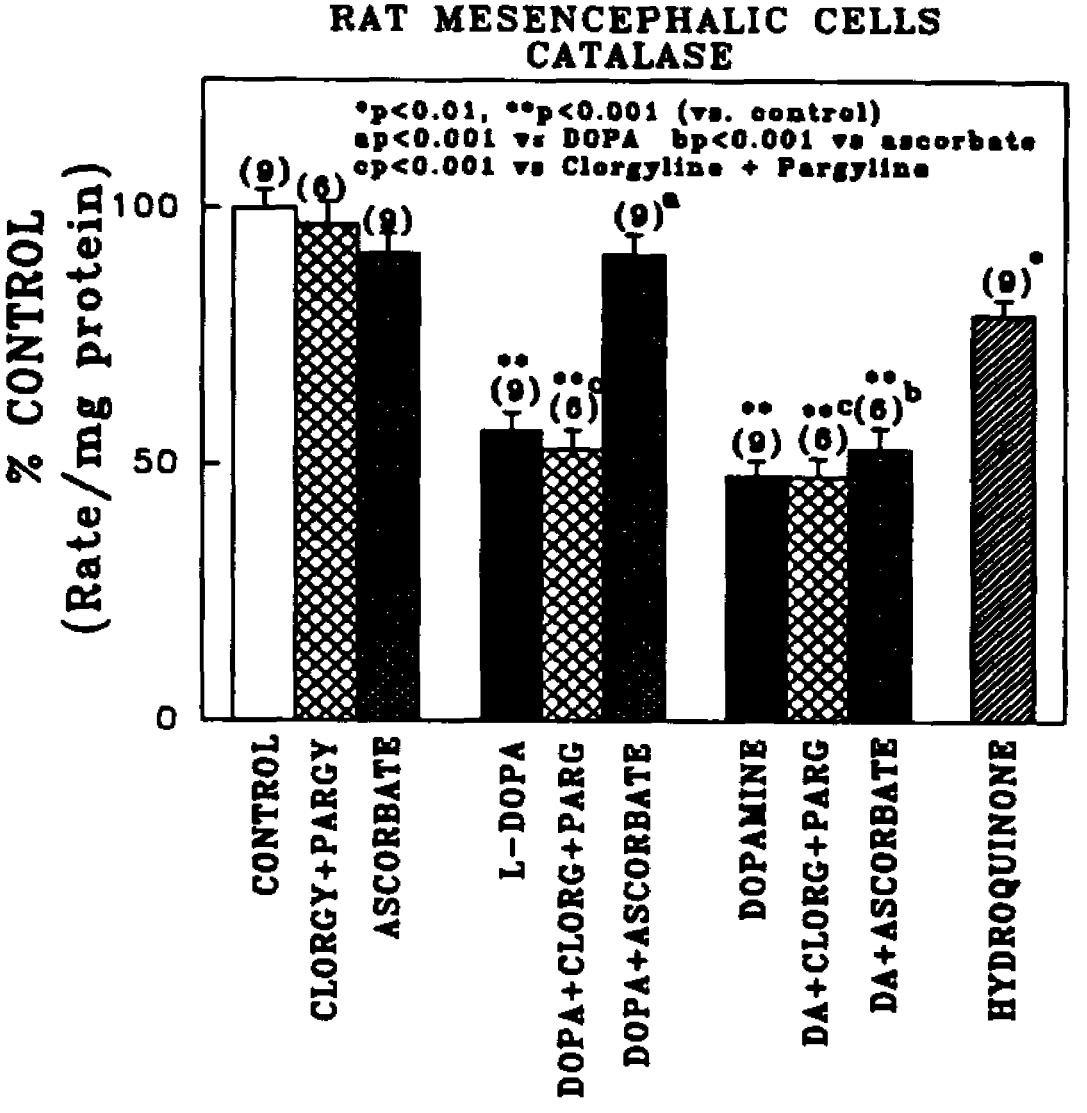

RAT MESENCEPHALIC CELLS
CATALASE
*p<0.01, **p<0.001 (vs. control)
ap<0.001 vs DOPA bp<0.001 vs ascorbate
cp<0.001 vs Clorgyline + Pargyline
% CONTROL
(Rate/mg protein)
100
50
0
CONTROL
CLORGY+PARGY
ASCORBATE
L-DOPA
DOPA+CLORG+PARG
DOPA+ASCORBATE
DOPAMINE
DA+CLORG+PARG
DA+ASCORBATE
HYDROQUINONE

Fig. 14

Part III: Biological importance of the rise in GSH

C11. Protection of cell cultures against oxidative stress.

It is generally assumed that elevated GSH can protect cells from an oxidant stress (Reed, 1990; Meister, 1991). Therefore, the effect of prior treatment with either L-DOPA (100 μM) or hydroquinone (10 μM) to elevate GSH on the subsequent viability of cell cultures was tested. Incubation with 400 μM t-butylhydroperoxide (t-BuOOH) was used as intense oxidant stress. It is known that organic peroxides such as t-BuOOH are normally detoxified by GSH peroxidase.

$$\text{t-BuOOH} + 2\text{GSH} \xrightarrow{\text{GSH peroxidase}} \text{GSSG} + \text{t-butyl alcohol} + H_2O \qquad \text{(Eqn. 6)}$$

After exposure to t-BuOOH, cell viability was assessed with the MTT assay. The results in Table 14 show that exposure of cell cultures to t-BuOOH for 120 min resulted in a significant decline in cell viability to 31.5% of control. Pretreatment with 100 μM L-DOPA did not by itself (no t-BuOOH) affect viability. However, cultures pretreated with L-DOPA and then exposed to t-BuOOH for 2 h showed 65.6 % viable cells, a doubling of the number of viable cells compared to cultures not pretreated with L-DOPA ($P < 0.001$).

Similar experiments were conducted with hydroquinone (Table 15). Again, exposure of cell cultures to t-BuOOH for 30-60 min resulted in progressive loss in cell viability to 68.1% of control at 30 min and 37.2% of control at 60 min. However, cells pretreated with hydroquinone were fully protected at 30 min ($p < 0.001$). At 60 min of exposure to t-BuOOH, partial protection was seen ($p < 0.001$). The results in Tables 14 and 15 show that prior treatment with either 100 μM L-DOPA or 10 μM

TABLE 14. Effect of pretreatment with L-DOPA (100 uM) on the viability of mesencephalic cultures exposed to t-butylhydroperoxide.

Pretreatment	Exposure Time (min)	Cell Viability	
		No t-BuOOH	400 μM t-BuOOH
		% Untreated Control ± SEM (N)	
None (Control)	120	100.0 ± 1.8 (36)	31.5 ± 1.9 (32)[a]
100 μM L-DOPA	120	100.1 ± 2.4 (35)	65.6 ± 3.3 (37)[a,b]

Mesencephalic cultures were treated with 100 μM L-DOPA for 48 h, rinsed, and then exposed to 400 μM t-BuOOH. Cell viability was assessed by the MTT assay. Results are expressed as a percent of the control cells not exposed to t-BuOOH. Results are pooled from 4 experiments. The absorbance values in the MTT assay for control samples (550 nm - 620 nm) was 0.702 ± 0.032 (N=36).

[a] $p < 0.001$ compared to corresponding cells not exposed to t-BuOOH.

[b] $p < 0.001$ compared to corresponding cells exposed to t-BuOOH, but not pretreated with L-DOPA.

TABLE 15. Effects of pretreatment with hydroquinone (10 uM) on the viability of mesencephalic cultures exposed to t-butylhydroperoxide.

Pretreatment	Exposure Time (min)	Cell Viability	
		No t-BuOOH	400 μM t-BuOOH
		% Untreated Control ± SEM (N)	
None (Control)	30	100.0 ± 1.9 (24)	68.1 ± 3.0 (20)[a]
10 μM hydroquinone	30	101.9 ± 2.0 (24)	101.6 ± 2.2 (21)[b]
None (Control)	60	100.0 ± 1.9 (24)	37.2 ± 4.7 (21)[a]
10 μM hydroquinone	60	101.9 ± 2.0 (24)	71.5 ± 3.8 (21)[a,b]

Mesencephalic cultures were treated with 10 μM hydroquinone for 48 h, rinsed, and then exposed to 400 μM t-BuOOH. Cell viability was assessed by the MTT assay. Results are expressed as a percent of the untreated control cells not exposed to t-BuOOH. Results are pooled from either 3 experiments. The absorbance values in the MTT assay for control samples (550 nm - 620 nm) was 0.871 ± 0.041 (N=24). In experiments, all samples were incubated for 60 min at 37°C and t-BuOOH was added either at zero time or at 30 min to yield exposure times of 60 or 30 min, respectively.

[a] $p < 0.001$ compared to corresponding cells not exposed to t-BuOOH.

[b] $p < 0.001$ compared to corresponding cells exposed to t-BuOOH, but not pretreated with hydroquinone.

hydroquinone provided substantial protection against cell damage by t-BuOOH.

C12. Ascorbate reverses the protection by L-DOPA.

As noted above, addition of ascorbate to the cell culture medium prevented the rise in GSH elicited by L-DOPA (Table 4). In additional experiments, cells were treated with L-DOPA both with and without ascorbic acid. Thus, two sets of cells were set up, both exposed to L-DOPA, but only one set contained elevated GSH. Then the viability of both sets of cells was compared during exposure to 400 μM t-BuOOH. The results in Table 16 show that pretreatment with L-DOPA or ascorbate, or a combination of the two, did not significantly affect cell viability (no t-BuOOH). When cells were subsequently exposed to 400 μM t-BuOOH, the loss in viability was only 25.9% in L-DOPA-treated cells, compared to 52.7% in control cells (a protection of better than 50%), in agreement with results shown in Table 16. However, when cells were pretreated with L-DOPA in the presence of ascorbate, the protective effect of L-DOPA was lost: These cells were no different in their response to t-BuOOH were than control cells (54.4% vs. 52.7% loss in viability). Moreover, cells pretreated with both ascorbate and L-DOPA and then exposed to t-BuOOH showed a small, but significantly greater loss in viability compared to cells pretreated with ascorbate alone. These experiments show that the protection from t-BuOOH correlates with elevated level of GSH. Exposure to L-DOPA is not protective unless the level of GSH rises.

TABLE 16. Protective effect of L-DOPA on mesencephalic cultures: Reversal by ascorbic acid.

Pretreatment	Cell Viability		Loss of viability
	No t-BuOOH	+ t-BuOOH	evoked by t-BuOOH
	% Untreated Control ± SEM		%
None (Control)	100.0 ± 1.5	47.3 ± 2.3[a]	52.7
100 μM L-DOPA	98.7 ± 1.2	73.1 ± 2.4[a,b]	25.9
200 μM Ascorbate	96.9 ± 1.7	54.6 ± 4.0[a]	43.7
L-DOPA + Ascorbate	95.1 ± 1.2	43.4 ± 1.9[a,c,d]	54.4

Mesencephalic cultures were treated as indicated for 48 h, rinsed, and then exposed to 400 μM t-BuOOH for 60 min. Cell viability was assessed by the MTT assay. Results are pooled from 3 experiments (N = 17-18 per group). The % loss in viability evoked by t-BuOOH is defined as: 100% x (MTT assay without t-BuOOH - MTT assay after t-BuOOH)/MTT assay without t-BuOOH.

[a] $p < 0.001$ compared to either untreated control or to cells after pretreatment with L-DOPA, ascorbate, or both (no t-BuOOH).

[b] $p < 0.001$ compared to control cells treated with t-BuOOH.

[c] $p < 0.001$ compared to L-DOPA alone with t-BuOOH.

[d] $p = 0.002$ compared to ascorbate alone with t-BuOOH.

D. DISCUSSION

D1. The rise in GSH

The main observation in this thesis is that exposure of cultures of embryonic rat mesencephalon to L-DOPA causes the level of cellular GSH to rise. The rise in GSH induced by L-DOPA is not unique for rat fetal mesencephalic cell cultures, nor for L-DOPA alone. Upregulation was seen with other cell types, such as Neuro-2A neuroblastoma and LLC-PK_1 porcine kidney epithelium cell lines, as well as primary cultures of glia (Table 3) and with other diphenolic compounds, such as dopamine, apomorphine, catechol and hydroquinone (Fig. 4).

This upregulation of GSH content was a surprise. Autoxidation was expected to lower GSH content. Scavenging of peroxides by GSH peroxidase, and reaction with oxy-radicals should lead to oxidation of GSH to GSSG (Eqn. 1). As a result, a portion of the GSH may be transformed via the reaction of GSSG with protein sulfhydryls to form mixed-disulfides (Eqn. 5) (Shivakumar and Ravindranath, 1993; Di Simplicio and Rossi, 1994; Werner and Cohen, unpublished observations). In addition, autoxidation of L-DOPA produces quinones, which remove glutathione irreversibly (Eqn. 7) by forming glutathionyl adducts (Ito, 1993). These reactions would be expected to lead to loss of GSH. Instead, a rise was observed.

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH peroxidase}} GSSG + 2\,H_2O \qquad \text{(Eqn. 1)}$$

$$\text{Protein-SH} + GSSG \longrightarrow \text{Protein-SSG} + GSH \qquad \text{(Eqn. 5)}$$

$$\text{L-DOPA-quinone} + GSH \longrightarrow \text{5-S-glutathionyl-DOPA} \qquad \text{(Eqn. 7)}$$

In an initial publication (Mytilineou, Han, and Cohen, 1993), the magnitude of the effect on cellular GSH in mesencephalic cultures was underestimated. It turned out that removal of L-DOPA after 48 hrs more than tripled the rise in GSH (73.6% vs. 19.7%; Table 7). Similar effects have been reported by other investigators who worked in different systems. For example, Shi et al., 1994 reported a doubling in GSH content when endothelial cells in culture were exposed to menadione (a redox-cycling quinone), followed by a washed out.

Other investigators also reported upregulation of GSH levels in response to an oxidant stress in other mammalian systems: GSH rises 2-3 fold in rat peritoneal macrophages exposed to glucose/glucose oxidase (an H_2O_2-generating system) (Bannai et al., 1991). Similar changes occur in intact kidney after chronic exposure to mercury in the drinking water (Woods et al. 1992). In a P-glycoprotein-negative (no overexpression of the MDR-1 [multidrug resistance] gene) cell line, GLC_4-Adr_{90}, a 75-fold acquired adriamycin resistance is accompanied by increased GSH (Meijer et al., 1991).

A rise in GSH in pure neurons in culture was not observed. However, a substantial amount of GSH in the neuronal cultures was observed (Tables 8 & 9). The levels of GSH in neuronal cells in culture have been the source of some controversy. A report by Raps et al. (1989) described an absence of GSH from pure neurons maintained in culture for 14 days (< 1.0 nmole/mg protein). We observed a mean level of 5.8 ± 0.2 nmole/mg protein in cultured neurons (legend to Table 9). The mean levels in undifferentiated astrocytes reported by Raps et al. (16.0 ± 5.0 nmole/mg) were similar to our values with cultured glia (17.9 ± 2.8 nmole/mg). Additional reports indicate measurable levels of GSH in chick neurons (13.4 ± 2.3 nmole/mg, Makar et al., 1994), as well as in neuronal cultures derived from mouse

brain (32.0 ± 9.0 nmole/mg, embryonic cerebral cortex, Bridges et al., 1991) and rat brain (21.1 ± 1.6 nmole/mg, embryonic cerebral cortex, Pileblad et al., 1991). Some of the previous investigators (Bridges et al., 1991; Pileblad et al., 1991) added serum to their growth medium for several days, permitting proliferation of glia before utilizing an inhibitor of glial division. It may be that the earlier glial proliferation added to the observed GSH levels. On the other hand, the GSH content of pure neurons in culture declines with age, while astrocytes maintain their GSH (Sagara et al., 1993b). The latter authors separated neurons and glia from mixed cultures and showed high levels in both the neuronal and glial populations (18.8 ± 4.2 and 28.0 ± 4.4 nmol/mg protein, respectively) at 9 days; the low levels observed by Raps et al. (1989) may reflect the age of their cultures. However, in vivo, an additional complexity is present. Histochemical analyses on fresh frozen tissue have shown that brain GSH in both the monkey and the rat is virtually absent from the cytosol of neuronal cell bodies, but appears to be localized to axons, nerve terminal regions, and glia (Slivka et al., 1987; Philbert et al., 1991).

Another role for glia in brain is protection from ammonia toxicity. Ammonia and H_2O_2 are both generated in the metabolism of L-DOPA and dopamine (Eqn. 8). In brain, only astrocytes contain the enzyme glutamine synthetase, which has an important role in ammonia detoxification.

$$\text{dopamine} + O_2 \xrightarrow{\text{monoamine oxidase}} H_2O_2 + NH_3 + \text{3,4-dihydroxyphenylacetaldehyde} \quad \text{(Eqn. 8)}$$

D2. Response of pure neurons to L-DOPA

As previously mentioned, pure neuronal cultures (absence of glia) failed to

increase their levels of GSH upon exposure to L-DOPA (Tables 8 and 9). At the same time, toxicity was evident from the release of LDH into the medium and from cellular damage and destruction, viewed by phase contrast microscopy. In this regard, pure neuronal cultures were very sensitive to the toxic effects of L-DOPA. Thus, although a mild oxidative stress derived from autoxidation reactions of L-DOPA is tolerated by primary cultures of rat fetal mesencephalon, it is not well tolerated by pure neuronal cultures from the same brain region. To diminish direct neural toxicity, the concentration of L-DOPA was decreased progressively. But there was no evidence for an upswing in GSH in the neuronal cultures (Table 5). It appears that neuronal cell cultures do not respond in the same way as mixed cultures, which are comprised of both neuronal and glial cells. However, pure neuronal cultures were protected from the toxic effects of L-DOPA (LDH release) by ascorbate and SOD (Table 6). Protection by ascorbate has been reported previously for neuroblastoma cells and mesencephalic cell cultures (Mena et al., 1992 and 1993). Protection is associated with suppression of melanization. Superoxide can participate in toxic events in several ways. A well-known effect of SOD is to suppress the superoxide-mediated catalysis of the oxidation of catechol compounds (e.g., Misra and Fridovich, 1972; Heikkila and Cohen, 1973); similar effects are seen with catechol amino acids (alpha-methyl-DOPA; Dybing et al., 1976). By suppressing oxidation reactions, SOD suppresses melanization. SOD does not, however, completely prevent melanization. The partial protection by SOD may be related to partial suppression of melanization.

The toxicity of L-DOPA to pure neuronal cultures also parallels a toxicity that appears to be more selectively targeted to tyrosine-hydroxylase positive neurons (i.e., DA neurons) in mesencephalic cell cultures. Mytilineou et al. (1993) reported that when mesencephalic cell culture incubated with L-DOPA (200 μM) for 48 hr, the number of tyrosine hydroxylase (TH^+)-positive neurons (DA neurons) was reduced to

69.7% of control values, accompanied by a decrease in [^{3}H]DA uptake to 42.3% of control values. The remaining DA neurons showed significantly reduced neurite length and overall deterioration. Lack of simultaneous change in the number of neurons stained with neuron-specific enolase indicated that toxicity was relatively specific for DA neurons. Other investigators have also observed toxicity directed at DA neurons in cell culture (Olney et al., 1990; Steece-Collier et al., 1990; Mena et al., 1993).

There is still one important question that needs to be asked: Why don't pure neuronal cultures upregulate their GSH content? It is known that the normal precursor for GSH in cell culture is cystine (cysteine disulfide), which is added as a component of the growth medium. Addition of cystine, rather than cysteine, mimics the condition present in extracellular fluids, such as cerebrospinal fluid, where the disulfide form predominates for both cysteine and glutathione. Recently, Sagara et al. (1993a and b) described a transport system present in both neurons and glia that takes up cysteine; cystine transport, by way of comparison, is much more prominent in glia. Following the reduction of cystine to cysteine within the glia, a portion is secreted into the medium, where it can be taken up by the neurons. In this way, glia will support the biosynthesis of GSH by neurons in cell culture (Sagara et al., 1993b). The apparent failure of pure cultured neurons to upregulate GSH in our experiments may be due, in part, to the absence of glia. Similarly, support of GSH synthesis may contribute to the long-range survival of neurons grown in the presence of glia (e.g., Schmalenbach and Muller, 1993).

D3. Mechanisms for the rise of GSH.

D3.1 *MAO, DOPA-decarboxylase, catalase, SOD*

A number of factors that could participate in the biological signaling mechanism were investigated. First, L-DOPA can be metabolized to dopamine and dopamine will be metabolized by monoamine oxidase (MAO). Therefore, the role of MAO activity was tested.

$$\text{L-DOPA} \xrightarrow{\text{DOPA decarboxylase}} \text{dopamine} \qquad \text{(Eqn. 4)}$$

$$\text{dopamine} + O_2 \xrightarrow{\text{monoamine oxidase}} H_2O_2 + NH_3 + \text{3,4-dihydroxyphenylacetaldehyde} \qquad \text{(Eqn. 8)}$$

Pargyline, an inhibitor of monoamine oxidase (MAO), had no effect on the rise in GSH content (Table 4). Therefore, the products formed intracellularly by MAO, which include H_2O_2, 3,4-dihydroxyphenylacetaldehyde, and ammonia, were not involved. In addition, DOPA-decarboxylase inhibitors did not prevent the rise in GSH (Table 1A and 1B). Therefore, formation of DA from L-DOPA is not necessary. However, an interesting finding was that decarboxylase inhibitors increased the effect of L-DOPA. The reason for this is not clear, but perhaps storage of dopamine in vesicles decreases the availability of dopamine for autoxidation.

SOD and catalase, which remove superoxide and H_2O_2 formed in the incubation medium during the autoxidation of L-DOPA, had no effect (Table 4). This does not mean that superoxide and H_2O_2 have no role. SOD and catalase do not penetrate into cells and therefore the experiment shows only that superoxide and H_2O_2 formed in the

medium have no effect. Intracellular superoxide and H_2O_2 are not eliminated by these experiments.

D3.2 *Ascorbate*

The elevation in GSH content was fully blocked by addition of 200 μM ascorbic acid (Table 4). Since ascorbic acid is an antioxidant, the data in Table 4 signify the presence of an oxidative stress. Autoxidation produces oxidizing species, such as H_2O_2, superoxide, hydroxyl radicals, semiquinones, and quinones (Figure 13). Ascorbic acid can suppress or reverse the autoxidation of L-DOPA.

D3.3 *GSSG*

Additional evidence for an oxidative stress is in the levels of glutathione disulfide (GSSG). GSSG is formed by the reaction of GSH with H_2O_2 catalyzed by GSH peroxidase (Eqn. 1); GSSG can also be formed after oxidation of GSH by other oxidizing species. Normally, GSSG levels are low (less than 10 % of the total glutathione) due to the activity of GSSG reductase. However, it is recognized that when GSSG levels increase, this indicates the presence of an oxidative stress (Spina and Cohen, 1988 & 1989; Werner and Cohen, 1993). The average concentration of GSH in brain is 1-2 mM (Folbergrova et al., 1979; Cooper et al., 1980; Slivka et al., 1987b). The average concentration of GSSG is 0.002-0.008 mM (Cooper et al., 1980; Slivka et al., 1987b). When GSSG levels reach 0.05-0.20 mM, this is generally associated with toxic signs (Gilbert, 1982). However, at lower levels, the elevated GSSG can act as a biological signal (Gilbert, 1982). One way this can happen is through the formation of protein mixed-disulfides (Eqn. 5), which can affect the biochemical properties of sulfhydryl-dependent proteins.

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH peroxidase}} GSSG + 2\,H_2O \qquad \text{(Eqn. 1)}$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} 2\,GSH + NADP^+ \qquad \text{(Eqn. 3)}$$

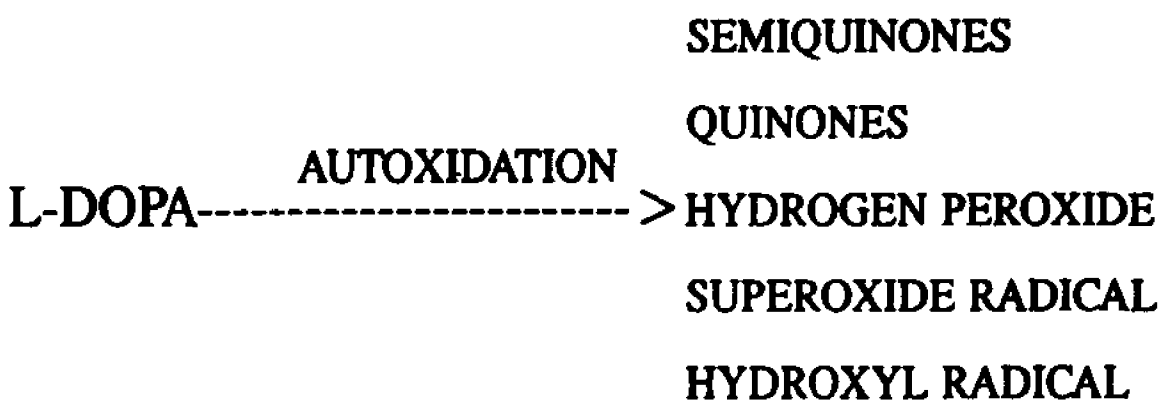
$$\text{Protein-SH} + GSSG \longrightarrow \text{Protein-SSG} + GSH \qquad \text{(Eqn. 5)}$$

It is interesting that in the present studies, the level of GSSG rose before the GSH became elevated (Table 2). This means that GSSG might be a biologic signal. To test the possibility that disulfides could be part of a signaling mechanism that causes GSH elevation, experiments were performed with two disulfides, GSSG and oxidized DTT. Both of them caused an elevation in cellular GSH. Although GSSG might be directly reduced to GSH within cells (Eqn. 3), oxidized DTT cannot lead directly to GSH. Therefore, these results are consistent with an oxidative stress mechanism and the involvement of GSSG.

D3.4 *Working hypothesis and structure-activity relationships.*

An oxidative signal for upregulation of GSH by L-DOPA was implicated by three observations. First, a change in cellular redox state was shown by a rise in cellular glutathione disulfide (GSSG), which preceded the rise in GSH. Second, an antioxidant (ascorbate) prevented the rise in GSH. Third, melanization of the cultures (oxidation and polymerization of L-DOPA) was observed visually and this could be blocked by ascorbate, simultaneous with prevention of the rise in GSH. It seemed that the relative ease of oxidation of the catechol structure of L-DOPA by molecular oxygen, with the formation of transient free radical species (semiquinones, hydroxyl radical, superoxide radical) and longer-lived oxidants (quinones and H_2O_2) (Fig. 15), could play a role. So, the working hypothesis was that autoxidation was required. To explore this

Fig. 15


SEMIQUINONES
QUINONES
AUTOXIDATION
L-DOPA
HYDROGEN PEROXIDE
SUPEROXIDE RADICAL
HYDROXYL RADICAL

possibility, a detailed study of structure-activity relationships was performed.

A number of amino acid structural analogs that do not undergo autoxidation, namely, 3-O-methyl-DOPA, 2,4-DOPA and tyrosine proved to be inactive, while alpha-methyl-DOPA, which retains both the catechol structure and the property of reacting with oxygen, did elevate cellular GSH levels (Fig. 1 and Fig. 2). In addition, dopamine and apomorphine, which are autoxidizable catechols, were active (Fig. 3). The effect of dopamine on cellular GSH, like that of L-DOPA, was blocked by ascorbate (results not shown), again implying a need for autoxidation of the parent compound. Finally, removal of the entire side-chain from L-DOPA/dopamine, as in 1,2-dihydroxybenzene (catechol), did not lessen the effect on cellular GSH (Fig. 4). However, the catechol structure per se was not an absolute requirement since hydroquinone also elevated GSH; hydroquinone, like catechol, reacts with oxygen to generate oxidant species. Lastly, resorcinol, which does not undergo autoxidation, was inactive in these experiments; resorcinol is the structure resulting from removal of the side chain of 2,4-DOPA. Therefore, it can be concluded that ease of reaction with oxygen is a requirement for the observed effects.

D3.5 *Dopamine receptor activation.*

From the structure-relationship study, it was found that the elevation of GSH can be induced by compounds that undergo autoxidation, However, the list of active compounds also includes DA and apomorphine, which are DA receptor agonists. Therefore, it was possible that activation of DA receptors could play a role. The receptor hypothesis was easily tested by studies with DA-receptor antagonists. It was found that D1 and D2 receptor antagonists such as SCH23390 (D1), spiperone (D2), and sulpiride (D2), had no blocking effects on the elevation of GSH by dopamine (see

Fig. 5 and Fig. 6). These data indicated that activation of DA receptors is not involved.

The R- and S- forms of apomorphine were also compared. Both contain the catechol structure that undergoes autoxidation. R-apomorphine is a well known DA-receptor agonist. However, S-apomorphine is inactive at dopamine receptors in the striatum, and is actually a DA-receptor antagonist in mesolimbic regions (Campbell et al., 1985). Since both R- and S-apomorphine were equally active in elevating the GSH level (Fig. 8), the data indicate that DA-receptor function is not required.

A possible exception is quinpirole, which is a D-2 and D-3 agonist. The structure of quinpirole does not contain an autoxidizable portion and yet quinpirole was active in elevating GSH (Fig. 12). Quinpirole was also used in later studies with inhibitors of protein kinase-C (see ahead).

Dopamine D-1 and D-2 receptors are linked to adenylate cyclase. D-1 activation increases adenylate cyclase activity, while D-2 activation suppresses it. We performed experiments with Sp-cAMP, which is a potent and specific membrane-permeable activator of cAMP-dependent protein kinases I and II. This compound bypasses receptor binding and directly activates the protein kinases. Sp-cAMP (as well as Rp-cAMP, an inhibitor of cAMP-dependent protein kinases I and II) failed to change the level of GSH (Table 9). Additional experiments were performed with forskolin, which is an activator of adenylate cyclase. Forskolin also failed to elevate GSH (Fig. 7). These results are in keeping with the observation that DA receptor antagonists did not alter the response of cell cultures to dopamine. Autoxidation seems to be the mechanism for GSH elevation.

Moullet et al. (1994) showed that p-quinone can inhibit adenylate cyclase in

human hepatoma cells (HepG2). They found that the quinone bound tightly to one major and two minor proteins in the plasma membrane. The inhibition was not affected by GTP, GDP or analogues, or by cholera and pertussis toxins. Quinone-binding and inhibition of adenylate cyclase was enhanced by membrane solubilization. They concluded that inhibition of adenylate cyclase by quinone was not mediated by a G-protein or by the activation of a defined receptor. The oxidized state of the quinone was required for inhibition; reduction of quinone (to hydroquinone) with sodium dithionite prevented the inhibition of adenylate cyclase, while re-oxidation with ferricyanide restored the inhibition. Finally, they found that inhibition by p-quinone competed with forskolin activation of adenylate cyclase: Addition of forskolin prevented inhibition by p-quinone.

We studied the effect of forskolin on the elevation of GSH by hydroquinone (Fig. 7). In three experiments, upregulation of GSH by 10 μM hydroquinone was completely blocked (returned to basal level) by 50 μM forskolin. As a control in these experiments, 1,9-dideoxy-forskolin was used. The latter compound does not stimulate adenylate cyclase and can be used as a negative control. 1,9-Dideoxy-forskolin had no effect on the elevation of GSH by hydroquinone in the mesencephalic cell cultures. These results are consistent with the observations of Moullet et al. (1994). The experiments appear to indicate that cAMP formation can block the upregulation of GSH by hydroquinone.

D3.6 *Protein kinase C.*

It was known that redox cycling compounds (such as menadione) activate protein kinase C (PKC; Kass et al., 1989). In addition, it is known that C6 glioma cells, which are characterized by high GSH levels, and which do not respond further to stimulation

of L-DOPA, also exhibit high levels of protein kinase C. Therefore, PKC inhibitors were tested for their effect on GSH levels in cell cultures exposed to redox cycling compounds, such as L-DOPA .

Unlike the dopamine receptor antagonists, the PKC inhibitors (staurosporine and H7) proved to be powerful blockers of GSH elevation. These observation agree with those of Kass et al. (1989) that the redox-cycling quinones can activate PKC. In their experiments, activation of PKC was blocked by addition of large (mM) amounts of ß-mercaptoethanol or DTT. This observation may be similar to the present finding that ascorbate (200 μM) also blocked up-regulation of GSH in cell cultures. In our experiments, activation of PKC appears to play an important role. It is of interest that even quinpirole, which does not autoxidize, appears to require PKC, since both PKC inhibitors (staurosporine and H-7) blocked the effect of quinpirole (Fig. 12). This observation reinforces the idea that PKC has a central role in regulating cellular GSH.

To sum up the findings: PKC activity is required for upregulation of GSH, but DA receptors are not involved.

D3.7 *Catalase activity.*

Although GSH levels rose on exposure to autoxidizing compounds, catalase activity declined. Perez-Polo et al. (1994) previously showed that nerve growth factor upregulated catalase mRNA and enzyme activity in PC-12 cells. The present study showed that L-DOPA and dopamine downregulated catalase activity (Fig. 14). Whereas ascorbate blocked the rise in GSH by L-DOPA or dopamine, it prevented only the effect of L-DOPA in decreasing catalase activity (Fig. 14).

Cohen and Hochstein (1963) have made a detailed exploration on the mechanism of catalase inhibition in erythrocytes. They showed that p-quinone and hydroquinone, a representative redox pair, can react with oxygen to produce H_2O_2 and inhibit catalase activity. The mechanism involves reaction of H_2O_2 with catalase (Fe^{3+} enzyme) to form compound I (Fe^{5+}) , followed by its transformation to compound II (Fe^{4+}), which is an inactive form of the enzyme. Resorcinol, which does not autoxidize, was used as a negative control, and was inactive as a catalase inhibitor.

Several authors have suggested that L-DOPA or dopamine is toxic to cells in culture via autoxidation and the generation of reactive oxygen (Mena et al., 1994, mesencephalic neurons; Basma et al., 1995, PC-12 cells). In our experimental system, consisting of mesencephalic cell cultures, L-DOPA was toxic specifically to the DA neurons (Mytilineou et al., 1993) while, concurrently, the level of GSH was upregulated. It is interesting that L-DOPA can simultaneously and reciprocally modulate an antioxidant like GSH and an antioxidant enzyme, catalase. Ascorbic acid, a well known antioxidant, prevented both the rise in GSH and the fall in catalase activity evoked by L-DOPA. On the other hand, ascorbic acid did not prevent the inhibition of catalase activity by dopamine.

D3.8 *Gamma-glutamylcysteine synthetase (gamma-GCS).*

Gamma-glutamylcysteine synthetase catalyzes the first and rate-limiting step of glutathione synthesis. Ohno et al.,(1990) reported that prostaglandin A_2 (PGA_2) induced marked elevation of cellular GSH in L-1210 cells in culture. This elevation was associated with an increase in the activity of gamma-GCS, but no change in glutathione synthetase activity. It was inhibited by L-BSO, an inhibitor of gamma-GCS, and also by actinomycin D, an inhibitor of transcription. Woods et al. (1992)

showed that prolonged administration of methyl mercury to rats causes a substantial increase in renal GSH associated with increased abundance of gamma-GCS mRNA in kidney cortex. Ogino et al., (1989) reported stimulation of GSH synthesis in iron-loaded mice. They used the ^{35}S-cysteine uptake method to study hepatic cysteine turnover. They found higher hepatic cysteine levels in the iron-loaded mice with no differences in cysteine turnover between the iron-loaded mice and control mice. They suggested that increasing gamma-GCS activity contributes to the elevation of GSH. Shi et al. (1994) also reported that both gamma-GCS and glutathione increased in bovine pulmonary artery endothelial cells after incubation with quinones.

In the present studies , gamma-GCS activity was measured by the modification method of Fernandez-Checa & Kaplowitz (1990). After 48 hr exposure of mesencephalic cell cultures to L-DOPA, DA or hydroquinone (Fig. 13), the GSH content was elevated simultaneously with increased gamma-GCS activity, as would be expected from the observations of Shi et al., (1994).

4. Biological significance of oxidative stress in mesencephalic cell cultures.

D4.1 *Elevated GSH protects cells from oxidative stress challenge.*

It is known that upregulation of antioxidant mechanisms is associated with protection from oxidative damage in both eukaryote and prokaryote cells. Several examples are: (1) A rise in the manganese form of SOD induced by exposure to a low concentration of tumor necrosis factor (10 ng/mL), which protects human embryonic kidney cells against subsequent exposure to a lethal concentration (100 ng/mL) (Wong et al. 1989); (2) a rise in copper-zinc SOD induced in bacteria by exposure to paraquat,

which protects against further paraquat toxicity (Hassan and Fridovich, 1977); (3) a rebound in GSH levels, which is associated with a sparing of renal cells from oxidative damage during chronic exposure to methyl mercury (Woods et al., 1992). The present study demonstrates that the same is true for neural tissue in the form of mesencephalic cell cultures.

On the other hand, many reports also have been concerned with the GSH-lowering properties and accompanying cellular damage by agents that undergo vigorous redox cycling, such as menadione, adriamycin, and hydroquinone (Meredith and Reed, 1983; Bellomo, 1990; Ohno et al., 1991; Li et al., 1994). These experiments produce an oxidative stress, which is as a danger to cells. Indeed, in our experiments, 100 μM hydroquinone proved lethal to the mesencephalic cultures. It was necessary to diminish the oxidative pressure by lowering the concentration to 10 μM in order to assess effects on GSH in viable cells.

The current results provide an example of a compensatory response to an oxidative stress that results in a longer-lasting protection for neural tissue. Thus, cultures previously exposed to the "mild" oxidative pressure caused by the autoxidation of 100 μM L-DOPA or 10 μM hydroquinone have elevated levels of GSH and are protected from loss in cell viability when incubated with t-butylhydroperoxide (Tables 14 and 15).

It is known that t-butylhydroperoxide is removed or detoxified by GSH peroxidase (Eqn. 6). Therefore, increased protection is expected when the levels of GSH rise. Upregulation of GSH provides an antioxidant buffer for peroxides. The observed protection of mesencephalic cultures correlates with increased levels of the co-substrate (GSH) for GSH peroxidase.

$$\text{t-BuOOH} + 2\text{GSH} \xrightarrow{\text{GSH peroxidase}} \text{GSSG} + \text{t-butyl alcohol} + H_2O \qquad \text{(Eqn. 6)}$$

A second enzyme that removes peroxides from cells is catalase. However, catalase activity did not rise. In fact, catalase levels fell during exposure to autoxidizable agents (Fig. 14). Also, catalase removes H_2O_2, but does not detoxify organic peroxides, such as t-butylhydroperoxide.

In experiments with PC-12 pheochromocytoma cells in culture, Pan and Perez-Polo (1993) studied antioxidant mechanisms that were altered by trophic factors. They found increases in GSH, GSH peroxidase, G-6-P dehydrogenase, and catalase (Cf. Sampath et al., 1994) when cells were incubated with nerve growth factor (NGF) or epidermal growth factor (EGF), but not other neurotrophic factors (brain-derived neurotrophic factor, insulin-like growth factor, basic fibroblast growth factor, or neurotrophin-3). NGF also protected the cells from damage when they were incubated with H_2O_2. Studies of time courses for the changes in antioxidant mechanisms showed that protection correlated with an early rise in the level of GSH, accompanied by increased gamma-GCS activity.

D4.2 *Ascorbate reverses the protection by L-DOPA* .

Ascorbate was found to prevent the rise in GSH when cells were incubated with L-DOPA (Table 4). Therefore, it was expected that ascorbate would interfere with the protective effect of L-DOPA on t-butylhydroperoxide toxicity. This expectation was confirmed by the observation that ascorbate neutralized the protective effect of L-DOPA (Table 16). In fact, cells treated with both L-DOPA and ascorbate were slightly, but significantly ($p=0.002$, Table 16) more damaged by t-butylhydroperoxide than cells pretreated with ascorbate alone (54.4% loss in viability for cells pretreated

with both ascorbate and L-DOPA vs. 43.7% loss for cells pretreated with ascorbate alone; Table 16). These results show that protection requires more than simply exposing cells to L-DOPA. Since ascorbate slows the autoxidation rate as monitored by the consumption of oxygen with an oxygen electrode (Pileblad et al., 1988) and also reverses the autoxidation by chemically reducing quinones and semiquinones, these experimental results appear to be related to the redox properties of L-DOPA. This is also in accord with the concept that autoxidation and cellular oxidation reactions are needed to obtain the rise in GSH.

D4.3 *Concluding Remarks.*

The current results show that an oxidative stress evokes a compensatory response of upregulating cellular GSH in several cell types, including fetal rat mesencephalon. It is concluded that L-DOPA can cause oxidative damage and also upregulation of critical antioxidant defenses (gamma-GCS and GSH). Whether or not damage occurs may depend on how strong the oxidative stress is and the threshold for damage in the nervous system. The threshold may be raised by upregulation of antioxidant defense. However, the upregulation mechanisms may be damaged if the stress is too strong. This occurred in our experiments with a high vs. low dose of hydroquinone, where the high dose (100 μM) killed the cells directly, while the low dose (10 μM) elevated GSH and protected the cells from t-butylhydroperoxide.

These experiments raise an interesting new question for future work. Does progressive damage to neurons in a neurodegenerative disease, such as Parkinson's disease, result from a failure to upregulate protective antioxidant mechanisms? Oxidant stress as an underlying factor in Parkinson's disease is strongly suspected (Fahn and Cohen, 1992). Evidence for oxidative stress is also available in Alzheimer's disease

and amyotrophic lateral sclerosis [ALS]) (reviewed by Cohen and Werner, 1993). In our experiments, upregulation of GSH provided significant protection against damage by an organic hydroperoxide. This may represent a model for protection against lipid peroxides. However, it is possible that upregulation mechanisms during an oxidative stress may fail due to genetic or environmental reasons. This would allow neuronal damage to occur.

FIGURE LEGENDS:

FIGURE 1: Effect of DOPA decarboxylase inhibitors (50 μM) on the rise in glutathione evoked by L-DOPA (200 μM x 48 hr) in cultures of Neuro-2A cells (Fig. 1A) and in cultures of LLC-PK_1 cells (Fig. 1B). The decarboxylase inhibitors and L-DOPA were added simultaneously. Numbers in parentheses are the number of cell cultures analyzed (n) in 2-5 experiments.

*Significantly different from control ($p < 0.001$)

**Significantly different from L-DOPA alone ($p < 0.001$)

FIGURE 2: Structure-activity relationships for the effect of L-DOPA (200 μM x 48 h) analogs on cellular glutathione in cultures of Neuro-2A cells (Fig. 2A) and LLC-PK_1 cells (Fig 2B). Numbers in parentheses are the number of cell cultures analyzed (n) in 2-5 experiments.

*Significantly different from control ($p < 0.001$)

FIGURE 3: Structures of the compounds studied. Autoxidizable compounds contain phenolic hydroxyl groups that are immediately adjacent (L-DOPA, alpha-methyl-DOPA, dopamine, apomorphine, catechol) or are para to one another (hydroquinone). Inactive compounds contain either 2 phenolic hydroxyls that are meta to one another (2,4-DOPA, resorcinol), or only one free phenolic hydroxyl group (tyrosine, 3-O-methyl-DOPA).

FIGURE 4: Structure-activity relationships for the effects of various phenolic compounds (10 and 100 μM) on cellular glutathione in mesencephalic cultures. CTRL (control), DA (dopamine), APO (apomorphine), CAT (catechol), HYD (hydroquinone), RES (resorcinol).

*Significantly different from control ($p < 0.001$)

FIGURE 5: Effect of dopamine and dopamine receptor antagonists (SCH-23390, sulperide, & spiperone) on GSH levels in mesencephalic cell cultures. Numbers in parentheses are the number of cell cultures analyzed (n) in 2-5 experiments.

FIGURE 6: Effect of high concentrations of DA receptor antagonists on GSH levels in mesencephalic cell cultures. Numbers in parentheses are the number of cell cultures analyzed (n) in 3 experiments.

FIGURE 7: Effect of 50 μM forskolin and 50 μM 1,9-dideoxyforskolin (1,9-D,D-forskolin) on changes in cellular GSH evoked by 100 μM dopamine or 10 μM hydroquinone. Numbers in parentheses are the number of cell cultures analyzed (n) in 3 experiments.

FIGURE 8: Comparison of the effects of R- and S-apomorphine on GSH levels in mesencephalic cell cultures. Numbers in parentheses are the number of cell cultures analyzed (n) in 3 experiments.

FIGURE 9: The chemical structure of quinpirole.

FIGURE 10: Effect of the disulfide forms of dithiothreitol and glutathione on GSH levels in mesencephalic cell cultures. Numbers in parentheses are the number of cell cultures analyzed (n) in 4 experiments.

FIGURE 11: Effect of protein kinase C inhibitors (H-7 and staurosporine) on the rise in cellular GSH evoked by dopamine in mesencephalic cell cultures. Numbers in parentheses are the number of cell cultures analyzed (n) in 4-6 experiments.

FIGURE 12: Effect of protein kinase C inhibitors (H-7 and staurosporine) on the rise in cellular GSH evoked by quinpirole in mesencephalic cell cultures. Numbers in parentheses are the number of cell cultures analyzed (n) in 2-5 experiments.

FIGURE 13: Effect of L-DOPA, dopamine (DA), and hydroquinone (HQ) on gamma-glutamylcysteine synthetase activity in mesencephalic cell cultures. Numbers in parentheses are the number of cell cultures analyzed (n) in 2 experiments.

FIGURE 14: Catalase activity in mesencephalic cell cultures. Clorgyline and pargyline are inhibitors of monoamine oxidase. Numbers in parentheses are the number of cell cultures analyzed (n) in 2-3 experiments.

FIGURE 15: The consequences of autoxidation of L-DOPA

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