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Spina, Mary Beth, Ph.D.

City University of New York, 1989

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A STUDY OF OXIDIZED GLUTATHIONE AS AN INDEX OF OXIDANT STRESS IN THE CENTRAL NERVOUS SYSTEM

by

MARY BETH SPINA

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

1989

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

A STUDY OF OXIDIZED GLUTATHIONE
AS AN INDEX OF OXIDANT STRESS
IN THE CENTRAL NERVOUS SYSTEM

by

Mary Beth Spina

Advisor: Professor Gerald Cohen

The oxidation of dopamine by monoamine oxidase generates hydrogen peroxide, which is potentially toxic to neurons. In the presence of hydrogen peroxide, reduced glutathione (GSH) is oxidized to glutathione disulfide (GSSG) by GSH peroxidase. A rise in the steady state level can be used as an index of oxidative stress. Dopamine metabolism was provoked both in vitro and in vivo and GSSG was measured.

Incubation of striatal synaptosomes with L-dopa or dopamine in either the presence or absence of 10 uM reserpine resulted in a rise in the level of (GSSG) within the isolated tissue pellet. This rise was dependent upon the concentration of DA or L-dopa. With 1.0 mM L-dopa in the presence of reserpine, GSSG was elevated by 7.0 $\pm$ 0.7 pmol/mg original striatal tissue; while, 10 uM DA in the presence of reserpine, elevated GSSG by 4.1 $\pm$ 0.5 pmol/mg original striatal tissue. In the presence of clorgyline or pargyline (monoamine oxidase inhibitors) the rise in GSSG caused by DA or L-dopa was suppressed. These data show that the metabolism of DA can evoke a significant rise in the

level of GSSG.

Experiments were also conducted in vivo in which mice were treated with either reserpine or haloperidol to induce DA metabolism, and GSSG levels were measured in the striata. Reserpine elevated GSSG by 6.1 $\pm$ 0.6 uM; and haloperidol increased GSSG by 12.3 $\pm$ 1.0 uM. The increase produced by reserpine was seen in the striatum but not in the frontal cortex, which receives a much sparser innervation by dopamine terminals. Similarly, when the dopamine terminals were destroyed in the striatum by 6-hydroxydopamine, the increase in GSSG caused by haloperidol was suppressed. Both an MAO-A inhibitor (clorgyline) and an MAO-B inhibitor (deprenyl) significantly suppressed the rise in GSSG caused by reserpine or haloperidol.

The data presented in this thesis indicates that the turnover of dopamine both in vitro and in vivo is associated with a significant oxidant stress. The data may shed some light on disease states which are accompanied by increased turnover of monoamines, such as Parkinson's disease.

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First I would like to thank Dr. Gerald Cohen, my preceptor. His dedication to science and to his students was an inspiration to me. His wealth of scientific knowledge and his unique approach to scientific design aided my understanding of science and was the driving force toward the completion of my doctorate.

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Chapter 1: Overview and Background

1.1: Overview

Oxidative stress is a state in which cells or organs are exposed to endogenous or exogenously-administered oxidants. Most often, the oxidant is a reduced form of oxygen, such as hydrogen peroxide, superoxide, or hydroxyl radical; or it may be a quinone or semi-quinone. These reactive species are generated during spontaneous or enzymatically-catalyzed reactions with molecular oxygen. As a result of an oxidant stress, the ratio of cellular pro-oxidants and anti-oxidants changes in favor of the pro-oxidant. An oxidant stress can be noted by an elevation in the level of oxidized product in a redox couple (e.g., elevation in oxidized glutathione), the disappearance of a cellular anti-oxidant (e.g., ascorbate), or the appearance of unusual oxidized products (e.g., lipid peroxides). Oxidative stress is thought to be involved in a variety of processes such as inflammation, ageing, carcinogenesis, and the toxicity of certain drugs. In the central nervous system, oxidative stress has been implicated in a variety of neuronal malfunctions such as Parkinson's disease, 6-hydroxydopamine toxicity and MPTP toxicity. In this thesis, I have evaluated peroxidative stress in the brain by measuring levels of oxidized glutathione (GSSG).

During intraneuronal metabolism of dopamine by monoamine oxidase, hydrogen peroxide is formed. Hydrogen peroxide is a strong oxidant and therefore it is potentially

hazardous to neurons. The major cellular mechanism for the detoxification of hydrogen peroxide is the glutathione peroxidase/glutathione reductase enzyme system. The reduction of hydrogen peroxide by reduced glutathione (GSH), catalyzed by glutathione peroxidase, results in the formation of oxidized glutathione (GSSG). An elevation in the level of GSSG can provide an index of oxidant stress.

In this thesis, the catabolism of dopamine by monoamine oxidase has been linked to an increase in GSSG. Studies were carried out both with isolated nerve terminals in vitro and with mice in vivo. Treatment with reserpine or with haloperidol (a neuroleptic drug) was used to increase dopamine turnover in nigrostriatal neurons. These experiments show that increased turnover of dopamine can evoke an oxidative stress through increased production of hydrogen peroxide and accumulation of oxidized glutathione. If normal catecholamine metabolism produces peroxide within catecholamine neurons, then increased neurotransmitter turnover may induce an oxidative stress. Increased dopamine turnover has been implicated in certain disease states, such as Parkinson's disease and schizophrenia. The observations in this thesis provide insights into mechanisms that may be responsible for neuronal senescence or alterations in neuronal function in CNS disorders that affect monoamine neurons.

1.2: Glutathione

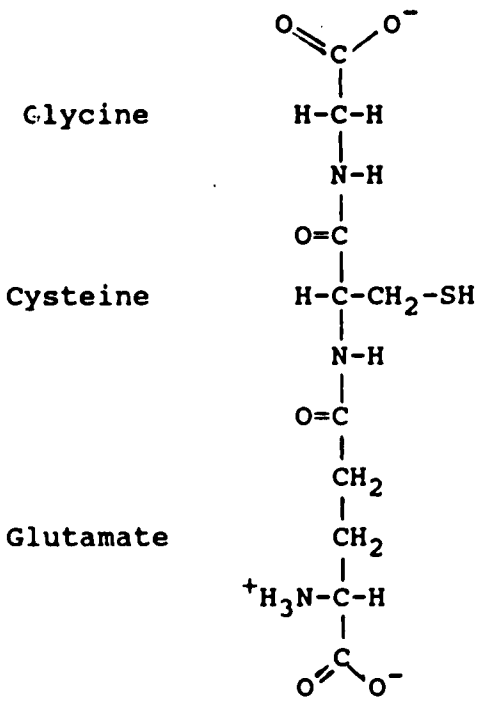
Glutathione is a tripeptide found in living cells. It contains the amino acids: glutamate, cysteine, and glycine (Figure 1). Glutathione was first discovered in yeast cells by de Rey-Pailhade (1888), who named it philthion. It was isolated in crystalline form by Hopkins (1921).

Glutathione has two structural features responsible for most of its chemistry: the sulfhydryl group (-SH) and the glutamyl bond. The sulfhydryl group allows glutathione to participate in oxidation-reduction processes; therefore it occurs in two forms, reduced glutathione (GSH) and oxidized glutathione (GSSG, glutathione disulfide). The glutamyl bond makes glutathione resistant to many peptidases. Glutathione serves many important functions, including keeping cellular components in their reduced form, transport of amino acids, and detoxification of hydrogen peroxide and organic peroxides.

High concentrations of glutathione are found in the brain. Levels are in the range of 1.0-2.0 mM in rodents and primates (Slivka et al., 1987). Subcellular localization of glutathione has also been studied. Glutathione is located primarily in the soluble supernatant fraction (62%) and partially in the crude mitochondrial fraction (34%) (Reichelt and Fonnum, 1969). Brain glutathione levels change during development. In the neonatal cat cortex, glutathione levels are about 80% of adult levels and

FIGURE 1

Structure of Glutathione



markedly increase during the postnatal period, reaching adult levels by the end of the second week (Berl and Purpura, 1963).

1.2.1: Glutathione synthesis

The pathway of glutathione biosynthesis was first described by Bloch in 1949. The overall reaction proceeds in two steps. The first step is catalyzed by the enzyme gamma-glutamylcysteine synthetase as shown in Equation 1 (where Pi denotes inorganic phosphate):

L-glutamate + L-cysteine + ATP ---------> L-gamma-glutamylcysteine + ADP + Pi (Eq. 1)

Gamma-glutamyl cysteine synthetase is a sulfhydryl-dependent enzyme that can be inhibited by thiol-blocking agents or buthionine sulfoximine. The second step is catalyzed by glutathione synthetase (Equation 2):

L-gamma-glutamylcysteine + glycine + ATP ----------> Glutathione + ADP + Pi (Eq. 2)

Glutathione synthetase requires Mg^{2+} ions for activity. The availability of gamma-glutamyl cysteine appears to limit the rate of GSH synthesis. This compound may be present in limiting amounts due to the low concentrations of cysteine in tissues (e.g. 40-60 uM in brain, Slivka et al., 1988).

1.2.2: Glutathione metabolism

The degradation of glutathione is initiated by the enzyme gamma-glutamyl transpeptidase. This enzyme catalyzes transfer of the gamma-glutamyl group of glutathione to amino acids or peptides as shown in Equation 3:

Glutathione + amino acid ---------->
gamma-glutamyl amino acid + cysteinylglycine (Eq. 3)

Gamma-glutamyl cyclotransferase catalyzes the conversion of gamma-glutamyl amino acids to pyrrolidone carboxylate and free amino acids (Equation 4):

Gamma-glutamyl amino acids ---------->
pyrrolidone carboxylate + amino acid (Eq. 4)

Cysteinylglycine dipeptidase breaks the peptide bond between cysteine and glycine and releases the free amino acids (Equation 5):

L-cysteinylglycine + H_2O ---------->
cysteine + glycine (Eq. 5)

Pyrrolidone carboxylate hydroxylase converts pyrrolidone carboxylate into glutamate in an ATP requiring step (Equation 6):

L-pyrrolidone carboxylate + ATP + $2H_2O$ ---------->
L-glutamate + ADP + Pi (Eq. 6)

It is this set of reactions, known as the gamma-glutamyl

cycle (Orlowski and Karkowsky, 1976), that degrades glutathione into its amino acids. It has been proposed that this cycle is important in amino acid transport. The first enzyme, gamma-glutamyl transpeptidase, is membrane bound and therefore functions in translocating amino acids across cellular membranes.

1.2.3: Reduced and oxidized forms

There are two enzymes which control the conversion of glutathione between its reduced and oxidized states. Glutathione peroxidase was first isolated by Mills (1957). GSH peroxidase is a selenium-containing enzyme that couples the reduction of H_2O_2 or organic peroxides to the oxidation of GSH (Orlowski and Karkowsky, 1976; Little and O'Brien, 1968) in the following reaction (Equation 7):

$$2GSH + ROOH \text{ ---------> } GSSG + H_2O + ROH \quad \text{(Eq. 7)}$$

In the above reaction, ROOH is either hydrogen peroxide (R=H) or an organic peroxide (R=alkyl group). GSH peroxidase activity depends upon changes in the oxidation state of selenium (Flohe et al., 1973). The activity of GSH peroxidase in brain is lower than that of other tissues such as liver, lung, and kidney (Lawrence et al., 1974; DeMarchena et al., 1974).

The enzyme responsible for maintaining cellular glutathione in its reduced form is GSSG reductase. The reductase is a flavo-enzyme and enzymatic activity is

coupled to the oxidation of NADPH (Equation 8):

$$GSSG + NADPH + H^+ \text{ ---------> } 2GSH + NADP^+ \quad \text{(Eq. 8)}$$

The reductase reaction is relatively specific for GSSG, though other disulfides can be used as substrates. Mixed disulfides of protein and glutathione, such as hemoglobin-glutathione, are substrates for glutathione reductase (Srivatava and Beutler, 1970; Srivastava, 1971). GSSG reductase contains one FAD molecule per subunit. The NADPH needed for reducing GSSG is provided mainly via the pentose phosphate shunt, although some is formed by the oxidation of malate by the malic enzyme. Cellular localization studies indicate that in rat brain both GSH peroxidase and GSSG reductase exhibit highest activity in the striatum (Brannan et al., 1980 a & b). Glutathione-S-transferase can also catalyze the reaction between organic peroxides and GSH in tissue such as liver (Prohaska and Ganther, 1977).

1.2.4: Levels of Glutathione

GSH is broadly distributed in relatively high concentrations (0.5 - 10 mM)in body tissues and serves, in part, to set the redox status of tissues. In liver, GSH is in the range of 2-10 mM (Meister, 1985), while in the brain GSH is approximately 2 mM (Cooper et al., 1980; Folbergrova et al., 1979; Renchrona et al., 1980). A review of the literature shows that levels in rat brain range from 1.4 to 3.3 mM (Slivka et al., 1987). GSSG, on the other hand, is

maintained at very low levels and constitutes only 0.5-1.2% of the total glutathione in rat, monkey, and human brain (Slivka et al., 1987). Low GSSG levels are also the rule in peripheral organs, such as liver (Adams et al., 1983; Akerboom and Sies, 1981). The efficiency of GSSG reductase is responsible for maintaining low GSSG levels in cells, even during exposure to peroxides. However, an elevation in the steady-state level of GSSG, which reflects a change in redox status, can occur during exposure to peroxides.

1.2.5: GSSG as an index of oxidative stress

Several workers have studied the rise in cellular GSSG, or an efflux of GSSG from tissues, during exposure to endogenous peroxides. Sies (1985) reported a rise in GSSG during infusion of organic peroxides or H_2O_2 into liver. Organic peroxides are good substrates for GSH peroxidase. Similarly, Oshino and Chance (1977) observed an efflux of GSSG during infusion of H_2O_2 into isolated rat liver. Sutherland et al. (1985) exposed rat alveolar macrophages to t-butyl hydroperoxide and observed increased GSSG levels. Adams et al. (1983) observed increased levels in GSSG in plasma and liver during exposure of rats to paraquat or related H_2O_2-generating compounds. A rise in tissue GSSG is generally taken as an index of oxidative stress (Adams et al., 1983; Sies, 1985).

1.2.6: GSSG as a damaging agent:

Numerous changes, such as inactivation of phosphofructokinase and glucokinase are seen in tissues when GSSG levels rise into the range 50-200 uM (Gilbert, 1982). Thus, although the activity of GSH peroxidase serves to protect cells from H_2O_2, there are biologic consequences when the flux of H_2O_2 is sufficient to alter the intracellular levels of GSSG. The formation of mixed disulfides with protein sulfhydryl groups can inhibit SH-dependent enzymes. Recent studies have indicated that increased mixed disulfide formation leads to inactivation of some enzymes and causes them to become more susceptible to proteolysis (Offermann et al., 1984). If GSH is markedly lowered, the protective role of GSH peroxidase will be impaired and oxidation of membrane lipids or toxic oxidative events may be promoted. In addition, GSSG can serve as a "third messenger" to evoke changes in cellular systems or enzymes (Gilbert, 1982).

1.2.7: Efflux of GSSG

As GSSG levels rise, the cell has two defense mechanisms. The enzyme, GSSG reductase, is very efficient in keeping most of the glutathione in the reduced form. Under certain conditions, however, the rise in intracellular GSSG leads to an export of the disulfide to extracellular fluid. It has been suggested that GSSG efflux may serve as a protective mechanism by keeping the GSSG level from rising

intracellularly. The export of GSSG from cells was first observed with the lens and with red cells exposed to H_2O_2 (Srivastava and Beutler, 1969).

In the perfused liver, the enzymatic generation of GSSG upon infusion of t-butyl hydroperoxide elicits GSSG release (Sies et al., 1972). Other organs, such as heart and lung release GSSG as well (Ishikawara and Sies, 1984; White et al., 1986). The relationship between the cellular level of GSSG and the rate of GSSG efflux is linear for liver; in other words, the efflux system is not saturable (Akerboom, 1982). However, the efflux system is saturable in perfused heart (Ishikura and Sies, 1984). Sies and Summer (1975) calculated that about 3% of the flux of GSSG in the liver is not recycled to GSH, but rather, is exported by the cells. In comparing the efflux mechanism of heart and liver, Sies (1975) reported that the export capacity of the liver is much greater than that of the heart, and he suggested that this factor could underlie the cardiac susceptibility to some types of oxidative stress. Brain has not been previously studied.

1.3: Dopamine (DA)

The experiments in this thesis focus on the dopaminergic innervation of the striatum. Cell bodies in the A8 and A9 regions of the substantia nigra give rise to axons that terminate in the striatum (nigrostriatal pathway). It is this pathway that undergoes degeneration in

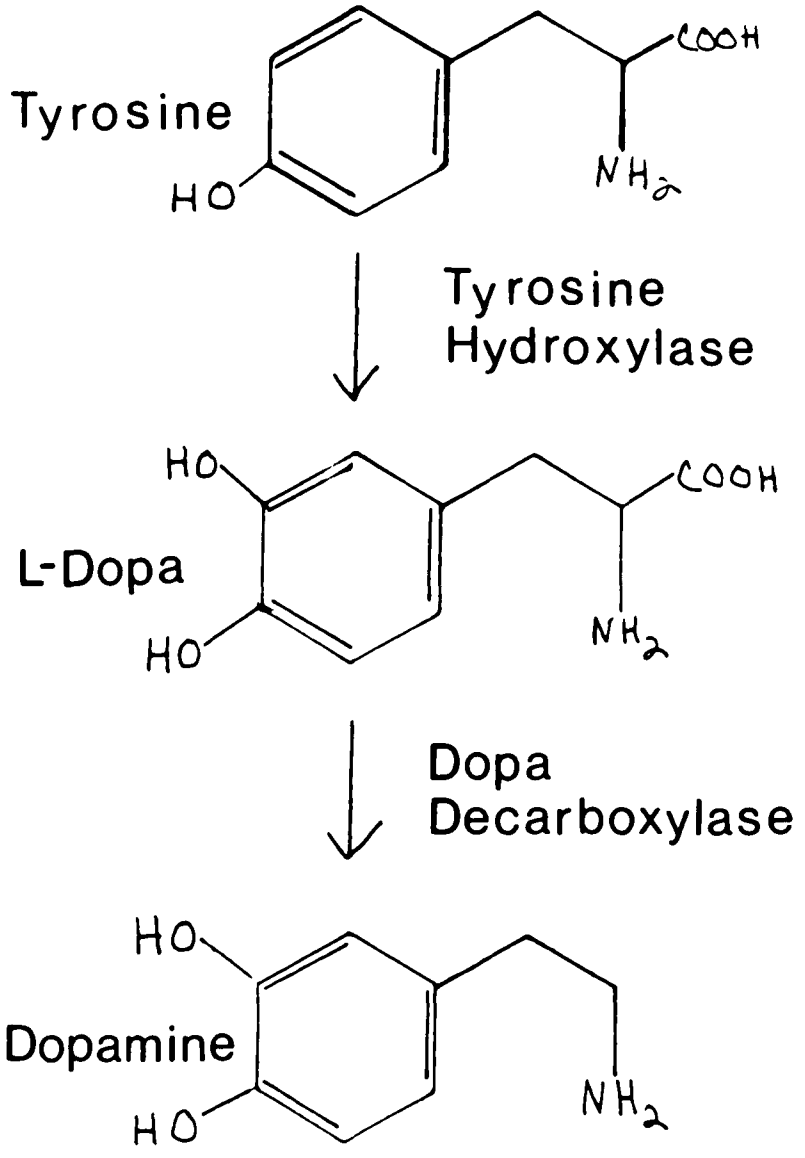
Parkinson's disease. The loss of DA in the caudate and putamen in Parkinson's disease reflects the loss of nigrostriatal DA neurons. Levels of DA in the striatum in rodents are in the range of 10 ug/g wet weight tissue (Kindt et al., 1988; Bradbury et al., 1986; Rollema et al., 1986). This represents an average concentration of 65 uM. However, studies by Anden et al. (1966) indicate that the concentration of DA within DA terminals is in the range of 50 mM.

1.3.1: Dopamine synthesis

DA, as well as other catecholamines (norepinephrine and epinephrine), are synthesized from the aromatic amino acid tyrosine. Tyrosine is transported across the blood brain barrier and then enters catecholamine neurons. Tyrosine hydroxylase catalyzes the first step in catecholamine synthesis, in which tyrosine is converted to L-dopa. Tetrahydrobiopterin is the cofactor for this enzyme. Dopa-decarboxylase subsequently catalyzes the formation of DA from L-dopa (See Figure 2). This enzyme requires pyridoxal phosphate as a cofactor. In the experiments that will be described, inhibition of dopa-decarboxylase is used as an experimental tool. A commonly used inhibitor of this enzyme is carbidopa. Carbidopa is a peripheral decarboxylase inhibitor that is used in combination with L-dopa in clinical treatment of Parkinson's disease; carbidopa blocks peripheral decarboxylation and allows more L-dopa to

FIGURE 2

Dopamine Synthesis



be transported into the brain. Centrally active decarboxylase inhibitors include NSD-1055 and NSD-1015.

1.3.2: Dopamine metabolism

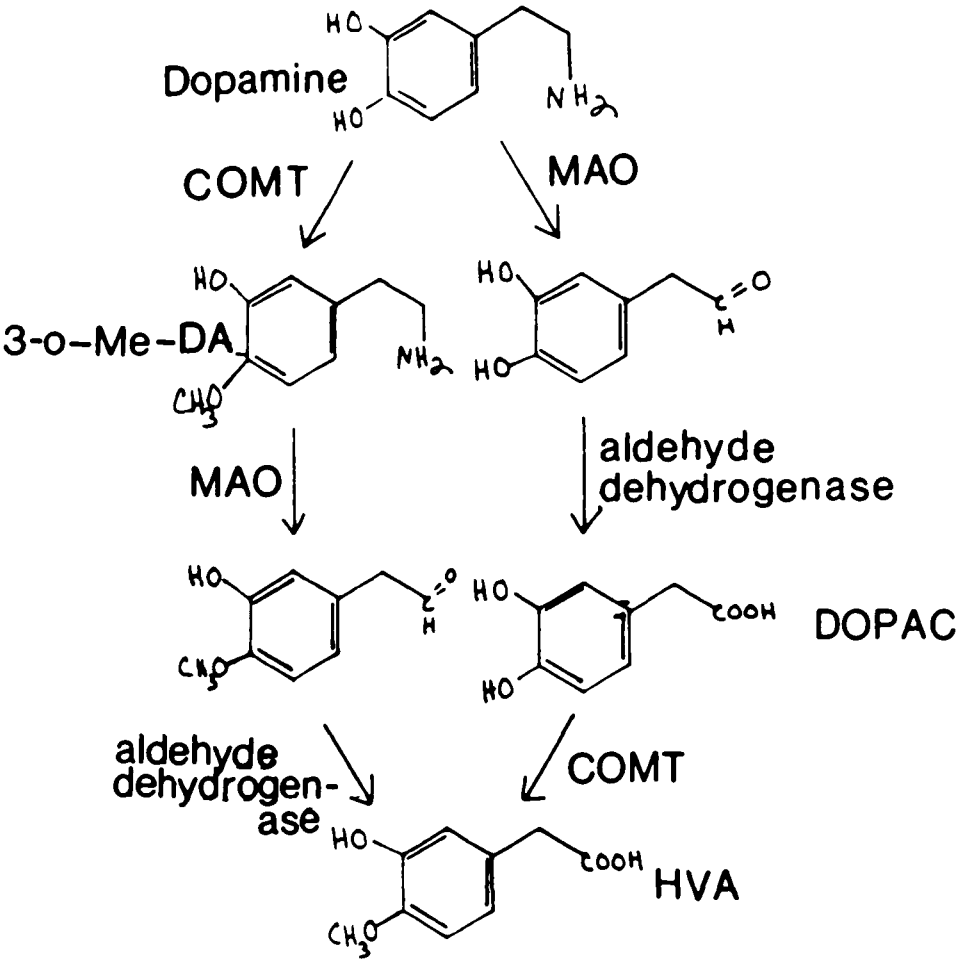
DA is metabolized by two enzymes: monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). MAO is a flavoprotein and is localized to the outer membrane of mitochondria in both neuronal and non-neuronal cells. It is present both in catecholamine nerve terminals and in postsynaptic elements. MAO catalyzes the oxidation of DA by oxygen to form the corresponding aldehyde, hydrogen peroxide, and ammonia, as shown in Equation 9:

$$DA + H_2O + O_2 \text{ ----------->}$$
$$3,4\text{-dihydroxyphenylacetaldehyde} + NH_3 + H_2O_2 \quad \text{(Eq. 9)}$$

The aldehyde product is then transformed to the corresponding acid, 3,4-dihydroxyphenylacetic acid (DOPAC), by aldehyde dehydrogenase, an NAD-dependent enzyme. The enzyme COMT is located outside the nerve terminal; therefore DA must be released from the nerve terminal before it will be O-methylated. After DA is released, methylation occurs in the 3 position to form 3-O-methyldopamine. 3-O-methyldopamine is subsequently metabolized by MAO and aldehyde dehydrogenase to yield homovanillic acid (HVA). If DA is first metabolized to DOPAC, COMT will O-methylate this acid to form HVA. In DA systems, DOPAC and HVA are the major metabolites (See Figure 3). It has been estimated

FIGURE 3

Dopamine Metabolism



that 80% of the HVA is formed from DOPAC and 20% from 3-O-methyldopamine (Westerink and Spaan, 1982a). 3-O-methyldopamine levels are sometimes used as an index of DA release (Boyar and Altar, 1987; Westerink and Spaan, 1982b).

1.3.3: MAO subtypes

In 1968, Johnston discovered two forms of monoamine oxidase. By definition, the A form of MAO is very sensitive to inhibition by the inhibitor clorgyline, whereas the B form is only inhibited at high concentrations of the inhibitor. On the other hand, deprenyl has the opposite selectivity (Knoll and Magyar, 1972). At low concentrations it produces selective inhibition of MAO-B. The two enzyme forms have also different substrate specificities; for example, 5-hydroxytryptamine (serotonin) is a preferred substrate for MAO-A and phenylethylamine for MAO-B (Johnston, 1968; Goridis and Neff, 1971). DA can be oxidized either by MAO-A or MAO-B.

1.3.4: Release of dopamine and synaptic transmission

DA is stored in nerve terminals within synaptic vesicles. Stored DA is not subject to metabolism by neuronal MAO. The uptake into vesicles is an active process which requires ATP. Uptake into vesicles can be inhibited irreversibly by reserpine and reversibly by tetrabenazine.

DA is released into the synapse where it interacts with receptors both postsynaptically and presynaptically (autoreceptors). A portion of the DA is O-methylated by

COMT to form 3-O-methyldopamine. However, most of the released DA is recaptured by the DA nerve terminal. This reuptake mechanism, which is associated with the axonal membrane, can be inhibited by many drugs, which include cocaine, mazindol, and nomifensine. During reuptake, a portion of the DA is metabolized presynaptically by monoamine oxidase, and results in an increased level of DOPAC. Levels of DOPAC serve to indicate presynaptic DA turnover.

1.4: Detoxification of H_2O_2

When DA is metabolized by MAO, H_2O_2 is formed. H_2O_2 is potentially toxic to the neuron and must be eliminated. H_2O_2 can be detoxified by two enzymatic systems, catalase and GSH peroxidase.

1.4.1: Catalase

Catalase is a heme enzyme that catalyzes the disproportionation of two moles of H_2O_2 to yield one mole of O_2 and two moles of H_2O (Equation 10):

$$H_2O_2 + H_2O_2 \text{ ---------> } O_2 + 2H_2O \quad \text{(Eq. 10)}$$

During this reaction, an intermediate is formed [catalase-H_2O_2 (compound I)] that can be identified spectrophotometrically (Chance, 1947). The appearance of compound I has been used to detect H_2O_2 (Oshino and Chance, 1977). Catalase is localized subcellularly in peroxisomes

(microperoxisomes in brain; DeDuve and Baudhuin, 1966). Levels of enzyme activity in the brain are low (Gaunt and DeDuve, 1976; Sinet et al., 1980; Brannan et al., 1981) but appear most concentrated in catecholaminergic neuronal cell bodies in anatomically defined nuclei, such as the locus coeruleus and the substantia nigra (McKenna et al., 1976).

1.4.2: GSH peroxidase

The reaction catalyzed by GSH peroxidase is shown in Equation 7.

$$2GSH + ROOH \text{ ---------> } GSSG + H_2O + ROH \quad \text{(Eq. 7)}$$

Using perfused liver and benzylamine as a substrate, Oshino and Chance (1977) showed that MAO activity was coupled to the production of GSSG. Since the appearance of GSSG was unaffected by inhibition of catalase with 3-amino-1,2,4-triazole, the authors concluded that GSH peroxidase, and not catalase, was responsible for removal of MAO-generated H_2O_2. Studies by Maker et al. (1981) showed that addition of serotonin or DA to perfused brain homogenates resulted in the oxidation of GSH to GSSG: oxidation of GSH was blocked by inhibition of MAO. From these and other studies, it seems probable that the H_2O_2 generated by MAO is preferentially detoxified by GSH peroxidase. Several reports have suggested that H_2O_2 generated during the metabolism of monoamines by MAO may be toxic (Graham et al., 1978; Sinet et al., 1980; Cohen, 1983). Allis and Cohen

(1977) administered the neurotoxin 5,7-dihydroxytryptamine to mice and inhibited the destruction of noradrenergic terminals in the left atrium with either an MAO inhibitor or a hydroxyl radical scavenger (.OH). This report provides evidence suggesting that the toxicity of MAO-generated H_2O_2 can be mediated via .OH formation.

1.5: Drugs that increase dopamine turnover

In the experiments that will be presented in this thesis, GSSG was measured after inducing increased DA turnover. In this way the consequences of increased MAO activity were evaluated. Experiments were conducted with rat striatal synaptosomes in vitro and also with in vivo experimental paradigms. DA turnover was provoked either with reserpine or haloperidol.

1.5.1: Reserpine

Reserpine blocks the storage of DA in synaptic vesicles. The action of reserpine occurs by inhibiting the ATP-dependent uptake into the vesicles. When this happens, the cytoplasmic pool of DA increases and thereby the metabolism of DA by MAO is potentiated.

1.5.2: Haloperidol

Acute administration of antipsychotic drugs, such as haloperidol (Haldol), increase dopaminergic cell activity and enhance DA turnover. DA catabolism is increased and, at the same time, DA biosynthesis is accelerated. These

antipsychotic drugs have potent DA receptor blocking capabilities, both at postsynaptic and presynaptic receptors. Blockade of postsynaptic receptors will increase dopaminergic activity via a neuronal feedback loop and also increase biosynthesis at the tyrosine hydroxylase step. Some of these observed effects are enhanced as a result of interaction with nerve terminal autoreceptors. Blockade of DA autoreceptors on nerve terminals increases both the synthesis and release of dopamine. Bannon et al. (1982) showed that acute haloperidol dramatically increased striatal DOPAC levels.

Chapter 2: Materials and methods

2.1: Source and preparation of drugs and chemicals

2.1.1: In-Vitro experiments

Drugs were obtained from the following sources: Reserpine phosphate (Ciba Pharmaceutical Co., Summit, NJ), clorgyline (May and Baker Ltd., Dagenham, England), NSD-1055 (brocresine, Lederle Laboratories, American Cyanamide, Pearl River, NY), carbidopa (alpha-methyldopahydrazine, Merck Sharp and Dohme, Rahway, NJ). The following drugs, chemicals, and enzymes were obtained from Sigma Chemical Co., St. Louis, MO: L-dopa, pargyline, dopamine, DOPAC, GSSG, 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB), GSSG reductase, reduced nicotinamide adenine dinucleotide phosphate (NADPH), diethylenetriamine pentaacetic acid (DTPA), and disodium ethylenediaminetetraacetate (EDTA). Catalase (crystalline suspension containing 74,600 units/mg) was obtained from the Millipore Corp., Freehold, NJ; the suspension was centrifuged at 700 x g for 10 minutes and the isolated crystals were dissolved in Krebs phosphate buffer to obtain a final concentration of 2 ug/ml. Buffer salts were the highest grade available. Solutions of drugs were prepared in the Krebs buffer.

2.1.2: In-Vivo experiments

Reserpine (Serpasil, Ciba Pharmaceutical Co., Summit, NJ) was dissolved in 20 ul glacial acetic acid and diluted into 0.3 mM dextrose in distilled water. Clorgyline (May

and Baker, Ltd., Dagenham, England), deprenyl (Medipex, Budapest, Hungary), and haloperidol (Sigma Chemical Co., St. Louis, Mo.) were prepared in isotonic saline.

2.2: Animal and tissue preparations

2.2.1: In-Vitro experiments: Tissue preparation

Male Sprague-Dawley rats (220-250 g; Ace Breeders, Boyertown, PA) were decapitated with a guillotine and the brains were rapidly excised. The striata were dissected over ice according to the method of Glowinski and Iversen (1966). In each experiment, the striata from 4-5 animals were pooled and a crude synaptosomal pellet was obtained. The striata were homogenized in 10 volumes of cold 0.32 M sucrose with a motor-driven homogenizer for 10 up-and-down strokes. The homogenate was then centrifuged at 700 x g for 10 min. The resultant supernatant was recentrifuged at 12,000 x g for 40 minutes (Sorvall Superspeed RC2-B centrifuge, Dupont, Wilmington, Delaware) and the P2 pellet was isolated; this pellet contains synaptosomes and free mitochondria. In a limited number of experiments, slices of striatum (1.0 x 0.4 x 0.4 mm) were prepared with a McIlwain-Mickel tissue chopper.

2.2.2: In-vitro experiments: Incubation procedures

The synaptosomal pellets (or striatal slices) were suspended in a modified Krebs-phosphate buffer (pH 7.4) containing: 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 0.8 mM $CaCl_2$, 16.2 mM Na_2HPO_4, 15.9 mM NaH_2PO_4, 0.1 mM ascorbic

acid, 0.1 mM diethylenetriaminepentaacetic acid (DTPA), and 5.5 mM glucose. Triplicate aliquots of synaptosomes were pipetted into 10 ml glass beakers on ice; each beaker contained 23-28 mg of original wet weight of tissue, depending upon the particular experiment, in a final volume of 1.5 ml after the addition of all other components. Samples were preincubated for 15 minutes at 37°C with 10 uM reserpine and, then, L-dopa or DA was added, generally to a final concentration of 1 mM or 10 uM respectively, and the incubation was continued for another 45 minutes. In some experiments, the L-dopa concentration was 0.2 mM or 0.04 mM or the DA concentration was 20 uM or 40 uM.

In separate experiments, the following agents were used in conjunction with reserpine plus L-dopa or DA, with incubation times as stated in the legends to the Tables: 10 uM clorgyline, 10 uM pargyline, 0.25 mM NSD-1055, 0.25 mM carbidopa, or 2 ug/ml catalase. These agents were added at zero time. At the termination of the incubation period, the beakers were chilled in an ice bath and the contents were transferred to cold 1.5 ml microfuge tubes and centrifuged in the cold at 12,000 x g for 40 minutes (Hill Scientific centrifuge MV15, Derby, CT). The supernatants were discarded and the pellets were homogenized in 400 ul of cold 0.4 M perchloric acid with pellet pestle mixers (Kontes, Vineland, NJ). The homogenates were recentrifuged at 11,000 x g for 15 minutes. When brain slices were used,

they were isolated with the aid of a Gooch crucible and rinsed once with fresh Krebs buffer. The slices were homogenized in 0.3 ml of cold perchloric acid and centrifuged (11,000 x g) for 15 minutes. The supernatant fluids were taken for assay of GSSG.

2.2.3: In-vivo experiments: Animal preparation

Male Swiss-Webster mice (25-30 g, Ace Breeders, Boyertown, PA) received intraperitoneal injections of reserpine (10 mg/kg) or haloperidol (1 mg/kg); control mice received the injection vehicle alone. Where indicated, mice received one of the following monoamine oxidase inhibitors: clorgyline (2.5 mg/kg, i.p.) or deprenyl (2.5 mg/kg, i.p.) at 18 hours prior to injection of reserpine. At two hours after reserpine or one hour after haloperidol, the mice were rapidly decapitated, the striatum and in some experiments the frontal cortex were dissected over ice, and tissues were homogenized in 10 volumes of cold 0.4 M perchloric acid, which contained 0.1 mM diethylenetriaminepentaacetic.

2.2.4: 6-hydroxydopamine lesions

Male Sprague Dawley rats (150-200 g, Ace Breeders) were anesthetized with pentobarbital and placed in a Kopf stereotaxic device. To obtain a nigrostriatal lesion, 8 ug of 6-hydroxydopamine (in 4 ul of saline containing 0.2 mg/ml ascorbic acid) was injected into the right substantia nigra (1.0 mm anterior to bregma, 2.5 mm lateral to bregma, incisor bar was 2.0 mm above horizontal plate, and 5.0 mm

from dura). Rats were allowed to recover for one week and then received an injection of haloperidol as described above. Successful 6-OHDA lesions were determined by the measurement of DA levels in the striata. DA levels were measured by HPLC techniques.

2.3: GSSG Assays

GSSG was assayed by the enzymatic recycling procedure of Tietze (1969), as modified by Cooper et al. (1980). This method utilizes GSSG reductase and is a sensitive assay for the measurement of GSSG. GSH was first removed by reaction with N-ethylmaleimide, as described by Adams et al. (1983). A 100 ul aliquot of the acidified supernatant was added to 900 ul of 11 mM N-ethylmaleimide in 100 mM potassium phosphate buffer, with 5 mM ethylenediaminetetraacetate (EDTA), at pH 7.5. After a 20 min incubation period at room temperature, the samples were passed over Sep-Pak C-18 cartridges (Millipore, Waters Associates, Milford, MA) to remove unreacted N-ethylmaleimide. Suction filtration was accomplished with the aid of Sep-Pak Cartridge Rack (Millipore Waters Associates, Milford, MA). The cartridges were rinsed once with an equal volume of buffer; the final pH was 7.0.

Spectrophotometric assays were performed with 1.5 ml of eluate, to which 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and GSSG reductase were added at concentrations

described by Cooper et al. (1980): 0.17 mM NADPH, 0.4 mM DTNB, and 16 ug/ml GSSG reductase. The final assay volume was 2.0 ml. The rate of color formation (rate of formation of 5-thio-2-nitrobenzoate) was monitored spectrophotometrically at 412 nm and ambient temperature for 5 min with a Stasar III flow-through spectrophotometer (Gilford Instruments, Oberlin, OH). Rates of color formation were corrected for the blank rate with DTNB alone, and compared to a standard curve obtained with known amounts of GSSG standards (See Figure 4).

2.4: DA, DOPAC, and HVA assays:

Synaptosomes from in-vitro experiments were homogenized in acid as described in Section 2.2.2, and the supernatants were analyzed for DA, DOPAC, and HVA. The supernatants were analyzed by high performance liquid chromatography (HPLC) with electrochemical detection (Bioanalytic Systems, West Lafayette, Ind.). The mobile phase contained 150 mM monochloroacetic acid (pH 3.0) with 7 mM EDTA and 1.8 mM sodium octyl sulfate, and 3.5% acetonitrile. The column used was 25 cm. long, containing octadecylsilane reverse-phase packing. Peak heights and retention times of standard solutions were compared with samples and used to calculate the concentrations of DA, DOPAC, and HVA (Sample Chromatogram- Figure 5).

2.5: Statistical analyses

Statistical analyses were conducted by 2-tailed paired or unpaired t-test, where appropriate. For multiple comparisons, ANOVA was followed by the Newman Keuls test.

FIGURE 4

Standard Curve for GSSG

Data shown are the pooled results from three experiments. Each experiment was analyzed in triplicate. The standard error for each concentration is less than 5% of the mean.

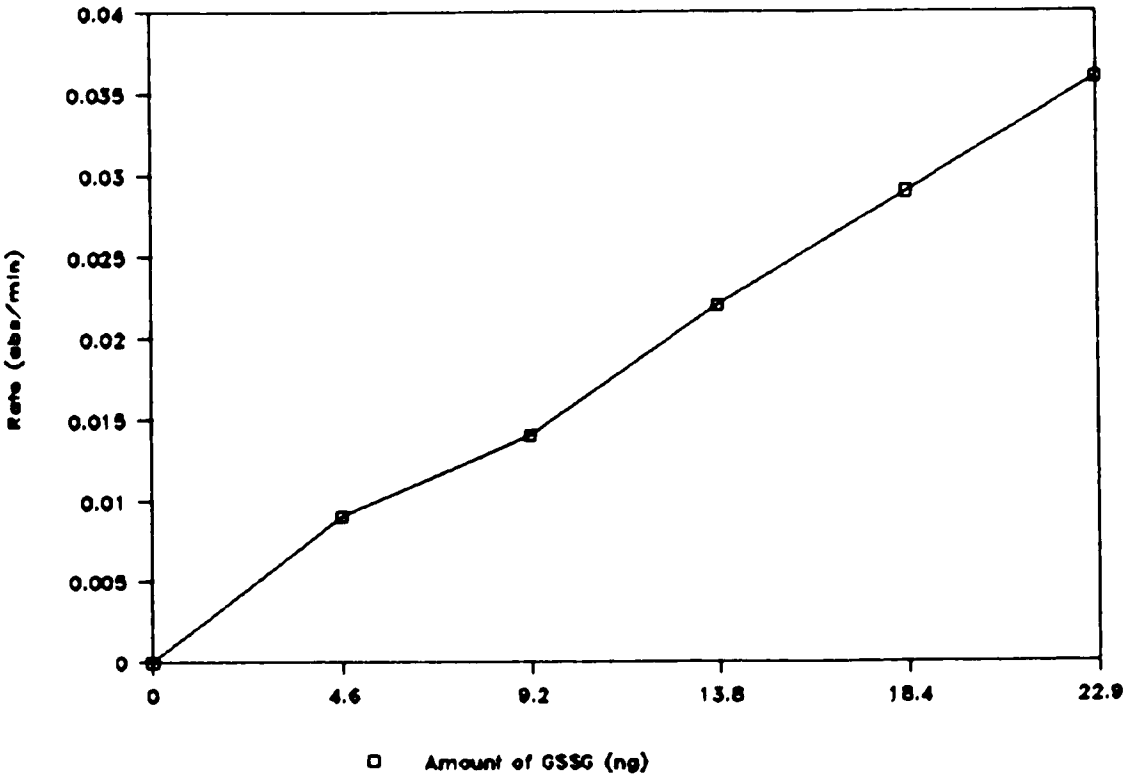
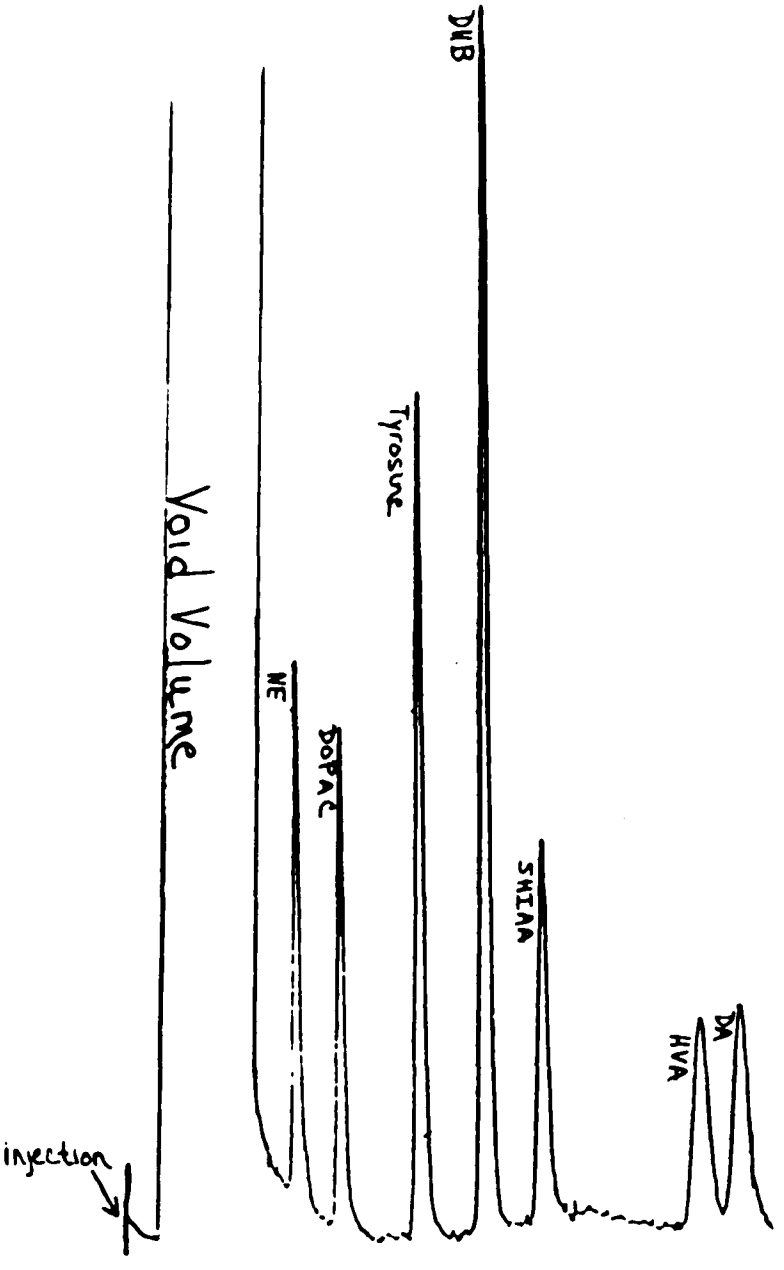


FIGURE 5

Sample Chromatogram



Separation of norepinephrine, tyrosine, dopac, dihydroxybenzylamine, dopamine, 5-hydroxyindoleacetic acid, and homovanillic acid by HPLC.

Chapter 3: In-vitro experiments: Results and discussion

3.1: Results

3.1.1: Preliminary experiments with added peroxides

In preliminary experiments, slices of rat striatum were incubated with t-butyl hydroperoxide, which is a substrate for GSH peroxidase, but not catalase. Incubation of 25 mg of tissue for 30 min in the Krebs phosphate buffer (2 ml) in the presence of 5.5 mM glucose resulted in levels of GSSG that rose from 6.0 $\pm$ 0.2 pmoles/mg striatal tissue (SEM, n=6) for control samples to 10.9 $\pm$ 0.7, 23.6 $\pm$ 0.7, and 38.5 $\pm$ 1.2 pmoles/mg, respectively, for t-butyl hydroperoxide concentrations of 10, 50 and 100 uM (pooled results from 2 experiments, Table 1). Slices were also incubated for 30 min with 100 uM H_2O_2 in the presence of 50 uM sodium azide to inhibit tissue catalase (particularly that present within entrapped erythrocytes in the tissue). At the end of 30 min, the GSSG level was 21.8 $\pm$ 0.3 pmoles/mg striatal tissue (SEM), compared to 1.0 $\pm$ 0.2 pmoles/mg striatal tissue for the control tissue. These results illustrate the rise in GSSG that takes place in striatal tissue during exposure to peroxides in the presence of glucose.

In succeeding experiments, synaptosomes were used for several reasons. Resuspended synaptosomes are cleared of contamination by erythrocytes; they represent a relative purification of nerve terminals, as opposed to cell bodies and glial cells; and they are easily distributed into replicate aliquots.

TABLE 1

GSSG levels in striatal slices after incubation with t-butyl hydroperoxide or hydrogen peroxide

Striatal slices (25 mg) were incubated in 2.0 ml of Krebs-phosphate buffer for 30 minutes at 37°C with various concentrations of t-butyl hydroperoxide or with 100 uM hydrogen peroxide plus 50 uM sodium azide. Experiments were performed in triplicate; n refers to the number of samples. The elevations in GSSG above control were all significant ($p < 0.001$)

Added Peroxide	pmoles GSSG/mg + SEM (n)
t-Butyl Hydroperoxide	
0 uM (Control)	6.0 ± 0.2 (6)
10 uM	10.9 ± 0.7 (6)
50 uM	23.6 ± 0.7 (6)
100 uM	38.5 ± 1.2 (6)
Hydrogen Peroxide	
0 uM (Control)	1.0 ± 0.2 (3)
100 uM	21.8 ± 0.3 (3)

3.1.2: Synaptosomal experiments

Experiments were conducted in which DA turnover was provoked with reserpine. Synaptosomes were pretreated with reserpine and subsequently DA or L-dopa was added. After incubation, GSSG levels were measured in the synaptosomal pellet. Various drugs were used to further elucidate the observed results.

3.1.2a:Effects of reserpine + dopamine or reserpine + L-dopa

Initial experiments were done in which GSSG was measured in striatal synaptosomes after incubation with reserpine plus DA or reserpine plus L-dopa. Reserpine was added in order to block the vesicular storage of DA within monoamine terminals and, thereby, facilitate the turnover of intrasynaptosomal DA (See Figure 6). During the preparation of the synaptosomal pellet most of the DA was lost; therefore it was necessary to replace DA either directly via uptake or indirectly by the addition of L-dopa. When L-dopa is added, decarboxylation by dopa-decarboxylase yields DA and, therefore, both the turnover of DA by MAO and the concurrent formation of H_2O_2 should be incremented. Reserpine (10 uM) was added first and the tissues were incubated for 15 min; then, DA (10 uM) or L-dopa (1 mM) was added and the incubation was continued for an additional 45 min.

Table 2 shows data from eleven experiments in which GSSG was measured in striatal synaptosomes after treatment

FIGURE 6

Schematic diagram of dopamine nerve terminal

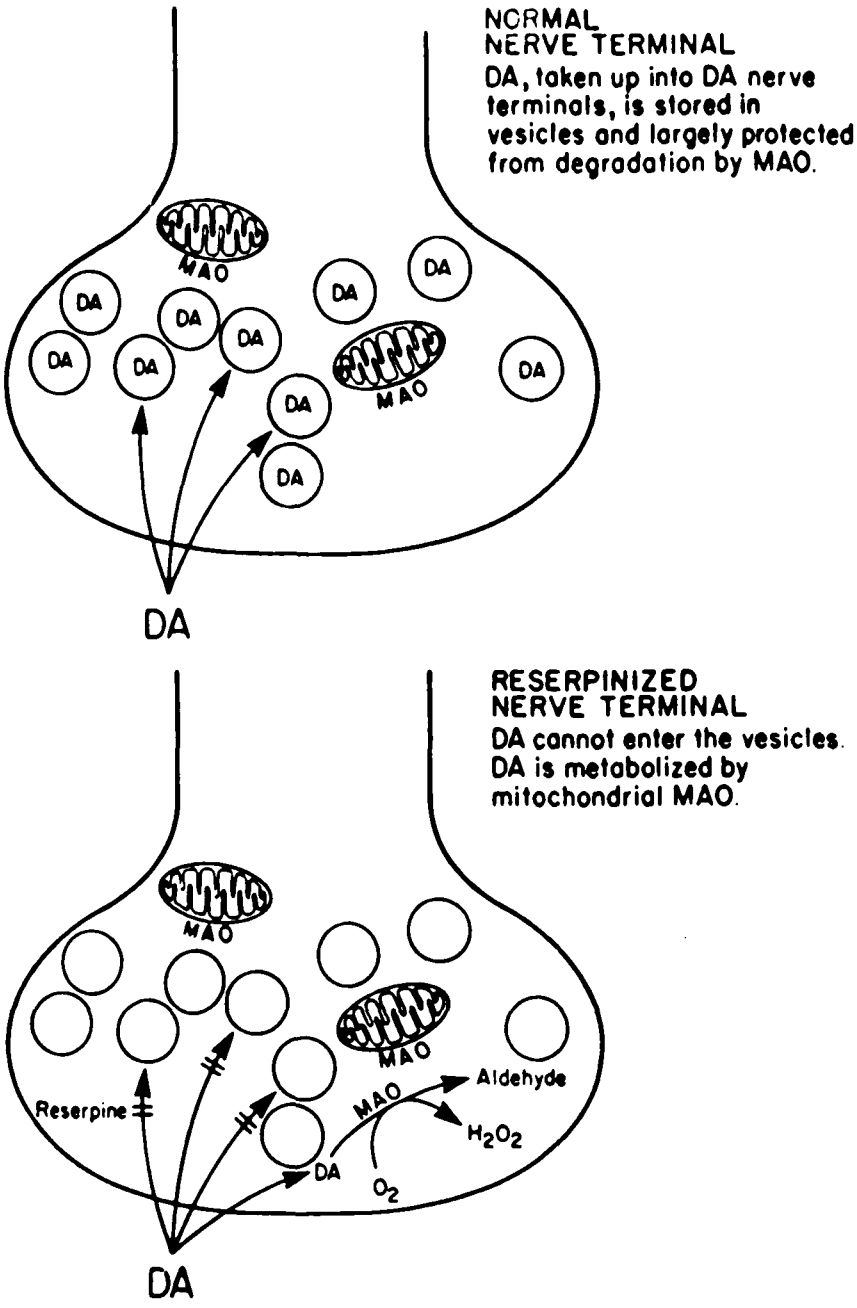


TABLE 2

GSSG levels after treatment of synaptosomes with reserpine and dopamine

Striatal synaptosomes were incubated at 37°C with reserpine (10 uM) for 15 min, followed by addition of dopamine (10 uM) for an additional 45 min. Data are presented in order of increasing % change in tissue GSSG. Individual experiments were compared by 2-tailed t-tests, and the pooled means by a paired t-test.

Expt.	Control (pmol GSSG/mg ± SEM)	Res + DA	Increase in GSSG pmoles/mg	%
1	20.0 ± 0.1	22.2 ± 0.2**	2.2	11.0
2	17.2 ± 0.3	19.6 ± 0.0**	2.4	14.0
3	26.5 ± 0.2	31.0 ± 0.5**	4.5	14.5
4	21.4 ± 0.0	25.3 ± 0.1**	3.9	18.2
5	17.9 ± 0.3	21.3 ± 0.2**	3.4	19.0
6	18.1 ± 0.2	21.9 ± 0.3**	3.8	21.0
7	28.5 ± 0.4	35.0 ± 0.2**	6.5	22.8
8	13.2 ± 0.2	16.4 ± 0.1**	3.2	24.1
9	19.9 ± 0.3	26.5 ± 0.2**	6.6	33.2
10	17.2 ± 0.2	23.0 ± 0.2**	5.8	33.7
11	6.6 ± 0.4	9.0 ± 0.1**	2.5	37.7
Mean ± SEM	18.8 ± 1.8	22.8 ± 2.0**	4.1 ± 0.5	22.9 ± 2.6

** ($p < 0.01$)

with reserpine and DA. The values shown are the means of the replicate samples in each experiment. Results were statistically significant in each experiment. Mean steady state levels of GSSG, pooled across eleven experiments, were increased by 22.9 ± 2.6% (SEM) during incubation with 10 uM reserpine and 10 uM DA. In these experiments, no GSSG was detected in the incubation medium; the GSSG remained in the tissue.

Table 3 shows results of experiments in which GSSG was measured in striatal synaptosomes after incubation with reserpine and L-dopa. In each of the eleven experiments a significant elevation in GSSG was detected after the incubation. The rise in GSSG ranged from 3.3 to 10.5 pmoles/mg original striatal tissue ($p < 0.001$-0.01), constituting an increment in the range 19.9-73.4% (mean 38.0% ± 4.5% SEM).

3.1.2b: Reserpine alone

In other experiments, the effect of reserpine alone was investigated. Incubation with reserpine for one hour in 4 experiments produced a mean rise of 2.7 ± 0.2 (SEM) pmoles GSSG/mg original striatal tissue, which was less than that seen in the 11 experiments with reserpine + 10 uM DA (4.1 ± 0.5 pmoles GSSG/mg, Table 2) or the 11 experiments with reserpine + 1.0 mM L-dopa (7.0 ± 0.7 pmoles GSSG/mg, Table 3). Moreover, although the mean rise in GSSG with reserpine alone did achieve statistical significance ($p < 0.005$;

TABLE 3

GSSG levels after treatment of synaptosomes with reserpine + L-dopa

Striatal synaptosomes were incubated at 37°C with reserpine (10 uM) for 15 min, followed by addition of L-dopa (1 mM) for an additional 45 min. Data are presented in order of increasing % change in tissue GSSG. Individual experiments were compared by 2-tailed t-tests, and the pooled means by a paired t-test.

Expt.	Control (pmol GSSG/mg ± SEM)	Res + L-dopa	Increase in GSSG pmoles/mg	%
1	16.6 ± 0.2	19.9 ± 0.3***	3.3	19.9
2	19.0 ± 0.8	23.6 ± 0.5**	4.6	24.2
3	19.8 ± 0.0	25.3 ± 0.9**	5.5	27.8
4	20.2 ± 1.0	26.2 ± 1.0**	6.0	29.7
5	21.0 ± 0.4	28.0 ± 0.6***	7.0	33.3
6	20.2 ± 1.2	27.0 ± 0.5**	6.8	33.7
7	19.5 ± 0.0	26.6 ± 1.0***	7.1	36.1
8	21.9 ± 0.8	31.8 ± 1.2**	9.9	45.2
9	15.6 ± 0.2	22.7 ± 0.2***	7.1	45.5
10	18.8 ± 0.7	28.1 ± 0.5***	9.3	49.5
11	14.3 ± 0.6	24.8 ± 0.0***	10.5	73.4
Mean ± SEM	18.8 ± 0.7	25.8 ± 0.9***	7.0 ± 0.7	38.0 ± 4.5

** ($p < 0.01$)
*** ($p < 0.001$)

paired 2-tailed t-test), significance was not always seen in the individual experiments. Hence, addition of DA or L-dopa provided a better working model for experiments with drug combinations (see ahead). The ineffectiveness of reserpine by itself may be due, in part, to a marked loss of tissue DA during isolation of the P2 pellet.

3.1.2c: Varying doses of dopamine or L-dopa

Table 4 shows a dose-response curve for varying doses of L-dopa or DA in the presence of reserpine. The rises in GSSG were 5.8, 9.5, 11.2 pmoles/mg tissue for the doses of DA (0.01 0.02, and 0.04 mM respectively) and 3.4, 4.9, and 9.5 for the doses of L-dopa (0.04, 0.20, and 1.00 mM respectively). These results show that the accumulation of GSSG was dependent upon the concentration of DA or L-dopa.

3.1.2d: L-dopa with and without reserpine

Table 5 shows experiments in which reserpine was present or absent during incubation with 0.2 mM or 1.0 mM L-dopa. With 0.2 mM L-dopa, the mean rise in GSSG produced by L-dopa in combination with reserpine (C-A) in 2 experiments shown in the table (6.7 pmoles GSSG/mg) was greater than that seen with L-dopa by itself (B-A) (3.8 pmoles GSSG/mg). The corresponding rises in GSSG with 1.0 mM L-dopa (12.5 pmoles/mg with reserpine (C-A) compared to 12.1 pmoles/mg without reserpine (B-A)) were greater than those observed with 0.2 mM L-dopa. These experiments show that reserpine amplified the response with 0.2 mM L-dopa ($p <$

TABLE 4

GSSG levels after treatment of synaptosomes with reserpine and varying doses of dopamine or L-dopa

Striatal synaptosomes were incubated at 37°C with reserpine (10 uM) for 15 min, followed by addition of DA or L-dopa at various concentrations for an additional 45 min.

Conc. of DA (mM)	Rise in GSSG (pmoles/mg tissue)
0.01	5.8
0.02	9.5
0.04	11.2

Conc. of L-dopa (mM)	Rise in GSSG (pmoles/mg tissue)
0.04	3.4
0.20	4.9
1.00	9.5

TABLE 5

GSSG levels after treatment of synaptosomes with L-dopa alone or reserpine + L-dopa

Striatal synaptosomes were incubated with reserpine (10 uM) or buffer for 15 min, followed by addition of L-dopa (0.2 mM or 1.0 mM) for an additional 45 min.

CONTROL (A)	L-DOPA (B)	(B-A)	RES/L-DOPA (C)	(C-A)
(pmoles GSSG/mg striatal tissue ± SEM)				
	..... 0.2 mM L-Dopa			
18.5 ± 0.5	22.4 ± 0.2	3.9	24.8 ± 0.3	6.3
16.9 ± 0.5	20.6 ± 0.0	3.7	24.0 ± 0.2	7.1
	..... 1.0 mM L-Dopa			
19.1 ± 0.3	32.3 ± 0.3	13.2	32.6 ± 0.6	13.5
18.0 ± 1.1	28.9 ± 0.5	10.9	29.5 ± 0.5	11.5

0.05), but not with 1.0 mM L-dopa ($p > 0.80$).

3.1.3: Drug manipulations

In the experiments that follow, in order to maintain a single cytoplasmic pool that would not be affected by changes in the amount of DA that can be stored in vesicles, reserpine was added along with DA or L-dopa.

3.1.3a: MAO inhibitors

In an attempt to link the increase in the level of GSSG to DA metabolism by MAO, experiments were performed in which MAO inhibitors were used. Clorgyline, in low concentration, acts as a selective inhibitor of MAO-A (Johnston, 1968). In the experiments shown in Figure 7 and Table 6, a relatively high concentration (10 uM) was used in order to achieve a non-selective inhibition of both the A and B forms of the enzyme. Experiments based on selective MAO-A and MAO-B inhibition were conducted in vivo and are discussed in Chapter 4.

Figure 7 shows results of experiments in which clorgyline was added along with reserpine 15 min prior to the addition of DA. The rise in GSSG caused by reserpine and DA was suppressed 85% by clorgyline.

Table 6 shows similar experiments with reserpine and L-dopa. The rise in GSSG induced by reserpine plus L-dopa was suppressed by clorgyline from 6.8-9.9 pmoles/mg tissue to 0.1-1.0 pmoles/mg, constituting a mean suppression of 92%. Therefore, GSSG accumulation requires MAO activity. In an

FIGURE 7

Effect of clorgyline on the GSSG rise produced by reserpine/dopamine

Striatal synaptosomes were incubated at 37°C with 10 uM reserpine. Clorgyline (10 uM) was added at zero time and DA (10 uM) was added at 15 min. Experiments were terminated at 45 min after the addition of DA.

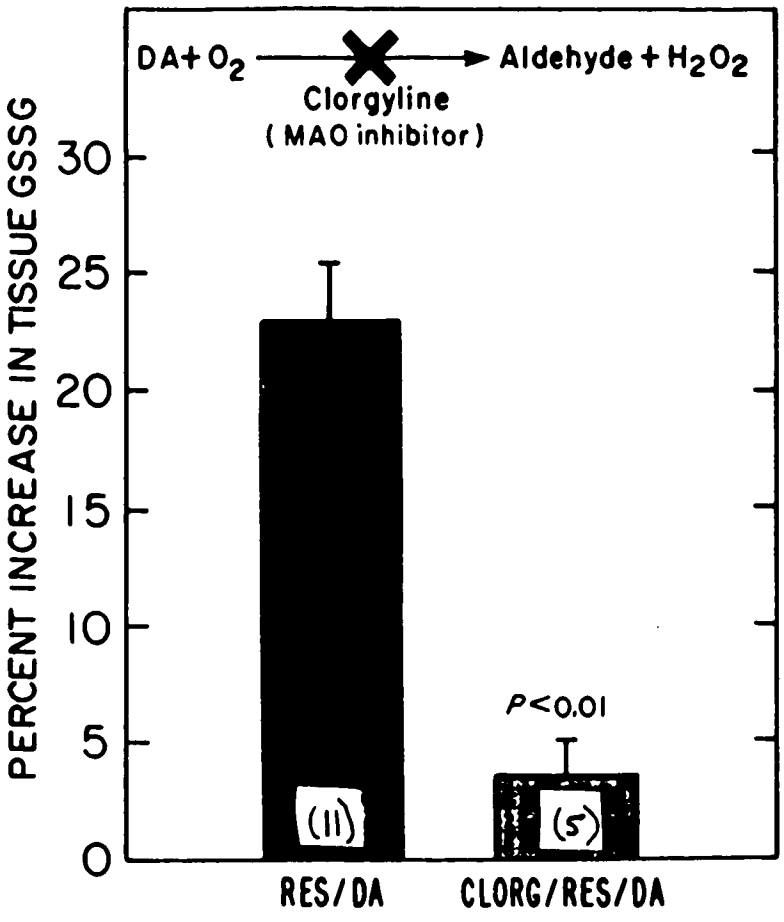


TABLE 6

GSSG levels after treatment with reserpine + L-dopa or reserpine + L-dopa + clorgyline

Striatal synaptosomes were incubated at 37°C with 10 uM reserpine. Clorgyline (10 uM) was added, where indicated, at zero time, and L-dopa (1 mM) was added at 15 min. Experiments were terminated at 45 min after the addition of L-dopa. In separate experiments, clorgyline alone had no effect on control levels of GSSG. In each experiment, the effect of reserpine plus L-dopa was significant ($p < 0.01$). Multiple comparisons were done by ANOVA followed by Newman-Keuls test.

pmoles GSSG/mg original tissue (± SEM)

	(A)	(B)	(C=B-A)	(D)	(E=D-A)
Expt	Control	Res/L-dopa		Res/L-dopa/clor	
1	15.6 ± 0.2	22.7 ± 0.2	7.1	16.4 ± 0.4**b	0.8
2	20.2 ± 1.2	27.0 ± 0.5	6.8	20.3 ± 0.2**b	0.1
3	21.9 ± 0.8	31.8 ± 1.2	9.9	22.9 ± 0.3**b	1.0
Mean ± SEM	19.2 ± 1.9	27.2 ± 2.7**a	7.9±1.0	19.9 ± 1.9**b	0.6±0.3***c

** $p < 0.01$; *** $p < 0.001$

[a]Compared to (A); [b]Compared to (B); [c]Compared to (C)

additional experiment, 10 uM pargyline was substituted for the clorgyline. Pargyline, in low concentration, is relatively selective for MAO-B; but at the dose employed, it inhibits both forms of MAO (Williams and Walker, 1984). Pargyline similarly suppressed the rise in GSSG during incubation with reserpine and L-dopa; the rise was suppressed from 6.7 pmoles/mg tissue to 0.8 pmoles/mg, constituting a suppression of 88% ($p < 0.001$, n=3 per group). Although L-dopa or DA can react spontaneously with oxygen to yield H_2O_2 (autoxidation), the results with MAO inhibitors indicate that production of H_2O_2 via oxidative deamination of DA by MAO was dominant in these experiments.

3.1.3b: Catalase

The resuspended P2 pellet contains MAO that is associated with both intrasynaptosomal mitochondria and free mitochondria in the medium. In order to distinguish the two sites for H_2O_2 production, catalase (2 ug/ml) was added to the medium. Catalase is a scavenger of H_2O_2; it can intercept the H_2O_2 produced in the medium by free mitochondria. The results in Table 7 show that catalase suppressed the rise in GSSG from 9.1 $\pm$ 0.7 pmoles/mg to 4.6 $\pm$ 0.4 pmoles/mg, in three experiments. The contribution to the GSSG level by intrasynaptosomal oxidation of dopamine by MAO was in the range 45-56%. The remainder derives from the penetration of externally generated H_2O_2 (oxidative deamination of dopamine in the medium) into the

TABLE 7

GSSG levels after treatment with reserpine + L-dopa, with and without catalase

Striatal synaptosomes were incubated at 37°C with 10 uM reserpine, with and without addition of catalase (2 ug/ml). L-Dopa (1 mM) was added at 15 min and the incubation was continued for an additional 45 min. In each experiment the effect of reserpine plus L-dopa was significant in both the presence and absence of catalase ($p < 0.001$).

pmoles GSSG/mg original tissue ± SEM

Without catalase			With catalase		
(A)	(B)	(C=B-A)	(D)	(E)	(F=E-D)
Control	Res + L-dopa		Control	Res + L-dopa	
17.5 ± 0.0	26.4 ± 0.0	8.9	13.1 ± 0.5	17.1 ± 0.0	4.0
16.5 ± 0.6	26.9 ± 0.9	10.4	14.3 ± 0.2	19.7 ± 0.9	5.4
16.6 ± 0.0	24.6 ± 0.2	8.0	13.7 ± 0.2	18.2 ± 0.4	4.5
Mean ± SEM					
16.9 ± 0.3	25.9 ± 0.7**a	9.1±0.7	13.7 ± 0.4**a	18.3 ± 0.8**b	4.6±0.4**c

** $p < 0.01$

[a]Compared to (A); [b]Compared to (D); [c]Compared to (C)

synaptosomes, with a minor contribution from the autoxidation of the added 1.0 mM L-dopa.

3.1.3c: Dopa-decarboxylase inhibitors

Experiments were performed with two inhibitors of dopa-decarboxylase, namely, NSD-1055 and carbidopa. Table 8 shows the results of experiments in which the tissue preparations were pre-incubated with the dopa-decarboxylase inhibitors for 30 min before adding 1.0 mM L-dopa and continuing the incubation for an additional 45 min. For both drugs, a significant suppression in GSSG was seen: 9.0 $\pm$ 1.0 pmole/mg without NSD-1055 vs 4.5 $\pm$ 0.7 pmoles/mg with NSD-1055 (50% suppression) and 8.7 $\pm$ 0.4 pmole/mg without carbidopa vs 3.5 $\pm$ 0.2 with carbidopa (60% suppression).

3.1.4: HPLC Analysis

Table 9 shows the levels of DA and DOPAC after incubation with the various drugs used in the prior experiments. In the presence of reserpine and 1 mM L-dopa, both DA and DOPAC levels rose significantly ($p < 0.001$). When clorgyline was added, the level of DOPAC was suppressed by 92% ($p < 0.001$) and simultaneously, the level of DA was increased by an equivalent amount. This result correlates with the 92% suppression of GSSG formation by clorgyline in Table 6 and verifies that GSSG formation reflects production of H_2O_2 by MAO. In the presence of 2 ug/ml catalase, the level of DOPAC was not altered (Table 9, $p > 0.20$). This result is as expected because catalase suppresses the rise

TABLE 8

GSSG levels after treatment with reserpine/L-dopa in the presence and absence of dopa decarboxylase inhibitors (DDI)

Striatal synaptosomes were incubated at 37°C with and without 0.25 mM NSD-1055 or 0.25 mM carbidopa. Reserpine (10 uM) and the dopa-decarboxylase inhibitor were added at zero time. L-Dopa (1 mM) was added after 30 min and the incubation was continued for an additional 45 minutes. In each experiment the effect of reserpine and L-dopa was significant ($p < 0.001$).

	pmoles GSSG/mg original tissue ± SEM				
(A)	(B)	(C=B-A)	(D)	(E)	(F=D-E)
Control	Res + L-dopa		Res+L-dopa+DDI	DDI	
				NSD-1055	
19.5 ± 0.0	26.6 ± 1.0	7.1	24.2 ± 0.3	18.9 ± 0.4	5.3
14.3 ± 0.6	24.9 ± 0.0	10.6	21.8 ± 0.3	16.7 ± 0.0	5.1
18.7 ± 0.7	28.1 ± 0.5	9.3	25.2 ± 0.4	22.1 ± 0.8	3.1
Mean 17.5	26.5**a	9.0	23.7**ab	19.2	4.5*c
±SEM ±1.6	±0.9	±1.0	±1.0	±1.6	±0.7
				Carbidopa	
16.6 ± 0.0	24.6 ± 0.2	8.0	22.7 ± 0.2	19.0 ± 0.4	3.7
17.5 ± 0.0	26.4 ± 0.0	8.9	24.4 ± 0.2	21.3 ± 0.2	3.1
16.0 ± 0.5	25.2 ± 0.3	9.2	22.8 ± 0.4	19.2 ± 0.3	3.6
Mean 16.7	25.4**a	8.7	23.3**ab	19.8	3.5**c
±SEM ±0.4	±0.5	±0.4	±0.6	±0.7	±0.2

* $p < 0.05$, ** $p < 0.01$

[a] Compared to (A); [b] Compared to (E); [c] Compared to (C)

TABLE 9

Dopamine and DOPAC levels after treatment with drugs

Quadruplicate suspensions of striatal synaptosomes were incubated at $37^{0}C$ with 10 uM reserpine and 1 mM L-dopa, and additional drugs added as described in the legends of the other tables.

Sample	Dopamine	DOPAC
	ug/ml (Mean ± SEM)	
Control	nd	0.20 ± 0.01
Reserpine/L-dopa	0.80 ± 0.02***a	1.40 ± 0.05***a
Clorgyline/Res/L-dopa	2.20 ± 0.06***b	0.11 ± 0.02***b
Catalase/Res/L-dopa	0.80 ± 0.01	1.50 ± 0.07
NSD/Res/L-dopa	nd***b	0.05 ± 0.00***b
Carbidopa/Res/L-dopa	nd***b	0.18 ± 0.03***b

*** $p < 0.001$

[a]Compared to control

[b]Compared to Reserpine/L-dopa

nd - not detectable, less than 0.04 ug/ml

in GSSG (Table 7) by scavenging H_2O_2 and not by a direct action on MAO. In the presence of the decarboxylase inhibitors, NSD-1055 and carbidopa, both DOPAC and DA levels were suppressed ($p < 0.001$), in keeping with the suppression of GSSG levels (Table 8). However, whereas decarboxylation of L-dopa was completely suppressed (Table 9), the rise in GSSG was only partially prevented (Table 8). The reason for the discrepancy is not immediately apparent. Additional experiments were performed in which the effect of reserpine alone was studied. In the presence of reserpine, levels of DOPAC were not significantly elevated (control 0.21 ± 0.02 ug/ml, compared to reserpine 0.20 ± 0.02 ug/ml, $n = 6$ per group, $p > 0.60$); these results are consistent with the very weak effect of reserpine alone on GSSG levels. In separate experiments, a direct effect of the drugs on the GSSG assay system was excluded.

3.2: Discussion

Incubation of striatal synaptosomes with reserpine and 10 uM DA or reserpine and 1 mM L-dopa induces a significant rise in tissue GSSG (22.9% for reserpine + DA, Table 2; 38.0% for reserpine + L-dopa, Table 3). The rise is due almost exclusively to MAO activity as seen by the suppression in GSSG rise by MAO inhibitors (Table 6 & Figure 7). The suppression by MAO inhibitors also rules out any significant contribution of H_2O_2 from a spontaneous reaction of DA or L-dopa with oxygen at neutral pH (autoxidation).

The rise in GSSG signifies a change in the steady state level of GSSG. Since the incubation medium does contain glucose, the NADPH needed for glutathione reductase can be made via the pentose phosphate shunt. Therefore, the increase in GSSG is seen even in the presence of an active GSSG reductase. A rise in the steady state level of GSSG reflects the presence of an oxidant stress (Adams et al., 1983; Sies, 1985).

Lesion studies in rats have shown that 80-85% of dopa-decarboxylase in the striatum is restricted to nigrostriatal DA terminals (Hefti et al., 1981a; Melamed et al., 1981). The suppressive action of dopa-decarboxylase inhibitors on GSSG formation produced by reserpine and L-dopa (Table 8) indicates that decarboxylation of L-dopa within DA terminals is required. Under the conditions of this study, one half of the GSSG arises from intrasynaptosomal H_2O_2 production (Table 7, catalase data), while the remainder can be

attributed to H_2O_2 production during the oxidative deamination of extracellular DA by free mitochondria. This is the first demonstration that the turnover of a natural transmitter, starting from precursor amino acid, can evoke an oxidant stress within isolated nerve terminals.

Although the rise in GSSG detected in these experiments is quite small, it may represent a greater increase in a smaller pool of tissue, specifically the DA nerve terminals. It can be calculated that the DA nerve terminals represent less than 1% of the weight of tissue in the striatum. The average concentration of DA in the striatum is 10 ug/g wet weight or 65 uM. The concentration of DA within varicosities in the striatum has been estimated by fluorescence microscopy to be 50 mM (Anden et al., 1966). Based upon these two figures, DA terminals represent about 0.13% by weight of the striatum (0.065 mM/50 mM x 100% = 0.13%). Other investigators (Levine et al., 1981) used a rough estimate of 1% DA terminals by weight in the striatum for other calculations. When the P2 pellet is isolated, then the relative proportion of DA nerve terminals in the pelleted tissue is increased, since the cell bodies and other cellular debris have been removed. If we use a rough estimate of 10% DA nerve terminals by weight in the synaptosomes, then the observed increases in GSSG (3.3-10.5 pmol/mg original tissue) represent increments of 33-105 uM within DA synaptosomes. If we assume only 50 % of the rise

is due to intraneuronal DA metabolism (based on the catalase data), then there is a rise of 15-50 uM GSSG within DA nerve terminals.

When GSSG levels rise, there may be biological consequences. If a major fraction of GSH is lost, the main mechanism for the detoxification of H_2O_2 (namely, GSH peroxidase) will be compromised. Therefore, levels of H_2O_2 will rise and lipid peroxidation may be induced. The overall integrity of the cell may be compromised. Beyond the consequences of a fall in GSH levels, an accumulation of GSSG can also induce biological changes. GSSG can inhibit key SH-dependent enzymes by making mixed disulfides with protein SH groups. Gilbert (1982) has shown that GSSG can act as a third messenger to evoke changes in cellular systems or enzymes. Such events as inactivation of phosphofructokinase and glucokinase are seen in tissues when GSSG levels rise into the 50-200 uM range. These levels of GSSG are within the range of those estimated to be formed in the nerve terminals. Based upon the observations with striatal synaptosomes, it seems possible that damaging events may occur when DA turnover is potentiated.

Many investigators routinely use experimental conditions such as those described here. Concentrations of L-dopa in the range 0.2-1.0 mM are not uncommon for in vitro studies with brain tissue (Ng et al., 1972; Heikkila et al., 1975); and catecholamines are often added in concentrations as high as 10 uM (Brannan et al., 1982; Olson and Malmfors,

1970). It is apparent that studies carried out under these conditions can induce an oxidative stress within the target neurons.

Doses of L-dopa in the range 50-250 mg/kg are often administered experimentally to rats (e.g., Buu et al., 1985; Hefti et al., 1981a; Lyles and Callingham, 1980). Peak levels of 0.05 mM L-dopa have been observed in the striatum of rats after 100 mg L-dopa/kg administered with carbidopa as a peripheral dopa decarboxylase inhibitor (Hefti et al., 1981a). In the absence of carbidopa, levels of 0.04 mM in the hippocampus and 0.006 mM in the striatum have been reported after 100 mg/kg L-dopa (Ehrenstrom and Johansson, 1985). Some of these values fall within the range of concentrations used in our experiments (0.04 mM, Table 4). Therefore, it is necessary to consider that an oxidant stress may be evoked in DA nerve terminals in vivo during treatment of experimental animals with L-dopa.

Chapter 4: In-vivo experiments: Results and discussion

4.1: Results

Experiments were conducted in which DA turnover was provoked in vivo by injection of either reserpine or haloperidol. Reserpine increases DA turnover by blocking the storage of DA in synaptic vesicles, while haloperidol increases DA turnover by increasing neuronal firing, DA synthesis and DA release. Mice were injected with either drug and levels of GSSG were measured in the striatum.

4.1.1: Reserpine experiments

4.1.1a: Reserpine alone

Initial experiments were performed in which GSSG was measured in the striatum of mice after treatment with reserpine. Mice were injected with 10 mg/kg reserpine and 2 hours later the striata were dissected and analyzed for GSSG. L-dopa was not injected in these experiments. Table 10 shows the results of 8 separate experiments. In each experiment there was a significant rise in GSSG. The increase in GSSG ranged from 4.3 to 10.0 uM (pmoles/mg striatum), producing a mean percentage increase of 96.5 $\pm$ 10.8%.

After treatment with reserpine, DA levels as well as the metabolites, DOPAC and HVA were measured by HPLC. The data in Table 11 permit the rise in GSSG to be compared to changes in DA and acid metabolites. The mean rise in GSSG was 5.1 uM. Over this time period, the mean DA level fell

TABLE 10

GSSG levels in mouse striata after treatment with reserpine

Mice were injected with 10 mg/kg reserpine. After 2 hours, the mice were sacrificed and the striata were dissected and analyzed for GSSG. Results are the means ± SEM for each experiment with 3-4 mice per group.

Expt.	Control	Reserpine	Increase in GSSG	
	(uM GSSG ± SEM)		(uM)	%
1	12.8 ± 0.2	17.1 ± 0.0***	4.3	34
2	6.4 ± 0.3	11.2 ± 0.3***	4.8	75
3	5.4 ± 0.5	10.6 ± 0.2***	5.2	96
4	6.1 ± 0.6	12.4 ± 0.1***	6.3	103
5	5.1 ± 0.4	10.4 ± 0.2***	5.3	104
6	5.2 ± 0.2	10.7 ± 0.3***	5.5	106
7	8.3 ± 0.9	18.3 ± 0.1***	10.0	120
8	5.3 ± 0.2	12.4 ± 0.3***	7.1	134
Mean	6.8 ± 0.9	12.9 ± 1.1***	6.1 ± 0.6	96.5 ± 10.8

*** $p < 0.001$, compared to control

TABLE 11

GSSG, DA, DOPAC, and HVA levels in mouse striata after treatment with reserpine

Mice were injected with 10 mg/kg reserpine. After 2 hours, the striata were dissected and analyzed for GSSG, DA, DOPAC, and HVA. Results are the mean ± SEM with n=3.

	GSSG	DA	DOPAC	HVA
		(uM)		
Control	7.6 ± 0.5	50.9 ± 1.4	4.8 ± 0.2	5.9 ± 0.2
Reserpine	12.8 ± 1.2	1.4 ± 0.2	8.8 ± 0.8	12.3 ± 1.1
Change (Res - Control)	+5.1*	-49.5*	+4.0*	+6.4*

* The changes are all significant ($p < 0.001$).

in the striatum from 50.9 to 1.4 uM, while the levels of DOPAC and HVA doubled. DOPAC levels rose from 4.8 to 8.8 uM and HVA from 5.9 to 12.3 uM. The latter results confirm the increase in MAO activity. The rise in GSSG (5.1 uM) can be accounted for by the H_2O_2 produced during the loss of DA (49.5 uM).

4.1.1b: Striatum vs. cortex

The striatum is an area enriched with DA nerve terminals, while the cortex is an area which receives a much sparser innervation. The effects of reserpine on GSSG levels in the striatum and frontal cortex are compared in Table 12. The striatum exhibited a significant rise in GSSG after reserpine, while the frontal cortex did not. The mean change in three experiments was +5.0 $\pm$ 0.3 uM in the striatum and -0.3 $\pm$ 0.6 uM in the cortex. Therefore, the response produced by reserpine on GSSG levels parallels the innervation by catecholamine neurons.

4.1.1c: MAO inhibitors

Table 13 shows the results of experiments in which animals were pretreated with clorgyline, a selective MAO-A inhibitor, 18 hours prior to treatment with reserpine. Clorgyline was given at a dose of 2.5 mg/kg, which is selective for inhibition of MAO-A in Swiss Webster mice (Kindt et al., 1988). In each of the four experiments, clorgyline suppressed the rise in GSSG produced by reserpine alone. Reserpine produced a rise of 5.2 $\pm$ 0.4 uM GSSG,

TABLE 12

GSSG levels in mouse striatum and frontal cortex after treatment with reserpine

Mice were injected with 10 mg/kg reserpine. After two hours, the mice were sacrificed and the striata and frontal cortex were dissected and analyzed for GSSG. Results are the mean ± SEM for each experiment with 3-4 mice per group.

Striatum

Expt.	Control	Reserpine	Increase in GSSG
	GSSG (uM ± SEM)		
1	5.2 ± 0.5	10.7 ± 0.3***	5.5
2	8.5 ± 0.3	13.4 ± 0.2***	4.9
3	3.6 ± 0.3	8.2 ± 0.3***	4.6
Mean	5.8 ± 1.4	10.8 ± 1.5***	5.0 ± 0.3

Frontal Cortex

Expt.	Control	Reserpine	Increase in GSSG
	GSSG (uM ± SEM)		
1	5.4 ± 0.6	4.1 ± 0.2	-1.3
2	8.3 ± 0.4	9.0 ± 0.2	0.7
3	3.6 ± 0.3	3.4 ± 0.2	-0.2
Mean	5.8 ± 1.4	5.5 ± 1.8	-0.3 ± 0.6

*** $p < 0.001$, compared to controls

TABLE 13

Effect of clorgyline on GSSG levels after pretreatment with reserpine

Mice were injected with 2.5 mg/kg clorgyline, and 18 hours later with 10 mg/kg reserpine. At two hours after reserpine, the mice were sacrificed and the striata were dissected and analyzed for GSSG. Results are the mean for each experiment with 3-4 mice per group.

	Rise in GSSG (uM)	
Expt.	Reserpine	Reserp/clor
1	5.3	-0.2
2	6.3	0.6
3	4.8	1.1
4	4.3	0.7
Mean	5.2 $\pm$ 0.4	0.6 $\pm$ 0.3***

***$p < 0.001$ compared to reserpine alone.

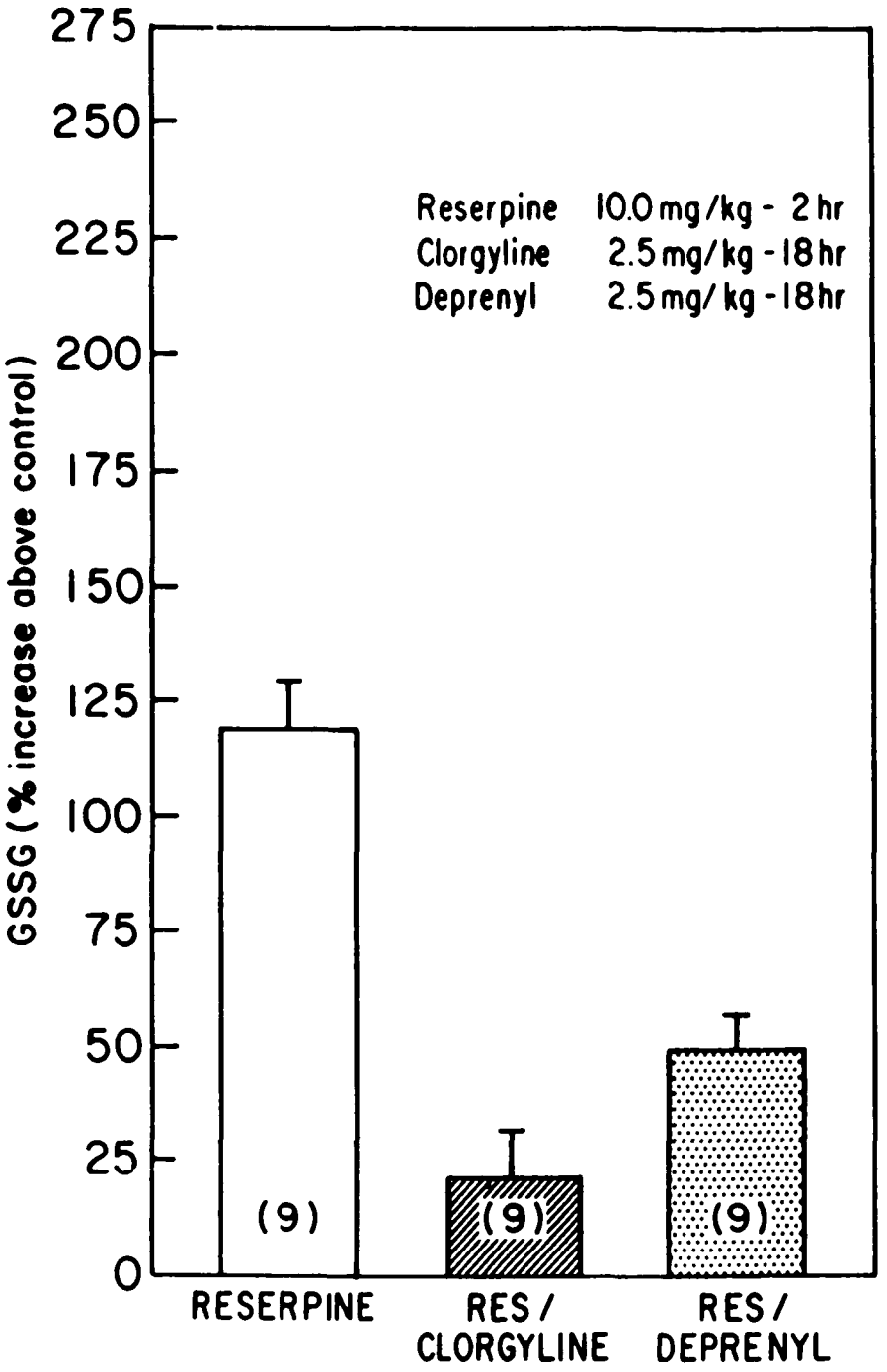
whereas with clorgyline pretreatment there was a rise of only 0.6 ± 0.3 uM GSSG. Clorgyline caused a mean suppression of 88% in the rise in GSSG levels. Clorgyline alone did not affect basal GSSG levels (control 5.6 ± 0.6 uM, and clorgyline-treated 5.8 ± 0.1 uM, n = 3). These results indicate that the rise in GSSG produced by reserpine is due to MAO activity.

Figure 8 shows the results in which the effects of deprenyl (a selective MAO-B inhibitor) and clorgyline were studied. The MAO inhibitors were administered 18 hours prior to reserpine at a dose of 2.5 mg/kg (a selective MAO-A or MAO-B dose, Kindt et al., 1988). Reserpine alone produced an increase in GSSG levels of 119 ± 10% (7.5 uM) above control. When the animals were pretreated with clorgyline, the increase in GSSG was suppressed by 82% (7.5 uM for reserpine alone vs. 1.4 uM for reserpine + clorgyline). Deprenyl suppressed the rise in GSSG by 58% (7.5 uM for reserpine alone vs. 3.2 uM for reserpine + deprenyl). Deprenyl alone, similar to clorgyline alone, did not have an effect on basal GSSG levels (control 7.0 ± 0.4 uM, and deprenyl-treated 7.0 ± 0.1 uM, n=4). The data in Figure 8 indicate that the rise in GSSG produced by reserpine is due to the metabolism of DA by both MAO-A and MAO-B, with MAO-A making a significantly stronger contribution.

FIGURE 8

Effects of clorgyline and deprenyl on the GSSG rise produced by reserpine

Error bars show the SEM for each group.



4.1.2: Haloperidol experiments

4.1.2a: Haloperidol alone

Experiments were performed in which GSSG was measured in the striatum of mice after treatment with haloperidol. Mice were injected with 1 mg/kg haloperidol and 1 hour later the striata were dissected and analyzed for GSSG. Table 14 shows the results of 8 independent experiments. In each experiment there was a significant rise in GSSG levels. The increase in GSSG ranged from 7.5 to 14.6 uM, producing a mean percentage increase of 210 $\pm$ 27%.

After treatment with haloperidol, DA, DOPAC, and HVA levels in the striatum were measured by HPLC. Table 15 compares the rise in GSSG to changes in the level of DA and metabolites. In these experiments, GSSG levels rose by 11.2 uM. DOPAC and HVA levels rose: DOPAC rose from 4.7 uM to 12.3 uM and HVA from 8.3 uM to 17.3 uM. These results confirm the expected increase in DA turnover. At the same time, DA levels rose from 43.7 uM to 56.4 uM. This is as expected from the increased biosynthetic activity.

4.1.2b: 6-Hydroxydopamine lesion experiments

In order to determine if the rise in GSSG produced by haloperidol was dependent on the presence of catecholamine nerve terminals, a lesion experiment was performed. Rats were injected with 6-hydroxydopamine in the right substantia nigra in order to destroy the dopaminergic innervation to the right striatum. In this way, the left (normal) and

TABLE 14

GSSG levels in mouse striata after treatment with haloperidol

Mice were injected with 1 mg/kg haloperidol. After 1 hour, the mice were sacrificed and the striata were dissected and analyzed for GSSG. Results are the means ± SEM with 3-4 mice per group.

Expt.	Control	Haloperidol	INCREASE IN GSSG	
	(uM GSSG ± SEM)		(uM)	%
1	11.4 ± 0.5	24.3 ± 0.3***	12.9	113
2	9.4 ± 0.3	25.6 ± 0.4***	14.2	151
3	5.7 ± 0.8	14.4 ± 0.1***	8.7	153
4	8.4 ± 1.8	23.0 ± 0.9***	14.6	174
5	5.8 ± 0.1	17.5 ± 0.3***	11.7	202
6	2.7 ± 0.5	10.2 ± 0.6***	7.5	278
7	4.5 ± 0.4	17.3 ± 0.8***	12.8	284
8	4.9 ± 0.5	20.7 ± 0.4***	15.8	322
Mean	6.6 ± 1.0	19.1 ± 1.9***	12.3 ± 1.0	210 ± 27

*** $p < 0.001$

TABLE 15

GSSG, DA, DOPAC, and HVA levels in mouse striata after treatment with haloperidol

Mice were injected with 1 mg/kg haloperidol. After 1 hour, the striata were dissected and analyzed for GSSG, DA, DOPAC, and HVA. Results are the mean ± SEM with n=3.

	GSSG	DA	DOPAC	HVA
		(uM)		
Control	7.4 ± 0.5	43.7 ± 1.3	4.7 ± 0.2	8.3 ± 0.3
Haloperidol	18.6 ± 1.4	56.4 ± 2.2	12.3 ± 1.2	17.3 ± 1.4
Change	+11.2*	+12.7*	+7.6*	+9.0*

* The rises in GSSG, DA, DOPAC, and HVA were all significant ($p < 0.001$).

and right (denervated) sides could be compared. Animals were allowed to recover for one week and then they were injected with haloperidol. The animals with successful lesions were identified by the loss of DA, which was assessed by HPLC at the termination of the experiments. The effect of haloperidol on GSSG was measured in the denervated striatum. The contralateral striatum was used as the control. Table 16 shows the results of these experiments. Haloperidol caused an increase in GSSG of 7.1 uM on the control side, while the rise was only 0.8 uM on the lesioned side. These results indicate that the response produced by haloperidol on GSSG levels is dependent on the presence of catecholamine neurons.

4.1.2c: MAO inhibitors

Figure 9 shows the results of experiments in which the effects of MAO inhibitors on the haloperidol-induced rise in GSSG were studied. Deprenyl and clorgyline were administered as previously described for experiments with reserpine. Haloperidol alone produced an increase in GSSG levels of 235 $\pm$ 30% (12.5 uM) above control. When animals were pretreated with clorgyline, the increase in GSSG was suppressed by 54% (12.5 uM for haloperidol alone vs. 5.8 uM for haloperidol + clorgyline). Deprenyl suppressed the increase in GSSG by 69% (12.5 uM for haloperidol alone vs. 3.8 uM for haloperidol + deprenyl). These data indicate that the rise in GSSG produced by haloperidol is due to the

TABLE 16

DA and GSSG levels in the striata of 6-OHDA lesioned rats after treatment with 1 mg/kg haloperidol

Rats were injected unilaterally with 6-OHDA in the right substantia nigra. After a recovery period of one week, haloperidol was administered. One hour later, the striata were dissected and analyzed for DA and GSSG levels. Results are the mean ± SEM with n=9.

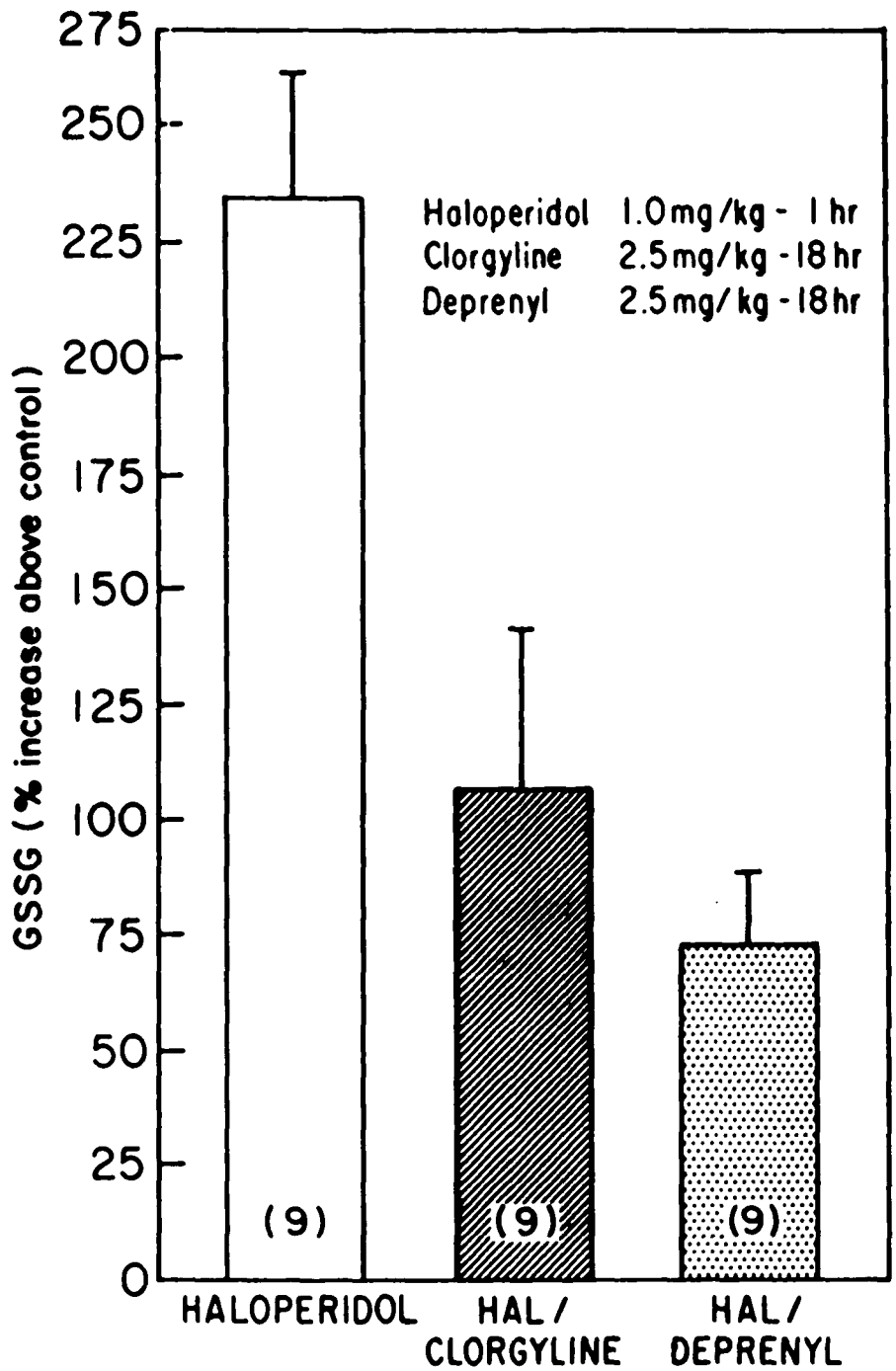
	GSSG	Increase in GSSG above control*	DA
	(uM)	(uM)	(uM)
Lesioned side	3.4 ± 0.9	0.8	4.0 ± 1.3
Contralateral Side	9.7 ± 0.3	7.1	42.0 ± 2.0

*Unlesioned animals were used as controls. GSSG levels in control animals were 2.6 uM (n=3 rats).

FIGURE 9

Effects of clorgyline and deprenyl on the GSSG rise produced by haloperidol

Error bars show the SEM for each group.



metabolism of DA by both MAO-A and MAO-B, with MAO-B making a somewhat stronger contribution.

4.2: Discussion

Treatment with reserpine or haloperidol causes large increases in the level of GSSG in the striatum of mice (96.5% above control after reserpine [Table 10] and 210% above control after haloperidol [Table 14]). The observed increases in GSSG are due to the metabolism of DA by MAO. This is the first demonstration that the turnover of a neurotransmitter in vivo is associated with a significant oxidant stress.

Reserpine increases DA turnover by interfering with the uptake and storage of DA in synaptic vesicles (Cooper et al., 1986). Hence, the level of DA in the cytoplasm rises and, as a result, DA is no longer protected from degradation by MAO. Haloperidol, on the other hand, is a DA receptor antagonist. It increases DA turnover by increasing both the synthesis and release of DA (Cooper et al., 1986). This drug mimics a condition in which DA turnover might be increased in vivo, such as Parkinson's disease (Hornykiewicz and Kish, 1986).

Haloperidol increases the level of GSSG by 210%, a value twice that seen with reserpine (97%). At the time point studied, reserpine causes a depletion of DA, while haloperidol actually increases the DA levels due to increased biosynthetic activity. Haloperidol also increases the levels of DOPAC and HVA (indices of DA turnover) to a greater extent than reserpine (Tables 12 & 15). Thus, the markedly greater elevation in GSSG after haloperidol

correlates with a greater increase in DA turnover.

Based on the pharmacological actions of reserpine and haloperidol, it is expected that the rises in GSSG would be dependent upon the presence of catecholamine nerve terminals. This is illustrated by two different experimental approaches. First, in studies with reserpine, the striatum and cortex were compared. The level of DA in the striatum is approximately 10 ug/g, whereas in the cortex, the levels of catecholamines, both DA and NE, are less than 1 ug/g. While the striatum showed a large increase in GSSG after reserpine, the cortex, on the other hand, showed a lack of response (Table 12). Second, in studies with haloperidol, the left and right striata were compared in rats with a unilateral (right) nigrotomy. The control striatum showed a large increase in GSSG after haloperidol, while, the denervated striatum showed a lack of response (Table 16). Therefore, it seems clear that the GSSG rises produced in the striatum in vivo are dependent on the presence of catecholamine nerve terminals.

Whereas the increase in GSSG caused by reserpine and haloperidol is due to the metabolism of DA by MAO, the basal levels of GSSG are not derived from MAO activity, since neither clorgyline nor deprenyl altered the control levels (See text). It would appear that the basal GSSG may be more heterogenously distributed in the striatum, so that the contribution from normal DA turnover is not detectable.

The experiments which compared the effect of selective MAO inhibitors on both the reserpine-induced and haloperidol-induced rises in GSSG indicate a difference between the two models of increased DA turnover. The reserpine experiments indicate a more prominent effect of MAO-A, compared to MAO-B. On the other hand, the effects of MAO-A and MAO-B were more comparable in the haloperidol model.

In the experiments with reserpine, inhibition of GSSG accumulation by clorgyline was 82% (See Figure 8). When deprenyl was substituted for clorgyline, the observed inhibitory effect was 52%, which was much greater than expected. One possible explanation for this result is that the accumulation of GSSG under these experimental conditions may not be linearly related to the rate of production of H_2O_2. The reason for suggesting a non-linear response is that MAO-derived H_2O_2 rises from undetectable, under basal conditions, to the major contributor when DA turnover is increased by haloperidol or reserpine. A simple linear response would not have produced such a large increase in MAO-derived GSSG when DA turnover is increased two or three-fold. In a tissue compartment, such as glia, where both MAO-A and MAO-B are present (Riederer, et al., 1986), the GSSG accumulation may require contributions from both enzymes. Since there is a measurable increase in GSSG after treatment with reserpine and haloperidol, the implication is that the GSSG reductase is overwhelmed by the rate of

formation of GSSG. Under this circumstance, a tissue compartment where H_2O_2 is produced by both MAO-A and MAO-B, may be markedly affected by inhibition of either form of the enzyme. In the reserpine experiments, post-synaptic sites containing both MAO-A and MAO-B may be strongly affected (strongly suppressed in GSSG accumulation) by inhibition of either MAO-A or MAO-B, giving rise to an unexpected large effect of deprenyl. In the haloperidol experiments, much more of the metabolism of DA takes place post-synaptically and the two types of MAO-inhibitors give comparable results.

In the in vivo experiments described in this chapter, similar to the in vitro experiments (Chapter 3), the increases in the GSSG levels appear, at face value, to be small (only uM concentrations). However, as discussed in Chapter 3, these small changes may represent much greater alterations in GSSG in a smaller pool of tissue associated with DA synapses. Although the entire striatum is subjected to analysis for GSSG, only 1% or less of the tissue is DA nerve terminals. Reserpine caused a doubling of the GSSG levels and haloperidol caused a tripling. If most of the increase in GSSG occurs in DA nerve terminals, then the observed increases (6.1 uM GSSG with reserpine and 12.3 uM GSSG with haloperidol) could represent increments of 610 and 1230 uM (0.6 and 1.2 mM). If, based on the data with the MAO inhibitors, we assume only 50% of the rise in GSSG is within the DA nerve terminals, then there may be an

accumulation of 0.3-0.6 mM GSSG within DA terminals. This represents a corresponding loss of GSH of 0.6-1.2 mM. As discussed in Chapter 3, the loss of GSH and the accumulation of GSSG can be damaging to the neuron and may lead to biologic consequences. Inactivation of SH-dependent enzymes, and other biologic consequences, are seen when GSSG rises into the range 0.05-0.2 mM (Gilbert, 1982).

Chapter 5: Summary and Conclusions

The data presented in this thesis indicates that the turnover of dopamine both in vitro and in vivo is associated with a significant oxidant stress. This study used reserpine and haloperidol to model increased turnover in the striatum. Both treatments were accompanied by the production of H_2O_2 and a measurable oxidative stress, namely increased GSSG.

The data may shed some light on disease states which are accompanied by increased turnover of monoamines, such as Parkinson's, disease. Parkinson's disease is a progressive neurodegenerative disorder in which the dopamine neurons of the nigrostriatal tract are destroyed. The loss of the dopamine neurons occurs naturally during the aging process; however in Parkinson's disease there is an accelerated loss (Agid and Blin, 1987). These DA neurons control motor functions. The expression of parkinsonian symptoms occurs when approximately 80% or more of the nigrostriatal neurons are destroyed (Hornykiewicz and Kish, 1986). The symptoms include akinesia, tremor, and rigidity. The mechanism responsible for the selective degeneration of the nigrostriatal neurons is unknown. An oxidant stress within DA neurons represents a possible mechanism of toxicity (Cohen, 1983). The turnover of increased amounts of DA by MAO and the formation of H_2O_2 may cause a peroxidative stress which may lead to the eventual demise of the neuron.

Observations on brain DA and homovanillic acid (HVA) in

autopsy specimens from patients with Parkinson's disease indicate a rise in the HVA/DA ratio in the putamen, which was interpreted as increased turnover of DA (Hornykiewicz and Kish, 1986). Studies in rats with extensive lesions of the substantia nigra indicate increased DA turnover (relative increases in acid metabolites) in remaining DA nerve terminals in the striatum when the lesion exceeds 80% (Hefti et al., 1981a; Altar et al., 1987). Increased release, reuptake, and presynaptic turnover of DA, in surviving nigrostriatal neurons, may contribute to the progressive loss of DA neurons in Parkinson's disease, as previously suggested (Cohen, 1983).

The predominant treatment of Parkinson's patients is L-dopa therapy. L-dopa is given in an attempt to raise the level of DA in the striatum of the patients. With the expected rise in DA levels, comes an increase in the turnover of DA. In this regard, treatment with L-dopa needs to be viewed with caution on theoretic grounds, even though no direct evidence exists for L-dopa-mediated damage (Cotzias et al., 1977; Hefti et al., 1981b; Perry et al., 1984). It remains to be determined if treatment with L-dopa can induce either physiologic changes or damaging effects in parallel with the observations on elevations in GSSG reported here.

A new therapeutic trial of "antioxidants" in Parkinson's disease (Lewin, 1987) is currently under

investigation. Deprenyl is one of the drugs being tested. This is based on the theory that an oxidant stress contributes to the progressive loss of dopamine neurons. Deprenyl is currently used as an adjunct to therapy with L-dopa in Parkinson patients (Birkmayer et al., 1975; Yahr and Kaufmann, 1988). The results reported here show that increased neurotransmitter turnover can indeed alter the redox state of dopamine nerve terminals. The studies with haloperidol indicate that deprenyl does substantially inhibit the increase in GSSG caused by haloperidol. Therefore, deprenyl may be able to protect against the oxidant stress caused by increased DA turnover in patients with Parkinson's disease.

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