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MPTP, L-Deprenyl and Parkinson's Disease::

Pharmacological Implications of Oxidative Stress

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"A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Ph.D."

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TABLE OF CONTENTS

CHAPTER 1

General Introduction

CHAPTER 2

Effect of MPTP and L-Deprenyl on Antioxidant Enzymes and Lipid Peroxidation Levels in Mouse Brain

---

CHAPTER 3

The Effect of L-Deprenyl, D-Deprenyl and MDL72974 on Mitochondrial Respiration: A Possible Mechanism Leading to an Adaptive Increase in Superoxide Dismutase Activity

---

CHAPTER 4

L-Deprenyl and MDL72974 Do Not Improve the Recovery of Dopaminergic Cells Following Systemic Administration of MPTP in the Mouse

---

CHAPTER 5

Effect of the MAO-B Inhibitor, MDL72974, on Superoxide Dismutase Activity and Lipid Peroxidation Levels in the Mouse Brain

---

CHAPTER 6

General Discussion

ABSTRACT // RÉSUMÉ

ABSTRACT

Excessive free radical formation or antioxidant enzyme deficiency can result in oxidative stress, a mechanism proposed in the toxicity of MPTP and in the etiology of Parkinson's disease (PD). To be effective as a toxin, MPTP must be metabolized by monoamine oxidase-B (MAO-B) to form MPP^+. The latter compound leads to the degeneration of the dopaminergic cell bodies of the substantia nigra (SN) and striatal dopamine (DA) depletion that are reminescent of PD. The toxic effects of MPP^+ are related to the inhibition of NADH dehydrogenase activity in the mitochondrial respiratory chain. This event leads to the rapid depletion of ATP synthesis and loss of membrane potential to further enhance free radical formations. Similar respiratory deficits are reported in PD. The major antioxidant enzymes in the CNS are superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT). SOD catalyses the dismutation of superoxide ($O_2^{\cdot}$) radical into H_2O_2, while GSH-PX and CAT convert H_2O_2 to H_2O. Lipid peroxidation and SOD activity are increased in PD brains, whereas CAT and GSH-PX remain unchanged or reduced. This observation is suggestive of a deficiency in dealing with excessive H_2O_2 formation which exacerbates iron-catalysed free radical generations. To date however, it is unclear if the alteration in SOD activity observed in PD is sufficient to enhance free radical formation and, consequently, to increase lipid peroxidation. We were able to demonstrate that, despite marked alterations in SOD and CAT activities in the SN and striatum of MPTP-treated mice, lipid peroxidation remained unaffected. These results suggest that factors other than antioxidant enzymes play a pivotal role in the modulation of peroxidative damage to lipids as observed in PD. Surprisingly, L-deprenyl, a potent MAO-B inhibitor known

to protect dopaminergic neurons against the deleterious effects of MPTP by blocking MPP^+ formation, failed to reverse the effect of MPTP on the activity of antioxidant enzymes in mouse brain. In fact, L-deprenyl lead to similar alterations as those observed in MPTP-treated mice, including enhanced SOD activities. Several studies suggest that SOD activity is up-regulated in response to $O_2^{\cdot}$, leading us to evaluate the effects of L-deprenyl on catecholamine levels and mitochondrial functions, the major intracellular sources of free radicals. Our results suggested that alterations in SOD activity as observed in the brain of PD as well as in L-deprenyl- and MPTP-treated mice represented an adaptive response due to a leakage of $O_2^{\cdot}$ radicals resulting from dysfunctions of the mitochondrial respiratory chain. This is of particular interest since L-deprenyl has been proposed to exert a "rescuing" effect in degenerating dopaminergic neurons. However, this hypothesis has not been substantiated in clinical trials or on the recovery of DA levels in MPTP-treated mice. In addition, our results demonstrated a reduction in tyrosine hydroxylase (TH) immunopositive cell density when L-deprenyl was administered to MPTP-treated mice compared to animals administered MPTP alone. Accordingly, the possibility that L-deprenyl induces low oxidative stress by altering mitochondrial functions may be masked by its acute stimulatory effects on dopaminergic transmission.

RÉSUMÉ

Le stress oxydatif causé par une production excessive de radicaux libres ou par une défaillance des enzymes antioxydants est proposé parmi les facteurs étiologiques de la maladie de Parkinson (MP) et comme mécanisme de toxicité du MPTP. Le MPTP est métabolisé par la monoamine oxydase type B (MAO-B) en MPP^+, le médiateur de la neurotoxicité du MPTP. L'administration du MPTP produit un état parkinsonien tel que démontré par la dégénérescence des neurones du système nigro-strié accompagnée par une perte de dopamine (DA) au niveau du striatum. La toxicité du MPP^+ est associée à l'inhibition de l'activité de la NADH deshydrogénase au niveau de la chaîne respiratoire mitochondriale. Une déficience respiratoire semblable est observée dans la MP. Il s'en suit une diminution de la synthèse de l'ATP et de la perte du potentiel électrochimique de la membrane augmentant ainsi la formation de radicaux libres. Les enzymes jouant un rôle fondamental dans l'élimination des radicaux libres sont la superoxide dismutase (SOD), la glutathione peroxidase (GSH-PX) et la catalase (CAT). La SOD catalyse la dismutation de radicaux supéroxyde ($O_2^{\cdot}$) en H_2O_2, tandis que la GSH-PX et la CAT convertissent H_2O_2 en H_2O. L'accumulation du taux de lipides péroxidés et une augmentation de l'activité de la SOD sont observées au niveau de la substance noire (SN) et du striatum des parkinsoniens, tandis que l'activité de la CAT et de la GSH-PX est réduite ou inaltérée. Cette observation suggère une défaillance dans l'élimination du H_2O_2 qui induit la formation de radicaux libres catalysés par le fer. Toutefois, il reste à préciser si une augmentation de l'activité de la SOD, en l'absence d'une augmentation de l'activité de la GSH-PX ou de la CAT, peut conduire à un état oxidatif et donc causer une augmentation de la péroxidation lipidique. Nos

résultats ont démontré qu'une altération de l'activité de la SOD, de la GSH-PX ou de la CAT est insuffisante pour induire la péroxidation lipidique. Il est donc fort probable que d'autre facteurs conduisent à une induction de la péroxidation lipidique telle qu'observée chez les parkinsoniens. Les changements de l'activité des enzymes antioxidants observés dans la SN et striatum de souris traitées au MPTP sont étonnamment similaires à ceux obtenus après l'administration du L-déprenyl, un inhibiteur de l'activité de la MAO-B. En effet, le L-déprenyl est aussi efficace que le MPP^+ à augmenter l'activité de la SOD. Plusieurs études ont démontré que l'activité de la SOD est induite par la formation de radicaux $O_2^{\cdot}$ ce qui nous a emmené à examiner les effets du L-déprenyl sur les sites majeurs de formations de radicaux libres, tels que l'oxydation des catécholamines et la chaîne respiratoire mitochondriale. Les résultats obtenus suggèrent que l'augmentation de l'activité de la SOD telle qu'observée chez les patients parkinsoniens ainsi que chez les souris traitées au L-déprenyl ou au MPTP représente une réponse adaptatrice dûe à une libération de radicaux libres suivant l'inhibition de la chaîne respiratoire. Ces observations sont particulièrement intéressantes puisque le L-déprenyl a été suggéré comme "salvateur" des neurones dopaminergiques en voie de dégénérescence. Par contre, cette hypothèse n'a pas été soutenue par des études cliniques ni par la normalisation de la DA dans le striatum de souris traitées au MPTP. De plus, nos résultats ont démontré que le L-déprenyl conduit à une réduction du nombre de neurones immunopositifs à la tyrosine

hydroxylase (TH) dans la SN de souris pre-traitées avec le MPTP comparativement au souris recevant seulement le MPTP. Par conséquent, la possibilité que le L-déprenyl induit un stress oxydatif en modifiant la fonction mitochondriale peut être masquée par la stimulation aiguë de la transmission dopaminergique induite par ce même L-déprenyl.

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To Wayne Rowe, Lyne Cleland and Marie-Anne De Santis:

Enceinte théâtrale arborant l'effigie d'une vie accomplie

Dans l'attente de la glorieuse dépêche

Encore faut-il s'y faire

Ce grand fracas, ombré d'incertitude

La force du torrent ne saurait emporter la semence

L'empreinte de la persévérance

Puisse la sagesse trouver le jour

Dans le raffinement de la ténacité et l'intégrité

Telle devrait être la quintessence de l'âme

En hommage à Jean-Louis, mon père

Your passage in time is crystallized in me

the silence of your violin echoes in my heart

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Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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In accordance with this passage, the text and figures included in chapters 2, 3, 4 and 5 of this thesis are duplications of manuscripts published, in press or submitted. Thus, the references of these are included at the end of each of their respective chapters. The references for the General Introduction and the General Discussion are presented at the end of the thesis. Written permission for the reproduction of published or in press manuscripts is provided in the Appendix.

CONTRIBUTION OF THE AUTHORS AND CO-AUTHORED PUBLICATIONS

As co-supervisors of my Ph. D. training, Judes Poirier and Rémi Quirion are co-authors on all the manuscripts.

(1) The effect of MPTP and L-deprenyl on antioxidant enzymes and lipid peroxidation levels in mouse brain

C. Thiffault, N. Aumont, R. Quirion and J. Poirier
(J. Neurochem. 65: 2725-2733, 1995)

Nicole Aumont: As a research assistant in J. Poirier's laboratory, N. Aumont provided technical support in performing the animal treatments and tissue preparations.

(2) The effect of L-deprenyl, D-deprenyl and MDL72974 on mitochondrial respiration: A possible mechanism leading to an adaptative increase in superoxide dismutase activity

C. Thiffault, R. Quirion and J. Poirier
(J. Pharmacol. Exp. Ther. Submitted, 1996)

(3) L-Deprenyl and MDL72974 do not improve the recovery of dopaminergic cells following systemic administration of MPTP in mouse

C. Thiffault, L. Lamarre-Théroux, R. Quirion and J. Poirier
(Brain Res. Mol. Brain Res. In press, 1996)

Louise Lamare-Théroux: As a part-time research technician in J. Poirier's laboratory, L. Lamarre-Théroux provided technical support in performing some of the experiments included in this manuscript.

(4) Effect of the MAO-B inhibitor, MDL72974, on superoxide dismutase activity and lipid peroxidation levels in the mouse brain

C. Thiffault, R. Quirion and J. Poirier
(Neurosci. Lett. Submitted, 1996)

ABBREVIATIONS

LIST OF ABBREVIATIONS

AAD ..... Aromatic Amino Acid Decarboxylase
Ach ..... Acetylcholine
ADP ..... Adenosine Diphosphate
ATP ..... Adenosine 5'-Triphosphate
BDNF ..... Brain-Derived Neurotrophic Factor
bFGF ..... Basic Fibroblast Growth Factor
ß-PEA ..... ß-Phenylethylamine
BSO ..... L-Buthionine Sulfoximine
CAT ..... Catalase
cDNA ..... Complementary Deoxyribonucleic Acid
CNS ..... Central Nervous System
CNTF ..... Ciliary Neurotrophic Factor
COMT ..... Catechol O-Methyltransferase
CSF ..... Cerebrospinal Fluid
DA ..... Dopamine
DOPAC ..... 3,4-Dihydroxyphenylacetic Acid
DSP-4 ..... N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine
EAA ..... Excitatory Amino Acid
ESR ..... Electron Spin Resonance
FAD ..... Flavin Adenine Dinucleotide
GFAP ..... Glial Fibrillary Acidic Protein
GSH ..... Glutathione
GSH-PX ..... Glutathione Peroxidase
GSSG ..... Glutathione Disulfide
HPLC ..... High Performance Liquid Chromatography
5-HT ..... 5-Hydroxytryptamine (Serotonin)
HVA ..... Homovanillic Acid

ICV ..... Intracerebroventricular

MAO ..... Monoamine Oxidase

$MPDP^+$ 1-Methyl-4-phenyl-2,3-dihydropyridinium

MPP^+ 1-Methyl-4-Phenylpyridinium

MPTP 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA ..... Messenger Ribonucleic Acid

NADH ..... Nicotinamide Adenine Dinucleotide

NE ..... Norepinephrine

NMDA ..... N-methyl-D-Aspartate

NO ..... Nitric Oxide

6-OHDA 6-Hydroxydopamine

PD ..... Parkinson's Disease

PET ..... Positron Emission Tomography

ROS ..... Reactive Oxygen Species

SN ..... Substantia Nigra

SOD ..... Superoxide Dismutase

TBN ..... Tetraphenylboron

TH ..... Tyrosine Hydroxylase

V ..... Velocity

CHAPTER 1

General Introduction

Preface to Chapter 1

The main objective of this General Introduction is to provide background information on oxidative stress proposed as a mechanism in the etiology of Parkinson's disease and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism. Cellular damage by oxidative stress involves numerous mechanisms including activation of stress activated protein kinases (Derijard *et al.*, 1994), activation of microglia (Giulian, 1993) and free radical formation (Beal *et al.*, 1993; Coyle and Puttfarcken, 1993; Halliwell, 1992). The focus of this work is on the role of free radicals and antioxidant defence systems. The first section refers to the cardinal neuropathological and biochemical features of Parkinson's disease. Sections 2, 3 and 4 contain discussions of experimental models such as 6-hydroxydopamine and MPTP, and their relevance to the study of Parkinson's disease. Growth factors, such as glia-derived neurotrophic factors, promote the survival of dopaminergic neurons and their use has been proposed as a novel therapeutic approach for the treatment of Parkinson's disease (Weiss, 1993); this general introduction will focus on current treatment strategy. L-Deprenyl, a monoamine oxidase type B (MAO-B) inhibitor, known to protect against the deleterious effects of MPTP, provides symptomatic relief in early and mild Parkinson's disease. However, the pharmacology of L-deprenyl is not restricted to MAO-B inhibition, and this aspect is reviewed in the fifth section. Finally, the last section of this introduction addresses the general objectives of the thesis.

1 Pathophysiological and Neuropathological Features of Parkinson's Disease

1.1 Historical Hallmarks

Parkinson's disease (PD) is a disorder of unknown etiology affecting about 1% of the general population over 50 years of age. The average age of onset is the late fifties, although 5% of patients are under age 40. The disease was originally described in 1817 by a British physician named James Parkinson (1755-1824) as *shaking palsy* or *paralysis agitans*, which incidentally is a misnomer since there is no paralysis. He listed its salient features as resting tremor, gait disturbance and bradykinesia. However, he overlooked an important feature, which was later recognized as rigidity. Interestingly, his observations were based on six patients and other occasional street encounters. The disorder was granted its eponymous title *la maladie de Parkinson* by Charcot in 1867.

The earliest reference to *shaking palsy* in the Western medical literature is by Galen (AD 138-201). Ancient historical texts refer to diseases which bear resemblance to the clinical features of *shaking palsy*. For example, ayurvedic treatises in Sanskrit (India, 5000 to 3000 BC) described the disorder *kampavata* (*kampa* meaning tremor and *vata* meaning psychomotor). Seeds of *mucuna prureins* were prescribed to alleviate *kampavata* symptoms and have now been shown

to possess antiparkinsonian capabilities (Gourie-Devi *et al.*, 1991; Vaidya *et al.*, 1978). Although it is difficult to interpret ancient texts accurately, nonetheless it is important to realize that PD existed long before industrialization and the extensive use of pesticides and herbicides in agriculture.

While the clinical description of PD was essentially complete by 1910, it was during subsequent years that its main neuropathological features were delineated. Lewy (1912) described in the substantia nigra (SN) the appearance of an inclusion body that was later to bear his name. Ultrastructurally, Lewy Bodies are composed primarily of a filament mesh that surrounds a compact core made up of basic proteins, phospholipids, tyrosine hydroxylase (TH), aromatic amino acids, aliphatic amino acids and ubiquitin (Bancher *et al.*, 1989; den Hartog Jager, 1969; Duffy and Tennyson, 1965; Forno *et al.*, 1986; Goldman *et al.*, 1983; Hirsch *et al.*, 1985; Kuzuhara *et al.*, 1988; Nakashima and Ikuta, 1984).

In 1919, Trétiakoff recognized the hallmark lesion in the SN. He noticed "a loss of black February 9, 1997pigment in the locus niger de Soemmering". However, it was not until the mid-1950s that the remaining clinical manifestations could be linked to neurochemical dysfunction. Among them are: 1) The observation of Montagu (1957) that dopamine (DA) is present in the brain in about equal concentrations to those of norepinephrine (NE). This significant finding suggested that DA could no longer be exclusively considered an intermediate in the synthesis of NE and epinephrine. 2) The proposal of Carlsson and his colleagues (1958), based on reserpine-induced parkinsonism and its effects on DA metabolism that DA might be

important to extrapyramidal function. The distinct distribution of DA, NE and epinephrine in the brain implied a role for DA as a neurotransmitter (Andén *et al.*, 1964). 3) The finding of Ehringer and Hornykiewicz (1960) as well as Barbeau, Murphy and Sourkes (1961a) of lowered DA in the basal ganglia and in urinary excretion led to the suggestion that DA was accountable for the symptomatic presentation of the disease. 4) The partial reversal of reserpine-induced extrapyramidal reactions by L-dopa (Carlsson *et al.*, 1957) led to its introduction in the treatment of PD.

The search was launched in the early 1960s for a clinical approach to the treatment of PD. In the spring of 1961, two groups reported simultaneously and independently on the use of L-dopa. In Montréal, Barbeau, Murphy and Sourkes, using oral L-dopa, noticed marked but short-lasting improvement in rigidity and tremor (Barbeau *et al.*, 1961b). Meanwhile in Vienna, Birkmayer and Hornykiewicz, using the intravenous route, observed changes in akinesia with L-dopa. So far, L-dopa remains the most effective treatment in PD.

Despite great strides made in the last 35 years, which have considerably improved the functional outlook of patients suffering from PD, and despite demonstration of a crucial DA deficiency in the basal ganglia, the etiologic factor or factors responsible for this condition remain an enigma. Since PD is primarily a disease of older people affecting only humans, it appears therefore that aging itself represents a risk factor. It is rare before the age of 40 years, and the incidence rate increases at least until the sixth or seventh decade of life.

1.2 Manifestations of Aging in the Nigrostriatal Dopaminergic System

A gradual decrease in TH –the rate limiting enzyme in the synthesis of DA– is reported to occur in the course of aging accompanied by a decline in striatal DA concentrations, which progresses at a rate of approximately 5 to 8% per decade (Bannon *et al.*, 1992; Carlsson and Winblad, 1976; Côté and Kremzner, 1983). Interestingly, DA transporter mRNA, responsible for the termination of neuronal signaling, falls precipitously at a time corresponding to the mean age of onset of PD (Bannon *et al.*, 1992). These observations led to the concept that PD presents some form of "accelerated aging phenomenon," first proposed by Barbeau (1973).

The notion of selective aging clearly implies an inherent and hence genetically determined predisposition. Although inheritance is only known to contribute to a subgroup of patients (Barbeau and Pourcher, 1982; Golbe *et al.*, 1990), three independent studies failed to reveal a high concordance rate for PD in monozygotic or dizygotic twins (Marsden, 1987; Marttila *et al.*, 1988a; Ward *et al.*, 1983). In addition, only 1 in 40 people over the age of 65 is affected by PD and the incidence of the disease declines past the age of 80 (Hoehn and Yahr, 1976; Kurland *et al.*, 1973). It is therefore unlikely that the functional changes that occur in these catecholamine-containing cells with aging alone would normally result in PD. Important differences in the pattern of dopaminergic degeneration and DA losses exist in normal aging as compared with PD (Fearnley and Lees, 1991; Kish *et al.*, 1992). A dorsolateral-to-ventromedial DA gradient is observed in the striatum of PD, with the greatest deficit occurring in the more dorsal parts; however, no such gradient is seen with normal aging (Kish *et al.*, 1992). In

addition, the decrease in DA levels in aging is similar for both the caudate and putamen, with a rostro-to-caudal gradient. Similarly, dopaminergic cell loss in the SN is most marked in the ventrolateral regions in PD pathology, whereas with age it is the dorsal tier that is more affected (Fearnley and Lees, 1991).

An alternative concept is that PD is due to a toxic insult occurring in the course of life that decreases the number of dopaminergic cells and that this decline is exacerbated by the aging process to produce the symptoms of the disease. This hypothesis is consistent with the variable rate of disease progression among patients (Barbeau and Roy, 1984; Birkmayer *et al.*, 1979; Mortimer *et al.*, 1982). An exponential decline in cell number best fits the rate of disease evolution and the progressive dopaminergic cell losses, suggesting that a putative neurotoxin (environmental or endogenous) alters the linear attrition in cell number expected from normal aging (Fig. 1) (McGeer *et al.*, 1988; Schulzer *et al.*, 1994). However,

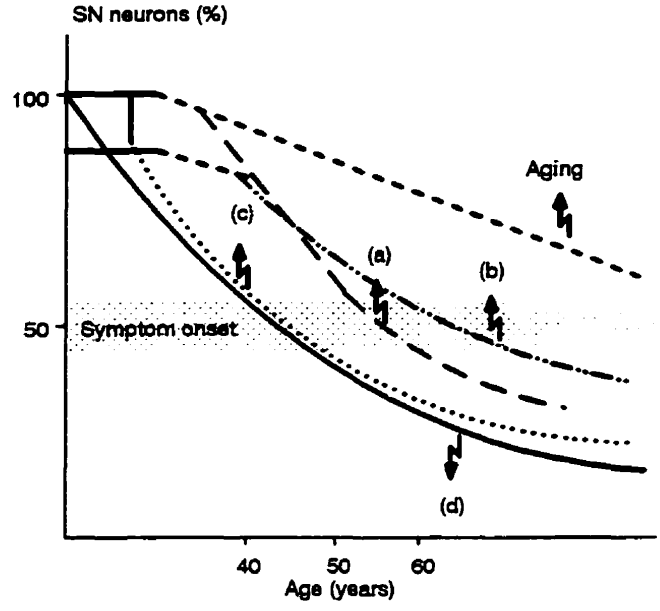

Fig. 1. Theoretical rate of dopaminergic neuron losses in normal aging and Parkinson's disease. A toxic insult superimposed on the normal age-related attrition (a). Neuronal cell losses taking place at the embryonic stage compounded to a toxic insult exacerbating the decline in cell numbers (b). Exposure to a neurotoxic agent resulting in a subclinical lesion and an exponential decline occurs thereafter (c). A rapid decline taking place early in life (d).
Modified from Schulzer *et al.*, 1994

attempts to correlate a single specific feature to the incidence of the disorder have so far been unsuccessful, despite observations that parkinsonian signs can be experimentally occasioned by selective neurotoxins.

A valid hypothesis of PD must therefore explain the preferential cell death as well as the almost universal appearance of Lewy bodies in the pigmented cells. A number of hypotheses have been proposed as a mechanism underlying the degeneration of the nigrostriatal dopaminergic pathway in PD. With respect to virology (Duvoisin, 1981; Elizan and Casals, 1983; Gibbs and Gajdusek, 1982), studies of aging, genetics (Golbe, 1990; Duvoisin, 1993), toxicology (Langston *et al.*, 1987b) and epidemiology (Zhang and Roman, 1993; Tanner, 1989; Lilienfeld *et al.*, 1990) have revealed that none of these concepts have proven completely satisfactory when taken as a single cause, possibly because all of these factors have a particular role, but are not exclusive elements in the etiology of PD (Barbeau, 1984; Poirier *et al.*, 1991). Accordingly, the development of the disease would be favoured by an interplay between genetic predisposition and exposure to putative environmental toxins compounded with the aging process. In this respect, parkinsonian patients have an inherent reduced capacity to metabolize xenobiotics (Green *et al.*, 1991; Heafield *et al.*, 1990; Steventon *et al.*, 1989; Waring *et al.*, 1989). These findings suggest a deficiency in the detoxification pathways of xenobiotics, which could have a major impact on a genetic predisposition to putative neurotoxins. In addition, neuropathological features provide additional insights into possible mechanism(s) underlying the degeneration of the nigrostriatal pathway in PD.

1.3 Neuroanatomical and Neurochemical Findings: Clues to the Cause of PD

1.3.1 The Significance of Lewy Body Inclusions

Lewy bodies, named after the man who first described them in 1912 (Lewy, 1912), represent another characteristic neuropathological feature of PD. They are usually found within cell bodies but may also lie in nerve cell processes (Forno, 1986). These cytoplasmic inclusions are present consistently and with greatest frequency in neurons of the SN and locus ceruleus of parkinsonian patients (Forno, 1986). The significance of Lewy bodies lies therefore in their intimate relation to neurodegeneration and PD. Lewy bodies are also observed in surviving neurons of PD patients and are consistent with the notion of an active process taking place until the end-stage of the disease (Lewy, 1912). Moreover, the presence of ubiquitinylated proteins in these structures (Bancher *et al.*, 1989) indicates that the neurons in which they are found are undergoing a degenerative process. Reactive microglial cells (Boka *et al.*, 1994) accompanied by the deterioration of the surviving dopaminergic neurons suggest that nerve cell death is still occurring at the patients' death, sometimes 20 years after the onset of PD. The incidence of Lewy bodies in asymptomatic individuals who also present some death of dopaminergic cells, known to degenerate in PD, suggests a preclinical phase (Forno, 1986; Gibb, 1986; Gibb and Lees, 1988). DA metabolism in the striatum of affected individuals is also consistent with the view that PD is a slow and progressive neurological disorder.

1.3.2 DA Metabolism in PD

Parkinsonian symptoms become evident only when striatal DA levels are reduced by approximately 80% corresponding to a decline in nigral neurons of about 50% (Bernheimer and Hornykiewicz, 1965; Fearnley and Lees, 1991; Hornykiewicz, 1966; Hornykiewicz and Kish, 1986; Sherman *et al.*, 1989). DA losses less than 80% seem to be very effectively compensated on the functional level by the remaining dopaminergic neurons prior to the appearance of symptoms. The increased DA metabolism and turnover rate, as demonstrated by a higher content of homovanillic acid (HVA, a DA metabolite) to DA, at autopsy is consistent with this view (Bernheimer and Hornykiewicz, 1965). Furthermore, a reduction in DA transporter mRNA per surviving neurons is also reported to contribute to these compensatory changes (Harrington *et al.*, 1996). This observation suggests that DA may diffuse outside the synaptic cleft to act at remote DA receptor sites. A reduction in nigral TH levels and its mRNA has been reported, confirming the altered metabolism of DA in the surviving dopaminergic neurons in PD (Javoy-Agid *et al.*, 1990; Kastner *et al.*, 1993a; 1993b).

1.3.3 Selective Vulnerability of Melanized Dopaminergic System

The massive cell death that occurs in DA-containing neurons of the mesencephalon accompanied by a loss of striatal DA represents the cardinal neurochemical and neuroanatomical features of PD. However, its pathology is not restricted to a dopaminergic deficit since neuronal degeneration is also observed to various degrees in noradrenergic neurons of the locus coeruleus (Forno, 1975; Forno and Norville, 1981) and in cholinergic neurons of the nucleus basalis of Meynert (Arendt *et al.*, 1983) and the hippocampus (Aubert *et al.*, 1992; Candy *et al.*, 1983; Smith *et al.*, 1988). Decreases in serotonin (5-HT) and peptides are also reported in several brain areas (Epelbaum *et al.*, 1983; Halliday *et al.*, 1990). Although changes in other neurotransmitter systems can be found in the parkinsonian brain, dopaminergic cell loss is by far the most prominent effect and it is present in every case with PD. In addition, the severity of symptoms correlates best with the degree of dopaminergic neuronal degeneration (Bernheimer *et al.*, 1973; Forno and Alford, 1971).

However, not all mesencephalic dopaminergic neurons degenerate to the same extent, as demonstrated by TH immunohistochemistry, indicating that a subpopulation is particularly vulnerable (Hirsch *et al.*, 1988). Accordingly, the loss of dopaminergic neurons is severe (77%) in the SN pars compacta (analogous to the catecholaminergic cell group A9 in the rat), intermediate in the peri- and retrorubral region (cell group A8) as well as in the ventral tegmental area (A10) (48 and 43% loss, respectively) and almost negligible in the central gray substance (3% loss). The retrorubral region and the ventral tegmental area are located adjacent

to the SN, with the ventral tegmental area located medially and the retrorubral region located caudally and dorsally (in the ventral reticular formation). Nigral dopaminergic cell bodies project widely to the caudate nucleus and putamen (the nigrostriatal pathway) (Andén *et al.*, 1964; Jimenez-Castellanos and Graybriel, 1989; Parent *et al.*, 1983; Smith and Parent, 1986). Within the SN pars compacta, the ventral cells project primarily to the dorsolateral striatum and are the most vulnerable, while the dorsal cells projecting to the ventromedial striatum are the least affected (Szabo, 1980). The intrinsic distribution of dopaminergic cell bodies in the SN pars compacta is consistent with the dorsolateral-to-ventromedial losses in DA observed in the striatum of PD, with the greatest deficit occurring in the more dorsal parts (Kish *et al.*, 1992). The midline cell group (ventral tegmental area) relatively spared in PD directs its efferent projections toward the ventral striatum, the nucleus accumbens and olfactory tubercle (the mesolimbic system) and frontal and entorhinal cortex (the mesocortical pathway) (Björklund and Lindvall, 1984; Fallon and Loughlin, 1987; Graybiel and Ragsdale, 1983). The retrorubral field, presenting moderate cell losses, projects to the caudate nucleus and putamen (Feigenbaum and Graybiel, 1988; Graybiel, 1989; Jimenez-Castellanos and Graybriel, 1987). One hypothesis to explain the pattern of DA cell loss is that the pathological process originates in the striatum and initially affects those DA cells that project to the striatum. As the disease progresses, non-striatal regions (e.g., nucleus accumbens and pre-frontal cortex) may also become involved in the pathological process and those DA neurons projecting to these non-striatal regions may be affected subsequently as well (German *et al.*, 1988).

Interestingly, 84% of catecholaminergic neurons in the SN are melanized, whereas 98% of these neurons in the central gray substance are devoid of neuromelanin (Hirsch, 1992). The ventral tegmental area and retrorubral field contain melanized neurons and non-melanized catecholaminergic neurons in similar proportions (Hirsch, 1992). Thus, the presence of neuromelanin in the nigrostriatal system is a key factor in determining the susceptibility of dopaminergic neurons to degeneration, as was first suspected by Trétiakoff (1919).

Neuromelanin, a brown-black deposit also called the aging pigment, is localized to the cytoplasm of neurons in the SN, locus ceruleus and additional loci in the midbrain (Bazelon *et al.*, 1967; Lillie, 1955; 1957). It is not present at birth and increases linearly with age until the sixth decade, a time which corresponds to the age of onset of the disease (Mann and Yates, 1974). A decline is seen thereafter as more pigmented neurons are lost (Mann and Yates, 1974). Therefore, the process of neuronal melanization implies some role for aging in the etiology of PD. Neuromelanin is known to be generated from the polymerization of oxidized products of DA, epinephrine and NE which leads to the formation of cytotoxic free radicals and H_2O_2 (Fig. 2) (Graham, 1978; 1979). Oxidized catechols have been shown to covalently crosslink neurofilaments, a mechanism thought to contribute to Lewy body formation (Montine *et al.*, 1995). Thus, free radicals generated from an increase in DA turnover and neuromelanin formation participate, at least in part, to the degeneration of the nigrostriatal pathway. This view is consistent with the increase in neuromelanin content with aging. As discussed above, however, aging itself cannot be solely accountable for the occurrence of the disease. These observations suggest that free radicals generated as a result of catecholamine oxidation and neuromelanin

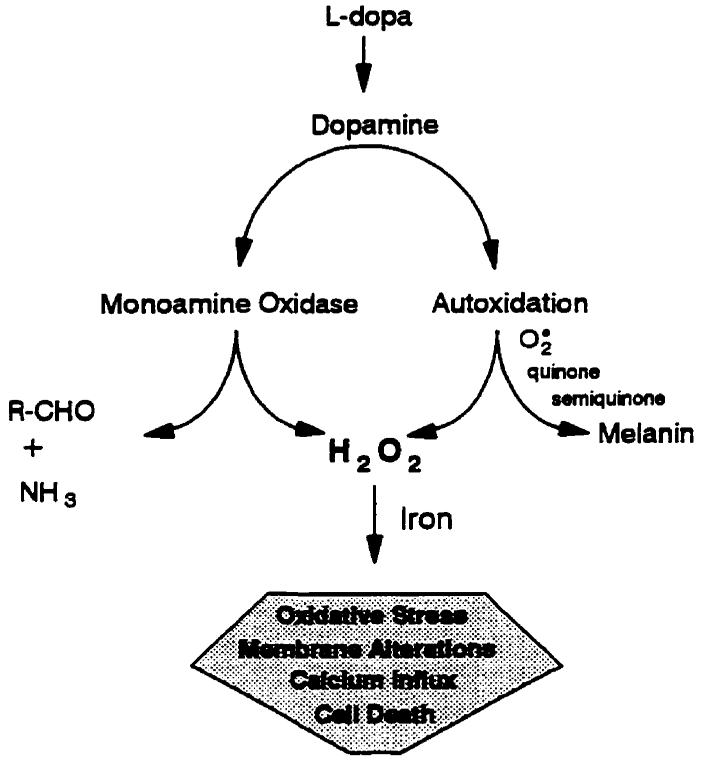

Fig. 2. Enzymatic and non-enzymatic oxidation of dopamine.

formation may not be the "event" that triggers initial cell death, but rather a mechanism which may contribute to the progressive degeneration of the neurons in which they are produced. In addition, there exist several potential sources for the generation of cytotoxic free radicals, other than catecholamine oxidation, and these sites are also affected in PD.

1.3.4 Antioxidant Enzymes and Free Radical Equilibrium: Evidence of Oxidative Stress in the Parkinsonian Nigrostriatal Pathway

Oxidative stress is thought to be the result of excessive free radical formation overwhelming the antioxidant defense capacity of the cell, a mechanism proposed in the etiology of PD. Free radicals are defined as any species having one or more unpaired electrons in their outer orbital, giving them an inherent ability to react with a variety of cellular constituents such as membrane lipids, the sulfhydryl group of proteins, polysaccharides and nucleic acids (for reviews Halliwell, 1992; Halliwell and Gutteridge, 1985). The gradual loss of membrane fluidity and potential, induced by a peroxidative damage to lipids, alters permeability to ions such as Ca^{2+} that in turn activates a number of degenerative processes involving enzymes such as peptidases, phospholipases and endonucleases, leading to neuronal death (for review Beal *et al.*, 1993; Coyle and Puttfarcken, 1993; Dawson *et al.*, 1995). A selective increase in lipid peroxidation levels and 8-hydroxy-2-deoxyguanosine concentrations, an oxidized product of a DNA base which results in strand breakage, is observed in the parkinsonian nigrostriatal pathway compared with other brain areas and age matched controls (Dexter *et al.*, 1989a; Pall *et al.*, 1986; Sanchez-Ramos *et al.*, 1994). These observations are consistent with a free radical- mediated neuronal degeneration in PD.

The major free-radical scavenging enzymes or antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT) (Fig. 3). Two major forms of SOD exist, mitochondrial or Mn-SOD and cytosolic or CuZn-SOD (Weisiger and Fridovich, 1973).

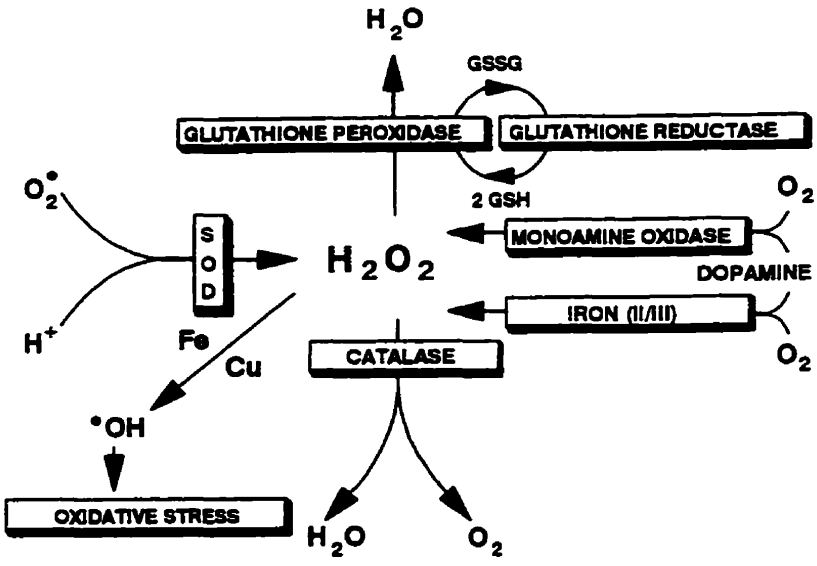

Fig. 3. Major free radical detoxifying enzymes.

Mn-SOD is present in both neuronal and glial cells, while CuZn-SOD is mostly neuronal and is found in large amounts in melanized nigral neurons of mice and humans as reported by us and others (Ceballos-Picot *et al.*, 1991; Poirier *et al.*, 1994; Rosenberg *et al.*, 1989; Takashima *et al.*, 1990; Zhang *et al.*, 1993). SOD catalyzes the dismutation of superoxide ($O_2^{\cdot}$) radical to O_2 and H_2O_2, while CAT converts H_2O_2 into O_2 and H_2O. Current knowledge is lacking on the distribution and the significance of CAT in the brain, considering its presence in minute amounts (Gaunt and de Duve, 1976). This enzyme is confined to peroxisomes of both neural and glial cells in the brain of rats and humans, albeit at different levels (Geremia *et al.*, 1990; Hassan and Fridovich, 1977; Houdou *et al.*, 1993; Moreno *et al.*, 1995; Victorica *et al.*, 1984). Glial cells show intense immunoreactivity, while neurons express a variable staining, with high signal in the brainstem, intermediate in the striatum and low in the SN of rat (Moreno *et al.*, 1995). The brain has acquired other means to deal with excess H_2O_2, which is achieved by the means of GSH-PX at the expense of glutathione (GSH) with a concomitant formation of

oxidized glutathione (GSSG) (Fig. 3). In addition, GSH-PX detoxifies lipid peroxides and is present in the cytosol and mitochondria of glial cells of rats and humans (Damier *et al.*, 1993; Geremia *et al.*, 1990; Prohaska and Ganthier, 1976). The possibility that GSH-PX is expressed in neurons, albeit in small amounts, should not be excluded (Geremia *et al.*, 1990).

We and others have observed an increase in SOD activity, whereas GSH-PX and CAT activities are unchanged or reduced, in the SN and striatum in PD (Ambani *et al.*, 1975; Kish *et al.*, 1985; Marttila *et al.*, 1988b; Poirier and Thiffault, 1993; Poirier *et al.*, 1994; Saggu *et al.*, 1989). Although the increase in overall SOD activity in PD is now well established, it is not clear if the CuZn-SOD, Mn-SOD or both types are affected. An increase in SOD without concomitant increments in GSH-PX or CAT activities (Ambani *et al.*, 1975; Kish *et al.*, 1985; Marttila *et al.*, 1988b; Poirier and Thiffault, 1993) results in the accumulation of H_2O_2. In addition, the enzymatic, by monoamine oxidases (MAO), and non-enzymatic oxidation of DA contributes to the levels of H_2O_2 formation (Fig. 2 section 1.3.3, p. 13). MAO-B activity increases with aging (Robinson, 1975), contributing to the levels of H_2O_2 produced. The latter may in turn react with transition metals, such as iron, via a Fenton reaction, and further increase the production of oxygen centred radicals (Fig. 4) (for review Halliwell, 1992). An increase in nigral iron concentrations is reported in PD (Dexter *et al.*, 1989b; Earle, 1968; Riederer *et al.*, 1989), whereas levels of GSH, a potent antioxidant, are reduced in the SN (Perry *et al.*, 1982; Perry and Yong, 1986; Sofic *et al.*, 1992). These results are consistent with a deficient ability to deal with excess H_2O_2. Taken together, this evidence suggests a widespread oxidative stress within the parkinsonian nigrostriatal pathway. In addition, the main form of iron found is Fe^{3+},

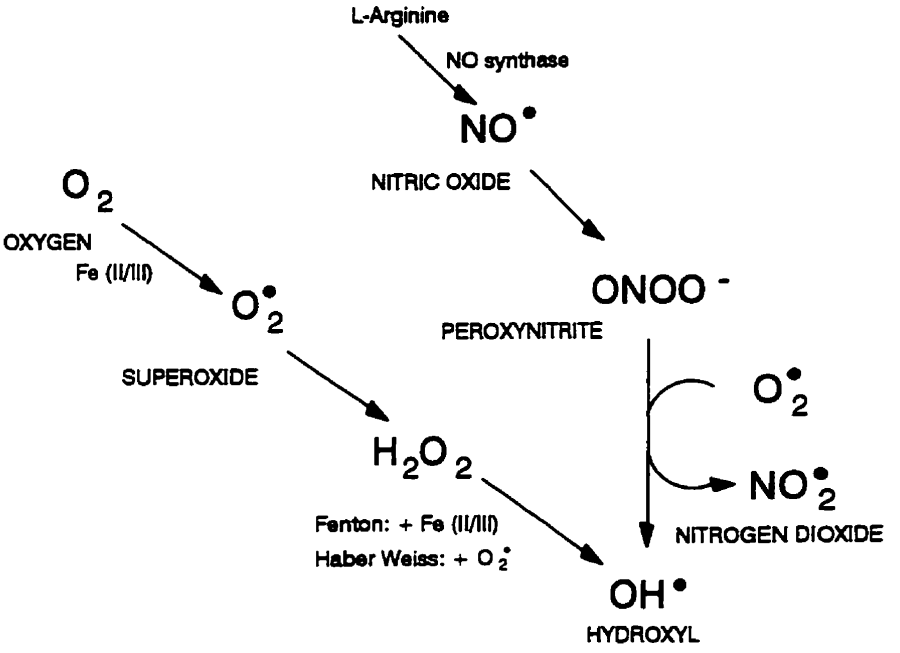

Fig. 4. Oxygen-derived free radicals and their reactions.

which results in a shift in the ratio of Fe^{2+}/Fe^{3+} from 2:1 to 1:2, known to optimize lipid peroxidation in brain synaptosomal preparations (Braughler *et al.*, 1986). Interestingly, iron levels are known to increase in the human brain with aging, and a plateau is seen around 50-60 years (Hallgren and Sourander, 1958), the age range at which PD becomes clinically detectable. Iron levels comparable with those of the liver can be found in the globus pallidus and SN, whereas intermediate levels are observed in the putamen and caudate nucleus of rat and humans (Barkai *et al.*, 1991; Connor *et al.*, 1990; Francois *et al.*, 1981; Hill and Switzer, 1984; Morris *et al.*, 1992; Uitti *et al.*, 1987).

The most reactive of the free radical species (Fig. 4) is the hydroxyl radical ($OH^{\cdot}$), which can also be formed from $O_2^{\cdot}$ and H_2O_2 (a non-radical) by the Haber-Weiss reaction to oxidize any molecule within a few Angströms from the site at which they are generated (for review Halliwell,

1992). However, $O_2^{\cdot}$ is much less reactive but can cross the cell membrane and act at a distance. It can react in the absence of transition metals with nitric oxide ($NO^{\cdot}$) to form peroxynitrite ($ONOO^-$), at an extremely fast rate ($6.7 \times 10^9\ M^{-1}$), and the latter can decompose to $OH^{\cdot}$ and nitrogen dioxide ($NO_2^{\cdot}$) radicals (Beckman *et al.*, 1990). $ONOO^-$ can diffuse over several cell diameters and, consequently, has been proposed as a prime candidate for mediating directly or indirectly oxidative damage *in vivo* (Beckman and Crow, 1993). In spite of its low reactivity, $O_2^{\cdot}$ is capable of inactivating a number of enzymes, including those with iron-sulphur clusters in which the oxidized cluster then loses Fe^{2+}, which is less tightly bound to sulfide ligands than Fe^{3+} (Gardner and Fridovich, 1991a, 1991b; Liochev and Fridovich, 1994). The former may be oxidized to Fe^{3+} through a Fenton type of reaction to participate in the cyclic production of free radicals (Fig. 4).

Although there are a number of intracellular sources of free radical formation, such as catecholamine oxidation and a reduced rate of H_2O_2 elimination, as discussed above, the mitochondria are thought to be the most important. An elegant demonstration was the finding that yeast with a complete absence of the electron transport chain grew normally in elevated O_2 levels (hyperoxia), which causes oxidative stress, whereas hyperoxia inhibited the growth of normal yeast (Guidot *et al.*, 1993). Isolated mitochondria generate 0.6 to 1.0 nmol of H_2O_2 min^{-1} mg of $protein^{-1}$, and this is estimated to account for 2 to 4% of oxygen uptake under physiological conditions (Boveris and Chance, 1973). The main sites where $O_2^{\cdot}$ is thought to escape are ubiquinone and NADH dehydrogenase (complex I, Fig. 5) (Boveris *et al.*, 1976;

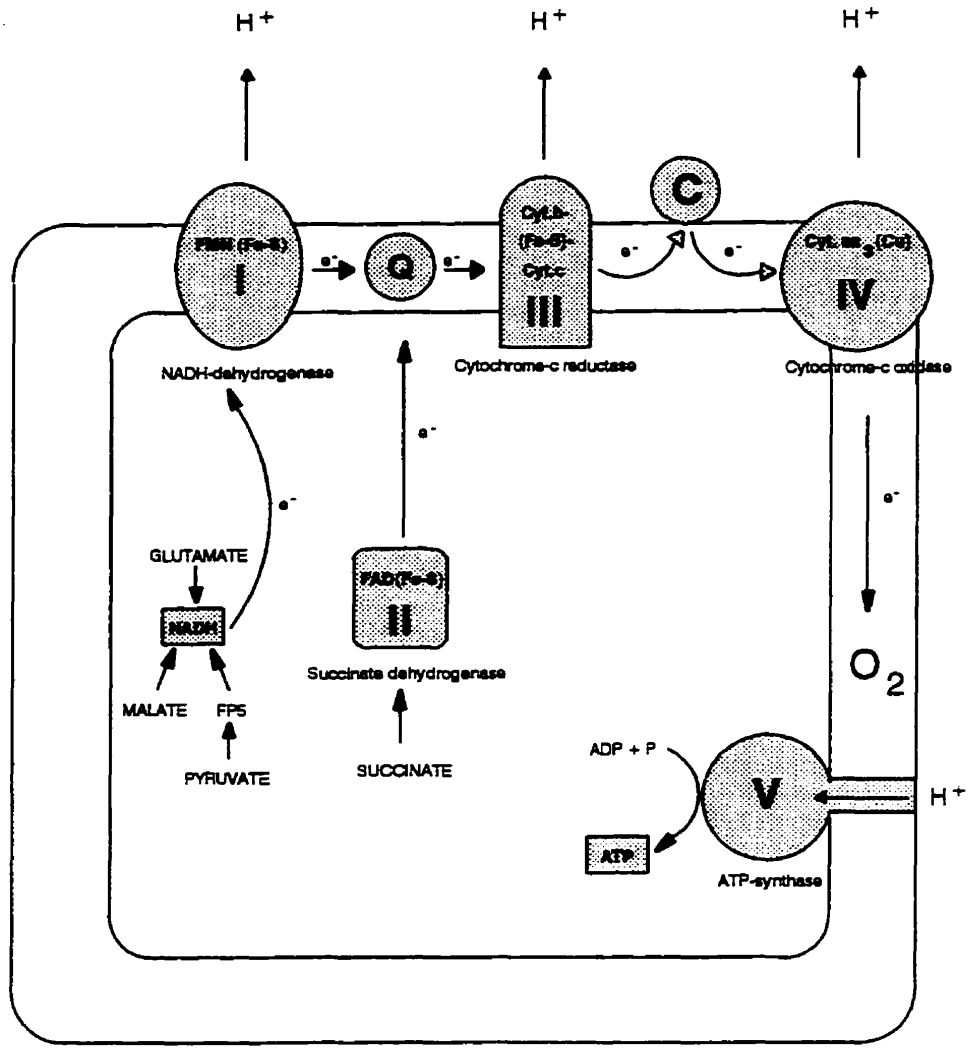

Fig. 5. Schematic diagram of the 4 major mitochondrial electron transporters. Electrons are transfered subsequently to molecular oxygen. A proton gradient generated by complexes I, III, and IV is used by complex V for ATP sythesis. Note that the major sites of electron leakage occur at complex I and ubiquinone (Q). C= cytochrome-C. Modified from Beal *et al.*, 1993

Paraidathathu *et al.*, 1992; Turrens and Boveris, 1980). Electrons are transferred one at a time to form ubisemiquinone, which can then react with O_2 to produce $O_2^{\cdot}$ (De Jong and Albracht, 1994). Evidence favouring ubiquinone as a major site of free radical generation is the observation that antimycin (a complex III inhibitor) leads to increased mitochondrial H_2O_2 production by blocking electron transfer from more proximal components of the respiratory

chain. Furthermore, depletion of ubiquinone from mitochondria results in a decrease in H_2O_2 generation (Patole *et al.*, 1986). Similarly, irreversible inhibition of NADH dehydrogenase by rotenone increases $O_2^{\cdot}$ formation in submitochondrial particles (Adams *et al.*, 1993; Krishnamoorthy and Hinkle, 1988; Ramsay and Singer, 1992; Turrens and Boveris, 1980). Production of $O_2^{\cdot}$ at the NADH dehydrogenase site accounts for approximately 50% of that generated at the ubiquinone pool (Kashkarov *et al.*, 1994; Turrens and Boveris, 1980).

Defects in mitochondrial electron transport chains have been reported in the SN and striatum of parkinsonian patients (Mizuno *et al.*, 1989; Schapira *et al.*, 1989; 1990), with a reduction in NADH dehydrogenase activity. These findings further support an association between free radical-mediated cellular injuries and dopaminergic degeneration as observed in PD. It is known that $O_2^{\cdot}$ has the ability to deactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain *in vitro* (Fukushima *et al.*, 1995; Zhang *et al.*, 1990), although its ability to do so *in vivo* remains to be demonstrated.

To date it is not clear whether altered antioxidant enzyme activities are sufficient to enhance free radical formation and, consequently, to an increase in lipid peroxidation levels and mitochondrial dysfunctions as observed in PD. Alternatively, changes in antioxidant capacity could merely reflect an adaptive response due to a leakage of oxygen-centered radicals resulting from the reduction of NADH-dehydrogenase activity. We have therefore undertaken the study of these possible relationships, which represent the central core of our work. Since PD is a disorder only observed in humans, it renders this analysis difficult. To address this issue it is

therefore important to be able to replicate biochemical and anatomical alterations observed in PD. The existence of selective neurotoxins which can mimic strikingly a number of deficits observed in PD have proven to be very useful tools in this respect.

2 Neurotoxins Provide Experimental Models with which to Study the Role of Oxidative Stress in the Etiology of PD

It has been known for some time that pharmacological agents that interfere with the production, storage, release or receptor recognition of DA cause a parkinsonian-like syndrome in animals and humans. For example, reserpine induces a reversible symptomatic condition by blocking the storage of DA in the synaptic vesicles in the terminals of nigrostriatal neurons (Carlsson *et al.*, 1958; Stitzel, 1976). This eventually leads to a hypodopaminergic state which is fully reversible after reserpine is discontinued. α-Methyl-p-tyrosine produces a similar state by inhibiting TH activity and by acting as a false dopaminergic neurotransmitter (Masserano *et al.*, 1989). By far the most common cause of drug-induced reversible parkinsonian-like syndrome in clinical practice results from the use of DA receptor antagonists for the treatment of psychiatric disorders (Baldessarini, 1996). While pharmacological manipulations of dopaminergic transmission tend to produce reversible effects, toxicological agents often lead to cell death. Accordingly, neurotoxins are considered the tools of choice for the study of the neurodegenerative process. An understanding of this process is critical to the development of strategies for altering or preventing neuronal degenerations in disease states such as PD. Furthermore, such studies could provide clues regarding the etiology of various

neurodegenerative diseases. The discovery that 6-hydroxydopamine (6-OHDA) destroys catecholaminergic systems (Tranzer and Thoenen, 1968) has given birth to the speculation that endogenous generation of this compound could be involved in the pathogenesis of PD. Finally, the more recent discovery of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston *et al.*, 1983) has created a dramatic resurgence of interest in the mechanisms of the neuronal degeneration observed in PD.

2.1 6-Hydroxydopamine

2.1.1 Neuropathology

In 1968, Tranzer and Thoenen published their monograph now considered classic detailing the selective destruction of peripheral nerve endings by 6-OHDA. To be effective as a neurotoxin in the central nervous system (CNS), 6-OHDA must be administered directly into the brain of animals, since it does not cross the blood-brain barrier. Intracerebroventricular (ICV) administration of large doses induces a widespread degeneration of dopaminergic and noradrenergic neurons that is well documented both neurochemically and morphologically (Blanchard *et al.*, 1995; Breese and Traylor, 1970; Hedreen and Chalmers, 1972; Pasinetti *et al.*, 1989; Sherman and Moody, 1995; Ungerstedt, 1968). In contrast, serotonergic, cholinergic and GABAergic neurons, among others, remain unaffected by 6-OHDA (Kostrzewa and Jacobowitz, 1974). The selectivity of 6-OHDA is related to its accumulation in dopaminergic and noradrenergic neurons through their high-affinity uptake systems (Kostrzewa and

Jacobowitz, 1974; Thoenen and Tranzer, 1973). This theory is supported by the use of antagonists of catecholamine uptake systems, which protect against 6-OHDA-induced neuronal losses (Kostrzewa and Jacobowitz, 1974; Thoenen and Tranzer, 1973). In order to achieve a degree of selectively towards dopaminergic systems, pre-treatment of rats with noradrenergic uptake inhibitors, such as desipramine, is required prior to an ICV administration of 6-OHDA (Breese and Traylor, 1971; Iversen and Uretsky, 1973). In addition, the selectivity of the lesion induced by 6-OHDA is dependent on the dose, volume and injection techniques (Agid *et al.*, 1973; Hökfelt and Ungerstedt, 1973). For example, Hökfelt and Ungerstedt (1973) reported a large area of necrotic tissue surrounding the site of injection after local administration of 6-OHDA into the SN. This led researchers to speculate that lesions produced by 6-OHDA may not be truly more selective than electrolytic, mechanical or chemical ones (Butcher *et al*, 1974; Poirier *et al.*, 1972).

2.1.2 Mechanism and Pharmacological Manipulation of 6-OHDA-Mediated Toxicity

Two mechanisms have been suggested to explain the toxicity of 6-OHDA, including oxidative stress and the impairment of mitochondrial functions. First, autoxidation of 6-OHDA leads to the generation $OH^{\cdot}$ and $O_2^{\cdot}$ radicals as well as H_2O_2 which results in an oxidative state, an effect potentiated by ascorbic acid present in mM concentrations in the brain (Fig. 6) (Heikkila and Cohen, 1972). Second, this neurotoxin is shown to uncouple mitochondrial oxidative phosphorylation and to inhibit mitochondrial activities of complexes I and IV *in vitro* (Glinka and Youdim, 1995; Wagner and Trendelenburg, 1971). However, its ability to alter

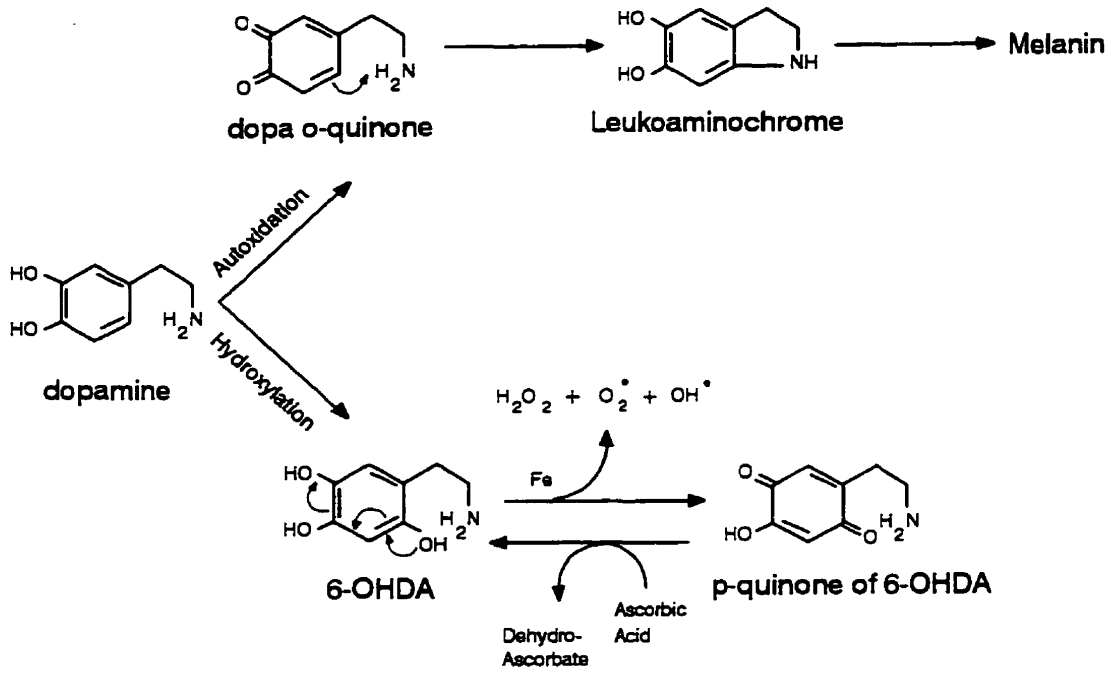

Fig. 6. Neuromelanin biosynthesis and proposed pathway for 6-hydroxydopamine formation from the 6-hydroxylation of dopamine in aqueous media. The autoxidation of 6-hydroxydopamine results in the production of cytotoxic free radicals.

mitochondrial functions *in vivo* has yet to be demonstrated. Interestingly, H_2O_2 induces similar mitochondrial alteration in neuronal cell lines as measured by a marker of complex I activity, suggesting that this effect is related to free radical-mediated injuries (Vroegop *et al.*, 1995). Lipid peroxidative damage to the cellular and mitochondrial membranes increases permeability to ions such as Ca^{2+} and consequently loss of membrane potential and ATP depletion. These events are considered the contributing factors to free radical-induced cell death. These observations are consistent with the increase in lipid peroxidation observed following ICV or intrastriatal injection of 6-OHDA to rodents (Ogawa *et al.*, 1994; Perumal *et al.*, 1995). Iron is also considered to play an important part in promoting oxidative damage induced by the autoxidation of 6-OHDA (Fig. 6). "Free" iron is not generally available under physiological conditions but is scavenged by the storage protein, ferritin, with high to moderate levels

confined to the SN and striatum, respectively, of rats and humans (Morris *et al.*, 1992). Ferritin iron can be released by $O_2^{\cdot}$ radicals (Biemond *et al.*, 1984; Bolann and Ulvik, 1987; Monteiro *et al.*, 1989; Thomas and Aust, 1986; Yoshida *et al.*, 1995) which can be formed from the autoxidation of 6-OHDA to promote lipid peroxidation as demonstrated by *in vitro* studies (Monteiro and Winterbourn, 1989; Reif *et al.*, 1988; 1989; Winterbourn *et al.*, 1991). This effect is inhibited by SOD, CAT or the iron chelator, desferroxamine (Monteiro and Winterbourn, 1989; Samokyszyn *et al.*, 1989). These results are consistent with the effects of 6-OHDA and iron mobilization *in vivo.* In rats, nigral iron levels are higher following a unilateral lesion induced by 6-OHDA compared to the ipsilateral intact side (Oestreicher *et al.*, 1994). Furthermore, degeneration of nigrostriatal neurons after ICV injections of 6-OHDA to rats is potentiated by the administration of iron (Ben-Shachar and Youdim, 1991b) or after depletion of brain GSH by ICV administration of L-buthionine sulfoximine (BSO), an inhibitor of GSH biosynthesis (Pileblad *et al.*, 1989). In contrast, vitamin E (Cadet *et al.*, 1989) or desferroxamine (Ben-Shachar *et al.*, 1991a) attenuated the 6-OHDA-induced depletion of striatal DA in rats. Vitamin E is a chain-breaking antioxidant that prevents the propagation of free radical damage to biological membranes by scavenging lipid-peroxyl radicals (Fig. 9 section 4.3, p. 48) (for review Saskia *et al.*, 1993; Traber and Packer, 1995). Thus, free radicals generated as a result of 6-OHDA oxidation and the consequential release of iron from ferritin play a role in the cytotoxicity of this compound *in vivo.*

An increase in SOD activity and its mRNA has been reported in the striatum of mice following an ICV administration of moderate levels of 6-OHDA (Ogawa *et al.*, 1994). However, one

group reported a decrease in striatal SOD activity after the delivery of large amounts of 6-OHDA into the ventricle known to cause a severe DA depletion (Ben-Shachar *et al.*, 1991b; Perumal *et al.*, 1989; 1992). As mentionned earlier, the nigrostriatal pathway is enriched with SOD enzymes (Ceballos-Picot *et al.*, 1991; Poirier *et al.*, 1994; Rosenberg *et al.*, 1989; Takashima *et al.*, 1990; Zhang *et al.*, 1993). Accordingly, the reduction in SOD activity appears to be associated with the severe degeneration of dopaminergic neurons. This observation is supported by the pre-treatment of rats with vitamin E, which partially protects against 6-OHDA-induced DA depletion to restore the increase in striatal SOD activity (Ben-Shachar *et al.*, 1991b; Perumal *et al.*, 1992).

Increases in SOD activity induced by 6-OHDA is consistent with an oxidative stress-inducing agent. Several studies have demonstrated that SOD activity is up-regulated when cells are exposed to excessive production of $O_2^{\cdot}$ radicals. For example, rats exposed to sublethal concentrations of O_2 (hyperoxia) showed elevations in CuZn-SOD and Mn-SOD activity in their lungs (Crapo and Tierney, 1973; Sjostrom and Crapo, 1981; Stevens and Autor, 1977). Free radicals have been implicated in the mechanism of toxicity of the herbicide paraquat, which interestingly bears a striking structural resemblance to the MPTP metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (Fig. 8 section 2.2.2, p. 33). Paraquat is rapidly reduced to form a bipyridyl radical through an oxidation-reduction cycle with O_2, leading to enhanced $O_2^{\cdot}$ formation with a concomitant increase in lipid peroxidation in rat lungs and purified rat lung microsomes (Bus *et al.*, 1975; 1976; Ilett *et al.*, 1974; Trush *et al.*, 1981). In addition,

mammalian cells exposed to paraquat demonstrated increases in CuZn-SOD and Mn-SOD activity (Frank, 1981; Krall *et al.*, 1988). Taken together, these results demonstrate that $O_2^{\cdot}$ itself has the ability to modulate SOD activity.

In spite of parkinsonmimetic effects induced by this neurotoxin, exogenous 6-OHDA has never been involved as an etiologic agent in PD due to its inability to cross the blood-brain barrier. Nonetheless, several reports suggested that 6-OHDA is formed as part of neuromelanin biosynthesis (Fig. 6) (Graham, 1978). It is suggested that the genesis of 6-OHDA could result *in vivo* from the 6-hydroxylation of DA in the presence of water (Senoh *et al.*, 1959). Tse and his colleagues (1976) noted that the latter reaction occurs far too slowly to compete with intracyclization of DA quinone to leukoaminochrome (Fig. 6). Identification of 6-OHDA in the cerebrospinal fluid (CSF) or in the SN of parkinsonians has never been reported. It is therefore unlikely that 6-OHDA participates in the degeneration of the nigrostriatal pathway in PD. Nevertheless, the above studies provide a rational basis for the use of antioxidants in the treatment of diseases suspected to involve oxidative stress.

2.2 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)

MPTP was discovered inadvertently as a byproduct of synthesizing heroin from Demerol. In 1979, Davis and his colleagues described the sudden onset of an irreversible parkinsonian-like syndrome in a 19-year-old man who was attempting to synthesize meperidine analogues (synthetic heroin) and was regularly injecting the product intravenously. After 2 years of

treatment with L-dopa and bromocriptine (a DA receptor agonist) he committed suicide. Neuropathological examination of his brain revealed loss of neurons in the SN, strikingly similar to changes observed in classical PD. Langston and co-workers (1983) have reported similar cases in Northern California. Interestingly, a progressive dopaminergic disturbance was observed in these patients demonstrating subclinical lesions following exposure to MPTP as revealed by positron emission tomography (PET) (Calne *et al.*, 1985). This decline in function was more rapid than would be predicted as a result of normal age-related neuronal attrition consistent with the rate of disease progression as observed in PD (Fig. 1 section 1.2, p. 6) (Vingerhoets *et al*, 1994). These reports suggested that there may be neurotoxic substances within our environment to which accidental exposure, and/or their improper metabolism or elimination, could gradually lead to irreversible damage to the dopaminergic nigrostriatal pathway.

2.2.1 Neuropathology

MPTP has also been demonstrated to induce an irreversible PD-like syndrome in several animal species, including monkeys (Burns *et al.*, 1983; Langston *et al.*, 1984a), mice (Sonsalla and Heikkila, 1986), cats (Schneider and Markham, 1986), dogs (Wilson *et al.*, 1987) and even frogs (Barbeau *et al.*, 1985). To date, the non-human primate remains the best animal model. When subjected to systemic administration of MPTP, these animals present dopaminergic deficits that are reminiscent of PD. For instance, striatal DA losses follow a dorsolateral-to-ventromedial gradient (German *et al.*, 1988; Pérez-Otaño *et al.*, 1994; Varastet *et al.*, 1994), a pattern typical of PD (Kish *et al.*, 1992). However, Hornykiewicz and his colleagues (1988)

have clearly demonstrated that the loss of DA in PD is greater in the putamen than the caudate nucleus, whereas in monkeys exposed to high levels of MPTP, they found no such differences. These differences may be attributed to the severity of the parkinsonian symptoms, which correlate best with the degree of DA losses (Hornykiewicz *et al.*, 1988; Pifl *et al.*, 1991). Alternatively, they could be related to varied schedules of MPTP treatment associated with its acute-versus-chronic administration. These observations are consistent with reports that acute and subacute MPTP administration to monkeys result in a striatal DA gradient opposite to that observed in PD: a dorsoventral but not rostrocaudal pattern (Deutch *et al.*, 1986; Elsworth *et al.*, 1987; 1989). In contrast, chronic administration of MPTP resulted in the classical striatal DA depletion characteristic of PD (German *et al.*, 1988; Pérez-Otaño *et al.*, 1994; Schneider, 1990; Varastet *et al.*, 1994).

Dopaminergic cell loss in the SN of monkeys exposed to MPTP strikingly resembles that observed in PD, with the ventral portion being most affected (German *et al.*, 1988; Rinne *et al.*, 1991; Varastet *et al.*, 1994). A correlation between neuromelanin content and the susceptibility of mesencephalic dopaminergic neurons to MPTP is also observed (Herrero *et al.*, 1993b), suggesting a role for this pigment in MPTP-induced neuronal degeneration. Thus, dopaminergic cell bodies of the SN pars reticulata, central gray substances and ventral tegmental areas are relatively unaffected by MPTP. However, reports of slight (Imai *et al.*, 1988; Kitt *et al.*, 1986), moderate (German *et al.*, 1988; Irwin *et al.*, 1990; Schneider *et al.*, 1987) to considerable (Elsworth *et al.*, 1990; Mitchell *et al.*, 1985) loss of dopaminergic neurons of the ventral tegmental areas are also seen. The apparent discrepancies among these studies may result

from differences in individual and subspecies sensitivity to the toxin, dosage or mode of administration of MPTP and from the experimental procedures used to visualize and count catecholaminergic neurons. Again, reported damage to noradrenergic neurons in the locus coeruleus has ranged from minimal (Imai *et al.*, 1988; Jacobowitz *et al.*, 1984; Kitt *et al.*, 1986) to severe (Forno *et al.*, 1988; Kitt *et al.*, 1986; Mitchell *et al.*, 1985). The vulnerability of noradrenergic neurons to MPTP increases with age, a phenomenon that may account for the differential effects observed in these experiments (Forno *et al.*, 1986; 1988; 1993). Lewy-like bodies are also observed in the SN and locus coeruleus of older primates and are a typical feature of PD (Forno *et al.*, 1986; 1993).

Although the primate represents one of the best available models of MPTP-induced parkinsonism, the rarity and cost of primates make their use difficult in extensive pharmacological studies. For this reason, mice subjected to MPTP offer an alternative model. Mice, like non-human primates, demonstrated striatal DA depletion and a concomitant decrease in nigral dopaminergic neurons following exposure to the neurotoxicant (Chiueh *et al.*, 1984; German *et al.*, 1992; Hallman *et al.*, 1985; Heikkila *et al.*, 1984a; Sundström *et al.*, 1988). However, mice are more resistant to the deleterious effects of MPTP than primates (Heikkila *et al.*, 1984a) and young mice have demonstrated some degree of recovery (Hallman *et al.*, 1985; Irwin *et al.*, 1992; Nishi *et al.*, 1989; Ricaurte *et al.*, 1986). In addition, the appearance of Lewy-like bodies has never been observed, even in older mice. Dosages and regimen schedules as well as the age of the animal result in varied susceptibility of midbrain neurons to MPTP as discussed above (Dawson *et al.*, 1995; German *et al.*, 1992; Seniuk *et al.*, 1990). Taken

together, these studies indicate that the neuropathological features observed in primates and mice exposed to MPTP are much closer to those observed in typical PD to those that can be reproduced using 6-OHDA.

2.2.2 Mechanism

To be effective as a neurotoxin, MPTP must be metabolized by MAO type B (MAO-B) to form the active metabolite MPP^+, as discussed in detail in section 3 (Chiba *et al.*, 1984; Markey *et al.*, 1984). MPP^+ has also been isolated in the brains of mice and monkeys following the systemic administration of MPTP, which favors MPP^+ but not MPTP as the neurotoxic mediator (Langston *et al.*, 1984b; Markey *et al.*, 1984). MAO-B activity is known to augment with age (Robinson, 1975), a factor contributing to the increased vulnerability to MPTP of older animals (Forno *et al.*, 1986; 1988; 1993; Irwin *et al.*, 1992; Nishi *et al.*, 1989; Tatton *et al.*, 1992; Tsai *et al.*, 1994). The high rate of oxidation of this tertiary amine was unexpected, despite extensive studies on the substrate specificities of MAO-A and B in numerous laboratories, but was soon confirmed (Fritz *et al.*, 1985). In addition, the selective degeneration of melanized dopaminergic neurons induced by MPTP is puzzling considering the low levels of MAO-B enzyme present outside dopaminergic neurons (Gutteridge and Toeg, 1982; Hirsch *et al.*, 1988; Westlund *et al.*, 1985).

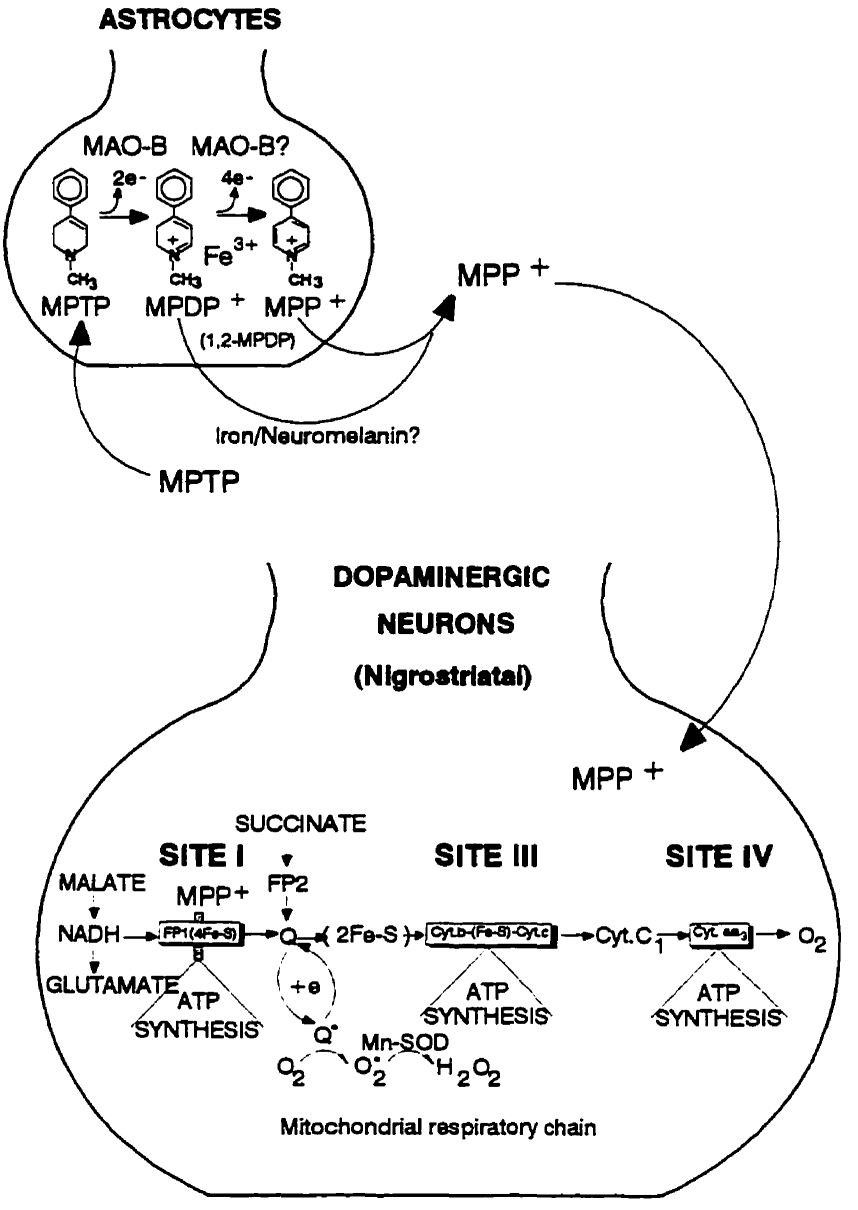

Fig. 7. Metabolic pathway for the activation of MPTP to MPP + and the site of MPP + toxicity.

The deleterious effects of MPP+ are related to the inhibition of the mitochondrial respiratory chain at complex I or NADH dehydrogenase (Fig. 7) (Nicklas *et al.*, 1985; Poirier and Barbeau, 1985b; Ramsay *et al.*, 1986b). This event leads to rapid ATP depletion and consequent losses of membrane potential, which in turn results in the degeneration of nigral dopaminergic cell

bodies (Bradbury *et al.*, 1986; Irwin and Langston, 1985; Javitch *et al.*, 1985). Interestingly, similar respiratory deficits are reported in the SN and striatum of parkinsonian patients with a reduction of NADH dehydrogenase activity (Mizuno *et al.*, 1989; Schapira *et al.*, 1989; 1990). These findings further support a role for an MPTP-like toxin in the etiology of PD.

Free radicals are also thought to play a role in the mechanism or mechanisms of MPTP-induced toxicity (Adams *et al.*, 1993; Chiueh *et al.*, 1992a; Poirier and Barbeau, 1985a; Poirier *et al.*, 1985; Przedborski *et al.*, 1992b; Rossetti *et al.*, 1988; Singer *et al.*, 1993; Wu *et al.*, 1993). Free radicals can induce lipid peroxidation, leading to an alteration in Ca^{2+} homeostasis and subsequent neuronal death. Johannessen and colleagues (1986) were the first to propose a role for oxidative stress based on the structural similarities between MPP^+ and paraquat (Fig. 8).

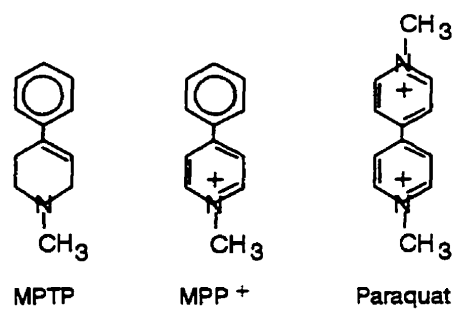

Fig. 8. Structures of MPTP, MPP + and paraquat. Note the structural similarity between MPP + and paraquat.

However, the electrochemical potential for the reduction of MPP^+ to form a free radical is greater than that of paraquat and cannot occur under physiological conditions (Elstner *et al.*, 1980; Frank *et al.*, 1987). In contrast, electron spin resonance (ESR) studies indicate that the

inclusion of MPP^+ in mitochondrial preparations induces the formation of $O_2^{\cdot}$ (Adams *et al.*, 1993; Rossetti *et al.*, 1988). Similarly, rotenone, which inhibits the respiratory chain at the same site as MPP^+, enhances ESR signals of $O_2^{\cdot}$ under these conditions (Adams *et al.*, 1993). Thus, disruption of the electron transport chain can lead to the appearance of incompletely reduced oxygen species, such as $OH^{\cdot}$ and $O_2^{\cdot}$ radicals, leading to an oxidative state. Reports that MPTP administration to mice induces an increase in free radical formation favor this view (Ali *et al.*, 1994). In addition, intrastriatal infusion of MPP^+ resulted in $OH^{\cdot}$ formation, as indicated by salicylate hydroxylation (Wu *et al.*, 1993). Accordingly, antioxidant defense systems can be overwhelmed by elevated levels of free radicals (Fig. 3 section 1.3.4, p. 15), which in turn could influence the levels of lipid peroxidation and consequently membrane integrity. We therefore made extensive use of MPTP as a model to study these possible relationships in the brains of mice.

3 Detailed Biochemical Mechanisms of MPTP-Induced Parkinsonism

3.1 MPTP Activation by Monoamine Oxidase and Passage of MPP^+ to the Locus of Neurotoxicity

The transformation of MPTP to MPP^+ involves 2 and 4 electron oxidation (Fig. 7 section 2.2.2, p. 32), forming 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) as an intermediate in the reaction (Chiba *et al.*, 1984). Two metabolic pathways have been proposed for the activation of MPTP. First, the obligatory conversion of MPTP to $MPDP^+$ requires the participation of MAO-B (Chiba *et al.*, 1984). The second 4 electron oxidation can be catalyzed by MAO-B,

transition metals (such as iron) or neuromelanin (Chiba *et al.*, 1984; Poirier and Barbeau, 1985a; Wu *et al.*, 1986). Although purified MAO-A is known to oxidized MPTP *in vitro*, the latter is a better substrate for MAO-B (Salach *et al.*, 1984). Moreover, MPTP, $MPDP^+$ and MPP^+ are, to a certain degree, inhibitors of both MAOs (Fuller *et al.*, 1985; Kinemuchi *et al*, 1985; Salach *et al.*, 1984; Singer *et al.*, 1985; 1986). MPTP is a suicidal inactivator (mechanism-based inactivation) of MAO-B, whereas $MPDP^+$ inhibits MAO-A activity more effectively (Salach *et al.*, 1984; Singer *et al.*, 1986). In addition, the rate of inactivation of MAO-B during the oxidation of $MPDP^+$ to MPP^+ is faster than during the oxidation of MPTP to $MPDP^+$ (Krueger *et al.*, 1990). Thus, the metabolism of MPTP is a self-limiting process in which the total concentration of MPP^+ formed depends on the amount of MAO-B present. This may contribute to the relative insensitivity of mice to MPTP toxicity, since the levels of MAO-B present in the brain are lower than those of MAO-A in mice and MAO-B in humans (Fowler and Strolin Benedetti, 1983; Garrick and Murphy, 1980; Saura *et al.*, 1992; Stenström *et al.*, 1987). The inter-species difference in relation to MPTP toxicity is discussed in more detail in section 3.4.

Evidence favouring MAO-B as a predominant enzymatic pathway is provided by the pre-treatment of mice and monkeys with selective and non-specific MAO-B inhibitors, L-deprenyl and pargyline, respectively, which protect against MPTP-induced striatal DA depletion (Heikkila *et al.*, 1984b; Langston *et al.*, 1984b; Markey *et al.*, 1984). In contrast, clorgyline, a selective MAO-A inhibitor, was ineffective (Heikkila *et al.*, 1984b). Accordingly, MPP^+, which could be formed by MAO-A *in vivo*, has no detectable effects on the toxicity of the nigrostriatal

pathway (Salach *et al.*, 1984). However, dopaminergic neurons of the nigrostriatal pathway of humans, monkeys and mice contain little or no MAO-B (Gutteridge and Toeg, 1982; Hirsch *et al.*, 1988; Stenström *et al.*, 1987; Westlund *et al.*, 1985). It therefore appears that the metabolic activation of MPTP must take place outside this system. This observation is consistent with the distribution of radiolabeled MPTP in the brain and the site of MPP^+ toxicity. Autoradiographic localisation of [^{3}H]-MPTP resembles that of MAO-B distribution in rodent and primate brains (Ekblom *et al.*, 1993; Javitch *et al.*, 1984; Konradi *et al.*, 1988; Parsons and Rainbow, 1987; Saura *et al.*, 1996; Westlund *et al.*, 1988; Willoughby *et al.*, 1988). Immunohistochemical and *in situ* hybridization studies revealed that MAO-B is confined to astrocytes and 5-HT-containing neurons, but not catecholaminergic neurons (Ekblom *et al.*, 1993; Konradi *et al.*, 1988; Saura *et al.*, 1996; Willoughby *et al.*, 1988). In contrast, systemic administration of radiolabeled MPTP in rodents and primates demonstrated intense labeling of the nigrostriatal dopaminergic neurons (Herkenham *et al.*, 1991; Takada *et al.*, 1991). Pre-treatment with pargyline, a non-selective MAO-B inhibitor, which blocks the conversion of [^{3}H]-MPTP to [^{3}H]-MPP^+ eliminated the retention of radiolabeled products. Moreover, the biotransformation of MPTP to MPP^+ as measured by high performance liquid chromatography (HPLC) remained unaffected in mice in spite of a prior denervation of catecholaminergic neurons with 6-OHDA (Nwanze *et al.*, 1995). Taken together, these results suggest that MPTP is converted to MPP^+ in MAO-B-containing cells and escapes from these sites to induce a selective degeneration of dopaminergic neurons of the nigrostriatal system.

Astrocytes are proposed as a major site of MPTP metabolism (Di Monte *et al.*, 1992; Wu *et al.*, 1992). The role played by serotonergic neurons in the production of MPP^+ may not be critical as lesions on these cells induced by 5,7-dihydroxytryptamine did not attenuate MPTP neurotoxicity (Brooks *et al.*, 1989). In addition, no loss in serotonergic neurons or 5-HT levels could be detected in the raphé nucleus following a systemic administration of MPTP, even though losses of striatal DA were apparent in cats (Lin *et al.*, 1995) and young mice (Date *et al.*, 1990). These observations suggest that MPTP is not preferentially metabolized to MPP^+, a potent mitochondrial toxin, in serotonergic neurons. It is therefore suggested that astrocytes represent the major site of MPTP activation. Since MPP^+ bears a positive charge, it poorly penetrates the blood-brain barrier and is therefore not expected to leave the astrocytic compartment. Alternatively, MPP^+ may kill glial cells, allowing it to escape in the CNS where it remains trapped and can diffuse to the site of toxicity. However, the degeneration of glial cells does not appear to be an apparent neuropathologic feature of MPTP exposure *in vivo* (Hess *et al.*, 1990; Schneider and Denaro, 1988), suggesting that astrocytes may be relatively resistant to MPTP/MPP^+ toxicity. Alternatively, gliosis may mask the damaging effects of MPP^+ toward astrocytes (Francis *et al.*, 1995; O'Callaghan *et al.*, 1990; Reinhard *et al.*,1988; Schneider and Denaro, 1988), thus explaining, at least in part, the failure to observe obvious cytotoxicity *in vivo*. This is consistent with the effect of MPTP in astrocytic cell cultures (Di Monte *et al.*, 1991; 1992; Wu *et al.*, 1992). Primary cultures of mouse astrocytes are shown to be sensitive to relatively high levels (250 μM) of MPTP present in the media, whereas lower concentrations (25 μM) were ineffective (Di Monte *et al.*, 1991; 1992; Wu *et al.*, 1992). In addition, MPP^+ and $MPDP^+$ are observed in the media of astrocytic cell cultures exposed to low levels of MPTP

and these compounds were noted even with MPTP removed from the media (Di Monte *et al.*, 1991; 1992; Wu *et al.*, 1992). It is therefore proposed that $MPDP^+$ generated within the cell crosses astrocytic membranes in the form of the lipophilic free base 1,2-MPDP and, finally, undergoes oxidation to MPP^+, known to be also catalized by transition metals or neuromelanin (Fig. 7 section 2.2.2, p. 32) (Di Monte *et al.*, 1987; 1991; Singer *et al.*, 1986; Wu *et al.*, 1992). Accordingly, the release of MPP^+ or $MPDP^+$ from astrocytes may contribute to the degeneration of DA-containing neurons of the nigrostriatal pathway.

3.2 MPP^+ Uptake by DA Transporter Explains the Selective Vulnerability of the Nigrostriatal System

The selectivity of MPP^+ as a neurotoxin is related to its active uptake by dopaminergic neurons of the nigrostriatal pathway, a mechanism that is analogous to 6-OHDA (Chiba *et al.*, 1985; Javitch *et al.*, 1985). An elegant demonstration of this is the finding that non-neuronal COS cells lacking catecholamine uptake are fairly resistant to MPP^+ toxicity (Kitayama *et al.*, 1992). Transfection of cDNA encoding the rat DA transporters in these cells confers toxicity to MPP^+ (Kitayama *et al.*, 1992). Similarly, mutant PC12 cells, derived from pheochromocytoma, devoid of catecholamine transporter are found to be resistant to MPP^+-induced toxicity (Bitler *et al.*, 1986). It has been demonstrated that DA uptake inhibitors protect against striatal DA depletion induced by systemic administration of MPTP in mice (Melamed *et al.*, 1985; Sundström and Johnson, 1985). In contrast, MPP^+ is a poorer substrate for adrenergic and serotonergic presynaptic uptake carriers (Chiba *et al.*, 1985; Javitch *et al.*, 1985; Sullivan and Tipton, 1988). MPTP itself is not accumulated into striatal or cortical synaptosomes by either DA or NE

uptake processes (Javitch *et al.*, 1985). In addition, radiolabeled MPP^+ is found to accumulate in synaptosomes prepared from brain striatal tissues and exhibited kinetic characteristics similar to those found for DA (Chiba *et al.*, 1985). Accordingly, the uptake of the toxicant is competitively inhibited by DA and DA uptake blockers such as mazindol (Chiba *et al.*, 1985; Javitch *et al.*, 1985). MPP^+ has been shown to be released from dopaminergic nerve terminals upon stimulation (Keller and Da Prada, 1985), suggesting that at least a portion may be stored in the dopaminergic vesicles. In addition, MPP^+ binds with high affinity to neuromelanin and therefore acts as a repository of this toxin (D'Amato *et al.*, 1986). Therefore, dopaminergic neurons retain MPP^+ avidly. Accordingly, MPP^+ acts as an effective substrate for DA uptake carriers and this provides a mechanism for its accumulation in nigrostriatal cells.

3.3 Biochemical Events Leading to the Degeneration of Nigrostriatal Dopaminergic Neurons

The hypothesis which has received extensive support from *in vitro* and *in vivo* studies suggests MPP^+ as an inhibitor of mitochondrial NADH dehydrogenase or complex I leading to the cessation of oxidative phosphorylation (Fig. 7 section 2.2,2, p. 32) (Nicklas *et al.*, 1985; Poirier and Barbeau, 1985b; Ramsay *et al.*, 1986b). Injection of rotenone, known to inhibit at the same site as MPP^+, into the medial forebrain bundle induces a selective degeneration of nigrostriatal dopaminergic neurons in rats (Heikkila *et al.*, 1985). Thus, inhibition of NADH dehydrogenase is sufficient on its own to induce neuronal cell losses. However, MPP^+ is several times less potent than rotenone at inhibiting mitochondrial complex I (Nicklas *et al.*, 1992). This apparent discrepancy was delineated upon astute observations.

Nicklas and his colleagues (1985) first demonstrated that a relatively high concentration (0.5 mM) of MPP^+ is required to block the rate of oxygen uptake in liver and brain mitochondrial preparations in the presence of pyruvate and malate (complex I substrates) during ATP synthesis. The inhibition of NAD^+-linked substrates was both concentration- and time-dependent, such that the initial rate of mitochondrial respiration was unaffected by MPP^+ and a progressive decline over several minutes was observed thereafter. In contrast, mitochondrial respiration, measured by a Clark oxygen electrode, was not inhibited by succinate (complex II substrate), suggesting that MPP^+ affected the transfer of electrons from complex I to complex II. However, the concentration ($\approx$ 50 μM) of MPP^+ found in whole nigral homogenates of MPTP-treated mice was found to be two orders of magnitude lower than that required to achieve respiratory inhibition *in vitro* (Irwin *et al.*, 1987; Langston *et al.*, 1987a; Nicklas *et al.*, 1985). It was also difficult to conceive how a cation could penetrate the mitochondrial membrane to gain access to NADH dehydrogenase.

Two major studies helped to explain this apparent paradox. First, dopaminergic transporters could accumulate sufficient MPP^+ to inhibit mitochondrial respiration, as discussed in section 3.2. Second, the pyridinium cation is concentrated in the matrix by the electrical gradient of the mitochondrial membrane at levels required to achieve inhibition at this locus (Davey *et al.*, 1992). This observation is consistent with the effects of tetraphenylboron anion (TBN^-) and respiratory chain uncouplers on MPP^+-induced inhibition of NADH dehydrogenase. TBN^- facilitated the transfer of MPP^+ accross the mitochondrial membrane by acting as an ion pairing agent. TBN^- therefore reduces considerably the concentration of MPP^+ required for this

inhibition by shielding the positive charge of the latter (Aiuchi *et al.*, 1988; Heikkila *et al.*, 1990; Ramsay *et al.* 1989). In contrast, this inhibition is prevented in the presence of mitochondrial uncouplers (Nicklas *et al.*, 1985). Uncouplers induce an efflux of accumulated pyridinium cations, suggesting that this inhibition is also reversible (Ramsay *et al.*, 1986a). The effect of uncouplers are remarkable as they reduce the electrochemical potential of mitochondria by acting as a proton sink. Thus, the transfer of electrons in the respiratory chain can no longer sustain ATP synthesis due to the collapse of the transmembrane potential. Therefore, the mitochondrial toxicity of MPP^+ appears to be self-limiting depending on the relationship between MPP^+ and ATP concentrations. Accordingly, its uptake slows down as ATP concentrations fall, lowering the intramitochondrial levels of this toxin. This observation is supported by the effect of MPP^+ on mitochondrial membrane potential and NADH dehydrogenase inhibition. A reduction in mitochondrial membrane potential is observed when this compound is incubated with mitochondria (Lambert and Bondy, 1989). Inhibition of NADH dehydrogenase has been shown to be of short duration in mice brains, returning to control levels within a few days of MPTP treatment (Mizuno *et al.*, 1988). Taken together, these observations clearly suggest that the half-life of MPP^+ within the nigrostriatal system is a crucial determining factor for its toxicity and may account for the inter-species differences in MPTP sensitivity.

3.4 Species Are Differentially Susceptible to MPTP-Induced Toxicity

A striking feature of MPTP is the marked inter-species vulnerability. Humans, monkeys and dogs exhibit the greatest vulnerability followed by cats and mice (Burns *et al.*, 1983; Chiueh *et al.*, 1984; Heikkila *et al.*, 1984a; Schneider and Markham, 1986; Wilson *et al.*, 1987). In contrast, rats and guinea pigs are more resistant (Chiueh *et al.*, 1984; Perry *et al.*, 1985b; Walters *et al.*, 1984). There are also considerable differences among various strains of mice (Giovanni *et al.*, 1991; Riachi and Harik, 1988; Sonsalla and Heikkila, 1988). Several explanations related to the strain and species differences have been put forward during the recent years. Differences in MPTP metabolism and MPP^+ retention as well as its distribution may be contributory factors. These observations are consistent with the half-life of MPP^+ in the brain after MPTP administration which is substantially longer in monkeys and mice than in rats (Giovanni *et al.*, 1994a; Hallmann *et al.*, 1985; Markey *et al.*, 1984; Nwanze *et al.*, 1995). Mice excrete 7-8 times the amount of unmetabolized MPTP than do monkeys (Lau *et al.*, 1988). The relative levels of MAO-B present in the brains of mice are lower than those of humans and monkeys (Fowler and Strolin Benedetti, 1983; Garrick and Murphy, 1980; Saura *et al.*, 1992; Stenström *et al.*, 1987) and contribute to the contrasting metabolism of MPTP in these species. In addition, MPP^+ is retained in mice brains for approximately 24 hrs with a half-life of approximately 2 to 4 hrs as opposed to several days for primates (Johannessen *et al*, 1985; Markey *et al.*, 1984). MPP^+ is found to bind to neuromelanin with high affinity (D'Amato *et al.*, 1986), suggesting that neuromelanin serves as a buffer, progressively liberating MPP^+. Interestingly, pigmentation of nigral neurons is an obvious characteristic in primates not evident

in rodents. Consistent with this is the direct correlation between neuromelanin content and the degree of dopaminergic cell loss in MPTP-treated monkeys (Herrero *et al.*, 1993b). Agents that compete with MPP^+ for neuromelanin binding, such as the antimalarian drug chloroquine, reduce or prevent MPTP-induced toxicity in monkeys (D'amato *et al.*, 1987). However, intracarotid injection of trace doses of [^{14}C]-MPTP in monkeys failed to produce substantial accumulation and retention of labeled MPP^+ in nigral neurons (Herkenham *et al.*, 1991). Perhaps the dose was too low to be toxic and to label neuromelanin intensely such that the radiolabeled product was sequestered mostly by vesicular DA transporters. Supporting the neuromelanin hypothesis is the observation that older mice and monkeys are more sensitive to MPTP treatment than younger ones (Nishi *et al.*, 1989) whose SN is not yet melanized (Herrero *et al.*, 1993a).

Although neuromelanin seems to underlie the unusual susceptibility of primates to MPTP, it does not, however, relate to the differential attributes of this neurotoxin among rodents. It has been suggested that high MAO-B activity in the brain capillary endothelium, creating a biochemical barrier to MPTP, might explain the resistance of rats to MPTP as compared to mice (Kalaria *et al.*, 1987; Riachi *et al.*, 1991). However, MPTP-induced DA loss in transgenic mice overexpressing neuronal MAO-B is surprisingly similar to that of their non-transgenic littermates (Andersen *et al.*, 1994). Astrocytes are the main contributors to the conversion of MPTP to MPP^+, whereas neuronal MAO-B participation is limited, as discussed in section 3.1, which may explain these findings. Other studies have demonstrated a high degree of correlation between striatal concentrations of MPP^+ and sensitivity to MPTP in several strains of mice

(Giovanni *et al.*, 1991; Sonsalla and Heikkila, 1988). To be effective as a neurotoxin, MPP^+ must accumulate and remain in high concentrations within the mitochondrial matrix since this inhibition is weak and reversible. Thus, the rate of MPP^+ elimination is a key factor in determining strain and species sensitivity to MPTP. However, striatal MPP^+ levels observed in rats following the systemic administration of very large doses of MPTP were higher than those observed following similar dosages in mice (Giovanni *et al.*, 1994a). These studies clearly indicate that MPP^+ reaches the nigrostriatal pathway in rats and that blood-brain barrier MAO-B activity may not be a critical factor. In addition, the affinity of the DA transporter for MPP^+ is similar to that of DA itself in the striatal tissue of both rats and mice (Javitch *et al.*, 1985; Mayer *et al.*, 1986; Sonsalla *et al.*, 1987). Interestingly, the EC_{50} required to induce striatal DA efflux in rats was 10 times higher than that of mice, as determined by intrastriatal infusion of MPP^+ (Giovanni *et al.*, 1994b). Thus, rats require exposure to a much higher concentration of MPP^+ than mice for the toxin to induce similar damage to dopaminergic neurons (Giovanni *et al.*, 1994b), and these effects cannot be attributed to differences in pharmacokinetic properties of DA transporters and MPP^+. Molecular differences in the vesicular monoamine transporter, which sequestered and stored MPP^+, could be responsible for the differential toxicity observed in mice and rats. The rat cDNA DA transporter encodes a 514 amino acid protein with twelve putative transmembrane segments that is 90.7% homologous to humans (Lesch *et al.*, 1993). However, a major sequence divergence (64%) is noted in the large luminal loop located between

the first two transmembrane domains (Lesch *et al.*, 1993). This loop may play an important role in determining the relative affinity to the toxicant for the transporter. Perhaps molecular differences in the vesicular monoamine transporter of rats and mice explain their relative sensitivity to the neurotoxic effects of MPP^+.

4 MPTP Alters the Antioxidant Capacity of the Nigrostriatal System: Evidence Supporting a Role for Oxidative Stress in MPTP-Induced Neurotoxicity

Although inhibition of mitochondrial function appears to be a sufficient explanation for the observed neurotoxic effect of MPTP administration, oxidative stress is thought to contribute to its long-term outcome. In this context, it is important to remember that inhibition of NADH dehydrogenase by MPP^+ is weak and reversible. As the levels of ATP fall, the uptake of MPP^+ slows and consequently leads to decreased intramitochondrial levels of the toxicant. This process may contribute to the regeneration of NADH dehydrogenase activity observed in mice brains (Mizuno *et al.*, 1988). Changes in the appearance of brain mitochondria following MPTP administration to monkeys but not to mice have been reported (Adams *et al.*, 1989a; Nakamura *et al.*, 1989). It is therefore possible that mitochondrial dysfunctions induced by MPTP are transient and may not fully explain the long-term effects of MPTP. Alterations in the antioxidant capacity of the nigrostriatal pathway observed as a result MPTP or MPP^+ administration seem to support this notion.

4.1 MPTP and Glutathione

GSH is a co-substrate of GSH-PX (Fig. 3 section 1.3.4, p. 15) and GSH-S-transferase is known to play an important role in protecting against oxidative stress and the detoxification of xenobiotics. MPTP administration is shown to deplete nigral and striatal levels of GSH in mice and rats (Desole *et al.*, 1993; 1995; Ferraro *et al.*, 1986; Yong *et al.*, 1986). This observation suggests a role for this antioxidant in MPTP-induced oxidative stress. Although GSH depletion can be a trait of some oxidative stress-inducing agents, such as 6-OHDA, it is more common with agents that deplete GSH by forming GSH conjugates (Perumal *et al.*, 1989). However, MPTP or MPP^+ are not known to form GSH conjugates (Adams and Odunze, 1991). GSH depletion produced by MPTP treatment may be related to ATP depletion, as ATP is known to be required by GSH synthetase and gamma-glutamylcysteine synthetase for the synthesis of GSH. This observation is supported by the use of mitochondrial respiratory inhibitors, such as potassium cyanide (complex IV inhibitor) and antimycin A (complex III inhibitor), known to induce ATP depletion that also results in GSH reduction (Mithöfer *et al.*, 1992). Pre-treatment with an antioxidant (α-tocopherol or ß-carotene) protects against GSH depletion but only partially against striatal DA and nigral cell loss, suggesting that some GSH reduction is related to oxidative stress consequential to respiratory chain inhibition (Perry *et al.*, 1985a; Yong *et al.*, 1986). It is therefore reasonable to assume that the manipulation of brain GSH content may alter MPP^+ toxicity. This hypothesis is consistent with the effect of carmustine and BSO, which reduce brain GSH content, on potentiating nigral DA depletion induced by MPTP in mice (Adams *et al.*, 1989b; Wüllner *et al.*, 1996). Carmustine is a known inhibitor of GSH-reductase

involved in the regeneration of GSH from its inactive form (GSSG) (Fig. 3 section 1.3.4, p. 15), whereas BSO blocks gamma-glutamylcysteine synthetase, the rate-limiting enzyme present in the synthesis of GSH. Moreover, administration of (-)-2-oxo-4-thiazolidine carboxylate, a GSH precursor known to increase brain GSH content, is found to attenuate the MPTP-induced reduction in striatal DA and its metabolites (Weiner *et al.*, 1988). Interestingly, GSH depletion has a differential effect on midbrain dopaminergic neurons. Depletion of brain GSH by BSO is known to decrease nigral DA and to induce neuronal hypertrophy not seen in the ventral tegmental area of mice (McNeill *et al.*, 1985). This observation suggests constitutive free radical formation within the SN that could be attributed to the degree of melanization. Accordingly, the antioxidant/oxidant balance appears to play a crucial role in nigral neuronal survival.

4.2 MPTP and Ascorbic Acid

Ascorbic acid (vitamin C) is a potent water-soluble antioxidant involved in the regeneration of α-tocopherol (vitamin E) and GSH from their oxidized forms through a series of oxidation-reduction reactions (Fig. 9) (Winkler *et al.*, 1994). Thus, brain ascorbic acid concentrations are maintained by an effecient homeostatic mechanism. This may contribute to lack of alterations in ascorbic acid levels, whereas its oxidized form (dehydroascorbic acid) is increased in MPTP-induced DA depletion in striatal synaptosomes prepared from rats (Desole *et al.*, 1993; 1995). Alterations in dehydroascorbic acid content are consistent with free radical-mediated

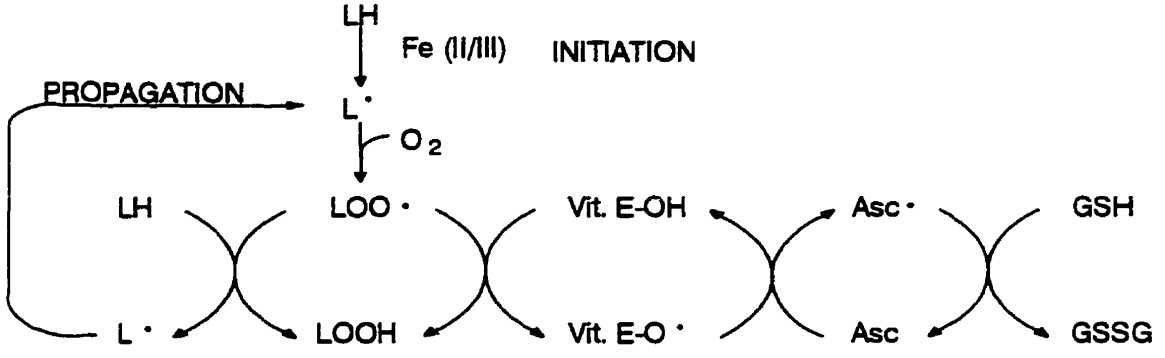

Fig. 9. Role of vitamine (Vit. E), ascorbic acid (Asc) and glutathione (GSH) in the detoxification of peroxidized lipids. The main features of the cellular antioxidant cascades are shown, with emphasis on the cyclical redox reactions. LH, polyunsaturated fatty acid; L˙ ,carbon-centered fatty acid radical; LOO ˙ , lipid peroxyl radical; LOOH, lipid hydroperoxide; vit-E-O ˙ ,α-tocopheryl radical; Asc ˙, ascorbyl radical.
Modified from Ernster and Dallner, 1995; Winkler *et al.*, 1994.

injuries. Ascorbic acid is also known to partially protect against MPTP neurotoxicity in mice, thereby substantiating this notion (Perry *et al.*, 1985a; Sershen *et al.*, 1985; Wagner *et al.*, 1985).

4.3 MPTP and α-Tocopherol

α-Tocopherol (vitamin E), a lipid soluble antioxidant, plays a critical role in the protection of lipid membranes from oxygen-derived free radicals (Fig. 9). Vitamin E-deficient animals have significantly higher basal levels of $O_2^{\cdot}$ radical formation than control animals. Nigral neurons are found to be more susceptible to MPTP neurotoxicity, as measured by alterations in the content of DA and its metabolites (LeBel *et al.*, 1989; Odunze *et al.*, 1990). An increase in lipid peroxidation levels is observed in the SN of vitamin E-deficient mice following MPTP

intoxication (Adams *et al.*, 1990). However, alterations in striatal lipid peroxidation, DA concentrations or DA metabolites were not exacerbated in these animals. These observations suggest that the antioxidative state of striatal dopaminergic neurons differs from that of the SN in terms of their susceptibility to free radicals generated by MPTP treatment. This is consistent with the lower content of GSH found in dopaminergic cell bodies of the SN as compared to their striatal terminal areas (Slivka *et al.*, 1987). Report that a small deficiency in vitamin E (3% of control levels) in rats resulted in a decrease in TH immunopositive neurons in the SN not observed in the ventral tegmental area is also consistent with the higher vulnerability of nigral dopaminergic cell bodies to free radical damage (Dexter *et al.*, 1994a). No alteration in DA concentrations or turnover could be detected in the striatum of these animals.

MPTP is also capable of producing changes in vitamin E in the brains of normal mice (Odunze *et al.*, 1990). A rapid increase in vitamin E concentrations is observed in the striatum of MPTP-treated mice; these values return to control values within a few hours. In contrast, the compensatory increase in nigral vitamin E levels in response to an oxidative challenge to the membrane by MPTP occurs very slowly and in a manner that is consistent with the higher susceptibility of this structure to free radical-inducing agents. Changes in vitamin E content may be responsible for the failure to observe an increase in striatal lipid peroxidation levels in MPTP-treated mice (Adams *et al.*, 1990; Corongiu *et al.*, 1987).

4.4 MPTP and Superoxide Dismutase

Mice pre-treated with diethyldithiocarbamate, an inhibitor of CuZn-SOD, are subject to enhanced striatal MPTP toxicity (Corsini *et al.*, 1985; Pikarsky *et al.*, 1985). The increase sensitivity to MPTP induced by this compound may indicate that SOD plays a role in the protection of cells from this neurotoxicant, presumably by detoxifying $O_2^{\cdot}$ radicals. Transgenic mice overexpressing CuZn-SOD, with a three-fold increase in striatal CuZn-SOD activity, are resistant to low doses of MPTP, thereby supporting this idea (Przedborski *et al.*, 1992b). However, increases in SOD activity in transfected cells and transgenic mice cause significant changes in the characteristics of the cell membrane as a consequence of increased lipid peroxidation (Ceballos-Picot *et al.*, 1991; Elroy-Stein *et al.*, 1986). In this context, it is important to remember that SODs scavenge $O_2^{\cdot}$ with a concomitant production of H_2O_2, and the accumulation of the latter participates in the cyclic production of oxygen-derived free radicals leading to oxidative stress (Fig. 3 and 4, section 1.3.4, p. 15 and 17, respectively). Transgenic mice overexpressing SOD are found to be more sensitive to the effect of hyperbaric O_2 than non-transgenic littermates (Oury *et al.*, 1992). Conversely, diethyldithiocarbamate increased resistance to hyperoxia (Oury *et al.*, 1992). Since manipulation of antioxidant systems also leads to enhanced MPTP-mediated toxicity (as discussed above), it is reasonable to expect a higher vulnerability to MPTP in transgenic mice overexpressing SOD. It may be that levels of SOD expression achieved in these mice have differential effects on models of free radical-induced toxicity. Alternatively, changes in other antioxidants, such as increases in GSH-PX or CAT activity, could have counteracted the elevated concentrations of H_2O_2. This assumption

is supported by the increase in CAT activity observed in transgenic mice found resistant to MPTP neurotoxicity and whose GSH-PX activity remained unaltered (Przedborski *et al.*, 1992a). To our knowledge, the effects of MPTP on SOD activity has not been examined. We therefore investigated the role of SOD in MPTP-induced toxicity in normal mice (Chapter 2).

4.5 MPTP and Calcium

A major consequence of MPP^+'s action is the inhibition of oxidative phosphorylation, if sustained for a long period, results in ATP depletion. ATP is required, among others things, for the regulation of ionic gradients (such as Na^+/K^+ ATPase) required for the passage of action potentials and the Ca^{2+}-dependent release of neurotransmitters. Failure to rapidly restore ionic homeostasis after depolarization leads to the activation of voltage-dependent Ca^{2+} channels and neuronal hyperexcitability. Another recurring characteristic of disruptions of the electron transport chain is the excessive formation of free radicals leading to oxidative stress. In turn, alterations in membrane integrity result in increased permeability to ions such as Ca^{2+}, leading to Ca^{2+} overload, further depleting energy reserves (Braughler *et al.*, 1985; Paraidathathu *et al.*, 1992). A common effector of intracellular Ca^{2+} overload is the activation of many Ca - dependent processes which contribute to cell damage, thus promoting a vicious cycle of oxidative stress and mitochondrial dysfunctions (Dykens, 1994; Kowaltowski *et al.*, 1995; Malis and Bonventre, 1986). Those most often quoted include activation of peptidases, phospholipases and endonucleases (Beal *et al.*, 1993; Coyle and Puttfarcken, 1993; Dawson *et al.*, 1995). For example, peptidases, such as calpain I, catalyze the enzymatic conversion of xanthine

dehydrogenase to xanthine oxidase involved in the catabolism of purine bases to yield $O_2^{\cdot}$ (McCord, 1985). In addition, phospholipases such as phospholipase A_2 release arachidonic acid, which in turn is metabolized by lipoxygenases and cyclo-oxygenases to form eiconasoids with concomitant $O_2^{\cdot}$ formation (Chan and Fishman, 1980). Furthermore, the rise in intracellular Ca^{2+} can lead to the up-regulation in the activity of NO synthase, an enzyme involved in the formation of $NO^{\cdot}$ that is expressed in striatal neurons (Beal *et al.*, 1993; Bredt *et al.*, 1990; Coyle and Puttfarcken, 1993; Dawson *et al.*, 1995; Lafon-Cazal *et al.*, 1993). $NO^{\cdot}$ reacts rapidly with $O_2^{\cdot}$ to form $ONOO^-$ which decomposes to $OH^{\cdot}$ to mediate neuronal injuries, as discussed in section 1.3.4 (Fig. 4 section 1.3.4, p. 17). Inhibition of NO synthase with nitro-L-arginine reduces $OH^{\cdot}$ radical formation produced by striatal infusion of MPP^+ in rats (Smith *et al.*, 1994). In addition, mice pre-treated with nitro-L-arginine showed attenuated striatal DA depletion resulting from MPTP administration (Smith *et al.*, 1994), suggesting a role for Ca^{2+}-induced free radical formation. Consistent with the role of Ca^{2+} in MPTP-induced oxidative stress is the increase in intracellular Ca^{2+} levels (Kass *et al.*, 1988) and ATP depletion (Di Monte *et al.*, 1986) in rat hepatocytes exposed to MPTP. Furthermore, MPP^+ is demonstrated to increase intracellular Ca^{2+} levels in mesencephalic cells, whereas this effect is not seen in cortical cultures devoid of dopaminergic cells (Chen *et al.*, 1995). Nimodipine, a blocker of voltage-gated Ca^{2+} channels (L-type), partially protects against MPTP-induced neurochemical changes in a dose-dependent manner in the striatum of mice (Gerlach *et al.*, 1993). The rise in intracellular Ca^{2+} levels will trigger the extrusion of Ca^{2+} from the cell, or its sequestration within the cell (endoplasmic reticulum and mitochondria). Ca^{2+} is extruded by a low-capacity high-

affinity ATPase (calmodulin-dependent) and by high-capacity low-affinity Na^+/Ca^{2+} exchangers that utilize the energy stored in the sodium gradient to expel Ca^{2+}. However, since the exchanger is electrogenic, carrying positive charges into the cell, it is also driven by membrane potential. Ion exchangers of this type are inherently bidirectional such that if the sodium gradient collapses, the membrane is depolarized, leading to a rise in intracellular Ca^{2+} levels. Similarly, failure to produce ATP may accelerate Ca^{2+} uptake, a mechanism thought to contribute to MPTP-toxicity. The sequestration of Ca^{2+} into the endoplasmic reticulum is ATP-dependent and its release occurs in response to the activation of a surface receptor on the endoplasmic reticulum. The degree of activation determines whether a net Ca^{2+} release or uptake takes place. The uptake of Ca^{2+} into the mitochondria is similarly regulated and driven by a transmembrane potential (the inside of the mitochondria being negative to the cytosol). However, the cycling of Ca^{2+} across the inner mitochondrial membrane may normally serve to regulate the mitochondrial Ca^{2+} gradient and, thereby, the activity of several Ca^{2+}-dependent dehydrogenases, including pyruvate dehydrogenase and isocitrate dehydrogenase (Denton and McCormack, 1985; Hansford, 1985), providing a means to control cellular ATP demands. Therefore, Ca^{2+} accumulation by mitochondria as a mechanism for regulating intracellular Ca^{2+} may only come into play if a pathological rise in intracellular Ca^{2+} occurs (Fig. 10). Calbindin-D_{28K}, a Ca^{2+} binding protein, may provide another means to deal with excessive Ca^{2+} accumulation by functioning as a Ca^{2+} buffer (Baimbridge *et al.*, 1992). This is of particular interest since calbindin-D_{28K} positive mesencephalic dopaminergic neurons are preserved in PD and in MPTP-treated animals (German *et al.*, 1992; Haber *et al.*, 1995; Lavoie and Parent, 1991; Yamada *et al.*, 1990). Accordingly, within the SN, the melanized dopaminergic neurons of the

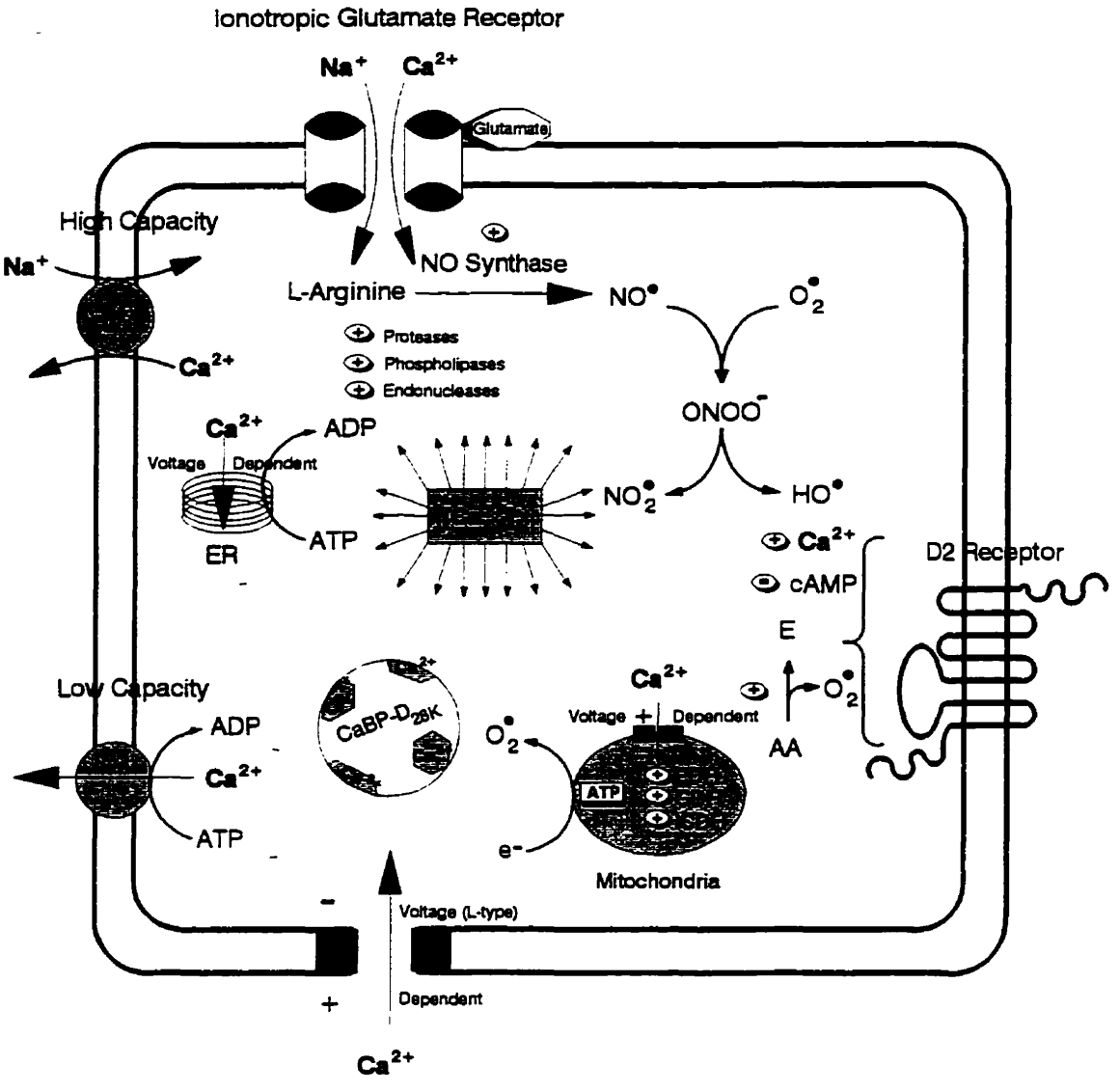

Fig. 10. Diagram demonstrating the effects of energy failure leading to increases in intracellular Ca^{2+} levels and oxidative stress. Impaired generation of ATP leads to membrane depolarization followed by relief of the Mg^{2+} block of the NMDA receptor and, consequently, loss in Ca^{2+} homeostatis (see text). Normally, Ca^{2+} can be sequestered by mitochondria and endoplasmic reticulum (ER). Ca^{2+} is extruded by low-capacity high-affinity ATPase (calmodulin-dependent) and by a high-capacity, low-affinity Na^{+} / Ca^{2+} exchanger. Influx of Ca^{2+} can also occur through the stimulation of NMDA and D2 receptors, leading to the activation of proteases, phospholipases and endonucleases. Ca^{2+} accumulates in mitochondria leading to $O_2^{\bullet}$ generations. It also activates NO synthase to further exacerbate free radical formation and ATP depletion, thereby promoting a vicious cycle. Modified from Beal, 1995; Chiueh *et al*., 1993; Coyle and Puttfarcken, 1993; Kennedy, 1989; Siesjö, 1990.

AA = arachidonic acid, E = eiconasoids, PDH = pyruvate dehydrogenase, GDH = glutamate dehydrogenase, ICDH = isocitrate dehydrogenase, CaBP = calbindin.

ventral tier which are the most susceptible to degeneration in PD and MPTP pathology are calbindin-D_{28K} negative, whereas the dorsal tier that is preserved demonstrates calbindin-D_{28K} immunostaining. Similarly, numerous dopaminergic neurons that are spared in the ventral tegmental area and peri- and retrorubral regions are calbindin-D_{28K} positive (German *et al.*, 1992). In the central gray substance, calbindin-D_{28K} is detected in all catecholaminergic neurons where cell loss is minimal.

Ca^{2+} influx can also occur through the activation of ionotropic N-methyl-D-aspartate (NMDA) receptors by excitatory amino acid (EAA) neurotransmitters such as glutamic acid. Under normal conditions, the ionic channel of the NMDA receptor complex is blocked by magnesium ions. Depolarization of the neuron removes the magnesium block and thus enhances the stimulatory effect of glutamic acid on this receptor. It is well known that the SN pars compacta receives a glutamatergic projection from the cerebral cortex (Fonnum, 1984) and that dopaminergic neurons of the SN pars compacta have NMDA receptors on their surface (Overton and Clark, 1992). This observation is also supported by the reduction in nigral NMDA binding sites following MPTP intoxication (Wüllner *et al.*, 1993). Moreover, it has been suggested that dopaminergic terminals in the striatum are part of a cortico-striato-thalamo-cortical negative feedback loop, with DA causing the release of EAA (Carlsson and Carlsson, 1990). Hence it seems that glutamic acid influences the nigrostriatal dopaminergic neurons at the level of both their cell bodies and terminals. A synergistic toxic effect is observed when the complex I inhibitor rotenone is incubated with glutamic acid in mesencephalic dopaminergic cultures (Marey-Semper *et al.*, 1995). Therefore the influence of NMDA antagonists on the

MPTP-induced neurotoxicity has been intensively studied. However, the involvement of EAA in this particular model where NMDA receptor antagonists have been used as protective agents against the toxic effects of MPTP is controversial (Chan *et al.*, 1993; Kupsch *et al.*, 1992; Santiago *et al.*, 1992; Sonsalla *et al.*, 1992; Storey *et al.*, 1992; Turski *et al.*, 1991; Zuddas *et al.*, 1992). The reasons for these conflicting results remain to be fully elucidated. For example, it has been reported that MK801 (6 x 3.4-5 mg/kg at 4 hrs interval starting 30 min prior to MPP^+) is effective against dopaminergic cell loss and DA depletion up to 24 hrs following intranigral or intrastriatal infusion of MPP^+ in rats (Storey *et al.*, 1992; Turski *et al.*, 1991). Moreover, MK801 (7 x 0.01 mg/kg/day for 5 days) administered in combination with a daily dose of MPTP prevented degeneration of dopaminergic neurons as measured by levels of DA and its metabolites in primates (Zuddas *et al.*, 1992). In contrast, Sonsalla *et al.* (1992) found no protection from MK801 (6 x 2.5 mg/kg at 4 hr interval starting 30 min prior to MPP^+ or MPTP) after intranigral infusion of MPP^+ in rats or 24 hrs after the systemic administration of MPTP in mice. Dosage regimens of MK801, rate of elimination and species differences may explain the contrasting results obtained in these experiments. In addition, MK801 has been reported to modify the rate of elimination of MPP^+ from mouse striatum (Chan *et al.*, 1993) and to block MPP^+ uptake by rat synaptosomal preparations (Clarke and Reuben, 1995). Thus, it may be that high levels of MK801 must be sustained for a long period in order to be effective against MPP^+ toxicity. However, chronic administration of MK801 may not be feasible given its known toxicity. Alternatively, it may be that MK801 delays the neurotoxic effects of MPP^+ by blocking the rise in Ca^{2+} influx which occur, in part, through the activation of NMDA receptors rendered more sensitive to EAA. In this context, ATP depletion will result in the

collapse of ionic gradients and the loss of membrane potential, leading to the activation of voltage-gated Ca^{2+} channels (L-type). This concept may be in line with the transient effects of MK801 on MPP^+-induced nigrostriatal toxicity, with protection seen up to 24 hrs, while impaired energy metabolism (ATP levels) continued for over 48 hrs (Chan *et al.*, 1993; Rouillard *et al.*, 1990; Storey *et al.*, 1992). Further studies are required to clarify these issues.

4.6 MPTP, Oxidative Stress and Parkinson's disease

In summary, the above studies demonstrate that MPTP intoxication in mice induces neurochemical, neuropathological and biochemical alterations that are reminiscent of PD. Table 1 shows the similarities among the alterations discussed so far between MPTP-induced parkinsonism and PD. Taken together, these observations lead researchers to rationalize that an antioxidative therapy could be beneficial in patients suffering from PD, as manipulation of antioxidant defense systems can, to a certain extent, attenuate or exacerbate the deleterious effects of MPTP in animal models. A large scale study began in 1989 to evaluate the effects of vitamin E and L-deprenyl on the progression of disabilities in early, untreated PD patients (The Parkinson Study Group, 1989a). Since oxidative stress is thought to play a significant role in the brains of PD patients, as demonstrated by, among other findings, higher lipid peroxidation levels (Dexter *et al.*, 1989a; Pall *et al.*, 1986), vitamin E, a lipid peroxyl radical scavenger, was considered of therapeutic value (The Parkinson Study Group, 1989a). However, vitamin E did not delay the onset of disability in these patients as measured by the length of time before L-dopa therapy was required (The Parkinson Study Group, 1993). The lack of effect of vitamin

Table 1.

Summary of evidence supporting a role for oxidative stress in the substantia nigra (SN) and striatum in PD and MPTP-induced parkinsonism.

	PD	MPTP	References
Melanized nigral neurons	selectively lost	selectively lost	Hirsch *et al.*, 1988 Herrero *et al.*, 1993
Oxidized DNA	increased	ND	Sanchez-Ramos *et al.*, 1994
Lipid peroxidation	increased	no change	Dexter *et al.*, 1989a Pall *et al.*, 1986 Adams *et al.*, 1990 Corongiu *et al.*, 1987
Reactive oxygen species	cannot be measured	increased	Ali *et al.*, 1994
GSH	reduced	reduced	Desole *et al.*, 1993, 1995 Ferraro *et al.*, 1986 Sofic *et al.*, 1992 Yong *et al.*, 1986
SOD activities	increased	ND	Marttila *et al.*, 1988b Poirier and Thiffault, 1993 Saggu *et al.*, 1989
GSH-PX activity	little or no change	ND	Marttila *et al.*, 1988b Poirier and Thiffault, 1993
CAT activity	little or no change	ND	Marttila *et al.*, 1988b Ambani *et al.*, 1975 Kish *et al.*, 1985
NADH dehydrogenase activity	reduced	reduced	Mizuno *et al.*, 1989 Schapira *et al.*, 1989, 1990

ND = Not Determined

E may be related to its low penetrance into the brain (Vatassery *et al.*, 1984). Conversely, the MAO-B inhibitor, L-deprenyl was found to delay the necessity of L-dopa therapy by 6 to 9 months in *de novo* parkinsonian patients (The Parkinson Study Group, 1989b). In addition, L-deprenyl, in combination to L-dopa, permits the dosage of L-dopa to be reduced by approximately 10-30% (Myllylä *et al.*, 1995; Tetrud and Langston, 1989; The Parkinson Study

Group; 1993). This aspect was considered significant by neurologists as loss of efficacy occurs during the prolonged use of L-dopa, characterized by wearing-off and by the "on and off" response fluctuations (abrupt swinging back and forth in response to L-dopa from clinical effectiveness to sudden akinesia). However, doubt has emerged in the clinical arena regarding the use of L-deprenyl as an adjunct to L-dopa therapy. For instance, L-deprenyl used in combination to L-dopa has been found of little benefit in patients with early and mild PD (The Parkinson's Disease Research Group, 1993). Increasing disability is also observed in patients after 2 years of combined therapy (Elizan *et al.*, 1989). Finally, the recent finding of a higher mortality rate during a prolonged period of L-deprenyl treatment raises some serious questions regarding the safety of this drug (Lees, 1995).

In addition, L-deprenyl is demonstrated to alter antioxidant enzyme activity, suggesting that the pharmacological profile of this compound is complex and extends beyond MAO-B inhibition. For instance, reports that L-deprenyl induces an increase in SOD activity (Carrillo *et al.*, 1991; Clow *et al.*, 1991; Knoll, 1988) are intriguing considering that SOD is up-regulated in several models of free radical-induced toxicity (hyperoxia, 6-OHDA, paraquat) (Crapo and Tierney, 1973; Frank, 1981; Krall *et al.*, 1988; Ogawa *et al.*, 1994; Sjostrom and Crapo, 1981; Stevens and Autor, 1977). Taken together, these studies led us to evaluate the possible mechanism leading to an increase in SOD activity and the relationship between increases in SOD activity and lipid peroxidation levels in PD.

5 The Pharmacology of the MAO-B Inhibitor L-Deprenyl

The incidental observation of the mood elevating effect of iproniazid, a MAO inhibitor used in the treatment of tuberculosis, led researchers to investigate the possible beneficial effects of MAO inhibitors in depressive patients (Crane, 1956; Fox, 1952; Kline, 1958; Zeller and Barsky, 1952). Accordingly, deprenyl (phenylisopropyl-N-methylpropinylamine, E-250) was synthesized as part of new spectrum of drugs developed for the treatment of depression some 30 years ago by Dr. Joseph Knoll of Budapest, Hungary (Fig. 11) (Knoll *et al.*, 1965). The

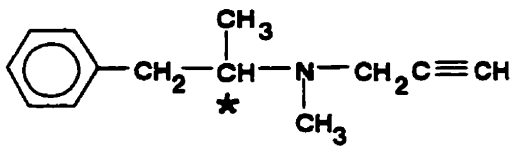

Fig. 11. Structure of L-deprenyl. The asterisk identifies the chiral carbon atom.

racemic mixture of deprenyl, or E-250 as it was then called, was reported to cause hyperthermia and excitation in rats and, most important, did not enhance the hypertensive effects of tyramine (so-called "cheese effects"), unlike the earlier MAO inhibitors (Knoll *et al.*, 1968). The psychostimulant effects of deprenyl lead to its introduction as an antidepressant and the first patients treated were depressed Soviet soldiers (Knoll *et al.*, 1965; Varga and Tringer, 1967). MAO located in the intestine and stomach offers a barrier against the abnormal absorption of tyramine contained in fermented products such as wine and cheese (Blackwell *et al.*, 1967; Hassan *et al.*, 1988). Tyramine is an indirectly acting sympathomimetic that causes the release of intra-neuronal NE, resulting in life threatening episodes of hypertension. A detailed analysis of the pharmacological and biochemical profile of the racemic mixture of deprenyl revealed that

the L-enantiomer (Eldeprenyl, Selegiline, Jumex, Movergan) was less toxic than the D-form and found to have150 times the potency to inhibit MAO (Knoll and Magyar, 1972; Magyar *et al.*, 1967). In 1968, Johnston developed clorgyline (2,4-dichlorophenoxypropyl-N-methylpropinylamine, MB 9302) and found it to be a surprisingly potent inhibitor of MAO that deaminated 5-HT. Conversely, L-deprenyl selectively inhibited the deamination of benzylamine and ß-phenylethylamine (ß-PEA) (Yang and Neff, 1973), leading Johnston to propose the existence of MAO subtypes. The nomenclature introduced by Johnston was based on substrate selectivity such that the clogyline-sensitive isoform was called MAO-A, whereas the L-deprenyl-inhibited type was referred to as MAO-B (Johnston, 1968). The association of MAO-A inhibition and the tyramine-induced hypertensive crisis was by then well established and discouraged clinicians from using these MAO antagonists in the treatment of depression. Since L-deprenyl was exempt from cardiovascular side effects, and could potentially reduce DA metabolism and oxidative stress associated with MAO metabolism (i.e., H_2O_2 formation) which increases with aging, this compound was introduced in the treatment of PD (Birkmayer *et al.*, 1975; Robinson, 1975). Birkmayer and his colleagues in 1974 were the first to report on the beneficial effects of L-deprenyl on a group of 223 parkinsonian patients. However, the clinical effects of L-deprenyl seemed to dissipate within a few days after therapy (Birkmayer *et al.*, 1977). Thus, the clinical efficacy of L-deprenyl may not be solely related to MAO-B inhibition as this block persists long after the treatment is discontinued. This review will therefore focus on the pharmacological aspects of L-deprenyl in the CNS.

5.1 Interactions of L-Deprenyl with Monoamine Oxidases

MAO contains a flavin-adenosine-dinucleotide (FAD) cofactor and is located on the outer mitochondrial membrane where it catalyzes the oxidative deamination of amines according to the overall equation:

$$R\text{-}CH_2NH_2 + O_2 + H_2O \rightarrow R\text{-}CHO + NH_3 + H_2O_2 \quad (1)$$

The enzymes occur both in the CNS and in peripheral tissues and are classified as two forms, A and B (Johnston, 1968). The natural substrates of MAO-A include epinephrine, NE and 5-HT, those of MAO-B are ß-PEA, benzylamine and tele-methylhistamine. Tyramine and DA are substrates for both forms of the enzyme (Glover and Sandler, 1986). Although the name monoamine seems implicit, it is not entirely appropriate since these enzymes are also capable of deaminating long-chain diamine compounds such as the anticonvulsant pro-drug 2-n-pentylaminoacetamide (milacemide) and even tertiary amines such as MPTP and 1-methyl-ß-carboline (harman) (Blaschko, 1952; Chiba *et al.*, 1984; De Varebeke *et al.*, 1988; May *et al.*, 1990). The relative substrate specificity of the two types of MAO is by no means universal, since it depends upon substrate concentrations and assay conditions (*in vitro* versus *in vivo*) (Fowler *et al.*, 1981). As such, the compounds described above will interact with both forms of MAO under favourable conditions. Thus, rat brain MAO-B has been shown to metabolize 5-HT but

with a much higher K_m value and at lower maximal velocity (V) than those of the A form (Fowler and Tipton, 1982). Conversely, ß-PEA is also a substrate for MAO-A but with a higher K_m value and lower V than those reported for the B type (Fowler and Strolin Benedetti, 1983).

The initial step of the interaction of L-deprenyl and MAO-B is reversible, forming a noncovalent complex within the active site. Subsequent reaction of L-deprenyl with that complex leads to the reduction of the enzyme-bound FAD with the concomitant oxidation of the inhibitor, which in turns reacts covalently with the N-5 residue of cysteinyl-flavin moiety (Maycock *et al.*, 1975; Salach *et al.*, 1979). It is therefore concluded that L-deprenyl belongs to the class of enzyme-activated irreversible inhibitors, also referred to as mechanism-based ("suicide") inhibitors. Such inhibitors, as in the case of MPTP discussed in section 3.1, can show a high degree of specificity towards a target enzyme since the effective inhibitory species is generated from an essentially unreactive compound from the catalytic domain of the enzyme. The potency of inhibition will be governed by two factors. First, the affinity of the inhibitor with the active site of the enzyme, which involves a non-covalent binding and second, the rate of reaction within that complex to form the irreversibly inhibited species. Thus, the degree of selectivity of an inhibitor towards MAO-A or MAO-B will depend on the relative magnitude of these two processes.

The IC_{50} values reported for the *in vitro* inhibition of mitochondrial MAO-B by L-deprenyl are about 1 and 4 nM for primate and rodent brains, respectively, with a selectivity of about two orders of magnitude for MAO-B over MAO-A (Riederer *et al.*, 1978; Terleckyj and Heikkila,

1992). The IC_{50} value observed after intraperitoneal injections of L-deprenyl in rodent brains is 0.36 mg/kg (Terleckyj and Heikkila, 1992) and displayed a 160-fold selectivity for MAO-B over MAO-A. According to Knoll (1978), the highest dose in rats that blocks MAO-B but leaves MAO-A relatively unaffected is about 0.25-0.5 mg/kg. This dose is less than 0.5% of the toxic dose or LD_{50}. A loss of selectivity towards MAO-B inhibition is observed in rat brains given a repetitive daily administration of a low dose of L-deprenyl. For instance, the first dose of 1 mg/kg inhibits by 10% the oxidation of 5-HT, which gradually increases to 70% following the 14th dose. Similarly, the metabolism of ß-PEA is inhibited by 30% with the first dose, increasing to about 90% by day 14 (Waldmeier and Felner, 1978). With more therapeutically relevant doses (0.25 mg/kg), the inhibition of 5-HT oxidation varies from 25 to 43% at the end of a 21-28 day period (Ekstedt *et al.*, 1979; Knoll, 1978; Zsilla *et al.*, 1986). Data are also available from the brains of patients receiving L-deprenyl at a daily dose of 10 mg (≈ 0.15 mg/kg) for approximately 6 days before death (Riederer *et al.*, 1978; Riederer and Youdim, 1986). MAO activity towards DA and 5-HT is inhibited by 90% (range 86-95%) and 65% (range 38-83%), respectively. The concentration of 5-HT remained unchanged at post-mortem, suggesting that the remainding MAO-A activity appeared to be sufficient for its metabolism. Blood platelets offer a practical tool to follow the kinetics of MAO-B inhibition in humans as they are enriched with the enzyme (Birkmayer *et al.*, 1977; Simpson *et al.*, 1985). Inhibition takes place rapidly after an oral dose of 10 mg of L-deprenyl in healthy volunteers and reaches 28% within 5 min. On average, 50% inhibition is attained in 15 min, and complete block is observed within an hour. During continuous treatment, the inhibition of platelet MAO-B is practically 100%.

The rate of recovery of MAO-B activity after irreversible inhibition by L-deprenyl is dependent upon the *de novo* enzyme synthesis. The turnover rate for MAO is found to vary from organ to organ and among species. For instance, the half-life for the recovery of MAO-B activity in rodent brains is reported to vary from 8-14 days even when a high dose of L-deprenyl (10mg/kg) is used (Felner and Waldmeier, 1979; Goridis and Neff, 1971), whereas in primate brains it is found to be as long as 30 days (Arnett *et al.*, 1987; Fowler *et al.*, 1994). These results are of profound impact with respect to the assessment of parkinsonian symptoms following L-deprenyl withdrawal in clinical trials (The Parkinson Study Group, 1993). The MAO-B inhibitory effects of L-deprenyl persist long after a wash-out period of 30 days, a criteria used by The Parkinson Study Group (1993). Thus, a drug-free interval of several months would be required for the recovery of MAO-B activity to more than 90% of control values (Fowler *et al.*,1994) in order to reduce the ambiguity in drawing conclusions concerning L-deprenyl's effects (symptomatic versus neuroprotection) in PD (section 5.4).

5.2 Distribution of MAOs in the CNS and Implications of MAO Oxidase Inhibition by L-Deprenyl on DA Metabolism

Although DA is a mixed substrate for both MAOs *in vitro* (Yu, 1986), it is preferentially metabolized *in vivo* by MAO-B in primates (Oreland *et al.*, 1983; Paterson *et al.*, 1995; Stenström *et al.*, 1987), whereas MAO-A is the predominant form involved in its metabolism in rodents (Butcher *et al.*, 1990; Finberg *et al.*, 1995; Kato *et al.*, 1986). These differences are attributed to the relative amounts of MAO subtypes in the brain and their respective

compartmentalization. For example, DA is metabolized by both MAOs in human cortical areas where the relative ratio of MAO-A to MAO-B is 1:1. However, DA is deaminated predominantly by MAO-B in the human basal ganglia and brainstem regions in which a ratio of 2:1 in favor of MAO-B is found (O'Carroll *et al.*, 1983). The distribution of MAO-A and B in the CNS is similar for both primates and rodents, whereas their relative quantity differs markedly (Konradi *et al.*, 1988; Saura *et al.*, 1996; Westlund *et al.*, 1988; Willoughby *et al.*, 1988). MAO-A predominates in neurons where NA is the primary neurotransmitter, such as the locus coeruleus, whereas MAO-B is situated in 5-HT-projection regions of the hypothalamus, raphé and area postrema of both rodents and primates. MAO-B is abundant in the ventricular and microvessel linings and rats appear to have remarkably higher levels of MAO-B activity than those found in humans (Kalaria and Harik, 1987). Astrocytes contain both MAO-A and MAO-B, with the latter predominant (Konradi *et al.*, 1988; Westlund *et al.*, 1988). The increase of MAO-B activity with aging appears to be associated with astrocytic proliferation. This observation is consistent with the relative increase in MAO-B activity observed in the SN of severely affected parkinsonians where intense gliosis is also observed (Riederer and Jellinger, 1983). In the human brain about 70% of total MAO activity is of type B, whereas it is reported to represent only 5% in rats (Oreland *et al.*, 1983; Riederer *et al.*, 1978). These are important aspects when evaluating the effects of MAO-inhibitors in rodents and extrapolating these findings to humans. For instance, the activity of MAO is about five times greater outside the neuron and the majority is of type B in human striatal synaptosomal preparations whereas in rat the opposite is seen (Oreland *et al.*, 1983). In contrast, the SN of human and rat appears to be virtually devoid of MAO-A and MAO-B immunolabeling.

The question then arises as to whether the extra-dopaminergic location of MAO-B could influence DA metabolism. Two studies have examined the contribution of intra- and extra-neuronal MAO with respect to DA metabolism in human and rat striatal synaptosomes, which included physiological DA concentrations in the assay (Oreland *et al.*, 1983; Stenström *et al.*, 1987). It is estimated that about 3% and 11% of DA is oxidized by extra-neuronal MAO-B and MAO-A, respectively, in the rats when taking into account neuronal DA reuptake mechanisms. However, MAO-B contributions can be moderately increased to approximately 10% when the DA reuptake process is inhibited (Liccione and Azzaro, 1988). This finding is also consistent with the lack of a significant effect observed on DA metabolism when MAO-B is inhibited by L-deprenyl, at doses which do not alter MAO-A activity, in the rat striatum (Berry *et al.*, 1994b; Butcher *et al.*, 1990; Paterson *et al.*, 1991) or in the unilaterally lesioned rats (Finberg *et al.*, 1995). In contrast, a high proportion of DA catabolism proceeds via MAO-B (66%) located outside the dopaminergic compartment in humans (Oreland *et al.*, 1983; Stenström *et al.*, 1987). Thus, it can be appreciated that MAO-B inhibition will have a differential impact with respect to DA metabolism in the striatum of rats than humans. This is consistent with the administration of L-deprenyl, at a dose that selectively blocked MAO-B, resulting in an increase in DA levels in the caudate nucleus of monkeys (Paterson *et al.*, 1995) and parkinsonian monkeys (Rausch *et al.*, 1990).

Although MAO inhibition can increase the concentration of DA at the synapse, other enzymes are also involved in its metabolism, such as membrane bound and soluble forms of catechol O-methyltransferase (COMT). Striatal COMT is located outside dopaminergic neurons and

inactivates DA and L-dopa by O-methylation (Kastner *et al.*, 1994). Large doses of 3-O-methyldopa, the major metabolite of L-dopa, given to rats resulted in an inhibition of L-dopa utilization in the striatum (Reches and Fahn, 1982) and blocked L-dopa-induced rotational behavior in rats with unilateral lesions to the nigrostriatal pathway (Reches *et al.*, 1982). These observations might explain why L-deprenyl itself is a less potent anti-parkinsonian or DA agonist drug than L-dopa. In addition to an alteration of DA metabolism, inhibition of MAO-B could also provide a symptomatic relief of PD through an elevation of ß-PEA. Whether ß-PEA has a physiological significance is still a matter of controversy, although it has been suggested that ß-PEA may act as an endogenous neuromodulator of dopaminergic functioning (Paterson *et al.*, 1990).

5.2.1 L-Deprenyl and ß-Phenylethylamine

ß-PEA is an indirectly acting sympathomimetic that induces catecholamine release from the presynaptic extragranular pool (Paterson *et al.*, 1990). ß-PEA is formed from the decarboxylation of phenylalanine catalyzed by aromatic amino acid decarboxylase (AAAD), and this represents the rate-limiting step. ß-PEA does not appear to be stored and its rate of synthesis in the brain is slow, whereas its catabolism to phenylacetic acid by MAO-B is extremely rapid. Thus, the turnover of ß-PEA is very rapid such that the steady state levels are kept at a low concentration (<5 ng/g tissue) (Paterson *et al.*, 1990).

Iontophoretically applied ß-PEA is demonstrated to potentiate nigral dopaminergic responses to DA (Paterson *et al.*, 1990), suggesting that ß-PEA may act as a modulator of dopaminergic transmission. Similar findings have also come from studies where changes in membrane fluidity caused by DA were found to be enhanced by ß-PEA (Harris *et al.*, 1988). Furthermore, presynaptic activation of DA receptors results in a decrease in ß-PEA synthesis and AAAD activity (Paterson *et al.*, 1990; Zhu *et al.*, 1992), whereas DA receptor antagonists increase both (Hadjiiconstantinou *et al.*, 1993; Zhu *et al.*, 1992). Destruction of nigral dopaminergic neurons with 6-OHDA leads to a decrease in both striatal DA and ß-PEA levels, suggesting that ß-PEA is localized in DA neurons (Boulton *et al.*, 1990). Thus, not only can ß-PEA modulate dopaminergic transmission, but a functional link may also exist between ß-PEA synthesis and dopaminergic activity.

Inhibition of MAO-B activity markedly increases striatal ß-PEA levels in several animals species including rats (Philips and Boulton, 1979), dogs (Milgram *et al.*, 1995) and monkeys (Berry *et al.*, 1994b). Consistent with these various findings is the reversal of this effect by NSD 1015, an inhibitor of AAAD, on alterations of ß-PEA levels caused by MAO-B inhibitors in rats (Berry *et al.*, 1994b). Interestingly, L-deprenyl is found to increase the expression levels of AAAD mRNA (Li *et al.*, 1992), which would presumably be seen as an increase in AAAD activity and further enhance striatal ß-PEA concentrations.

Urinary excretion of ß-PEA is increased during L-deprenyl therapy (Karoum *et al.*, 1982). The concentrations of ß-PEA in *postmortem* brains of PD patients receiving L-deprenyl therapy are

approximately 1000% and 3000% higher in the striatum and limbic areas, respectively, than those observed in control brains (Riederer *et al.*, 1987). In comparison, the increase of DA concentrations was 23% in the striatum and 41% in the limbic areas (Riederer *et al.*, 1987). Therefore the relatively higher increase in ß-PEA may contribute to the therapeutic efficacy of L-deprenyl which reduces the dose of L-dopa required in the combined treatment and may also explain the occasional mood elevation effects reported by patients.

5.2.2 Other Effects of L-Deprenyl on Striatal DA Levels that Appear to be Independent of MAO-B Inhibition

L-Deprenyl is found to block the reuptake of [^{3}H]-DA in rat striatal slices in a dose-dependent manner with an IC_{50} of 54 µM (Bondiolotti *et al.*, 1995; Fang and Yu, 1994; Lai *et al.*, 1980). In comparison, methylphenidate, nomifensine and GBR12909 are more potent inhibitors of the DA reuptake carrier, with IC_{50} values of 0.64, 0.11 and 0.016 µM, respectively. In addition, chronic low dose L-deprenyl (0.25 mg/kg/day for 3 weeks) protected striatal dopaminergic neurons against the deleterious effects of 6-OHDA and this effect was attributed to DA reuptake block (Hársing *et al.*, 1979; Knoll, 1987). In these studies, L-deprenyl prevented the enhancement of acetylcholine (ACh) release caused by 6-OHDA-induced striatal DA depletion. It is well known that the loss of DA inhibitory input causes hyperactivity of cholinergic interneurons, such an imbalance being typical of PD. Although this method is indirect, a more direct approach also supports this finding. For instance, L-deprenyl is found to block [^{3}H]-DA reuptake and to increase DA turnover rate in striatal slices prepared from L-deprenyl-treated rats

(0.25 mg/kg/day for 2 weeks or 3 weeks) (Knoll, 1987; Knoll and Miklya, 1994; Tekes *et al.*, 1988; Zsilla *et al.*, 1986). Inhibition of DA reuptake is observed at 1 hr but not at 24 hrs after a single dose, indicating that the reuptake block was reversible and therefore appeared to act independently of MAO-B inhibition (Knoll, 1987). L-Deprenyl up-regulated binding of mazindole, a marker of DA uptake sites; this is a phenomenon associated with DA uptake antagonists (Wiener *et al.*, 1989a). Furthermore, L-deprenyl pre-treatment decreased the intensity of amphetamine-induced stereotypy behavior, indicating that L-deprenyl may reduce the uptake of amphetamine (Timár *et al.*, 1993). Alterations in DA turnover rate could have resulted from MAO-A inhibition as the dosage of L-deprenyl used was shown to inhibit MAO-A by 20% at the end of the 2 week treatment. This observation appears unlikely since the MAO enzyme needs to be inhibited by about 85% to obtain quantitative alterations in DA metabolism in rats (Hársing *et al.*, 1979). Pargyline, a non-selective MAO inhibitor, is devoid of effects on the dynamic of DA transmission (Knoll, 1987). In addition, L-amphetamine metabolites of L-deprenyl (section 5.3 and 5.3.1) may not be responsible for the increase in DA turnover rate (as estimated by blocking the *de novo* DA synthesis with α-methyl-p-tyrosine) since D-amphetamine (0.25 mg/kg/day s.c. for two weeks) has an opposite effect (Zsilla and Knoll, 1982). Chronic L-deprenyl administration is also found to enhance DA release from striatal slices upon KCl stimulation (Knoll, 1987; Knoll and Miklya, 1994; Zsilla *et al.*, 1986). Taken together, the clinical efficacy of a chronic L-deprenyl treatment appears to be related to its stimulatory effects on dopaminergic transmission and seems to play a more important role than

MAO-B inhibition. This observation is consistent with the loss of symptomatic effects of L-deprenyl after a few days of treatment in PD, while MAO-B inhibition is known to persist long after this period (Birkmayer *et al.*, 1977).

5.3 Pharmacokinetic Aspects and Metabolism of L-Deprenyl

L-Deprenyl is readily absorbed from the gastrointestinal tract as shown in animal and human studies (Magyar and Tóthfalusi, 1984). The maximal plasma concentration is reached within 0.5 to 2 hrs in humans and rats following an oral dose with a half-life of about 1 to 2 hrs, indicating that L-deprenyl is rapidly metabolized (Mahmood *et al.*, 1994; Michaelis *et al.*, 1993). An apparent volume of distribution of 150 to 300 L is achieved after an intravenous administration and 94% is found to be bound to serum proteins, especially to macroglobulins of mice and humans, with a bioavailability of less than 10% (Heinonen *et al.*, 1992; Magyar and Tóthfalusi, 1984; Mahmood *et al.*, 1994; Szökö *et al.*, 1984). L-deprenyl rapidly penetrates tissues due to its high degree of lipophilicity. High radioactive levels are detected within seconds of its administration in the brains of mice (autoradiography) and humans (PET) at levels 3.5 times higher than those found in plasma (Fowler *et al.*, 1987; Magyar and Tóthfalusi, 1984; Mahmood *et al.*, 1994). The uptake of [^{11}C]-L-deprenyl is more than twice as high in the thalamus and striatum than the cortical areas, reflecting a preponderance of MAO-B in the former areas (Fowler *et al.*, 1987).

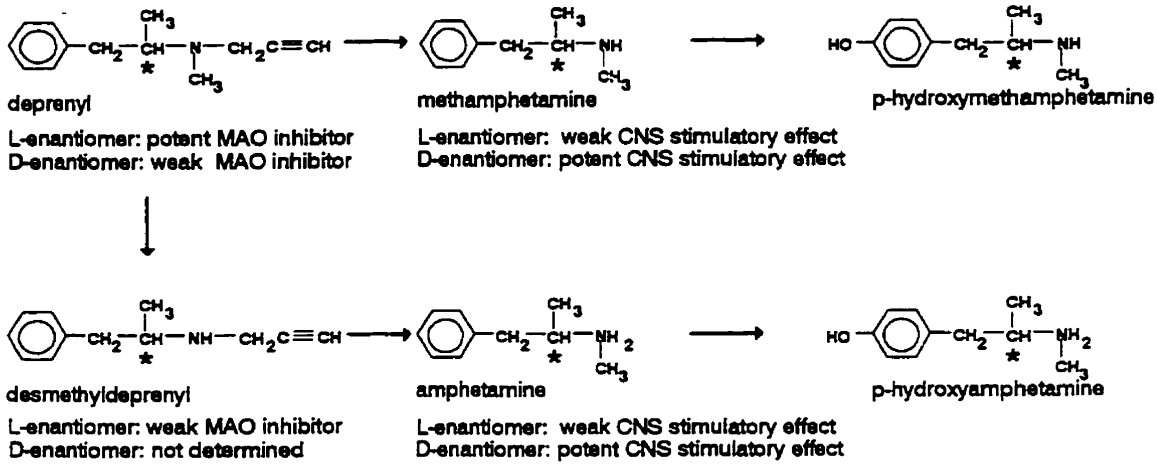

Fig. 12. Metabolism of L-deprenyl. Note that the stereoselectivity is maintained during metabolism. The enantiomeric carbon atom is identified by an asterisk. Modifed from Yoshida *et al* ., 1986.

The first-pass metabolism of L-deprenyl is intense and takes place mainly in the liver through the microsomal cytochrome P-450 system (Fig. 12) (Yoshida *et al.*, 1986). L-deprenyl is metabolized to L-desmethyldeprenyl (also called nordeprenyl) and L-methamphetamine. L-Desmethyldeprenyl is further metabolized to L-amphetamine, while L-amphetamine and L-methamphetamine are converted to their corresponding p-hydroxylated forms in rats and humans (Kalász *et al.*, 1990; Magyar and Táthfalusi, 1984; Yoshida *et al.*, 1987). The formation of L-amphetamine is also partially catalyzed by the monooxygenase system. Metabolism of L-deprenyl can also result from the *in vivo* cleavage of the inhibitory molecule from MAO-B with the release of the metabolites L-methamphetamine and L-amphetamine (Reynolds *et al.*, 1978a). All three metabolites of L-deprenyl have been detected in human plasma, CSF and urine after single or continous administration of the drug (Heinonen *et al.*, 1989). L-methamphetamine accounts for the majority of the metabolites and the parent drug has not been detected in urine. In humans, the highest median concentration of L-methamphetamine after a 1 week administration of a daily dose of 10 mg L-deprenyl is about 30 ng/mL, seen approximately 5 hrs

after the administration of the compound. The median peak concentration of L-desmethyldeprenyl and L-amphetamine is approximately 15 ng/mL and is detected at around 1 hr and 5 hrs, respectively. No significant differences are found in the plasma levels of the metabolites after a single or multiple dosage; thus no accumulation of the metabolites occurs (Heinonen *et al.*, 1992). The concentrations of L-desmethyldeprenyl, L-amphetamine and L-methamphetamine in the CSF are 1, 7 and 15 ng/mL, respectively, after multiple dosing (Heinonen *et al.*, 1989; 1992). In addition, L-amphetamine has been detected in the striatum and various brain regions in PD (Reynolds *et al.*, 1978b).

The metabolites of L-deprenyl are to a certain degree inhibitors of MAO-B. The inhibitory potencies of L-desmethyldeprenyl, L-amphetamine and L-methamphetamine are reported to be 30, 500 and 1000 times, respectively, lower than that of L-deprenyl *in vitro* (Borbe *et al.*, 1990). However, *in vivo*, L-desmethyldeprenyl is found to inhibit MAO-B irreversibly with a potency that is only 3 times less than that of L-deprenyl (Borbe *et al.*, 1990).

5.3.1 Do the Metabolites of L-Deprenyl Play a Role in L-Deprenyl Therapeutic Actions?

The basic pharmacological action of D-amphetamine and D-methamphetamine is to release catecholamines from the presynaptic nerve terminals (Fischer and Cho, 1979). Both compounds inhibit DA uptake (Schaeffer *et al.*, 1976) and MAO activity (Robinson, 1985). Chronic administration of high doses (> 16 mg/kg/day) of D-amphetamine or D-methamphetamine results in a neurotoxicity characterized by a loss of DA nerve terminals (Fuller and Hemrick-

Leucke, 1982; O'Callaghan and Miller, 1994; Robinson and Camp, 1987; Schmidt *et al.*, 1985). In addition, D-amphetamine induces an increase of blood pressure (Simpson, 1978), stereotypic locomoter behavior in rats (Segal, 1975) and self-administration in monkeys (Balster and Schuster, 1973). Similarly, D-methamphetamine has acknowledged abuse potential in humans and is an effective reinforcer of intravenous self-administration behavior in rats (Yokel and Pickens, 1973).

To be effective as a psychostimulant, the racemic mixture of deprenyl (E-250) must be administered at very high doses (50-100 mg daily) (Knoll *et al.*, 1965; Varga and Tringer, 1967). These effects have been attributed to D-amphetamine metabolites formed from D-deprenyl (Varga and Tringer, 1967). Thus, the stereoselectivity of the metabolites maintained during L- and D-deprenyl metabolism is a key factor in determining their properties (Fig. 12). L-amphetamine and L-methamphetamine possess about one-tenth the amphetamine efficacy of the corresponding D-isomer (Chiueh and Moore, 1974) and the former substances have been detected in the brains of patients treated with L-deprenyl (Reynolds *et al.*, 1979b). L-deprenyl is found to substitute for D-amphetamine in drug discrimination studies in rats and monkeys at doses of 17 mg/kg and 5.6 mg/kg of L-deprenyl, respectively (Yasar and Bergman, 1994). It is noteworthy that ß-PEA has previously shown to produce amphetamine-like behavioral effects (Borison *et al.*, 1977; Dourish, 1982). Conceivably, increased circulating levels of ß-PEA after the administration of L-deprenyl may contribute to its behavioral effects. However, doses of L-deprenyl which maximally increase ß-PEA concentrations in rats and monkeys are about one tenth those that substitute for D-amphetamine or D-methamphetamine (Buu and Angers, 1987;

Yasar and Bergman, 1994). These observations suggest that MAO-B inhibition does not play a major role in these effects. MDL72974 (4-fluoro-ß-(fluoromethylene)-benzenebutanamine, mofegiline hydrochloride), a selective irreversible MAO-B which, unlike L-deprenyl, is not metabolized to amphetamine (Dow *et al.*, 1994), and presumably increases the levels of ß-PEA, failed to produce amphetamine-like discriminative stimulus effects in rats at doses as high as 10 mg/kg (Moser, 1990). Therefore, ß-PEA as a result of MAO-B inhibition may not be the mechanism leading to the generalization of amphetamine-like behaviour. Increases in locomotor activity in rats administered L-deprenyl is blocked by prodifen, a microsomal enzyme inhibitor, supporting the notion that these effects are related to the metabolism of L-deprenyl (Engberg *et al.*, 1991).

With a more therapeutically relevant dose (0.15 mg/kg), self-administration of L-deprenyl is not an apparent behavior in primates at levels up to 1 mg/kg (Winger *et al.*, 1994), and no increase in locomotor activity has been observed in dogs following chronic L-deprenyl treatment (1 mg/kg/day for 3 weeks) (Milgram *et al.*, 1995). Taken together, these results suggest that the levels of L-amphetamine and L-methamphetamine achieved by a therapeutic dose of L-deprenyl are insufficient to alter dopaminergic functions. This observation is consistent with plasma levels observed in D-amphetamine- and D-methamphetamine-treated animals at doses known to cause stereotypic behavior. For instance, the highest levels of L-amphetamine recorded following 3 weeks of L-deprenyl (1 mg/kg) in dogs were 20-40 ng/mL (Milgram *et al.*, 1995). These levels are less than one-tenth of the levels reported by Bareggi and colleagues (1979) who observed hyperthermia and stereotypy when D-amphetamine was administered at 2 mg/kg orally,

producing approximately 500-600 ng/mL of serum D-amphetamine. In addition, the threshold dose of L-methamphetamine to induce spontaneous locomotor activity in rats is estimated to be around 0.5 mg/kg/day s.c. (Knoll and Miklya, 1994). This dose resulted in a plasma concentrations of about 100-200 ng/mL plasma (Cook *et al.*, 1992; Melega *et al.*, 1995) and is higher than that produced by L-deprenyl at levels up to 3 mg/kg (Heinonen *et al.*, 1994). Furthermore, patients do not exhibit amphetamine-associated behavior while receiving L-deprenyl or withdrawal symptoms after discontinuing its use (Birkmayer *et al.*, 1982; Thornton *et al.*, 1980). Substitution of L-methamphetamine and L-amphetamine for L-deprenyl have proved ineffective clinically (Stern *et al.*, 1983). In addition, AGN-1135, another selective MAO-B inhibitor, is not metabolized to amphetamine, yet shares the antiparkinson effect of L-deprenyl (Youdim and Finberg, 1987). Taken together, these results suggest that the metabolites of L-deprenyl do not contribute significantly to its clinical efficacy in PD.

5.4 The Neuroprotective Effects of L-Deprenyl in Models of Neuronal Injuries

In recent years L-deprenyl has been shown to protect neurons from a variety of injuries that would normally result in neurodegeneration. A protective effect of L-deprenyl is observed against the neurotoxins MPTP, 6-OHDA and N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4). In addition, L-deprenyl is found to improve the survival of motoneurons caused by axotomy. The mechanism underlying the protective effects of L-deprenyl against these various neuronal insults appears to be different.

L-deprenyl prior to MPTP administration is known to protect nigrostriatal dopaminergic neurons against degeneration, a mechanism related to MAO-B inhibition as discussed in detail in section 2.2 and 3.1. Conversely, L-deprenyl administered to mice 72 hrs following MPTP significantly improved the recovery of dopaminergic neurons of the SN as demonstrated by TH immunopositive cell counts (Tatton and Greenwood, 1991). This effect does not appear to be related to MAO-B inhibition since MPTP (30 mg/kg/day) was administered for 5 consecutive days and 72 hrs prior to L-deprenyl treatment. In addition, it appears unlikely that L-deprenyl protected against the deleterious effects of MPP^+ by blocking the DA reuptake system (see section 5.2.2) since the half-life of the neurotoxicant in the CNS of mice varies from 2-4 hrs (Johannessen *et al.*, 1985; Markey *et al.*, 1984). The mechanism by which L-deprenyl exerts a trophic-like action is unclear at present. However, two studies failed to observe a significant effect of L-deprenyl on the restoration of the levels of striatal DA and its metabolite in MPTP-lesioned mice (Gupta and Wiener, 1995; Wiener *et al.*, 1989b). Furthermore, there is no evidence suggesting that L-deprenyl rescues degenerating dopaminergic neurons in early and untreated PD patients (Lees, 1995; Olanow *et al.*, 1995; Schneider, 1995; The Parkinson's Disease Research Group, 1993; The Parkinson Study Group, 1989b; 1993; Ward, 1994). These apparent discrepancies prompted us to re-evaluate the long term impact of L-deprenyl on MPTP-induced dopaminergic cell loss in mice.

Intracerebral administration of 6-OHDA induces the degeneration of noradrenergic and dopaminergic systems in various animal models (section 2.1.1). The deleterious effects of this neurotoxicant are related to its selective uptake by catecholaminergic neurons and the formation

of cytotoxic free radicals (Fig. 6 section 2.1.2, p. 24 and see section 2.1.2). A single intraperitoneal injection of L-deprenyl (5 mg/kg) or pre-administration over a 3 weeks period (0.25 mg/kg) led to a normalization of ACh release in striatal slices prepared from 6-OHDA-treated rats (Knoll, 1987). In contrast, the striatum of 6-OHDA-treated animals released twice as much ACh upon stimulation and this effect was antagonized by adding DA. This observation suggested that 6-OHDA resulted in striatal DA depletion because of a deafferentation of dopaminergic neurons. Since MAO-B is not implicated in the mechanism of 6-OHDA cytotoxicity, Knoll concluded that the protective effect afforded by L-deprenyl is related to the inhibition of 6-OHDA uptake by DA neurons (see section 5.2.2). Whether an actual protective effect occurred in this study is difficult to ascertain, since a rather indirect measure of nigrostriatal degeneration was used. However, the reuptake inhibition provided by L-deprenyl is reported to be reversible *in vitro* (Knoll, 1987), while 37% inhibition is present at 24 hrs following the last dose of a similar dosing schedule (over a 3 weeks period) in the rat striatum (Tekes *et al.*, 1988). It seems unlikely that a 37% inhibition of reuptake could account entirely for the complete protection against neuronal degeneration as reported (Knoll, 1987). Alternatively, L-deprenyl administered to rats could have provided protection against 6-OHDA by inducing an increase in CuZn-SOD, Mn-SOD and CAT activities (Carrillo *et al.*, 1991; Clow *et al.*, 1991; Knoll, 1988; Vizuete *et al.*, 1993). Consequently, free radicals and H_2O_2 generated by the toxin may have been eliminated before they cause any damage to the neurons (Fig. 6 section 2.1.2, p. 24 and section 2.1.2). More direct evidence (such as TH immunoreactive cell density) is required to confirm whether L-deprenyl can effectively protect against 6-OHDA-mediated dopaminergic degeneration.

L-Deprenyl, but not the more potent MAO-B inhibitor MDL72974, is found effective against the deleterious effect of DSP-4 on central noradrenergic neurons (Finnegan *et al.*, 1990). This observation suggests that MAO-B inhibition is not critical to the mechanism of DSP-4 toxicity and that another pharmacological action or actions of L-deprenyl explain the reported protective effect. It is well known that a single systemic administration of DSP-4 produces a long-lasting reduction of NE, dopamine-ß-hydroxylase (a noradrenergic marker) activity, and a profound inhibition of [^{3}H]-NE uptake in both the central and peripheral nervous systems of rodents (Hallman and Jonsson, 1984; Hallman *et al.*, 1985; Jaim-Etcheverry and Zeiher, 1980; Jonsson *et al.*, 1981; Ross *et al.*, 1973; Ross, 1976; Ross and Renyi, 1976). A prominent feature of DSP-4-induced lesions is that brain regions innervated with noradrenergic axons of the locus coeruleus are damaged by the toxin, such as hippocampal and cortical regions (Fritschy and Grzanna, 1989, 1991, 1992; Fritschy *et al.*, 1990; Grzanna *et al.*, 1989). It has been proposed that DSP-4 undergoes spontaneous cyclization to from a positively charged aziridinium ion (Ross *et al.*, 1973), which leads to the irreversible alkylation of the NE uptake carrier and cell death (Zieher and Jaim-Etcheverry, 1980). Desipramine, a NE reuptake inhibitor, is demonstrated to protect against DSP-4-induced NE depletion in rats (Jonsson *et al.*, 1982; Ross, 1976). Thus, it appears that the protection afforded by L-deprenyl may be related to the inhibition of the noradrenergic reuptake system as this effect is also reported to occur in rat brain synaptosomal preparations (Bondiolotti *et al.*, 1995; Lai *et al.*, 1980). Consistent with this interpretation is the fact that Finnegan and co-workers reported a maximum protective effect of L-deprenyl 1 hr prior to DSP-4. Less protection is given with a 24 hrs pre-treatment, while the administration of L-deprenyl 4 days prior to DSP-4 failed to provide protection (Finnegan

et al., 1990). This occurred in spite of a marked MAO-B inhibition, over 90% after 1 hr and 24 hrs pre-treatment, and over 70% at 4 days pre-treatment, for both L-deprenyl and MDL72974. However, this observation is difficult to reconcile with the fact that other MAO-B inhibitors, MDL72145, N-methyl-N-(2-pentyl)-propargylamine and N-methyl-N-(2-hexyl)-propargylamine shown to protect against DSP-4 (Bertocci *et al.*, 1988; Yu *et al.*, 1994) do not inhibit NE reuptake systems (Bey *et al.*, 1984; Fang and Yu, 1993). To these apparently contradictory results must be added the fact that clorgyline and D-amphetamine do not protect against DSP-4 toxicity and yet are more powerful inhibitors of catecholamine uptake mechanisms than L-deprenyl (Hallman and Jonsson, 1984).

Chronic treatment with L-deprenyl, but not D-deprenyl, is also found to increase the number of motoneurons that survive a facial nerve transection by a factor of 2.2 in 14 days in older rats (Salo and Tatton, 1992). A plateau effect is seen at dosages of 0.01 and 10 mg/kg (every 2 days for 21 days), indicating that higher concentrations do not enhance survival (Ansari *et al.*, 1993). The mechanism whereby L-deprenyl improves the survival of motoneurons following axotomy is not clear and may depend upon the stimulation of the production of neurotrophic factors or regenerative processes.

The lesion of cholinergic motoneurons from their target muscles by axotomy is often used to test the dependence of motoneuron survival on muscle-derived trophic support (Crews and Wigston, 1990; Lowrie and Vrbova, 1992). Accordingly, application of neurotrophic factors such as ciliary neurotrophic factor (CNTF) (Sendtner *et al.*, 1990), basic fibroblast growth

factor (bFGF) (Grothe and Unsicker, 1992) and brain-derived neurotrophic factor (BDNF) (Sendtner *et al.*, 1992; Yan *et al.*, 1992) to the lesioned ends of motoneuron axons reduces the death of hypoglossal or facial motoneurons. Glial cells that accumulate at brain injury sites are thought to be the source of neurotrophic factors (Nieto-Sampedro *et al.*, 1983). It is well known that the percentage of motoneurons that survive axotomy increases from 25% to 70% during early postnatal life (14-21 days old) to reach approximately 90% in adults rodents (3-4 weeks old) (Ansari *et al.*, 1993; Pollin *et al.*, 1991; Salo and Tatton, 1992; Sendtner *et al.*, 1990; Snider and Thanedar, 1989). The differential vulnerability to axotomy between immature and adult animals appears to be related to the stage of development and differentiation of astrocytes and Schwann cells (Jesson and Mirsky, 1991; Lillien *et al.*, 1990; Raff, 1989), which constitute an alternative source of trophic factor for older animals.

Whether L-deprenyl alters astrocytic differentiation and proliferation or the secretion of neurotrophic factors in response to neuronal injuries is an intriguing possibility. Interestingly, L-deprenyl increases glial fibrillary acidic protein (GFAP) immunoreactivity, an astrocytic marker, after lesioning the rat striatum with an injection cannula, whereas no such effect of the drug is detected in unlesioned animals (Biagini *et al.*, 1993). CNTF gene expression, bFGF and GFAP immunoreactiviy are enhanced by L-deprenyl following mechanical lesions inflicted to the rat striatum and astrocytic cell cultures (Biagini *et al.*, 1994; Seniuk *et al.*, 1994). Thus, it would appear that L-deprenyl exerts an indirect effect by increasing astrocytic response and consequently, trophic support to injured neurons through an as yet undefined mechanism. These observations may be relevant to the increase in TH immunopositive neurons in

mesencephalic cell cultures exposed to L-deprenyl (Roy and Bédard, 1993) where the ratio of astrocytes to neurons is found altered (Koutsilieri *et al.*, 1996). It has been suggested that L-deprenyl acts on the G_1-G_o boundary of the cell cycle by preventing astroglia from entering the non-proliferative G_o phase of the cycle (Skibo *et al.*, 1993). Polyamines such as putrescine, spermine and spermidine, play an important role in cell cycle regulation and are known to stimulate astrocytic response to neuronal injuries (Pegg, 1986). Consistent with this role is the report that glial activation can be blocked through inhibition of polyamine synthesis and such an effect can be restored by the inclusion of putrescine in the lesioned rat striatum (Zini *et al.*, 1990). Perhaps more important is the fact that N-acetylated putrescine, spermine and spermidine are good substrates of MAO-B and act as positive feedback on the synthesis of the normal polyamine (Youdim *et al.*, 1991). MAO-B inhibition as a result of L-deprenyl treatment is expected to decrease N-acetylpolyamine metabolism, which in turn will result in increases in polyamine synthesis, glial activation and a reduction in neuronal losses (Zoli *et al.*, 1993).

5.5 L-Deprenyl and Aging

It has been demonstrated that rats receiving L-deprenyl (3 x 0.25 mg/kg/week) at 24-months of age lived an average of about 1 year longer than the saline-treated group (Knoll, 1988). This effect was not associated with reduced food intake, a consequence which may be attributed to the amphetamine-like metabolites of L-deprenyl. In fact, the weight decrease was lower in the L-deprenyl-treated group. This remarkable increase in life expectancy has been partly confirmed, although the increase was drastically smaller (20 days) than previously observed (Milgram *et al.*,

1990), and others reported no effect of the drug (Ingram *et al.*, 1993; Piantanelli *et al.*, 1994). Treatment duration, age of animal at the beginning of treatment, species and strain differences may account for the variability in the results. Kitani and colleagues (1994) demonstrated that the impact of L-deprenyl on the mean survival times of rodents is more pronounced when the drug is administered at a later age (24 compared to 18 months and at birth). If this effect of L-deprenyl is real, it may be relevant to the recent findings in which this drug was demonstrated to improve neuronal deficits of older animals. L-Deprenyl is found to increase nerve cell density in the hippocampus and frontal cortex regions of aged rats that would normally have declined with aging (Amenta *et al.*, 1994; Yong-Chun *et al.*, 1995). Interestingly, a concomitant reduction in GFAP immunoreactivity is also observed in these animals. The latter observation is even more difficult to reconcile with the increase in GFAP immunolabeling observed as a result of L-deprenyl treatment (Biagini *et al.*, 1993; 1994; Koutsilieri *et al.*, 1996; Seniuk *et al.*, 1994; Skibo *et al.*, 1993). In addition, Birkmayer and his co-workers (1985) reported an increase in life expectancy in PD patients receiving a combined L-deprenyl/L-dopa therapy. Although this study was retrospective and uncontrolled, such an effect of L-deprenyl in humans has not been confirmed (Elizan *et al.*, 1989; Lees, 1995). Thus, further studies are required to explore these various issues.

5.6 L-Deprenyl and Oxidative Stress

L-Deprenyl is also found to suppress oxidative stress associated with an increase in DA turnover known to alter GSSG levels in mice striatum (Cohen and Spina, 1989). Such a compensatory

mechanism is also observed in PD. It is proposed that DA or its precursor L-dopa can greatly exacerbate GSH losses at the expense of a raise in GSSG concentrations due to enhanced $O_2^{\cdot}$ formation (Spencer *et al.*, 1995) or from H_2O_2 generated from the enzymatic oxidation of DA (Maker *et al.*, 1981), a mechanism thought to contribute to the loss of GSH levels (≈ 40%) in the SN of parkinsonian patients (Perry *et al.*, 1982; Sofic *et al.*, 1992). This effect of L-deprenyl appears to be independent of MAO inhibition since the dose used is insufficent to block MAO-A known to play a major role in DA metabolism in rodent brains (Cohen and Spina, 1989; Oreland *et al.*, 1983; Stenström *et al.*, 1987). This observation is also consistent with the effect of L-deprenyl on MPP^+-induced free radical formation in rats using a microdialysis approach. Intrastriatal infusion of L-deprenyl, at a dose lower than that required to inhibit MAO-B, decreases significantly $OH^{\cdot}$ elicited by the perfusion of MPP^+ or its analogue, as measured by a salicylate hydroxylation trapping procedure (Chiueh *et al.*, 1992a; Wu *et al.*, 1993). Whether L-deprenyl scavenges free radicals directly or indirectly through alterations of the antioxidant defense system is unclear at this time. The literature suggests that the antioxidant effect of L-deprenyl may be indirect. Indeed, chronic L-deprenyl administration has been shown to increase striatal SOD activity by as much as 10 fold in rats (Knoll, 1988). This finding has been confirmed, albeit to a lesser extent (Carrillo *et al.*, 1991; Clow *et al.*, 1991; Vizuete *et al.*, 1993). In addition, an increase in CAT activity is observed in the striatum and SN of rats following 3 weeks of L-deprenyl treatment (2 mg/kg) (Carrillo *et al.*, 1992b; Pattichis *et al.*, 1995; Vizuete *et al.*, 1993). Thus, the removal of $O_2^{\cdot}$ and H_2O_2 will interrupt the cyclic formation of oxygen-derived free radicals which could be responsible for the reduction in salicylate hydroxylation (Chiueh *et al.*, 1992a; Wu *et al.*, 1993). The question that remains as to why L-deprenyl should

alter SOD activity since it is shown to be up-regulated in response to enhanced $O_2^{\cdot}$ formation in various animal models of free radical-induced toxicity (hyperoxia, paraquat, 6-OHDA) (Crapo and Tierney, 1973; Frank, 1981; Ogawa *et al.*, 1994; Sjostrom and Crapo, 1981; Stevens and Autor, 1977).

6 General Objective

PD is characterized by the massive degeneration of melanized dopaminergic neurons of the SN, accompanied by striatal DA depletion. Although significant progress has been made in understanding the pathophysiology of PD, the most important question is still unanswered: What is the *primum movens* of the degeneration of nigral dopaminergic neurons? Oxidative stress is thought to participate in the mechanism of nerve-cell death in PD for several reasons. (1) Catecholaminergic neurons containing neuromelanin, an autoxidation by-product of catecholamines, are more vulnerable to degeneration than are non-melanized dopaminergic neurons (Hirsch *et al.*, 1988). (2) The content of iron, which exacerbates the production of free radicals in the presence of H_2O_2, is increased in the SN (Dexter *et al.*, 1989b). (3) Free radical detoxification pathway, including SOD activity and GSH levels (Ambani *et al.*, 1975; Kish *et al.*, 1985; Marttila *et al.*, 1988b; Perry *et al.*, 1982; Poirier and Thiffault, 1993; Saggu *et al.*, 1989; Sofic *et al.*, 1992) are altered. (4) Lipid and DNA peroxidation are elevated in the SN (Dexter *et al.*, 1989a; Pall *et al.*, 1986; Sanchez-Ramos *et al.*, 1994). However, it remains to be determined whether oxidative stress contributes to the disease or is merely a consequence of nerve-cell death. In this respect, increases in SOD activity could represent an adaptive mechanism caused by a mitochondrial respiratory deficit. To address this issue it is therefore important to replicate biochemical and anatomical alterations observed in PD. An accidental

contribution came in the early 1980s, when numerous young adults presented a PD-like syndrome. Extensive investigation revealed the phenomenon was caused by the unintentional self-administration of MPTP, a by-product of meperidine synthesis (Davis *et al.*, 1979; Langston *et al.*, 1983).

MPP^+, the neurotoxic metabolite of MPTP, induces nigrostriatal dopaminergic degeneration reminiscent of PD (German *et al.*, 1988; Langston *et al.*, 1983; Markey *et al.*, 1984). The deleterious effects of MPP^+ are related to its selective capture by the DA uptake system and its further accumulation in mitochondria (mM range), which leads to the inhibition of NADH-dehydrogenase or complex I activity (Chiba *et al.*, 1985; Nicklas *et al.*, 1985); Poirier and Barbeau, 1985b). Interestingly, a similar respiratory deficit is reported in the SN and striatum of parkinsonians, with a reduction in NADH dehydrogenase (Mizuno *et al.*, 1989; Schapira *et al.*, 1989; 1990). This observation supports the speculation that an MPTP-like toxin may be responsible for PD and lead to the introduction of L-deprenyl as an adjunct to L-dopa therapy. Free radicals are also thought to play a key role in the mechanism or mechanisms of MPP^+-induced toxicity (Adams *et al.*, 1993; Ali *et al.*, 1994; Poirier and Barbeau, 1985a; Poirier *et al.*, 1985; Rossetti *et al.*, 1988; Przedborski *et al.*, 1992b; Singer *et al.*, 1993; Wu *et al.*, 1993) Accordingly, our **first objective** was to investigate whether administering MPTP to mice results in the changes in SOD, CAT and GSH-PX activity and increased lipid peroxidation levels observed in PD. L-Deprenyl, prior to MPTP administration, was used to inhibit MPP^+ formation and its subsequent effect on antioxidant systems.

L-Deprenyl has also been found to suppress oxidative stress associated with an increase in DA turnover (Cohen and Spina, 1989) and free radicals generated from an intrastriatal infusion of MPP^+ (Chiueh *et al.*, 1992; Wu *et al.*, 1993). It is suggested that this effect of L-deprenyl occurs independently of MAO-B inhibition (Chiueh *et al.*, 1992; Wu *et al.*, 1993). The mechanism whereby L-deprenyl acts as a free radical scavenger may be indirect. Indeed, chronic L-deprenyl administration has been shown to increase striatal SOD and catalase activity(Carrillo *et al.*, 1991; Clow *et al.*, 1991; Knoll, 1988; Vizuete *et al.*, 1993). Thus, the removal of $O_2^{\cdot}$ and H_2O_2 will interrupt the cyclic formation of oxygen-derived free radicals responsible for the reduction in salicylate hydroxylation (Chiueh *et al.*, 1992; Wu *et al.*, 1993). However, SOD is reported to be up-regulated in response to enhanced $O_2^{\cdot}$ formation in various models of free radical-induced toxicity (hyperoxia, paraquat, 6-OHDA) (Crapo and Tierney, 1973; Frank, 1981; Ogawa *et al.*, 1994; Sjostrom and Crapo, 1981; Stevens and Autor, 1977). Thus, our **second objective** was to investigate the possible relationship between $O_2^{\cdot}$ formation and enhanced SOD activity. The major intracellular sources of $O_2^{\cdot}$ radical formation are catecholamine autoxidation and the mitochondrial respiratory chain. We therefore examined the effect of L-deprenyl on striatal and cerebellar catecholamine levels in mice, and the rate of oxygen consumption, a measure of respiratory functions, in fresh mitochondrial preparations. The effects of D-deprenyl, a weak MAO-B inhibitor, are contrasted with those of MDL72974 (4-Fluoro-ß-(fluoromethylene)-benzenebutanamine or mofegiline), a potent MAO-B inhibitor, in the same experimental paradigms in order to establish the role of MAO-B.

L-Deprenyl administration has also been demonstrated to significantly improve the recovery of dopaminergic neurons of the SN in MPTP-lesioned mice as demonstrated by TH immunopositive cell counts (Tatton and Greenwood, 1991). This observation leads to the proposal that L-deprenyl "rescues" degenerating neurons. The mechanism by which L-deprenyl exerts a trophic-like action is unclear at present. However, two studies failed to show a significant effect of L-deprenyl on the restoration of striatal DA and DA metabolites in MPTP-lesioned mice (Gupta and Wiener, 1995; Wiener *et al.*, 1989b). Furthermore, no data exist to suggest that L-deprenyl can "rescue" degenerating dopaminergic neurons in early and untreated PD patients (Lees, 1995; Olanow *et al.*, 1995; Schneider, 1995; The Parkinson Study Group, 1989b; 1993a; 1993b; Ward, 1994). Although L-deprenyl is well known for its short term beneficial effects in PD (Elizan *et al.*, 1989; The Parkinson Study Group 1993a), the recent finding of a higher mortality rate during a prolonged period of treatments raises some serious questions regarding the safety of this drug (Lees, 1995). Our **third objective** was to re-evaluate the long term impact of L-deprenyl on MPTP-induced dopaminergic cell loss in mice in order to clarify these issues.

CHAPTER 2

Effect of MPTP and L-Deprenyl on Antioxidant Enzymes and Lipid Peroxidation Levels in Mouse Brain

Preface to Chapter 2

Oxidative stress is suggested as a major factor in the etiology of PD and in MPTP-induced neurotoxicity. The increase in lipid peroxidation levels and alterations in antioxidant defence systems (SOD, GSH and GSH-PX) in the SN of PD patients support this hypothesis. These observations also suggest a deficiency in dealing with excess H_2O_2. The latter may in turn react with transition metals, such as iron, and further increase the production of oxygen-centered radicals to alter membrane integrity and Ca^{2+} homeostatis, resulting in cell death. Interestingly, an increase in nigral iron concentrations is reported in PD. The main objective of this study was to determine if altered antioxidant enzyme activity was sufficient to enhance free radical formation and consequently increase lipid peroxidation levels. This is important since alterations in lipid peroxidation levels observed in PD could be merely the consequence of dopaminergic neurodegeneration and not involved in cell death.

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THE EFFECT OF MPTP AND L-DEPRENYL ON ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION LEVELS IN MOUSE BRAIN.

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ABSTRACT

Excessive free radical formation or antioxidant enzyme deficiency can result in oxidative stress; a mechanism proposed in the toxicity of MPTP and in the etiology of Parkinson's disease (PD). However, it is unclear if altered antioxidant enzyme activity is sufficient to increase lipid peroxidation in PD. We therefore investigated if MPTP can alter the activity of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-PX) and the level of lipid peroxidation. L-Deprenyl, prior to MPTP administration, is used to inhibit MPP^+ formation and its subsequent effect on antioxidant enzymes. MPTP induced a three-fold increase in SOD activity in the striatum of C57Bl/6 mice. No parallel increase in GSH-PX or CAT activities was observed while striatal lipid peroxidation decreased. At the level of the substantia nigra (SN), even though increases in CAT activity and reduction in SOD and GSH-PX activities were detected, it did not result in altered lipid peroxidation. Interestingly, L-deprenyl induced similar changes in antioxidant enzymes and lipid peroxidation levels, as did MPTP. Taken together, these results suggest that an alteration in SOD activity, without compensatory increases in CAT or GSH-PX activities, is not sufficient to induce lipid peroxidation.

INTRODUCTION

N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces an irreversible Parkinson's disease (PD)-like syndrome in several animal species including primates (Langston *et al.*, 1983) and mice (Sonsalla and Heikkila, 1986). MPTP is metabolized by monoamine oxidase-B (MAO-B; EC 1.4.3.4) to form 1-methyl-4-phenylpyridinium (MPP^+) (Chiba *et al.*, 1984; Markey *et al.*, 1984). The latter leads to the degeneration of dopaminergic cell bodies in the substantia nigra (SN) (Bradbury *et al.*, 1986; Irwin and Langston, 1985; Javitch *et al.*, 1985). L-Deprenyl, an inhibitor of MAO-B, blocks MPP^+ formation and thus protects neurons against the deleterious effects of MPTP (Chiba *et al.*, 1984; Heikkila *et al.*, 1984). MPP^+ toxicity involves the inhibition of NADH dehydrogenase (EC 1.6.5.3) or complex I activity, the first enzyme of the mitochondrial respiratory chain (Nicklas *et al.*, 1985; Poirier and Barbeau, 1985a; Ramsay *et al.*, 1986). This event leads to the rapid depletion of ATP synthesis, loss of the membrane potential and neuron degeneration. Similar respiratory chain dysfunctions are reported in PD, with a reduction in NADH dehydrogenase activity being found in the substantia nigra (SN) (Schapira *et al.*, 1989) and striatum (Mizuno *et al.*, 1989) of Parkinsonian patients.

Free radicals are also thought to play a key role in the mechanism(s) of MPP^+-induced toxicity (Adams *et al.*, 1993; Poirier and Barbeau, 1985b; Poirier *et al.*, 1985; Przedborski *et al.*, 1992; Rossetti *et al.*, 1988; Singer *et al.*, 1993), as they can induce lipid peroxidation leading to an alteration in Ca^{++} homeostasis and subsequent neuronal death.

The major free radical scavenging enzymes or antioxidants are superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidase (GSH-PX; EC 1.11.1.9) and catalase (CAT; EC 1.11.1.6). Two major forms of SOD exist, a mitochondrial or Mn-SOD and a cytosolic or CuZn-SOD types (Weisiger and Fridovich, 1973). SOD catalyses the dismutation of superoxide ($O_2^{\cdot}$) to hydrogen peroxide (H_2O_2) while CAT and GSH-PX convert H_2O_2 into H_2O. A deficiency in antioxidant enzymes or an excessive production of free radicals overwhelm the antioxidant defence system, resulting in oxidative stress. Such a mechanism is proposed in the etiology of PD. Lipid peroxidation (Dexter *et al.*, 1989a; Pall *et al.*, 1986) and SOD activity (Marttila *et al.*, 1988; Poirier *et al.*, 1994; Saggu *et al.*, 1989) are increased in the SN and striatum in PD whereas GSH-PX and CAT activities are unchanged or reduced (Ambani, *et al.*, 1975; Kish *et al.*, 1985; Poirier and Thiffault, 1993). While the increase in overall SOD activity in PD is now well established, it is not clear if the CuZn-SOD, Mn-SOD or both types are affected. An increase in SOD without concomitant increments in GSH-PX or CAT activities (Ambani *et al.*, 1975; Kish *et al.*, 1985; Marttila *et al.*, 1988; Poirier and Thiffault, 1993) results in the accumulation of H_2O_2. The latter may in turn react with transition metal such as iron via a Fenton reaction and further increase the production of free radicals. Interestingly, iron is also found to be increased in the SN of Parkinsonian patients (Dexter *et al.*, 1989b; Earle, 1968; Riederer *et al.*, 1989). To date, it is not clear if the increase in SOD activity observed in PD alters the balance between oxidants and antioxidants and causes an increase in lipid peroxidation, or if the increase in SOD activity is only a consequence of the inhibition of the respiratory chain.

In this study, we investigated if MPP^+ leads to a variation in the activity of antioxidant enzymes and lipid peroxidation levels. L-Deprenyl, prior to MPTP administration, was used to inhibit MPP^+ formation, and its subsequent effect on the antioxidant system. We report that inhibition by MPP^+ of complex I activity in the mitochondrial respiratory chain leads to alterations of antioxidant enzymes without affecting lipid peroxidation.

MATERIALS AND METHODS

Materials

Three-month-old male (25-30 g) C57BL/6 mice (Charles River, St-Constant, Qué, Canada) with access to food and water ad libitum were maintained under a 12-h light/dark cycle. Animal care was according to protocols and guidelines approved by McGill University and the Canadian Council for Animal Care. MPTP HCl and R(-)-deprenyl HCl were obtained from RBI (Natick, MA, USA); (-)-epinephrine-(+)-bitartrate, Triton X-100, brij 96, t-butyl hydroperoxide and-1,1,3,3-tetramethoxypropane were purchased from Sigma (St-Louis, MO, USA); H_2O_2, n-butanol and pyridine were from Fisher (Fair Lawn, NJ, USA); CuZn-SOD from bovine erythrocytes and glutathione reductase were obtained from Boehringer Mannheim (Mannheim, Germany). All reagents were of the highest purity available commercially.

Animal Treatments

A total of 1250 animals were divided into four groups; one group was treated with 3 x 20 mg/kg MPTP (i.p.) at two hrs interval, a second group was administered L-deprenyl at a dosage of 10

mg/kg and every 48 hrs. At this dosage, L-deprenyl inhibits more than 95% MAO-B as well as some of MAO-A activity (Knoll, 1978; Heikkila *et al.*, 1990). A third group received a combination of both treatments in which L-deprenyl was given one hr prior to MPTP and finally, a fourth group received vehicle (0.9% saline) only. Animals were sacrificed by cervical dislocation at 2 and 36 hrs, and 15 days following the last injection. A total of eight doses of L-deprenyl was given and animals were killed 24 hrs after the last L-deprenyl injection for the 15 days time point. Brains were rapidly removed and cut in 1 mm coronal sections on ice. The striatum was carefully excised and the substantia nigra was punched using a fine pasteur pipet. The cerebellar cortex was isolated from the white matter and blood vessels. The SN, striatum and cerebellum from 15, 6 and 5 animals respectively, within the same treatment group were pooled, quickly homogenized in potassium phosphate buffer (no sodium) containing 0.154 M KCl pH 7.4 and frozen at -80 °C for one measurement of SOD, GSH-PX and CAT activities as indicated below (n=5 per time point). The SN, striatum and cerebellum from 10, 2 and 2 animals respectively, were pooled and quickly frozen at -80 °C for one evaluation of thiobarbituric reactivity as a measure of lipid peroxidation (n=5 per time point).

Superoxide Dismutase

CuZn-SOD and Mn-SOD were assayed with the method of Misra and Fridovich (1972) which is based on the ability of SOD to inhibit the autoxidation of (-)-epinephrine-(+)-bitartrate to adrenochrome at pH 10.2 and 30°C. The rate of adrenochrome formation monitored at 480 nm (Spectronic model 1201, Milton Roy Co., Quarry Bay, Hong Kong) was on average 0.025 optical density/min. Fifty percent inhibition of the autoxidation is defined as one unit of

enzymatic activity. One unit was reached with 48 ng/mL CuZn-SOD from bovine erythrocytes. Aliquots of homogenate were chosen such that between 20-60% inhibition was attained and incubated in the carbonate buffer (pH 10.2) for 10 min at 37°C with/without 5mM CN^-. CN^- is known to inhibit CuZn-SOD and allowed the measurement of Mn-SOD. Total SOD activity was subtracted from Mn-SOD measurement to obtain CuZn-SOD.

Catalase

The enzymatic activity of catalase was determined using a Clark oxygen electrode as described by Del Rio *et al.* (1977). Briefly, 50mM Na_2HPO_4/KH_2PO_4 buffer (pH 7.0) was added to a reaction vial and degassed with a stream of N_2 for 5 min at 25°C to remove O_2. 100µL H_2O_2 (33.5 mM final concentration) was included and the linear rate of O_2 released was recorder on a chart. Homogenates were incubated in the above buffer containing 0.65% (vol/vol) Triton X-100 for 1 min at 37°C. Catalase activity was calculated in O_2 ppm/min and subtracted from the spontaneous rate of H_2O_2 decomposition.

Glutathione Peroxidase

Selenium-dependent peroxidase was measured by a coupled enzyme procedure with glutathione reductase and NADPH (Günzler *et al.*, 1974). The enzyme was solubilized in 0.1% (vol/vol) brij 96 and 0.154 M KCl and centrifuged at 16000 x g for 10 min. The supernatant was assayed

in 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA, 1.4 mM GSH, 0.25 mM NADPH, 1 U/mL glutathione reductase. 1.2 mM t-butyl hydroperoxide was utilized as a substrate. The unit of activity was calculated from the rate of NADPH consumption monitored at 366 nm and 37°C.

Lipid Peroxidation

Thiobarbituric acid (TBA) was reacted colorimetrically with a product of lipid peroxidation, malondialdehyde (MDA), according to the methodology of Ohkawa *et al.* (1979). Brain tissue was sonicated in 0.154 M KCl on ice and refluxed for 60 min at 95 °C in the presence of 0.405% (wt/vol) sodium dodecyl sulphate (SDS), 0.3% (wt/vol) TBA and 7.5% (vol/vol) acetic acid at pH 3.5. The colored product was extracted with n-butanol:pyridine (15:1, v/v) and optical density read at 532 nm. 1,1,3,3-Tetramethoxypropane (TMP) was used as the standard curve and the results were reported as nmol MDA/mg protein.

Protein Assay

The protein concentration was determined via Pierce BCA Protein Assay Kit (Rockford, Il, USA) with bovine serum albumin as the standard and absorbance measured at 562 nm.

Statistics

Statistical analysis was determined non-parametrically by Wilcoxon test and value of $p<0.05$ was utilized as the criterion for statistical significance. Each bar represented a total of 5 independent experiments.

RESULTS

SOD

Fig. 1 summarizes CuZn- and Mn-SOD activities in the SN (a), striatum (b) and cerebellum (c). A significant ($p<0.05$) reduction in Mn-SOD activity to 50% of control levels was observed in L-deprenyl, MPTP and L-deprenyl/MPTP treatments in the SN of C57Bl/6 mice. L-Deprenyl treatment lead to a modest reduction ($p<0.05$) in CuZn-SOD activity at 36hrs. No alteration in this measurement was detected in the MPTP treated group. A combined L-deprenyl/MPTP treatment caused a moderate increase ($p<0.05$) in CuZn-SOD activity at 2 hrs whereas the enzymatic activity was reduced at 15 days in this brain region. In contrast, striatal SODs were elevated above control levels by L-deprenyl, MPTP and L-deprenyl/MPTP treatments (fig. 1b). Significant 1-3 fold increases in Mn-SOD and CuZn-SOD were found in the striatum of C57Bl/6 mice (fig. 1b). In the cerebellum however, the augmentation in SOD activity was not as marked as that seen in the striatum (fig. 1c).

Overall the results show that L-deprenyl was ineffective in blocking the changes in the activities of CuZn-SOD and Mn-SOD induced by a MPTP treatment. In fact, L-deprenyl itself lead to similar changes in the activitites of the above enzymes as compared to that seen with MPTP. Significant differences ($p<0.05$) in the activities of CuZn- and Mn-SOD were found between L-deprenyl or MPTP and the combined L-deprenyl/MPTP treatment in the brain structures examined (fig. 1). In addition, these alterations extended beyond the nigrostriatal dopaminergic pathway (fig. 1c).

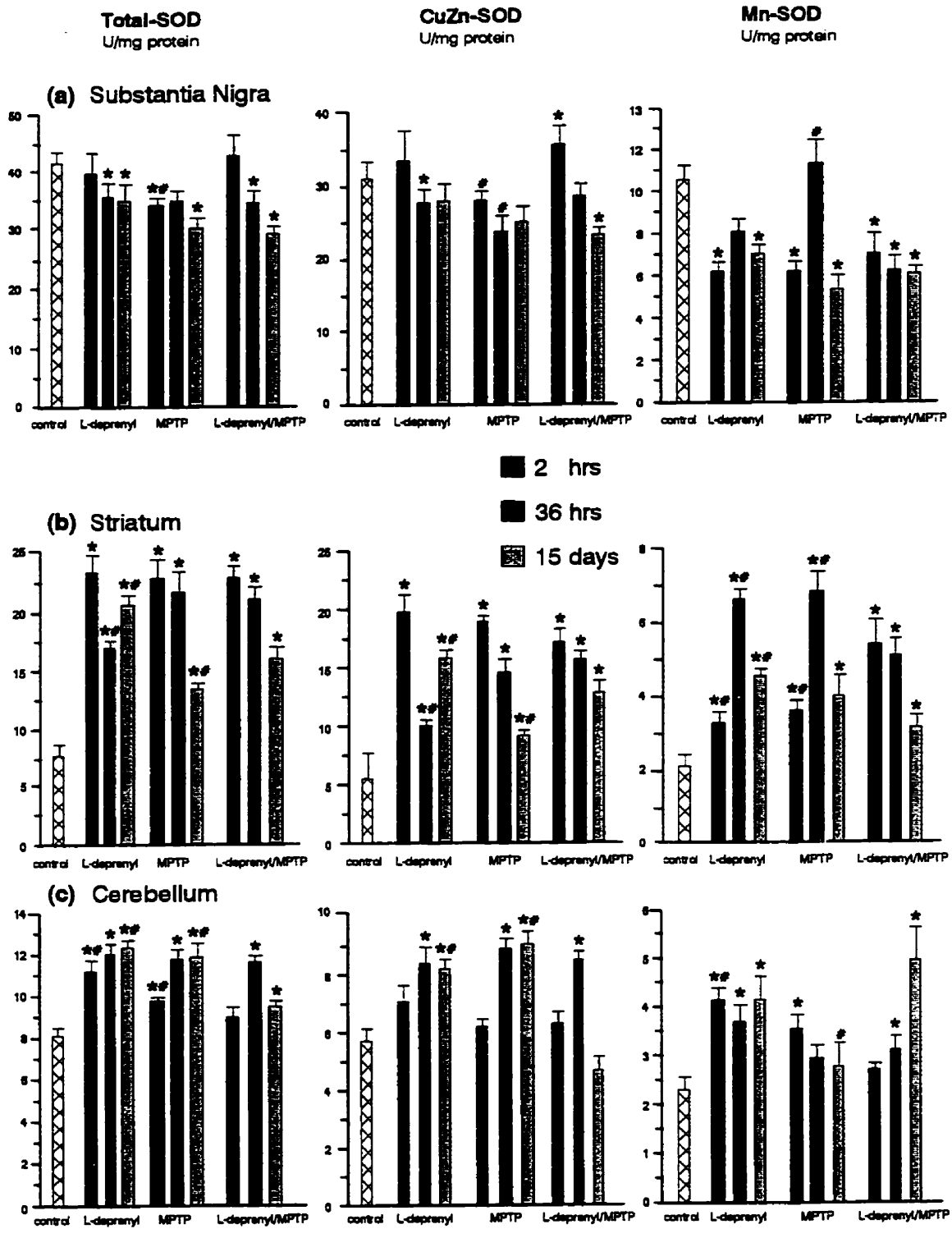

Fig. 1. SOD activity in the SN (a), striatum (b) and cerebellum (c) of C57Bl/6 mice at 2 h (■), 36 h (■) and 15 days (▨) post-treatment. Mice were treated with L-deprenyl, MPTP, and L-deprenyl/MPTP, and controls (▨) received 0.9% saline only (vehicle). Enzymatic activity measurements and animal treatments are described in Materials and Methods. Stars and pound signs indicate significant differences ($p<0.05$), compared with controls and corresponding L-deprenyl/MPTP time point, respectively. Mean ± SE values of five independent experiments are shown.

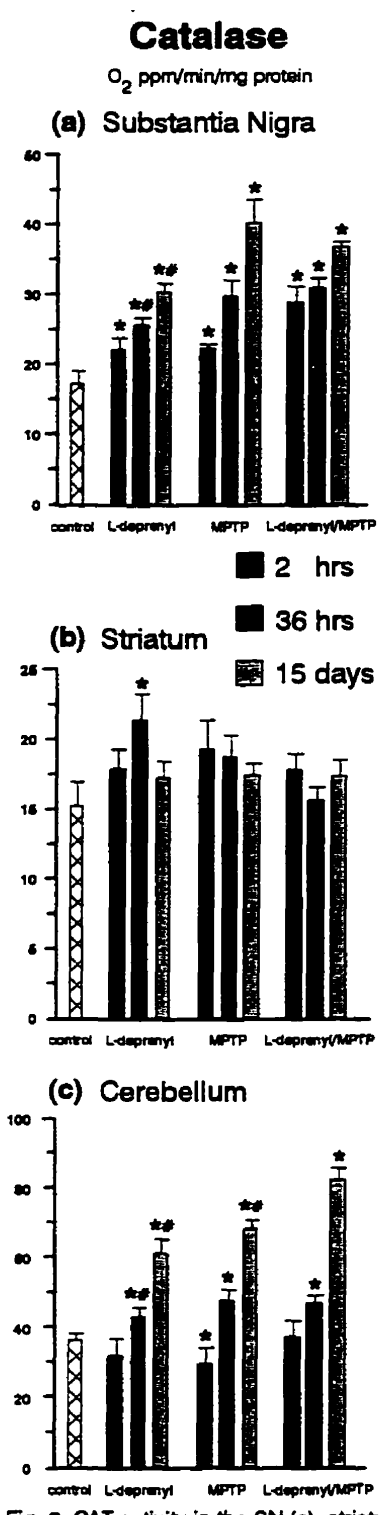

Fig. 2. CAT activity in the SN (a), striatum (b) and cerebellum (c) of C 57Bl/6 mice at 2 h (■), 36 h (■) and 15 days (▨) post-treatment. Mice were treated with L-deprenyl, MPTP, and L-deprenyl/MPTP, and controls (☒) received 0.9% saline only (vehicle). Enzymatic activity measurements and animal treatments are described in Materials and Methods. Stars and pound signs indicate significant differences ($p < 0.05$), compared with controls and corresponding L-deprenyl/MPTP time points, respectively. Mean ± SE values of five independent experiments are are shown.

CAT

Fig. 2 illustrates the extents of alterations of CAT activity in the SN (a), striatum (b) and cerebellum (c) of C57Bl/6 mice. L-Deprenyl, MPTP and L-deprenyl/MPTP treatments induced 1-2 fold increases ($p<0.05$) in CAT activity in the SN (fig. 2a). In the cerebellum however, an initial 20% decrease in CAT activity was observed in the MPTP group at 2 hrs and a 50 to 100% increase was seen thereafter (fig. 2c). L-Deprenyl and L-deprenyl/MPTP treatments resulted in 20 to 100% augmentation in CAT activity at 36 hrs and 15 days, respectively. The enzymatic activity remains relatively unchanged in the striatum except for a transient 40% increase in the L-deprenyl group.

As previously noted, L-deprenyl lead to parallel changes in the activity of CAT as compared to that of MPTP treatment in the SN and cerebellum. Significant differences ($p<0.05$) in enzymatic activity were observed in the L-deprenyl group as compared to the combined L-deprenyl/MPTP treated animals (fig. 2). Accordingly, alterations in CAT activity seen with L-deprenyl/MPTP could not solely be accounted for by L-deprenyl.

Table 1. GSH-PX activity in the SN, striatum and cerebellum of C57BL/6 mice.

Treatment	SN (U/mg of protein)	Striatum (U/mg of protein)	Cerebelum (U/mg of protein)
control (0.9% saline)	1.37 ±0.07	1.01 ±0.02	1.55 ±0.16
L-deprenyl			
2 hrs	1.47 ±0.03 b	1.04 ±0.02	1.52 ±0.05 b
36 hrs	1.20 ±0.04 b	1.02 ±0.03	1.36 ±0.03
15 days	1.07 ±0.03 a	1.00 ±0.04	1.31 ±0.02
MPTP			
2 hrs	1.41 ±0.07	1.12 ±0.05	1.52 ±0.08
36 hrs	1.30 ±0.02	1.08 ±0.03	1.27 ±0.01 a
15 days	1.22 ±0.08	1.06 ±0.02	1.29 ±0.04
L-deprenyl/MPTP			
2 hrs	1.43 ±0.03	1.14 ±0.10	1.43 ±0.02
36 hrs	1.31 ±0.03	0.99 ±0.02	1.36 ±0.04
15 days	1.20 ±0.06	0.98 ±0.04	1.31 ±0.02

Enzymatic activity analysis and animal treatments are described in Materials and Methods. Means ± SE values of five independent determinations are shown.

a $p<0.05$ as compared to control.

b $p<0.05$ as compared to L-deprenyl/MPTP treatment at respective time points.

GSH-PX and Lipid Peroxidation

GSH-PX and lipid peroxidation determinations in the SN, striatum and cerebellum are shown in Table 1 and 2, respectively. A modest decrease ($p<0.05$) in nigral GSH-PX activity was observed in the L-deprenyl- and L-deprenyl/MPTP-treated groups at 15 days post-treatment. A similar reduction in cerebellar GSH-PX activity was seen in the MPTP-treated animals. No alteration in GSH-PX activity could be detected in the striatum.

Table 2. Malondialdehyde (MDA) formation as a measure of lipid peroxidation in the SN, striatum and cerebellum of C57BL/6 mice.

Treatment	SN (nmol MDA/mg of protein)	Striatum (nmol MDA/mg of protein)	Cerebelum (nmol MDA/mg of protein)
control (0.9% saline)	3.23 ±0.25	3.78 ±0.28	5.64 ±0.72
L-deprenyl			
2 hrs	ND	3.45 ±0.08	7.15 ±0.37
36 hrs	3.66 ±0.39 b	3.10 ±0.30	6.83 ±0.33
15 days	3.27 ±0.32	2.98 ±0.17 a	5.87 ±0.46
MPTP			
2 hrs	ND	2.91 ±0.12	5.30 ±0.23
36 hrs	3.04 ±0.22	2.63 ±0.06 a	5.83 ±0.21 b
15 days	3.02 ±0.20	3.23 ±0.21	6.42 ±0.40
L-deprenyl/MPTP			
2 hrs	ND	3.14 ±0.08	5.85 ±0.40
36 hrs	2.93 ±0.09	2.58 ±0.11 a	6.55 ±0.36
15 days	2.80 ±0.09	2.67 ±0.17 a	5.64 ±0.32

MDA measurement and animal treatments are described in Materials and Methods. Mean ± SE values of five independent determinations are shown.

a $p<0.05$ as compared to control.

b $p<0.05$ as compared to L-deprenyl/MPTP treatment at respective time points.

ND Not determined.

Lipid peroxidation was unaltered in the SN and cerebellum of C57Bl/6 mice as compared to saline treated animals (Table 2). However, L-deprenyl and MPTP treatments resulted in a 30% decrease ($p<0.05$) in striatal lipid peroxidation at 36 hrs and 15 days, respectively. A similar reduction was observed at both time points in the L-deprenyl/MPTP-treated group.

Significant differences ($p<0.05$) in GSH-PX activity and lipid peroxidation levels were observed between L-deprenyl or MPTP and L-deprenyl/MPTP at two time points in the SN and cerebellum (Table 1 and 2).

DISCUSSION

We have shown that L-deprenyl, MPTP and L-deprenyl/MPTP treatments result in alterations of the antioxidant defense system. Mn-SOD, CuZn-SOD and CAT activities were the most affected in three brain areas examined, with the exception of CAT activity which remained relatively unchanged in the striatum.

Despite alterations in antioxidant enzymes, we did not observe an increase in lipid peroxidation in the SN, striatum and cerebellum of mice. Moreover, in spite of a 50% decrease in Mn-SOD activity in the SN, lipid peroxidation remained relatively unchanged in the MPTP- or L-deprenyl-treated mice. Lipid peroxidation, as measured by malondialdehyde (MDA) formation, has received a number of criticisms (Draper *et al.*, 1993; Gray, 1978; Gutteridge and Quinlan, 1983). MDA formed *in vivo* can complex with various molecules such as nucleic acids, proteins and phospholipids, leading to the underestimation of endogenous lipid peroxidation (Draper *et al.*, 1988). In addition, thiobarbituric acid (TBA) can react with aldehydes other than MDA to give a maximal absorption at 532 nm (Gutteridge and Tickner, 1978). In spite of these critiques, the TBA test has been widely used to assess oxidative damage to membrane lipids (Janero, 1990). In fact, Götz *et al.* (1993) concluded that this assay procedure is a reflection of the susceptibility of lipids to undergo peroxidation *in vitro* at ambient air. This aspect was considered important since it allowed us to indirectly evaluate lipid hydroperoxides that break down during the assay to yield MDA. Our failure to observe an increase in lipid peroxidation following MPTP treatment is confirmed by other reports using other methodologies. MPTP did

not induce lipid peroxidation in the striatum of mice with physiologically adequate levels of vitamin E (Adams *et al.*, 1990, Corongiu *et al.*, 1987), whereas an increase is found in vitamin E-deficient mice (Adams *et al.*, 1990). This suggests that factors other than antioxidant enzymes play a pivotal role in protection against membrane peroxidation. Our results are also consistent with alterations in the activities of antioxidant enzymes in Parkinson's disease brains, SOD being the most affected, but to extents that are insufficient to lead to increases in lipid peroxidation levels.

MPTP treatment resulted in a 1-3 fold increase in Mn-SOD and CuZn-SOD activities in the striatum, which is consistent with acute oxidative stress. Several lines of evidence have demonstrated that SOD is upregulated when cells are exposed to excessive production of superoxide ($O_2^{\cdot}$) radicals. For instance, rats exposed to sublethal concentrations of oxygen showed elevations in CuZn-SOD and Mn-SOD activities in their lungs (Crapo and Tierney, 1973; Sjostrom and Crapo, 1981; Stevens and Autor, 1977). Free radicals have been implicated in the mechanisms of toxicity of the herbicide paraquat, which structurally resembles MPP^+. Paraquat induces $O_2^{\cdot}$ formation with a concomitant increase in lipid peroxidation in rat lung and purified rat lung microsomes (Bus *et al.*, 1975; Bus *et al.*, 1976; Ilett *et al.*, 1974; Trush *et al.*, 1981). In addition, mammalian cells exposed to paraquat demonstrated increases in CuZn-SOD and Mn-SOD activities (Frank, 1981; Krall *et al.*, 1988). Electron spin resonance (ESR) studies indicated that the inclusion of MPP^+ in mitochondrial preparations induces the formation of $O_2^{\cdot}$ (Adams *et al.*, 1993; Rossetti *et al.*, 1988). Interestingly, rotenone is known to inhibit the respiratory chain at the same site as MPP^+, and to enhance ESR signals ($O_2^{\cdot}$)

under the same conditions (Adams *et al.*, 1993). Rotenone has a chemical structure distinct from that of MPTP or MPP^+. Thus, our results suggest that the increase in Mn-SOD activity in the striatal mitochondria could be the consequence of a leakage of $O_2^{\cdot}$ due to MPP^+ inhibition of NADH dehydrogenase within the respiratory chain. On the other hand, this increase could also be the result of a redox reaction between MPP^+ in the presence of mitochondrial enzymes or with another MPTP metabolite, $MPDP^+$ (Adams *et al.*, 1993; Klaidman *et al.*, 1993; Rossetti *et al.*, 1988).

The increase in CuZn-SOD activity seen following MPTP treatment is significant since MPP^+ can generate $O_2^{\cdot}$ in the presence of cytosolic enzymes (Klaidman *et al.*, 1993). Transgenic mice with a 1.5- to 5-fold increase in the activity of CuZn-SOD were protected against dopamine depletion when moderate levels of MPTP (3 x 30 mg/kg, 24 hrs interval) were administered (Przedborski *et al.*, 1992). In addition, White and co-workers (1993) have shown that the expression of Mn-SOD is not altered in CuZn-SOD transgenic mice. In our study, the mitochondrial form of SOD, Mn-SOD, was also induced following MPTP treatment. This observation suggests a role for this enzyme during an acute MPTP treatment. It would be of interest to study the effect of higher levels of MPTP on dopamine depletion in transgenic mice, considering the differential compartmentalization of CuZn-SOD and Mn-SOD.

Changes in SOD activities induced by free radicals following MPTP administration were not confined to the nigrostriatal pathway (fig. 1c). MPTP toxicity, on the other hand, is somewhat specific to this pathway (Herkenham *et al.*, 1991; Takada *et al.*, 1991; Langston *et al.*, 1983;

Langston *et al.*, 1984). Moreover, in spite of the formation of free radicals we did not observe a significant increase in lipid peroxidation levels. In fact, a decrease was detected in the striatum of MPTP-treated animals (Table 2). The induction of antioxidant enzymes is therefore adequate to curtail free radicals produced as a result of MPTP treatment. This observation is supported by earlier reports in which oxygen-derived reactive species are detected 2hrs but not 9 hrs after MPTP administration (Ali *et al.*, 1993; 1994). Taken together, this suggests that the generation of free radicals does not seem to play a major role in the toxicity of MPTP. Accordingly, inhibition of NADH dehydrogenase within the mitochondrial respiratory chain is likely the predominant mechanism by which MPP^+ exerts its toxic effect.

Surprisingly, L-deprenyl was unable to reverse the effect of MPTP on the activities of antioxidant enzymes. L-Deprenyl is known to protect dopaminergic neurons against MPTP toxicity through its inhibition of MAO-B activity (Chiba *et al.*, 1984; Heikkila *et al.*, 1984). Unexpectedly, results obtained with L-deprenyl paralleled observations made using MPTP. The combined administration of L-deprenyl/MPTP lead to alterations in CuZn-SOD, Mn-SOD and CAT activities in the three brain regions examined which could not solely be accounted for by L-deprenyl treatment. Although L-deprenyl, at the regimen used in our experiments, is known to block MAO-B activity by more than 95% (Knoll, 1978; Heikkila *et al.*, 1990), it is conceivable that MPP^+ can still be formed, albeit in minute amounts, to induce some effects. Alternatively, MPTP itself may be able to modulate the activity of these enzymes. The latter appears unlikely since L-deprenyl is able to suppress the production of free radicals when MPTP is incubated with mitochondrial fractions, suggesting that MPTP metabolites are responsible for

the formation of free radicals (Adams *et al.*, 1993; Rossetti *et al.*, 1988). However, MPTP by itself can not alter oxygen uptake in subcellular mitochondrial preparations (Nicklas *et al.*, 1985; Thakar *et al.*, 1988). Therefore, the induction of Mn-SOD by MPTP may not be through a free radical mechanism.

Administration of L-deprenyl to rats is known to increase striatal SOD activity at concentrations lower than that required to fully inhibit MAO-B (Carrillo *et al.*, 1991; Clow *et al.*, 1991; Knoll, 1988). Hence, mechanisms by which L-deprenyl can induce SOD activity appear to be independent from the inhibition of MAO-B. As discussed above, the literature suggests that the induction of SOD may occur via a free radical mechanism. However, several lines of evidence indicate that L-deprenyl acts as an antioxidant. L-Deprenyl is shown to suppress the formation of free radicals of an analog of MPTP (Chiueh *et al.*, (1992). In addition, Cohen (1989) has demonstrated that L-deprenyl can suppress oxidative stress induced by the oxidation of dopamine. Although it is clear that L-deprenyl has some antioxidant properties, a chronic L-deprenyl treatment may have a differential effect. This aspect is currently being investigated in detail in our laboratory.

L-Deprenyl, MPTP and L-deprenyl/MPTP treatments resulted in increases in CAT activity in the SN and the cerebellum. Current knowledge is lacking on the real significance of CAT in the brain, considering its presence in minute amounts, likely confined to peroxisomes (Gaunt *et al.*, 1976). However, our results suggest that this enzyme may be important in the process of eliminating H_2O_2.

In summary, our data have shown that alterations in antioxidant defence systems do not necessarily lead to an increase in lipid peroxidation. Interestingly, L-deprenyl was unable to attenuate the effect of MPTP on antioxidant enzymes. Although L-deprenyl has been shown to have antioxidant property, our results suggest that the effect of chronic L-deprenyl administration should be carefully assessed. Alterations in mitochondrial Mn-SOD activity may occur in response to $O_2^{\cdot}$ leakage resulting from the inhibition of the respiratory chain or, alternativly, from a redox reaction induced by MPP^+. Finally, we propose that the increase in nigral Mn-SOD activity reported in idiopathic PD (Saggu *et al.*, 1989) may represent an adaptive response due to a leakage of oxygen-centered radicals resulting from dysfunctions of the mitochondrial respiratory chain.

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CHAPTER 3

The Effect of L-Deprenyl, D-Deprenyl and MDL72974 on Mitochondrial Respiration: A Possible Mechanism Leading to an Adaptive Increase in Superoxide Dismutase Activity

Preface to Chapter 3

As seen in the previous chapter, L-deprenyl failed to reverse the effect of MPTP on the antioxidant enzyme activity and lipid peroxidations levels. Unexpectedly, results obtained with L-deprenyl were similar to those observed with MPTP, despite the regional differences seen in MPTP- and L-deprenyl-treated mice. The mechanism by which L-deprenyl induces an increase in SOD activity is intriguing considering that these enzymes are up-regulated in response to $O_2^{\cdot}$ in various models of free radical-mediated injuries (such as hyperoxia, 6-OHDA, paraquat). Taken together, these results lead us to evaluate the possible relationship between L-deprenyl, $O_2^{\cdot}$ formation and enhanced SOD activity. In this series of studies, we investigated the effects of L-deprenyl on catecholamine levels and mitochondrial electron transport chains, the major source of $O_2^{\cdot}$ formations. In addition, D-deprenyl, a weak MAO-B inhibitor, and MDL72974 (4-fluoro-ß-(fluoromethylene)-benzenebutanamine or mofegiline), a more potent MAO-B blocker that is not metabolized to amphetamines (unlike D-deprenyl and L-deprenyl), were investigated using the same experimental paradigms to establish the putative, respective roles of MAO-B and amphetamine in the effects of L-deprenyl.

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THE EFFECT OF L-DEPRENYL, D-DEPRENYL AND MDL72974 ON MITOCHONDRIAL RESPIRATION: A POSSIBLE MECHANISM LEADING TO AN ADAPTATIVE INCREASE IN SUPEROXIDE DISMUTASE ACTIVITY

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ABSTRACT

L-Deprenyl is an irreversible monoamine oxidase-B inhibitor with a complex pharmacological profile. For instance, L-deprenyl administration to rats and mice increases cytosolic CuZn- and mitochondrial Mn-superoxide dismutase activities in the striatum. CuZn- and Mn-superoxide dismutase are enzymes involved in defense against superoxide ($O_2^{\cdot}$) radicals. Hence, an increase in CuZn- and Mn-superoxide dismutase activities is suggestive of oxidative stress. The major intracellular site of $O_2^{\cdot}$ radical formation is the mitochondrial respiratory chain. Several reports indicated that alterations in mitochondrial respiratory functions enhance $O_2^{\cdot}$ production. We observed that L-deprenyl induced a dose-dependent inhibition of oxygen (O_2) consumption (state 3) during ATP synthesis in presence of complex I (pyruvate and malate) and complex II (succinate) substrates in fresh mitochondrial preparations. D-Deprenyl produced a similar inhibitory profile, whereas MDL72974, a selective monoamine oxidase-B inhibitor, was less effective. Administration of D-deprenyl or MDL72974 to mice resulted in an increase in both striatal CuZn- and Mn-superoxide dismutase activities. Accordingly, we propose that the impairment of mitochondrial respiratory functions which stimulate $O_2^{\cdot}$ formation could modulate CuZn- and Mn-superoxide dismutase activities, through a mechanism that appears to be independent of monoamine oxidase-B inhibition.

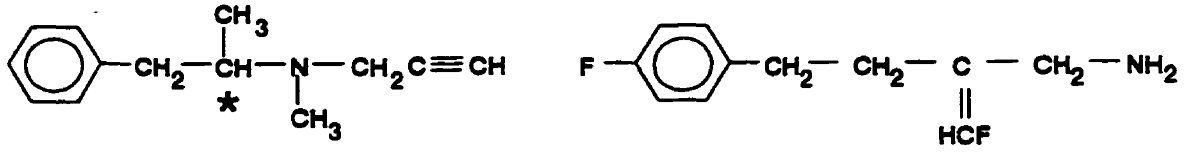
INTRODUCTION

L-Deprenyl was originally introduced as a new class of irreversible monoamine oxidase-B (MAO-B; EC 1.4.3.4) inhibitor devoid of the hypertensive side effects associated with MAO-A inhibition (Knoll, 1978). MAO-B inhibition results in a reduction in dopamine (DA) catabolism and an increase in endogenous levels of β-phenylethylamine (β-PEA) (Zsilla *et al.*, 1986; Boulton *et al.*, 1990; Knoll and Miklya, 1994; Paterson *et al.*, 1995). The pharmacology of L-deprenyl was later found to extend beyond MAO-B inhibition (Knoll, 1978; Berry *et al.*, 1994). For instance, L-deprenyl is shown to block DA uptake and to induce an increase in superoxide dismutase activity in the rat striatum (SOD; EC 1.15.1.1) (Clow *et al.*, 19 91; C arrillo *et al.*, 1992; Fang and Yu, 1994; Bondiolotti *et al.*, 1995). L-Deprenyl is also reported to act as a potent antioxidant (Cohen and Spina 1989; Chiueh *et al.*, 1992) and to protect dopaminergic neurons of the nigrostriatal pathway against the neurotoxicity induced by MPTP, by blocking its metabolism via MAO-B to form the neurotoxicant MPP^+ (Chiba *et al.*, 1984).

Since L-deprenyl stimulates dopaminergic transmission and demonstrates antioxidant properties, this compound was considered as a potential therapeutic agent in the treatment of Parkinson's disease (PD) (The Parkinson Study Group, 1989). However, results from clinical trials in which L-deprenyl is used as an adjunct to L-dopa therapy have been controversial. Although L-deprenyl is well known for its short term beneficial effects in PD (Elizan *et al.*, 1989; The Parkinson Study Group, 1993), the recent finding of a higher mortality rate during a prolonged period of treatments raises some serious questions regarding the safety of this drug (Lees, 1995).

Several studies suggest that SOD activity is up-regulated in response to enhanced superoxide ($O_2^{\cdot}$) formation in various animal models of free radical-induced toxicity (hyperoxia, paraquat, 6-hydroxydopamine (6-OHDA) (Stevens and Autor, 1977; Frank, 1981; Sjostrom and Crapo, 1981; Ogawa *et al.*, 1994). An increase in SOD activity as a result of L-deprenyl administration is consistent with an oxidative stress-inducing agent. SOD is an antioxidant enzyme which converts superoxide radical ($O_2^{\cdot}$) to hydrogen peroxide (H_2O_2) and O_2 (McCord and Fridovich, 1969; Fridovich, 1978). Two major forms of SOD exist, a mitochondrial or Mn-SOD and a cytosolic or CuZn-SOD type (Weisiger and Fridovich, 1973), and represent the first line of defense against free radical injuries. The latter involves peroxidative damage to cellular components such as nucleic acids, proteins and lipids, leading to increases in membrane permeability, alterations in calcium homeostasis and cell death (Coyle and Puttfarcken, 1993).

The observation that L-deprenyl administration to rodents induces alterations in SOD activity in the striatum (Knoll, 1988; Carrillo *et al.*, 1991; Clow *et al.*, 1991; Thiffault *et al.*, 1995) lead us to investigate possible relationships between $O_2^{\cdot}$ formation and enhanced SOD activity. Furthermore, since catecholamine oxidation and mitochondrial respiration are known to be the major source of $O_2^{\cdot}$ formation *in vivo* (Boveris and Chance, 1973; Turrens and Boveris, 1980; Graham, 1984; Sohal and Brunk, 1992), we examined the effect of L-deprenyl on striatal and cerebellar catecholamine levels in mice and on the rate of oxygen consumption in fresh brain mitochondrial preparations. The effects of D-deprenyl, a weak MAO-B inhibitor, was contrasted with those of MDL72974, a potent MAO-B inhibitor, in the same experimental



L-deprenyl and D-deprenyl MDL72974

Fig. 1. Structures of L-deprenyl, D-deprenyl and MDL72974 (Mofegiline). The asterisk identifies the chiral carbon atom.

paradigms. The chemical structures of L-deprenyl, D-deprenyl and MDL72974 are shown in Fig. 1.

MATERIALS AND METHODS

Materials

Three-month-old male (25-30 g) C57BL/6 and CD1 mice (Charles River, St-Constant, Qué., Canada) with access to food and water ad libitum were maintained under a 12-h light/dark cycle. Animal care was provided according to protocols and guidelines approved by McGill University and the Canadian Council for Animal Care. L-Deprenyl and D-deprenyl HCl were obtained from Research Biochemicals International (Natick, MA, USA); MDL72974 was a generous gift from Marion Merrell Dow Research (Cincinnati, OH, USA); β-(ethyl-1-^{14}C)-phenylethylamine hydrochloride was obtained from Dupont (Boston, MA, USA); Ficoll type 400, (-)-epinephrine-(+)-bitartrate, β-phenylethylamine, malic acid, succinic acid, pyruvic acid and adenosine 5'-diphosphate were purchased from Sigma (St. Louis, MO, USA); n-heptane was from Fisher (Fair Lawn, NJ, USA); CuZn-SOD from bovine erythrocytes was obtained from Boehringer Mannheim (Mannheim, Germany).

Animal Treatments

A total of 30 C57BL/6 mice were divided into three groups; each group received either L-deprenyl, D-deprenyl, or MDL72974 at a dosage of 10 mg/kg every 2 days for 15 days. At this dosage, L-deprenyl inhibits 95% of brain MAO-B activity as well as some of MAO-A activity (Knoll, 1978; Heikkila *et al.*, 1990). A fourth group consisting of 10 animals was administered vehicle (0.9% saline) only. Animals were sacrificed by cervical dislocation 24 hr following the last injection. Consequently a total of eight doses of L-deprenyl, D-deprenyl and MDL72974 were given. Brains were rapidly removed and cut in 1 mm coronal sections on ice. The striatum was carefully excised and the cerebellar cortex was isolated from the white matter and blood vessels. The striatum and cerebellum were frozen at -80°C for the determination of SOD activity and catecholamine levels as indicated below (n=5). Finally, a total of 12 C57BL/6 mice were distributed into three groups for MAO activity determination (see below). Six animals were treated with L-deprenyl as indicated above and killed at 2 hr and 15 days. Three others received a single dose of L-deprenyl (10 mg/kg) and were sacrificed 20 days later. This time point was selected since the recovery of MAO-B activity in rodent brains varies from 8-14 days (Goridis and Neff, 1971; Felner and Waldmeier, 1979). The remaining animals were given vehicle only and sacrificed 15 days later (n=3).

Superoxide Dismutase

CuZn-SOD and Mn-SOD were assayed with the method of Misra and Fridovich (1972) which is based on the ability of SOD to inhibit the autoxidation of (-)-epinephrine-(+)-bitartrate to adrenochrome at pH 10.2 and 30°C. The rate of adrenochrome formation monitored at 480

nm (Spectronic model 1201, Milton Roy Co., Quarry Bay, Hong Kong) was on average 0.025 optical density/min. Fifty percent inhibition of the autoxidation is defined as one unit of enzymatic activity. One unit was reached with 48 ng/ml CuZn-SOD from bovine erythrocytes. Aliquots of homogenates were chosen such that between 20-60% inhibition was attained and incubated in the carbonate buffer (pH 10.2) for 10 min at 37°C with/without 5 mM CN^-. CN^- is known to inhibit CuZn-SOD and allowed the measurement of Mn-SOD. Total SOD activity was subtracted from Mn-SOD measurement to obtain CuZn-SOD.

Isolation of Mitochondria for Monoamine Oxidase Measurement

Mitochondria were isolated from the brain of C57BL/6 mice according to a procedure described by Nyman and Whittaker (1963). In brief, brain (without the cerebellum) was homogenized in 5 ml 0.32 M sucrose/0.1 mM EDTA and centrifuged at 1000 x g for 10 min at 4°C. The supernatant was removed and centrifuged at 100 000 x g for 30 min at 4°C. The pellet containing the intact mitochondria, was removed and carefully suspended in 0.32 M sucrose/0.1 mM EDTA, layered on 0.88 M sucrose and centrifuged at 100 000 x g for 60 min at 4°C. The mitochondrial fraction (P3) was washed in 30 mM potassium phosphate buffer (pH 7.4) and kept frozen (-80°C) until the determination of MAO-B activity.

The enzymatic activity of MAO-B was determined according to Wurtman and Axelrod (1963) as modified by Da Prada *et al* (1989). Twenty µl of mitochondrial suspension (0.5-1.0 mg protein/ml) was incubated with 100 µl of 0.08 mM β-phenylethylamine (β-PEA) in 30 mM potassium phosphate buffer (pH 7.4) containing 0.1 µCi/ml of ^{14}C-β-PEA. The reaction

mixture was incubated under constant agitation at 37°C in a final volume of 200 µl of phosphate buffer. The enzymatic oxidation was stopped at 0, 10, 15 or 20 minutes by the addition of 200 µl of 2 N HCl. The deaminated metabolite was extracted with 5 ml of n-heptane, centrifuged (1000 x g for 5 min) and the tubes placed into a bath of dry ice and isopropyl alcohol. The organic layer was poured into a vial containing 8 ml of scintillation fluid and counted. Incubation mixture as described above but lacking homogenates was used as background sample and contained less than 0.1% of total substrate added. Incubation times were selected so as to maintain a linear rate of substrate utilization (10-30%). MAO-B activity was reported as percent of control values.

Catecholamine Levels

Catecholamines were quantified by reverse phase HPLC coupled to electrochemical detection (EC) (Renner and Luine, 1984). Briefly, striatal DA was extracted by homogenizing in 0.022 M sodium acetate (pH 5.0) containing 1 mM EDTA, 0.045 mM 3,4-dihydroxybenzylamine (DHBA, internal standard) and 0.05 mg/ml ascorbic acid oxidase. The mixture was centrifuged and the supernatant was analyzed for DA content. The removed supernatant was replaced by an equal volume of PBS (10 mM phosphate saline buffer pH 7.4) for protein analysis (see below). Cerebellar catecholamines were extracted onto alumina and recovered into 0.1 M perchloric acid with DHBA as internal standard according to the methods of Anton and Sayre (1962) as modified and described in detail by Müller and Unsicker (1981). Using this extraction procedure, the recovery of noradrenaline (NE) averaged 64.8%.

Samples and external standards were placed in a sample processor (Waters model 710B WISP). The HPLC (Waters, Milford, MA, USA) conditions were as follows: samples and external standards were eluted at a flow rate of 1 ml/min (Waters pump model 510) from a C18 column (Waters, 25 x 0.46 cm with 5 µm packing) with a mobile phase consisting of 0.3 M sodium acetate buffer pH 3.8, 100 mg/L EDTA, 47 mg/L sodium octyl sulfate and 1% acetonitrile. The electrochemical detector (Mandel model L-C 4B, Guelph, Ont., Canada) was set at a potential of +0.740 V versus a Ag/AgCl reference electrode. The chromatograms were recorded and integrated (Waters data module model 740). Striatal DA and cerebellar NE concentrations (n=5) were reported in ng/mg protein after corrections were made by means of DHBA, used as an internal standard.

Isolation of Mitochondria for Oxygen Uptake Measurement

Brain mitochondria with intact respiratory function were prepared from CD1 mice using the Ficoll-gradient procedure (Clark and Nicklas, 1970). Briefly, cerebral hemispheres were rapidly removed and homogenized in 0.25 M sucrose, 10 mM Tris and 0.5 mM EDTA at pH 7.4 (no sodium) and centrifuged at 2 000 x g for 3 min. The supernatant was centrifuged for 8 min at 12 500 x g and the crude mitochondrial pellet was suspended in 3% Ficoll, 0.12 M mannitol, 0.03 M sucrose and 25 µM EDTA at pH 7.4 (no sodium). This suspension was carefully layered on top of 6% Ficoll, 0.24 M mannitol, 0.06 M sucrose and 50 µM EDTA at pH 7.4 (no sodium) and centrifuged at 11 500 x g for 30 min. The layer containing the active mitochondria was removed from the pellet and washed in 0.25 M sucrose, 10 mM Tris and 0.5 mM EDTA at pH 7.4 (no sodium).

O_2 consumption was measured in fresh mitochondrial preparations (0.3-0.5 mg protein) using a Clark oxygen electrode fitted in a 2 ml conical tube to minimize air diffusion and equipped with a stirring bar (Nicklas *et al.*, 1985). Mitochondria were incubated at ambient temperature (23-25°C) in a final volume of 1 ml containing 25 mM sucrose, 75 mM mannitol, 50 µM EDTA, 95 mM KCl, 5 mM potassium phosphate and 20 mM Tris HCl at pH 7.4. All respiratory substrates were dissolved in the above buffer and adjusted to pH 7.4 with 1 M Tris buffer. The resting state of O_2 uptake (state 4) was measured in ppm/min in the presence of 10 µl of 250 mM pyruvate and malate (complex I substrates). After a few minutes 10 µl of 25 mM ADP was added to allow the measurement of O_2 consumption (ppm/min) during ATP synthesis (state 4). The test substances were included as indicated in Fig. 4 and the slope was quantitated (O_2 ppm consumed/min) over a 3-4 min period. Ten µl of 2 M succinate (complex II substrate) was then added in an attempt to bypass the inhibition induced at complex I by the test substance. All additions were made using a Hamilton microsyringe. The pH of the final mixture was always maintained between 7.2 and 7.5 in the course of the measurement.

Protein Assay

The protein concentration was determined using a Pierce BCA Protein Assay Kit (Rockford, II, USA) except that protein extractions for cerebellar catecholamines were quantified according to a method described by Bradford (1976) with bovine serum albumin as the standard and absorbance measured at 562 and 595 nm, respectively.

Statistics

Statistical analysis was determined by the Wilcoxon test (n=5) followed by Dunn's test for pair-wise comparison. Values of $p<0.05$ and were utilized as the criteria for statistical significance.

RESULTS

Fig. 2 summarizes CuZn-SOD and Mn-SOD activities in the striatum and cerebellum of C57BL/6 mice following L-deprenyl administration. We observed a significant increase ($p<0.05$) in Mn-SOD and CuZn-SOD activities in the striatum and cerebellum of L-deprenyl- treated mice (Fig. 2). The extent of changes in CuZn-SOD activity seen in the striatum was more pronounced than that seen in the cerebellum. Striatal SOD activities remained elevated 20 days after a single i.p. administration of L-deprenyl to mice (Fig. 2). These alterations in CuZn-SOD and Mn-SOD activities were observed even though MAO-B activity had recovered by

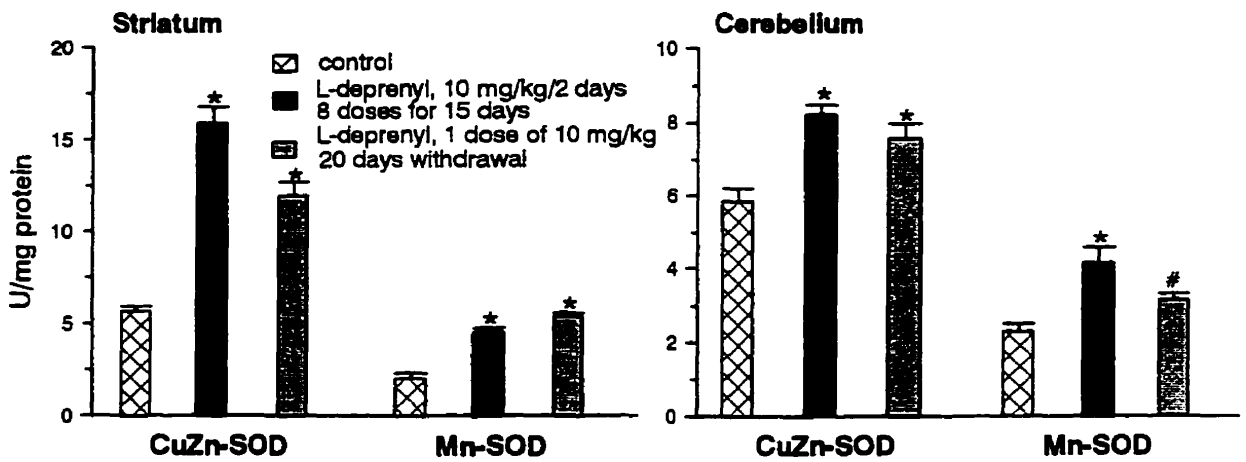


Fig. 2. SOD activity in the striatum and cerebellum of C57Bl/6 mice at 15 days (8 x 10 mg/kg at 2 days interval) and 20 days (1 x 10 mg/kg) post-treatment. Control mice received 0.9% saline only (vehicle). Enzymatic activity measurements and animal treatments are described in Materials and Methods. Asterisks and number signs indicate significant differences (Wilcoxon test, $p < 0.05$) as compared to controls and L-deprenyl at 15 days, respectively. Means ± SE values of five independent experiments are shown.

TABLE 1. MAO-B activity in whole brain homogenates after L-deprenyl treatment (10 mg/kg/2 days; i.p.) and up to 20 days post-treatment.

Number of doses		MAO-B activity % of control
1	2 hrs	16.6 ± 1.4
8	15 days	10.4 ± 0.5
1	20 days	49.2 ± 0.8 *

Analysis of enzymatic activity and animal treatments are described in Materials and Methods. Means ± SE values of three indepedent determinations are shown. * $p<0.05$ (Wilcoxon test) as compared to 2 hrs and 15 days post-treatment.

approximately 50% at this time point (Table 1). However, one dose of L-deprenyl normalized cerebellar Mn-SOD activity but not CuZn-SOD activity in mice after a 20 day L-deprenyl washout.

To determine whether increased SOD activities are associated with $O_2^{\cdot}$ formed as a consequence of enhanced catecholamine oxidation, we examined catecholamine levels in the striatum and cerebellum of L-deprenyl, D-deprenyl and MDL72974 treated mice (Fig. 3). L-Deprenyl and MDL72974 but not D-deprenyl induced a four-fold increase ($p<0.05$) in striatal DA levels. In contrast, NE remained unaltered in the cerebellum following L-deprenyl, D-deprenyl and MDL72974 treatments.

In an earlier report, we observed that MPTP administration (3 x 20 mg/kg/2 hr) led to a significant increase in striatal and cerebellar CuZn-SOD and Mn-SOD at 15 days post-treatment (Thiffault *et al.*, 1995). The MPTP metabolite, MPP^+, is known to inhibit NADH

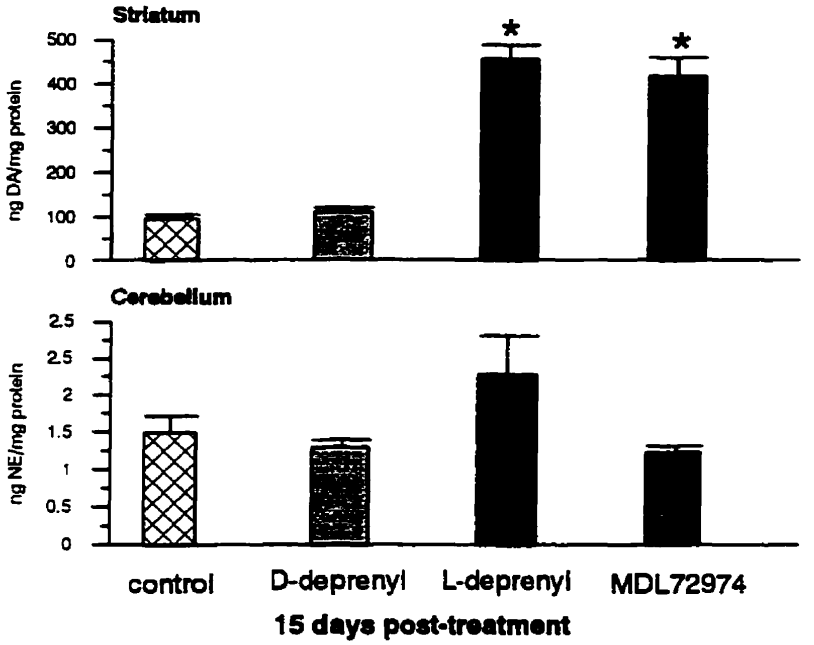


Fig. 3. Striatal and cerebellar catecholamine contents following D-deprenyl, L-deprenyl and MDL72974 administration in C57Bl/6 mice at 15 days post-treatment. HPLC analysis of catecholamines and animal treatments are described in Materials and Methods. Asterisks indicate significant differences (Wilcoxon test, $p < 0.05$) as compared to controls. Mean ± SE values of five independent experiments are shown.

dehydrogenase activity within the respiratory chain (Nicklas *et al.*, 1985). As a result, $O_2^{\cdot}$ formation is enhanced which in turn may be responsible for alterations in SOD activities observed following MPTP treatment (Rossetti *et al.*, 1988; Adams *et al.*, 1993; Thiffault *et al.*, 1995). These observations prompted us to investigate the effect of L-deprenyl on respiratory function in fresh mouse brain mitochondria. We made use of another selective MAO-B inhibitor, MDL72974 since, unlike L-deprenyl, it is not metabolized to amphetamine and methamphetamine (Dow *et al.*, 1994; Heinonen *et al.*, 1994). D-Deprenyl was also examined since it is approximately 150 times less potent than its isomer at inhibiting MAO-B (Magyar *et al.*, 1967) and therefore allowed us to assess the involvement of MAO-B inhibition in this

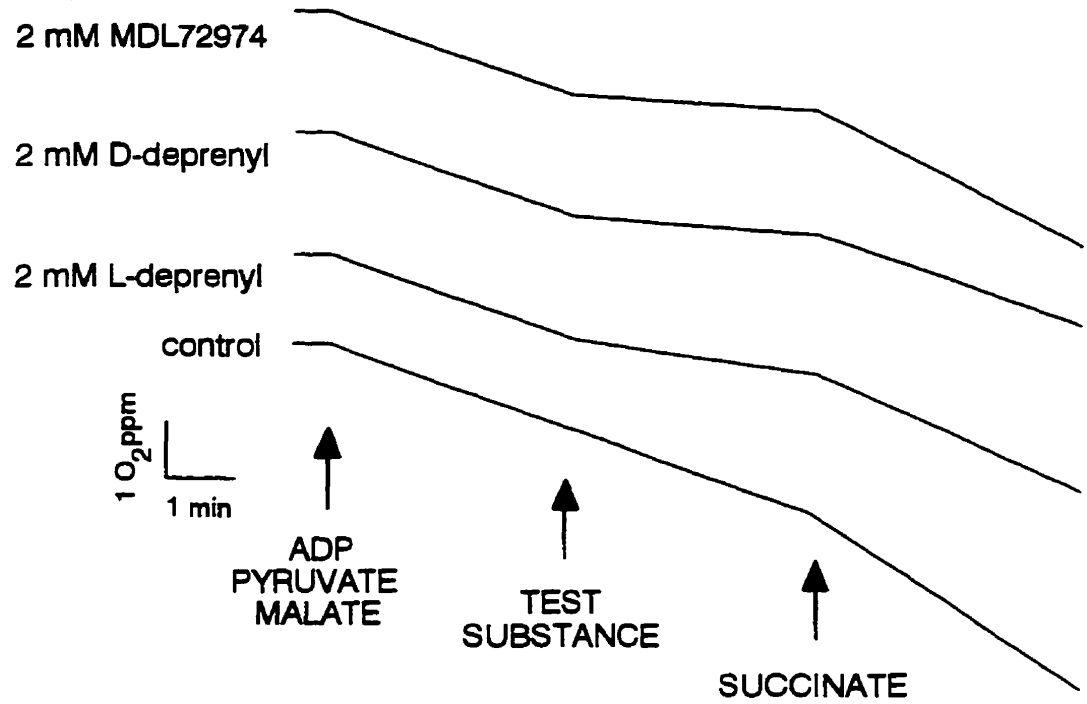


Fig. 4. Effects of L-deprenyl, D-deprenyl and MDL72974 on the rate of oxygen uptake in fresh mitochondrial preparations from CD1 mouse brain tissue as described in Materials and Methods. The curves are representative of individual experiments and were used to construct the inhibition curves of Figure 5. The arrows indicate the time at which additions were made.

paradigm. The chemical structures of L-deprenyl, D-deprenyl and MDL72974 are shown in Fig. 1.

Fig. 4 shows the rate of O_2 consumption (in ppm/min) observed in a typical experiment. ADP was added to pyruvate and malate to promote ATP synthesis (state 3) and O_2 utilization. The slope (O_2 in ppm/min) obtained from Fig. 4 is representative of mitochondrial respiratory activity. Fig. 5 illustrates the effect of increasing concentrations of L-deprenyl, D-deprenyl and MDL72974 on O_2 utilization (state 3) in fresh mitochondrial preparations. L-Deprenyl, D-deprenyl and MDL72974 induced a dose-dependent inhibition of O_2 uptake during ATP

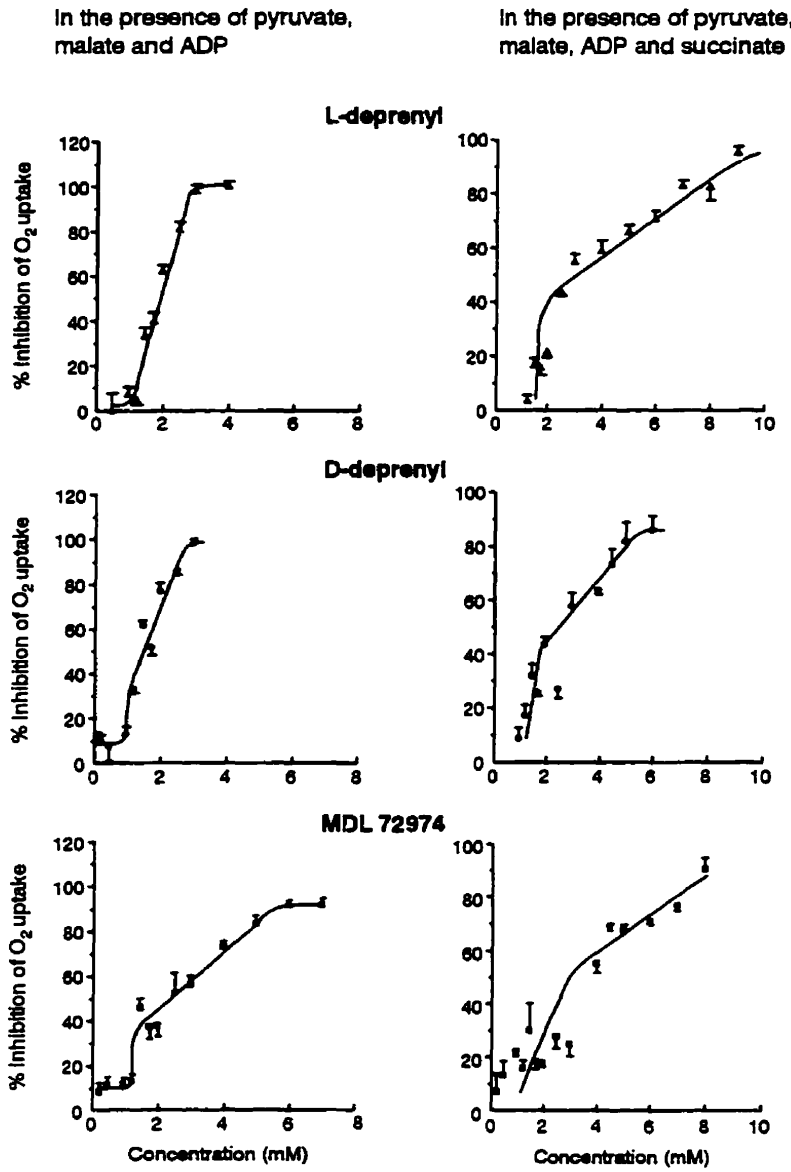


Fig. 5. Inhibition curves of the effects of L-deprenyl, D-deprenyl and MDL72974 on the rate of oxygen consumption in fresh mitochondrial preparations from CD1 mouse brain tissue. Measurements were made in presence of 2.5 mM pyruvate, 2.5 mM malate and 2 mM ADP followed by the addition of 20 mM succinate as described in Materials and Methods. Mean ± SE values of four independent experiments are shown.

synthesis in the presence of complex I substrates (pyruvate and malate, Fig. 5). IC_{50}s ranged from 1.9, 1.5 and 2.4 mM for L-deprenyl, D-deprenyl and MDL72974, respectively. O_2 utilization could not be fully restored upon the addition of succinate (complex II substrate, Fig. 5), suggesting that electrons transferred to O_2 are impaired at two sites.A dose-dependent inhibition occurred, albeit at higher doses for L-deprenyl and D-deprenyl, with an IC_{50} of 2.8 and 2.5 mM, respectively (Fig. 5). In contrast, the rate of O_2 uptake in presence of pyruvate, malate and ADP inhibited by MDL72974 remained unaffected by the addition of succinate (IC_{50}=2.6 mM), suggesting that the flow of electrons is blocked at only one site.

The effect of L-deprenyl, D-deprenyl and MDL72974 on respiratory function led us to investigate whether D-deprenyl and MDL72974 could, in turn, modulate SOD activity in the striatum and cerebellum of C57BL/6 mice. Administration of D-deprenyl and MDL72974, (10 mg/kg/2 days) resulted in a significant increase ($p<0.05$) in SOD activities in the striatum of mice at 15 days post-treatment (Fig. 6). MDL72974 was more potent than L-deprenyl at inducing changes in CuZn-SOD activity, whereas D-deprenyl was the least effective (Fig. 6). Increases in striatal Mn-SOD activity following D-deprenyl and MDL72974 administration to mice were very similar to those of L-deprenyl (Fig. 6). Cerebellar CuZn-SOD activity was increased in L-deprenyl- and MDL72974-treated mice, whereas a modest reduction ($p<0.05$) could be detected following D-deprenyl treatment. An alteration in Mn-SOD activity in the cerebellum of mice was observed only in the L-deprenyl treated group (Fig. 6).

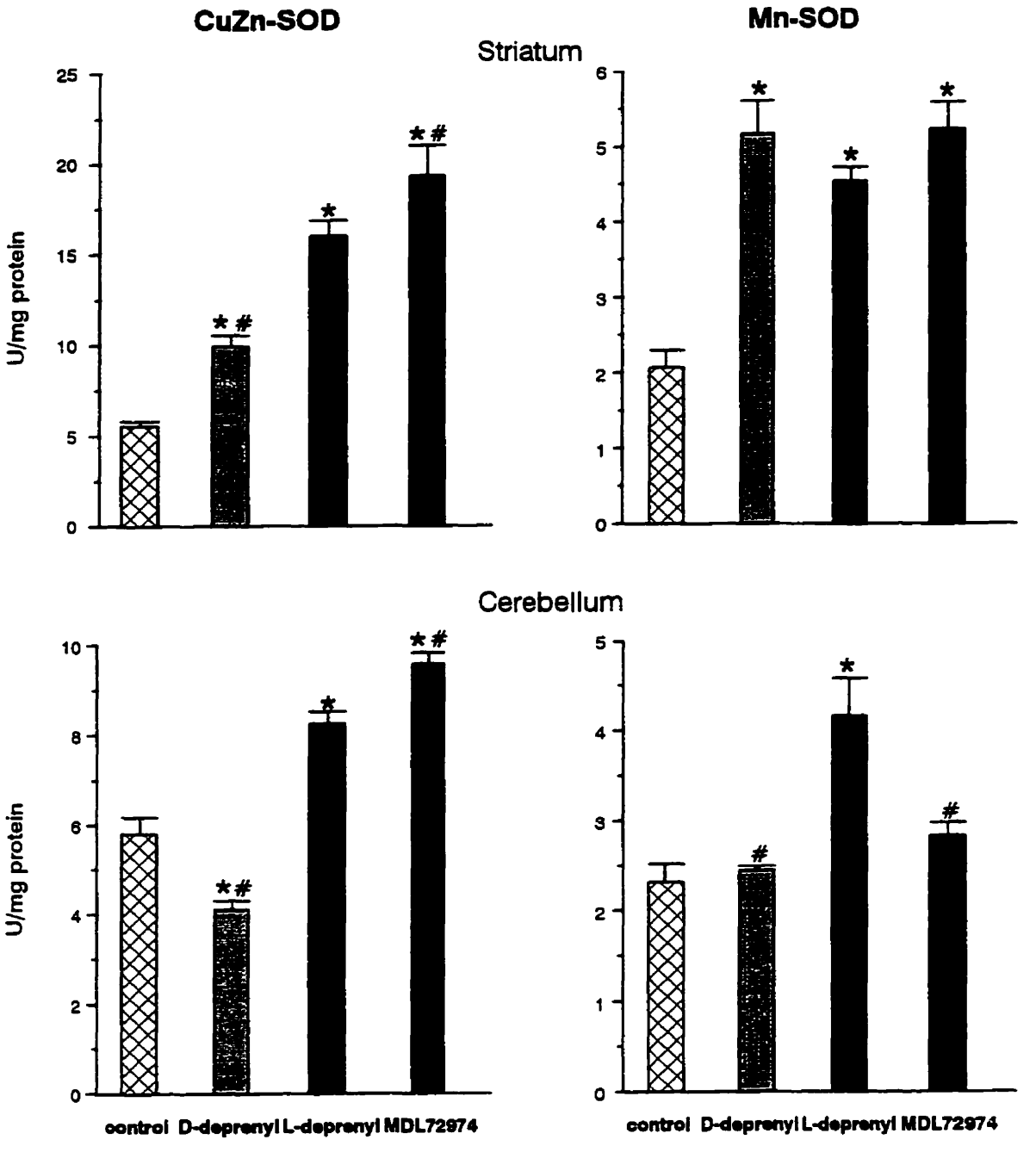


Fig. 6. Effects of D-deprenyl and MDL72974 on SOD activity in the striatum and cerebellum of C57Bl/6 mice at 15 days post-treatment. Control mice received 0.9% saline only (vehicle). Enzymatic activity measurements and animal treatments are described in Materials and Methods. Asterisks and number signs indicate significant differences (Wilcoxon test, $p<0.05$) as compared to controls and L-deprenyl, respectively. Mean ± SE values of five independent experiments are shown.

DISCUSSION

In the present study, L-deprenyl (10 mg/kg) caused a marked increase in striatal CuZn-SOD and Mn-SOD activities in the striatum of C57BL/6 mice. This observation is consistent with previous reports in which lower L-deprenyl concentrations were shown to be sufficient to induce SOD activity in the rat striatum (Knoll, 1987; Carrillo *et al.*, 1991; Clow *et al.*, 1991; Clow *et al.*, 1992). To our knowledge, L-deprenyl is not known to act as a direct modulator of SOD activity and we failed to observe any effect of this MAO-B inhibitor on purified SOD activity *in vitro* with concentration as high as 4 mM. Striatal CuZn-SOD and Mn-SOD activities remained elevated following L-deprenyl withdrawal even though MAO-B activity had recovered by approximately 50%. This observation suggests that the modulation of striatal SODs by L-deprenyl is not readily reversible and appears to be independent, at least in part, from the inhibition of MAO-B.

We have previously demonstrated that MPTP and L-deprenyl administration to mice induced similar changes in striatal, nigral and cerebellar CuZn-SOD and Mn-SOD activities (Thiffault *et al.*, 1995). Several studies have shown that SOD is up-regulated in animals exposed to $O_2^{\cdot}$ radical (Stevens and Autor, 1977; Frank, 1981; Sjostrom and Crapo, 1981; Ogawa *et al.*, 1994). Accordingly, the mechanism by which L-deprenyl modulates SOD activities within the nigrostriatal pathway and the cerebellum may represent an adaptive mechanism reacting to the endogenous formation of $O_2^{\cdot}$ radical.

Numerous potential sources of $O_2^{\cdot}$ exist in the cell, including the electron transport chain and the autoxidation of catecholamines (Boveris and Chance, 1973; Turrens and Boveris, 1980; Graham, 1984; Sohal and Brunk, 1992). In this respect, L-deprenyl is shown to up-regulate to various degree SOD activities in brain areas enriched with catecholamines (Carrillo *et al.*, 1992; Thiffault *et al.*, 1995). The present study, as well as others, have shown that L-deprenyl increases DA content in the striatum. This increase may be through a) the inhibition of DA uptake and metabolism (Oreland *et al.*, 1983; Knoll and Miklya, 1984; Zsilla *et al.*, 1986; Knoll, 1987); b) increased levels of β-phenylethylamine (an indirectly acting sympathomimetic) (Boulton *et al.*, 1990; Paterson *et al.*, 1990); and/or c) its amphetamine metabolites (Reynolds *et al.*, 1978; Karoum *et al.*, 1982). In addition, L-deprenyl is known to block the uptake of NE in rat brain synaptosomal preparations (Lai *et al.*, 1980; Bondiolotti *et al.*, 1995). However, we could not detect any alterations in NE levels in the cerebellum of L-deprenyl, D-deprenyl and MDL72974 treated mice. The concentration of L-deprenyl used in the present study appears therefore insufficient to inhibit NE uptake *in vivo*. In addition, DA levels remained unchanged in the striatum following D-deprenyl administration. Thus, free radicals generated as a result of the non-enzymatic oxidation of catecholamines may not be the key mechanism underlying the modulation of SOD activities observed in the present study. This observation is consistent with the effect of clorgyline, a MAO-A inhibitor, and L-dopa, a DA precursor, on DA levels and SOD activity in the rat striatum. Clorgyline has been shown to increase DA levels in the rat

striatum and DA released by rat striatal slices, but this MAO-A inhibitor failed to induce an increase in SOD activity in the rat striatum (Knoll, 1988; Zsilla *et al.*, 1986; Fang and Yu, 1994). Similarly, striatal SOD activity remained unaltered following L-dopa administration to rats (Clow *et al.*, 1992).

Alternatively, L-deprenyl could directly modulate free radical production by altering respiratory function within mitochondria, the major site of intracellular $O_2^{\cdot}$ formation (Boveris and Chance, 1973; Turrens and Boveris, 1980; Sohal and Brunk, 1992). For instance, rotenone and MPP^+ inhibit NADH dehydrogenase (complex I) in the respiratory chain to stimulate $O_2^{\cdot}$ production (Turrens and Boveris, 1980; Rossetti *et al.*, 1988). MPTP administration to mice resulted in an increase in striatal CuZn-SOD and Mn-SOD activities (Thiffault *et al.*, 1995). Interestingly, although in a different model, Mn-SOD activity is increased when procaryotes are exposed to classical respiratory chain inhibitors (Brown-Paterson *et al.*, 1995). Accordingly, we propose that alterations in respiratory function result in a leakage of oxygen-centered radicals which in turn leads to adaptive increases in CuZn-SOD and Mn-SOD activities. Taken together, these observations led us to investigate the effect of L-deprenyl on O_2 utilization in fresh mouse brain mitochondrial preparations.

We observed that L-deprenyl induced a marked reduction in O_2 consumption (state 3) during ATP synthesis which could be partially restored with added succinate (complex II substrate, Fig. 4). Analysis of dose-response curves also indicates that L-deprenyl is more potent at inhibiting O_2 utilization in the presence of complex I substrates than in the presence of complex II

substrates (Fig. 5). These observations suggest that the impairment of electron flow may occur at complex I (NADH dehydrogenase) and complex III or IV. We also evaluated the relationship between MAO-B inhibition and respiratory chain impairment *in vitro*. As mentioned earlier, D-deprenyl is about 150 times less potent than L-deprenyl at inhibiting MAO-B, whereas MDL72974 is 25 times more effective (Magyar *et al.*, 1987; Zreika *et al.*, 1989). D-Deprenyl resulted in a similar inhibitory profile as compared to L-deprenyl, suggesting that the impaired sites are similar and that these effects are not related to MAO-B inhibition. However, MDL72974 has equal potency at reducing O_2 consumption during ATP synthesis whether complex I or complex II substrates are included. This observation suggests that sites other than complex I and II are affected by MDL72974. Taken together, these results imply that structural similarities among these three compounds could be responsible for the effects seen on O_2 utilization and $O_2^{\cdot}$ generation (Fig. 1).

A recent report showed that administration of L-deprenyl to mice altered the redox state of ubiquinone, suggesting that the flow of electrons is impaired in the respiratory chain (Götz *et al.*, 1995). Götz and colleagues (1995) observed a decrease in ubiquinone levels, whereas ubiquinol (reduced ubiquinone) concentrations are increased in the mice striatum. Ubiquinol levels have been shown to be augmented as a result of impaired mitochondrial respiration. For instance, ubiquinol concentrations were demonstrated to increase in tubular kidney cells exposed to complex IV inhibitors and in disease states with defects in respiratory chain components

(Ernster and Dallner, 1995; Van de Water *et al.*, 1995). These results are also consistent with the hypothesis that L-deprenyl enhances $O_2^{\cdot}$ formation by altering the rate of electron transfer within the respiratory chain, leading to increases in SOD activities in the mouse striatum.

We observed that MDL72974 is more potent at inducing an increase in CuZn-SOD activity in the striatum than in the cerebellum. In an earlier study, Clow *et al* (1993) failed to observe any effect of MDL72974 on SOD in the striatum of rat using doses of 1.25 and 25 mg/kg. This may be explained by the fact that Carrillo and colleagues (1993) reported the bell shape nature of the effects of L-deprenyl on SOD activity, with higher doses being less effective than intermediate ones. Similar dose-response curves for MDL72974 could explain the positive results obtained in the present study.

Interestingly, L-deprenyl, D-deprenyl and MDL72974 treatments have a differential impact on striatal and cerebellar SOD activities in mice. The extent of alterations in SOD activities observed in the striatum were more pronounced than those obtained in the cerebellum, suggesting that the former is particularly vulnerable to these compounds. These observations are consistent with the effect of respiratory chain inhibitors on nigrostriatal dopaminergic functions as compared to those of other neuronal systems. For example, stereotaxic injection of rotenone into the medial forebrain bundle has been shown to induce a selective nigrostriatal lesion in rats (Heikkila *et al.*, 1985). The uptake mechanism, which depends on ATP production, of DA was also more affected by rotenone, antimycin and cyanide as compared to other neurotransmitters in mouse striatal synaptosome preparations and in cultured mesencephalic neurons (Marey-

Semper *et al.*, 1993). Interestingly, rotenone was more effective at inhibiting DA uptake in striatal versus nucleus accumbens synaptosomal preparations (Marey-Semper *et al.*, 1993). Thus, alterations in ATP synthesis as a result of chronic L-deprenyl administration could be detrimental to dopaminergic neurons. The effect of L-deprenyl on energy metabolism reported by us and others (Götz *et al.*, 1995) could be relevant to the observed increase in mortality rate reported in parkinsonian patients treated with a combination of L-deprenyl and L-dopa (Lees, 1995).

In summary, our results suggest that a deficiency in mitochondrial respiration induced by L-deprenyl increases SOD activities. However, it is not clear if L-deprenyl can accumulate within the nigrostriatal pathway to levels required to inhibit mitochondrial functions (Heinonen *et al.*, 1994; Mahmood *et al.*, 1994). It is known that L-deprenyl, at doses lower than that required to inhibit MAO-B, leads to an increase in striatal SOD activities in the rat (Knoll, 1988; Carrillo *et al.*, 1991; Clow *et al.*, 1991). In addition, administration of a high dose (100 mg/kg) of L-deprenyl has been shown to alter ubiquinone redox state in mouse striatum within 8 days post-treatment, suggesting an electron transfer flow deficiency within the respiratory chain (Götz *et al.*, 1995). Accordingly, successive administrations of L-deprenyl may impair the activities of respiratory chain complexes within dopaminergic neurons of the nigrostriatal pathway.

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CHAPTER 4

L-Deprenyl and MDL72974 Do Not Improve the Recovery of Dopaminergic Cells Following Systemic Administration of MPTP in Mouse

Preface to Chapter 4

It has been demonstrated that L-deprenyl administered to mice following MPTP treatment significantly improved the recovery of nigral dopaminergic neurons as measured by TH immunopositive cell counts. This observation led to the proposal that L-deprenyl exerted a "neurorescue" effect. However, two studies failed to show that L-deprenyl had a significant effect on the restoration of striatal levels of DA and DA metabolites in MPTP-lesioned mice. Furthermore, there is no evidence suggesting that L-deprenyl can in fact "rescue" degenerating dopaminergic neurons in early and untreated PD patients. Accordingly, in this chapter we re-evaluated the long-term impact of L-deprenyl on MPTP-induced dopaminergic cell loss in mice in order to clarify these issues.

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L-DEPRENYL AND MDL72974 DO NOT IMPROVE THE RECOVERY OF DOPAMINERGIC CELLS FOLLOWING SYSTEMIC ADMINISTRATION OF MPTP IN MOUSE.

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Key Words: Deprenyl, MDL72974, Neurorescue, Tyrosine hydroxylase, Monoamine oxidase, MPTP.

ABSTRACT

L-Deprenyl, a monoamine oxidase B (MAO-B) inhibitor, administered prior to MPTP protects dopaminergic neurons against degeneration in several animal species including mice. L-Deprenyl inhibits formation of MPP^+, the mediator of MPTP toxicity. In addition, L-deprenyl, administered 72 hrs following MPTP, improves the recovery of tyrosine hydroxylase (TH) immunopositive neurons in the substantia nigra (SN) of mice. This observation lead to the proposal that L-deprenyl exerts a "neurorescue" effect. However, clinical trials failed to demonstrate that L-deprenyl can effectively "rescue" degenerating dopaminergic neurons in early untreated Parkinson's disease (PD) patients. These observations prompted us to reevaluate the long term impact of L-deprenyl on MPTP-induced dopaminergic cell loss in mice. In addition, we made use of another MAO-B inhibitor, MDL72974, to assess MAO-B participation in this paradigm. Our results suggest that L-deprenyl does not improve the recovery of TH immunopositive neurons in MPTP-treated mice. An apparent reduction in TH^+ neurons is observed in the SN of MDL72974- and L-deprenyl/MPTP-treated mice at 30 days post-treatment. The possible implication of these findings in relation to the use of MAO-B inhibitors in PD is discussed.

INTRODUCTION

The discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces an irreversible Parkinson's disease (PD)-like syndrome in several animal species including primates and mice provided an animal model of PD [22,38]. The mechanisms by which systemic administration of MPTP results in the degeneration of the nigrostriatal pathway have been described in detail elsewhere [1,12,29,47]. Briefly, MPTP is metabolized by monoamine oxidase-B (MAO; E.C. 1.4.3.4) to form 1-methyl-4-phenylpyridinium (MPP^+) [6,8,18,24]. MPP^+ toxicity involves the inhibition of NADH dehydrogenase (EC 1.6.5.3), a component of the mitochondrial respiratory chain [25,30,32], which results in an energy crisis. Administration of L-deprenyl (Selegiline), a MAO-B inhibitor, prior to MPTP has been shown to block MPP^+ formation and thus protect neurons against the deleterious effects of MPTP in mice [16].

In addition, it has been shown that L-deprenyl administration to mice following MPTP treatment significantly improves the recovery of dopaminergic neurons of the substantia nigra (SN) [40]. This observation lead to the proposal that L-deprenyl exerts a "neurorescue" effect. The mechanism by which L-deprenyl exerts a trophic-like action is unclear and appears to be associated with astroglial hypertrophy [4]. However, one report failed to observe the restoration of striatal dopamine levels in L-deprenyl-treated mice after lesioning the nigrostriatal pathway with MPTP [49].

Clinical trials have also failed to demonstrate that L-deprenyl "rescues" degenerating dopaminergic neurons in early untreated PD patients [23,26,43,44,45,48]. However, the use of L-deprenyl in PD has been the subject of controversy. For instance, the DATATOP multicenter study reported that L-deprenyl improves motor disabilities in *de novo* parkinsonians [45,48]. The mechanism by which L-deprenyl alleviates parkinsonian symptoms may be associated with MAO-B inhibition, dopamine reuptake blockade [11] and possibly an amphetamine-like action [19,35]. MAO-B inhibition results in a reduction of dopamine catabolism [3,27] and an increase in the endogenous levels of ß-phenylethylamine (ß-PEA), an indirectly acting sympathomimetic [5,17]. In contrast, the Parkinson's Disease Research Group of the United Kingdom reported an increase in mortality rate in L-deprenyl-treated parkinsonians [23]. The mechanism by which L-deprenyl treatment resulted in an increase in death rate is unclear at this time.

Taken together, these observations lead us to reevaluate the long term impact of L-deprenyl on MPTP-induced dopaminergic cell loss in C57Bl/6 mice [40]. We also used another selective MAO-B inhibitor, MDL72974 (mofegiline hydrochloride), in order to assess the role of MAO-B inhibition in neurorescue.

MATERIALS AND METHODS

Materials

Three-month-old male (25-30 g) C57BL/6 mice (Charles River, St-Constant, Qué, Canada) with access to food and water ad libitum were maintained under a 12-h light/dark cycle. Animal care was according to protocols and guidelines approved by McGill University and the Canadian Council for Animal Care. R(-)deprenyl HCl was obtained from RBI (Natick, MA, USA); MDL72974 was a generous gift from Marion Merrell Dow Research (Cincinnati, OH, USA). HPLC grade sodium acetate trihydrate and acetonitrile were from Fisher (Fair Lawn, NJ, USA); ascorbic acid oxidase was obtained from Boehringer Mannheim (Mannheim, Germany). Homovanillic acid, dopamine HCl, 3,4-dihydroxyphenylacetic acid and 3,4,-dihydroxybenzylamine were purchased from Sigma (St. Louis, MO, USA). Octyl sodium sulfate, HPLC grade, was from KODAK (Rochester, NY, USA). Rabbit polyclonal antisera to tyrosine hydroxylase was purchased from Eugene Tech (Ridgefield Park, NJ, USA). All reagents were of the highest purity commercially available.

Animal Treatments

A total of 42 C57Bl/6 mice were administered MPTP at 30 mg/kg/day (i.p.) for 5 consecutive days. Day 1 represents 24 hrs following the last of MPTP injections and indicates the time at which the recovery of catecholamine levels and TH^+ immunostaining begins. Six animals were sacrificed on each of day 1, day 7 or day 30 (or 35 days from the beginning of the experiment. Of the remaining 24 MPTP-treated mice, 4 groups of 6 animals were treated subsequently (day

3 or 72 hrs following the of last MPTP injections) with either 0.025 mg/kg of L-deprenyl (M-Dep1), 0.25 mg/kg of L-deprenyl (M-Dep 2), 0.025 mg/kg of MDL72974 (M-MDL 1) or 0.25 mg/kg of MDL72974 (M-MDL 2) every 2 days for 28 days. Mice were then sacrificed by cervical dislocation at 30 days post-MPTP-treatment (or 28 days from the start of L-deprenyl or MDL72974 treatment).

Another group of 12 mice did not receive any MPTP. These mice were treated with either 0.25 mg/kg L-deprenyl (Dep 2) or MDL72974 (MDL 2) as above and sacrificed 28 days later. The total number of L-deprenyl or MDL72974 doses received was 14. Tissues were collected 24 hrs following the last L-deprenyl or MDL72974 injection. Finally, another 6 mice were given vehicle (0.9% saline) and sacrificed 30 days later. Brains were rapidly removed and sectioned between the SN and striatum. The rostral portion was cut in 1 mm coronal slices on ice; the striatum was carefully excised and frozen at -80°C for dopamine (DA), homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) determinations as indicated below. The caudal portion was immersed in 2-methylpentane (Fisher, Fair Lawn, NJ, USA) on dry ice at -40°C for 15 sec, and stored at -80°C for cryostat sectioning for tyrosine hydroxylase (TH) immunocytochemistry as described below.

Catecholamine Determination by HPLC

Catecholamines were quantified by reverse phase HPLC coupled to electrochemical detection (EC) [34]. Briefly, striatal DA and its metabolites were extracted by homogenizing in 0.022 M sodium acetate (pH 5.0) containing 1 mM EDTA, 0.045 mM 3,4,-dihydroxybenzylamine

(DHBA, internal standard) and 0.05 mg/mL ascorbic acid oxidase. The mixture was centrifuged and the supernatant was analyzed for DA, HVA and DOPAC contents. The removed supernatant was replaced by an equal volume of PBS (10 mM phosphate saline buffer pH 7.4) for protein analysis (see below). Samples and external standards were placed in a sample processor (Waters model 710B WISP). The HPLC (Waters, Milford, MA, USA) conditions were as follow: samples and external standards were eluted at a flow rate of 1 mL/min (Waters pump model 510) from a C18 column (Waters, 25x0.46 cm) with a mobile phase consisting of 0.3 M sodium acetate buffer pH 3.8, 100 mg/L EDTA, 47 mg/L sodium octyl sulfate and 1% acetonitrile. The electrochemical detector (Mandel model L-C 4B, Guelph, Ont, Canada) was set at a potential of +0.740 V versus a Ag/AgCl reference electrode. The chromatograms were recorded and integrated (Waters data module model 740). DA, HVA and DOPAC concentrations (n=6) were reported in ng/mg protein after corrections were made by means of 3,4,-dihydroxybenzylamine, used as an internal standard.

Tyrosine Hydroxylase Immunocytochemistry and Cell Surface Area Determinations

Sections (15 µm thick) of the SN were prepared on a Bright cryostat (Huntingdon, England), air dried overnight and stored at -80°C. We obtained between 45 and 55 sections per SN per animal. Every fifth section was processed for TH immunocytochemistry as described by Pasinetti et al. (1989) [28]. Tissue sections were thawed at room temperature, washed in PBS, fixed in 4% paraformaldehyde and briefly treated with 3% H_2O_2 in PBS. Non-specific staining was blocked by exposure to 1% goat antisera for 30 min. Sections were incubated in a primary TH rabbit antibody (1:5000 dilution; Eugene Tech, Ridgefield Park, NJ, USA) for 18 hrs at

4°C and then rinsed in PBS. Biotinylated secondary antibody (Vectastain ABC kit, Burlingame, CA, USA) and avidin complexed to biotinylated-horseradish peroxidase were subsequently applied and visualized by addition of H_2O_2 and 3,3'-diaminobenzidine (Sigma, St-Louis, MO, USA). Sections were rinsed, dehydrated by stepwise exposure to an increasing series of ethanol concentrations, and protected by coverbond-linked coverslips. TH-positive neurons of the SN were counted independently on both sides by means of a 25X objective on a Leitz microscope (Wetzlar, Germany) for each animal (n=6). Data are presented as an average of cell densities per nigral section since the number of immunostained sections varied from 8 to 11 per animal.

The average of TH-immunopositive cell surfaces (μm^2) was calculated by means of an image analyser system (MCID model M4, Imaging Research Inc., St. Catherine, Ont, Canada) coupled to a Leitz microscope (Wetzlar, Germany) equipped with a 40X objective. Each bar (n=6) represents an average of eight sections (10 cells/section) per treatment condition.

Protein Assay

Protein concentration was determined by a Pierce BCA Protein Assay Kit (Rockford, Il, USA) with bovine serum albumin as the standard and absorbance measured at 562 nm.

Statistics

Statistical analysis was determined non-parametrically by Wilcoxon followed by Dunn's for pair-wise comparison and test, and a value of $p<0.05$ was utilized as the criteria for statistical significance. Each bar represents a total of 6 independent experiments.

RESULTS

Figures 1 and 2 summarize the levels of DA (A) DOPAC (B) and HVA (C) in the striatum of C57Bl/6 mice. At this dosage regimen, MPTP treatment lead to modest alterations in striatal catecholamines levels (Fig. 1). A significant ($p<0.05$) reduction in the concentration of DA to 65% of control levels was observed following MPTP, while HVA and DOPAC contents remained unaltered up to 7 days post-treatment (Fig. 1). In contrast, DA, HVA and DOPAC levels were increased following MPTP administration at 30 days. L-Deprenyl when administered alone or 72 hrs following the last MPTP injections induced an increase in DA and HVA as compared to levels in controls and in MPTP-treated mice (Fig 2A and 2C). MDL72974 was not as potent as L-deprenyl at inducing alterations in DA levels. However, a 50% reduction in striatal DOPAC concentrations was seen with MDL72974 whether this coumpound was given alone or following MPTP administration at both dosage regimens (Fig. 2B). An increase in HVA content was seen only when MDL72974 was administered after MPTP as compared to control and MPTP-treated animals (Fig. 2C).

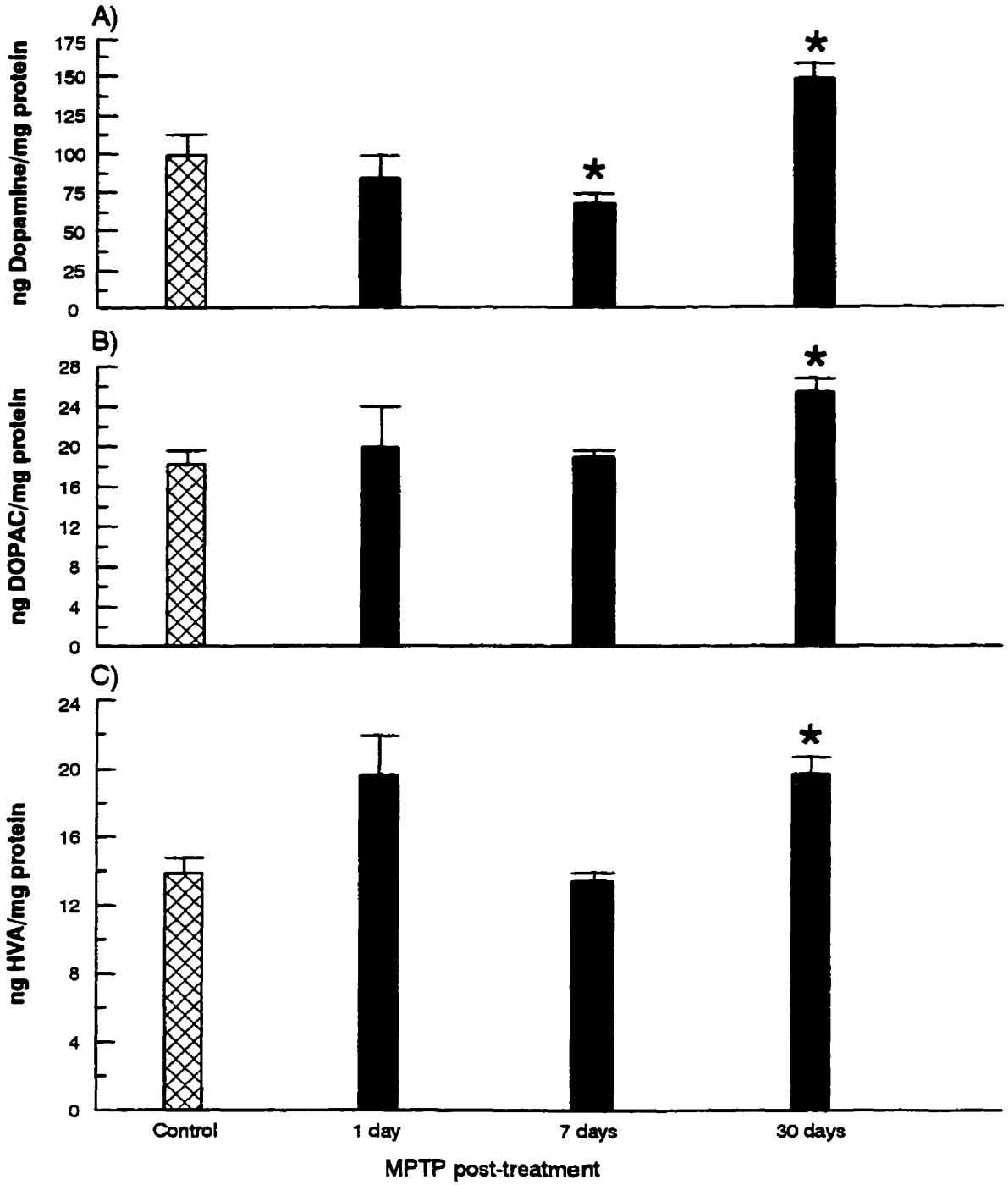

Fig. 1. Striatal catecholamine contents following MPTP administration in C57Bl/6 mice. HPLC analysis of catecholamines and animal treatments are described in Materials and Methods. Asterisks indicate significant differences ($p<0.05$) as compared to controls. Mean ± SE values of six independent experiments are shown.

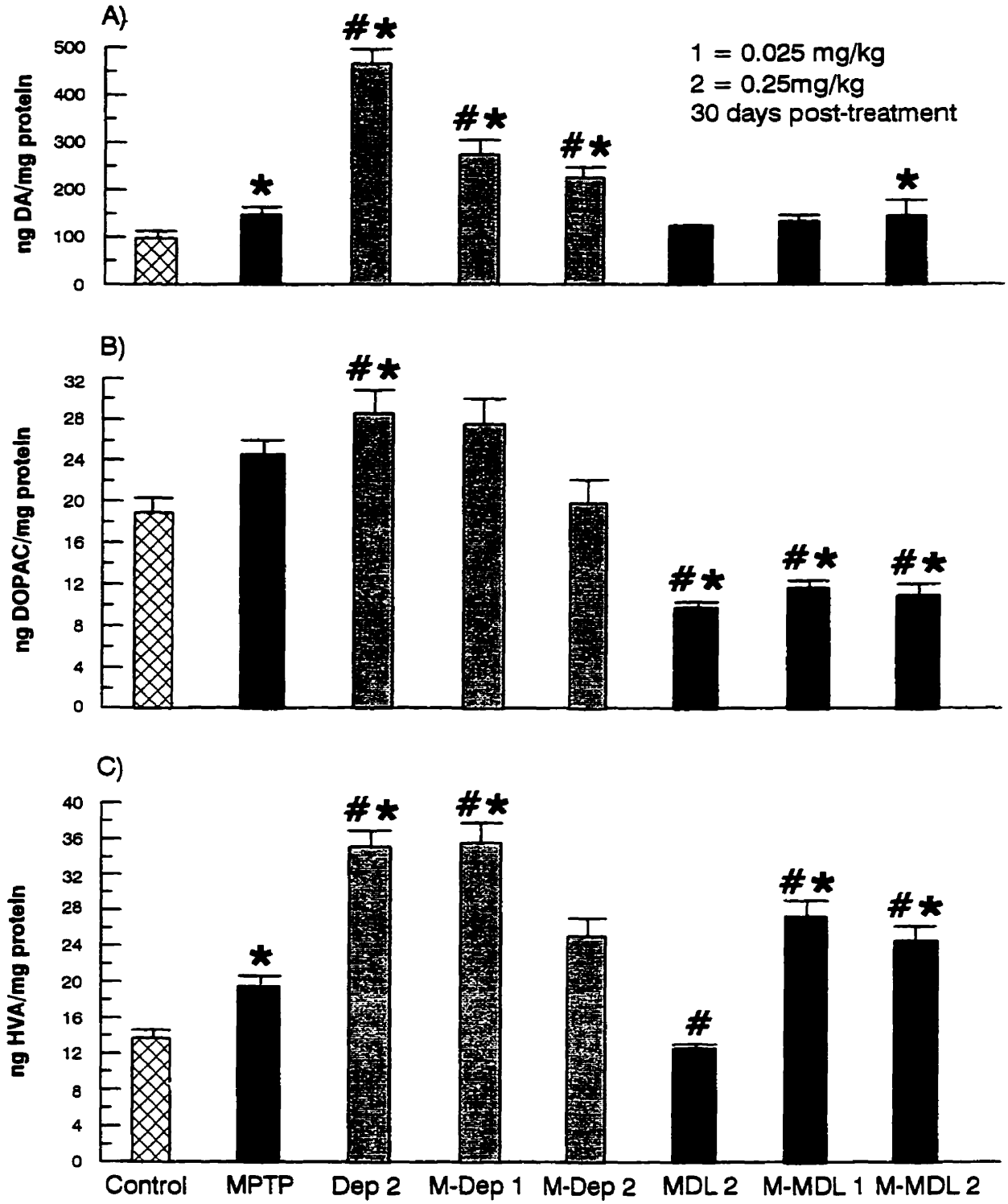

Fig. 2. Effect of MPTP (M), L-deprenyl (dep), MDL72974 (MDL), MPTP/L-deprenyl (M-dep) and MPTP/MDL72974 (M-MDL) on catecholamine contents in the striatum of C57Bl/6 mice at 30 days post-treatment. HPLC conditions and animal treatments are described in Materials and Methods. Asterisks and number signs indicate significant differences ($p<0.05$) as compared to controls and MPTP, respectively. Mean ± SE values of six independent experiments are shown.

Fig. 3 and 4 illustrate the effect of L-deprenyl and MDL72974 on TH immunostaining in the SN following MPTP treatment. The relative density of TH^+ cells was similar on both sides of the SN in the control and treated groups. TH^+ cell immunostaining returned to control levels at 30 days post-treatment following intraperitoneal injection of MPTP (5 x 30 mg/kg/day, Fig. 3 and 4B). MDL72974 induced an apparent reduction in TH^+ neurons as compared to control levels, whereas no alteration in cell density was observed when this compound was given in combination with MPTP (Fig. 3 and 4 E,F). In contrast, L-deprenyl alone had no effect on TH^+ cells density as compared to control groups (Fig. 3 and 4C). However, a modest reduction was seen when L-deprenyl was administered at 0.25 mg/kg/2 days in MPTP-treated mice (Fig. 3 and 4D).

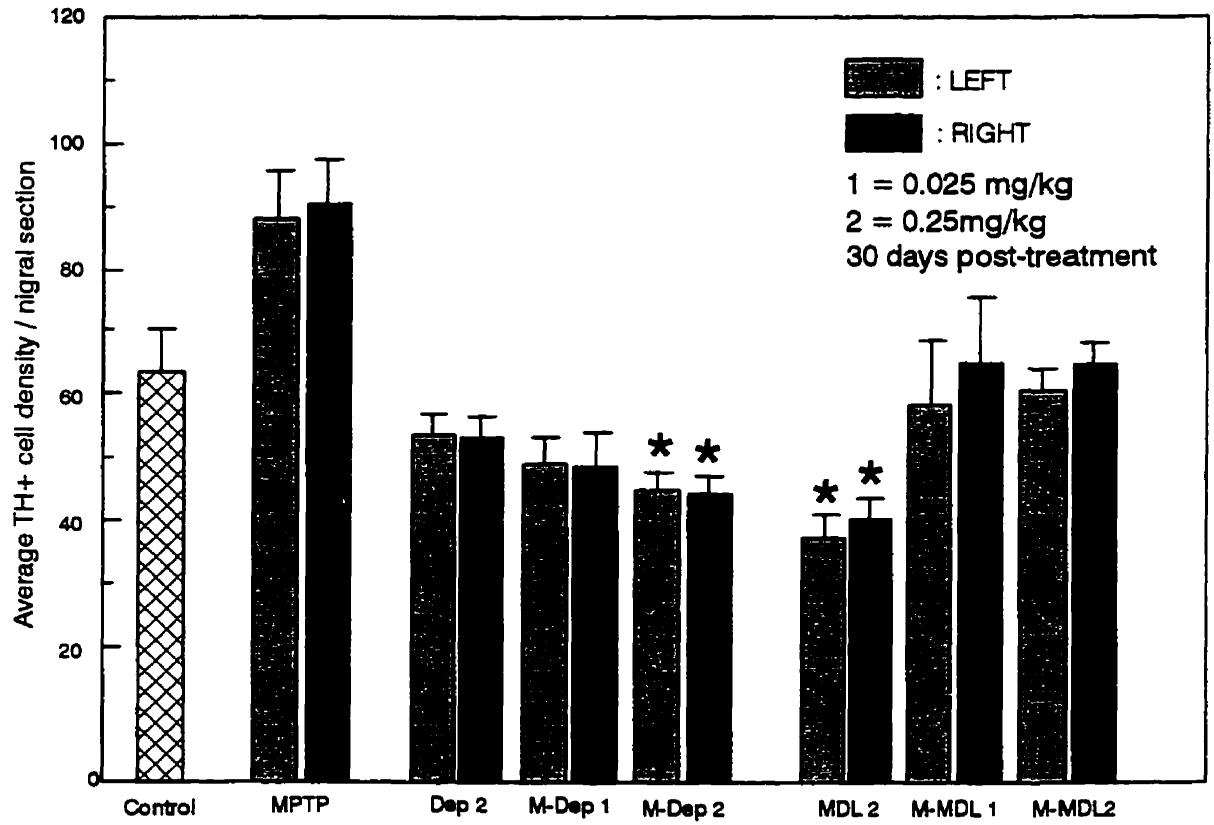

Fig. 3. Tyrosine hydroxylase immunopositive cell densities in the SN following MPTP (M), L-deprenyl (dep), MDL72974 (MDL), MPTP/L-deprenyl (M-dep) and MPTP/MDL72974 (M-MDL) treatments in the striatum of C57Bl/6 mice at 30 days post-treatments. Immunohistochemistry techniques and animals treatments are described in Materials and Methods. Asterisks indicate significant differences ($p<0.05$) as compared to controls. Mean ± SE values of six independent experiments are shown.

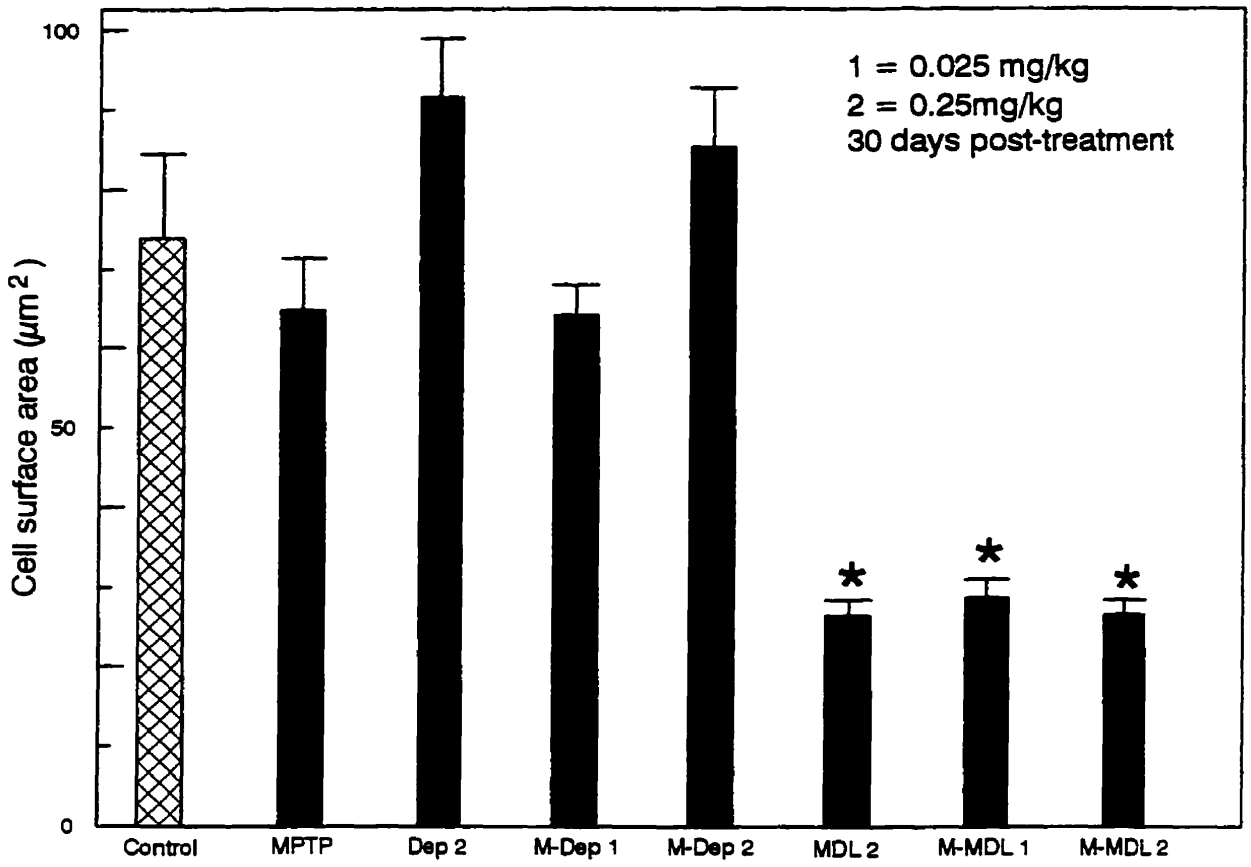

Fig. 5. Average of tyrosine hydroxylase immunopositive cell surfaces (μm^2) performed by the image analysis system after MPTP (M), L-deprenyl (dep), MDL72974 (MDL), MPTP/L-deprenyl (M-dep) and MPTP/MDL72974 (M-MDL) treatments in the SN of C57Bl/6 mice at 30 days post-treatment. The surface area calculations and animal treatments are described in Materials and Methods. Asterisks indicate significant differences ($p<0.05$) as compared to controls. Mean ± SE values of six independent experiments are shown.

To determine whether the relative density of TH^+ cell immunolabeling was not due to changes in cellular morphology, we calculated the average cell surface area by means of a computerized image analysis system (Fig. 5). A significant reduction (60%, $p<0.05$) in the mean cell area was observed in MDL72974- and MPTP/MDL72974-treated mice.

DISCUSSION

The most significant finding presented in this report is that a recovery of TH immunopositive cell density is observed in the SN at 30 days following the administration of 5 x 30 mg MPTP/kg/day. This dosage regimen was selected to replicate the experimental protocol described previously [40]. Modest alterations in DA and its metabolites are also found in the striatum of MPTP-treated mice (Fig. 1). Our results suggest that this MPTP dose regimen has a limited impact on the dopaminergic nigrostriatal pathway. This observation is consistent with earlier reports demonstrating that mice are resistant to low concentrations of MPTP [14,15,38]. A peak concentration of MPP^+ is observed at 3 hrs following a single administration of 40 mg/kg of MPTP, with a half-life of approximately 2 hrs [15]. A correlation between MPP^+ content and the degree of DA loss has been reported in mice striatum [14]. Accordingly, to be effective, MPTP must be administered repetitively at short time intervals to allow MPP^+ to accumulate at concentrations required to inhibit NADH dehydrogenase within mitochondria [31,38].

Tatton and Greenwood reported that MPTP (5 x 30 mg/kg/day) induced a 40-50% reduction in TH^+ neurons in the SN of C57Bl/6 mice at 20 days post-treatment [40]. Our results suggest that the loss of TH^+ immunostaining is transient since the density of TH^+ neurons recovered to near control levels at 30 days following MPTP administration. Accordingly, the loss of TH^+ neurons does not seem to be related to dopaminergic cell death in the SN at 20 days post-treatment [40]. It has been demonstrated that neurons are in the reinnervation phase in the

striatum at 20 days post-MPTP-treatment [41]. This observation is supported by earlier reports which demonstrated that striatal DA content can recover, at least partially, by 2 to 3 months in MPTP-treated mice [36,49].

In addition, we observed a significant increase in levels of DA and its metabolites at 30 days post-treatment in MPTP-treated mice, which suggests a rebound in the activity of DA synthetic enzymes. This finding is consistent with the effect of reserpine on dopaminergic system. Reserpine, which depletes DA strorage within terminal vesicles, also decreases TH^+ immunostaining [33]. In response to this insult, neurons not only replenish the empty vesicles but also induce *de novo* synthesis of TH. As a result, TH synthesis is enhanced for several days in dopaminergic neurons following reserpine treatment.

Taken together, our results do not support the notion that L-deprenyl promotes the recovery of TH^+ neurons following MPTP administration [40]. In fact, we observed that TH^+ levels return to control values in MPTP-, MPTP/L-deprenyl (0.025 mg/kg)- and MPTP/MDL72974 (0.025 and 0.25 mg/kg)-treated animals. Surprisingly, an apparent reduction in the number of TH^+ immunolabeled cells is seen following MPTP/L-deprenyl (0.25 mg/kg) treatment without concomitant reduction in striatal DA or its metabolites or changes in the morphological appearance of nigral neurons (Fig. 2 ,3, 4 and 5). Thus, L-deprenyl (0.25 mg/kg), when given after MPTP, seems to interfere with the recovery of TH protein levels in neurons of MPTP-treated animals.

A reduction in the number of TH^+ neurons without a concomitant reduction in DA levels is also observed in the SN of the MDL72974 (0.25 mg/kg)-treated group . This MAO-B inhibitor causes a 60% reduction in the average TH^+ cell area when given alone or following MPTP administration in mice. These observations suggest that the MAO-B inhibitor, MDL72974, may be detrimental to dopaminergic neurons through an as yet undetermined mechanism.

It is well known that MPTP, L-deprenyl and MDL72974 are inhibitors of flavin-containing MAO-B [13,47,50]. Whether the inhibition of MAO-B or other flavoproteins are responsible for the down-regulation of TH levels is unclear at this time. Interestingly, MPTP, L-deprenyl and MDL72974 induce an increase in superoxide dismutase activity in the striatum of rat and mice [7,9,20,46 and in preparation]. Several lines of evidence have demonstrated that superoxide dismutase is upregulated in cells exposed to excessive levels of superoxide radicals [10,37,39]. Thus, the possibility that L-deprenyl induces a low oxidative stress level within the nigrostriatal pathway may be masked by the observation that this compound stimulates dopaminergic transmission [21]. This is consistent with the observation that L-deprenyl increases the levels of DA and its metabolites in mice striatum.

MDL72974 caused a significant reduction in DOPAC, but not DA or HVA, levels at 30 days post-treatment. DA appears to be preferentially metabolized by MAO-A *in vivo* in rat and mice [2]. Accordingly, our results suggest that multiple low doses of MDL72974 inhibit MAO-B activity as well as some MAO-A activity. In this respect, MDL72974 inhibits MAO-A more

effectively than L-deprenyl at the dosage of 0.25 mg/kg [42]. Changes in striatal DA, DOPAC, and HVA contents in L-deprenyl-treated mice are consistent with the notion that L-deprenyl blocks DA uptake, whereas MDL72974 is devoid of such an effect [11].

In conclusion, L-deprenyl and MDL72974 do not improve TH^+ cell recovery in the SN of MPTP-treated mice. This observation is consistent with an earlier report demonstrating that L-deprenyl fails to restore DA levels in MPTP-lesioned mice [49]. Furthermore, MPTP/L-deprenyl (0.25 mg/kg) and MDL72974 (0.25 mg/kg) appears to interfere with TH protein levels in neurons *in vivo*. L-Deprenyl and MDL72974 have been shown to induce superoxide dismutase activity [46 and in preparation] in a manner that is consistent with an oxidative stress-inducing agents. Taken together, these results suggest that the chronic use of MAO-B inhibitors in PD should be carefully reconsidered.

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CHAPTER 5

Effect of the MAO-B Inhibitor, MDL72974, on Superoxide Dismutase Activity and Lipid Peroxidation Levels in the Mouse Brain

Preface to Chapter 5

In chapter 2 we observed that alterations in antioxidant defense systems do not necessarily lead to an increase in lipid peroxidation. The effect of MPP^+, L-deprenyl and MDL72974 on the rate of mitochondrial O_2 consumption (chapter 3) suggested that alterations in antioxidant enzyme activity may be an adaptive response caused by a leakage of oxygen-centered radicals resulting from dysfunctions of the mitochondrial respiratory chain. L-Deprenyl was more effective than MDL72974 in inhibiting the rate of O_2 uptake in the brain of mice with intact mitochondria. Thus, in chapter 4 L-deprenyl was expected to be more potent than MDL72974 at inducing alterations in TH-immunopositive neurons and average cell surface area. In this chapter, we therefore turn our attention to the effect of MDL72974 on striatal and cerebellar lipid peroxidation levels. This study may reveal that MDL72974 is detrimental to dopaminergic neurons through an as yet undetermined mechanism.

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EFFECT OF THE MAO-B INHIBITOR, MDL72974, ON SUPEROXIDE DISMUTASE ACTIVITY AND LIPID PEROXIDATION LEVELS IN THE MOUSE BRAIN.

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Key Words: Lipid Peroxidation, Superoxide Dismutase, MDL72974, Monoamine Oxidase Inhibitor, Mofegiline, Oxidative stress,

ABSTRACT

MDL72974 is a member of a series of MAO-B inhibitors to be used as potential therapeutic agents in the treatment of Parkinson's and Alzheimer's diseases. However, we have recently observed a reduction in the density of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra of mice treated with MDL72974. As oxidative stress is known to play a significant role in the nigrostriatal pathway, analysis of the relationship between TH^+ cell losses induced by MDL72974 and free radical production was investigated in the present study. Results demonstrate a significant increase in superoxide dismutase (SOD) activity, a key antioxidant, in the striatum and cerebellum of MDL72974-treated mice, presumably in response to free radical production. An increase in lipid peroxidation levels was also observed in the striatum of these animals in a manner which is consistent with oxidative stress-inducing agents. We therefore suggest that MDL72974 may be detrimental to dopaminergic neurons of the nigrostriatal pathway via free radical-mediated reactions.

INTRODUCTION

Monoamines are metabolized by two distinct monoamine oxidase (MAO) subtypes, A and B, based on substrate and inhibitor specificities [5]. For example, serotonin is preferentially deaminated by the clorgyline-sensitive MAO-A isoform. In contrast, ß-phenylethylamine is metabolized by MAO-B and blocked by L-deprenyl. In addition, significant inter-species differences exist. The case of dopamine is particularly interesting as it is preferentially oxidized by MAO-A in rodents [14,25], whereas MAO-B is the predominant form involved in its metabolism in primates [26]. Since MAO-B inhibitors can alter dopamine metabolism, they have gained popularity as therapeutic agents in the treatment of Parkinson's disease (PD) [4,30]. MDL72974 (mofegiline) is a member of an extensive series of selective and irreversible MAO-B inhibitors. MDL72974, in combination with the dopamine precursor, L-dopa, demonstrated antiparkinsonian potential in a four weeks open-study involving thirty-two patients [21].

In contrast, however, evidence obtained in C57BL/6 mice suggested that MDL72974 markedly altered tyrosine hydroxylase (TH^+) immunostaining following 30 days of treatment [Thiffault *et al.*, unpublished observations]. Since the nigrostriatal pathway is particularly vulnerable to free radical-mediated injuries [1,23,31], we investigated here if MDL72974 could induce an oxidative stress which may be responsible for TH^+ cell losses.

Accordingly, lipid peroxidation was measured following a chronic MDL72974 treatment. In addition, superoxide dismutase (SOD) activity was assessed since this enzyme has been shown to be up-regulated in several models of free radical-induced toxicity (hyperoxia, paraquat, 6-hydroxydopamine) [12,15,23,28,29]. SOD is an enzyme which catalyzes the dismutation of superoxide radical ($O_2^{\cdot}$) to hydrogen peroxide and O_2 [16,19]. Two major forms of SOD exist, mitochondrial or Mn-SOD and cytosolic or CuZn-SOD types [33].

MATERIALS AND METHODS

Materials

Three-month-old male (25-30 g) C57BL/6 mice (Charles River, St-Constant, Qué, Canada) with access to food and water ad libitum were maintained under a 12-h light/dark cycle. Animal care was according to protocols and guidelines approved by McGill University and the Canadian Council for Animal Care. MDL72974 was a generous gift from Marion Merrell Dow Research (Cincinnati, OH, USA). All reagents were of the highest purity available commercially.

Animal Treatment

Mice were treated with MDL72974 at a dosage of 10 mg/kg (i.p.) every 48 hrs. A second group received vehicle (0.9% saline) only. Animals were sacrificed by cervical dislocation at 15 days post-treatment and 24 hrs following the last MDL72974 injection. A total of eight doses of MDL72974 was administered. Brains were rapidly removed and cut in 1 mm coronal

sections on ice. The striatum was carefully excised and the cerebellar cortex was isolated from the white matter and blood vessels. The striatum and cerebellum from two animals were pooled, quickly homogenized in 10 mM potassium phosphate buffer pH 7.4 (no sodium) containing 0.154 M KCl and frozen at -80°C for SOD and lipid peroxidation determinations as indicated below (n=5).

SOD

CuZn-SOD and Mn-SOD were assayed using the method of Misra and Fridovich [20] which is based on the ability of SOD to inhibit the autoxidation of (-)-epinephrine-(+)-bitartrate to adrenochrome at pH 10.2. Fifty percent inhibition of the autoxidation is defined as one unit of enzymatic activity. Total SOD activity was subtracted from CN^- insensitive SOD or Mn-SOD measurement to obtain CuZn-SOD.

Lipid Peroxidation

Thiobarbituric acid (TBA) was reacted colorimetrically with a product of lipid peroxidation, malondialdehyde (MDA), according to the methodology described in detail by Ohkawa *et al.* [24]. 1,1,3,3-Tetramethoxypropane (TMP) was used as the standard curve and results are reported in nmol MDA/mg protein. Protein concentration was determined by Pierce BCA Protein Assay Kit (Rockford, Il, USA) with bovine serum albumin as standard and absorbance measured at 562 nm. Statistical analysis was determined non-parametrically using the Wilcoxon test, and a value of $p<0.05$ was utilized as the criteria for statistical significance.

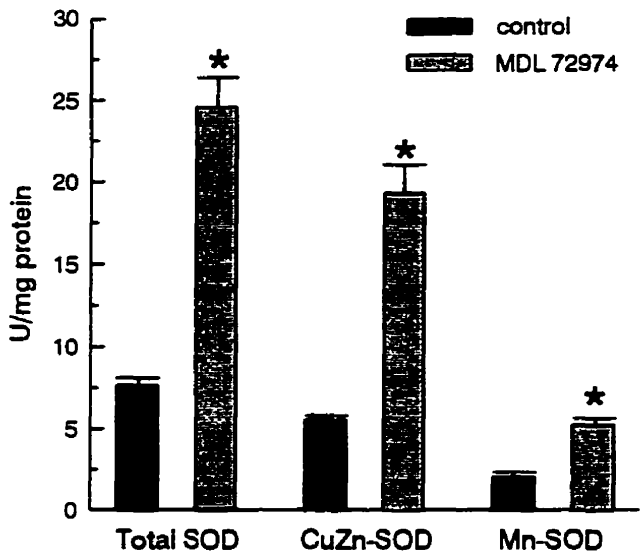

Fig. 1. SOD activity in the striatum of C57Bl/6 mice following MDL72974 administration (i.p.) at 15 days post-treatment. Enzymatic activity measurement and animal treatments are described in Materials and Methods. Asterisks indicate significant differences ($p<0.05$) as compared to controls. Mean ± SE values of five independent experiments are shown.

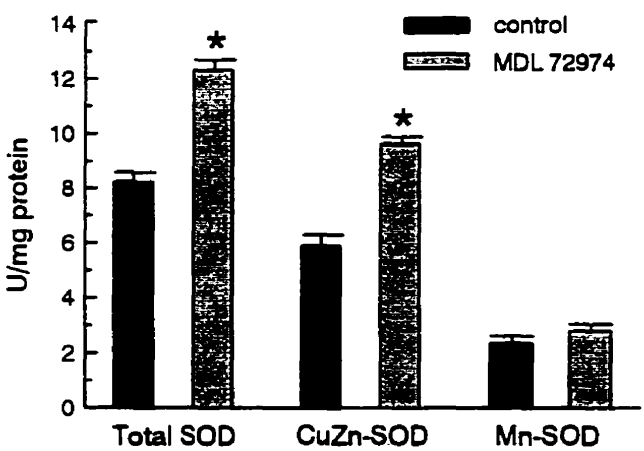

Fig. 2. SOD activity in the cerebellum of C57Bl/6 mice following MDL72974 administration (i.p.) at 15 days post-treatment. Enzymatic activity measurement and animal treatments are described in Materials and Methods. Asterisks indicate significant differences ($p<0.05$) as compared to controls. Mean ± SE values of five independent experiments are shown.

RESULTS

Fig. 1 and 2 summarize CuZn-SOD and Mn-SOD activities in the striatum and cerebellum of C57BL/6 mice following MDL72974 administration. Two- and five-fold increases in Mn-SOD and CuZn-SOD activities, respectively, were observed in the mouse striatum. MDL72974 administration indicated a significant 50% increase in CuZn-SOD activity in the cerebellum, whereas Mn-SOD activity remained unaltered in this region. Fig. 3 illustrates the effect of MDL72974 on malondialdehyde produced from lipid peroxidation in the striatum and cerebellum of C57BL/6 mice. An increase in malondialdehyde formation was observed in the striatum of MDL72974 treated mice, whereas a reduction (20%, $p<0.05$) was noted in the cerebellum.

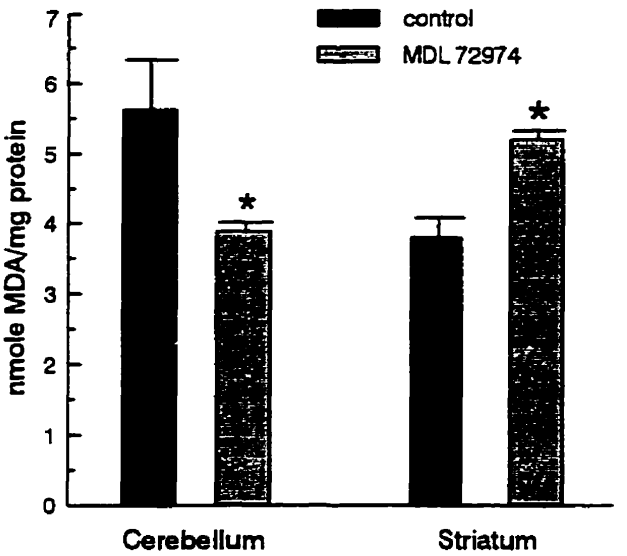

Fig. 3. Thiobarbituric reactive substances as a measure of lipid peroxidation levels in the striatum and cerebellum of C57Bl/6 mice following MDL72974 administration (i.p.) at 15 days post-treatment. Lipid peroxidation measurements and animal treatments are described in Materials and Methods. Asterisks indicate significant differences ($p<0.05$) as compared to controls. Mean ± SE values of five independent experiments are shown.

DISCUSSION

Hence, MDL72974 induced an increase in lipid peroxidation in the striatum of C57BL/6 mice at 15 days post-treatment. This may suggest that this MAO-B inhibitor is detrimental to dopaminergic neurons of the nigrostriatal pathway. This observation is consistent with an earlier investigation which demonstrated a 30% decrease in TH^+ neurons in the substantia nigra of MDL72974 treated mice (Thiffault *et al.*, unpublished results). In addition, two- to five-fold increases in SODs activities were observed in the striatum following MDL72974 administration, the CuZn-SOD subtype being most affected. However, Clow and co-workers observed that SOD activity was unaltered in the striatum of MDL72974 treated rats at doses of 1.25 and 25

mg/kg [11] while Carrillo *et al.* reported the bell shape nature of the effects of L-deprenyl, a MAO-B inhibitor, on SOD activity with higher doses being less effective than intermediate ones [7]. Similar bell shape dose-response curves could explain the positive results obtained in the present study with MDL72974.

In the cerebellum, a modest increase in Mn-SOD activity was observed while lipid peroxidation was decreased in MDL72974 treated mice. Taken together, these data suggest that SOD activity, as a result of MDL72974 treatment, has a differential effect on lipid peroxidation levels. This is consistent with another study in which the effect of SOD on lipid peroxidation levels was investigated in isolated myocardium following transient ischemia [22]. Reoxygenation was shown to result in an increase in lipid peroxy radicals in post-ischemic heart [2]. Nelson *et al.* observed that the presence of a low concentration of SOD in the perfusate limited the extent of oxidative damage to lipids while elevated concentrations potentiated lipid peroxidation [22]. Similarly, mammalian cells and mouse brain overexpressing human CuZn-SOD demonstrated increased levels of peroxidized lipids [8,9,13].

Our results also suggest that the striatum may be more vulnerable than the cerebellum to the effect of MDL72974 administration as indicated by the extent of changes in CuZn-SOD, Mn-SOD and lipid peroxidation. Levels of MAO-B and Mn-SOD are fairly similar in these brain regions in rodents and primates [5,34,35]. In contrast, CuZn-SOD is expressed predominantly in the nigrostriatal pathway [35]. A lack of correlation was observed between CuZn-SOD expression and pattern of neurodegeneration seen in Parkinson's disease, Alzheimer's disease and

amyotrophic lateral sclerosis [3]. Thus, factor(s) other than CuZn-SOD may be responsible for the differential effect of MDL72974 administration in the mouse striatum and cerebellum. Interestingly, it has been reported that dopaminergic neurons of the nigrostriatal pathway are more vulnerable to respiratory chain inhibitors than other neuronal populations [10,17,18]. Inhibition of mitochondrial respiration which induces superoxide formation has been suggested as being responsible for the increase in SOD activity [6,27,31,32]. We also recently observed that high concentrations of MDL72974 inhibit the mitochondrial respiratory chain *in vitro* [unpublished observations]. Such a mechanism may underlie the differential effects observed here between the striatum and cerebellum of MDL72974-treated mice.

In summary, our results show that administration of MDL72974 induces an increase in SOD activities and lipid peroxidation in the mouse striatum. These observations are consistent with properties of various oxidative stress-inducing agents [12,15,23,28,29].

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CHAPTER 6

General Discussion

GENERAL DISCUSSION

1 Effects of L-Deprenyl on MPTP-Induced Oxidative Stress

1.1 MPTP Alters Antioxidant Enzymes Activities and Lipid Peroxidation in Mouse Brain: A Model Relevant to PD?

We have shown that MPTP administration to mice resulted in alterations of antioxidant defence systems. Mn-SOD, CuZn-SOD and CAT activities were the most affected in the three brain areas examined (SN, striatum and cerebellum), with the exception of CAT activity in the striatum, which remained unchanged. Despite alterations in antioxidant enzyme activities, we did not observe an increase in nigral, striatal and cerebellar lipid peroxidation levels in MPTP-treated mice. Moreover, in spite of a 50% decrease in Mn-SOD activity in the SN, lipid peroxidation remained relatively unaffected. This observation is confirmed by other reports in which MPTP did not induce lipid peroxidation in the striatum of mice with physiologically adequate levels of vitamin E (Corongiu *et al.*, 1987; Adams *et al.*, 1990), whereas an increase was found in vitamin E-deficient mice (Adams *et al.*, 1990). Lipid peroxidation has also been examined *in vitro* following exposure to MPTP or MPP^+. MPTP was found to inhibit lipid peroxidation (Lambert and Bondy, 1989; Poirier and Barbeau, 1987; Rios and Tapia, 1987), while MPP^+ stimulated it (Rios and Tapia, 1987) in brain and erythrocyte preparations. However MPTP potentiated iron-induced lipid peroxidation (Lambert and Bondy, 1989; Poirier and Barbeau, 1987). Perhaps, the concentrations of MPP^+, MPTP and the redox state

of iron required to promote lipid peroxidation *in vitro* were inadequate under *in vivo* conditions at the dosage used (3 x 20 mg/kg MPTP at 2 hr intervals). Taken together, these observations suggest that factors other than antioxidant enzymes play a pivotal role in the modulation of peroxidative damage to membrane lipids. Our results are also consistent with alterations in the activities of antioxidant enzymes in the PD brain, SOD (Marttila *et al.*, 1988b; Saggu *et al.*, 1989; Poirier and Thiffault, 1993) being the most affected, but to levels that are insufficient to lead to increases in lipid peroxidation.

Interestingly, we observed changes in cerebellar antioxidant enzyme activity in MPTP-treated mice. This brain area was selected as a control since, to our knowledge, no known pathological deficits have been reported in this structure in PD. In addition, MPTP toxicity is somewhat specific to the nigrostriatal pathway (Langston *et al.*, 1984a; Heikkila *et al.*, 1984a; Hallman *et al.*, 1985; Sundström *et al.*, 1988; Takada *et al.*, 1991; Varastet *et al.*, 1994). This observation suggests that MPTP is metabolized to MPP^+ in the cerebellar cortex to an extent that appears insufficient to be cytotoxic. Consistent with this interpretation is the presence of MAO-B in the cerebellar purkinje cell layer as revealed by immunohistochemical and *in situ* hybridization studies (Konradi *et al.*, 1988; Saura *et al.*, 1994; 1996; Westlund *et al.*, 1988; Willoughby *et al.*, 1998). Interestingly, administration of radioactive MPTP to rodents and primates labelled the cerebellum (Herkenham *et al.*, 1991; Takada *et al.*, 1991), whereas pre-treatment with a non-selective MAO-B inhibitor, pargyline, eliminated the retention of the radiolabelled products (Takada *et al.*, 1991), indicating that MPTP is also converted by MAO-B to MPP^+ in this

structure. It is therefore apparent that concentrations of MPP^+ present in the cerebellum, as well as in various brain areas (Herkenham *et al.*, 1991), are not sufficient to significantly impair cellular energy demands, even in the presence of an oxidative stress.

MPTP administration to mice had a differential impact on striatal and nigral CuZn-SOD, Mn-SOD and CAT activities. Increases in CuZn-SOD and Mn-SOD activities were observed in the striatum of MPTP-treated mice, whereas no change in CAT activity was detected. Conversely, a reduction in nigral CuZn-SOD and Mn-SOD activities was seen, while CAT activities remained elevated in the SN. These divergences are unlikely to be attributed to MPTP metabolism by MAO-B (Nwanze *et al.*, 1995; Yang *et al.*, 1988) since this enzyme is present in minute amounts in dopaminergic nigrostriatal neurons of rats and mice (Nakamura *et al.*, 1995; Saura *et al.*, 1994; Saura Marti *et al.*, 1990; Willoughby *et al.*, 1998). The mechanism underlying the biochemical differences in the SN and the striatum in response to MPTP-induced oxidative stress is not clear at present. Our results are consistent with other investigations showing that antioxidant manipulations have a distinct influence on nigral and striatal biochemistry. Reduction in GSH concentrations in mice exacerbated nigral DA losses, while striatal levels remained unaffected by low doses of MPTP (Adams *et al.*, 1989; Perry *et al.*, 1986; Weiner *et al.*, 1988). Furthermore, an increase in lipid peroxidation levels was observed in the SN of vitamin E-deficient mice, whereas this effect is not seen in the striatum of mice following MPTP intoxication (Adams *et al.*, 1990). These observations may be explained by the fact that dopaminergic terminals represent only a small fraction of the tissue present in the striatum. It was difficult to ascertain through our studies whether alterations in antioxidant

enzyme activities occur in dopaminergic neurons as nigral and striatal homogenates were used. It is well known that Mn-SOD is expressed in both neuronal and glial cells, while CuZn-SOD is mostly neuronal and found in large quantities in melanized dopaminergic neurons of mice and humans (Ceballos-Picot *et al.*, 1991; Poirier *et al.*, 1994; Rosenberg *et al.*, 1989; Takashima *et al.*, 1990; Zhang *et al.*, 1993). CAT is found in peroxisomes of both neural and glial cells, albeit in different amounts in the brains of rodents and primates (Geremia *et al.*, 1990; Hassan and Fridovich, 1977; Houdou *et al.*, 1993; Moreno *et al.*, 1995; Victorical *et al.*, 1984). The reduction in nigral CuZn-SOD activities may be related to the degeneration of dopaminergic neurons. Consistent with this hypothesis was the reduction (≈40%) in TH immunopositive cells observed in MPTP-treated mice (Thiffault *et al.*, unpublished). Possible alterations in nigral and striatal Mn-SOD and CAT activities in dopaminergic neurons as a result of MPTP may have been masked by astrocytic proliferation that is known to occur in response to this neurotoxicant (Francis *et al.*, 1995; O'Callaghan *et al.*, 1990; Reinhard *et al.*, 1988; Schneider and Denaro, 1988). *In situ* hybridization and histochemical analyses of Mn-SOD, CuZn -SOD and CAT in the SN and striatum of MPTP- and saline-treated mice are required in order to establish the neuronal or glial involvement in these alterations. However, the degree of antioxidant enzyme activation is not always accompanied by changes at the protein or mRNA levels.

Although the MPTP model is widely used to study the putative mechanism or mechanisms of cell death as observed in PD, our results demonstrated significant differences in alterations of antioxidant enzyme activity as described above, and those observed in the SN and striatum of PD patients (Table 2). For example, increases in CuZn-SOD and Mn-SOD are observed in the

SN and striatum of PD patients (Marttila *et al.*, 1988b; Saggu *et al.*, 1989; Poirier and Thiffault, 1993). There have also been reports of small reductions in CAT and GHH-PX activity(Ambani *et al.*, 1975; Kish *et al.*, 1985), while other studies reported no change (Marttila *et al.*, 1988b; Poirier and Thiffault, 1993). In this context, it is important to remember that PD spans much of the lifetime of afflicted individuals (McGeer *et al.*, 1988; Schulzer *et al.*, 1994), whereas MPTP produces a parkinsonian-like syndrome in a matter of hours (Irwin *et al.*, 1990; Nishi *et al.*, 1989; Sundström *et al.*, 1988; Tatton *et al.*, 1992). Additional differences may be attributed to the age of the animals used and acute-versus-chronic rate of cellular degeneration. In addition, dopaminergic deficits in MPTP-treated mice are shown to recover, at least partially, in 2 to 3 months (Ricaurte *et al.*, 1986; Tatton *et al.*, 1992; Weiner *et al.*, 1989). Accordingly,

Table 2. Alterations in antioxidant enzyme activity and lipid peroxidation levels observed in the nigrostriatal pathway at *post mortem* in PD and MPTP-induced parkinsonism.

	PD	MPTP	References
Lipid peroxidation			
SN	increase	no change	Adams *et al.*, 1990
Striatum	no change	small decrease	Corongiu *et al.*, 1987
			Dexter *et al.*, 1989a
			Pall *et al.*, 1986
			Thiffault *et al.*, 1995
SOD activities			
SN	increase	decrease	Marrtila *et al.*, 1988b
Striatum	increase	increase	Poirier and Thiffault, 1993
			Saggu *et al.*, 1989
			Thiffault *et al.*, 1995
GSH-PX activity			
SN	small decrease/no change	no change	Marrtila *et al.*, 1988b
Striatum	no change	no change	Poirier and Thiffault, 1993
			Thiffault *et al.*, 1995
CAT activity			
SN	small decrease/no change	increase	Ambani *et al.*, 1975
Striatum	small decrease/no change	no change	Kish *et al.*, 1985
			Marrtila *et al.*, 1988b
			Thiffault *et al.*, 1995

the MPTP mouse model presents some limitations to the study the neurodegenerative features of PD. However, our results revealed the usefulness of this approach in the study of the pharmacological implications of oxidative stress on antioxidant enzymes and lipid peroxidation.

1.2 MPTP and Free Radicals

Several sources of free radicals are proposed to be generated as a result of MPTP administration to induce oxidative stress. Because of its structural similarity to paraquat, MPP^+ was proposed to undergo spontaneous oxidation (Fig. 8 chapter 1, p. 33) (Johannessen *et al.*, 1985). However, this possibility appears unlikely because the electrochemical potential for reducing MPP^+ to form a free radical under physiological conditions is too great (Elstner *et al.*, 1980; Frank *et al.*, 1987). A number of reports demonstrate that free radicals are in fact formed in the presence of MPTP and its MAO metabolites: $MPDP^+$ and MPP^+. For instance, incubation of MPDP in the presence of MPP^+ in an aqueous solution led to a time-dependent increase in free radicals as monitored by ESR, whereas no such signal arose when either of these compounds was used alone (Rossetti *et al.*, 1988). Although the concentrations used were relatively high (10 mM), the suggestion remained of a radical-generating redox cycling between $MPDP^+$ and MPP^+. In addition, MPP^+ is known to catalyze the formation of $O_2^{\cdot}$ and $OH^{\cdot}$ radicals in the presence of microsomal (Sinha *et al.*, 1986) and cytosolic (Klaidman *et al.*, 1993) enzymes. This is significant since free radicals have been demonstrated to deactivate the NADH dehydrogenase complex *in vitro* (Cleeter *et al.*, 1992; Fukushima *et al.*, 1995; Zhang *et al.*, 1990). Similar inhibitory action was also reported following exposure to MPP^+ (Nicklas *et al.*, 1985; Poirier and

Barbeau, 1985b; Ramsay *et al.*, 1986b), and in the SN and striatum of PD (Mizuno *et al.*, 1989; Schapira *et al.*, 1989; 1990). However, neither MPTP (Vyas *et al.*, 1986) nor $MPDP^+$ (Walker *et al.*, 1991) had a significant impact on the inhibition of mitochondrial function induced by MPP^+. Our results were consistent with this observation, suggesting free radicals generated from the systemic administration of MPTP to mice do not play a major role in neurotoxicity as we did not observe an increase in nigral, striatal or cerebellar lipid peroxidation levels. Accordingly, the inhibition of NADH dehydrogenase within the mitochondrial respiratory chain is likely the predominant mechanism by which MPP^+ exerts its toxic effect. Consistent with this hypothesis is the observation that when injected into the medial forebrain bundle, rotenone, known to inhibit the respiratory chain at the same site as MPP^+, induces a selective degeneration of nigrostriatal dopaminergic neurons in the rats (Heikkila *et al.*, 1985). It is well known that the mitochondrial respiratory chain (NADH dehydrogenase and ubiquinone sites) is the major intracellular source of free radical formation (Adams *et al.*, 1993; Boveris and Chance, 1973; Boveris *et al.*, 1976; De Jong and Albracht, 1994; Guidot *et al.*, 1993; Kashkarov *et al.*, 1994; Krishnamoorthy and Hinkle, 1988; Paraidathathu *et al.*, 1992; Patole *et al.*, 1986; Ramsay and Singer, 1992; Turrens and Boveris, 1980). For instance, the addition of MPP^+ to mitochondrial preparations enhanced the $O_2^{\cdot}$ ESR signal (Adams *et al.*, 1993; Rossetti *et al.*, 1988). Furthermore, rotenone increased the ESR signal of $O_2^{\cdot}$ under similar conditions (Adams *et al.*, 1993). Thus, free radicals generated from the systemic administration of MPTP appear to be consequential to MPP^+-induced NADH dehydrogenase inhibition. Consistent with this observation is the detection of reactive oxygen species (ROS) (including H_2O_2) at 2 hrs but not

9 hrs post-MPTP administration (Ali *et al.*, 1993; 1994). This is significant since the inhibition of NADH dehydrogenase by MPP^+ is reported to be reversible and of short duration (Mizuno *et al.*, 1988; Nicklas *et al.*, 1985; Ramsay *et al.*, 1986a).

The enzymatic and non-enzymatic oxidation of catecholamines is known to represent an alternative source of ROS (Graham, 1978; 1979). In this respect, MPP^+ and $MPDP^+$ and their 2'-methyl derivatives have been found to evoke DA release in the brain (Chiueh *et al.*, 1992b; 1994; Wu *et al.*, 1993). Chiueh and his colleagues (1992b) concluded that the oxidation of this released DA resulted in enhanced $OH^{\cdot}$ radical generation since this could be blocked by free radical scavengers. Because DA autoxidation results in the formation of semiquinone radicals and melanin, as well as other ROS, it has been suggested that neuromelanin content in neurons of the SN is an indicator of oxidative stress (Chiueh *et al.*, 1994). In addition, MPTP can produce an aminochrome pigment in the presence of DA, this black polymer serving as a substrate in the process of melanization of nigral neurons (Das *et al.*, 1978). Moreover, DA neuromelanin is shown to promote the transformation of $MPDP^+$ to MPP^+ (Wu *et al.*, 1986). Interestingly, melanized dopaminergic neurons are the ones most susceptible to degeneration, not only in MPTP-treated animals (Herrero *et al.*, 1993b) but also in PD (Hirsch *et al.*, 1988). However, manipulation of endogenous antioxidant contents or their use in pre-treatment partially protected against MPTP-induced dopaminergic cell death (Adams 1989; Perry *et al.*, 1985a; Sershen *et al.*, 1985; Wagner *et al.*, 1986; Weiner *et al.*, 1988; Yong *et al.*, 1986) while others reported no effects (Martinovits *et al.*, 1986; Mihatsch *et al.*, 1991; Perry *et al.*, 1985b; 1986; 1987; Sutphin and Buckman, 1991). It appears, therefore, that inhibition of the

mitochondrial respiratory chain is sufficient to account for MPTP neurotoxicity and its effect on antioxidants enzymes and lipid peroxidation, as observed in our studies. Accordingly, we proposed that alterations in SOD activity and lipid peroxidation reported in the SN of PD patients may be consequential to free radicals generated as a result of mitochondrial respiratory chain dysfunctions. Consistent with this interpretation is the reduction of NADH dehydrogenase observed in the SN of preclinical parkinsonians with incidental Lewy body inclusions (Dexter *et al.*, 1994), suggesting that defects in the mitochondrial respiratory chain may represent an early event leading to widespread oxidative stress.

1.3 L-Deprenyl and its Effects on MPTP-Induced Alterations in Antioxidant Enzyme Activity and Lipid Peroxidation

We were surprised that L-deprenyl failed to reverse the effect of MPTP on the activity of antioxidant enzymes, since this molecule is known to protect dopaminergic neurons against MPTP toxicity, possibly via its inhibition of MAO-B activity (Chiba *et al.*, 1984; Heikkila *et al.*, 1984b). Unexpectedly, our results with L-deprenyl were rather similar to those obtained with MPTP, despite the regional differences observed in MPTP- and L-deprenyl-treated mice. L-Deprenyl treatment resulted in one- to fourfold increases in striatal CuZn-SOD and Mn-SOD activity, whereas a modest decrease was detected in the mouse SN. This observation was confirmed by other reports in which L-deprenyl, at doses (2 mg/kg/day for 3 weeks) lower than those used in our studies, were shown to be sufficient to increase SOD activity in the rat striatum (Carrillo *et al.*, 1991; Clow *et al.*, 1991; Knoll, 1988; Vizuete *et al.*, 1993).

Interestingly, Carrillo and her colleagues (1993) reported a differential effect of L-deprenyl treatment on SOD activity in the SN and striatum of rats. For instance, increases in SOD activity were detected in the striatum, whereas a similar dosing schedule (2 mg/kg/day for 3 weeks) was ineffective on nigral SODs (Carrillo *et al.*, 1993). In addition, increases in nigral SOD activities can be achieved at lower doses, e.g. 0.5 mg/kg (Carrillo *et al.*, 1993). Furthermore, while an increase in SOD activity was observed in the SN of young male rats, a reduction was seen in young females using a similar dosage regimen (Carrillo *et al.*, 1992a; 1993). Similarly, we and another researcher (Knoll, 1988) reported relatively no change in striatal CAT activity, while another group found an increase at 3 but not 2 weeks post-L-deprenyl treatment (Carrillo *et al.*, 1992b; 1993). Accordingly, dosage schedule, sex, age, species and route of L-deprenyl administration, as well as methodological approach to measurement of SOD and CAT activity may explain the various results obtained in these experiments.

To our knowledge, L-deprenyl is not known to act as a direct modulator of SOD activity and we failed to observe any effects of this MAO-B inhibitor on purified SOD activity *in vitro* using concentrations as high as 4 mM (Thiffault *et al.*, unpublished). In addition, we demonstrated that one single dose of L-deprenyl (10 mg/kg) at 20 days post-treatment was as effective at increasing striatal SOD activity as eight doses administered to mice over 15 days at 2 day intervals. This observation suggests that the modulation of striatal SODs by L-deprenyl is not readily reversible and appears to be independent, at least in part, from MAO-B inhibition. Consistent with this hypothesis is the 50% recovery of MAO-B activity that we observed at 20 days following one dose of L-deprenyl in the mouse brain. The half-life for the recovery of

MAO-B activity in rodent brains is known to vary from 8-14 days, even when high doses of L-deprenyl (10mg/kg) are used (Felner and Waldmeier, 1979; Goridis and Neff, 1971). The mechanism by which L-deprenyl induces an increase in SOD activity is intriguing, considering that these enzymes are up-regulated in response to $O_2^{\cdot}$ in various models of free radical-mediated injuries (such as hyperoxia, 6-OHDA, paraquat) (Crapo and Tierney, 1973; Frank, 1981; Krall *et al.*, 1988; Ogawa *et al.*, 1994; Sjostrom and Crapo, 1981; Stevens and Autor, 1977). Taken together, these studies led us to evaluate the possible relationship between L-deprenyl, $O_2^{\cdot}$ formation and enhanced SOD activity.

2 Possible Mechanism Underlying the Modulation of SOD Activity by L-Deprenyl

In this series of studies, we investigated the effects of L-deprenyl on catecholamine levels and mitochondrial electron transport chains, since these sites are a major source of $O_2^{\cdot}$ production, as discussed in section 1.2 (Adams *et al.*, 1993; Boveris and Chance, 1973; Boveris *et al.*, 1976; De Jong and Albracht, 1994; Graham, 1978; 1979; Guidot *et al.*, 1993; Kashkarov *et al.*, 1994; Krishnamoorthy and Hinkle, 1988; Paraidathathu *et al.*, 1992; Patole *et al.*, 1986; Ramsay and Singer, 1992; Turrens and Boveris, 1980). In addition, D-deprenyl, a weak MAO-B inhibitor, and MDL72974 (4-fluoro-ß-(fluoromethylene)-benzenebutanamine or mofegiline), a more potent MAO-B blocker not metabolized to amphetamines (unlike D-deprenyl and L-deprenyl) were investigated in the same experimental paradigms to establish the putative, respective role of MAO-B and amphetamines in the effects of L-deprenyl.

2.1 $O_2^{\cdot}$ Generated from Catecholamine Autoxidation: Relationship With SOD Activities

L-Deprenyl administration resulted in a five-fold increase in striatal DA concentrations, whereas no alteration in cerebellar NE could be detected in mice. This observation is consistent with previous reports showing L-deprenyl to enhance striatal DA content (Knoll, 1987; Zsilla *et al.*, 1986) while NE levels remained unaltered in cortical areas in rodents (Gupta and Wiener, 1995). In addition, at this dosage, an L-deprenyl (8 x 10 mg/kg, every 2 days) treatment produced a marked inhibition of mouse-brain MAO-B (89.6% ± 0.7) and MAO-A (49.8% ± 1.4). It is known that DA and NE are preferentially metabolized by MAO-A in rodent brains (Butcher *et al.*,1990; Fagervall and Ross, 1986; Finberg *et al.*, 1995; Gupta and Wiener, 1995; Oreland *et al.*, 1983; Stenström *et al.*, 1987). Our results suggest that the remainder of MAO-A activity (over 50%) is sufficient to maintain a normal NE metabolism. This hypothesis does not, however, entirely explain the effect of L-deprenyl on DA metabolism. Interestingly, L-deprenyl was shown to block DA uptake in synaptosomes prepared from the striatum of L-deprenyl-treated rats (Fang and Yu, 1994; Knoll, 1987; Knoll and Myklya, 1994; Zsilla *et al.*, 1986). Other reports indicated that the extraneuronal metabolism of DA by MAO-A and B is highly significant when DA uptake is inhibited (Oreland *et al.*, 1983; Stenström *et al.*, 1987). Furthermore, MAO-B inhibition is known to increase striatal levels of ß-PEA, an indirectly acting sympathomimetic trace amine (Paterson *et al.*, 1990; Philips and Boulton, 1979). Taken together, these observations suggest that the effects of L-deprenyl on DA reuptake, MAO activity and ß-PEA levels play a role in the modulation of striatal DA content in mice.

L-Amphetamine and L-methamphetamine, two metabolites of L-deprenyl, could potentially mediate its effects on catecholamine levels in mice (Fig. 12 chapter 1, p. 73). These compounds are well known to alter the dynamics of dopaminergic transmission (Balster and Schuster, 1973; Harris and Baldessarini, 1973; Miller *et al.*, 1980; Thornburg and Moore, 1973) and, at higher doses, can induce neurotoxicity through a mechanism that may involve oxidative stress (Cadet *et al.*, 1994; Hirata *et al.*, 1995; Jonnsson and Nwanze, 1982; O'Callaghan and Miller, 1994). Interestingly, D-deprenyl is metabolized to D-amphetamine and D-methamphetamine (Reynolds *et al.*, 1978a), which both have approximately 10 times the potency of the corresponding L-enantiomer (Chiueh and Moore, 1974; Taylor and Snyder, 1974). However, DA and NE levels remained unaltered in mice following D-deprenyl treatment (10 mg/kg). In addition, MDL72974 is not metabolized to amphetamines (Dow *et al.*, 1994), and yet resulted in similar changes in striatal and cerebellar catecholamine levels as observed in L-deprenyl-treated mice. Taken together, these results suggest that amphetamine metabolites do not mediate the effects of L-deprenyl and D-deprenyl on catecholamine levels at the concentrations used in our studies.

We and others have shown that L-deprenyl can up-regulate SOD activity in brain areas enriched with catecholamines (Carrillo *et al.*, 1992c; Thiffault *et al.*, 1995). Although an increase in striatal DA levels was observed in L-deprenyl- and MDL72974-treated mice, cerebellar NE levels remained unaffected while SOD activity was enhanced in this structure. In addition, D-deprenyl is shown to up-regulate striatal SOD activity in the absence of altered DA contents. Accordingly, our data suggest that catecholamine oxidation, known to result in $O_2^{\cdot}$ formation

(Graham, 1978; 1979), may not be the key mechanism underlying the modulation of striatal and cerebellar SOD activity. Moreover, this effect may not be attributed to MAO inhibition or the amphetamine-like metabolites of L-deprenyl and D-deprenyl discussed above. This is also consistent with the effects of clorgyline, a MAO-A inhibitor and L-dopa, a DA precursor, on DA levels and SOD activity in the rat striatum. Clorgyline was shown to increase stratial DA levels in rats and the release of DA from striatal slices. However, this MAO-A inhibitor failed to induce an increase in SOD activity in the rat striatum (Knoll, 1988; Zsilla *et al.*, 1986; Fang and Yu, 1994). Similarly, striatal SOD activity remained unaltered following L-dopa administration in rats (Clow *et al.*, 1992).

2.2 $O_2^{\cdot}$ Leakage from the Mitochondrial Respiratory Chain: Relationship with SOD Activity

We observed that L-deprenyl induced a dose-dependent reduction in O_2 consumption during ATP synthesis (state 3 with an IC_{50} of 1.9 mM), which could be partially restored by adding succinate (IC_{50}= 2.8 mM). $O_2^{\cdot}$ radical formation is enhanced by mitochondrial respiratory chain inhibitors and represents the major intracellular source of free radicals, as discussed in section 1.2. D-Deprenyl resulted in a similar inhibitory profile, suggesting that the transfer of electrons is impaired at more than one site which include NADH dehydrogenase (complex I) and cytochrome-c reductase (complex III) or cytochrome-c oxidase (complex IV) (Fig. 5 chapter 1, p. 19). This observation is consistent with the effect of respiratory chain inhibitors on the rate of O_2 consumption in fresh mitochondria prepared from rodent brains. Rotenone and MPP^+ were shown to inhibit ADP-stimulated O_2 consumption in the presence of pyruvate and malate

(two NADH dehydrogenase substrates) (Nicklas *et al.*, 1985). However, this effect was fully reversed by introducing succinate, suggesting that the flow of electrons was blocked solely at the point of entry, or at the NADH dehydrogenase (Nicklas *et al.*, 1985). In contrast, MDL72974 was equally effective at reducing O_2 consumption during ATP synthesis, whether NADH dehydrogenase or succinate dehydrogenase substrates were present, with an IC_{50} of 2.4 and 2.6 mM, respectively. This observation suggests that the inhibition is taking place at only one site, namely the cytochrome-c reductase or cytochrome-c oxidase. Moreover, MDL72974 may not alter succinate dehydrogenase (complex II) activity since O_2 utilization is not inhibited when pyruvate and malate are included and since NADH dehydrogenase (complex I) can directly donate its electron to ubiquinone (Fig. 5 chapter 1, p. 19) (Ernster and Dallner, 1995). Interestingly, a recent report demonstrated that the administration of a very large dose of L-deprenyl (100 mg/kg) altered the redox state of ubiquinone, suggesting that the flow of electrons is impaired in the respiratory chain (Götz *et al.*, 1995). In this model, Götz and colleagues (1995) observed a decrease in ubiquinone levels, while ubiquinol (reduced ubiquinone) was increased in the mouse striatum. Ubiquinol concentrations have been shown to be altered as a result of an impairment in mitochondrial respiration. For example, the inhibition of NADH-ubiquinone oxidoreductase activity of complex I by MPP^+ or rotenone resulted in the depletion of ubiquinol content (Fariello *et al.*, 1987; Mizuno *et al.*, 1987; van de Water *et al.*, 1995). In contrast, complex III (antimycin A) and IV (CN^-) inhibitors increased ubiquinol levels (Kowaltowski *et al.*, 1995; Takahashi *et al.*, 1995) while a synergistic effect is seen when a complex I (rotenone) inhibitor is included (Takahashi *et al.*, 1995). Our observations further confirm these reports which demonstrate changes in ubiquinol levels as a

characteristic of respiratory chain inhibitors such as L-deprenyl. Taken together, our results suggest that structural similarities exist among these compounds which could be responsible for the effects seen following the O_2 utilization and $O_2^{\cdot}$ generation (Fig. 13). The aromatic structure, which is devoid of enantiomeric carbon atoms, could be involved, as suggested by the effect of L- and D-deprenyl on mitochondrial functions. In addition, the presence of a fluorin atom on the aromatic ring may reduce the capacity of MDL72974 to impair the mitochondrial respiratory chain.

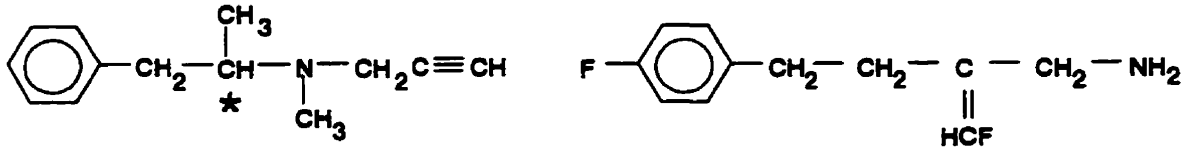

Fig. 13. Structures of L-deprenyl, D-deprenyl and MDL72974 (Mofegiline).The asterisk identifies the chiral carbon atom.

It has been suggested that MAO activity influences the state of mitochondrial respiration (Smith and Reid, 1978; Wojtczak *et al.*, 1995), indicating a functional link between the inner mitochondrial membranes and the outer (where MAO is located). To date, however, this putative regulatory mechanism has not been confirmed (Wojtczak *et al.*, 1995). Our results also suggest that, for at least three reasons, the effect of L-deprenyl on mitochondrial respiratory function is independent of MAO-B. First, the inhibitory potency (IC_{50}) of L-deprenyl toward MAO-B *in vitro* (Terleckyj and Heikkila, 1992) is 6 orders of magnitude below that observed for the rate of oxygen consumption. Second, D-deprenyl is approximately 150 times less potent than its L-isomer at inhibiting MAO-B (Magyar *et al.*, 1967), and yet exhibits a similar inhibitory profile towards the respiratory chain. Finally, MDL72974 is 25 times more effective as a MAO-B inhibitor than L-deprenyl (Terleckyj and Heikkila, 1992; Zreika *et al.*,1989), while

being much less potent in altering mitochondrial O_2 uptake. Our results also suggest that these effects are somewhat selective as we observed a differential impact of L-deprenyl on the rate of mitochondrial O_2 consumption depending on whether complex I or II was included in intact mitochondria prepared from mouse brains.

We therefore propose that alterations in respiratory function caused by MDL72974, L-deprenyl and D-deprenyl result in a leakage of oxygen-centered radicals, which in turn lead to adaptive increases in CuZn-SOD and Mn-SOD (Fig. 14). Consistent with this interpretation is the effect of MPP^+ and rotenone on $O_2^{\cdot}$ formation and SOD activity. MPP and rotenone, known to inhibit NADH dehydrogenase activity, stimulated $O_2^{\cdot}$ production in mitochondrial preparations (Adams *et al.*, 1993; Rossetti *et al.*, 1988; Turrens and Boveris, 1980). In addition, we demonstrated that the systemic administration of MPTP to mice resulted in an increase in

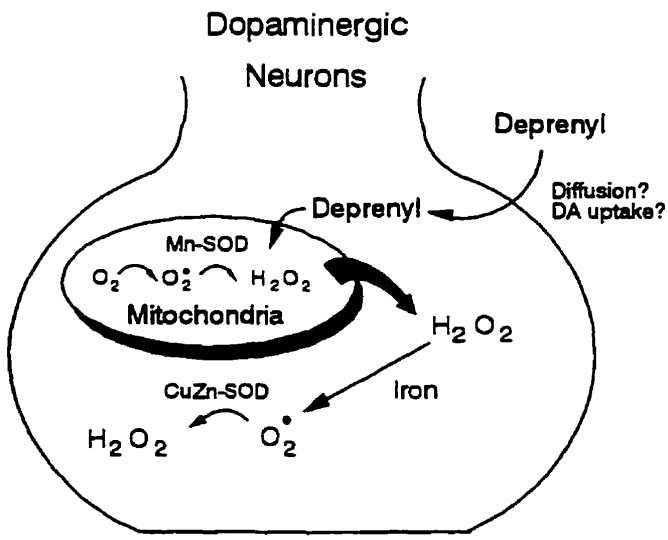

Fig. 14. Proposed mechanism leading to the generation of free radicals and the up-regulation of SOD activities induced by L-deprenyl.

CuZn-SOD and Mn-SOD activity. Interestingly, although in a different model, Mn-SOD activity is increased when procaryotes are exposed to classical respiratory chain inhibitors (Brown-Peterson *et al.*, 1995).

3 Is L-Deprenyl Neurotoxic?

It is known that the pharmacological profile of L-deprenyl is complex and extends beyond MAO-B inhibition (Berry *et al.*, 1994b; Knoll, 1988). For example, L-deprenyl was found to block DA uptake (Fang and Yu, 1994), to up-regulate SOD activity (Carrillo *et al.*, 1991; Clow *et al.*, 1991; Knoll, 1988; Thiffault *et al.*, 1996; Vizuete *et al.*, 1993) and to induce increases in levels of mRNA for AAAD, the enzyme involved in the conversion of L-dopa to DA (Li *et al.*, 1992). Some of these actions may contribute to the symptomatic relief observed in PD patients receiving L-deprenyl (Birkmayer *et al.*, 1975; Elizan *et al.*, 1989; Schulzer *et al.*, 1992; The Parkinson's Study Group, 1989b, 1993). Interestingly, MPP^+ is found to inhibit MAO-B (Salach *et al.*, 1984; Singer *et al.*, 1985), to block DA reuptake (Chiba *et al.*, 1985; Javitch *et al.*, 1985) and to increase SOD activity(Thiffault *et al.*, 1995). However, our results do not suggest that L-deprenyl possesses the neurotoxic properties associated with the systemic administration of MPTP or the intracerebral injection of MPP^+. The latter (IC_{50}= 60 μM) has 30 times the capacity of L-deprenyl (IC_{50}= 1.9 mM) to inhibit the rate of O_2 consumption in intact mitochondria prepared from mouse brain when pyruvate and malate are used as substrates (Nicklas *et al.*, 1985). Consistent with this observation was a study on the threshold effect of respiratory chain inhibitors on oxidative phosphorylation in intact mitochondria prepared from

rat brain (Davey and Clark, 1996). In that study, 72, 70 and 60% reductions in complexes I, III and IV activities, respectively, could be achieved before compromising cellular energy demands as demonstrated by the rate of O_2 uptake in intact mitochondria prepared from rat brain. Interestingly, dopaminergic neurons of the nigrostriatal pathway are more vulnerable to ATP depletion than other neuronal systems. For instance, DA uptake mechanisms, which depend on ATP supply, are more effectively blocked by rotenone, antimycin and CN^- than other neurotransmitter uptake systems in mouse striatosomal preparations or mesencephalic cells cultures (Marey-Semper *et al.*, 1993). In addition, rotenone more effectively inhibits DA uptake in striatal versus nucleus accumbens synaptosomal preparations (Marey-Semper *et al.*, 1993). Taken all together, these results suggest that the chronic use of L-deprenyl could compromise ATP synthesis in dopaminergic neurons already impaired by a reduction in NADH dehydrogenase activity as observed in PD.

Although L-deprenyl is well known for its short term symptomatic effects in early and untreated PD (Elizan *et al.*, 1989; The Parkinson Study Group, 1989b, 1993), concerns have recently been raised in the clinical arena regarding its long-term use as an adjunct to L-dopa therapy. For instance, The Parkinson Study Group (DATATOP) observed that the clinical efficacy of L-deprenyl is lost after a year of treatment (Shoulson *et al.*, 1996), and another group even reported a worsening of symptoms after a 2 year treatment (Elizan *et al.*, 1989). Moreover, the British counterpart of DATATOP failed to observe any significant effect of L-deprenyl as an adjunct to L-dopa therapy in mild PD (The Parkinson's Disease Research Group, 1993). Finally, the recent finding of a higher mortality rate reported during a prolonged period of L-

deprenyl treatment raises some serious questions regarding the safety /toxicity profile of this drug (Lees, 1995). This observation is consistent with the effects of L-deprenyl on energy metabolism reported by us and others (Götz *et al.*, 1995).

3.1 L-Deprenyl and the Issue of Neuroprotection

L-Deprenyl administration to mice after MPTP treatment significantly improved the recovery of dopaminergic neurons of the SN as demonstrated by TH immunopositive cell counts at 20 days post-treatment (Tatton and Greenwood, 1991). This effect did not appear to be related to MAO-B inhibition since MPTP (30 mg/kg/day) was administered for 5 consecutive days and 72 hrs prior to L-deprenyl treatment. In addition, it seems unlikely that L-deprenyl protected against the deleterious effects of MPP^+ by blocking DA reuptake (Fang and Yu, 1994) since the half-life of the neurotoxicant in the mouse CNS varies between 2 and 4 hrs (Johannessen *et al.*, 1985; Markey *et al.*, 1984). However, two reports failed to observe a significant effect of L-deprenyl on the restoration of striatal levels of DA and its metabolites in MPTP-lesioned mice (Gupta and Wiener, 1995; Wiener *et al.*, 1989b). Furthermore, there is no evidence suggesting that L-deprenyl can in fact "rescue" degenerating dopaminergic neurons in early and untreated PD patients (Lees, 1995; Olanow *et al.*, 1995; Schneider, 1995; The Parkinson's Disease Research Group, 1993; The Parkinson Study Group, 1989b; 1993; Ward, 1994). Our results are also consistent with these observations which do not support the notion that L-deprenyl can effectively "rescue" degenerating dopaminergic neurons following MPTP treatment.

We observed a spontaneous recovery (without L-deprenyl treatment) of TH immunopositive cell density in the SN of MPTP-treated mice after 30 days using a previously described dosing regimen (5 x 30 mg MPTP/kg/day) (Tatton and Greenwood, 1991). Modest alterations in levels of striatal DA and its metabolites were found in these mice. This observation confirmed other reports in which striatal DA content and nigral TH immunostaining can spontaneously recover, at least partially, by 2 to 3 months in MPTP-treated mice (30 mg MPTP/kg/day for 2-10 days) (Hallman *et al.*, 1985; Ricaurte *et al.*, 1986; Wiener *et al.*, 1989b). Accordingly, to be effective as a neurotoxin, MPTP must be administered repeatedly at short intervals to allow MPP^+ to accumulate at concentrations required to inhibit NADH dehydrogenase (Giovanni *et al.*, 1991; 1994; Ramsay *et al.*, 1986; Sonsalla and Heikkila, 1986).

Surprisingly, an apparent greater reduction in the number of TH immunopositive neurons was seen when L-deprenyl (0.25 mg/kg/2 days for 28 days) was administered to mice after MPTP. This observation may not be related to changes in the morphological appearance of nigral neurons as changes in the average cell surface area in MPTP/L-deprenyl-treated mice did not reach statistical significance. Thus, L-deprenyl (0.25 mg/kg) given after MPTP apparently interfered with the recovery of TH protein levels in neurons. Alternatively, alterations in the average neuronal density in MPTP/L-deprenyl-treated mice may have been influenced by astroglial proliferations known to occur in response to L-deprenyl (Biagini *et al.*, 1993; 1994). In this respect, an increase in the ratio of glial cells to neurons may lead to an alteration in the volume of the SN and therefore the number of TH immunopositive neurons per nigral section. A reduction in the number of TH^+ neurons was also observed in the SN of mice receiving

another MAO-B inhibitor, MDL72974 (0.25 mg/kg). However, no concomitant reduction in striatal DA levels could be detected in these mice. Interestingly, we did not observe a reduction in TH immunopositive cell numbers when this compound is given after MPTP. This effect is seen even though a 60% reduction in the average cell surface area is detected in MDL72974- and MPTP/MDL72974-treated mice. In spite of a necrotic appearance, we cannot ascertain from our experiments whether these cells undergo degenerative processes.

It is well known that MPTP, L-deprenyl and MDL72974 are inhibitors of flavin-containing MAO-B (Magyar *et al.*, 1967; Riederer *et al.*, 1978; Salach *et al.*, 1984; Singer *et al.*, 1985; Terleckyj and Heikkila, 1992; Zreika *et al.*, 1989). Whether the inhibition of MAO-B or other flavoproteins are responsible for the down-regulation of TH immunopositive cells is unclear at this time. Interestingly, MPP^+, L-deprenyl and MDL72974 can enhance striatal SOD activity in rodents (Carrillo *et al.*, 1991; Clow *et al.*, 1991; Knoll, 1988; Thiffault *et al.*, 1995), and alter the rate of mitochondrial respiration (Nicklas *et al.*, 1985; Thiffault *et al.*, 1996), albeit to variable degrees. Their rank of order was MPP^+ < L-deprenyl < MDL72974 to induce increases in striatal SOD activity in mice at 15 days post-treatment. Conversely, their relative potency in inhibiting the rate of O_2 uptake in intact mitochondria prepared from mouse brain was MPP^+ > L-deprenyl > MDL72974. Accordingly, L-deprenyl was expected to be more potent than MDL72974 in inducing alterations in TH immunopositive neurons and average cell surface area. The increase in striatal lipid peroxidation observed in MDL72974-treated mice suggests that this MAO-B inhibitor could be detrimental to dopaminergic neurons through an as yet undetermined mechanism. Alternatively, it may be that the metabolites of MDL72974 (Dow

et al., 1994; Stoltz *et al.*, 1995) mediated some of these effects. Unfortunately, there is little information regarding the pharmacological and pharmacokinetic profile of this compound in the literature.

4 General Conclusion

MPTP, L-deprenyl and MDL72974 induced alterations in striatal SOD activity in a manner that is consistent with oxidative stress-inducing agents. Since SODs are up-regulated in response to enhanced $O_2^{\cdot}$ production, we investigated the possible effects of L-deprenyl, D-deprenyl and MDL72974 on catecholamine levels and mitochondrial functions which represent the major sites of intracellular free radicals. A lack of parallelism was observed between alterations in catecholamine levels and increases in SOD activity in the striatum and cerebellum of L-deprenyl-, D-deprenyl- and MDL72974-treated mice. These observations suggest that catecholamine autoxidation, known to generate $O_2^{\cdot}$ radicals, may not be the key mechanism underlying the modulation of SOD activity by these compounds. Interestingly, we observed that L-deprenyl, D-deprenyl and MDL72974 induced alterations in the rate of O_2 consumption in intact mitochondria prepared from mouse brain. These effects appear to be independent of MAO-B inhibition since D-deprenyl, a weak MAO-B inhibitor, but not MDL72974, a potent MAO-B inhibitor, resulted in a similar dose response inhibition of O_2 uptake by mitochondria. These results suggest that structural similarities exist among these three compounds that could

be responsible for their effects on O_2 utilization and $O_2^{\cdot}$ generation (Fig. 13). Thus, alterations in SOD activity may represent an adaptive response due to a leakage of $O_2^{\cdot}$ radicals resulting from dysfunctions of the mitochondrial respiratory chain (Fig. 14).

In an earlier study, L-deprenyl administration to mice was shown to significantly improve the recovery of TH immunopositive nigral neurons in mice pre-treated with MPTP (Tatton and Greenwood, 1991). This observation lead to the proposal that L-deprenyl could exert a "rescuing" effect in these cells. However, our results do not support this notion. In fact, an apparent reduction in TH immunopositive cell density was observed when this compound was given to mice following MPTP compared to animals administered MPTP alone. Alternatively, increases in GFAP immunoreactivity which is known to be enhanced by L-deprenyl treatment following an injury (Biagini *et al.*, 1993; 1994) may have altered the average number of TH^+ neurons per nigral section. Whether this effect is related to changes in the cell cycle of astrocytes (Skibo *et al.*, 1993) is an intriguing area of research. The possibility that L-deprenyl induces low oxidative stress, as demonstrated by its effect on SOD activity and mitochondrial respiratory function in the nigrostriatal pathway, may be masked by its acute stimulatory effects dopaminergic transmission.

MDL72974, but not L-deprenyl, reduced the number of nigral TH immunopositive neurons, which was accompanied by an increase in lipid peroxidation in the mouse striatum. This

observation suggests that MDL72974 may be detrimental to dopaminergic neurons through an as yet undetermined mechanism. Accordingly, this drug should not be considered as an adjunct to L-dopa therapy in PD patients.

The observation that a recovery of TH^+ neuronal density occurred in MPTP-treated mice was interesting since this particular strain was long considered vulnerable to the deleterious effects of MPP^+ toxicity (Giovanni *et al.*, 1991; Riachi and Harik, 1988; Sonsalla and Heikkila, 1988). Whether this process was accompanied by neurotrophic support to injured dopaminergic neurons is unknown at this time. Such a study could provide a novel therapeutic approach to the treatment of PD.

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Contributions to Original Knowledge

As stated in the "Guidelines Concerning Thesis Preparation", Faculty of Graduate Studies and Research, McGill University:

Elements of the thesis that are considered to constitute original scholarship and an advancement of knowledge in the domains in which the research was conducted must be clearly indicated. This requirement is mandatory for doctoral theses.

CHAPTER 2

Oxidative stress has been studied in some detail in MPTP-treated animals including mice, rats and primates and is proposed as a mechanism underlying MPTP toxicity. The manuscript presented in chapter 2 contributes to original knowledge with regard to the effect of MPTP on SOD, CAT and GSH-PX activities in the SN, striatum and cerebellum of mice. This is significant since alterations in antioxidant enzyme activities as a result of MPP^+-induced oxidative stress have never been demonstrated to occur within and outside of the nigrostriatal dopaminergic pathway. On the other hand, MPTP toxicity is somewhat specific to this pathway, suggesting that oxidative stress does not play a major role in MPTP-induced neurodegeneration. In addition, increases in SOD activities without concomitant increments in GSH-PX or CAT activity is thought to be responsible for the accumulations peroxidized lipids observed in the brain of parkinsonian patients. We were the first to demonstrate that marked alterations in SOD, CAT and GSH-PX activities do not result in enhanced lipid peroxidations.

L-Deprenyl, a MAO-B inhibitor known to protect against the deleterious effects of MPTP, when administered to mice resulted in alterations in SOD, CAT, GSH-PX and lipid

peroxidation levels similar to those observed in MPTP-treated mice. This observation was surprising considering that this MAO-B inhibitor is used as an adjunct to L-dopa therapy in Parkinson's disease.

CHAPTER 3

The mechanism by which L-deprenyl induces an increase in SOD activities in several rat brain regions has never been investigated. Interestingly, several studies demonstrated that SOD activities are up-regulated in mammalian cells exposed to hyperoxia, 6-OHDA and paraquat, known to result in enhanced $O_2^{\cdot}$ radical formations. The original contribution to knowledge of this manuscript relates to the evaluation of the relationship between L-deprenyl, $O_2^{\cdot}$ and enhanced SOD activities. Morover, the finding that L-deprenyl reduces O_2 uptake in intact mouse brain mitochondria contributes to original knowledge by suggesting that alterations in SOD activities are due to a leakage of $O_2^{\cdot}$ radicals which results from dysfunctions of the mitochondrial respiratory chain. This is significant since increases in SOD activities and respiratory deficits are observed not only following MPTP intoxication but are also reported in the SN and striatum in PD. The effects of L-deprenyl on energy metabolism reported by us seemed relevant to the increase in mortality rate recently reported during a prolonged period of L-deprenyl treatment in PD.

CHAPTER 4

L-Deprenyl administration to mice has been reported to significantly improve the recovery of TH immunopositive nigral neurons in mice pre-treated with MPTP. This observation lead to

the proposal that L-deprenyl could exert a "rescuing" effect in these cells. However, two reports failed to observe a significant effect of L-deprenyl on the restoration of striatal DA and DA metabolites in MPTP-lesioned mice. Furthermore, there is no evidence suggesting that L-deprenyl could in fact "rescue" degenerating dopaminergic neurons in early and untreated PD patients. The long term impact of L-deprenyl on MPTP-induced dopaminergic cell loss in mice was therefore investigated in order to clarify these issues. The reduction in TH immunopositive cell density observed when L-deprenyl was administered to MPTP-treated mice compared to animals administered MPTP alone contributed to original knowledge. Thus, L-deprenyl given after MPTP apparently interfered with the recovery of TH protein levels in neurons. In addition, a reduction in the number of TH^+ neurons was also observed in the SN of mice receiving another MAO-B inhibitor, MDL72974, suggesting that the latter is detrimental to the nigrostriatal dopaminergic neurons, which contributes to original knowledge. Accordingly, this drug should not be considered as an adjunct to L-dopa therapy in PD patients.

CHAPTER 5

The manuscript presented in chapter 5 provides an original contribution to knowledge with respect to the effects of MDL72974 on lipid peroxidation levels in mouse brain. The relative potencies of MPP^+, L-deprenyl and MDL72974 in altering the rate of mitochondrial O_2 consumption and SOD activities lead us to postulate that the number of TH immunopositive neurons in mouse brain would be more affected by L-deprenyl than by MDL72974 treatment. However, our results did not support this possibility. Accordingly, the increase in lipid peroxidation observed in MDL72975-treated mice is significant since it suggested that this

MAO-B inhibitor is detrimental to dopaminergic neurons through an as yet undetermined mechanism.

Appendix I

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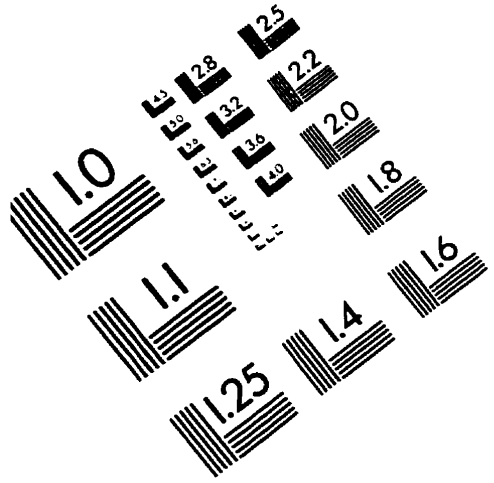
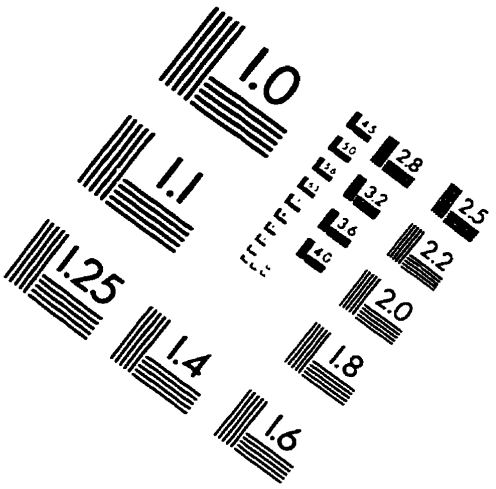
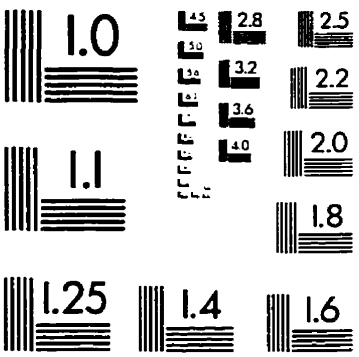
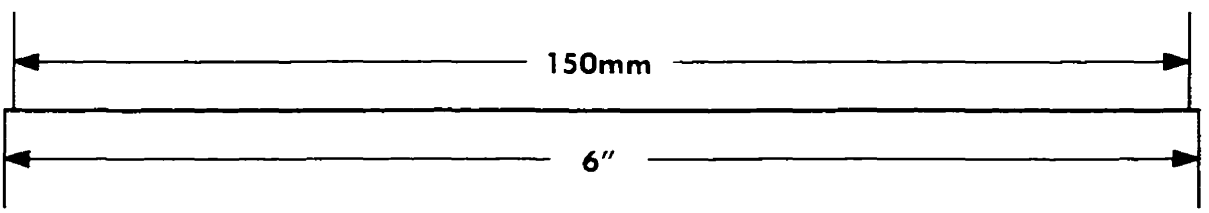
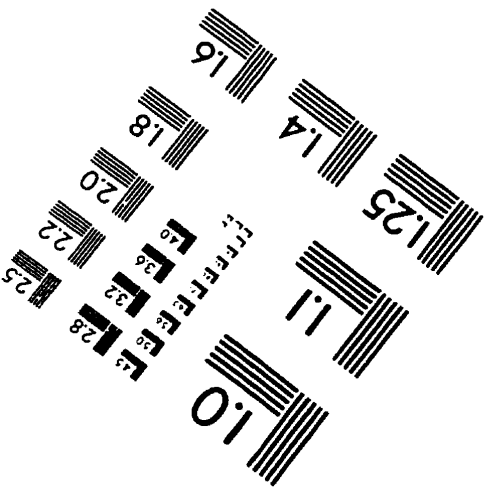
IMAGE EVALUATION
TEST TARGET (QA-3)

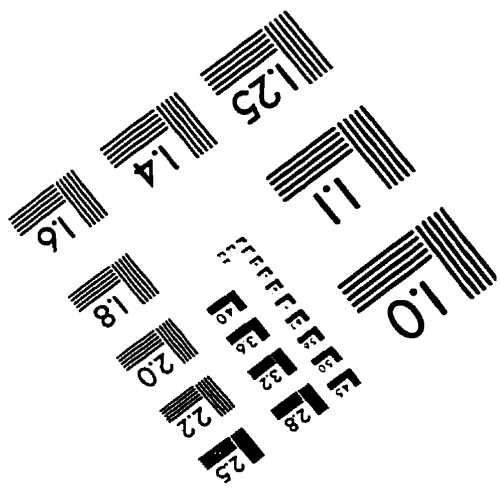












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