erability of the basal ganglia

by Willem N. Sloot

RIJKSUNIVERSITEIT GRONINGEN

Oxidant stress and vulnerability of the basal ganglia

Proefschrift

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Willem Nico Sloot

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te Ermelo

Promotor

Prof.Dr. J. Korf

Referent

Dr. J-B. P. Gramsbergen

Stellingen

behorend bij het proefschrift
"Oxidant stress and vulnerability of the basal ganglia"

o Vrij ijzer of 'low-molecular-weight' ijzercomplexen spelen geen direkte rol bij de toxiciteit van mangaan *(dit proefschrift)*.

o Oxidatie van dopamine door mangaan leidt niet (direkt) tot produktie van vrije zuurstofradikalen, en lijkt derhalve een ondergeschikte rol te spelen bij de neurotoxiciteit van mangaan *(dit proefschrift; bevestigt het in vitro werk van Archibald and Tyree: Arch. Biochem. and Biophys.* **256,** *638-650: 1987 en Segura-Aguilar and Lind: Chem.-Biol. Interactions* **72,** *309-324: 1989)*.

o De straatdrug 'angeldust' (phencyclidine) zou als medicijn voor de behandeling van de ziekte van Parkinson moeten worden overwogen.

o Het artikel waarin Ben-Shachar et al. beweren, dat een intranigrale Fe^{3+}-injektie selectief schade geeft van het nigrostriatale dopamine systeem is ten onrechte gepubliceerd *(J. Neurochem.* **57,** *2133-2135: 1991)*.

o Het nuttigen van voedingsvezels uit granen tijdens tenminste de puberale en vroeg adolescente periode van de vrouw zal de kans op het krijgen van borstkanker op latere leeftijd verminderen *(C.J.M. Arts, Proc. 5th Symp. on the Analysis of Steroids, Hungary, 1993)*.

o De stelling 'Daar kun je vergif op innemen' is binnen de toxicologie minder stellig.

o Het is taalkundig gezien een geruststellende gedachte dat de 'reserpinized rat' nog geen gezelschap heeft gekregen van de 'haloperidolized rat' en de bromocryptinized rat'.

o De uitspraak: 'Budgetary constraints applied thoughtlessly can ruin a research organization; if applied diligently, however, financial pressures are the most powerful tool to set priorities, to eliminate nonsense and to improve processes' *(Jürgen Drews, Hoffmann la Roche, at the International Pharmaceutical Growth Strategies conference, 1993)* is een terecht pleidooi tegen zowel de kaasschaaf als de botte bijl.

o Zonder neuronen bestaat er geen communicatie.

o De term 'junk-DNA' staat voor 'Ik weet het niet'.

o Het bevorderen van de anonimiteit van auteurs naar de referees toe bij het beoordelen van ingediende manuscripten is geen garantie voor een objektieve beoordeling.

o Het promotieproces heeft bepaalde socio-pathische trekjes.

Willem N. Sloot,

Promotiecommissie:

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voor San

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Grafisch Bedrijf Ponsen & Looijen B.V. Wageningen, 1995

List of abbreviations

AD	: Alzheimer's disease
ALS	: amyotrophic lateral sclerosis
AMPA	: α-amino-3-hydroxy-5-methyl-4-isoxasole-proprionic acid
BBB	: blood-brain barrier
CB	: cerebellum
CNS	: central nervous system
COL	: colchicine
COMT	: catechol-*o*-methyltransferase
CSF	: cerebrospinal fluid
CTX	: cerebral cortex
DA	: dopamine
DFX	: deferoxamine (desferal)
2,3-DHBA	: 2,3-dihydroxybenzoic acid
2,5-DHBA	: 2,5-dihydroxybenzoic acid
DOPA	: see L-DOPA
DOPAC	: 3,4-dihydroxyphenylacetic acid
DSN	: area dorsal to substantia nigra
EC(D)	: electrochemical (detection)
EDTA	: ethylenediaminetetracetate
EP	: entopeduncular nucleus
ESR	: electron spin resonance
FC	: frontal cortex
Fe	: iron
Ferrozine	: 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine monosodium salt
FX	: ferrioxamine
GABA	: γ-aminobutyric acid
GAD	: glutamic acid decarboxylase
Glu	: glutamate
GP	: globus pallidus
GPe	: globus pallidus externa
GPi	: globus pallidus interna
Gpx	: glutathione peroxidase
GSH	: reduced gluthatione
GSSG	: oxidized glutathione
HD	: Huntington's disease
5-HIAA	: 5-hydroxyindole-3-acetic acid
HMW	: high-molecular-weight
H_2O_2	: hydrogen peroxide
HP	: hippocampus
5-HT	: 5-hydroxytryptamine (serotonin)
HVA	: 4-hydroxy-3-methoxy-phenylacetic acid (homovanillic acid)
KA	: kainate
L-DOPA	: L-3,4-dihydroxy-phenylalanine
LMW	: low-molecular-weight
MANEB	: manganese ethylene-bis(dithiocarbamate)
MAO	: monoamine oxidase
MFB	: medial forebrain bundle
MMT	: methylcyclopentadienyl manganese tricarbonyl
Mn	: manganese
M(N)RI	: magnetic (nuclear) resonance imaging
MPP^+	: 1-methyl-4-phenylpyridinium
MPTP	: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	: multisystem atrophy
3-MT	: 3-methoxytyramine
NA	: nucleus accumbens
NE	: norepinephrine
NMDA	: N-methyl-D-aspartate
NO	: nitric oxide
NOS	: nitric oxide synthase
$O_2^{\cdot-}$	: superoxide anion radical
$^{\cdot}OH$	: hydroxyl radical
6-OHDA	: 6-hydroxydopamine
6-OH-DOPA	: 2,4,5-trihydroxy-phenylalanine
ONOOH	: peroxynitrite
PCA	: perchloric acid
PET	: positron emission tomography
PD	: parkinson's disease
PPN	: pedunculopontine nucleus
PSP	: progressive supranuclear palsy
QUIN	: quinolinic acid
ROD	: relative optical density
SA	: salicylic acid
SN	: substantia nigra
SNc	: substantia nigra pars compacta
SNr	: substantia nigra pars reticulata
SOD	: superoxide dismutase
ST	: (neo)striatum
STN	: subthalamic nucleus
TH	: tyrosine hydroxylase
THAL	: thalamus
UV	: ultraviolet
VTA	: ventral tegmental area

Contents

Scope and aim

This thesis focuses on the relationship between the vulnerability of the basal ganglia and oxidant stress. *Chapter 1* introduces the reader in general to oxidative stress (Part I), the neurobiology of the basal ganglia (Part II), and finally the (possible) links between (factors that induce) oxidative stress and manganese (Mn) neurotoxicity as well as idiopathic Parkinson's disease (iPD) and its related experimental models (Part III). Mn-intoxication is well recognized for its selective pathology of the basal ganglia, whereas iron is known for its free radical-related pathology. Therefore, acute Mn^{2+} and Fe^{2+} injections into the rat brain served as working models to test hypotheses with regard to oxidative stress and (non)-selective damage of the basal ganglia. Hence, these models will not provide direct answers regarding the pathogenesis of iPD, but may help to improve current insights into the significance of oxidative stress in basal ganglia pathology as well as the feasibility or benefit of anti-oxidant therapies.

Chapter 2 describes a further developed and characterized Mn model to produce selective lesions of the basal ganglia by means of micro-injections into rat striatum as a relatively cheap, reproducible and rapid technique. Dose-response and time-course studies were designed to test the selectivity and possible role of dopamine (DA) and endogenous iron in precipitating cell death by comparing Mn^{2+}, Fe^{2+} and 6-hydroxydopamine (6-OHDA) injections into various brain regions using different biochemical endpoints and combined histology and ^{45}Ca autoradiography. The latter technique was used as a marker of cell death. In addition, as described in *Chapter 3*, Mn kinetics and dynamics in the brain were studied using local tracer injections of ^{54}Mn and blockade of axonal transport through the medial forebrain bundle by mechanical and chemical ways, and selective destruction of intrinsic pathways within the basal ganglia. These experiments were conducted to reveal some aspects of the largely unknown physiology of Mn in the brain and to enable us to design experimental (or future therapeutic) drug manipulation studies with regard to the retention of Mn in this model as performed in Chapter 6.

Once the model had been sufficiently characterized, the next goal was to develop a method to demonstrate *in vivo* free-radical formation during neurodegeneration induced by Mn^{2+} or Fe^{2+}. *Chapter 4* describes a method to detect hydroxyl radicals (·OH) based upon the non-enzymatic reaction of these

radicals with salicylic acid (SA) to form its stable adduct 2,3-dihydroxybenzoic acid (DHBA), which can be simultaneously analyzed with biogenic amines and related metabolites. With this method, time-course and dose-response studies with intrastriatal Fe^{2+} or Mn^{2+} injections were designed to find out whether free oxygen radicals are a cause or a consequence of tissue damage as determined by biochemical endpoints.

Results of these studies with Fe^{2+} and Mn^{2+} are discussed in the *Chapter 5 and Chapter 6* respectively, and revealed interesting differences. Since it was concluded from the delayed production of 2,3-DHBA after Mn^{2+} that Mn^{2+} does not trigger $^{\cdot}OH$ itself, Chapter 6 also deals with the *in vivo* role of DA (or indirectly DA autoxidation) and endogenous iron in Mn^{2+}-induced free radicals. For the first goal, rats were pre-treated with reserpine to deplete striatal DA and tested for $^{\cdot}OH$ formation by Mn^{2+}. Since Mn interacts in several ways with iron homeostasis and released iron may explain the delayed Mn^{2+}-induced oxidative stress, studies were designed to analyze different endogenous iron pools in rat striatum after local Mn^{2+} injection. Another approach for this issue was chosen by using co-injections with deferoxamine to chelate "free" iron.

Finally, in *Chapter 7* the main conclusions and findings from the studies described in Chapters 2 to 6 are discussed and summarized.

Contents Chapter 1

Oxidant stress and vulnerability of the basal ganglia: Implications for neurodegenerative diseases

1.0. General introduction

The biochemical, anatomical and physiological organization of the central nervous system (CNS) renders it potentially more vulnerable to irreversible damage then other mammalian organs. In particular, the limited capacity of the CNS to regenerate is pertinent for its vulnerability. Some external influences that may affect the brain are prevented by the blood-brain barrier, which consists of a blood-cerebrospinal fluid (CSF) barrier, the choroid plexus, and a blood-brain barrier (BBB): the tight junctions between endothelial cells of the brain vasculature (Kandel and Schwartz, 1985). Among the other organs, only the testis and thymus possess a blood-organ barrier, probably to protect against auto-immunological attack.

The intriguing question to address is what intrinsic characteristics can be identified that could make the brain potentially vulnerable to metabolic and neurotoxic insults. For instance, the brain is highly dependent on oxygen and glucose, as the main source of combustion, because of its high energy need to exert its function as signalling organ via electrical conduction and neurochemical transmission, and its virtually lack of energy reserves. For this purpose, ATP-dependent pumps are constantly needed to maintain sodium/ potassium, calcium and chloride gradients between intra- and extra-neuronal compartments. Therefore, any energy failure may become critical. Also the neural release of neurotransmitters and binding to receptors is a critical process that needs a constant and stable micro-environment and can lead to substantial energy demands. Further, the salvatory transmission needed for rapid signalling is enabled by an insulator called myeline. Myeline contains not only plenty of easily, and potentially oxidizable, unsaturated lipids, but also has a complex geometry. So, it is not surprising that neurons are highly dependent on the support of glia (astroglia, microglia, ependymal cells and oligodendrocytes). Moreover, neurons in the CNS do not show mitosis after birth, so they cannot be replaced after injury. Despite this fact, the functional plasticity of the brain is not to be underestimated (i.e. hydrocephalis patients).

The brain also endures periods of sensitivity to insults, in particular during ontogeny and aging. During ontogeny, an immature BBB and the

complex process of rapid growth and constructing neuronal connections may be the main causes of vulnerability. On the other hand, changing intrinsic factors, i.e presence or absence, at certain developmental stages (including aging) may also result in a less or more vulnerable state (i.e. with hypoxia, glutamate neurotoxicity). During aging the inability of neurons to divide may also lead to all kinds of imperfections and/or storage of (degradation) products from aberrant metabolism (under specific circumstances). For instance, the formation of tangles and plaques in Alzheimer's disease, and Lewy bodies in Parkinson's disease (PD) may represent such phenomena.

The vulnerability of the brain, however, is not homogeneous, and intoxications or other pathologies are often characterized by selective brain damage. This suggests that specific (endogenous) factors within a given area may determine its selective vulnerability. Several major chronic neurodegenerative diseases with unknown cause(s) such as PD are characterized by lesions of the basal ganglia. The basal ganglia are, however, also injured under a wide variety of other acute and chronic conditions, including several intoxications, ischemia/hypoxia and head trauma (Jellinger, 1986a, b). Oxidative stress may be an important contributing factor, or even a common unifying mechanism, to explain the vulnerability of the basal ganglia as has been proposed for iPD (Olanow et al., 1992; Fahn and Cohen, 1992; Olanow, 1993; Coyle and Puttfarcken, 1993).

This thesis is focused on the relationship between the vulnerability of the basal ganglia and (factors that may cause) oxidative stress as studied by intoxications with two chemically related transition metals, iron and manganese.

Part I

1.1. Oxidative stress

1.1.1. Iron and oxygen: A dangerous pair

The damaging effect of oxygen in aerobic organisms is highly dependent on the presence of a transition metal such as iron, titanium, copper, vanadium, or cobalt, of which iron and, to a lesser extent, copper are physiologically the most relevant (Koster and Sluiter, 1994; Halliwell and Gutteridge, 1990; Ryan and Aust, 1992). The essential need of oxygen and iron for aerobic organisms forced them to develop defenses against reactive oxygen species, i.e. superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$), and to regulate the transport and storage of iron strictly (Crichton and Charloteaux-Wauters, 1987; Octave et al., 1983; Ponka et al., 1990). Since the mitochondrial respiratory chain is believed to be one of the most important sources of $O_2^{\cdot-}$ and H_2O_2, the latter oxygen species can be assumed to be present at all times in aerobic cells (Halliwell and Gutteridge, 1990; Halliwell, 1992; Boveris and Cadenas, 1982; Freeman and Crapo, 1982; Dykens, 1994). *Oxidative stress may be defined as a disbalance between the pro- and anti-oxidant state in aerobic organisms.* Oxidative stress is now believed to be an important process in a wide variety of diseases (see Table I), including several diseases and traumas of the CNS (Halliwell, 1992; Hall and Braughler, 1993).

1.1.1.1. Oxygen toxicity: Sources of deleterious oxygen radicals

A free radical is defined in a broad sense as any molecule or atom that contains one or more orbitals with an unpaired electron (usually the outer orbit), which makes it unstable and suitable for one-electron transfer reactions (Halliwell and Gutteridge, 1990). Among the reactive oxygen species ($O_2^{\cdot-}$, H_2O_2, $HO_2^{\cdot}$, $NO^{\cdot}$, $NO_2^{\cdot}$, etc.), $^{\cdot}OH$ are the most toxic, and react at great speed with several molecules found in living cells, including DNA, membrane lipids, proteins and carbohydrates.
There are now two processes known to be able to generate $^{\cdot}OH$:

(1) the iron-catalyzed Haber-Weiss reaction,
(2) the decomposition of peroxynitrite (ONOOH)

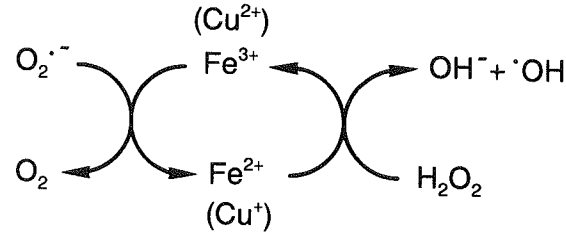
Table I: *Clinical conditions in which involvement of oxygen radicals has been suggested (adapted from Halliwell and Gutteridge, 1990).*

- Inflammatory-immune injury
  - Glomerulonephritis (idiopathic, membr.)
  - Vasculitis (hepatites B virus, drugs)
  - Autoimmune diseases
  - Rheumatoid arthritis
- Ischemia: reflow status
  - Stroke/myocardial infarction/arrhythmias
  - Organ transplantation
  - Inflamed rheumatoid joint?
  - Frostbite
  - Dupuytren's contracture
  - Dysbaric osteonecrosis
- Drug- and toxin-induced reactions
  - Iron overload
    - Idiopathic hemachromatosis
    - Dietary iron overload (Bantu)
    - Thalassemia and other chronic anemias treated with multiple blood transfusions
    - Nutritional deficiencies (kwashiorkor)
  - Alcoholism
    - Alcohol-induced iron overload
    - Alcoholic myopathy
  - Radiation injury
    - Nuclear explosions
    - Accidental exposure
    - Radiotherapy
    - Hypoxic cell sensitizers
  - Aging
    - Disorders of premature aging
  - Red blood cells
    - Phenylhydrazine
    - Primaquine, related drugs
    - Lead poisoning
    - Protoporphyrin photoxidation
    - Malaria
    - Sickle cell anemia
    - Favism
    - Fanconi's anemia
    - Hemolytic anemia of prematurity
  - Lung
    - Cigarette smoke effects
    - Emphysema
    - Bronchopulmonary dysplasia
    - Paraquat toxicity
  - Heart and cardiovascular system
    - Alcoholic cardiomyopathy
    - Keshan disease (selenium deficiency)
    - Atherosclerosis
    - Adriamycin cardiotoxicity
  - Kidney
    - Autoimmune nephrotic syndromes
    - Aminoglycoside nephrotoxicity
    - Heavy metal nephrotoxicity
  - Gastrointestinal tract
    - Endotoxic liver injury
    - Halogenated hydrocarbon liver injury (e.g. bromobenzene, carbon tetrachloride, halothane)
    - Diabetogenic action of alloxan
    - Pancreatitis
    - NSAID-induced gastrointestinal tract lesions
    - Oral iron poisoning
  - Brain/nervous system/neuromuscular disorders
    - Hyperbaric oxygen
    - Vitamin E deficiency
    - Neurotoxins, including lead
    - Parkinson's disease
    - Hypertensive cerebrovascular injury
    - Neuronal ceroid lipofuscinosis
    - Allergic encephalomyelitis and other demyelinating diseases
    - Aluminum overload (Alzheimer's disease)
    - Potentiation of traumatic injury
    - Muscular dystrophy
    - Multiple sclerosis
  - Eye
    - Cataractogenesis
    - Ocular hemorrhage
    - Degenerative retinal damage
    - Retinopathy of prematurity (retrolental fibroplasia)
    - Photic retinopathy
  - Skin
    - Solar radiation

Table I (continued)

Oxidant pollutants (O_3, NO_2)	Thermal injury
ARDS (some forms)	Porphyria
Mineral dust pneumoconiosis	Hypericin, other photosensitizers
Asbestos carcinogenicity	Contact dermatitis
Bleomycin toxicity	
SO_2 toxicity	

The first process is called the metal- or iron-catalyzed Haber-Weiss reaction (1) (Haber and Weiss, 1934), that consists of two major parts in which iron (or copper) and oxygen species react (which is a simplification of several intermediate reactions). One part of the reaction, the decomposition of H_2O_2 by Fe^{2+}, is called the Fenton reaction that leads to Fe^{3+}, OH^- and $^{\cdot}OH$. The initial product of reaction of Fe^{2+} and H_2O_2 may be another highly oxidizing species, an iron-oxygen complex called ferryl that then decomposes to yield $^{\cdot}OH$. The other part of the reaction combines Fe^{3+} with $O_2^{\cdot -}$ that produces Fe^{2+} and O_2 via a possible intermediate called perferryl. The overall reaction is (1):

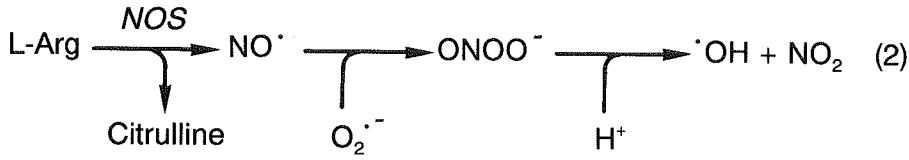


Fenton Reaction

The Haber-Weiss reaction is well established and is generally believed to occur *in vivo* in the presence of free iron or low-molecular-weight forms of iron (or copper) as the catalyst (Halliwell and Gutteridge, 1990).

The second process represents a recently discovered source of $^{\cdot}OH$ production independent of iron, namely via the L-arginine:NO pathway. NO is synthesized from L-arginine by NO synthase, and acts as a physiological signalling molecule in vascular tissue (blood vessel relaxation and platelet aggregation) and brain through diffusion and subsequent stimulation of (soluble) guanylate cyclase in the target cells. NO is also the effector molecule produced by activated immunological cells for killing microorganisms and tumor cells (reviewed by Moncada et al., 1991). In the brain, NO has probably a role in short-term effects of excitatory amino acids as well as long-term effects on brain development, learning and memory functioning. NO-synthase structually resembles P-450 reductase (Bredt et

al., 1991) and is widespread in brain tissue (Hope et al., 1991) with the highest concentrations in cerebellum, followed by hypothalamus and midbrain, striatum, and hippocampus. Under (excito)toxic conditions, NO signalling may be overexpressed (Halliwell, 1992; Garthwaite, 1990; Moncada et al., 1991), and/or NO may react with (excessive) $O_2^{\cdot -}$ to form peroxynitrite (ONOOH), which partially decomposes to $^{\cdot}OH$ and NO_2 (Beckman et al., 1990):



Peroxynitrite itself is also a highly oxidizing agent capable of causing tissue damage, i.e. protein oxidation and tyrosine residue nitration of, for example, receptors (Olanow, 1993). Noteworthy, the cytotoxic action of macrophages results in inhibition of complex I and II of the mitochondrial electron transport system of the target cells and macrophages themselves. In addition, it has been demonstrated with activated macrophages and other cells that NO causes inhibition of mitochondrial respiration, release of iron, and inactivation of, in particular, non-heme iron-sulfur-containing enzymes, which is probably due to formation of nitrosyl-iron-sulfur complexes (Moncada et al., 1991). Others have mentioned the possible neuroprotective effect of NO· by scavenging the more toxic reactive oxygen species (Chiueh, 1994).

1.1.1.2. How to measure free radicals?

The most direct and specific way to measure free (oxygen) radicals is by electron spin resonance (ESR) spectroscopy using specific radical trapping agents. However, ESR has limited utility in biological systems (Pou et al., 1989; Mason and Chignell, 1994). Since (oxygen) radical reactions occur very fast (10^{-10} s) and thus radicals themselves cannot be detected in living organisms, detection is historically based on indirect methods related to oxidation products of biological molecules, including DNA adducts (chemical alterations of deoxyribose and of purine and pyrimidine bases, and strand breakage), protein oxidations (carbonyl content assay and other assays; Levine et al., 1990; Packer and Glazer, 1990), and, most often, lipid peroxidation products of unsaturated fatty acids (diene conjugation, TBA test: reaction of malonaldehyde and thiobarbituric acid, and other assays)

(Halliwell and Gutteridge, 1985 and 1990; Packer and Glazer, 1990). Iron and copper are the only metals considered capable of initiating the process of lipid peroxidation *in vivo*, and that the (at all times) presence of trace amounts of these metals in samples are the principal basis for demonstrating lipid peroxidation in *in vitro* assays (Minotti and Aust, 1989; Halliwell and Gutteridge, 1990; Halliwell, 1992). Some agents such as nitroblue tetrazolium can be used to detect $O_2^{\cdot-}$, whereas deoxyribose degradation and aromatic hydroxylation assays (benzenes, phenols, salicylate, phenylalanine) have been used to detect $^{\cdot}OH$ (Halliwell et al., 1989). Salicylate is increasingly being used as a relatively safe compound to trap $^{\cdot}OH$ in living organisms, and thus may serve as an index of *in vivo* $^{\cdot}OH$ formation (Floyd et al., 1984 and 1986; Grootveld and Halliwell, 1986; Maskos et al., 1990; Ingelman-Sundberg et al., 1991). Both hydroxylation products of salicylate, the 2,3- and 2,5-dihydroxybenzoates, are used for this purpose, although only the 2,3 isomer is believed to be a strict non-enzymatic product, while the 2,5 isomer can also be formed enzymatically via cytochrome P-450 oxidation. Furthermore, oxidized glutathione (GSSG) and the ascorbyl radical have been suggested to serve as physiological indicators of *in vivo* oxidant stress, reflecting the activity of the intracellular defense system against reactive oxygen species (Hughes et al., 1990; Werner and Cohen, 1993; Roginsky and Stegmann, 1994).

Evidence of the occurence of oxidative stress under various toxic and pathological conditions is based on (partial) attenuation of tissue damage by antioxidants, including free-radical scavengers (phenol, salicylate, mannitol, uric acid, N-*tert*-butyl-α-phenylnitrone (spin trapping agent PBN), etc.), antioxidant vitamines (A, β-carotene, C and E), antioxidant enzymes (SOD, catalase, Gpx), and iron chelators (Desferal, ceruloplasmin, transferrin, etc.) (Halliwell, 1989; Halliwell and Gutteridge, 1986; Hall, 1992). Some biologically important antioxidants are summarized in Table II (adapted from Gutteridge and Halliwell, 1990).

Antioxidants can act by (Gutteridge, 1994):
1. removing oxygen (or decreasing local O_2), catalytic metal ions, or key reactive oxygen species such as $O_2^{\cdot-}$ and H_2O_2,
2. scavenging or quenching singlet oxygen or initiating radicals such as $^{\cdot}OH$, $RO^{\cdot}$, $RO_2^{\cdot}$,
3. breaking the chain of an initiated sequence

It should be noted, that many antioxidants have more than one mechanism of action (Desferal, propyl gallate, 21-aminosteroids).

Adverse effects of (excessive) NO can be inhibited by using hemoglobin or other compounds that react avidly with NO (Moncada et al., 1991), or by using specific NO-synthase inhibitors to block its formation. The latter has been used recently to attenuate the neurotoxicity of several mitochondrial toxins (Schulz et al., 1994).

Table II: *Some biologically important antioxidants (adapted from Gutteridge and Halliwell, 1990)*

Site	Mode of action
Extracellular	
Transferrin	Binds ferric ions
Lactoferrin	Secreted by phagocytic cells, binds ferric ions and retains them at low pH
Haptoglobins	Bind hemoglobin and deter hemoblobin from decomposing lipid peroxides
Hemopexin	Binds heme and prevents it from decomposing lipid peroxides
Albumin	Binds copper ions and heme tightly and iron ions weakly. Probably a site-specific sacrificial antioxidant. Scavenges hypochlorous acid and protects α-antiproteinase against it.
Ceruloplasmin	Catalyzes oxidation of ferrous ions and ferrous complexes to the ferric state for binding to transferrin (ferroxidase I activity). Ferroxidase activity involves the four-electron reduction of O_2 to H_2O with no reactive oxygen intermediates released. Binds copper ions non-specifically and inhibits copper-dependent radical reactions.
Superoxide dismutases	High-molecular-mass glycoproteins catalytically removes O_2, probably from endothelial cell surface (Only trace amounts present in bulk fluid).
Glutathione peroxidase	Large molecular mass selenium containing glycoprotein that can remove H_2O_2 and lipid peroxide (low concentration, function not yet clear).
Urate (uric acid)	Scavenges organic and inorganic oxygen radicals and can bind iron and copper ions and stop or slow their catalysis of free radical reactions.
Glucose	Rate constant for reaction with $^{\cdot}OH$ radicals ca. 1.0×10^9 M^{-1} s^{-1}. Present at 4-6 mM or higher after a carbohydrate-rich meal.
Bilirubin	Scavenges peroxyl radicals; may protect albumin-bound fatty acid molecules from oxidation.
Mucins	Scavenge $^{\cdot}OH$ radicals with high rate constant (ca. 5×10^9 M^{-1} s^{-1}) and bind metal ions.

Table II (continued)

Site	Mode of action
Membrane	
Vitamin E	Lipid-soluble, chain-breaking antioxidant. May also protect lipoprotein lipids in the plasma.
β-Carotene	Singlet oxygen and ·OH radical scavenger, inhibitor of lipid peroxidation under certain conditions.
Coenzyme Q	In the reduced state it may act as an antioxidant in addition to its major roles in energy metabolism.
Intracellular	
Superoxide dismutases (Cu and Mn enzymes in animals)	Catalytic removal from cells of $O_2^{\cdot-}$.
Catalase (contains four NADPH molecules)	Catalytic removal of H_2O_2 when H_2O_2 is at high concentrations (catalytic activity). Has a peroxidatic activity when methanol, ethanol, formate, and nitrite are electron donors.
Glutathione peroxidase (Se enzyme)	Catalytic removal of H_2O_2 and lipid hydroperoxides. Can effectively remove low steady-state levels of H_2O_2.

1.1.1.3. Iron toxicity

The toxicity of iron and hemoglobine (Hb) in brain has been demonstrated by local experimental injections of these agents, which serve as models for head trauma (from bleedings) (Thulborn et al., 1989; Triggs and Wilmore, 1984; Liu et al., 1994) and epilepsy as a result of head trauma (Willmore et al., 1986; Willmore, 1990). Brain trauma initiates a sequence of events that includes disturbances in blood flow (vasoregulation), BBB, intracranial pressure, as well as focal or diffuse ischemia, contusion, laceration, hematoma formation, and hemorrhagic infarction. Extravasation of blood followed by hemolysis and deposition of heme-containing compounds (hemoglobin) and/or release of iron is supposed to cause generation of free oxygen radicals (Willmore et al., 1983; Puppo and Halliwell, 1988) and lipid peroxidation (Triggs and Willmore, 1984; Willmore and Rubin, 1984; Willmore, 1990; Braughler and Hall, 1989; Hall and Braughler, 1993).

Under non-physiological circumstances (e.g. low pH inducing release of iron from proteins (Bralet et al., 1992), or imbalance of redox homeostasis) iron-containing proteins, including the hemeproteins hemoglobin, myoglobin and cytochromes, as well as transferrin, lactoferrin, and ferritin, have been suggested to participate in free oxygen radical

formation. Both the iron protein itself and iron mobilized from proteins have been suggested to produce oxyradical-induced tissue damage, although the first option for proteins such as transferrin, lactoferrin and ferritin has been disproved (Halliwell and Gutteridge, 1990). For instance, iron is mobilized at low pH (< 6) from transferrin, and from ferritin by strong reducing agents (6-OHDA, poorly by $O_2^{\cdot-}$, probably by paraquat and other redox-cycling compounds) or radicals generated during lipid peroxidation (Biemond et al., 1984; Halliwell and Gutteridge, 1986 and 1990; Bolann and Ulvik, 1990; Halliwell, 1992).

In addition, heme proteins (Oxy or Met) interact with H_2O_2 to form oxo-heme-oxidants or some other postulated heme-associated ferryl radical (Sadrzadeh et al., 1984; Grisham and McCord, 1986; Puppo and Halliwell, 1988), which may be the damaging radical (Halliwell and Gutteridge, 1990) These 'caged' ferryl-type radical species (i.e. the radical stays firmly within the protein) are as potent as $^{\cdot}OH$, stimulate lipid peroxidation and deoxyribose degradation, and will oxidize compounds such as phenols, aminopyrine and aromatic amines (Kalyanaraman et al., 1987; Puppo and Halliwell, 1988), but cannot be inhibited by mannitol or probably deferroxamine (Grisham 1985; Grisham and McCord, 1986). An excess of H_2O_2 degrades hemoglobin releasing iron that reacts with H_2O_2 to form deleterious $^{\cdot}OH$ (Sadrzadeh et al., 1984; Puppo and Halliwell, 1988; Halliwell and Gutteridge, 1990; Halliwell, 1992; Gutteridge, 1994).

Plasma contains proteins such as haptoglobins and hemopexin to bind and conserve free hemoglobin and heme iron, which diminishes heme protein-induced reactions (Gutteridge, 1994). Hemosiderin, probably some kind of breakdown product of ferritin, is also incapable of catalyzing Haber-Weiss-like reactions or demobilizing iron.

Beside iron-containing proteins, there exists a pool of small simple iron chelates (or transit, intermediary or low-molecular-weight pool), probably representing iron attached to phosphate esters (ATP/ADP, GTP), to organic acids (such as citrate), and perhaps to the polar head groups of membrane lipids, or DNA. The exact chemical nature and extent of this pool, and its possible participation in mediating oxidative damage -although often suggested- is far from clear (Crichton and Charloteaux-Wauters, 1987). Table III summarizes biological iron complexes and their possible participation in oxygen radical reactions (adapted from Halliwell and Gutteridge, 1990).

Table III: *Biological iron complexes and their possible participation in oxygen radical reactions (adapted from Halliwell and Gutteridge, 1990)*

Type of iron complex	Decomposition of lipid peroxides to form alkoxyl and peroxyl radicals	Hydroxyl radical formation by Fenton chemistry
Loosely bound iron		
Iron ions attached to		
Phosphate esters (eg. ATP)	Yes	Yes
Carbohydrates and organic acids (e.g. citrate, picolinic acid, deoxyribose)	Yes	Yes
DNA	Probably yes	Yes
Membrane lipids	Yes	Yes
Mineral ores, e.g. asbestos, silicates	Yes	Yes
Iron tightly bound to proteins		
Non-heme iron		
Ferritin (4500 mol Fe per mol protein)	Yes	Yes (when Fe is released)
Hemosiderin	Weakly	Weakly (when Fe is released)
Lactoferrin (iron saturated, 2 mol Fe^{3+} per mol protein)	No	No (only if Fe is released)
Transferrin (iron saturated, 2 mol Fe^{3+} per mol protein)	No	No (only if Fe is released)
Heme iron		
Hemoblobin	Yes	Yes (when Fe is released)
Leghemoglobin	Yes	Yes (when Fe is released)
Myoblobin	Yes	Yes (when Fe is released)
Cytochrome c	Yes	Yes (when Fe is released)
Catalase	Weakly	Not observed (theoretically possible if the enzyme were inactivated and Fe were released)

1.1.2. Brain defense mechanisms against oxidative stress

In general, the defense mechanism against iron and oxygen toxicity is to limit reactive oxygen species, and to handle iron (and copper) safely.

1.1.2.1. Oxidative defense enzymes

In every cell consuming oxygen, $O_2^{\cdot-}$ is dismutated to H_2O_2 and oxygen

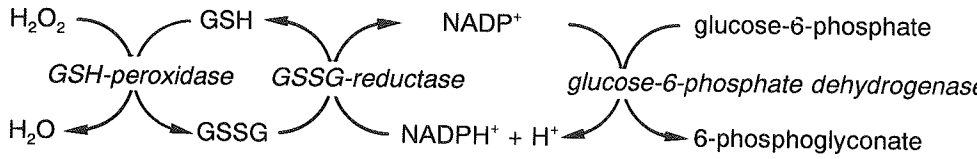
by superoxide dismutase (SOD). The first line of defense against oxygen toxicity exists in mainly two forms, an inducible Mn-containing SOD (in response to oxygen challenge) that is located in mitochondria, and a Cu/Zn-containing SOD that is located in the cytosol and in small amounts in extracellular fluids. The latter is glycosylated.

$$2\ O_2^{\cdot -} \xrightarrow{SOD} O_2 + H_2O_2$$

Subsequently, hydrogen peroxide is converted into oxygen and water by catalase, a heme protein containing four NADPH molecules, which is most likely located in peroxysomes:

$$2\ H_2O_2 \xrightarrow{catalase} O_2 + H_2O$$

Or, H_2O_2 reacts with glutathione peroxidase (Gpx), a selenium containing enzyme in the cytosol and mitochondria (~10%), to form oxidized gluthatione (GSH) and water. Oxidized GSH (GSSG) is reduced back to GSH by glutathione reductase notably at the expense of NADPH, of which the pool is limited compared to the GSH pool in liver (Reed, 1986).



The distribution of antioxidant enzymes in the brain may in part determine selective regional damage. In general, the rat brain is poor in catalase activity, the highest activity having been reported in substantia nigra (Brannan et al., 1981). Others have reported abundant SOD levels in rat brain and liver (Asayama and Burr, 1985; Inagaki et al., 1991). *In vitro* studies have shown, that in glia antioxidant properties are better represented than in neurons with regard to activities and contents of SOD and enzymes of GSH metabolism as well as vitamin E levels (Savolainen, 1977; Makar et al., 1994).

In contrast, antibodies directed against Mn-SOD stained neurons more abundantly and more intensively than glia in the human brain. The distribution was heterogenous with the most intense staining in caudate nucleus and putamen (Zhang et al., 1994; Dobashi et al., 1989; Akai et al., 1990 Inagaki et al., 1991a; Inagaki et al., 1991b). Since Gpx is exclusively

located in glial cells to eliminate H_2O_2, it is most likely that glial cells lacking Mn-SOD contain Cu/Zn-SOD (Damier et al., 1993). In rat striatum, Mn-SOD is present in several types of neurons as evidenced by double-immunostaining (cholinergic, somatostatinergic) and morphology (GABAergic) (Inagaki et al., 1991b). With respect to catalase, Gpx, Mn-SOD or Cu/Zn-SOD activity, quite comparable amounts and ontogenic patterns are found in various dissected regions of the rodent brain (Del Maestro and McDonald, 1987; Przedborski et al., 1992). Elevated Gxp, Mn-SOD and Cu/Zn-SOD levels are found in rat cerebral cortex of aged rats (Scarpa et al., 1987), whereas in whole mice brain mRNA levels of Cu/Zn-SOD increased without changes in mRNA Gpx (de Haan et al., 1992). In the latter study, the increased SOD levels were associated with an enhanced susceptibility to lipid peroxidation with age.

Both the concept of a high regional content or activity of antioxidant enzymes that protect against oxidative damage, and a low regional content or activity that render such cells susceptible to oxidative stress have been suggested and speculatively linked to different, selective brain pathologies (Inagaki et al., 1991a,b; Przedborski et al., 1992; de Haan et al., 1992; Zhang et al., 1994). Additional research is required to test these concepts.

Besides selectivity after oxidative challenge through normally occurring differences in regional antioxidant enzyme expression, selective neuropathology may also occur after a lack or dysfunction of antioxidant enzymes. A striking example of the latter is the recently discovered Cu/Zn-SOD mutation in familial amyotrophic lateral sclerosis (ALS), which is associated with a decreased enzyme activity (not in sporadic ALS)(Rosen et al., 1993; Bowling et al., 1993; Robberecht et al., 1994). ALS patients (both familial and sporadic) have selective lesions in the anterior horn of the spinal cord and motor cortex.

1.1.2.2. Brain iron homeostasis

Since free iron and, to a lesser extent, copper are necessary to catalyze the Haber-Weiss reaction, the nature of oxidative damage by excess formation of $^{\cdot}OH$ through this reaction is dependent on the localization and sequestration of these metals.

It is clear that organisms require iron for a large number of biological processes, including electron transfers in mitochondria (Fe-S clusters, cytochromes), the transport, storage and activation of oxygen, important enzyme functions, and many other functions (reviewed by Ponka et al.,

1990). Iron deficiency in utero or during the first three weeks of life leads to a permanent deficit in cerebral iron even if hematologic parameters are restored by supplementation. Various alterations in behavior, endocrinologic and neurochemical functions have been observed in iron-deficient rats as well as in formerly iron-deficient rats (Dallman et al., 1975; Dallman and Spirito, 1977; Ben-Shachar et al., 1986; Ben-Shachar and Youdim, 1990; Youdim et al., 1980, 1983, 1989 and 1990; Dwork et al., 1990). However, the specific functions of iron in the brain are largely unknown.

The heterogeneous distribution of non-heme iron as shown by Perl's blue staining in the rat and primate brain differs from the regional distribution of its transport protein transferrin (Francois et al., 1981; Hill and Schwitzer, 1984; Dwork et al., 1988; Mash et al., 1990; Connor et al., 1990; Morris et al., 1992; Benkovic and Connor, 1993). The regional distribution of ferritin and non-heme iron, however, are very similar and suggest that non-heme iron is essentially stored in ferritin. A single protomer molecule containing 24 subunits of ferritin can sequester up to 4500 mol iron as ferrihydroxyphosphate per mol protein (Joshi and Zimmerman, 1988).

The highest intensity of non-heme iron staining in brain is found in, for instance, the insulae of Calleja, circumventricular organs, and olfactory tubercle, followed by high to very high levels in all regions belonging to the basal ganglia (Hill, 1990). At present it seems that neuroglia (including choroid plexus and ependymal cells) have important iron-regulating functions, in particular oligodendrocytes which contain ferritin and transferrin and synthesize transferrin receptors, while the role of microglia may be significant in the sequestration and detoxification of iron (Benkovic and Connor, 1993). In addition, although generally not much iron is observed in neurons, this study showed an ferritin-independent accumulation of neuronal iron with age. There is little overlap in distribution of transferrin receptors and iron, and most areas with a high concentration of iron receive (often GABAergic) input from sites with dense transferrin receptors, suggesting neuronal transport of iron (Dwork et al., 1990; Hill, 1990).

Iron is distributed in the body predominantly attached to transferrin, which contains two binding sites for ferric iron and binds to the transferrin receptor which is internalized, followed by iron release in an intracellular acidic vescicle (lysosome or other endosome) and subsequent sequestration to cytosol ferritin (Octave et al., 1983; Crichton and Charloteaux-Wauters, 1987; Roberts et al., 1993). The exact sequestration and mobilization of iron to and from ferritin and the existence of a so-called transit (or low-

molecular-weight) iron pool (an intermediate between ferritin and pool of iron-sulfur proteins, heme proteins and non-heme enzyme iron) are largely unknown.

1.1.2.3. Copper homeostasis

The brain contains relatively high copper levels (Prohaska, 1987), but little is known about copper or whether it becomes available to stimulate free-radical reactions. If so, the Cu-catalyzed Haber-Weiss reaction is much slower than that of iron (Halliwell and Gutteridge, 1985). Cu-containing proteins in the brain are mitochondrial cytochrome-c oxidase, Cu/Zn-SOD, dopamine-β-monooxygenase (for the synthesis of norepinefrine), neurocuprein and other proteins (Prohaska, 1987). Cu is enriched in the locus ceruleus, substantia nigra and hypothalamus, which may be related to noradrenergic functions.

In plasma copper is transported to albumin and the copper-containing protein ceruloplasmin, which also quickly oxidizes ferrous to ferric iron that can bind to transferrin (Halliwell and Gutteridge, 1986; Gutteridge, 1994).

Wilson's disease is a well-known Cu-storage disease with pathology in the basal ganglia that can be treated by chelation therapy (Mena et al., 1970).

1.1.3. Mitochondrial (dys)functions and oxidant stress

The electron transport chain that is incorporated in the inner membrane of mitochondria consists of five major multiple subunit enzyme complexes (I-V) to generate ATP from ADP, while reducing oxygen to water, a process called oxidative phosphorylation (see Figure 1).

Beside the mentioned leakage of reduced oxygen species (H_2O_2, $O_2^{\cdot-}$) of mitochondria during this process, there appears to exist a close interrelationship between mitochondrial energy impairment and oxidative stress in disease and toxicology. For instance, each site or complex of the electron transport chain can be specifically blocked by mitochondrial toxins such as rotenone, antimycin A, KCN, and CO. Strikingly, these and other mitochondrial toxins, including rotenone (Heikkila et al., 1985), CN, CO, Mn, MPTP, azide, and 3-nitropropionic acid, often cause selective (experimentally induced) lesions in the basal ganglia (see also section 1.2.3), which are often accompanied by an excitoxic component (Beal, 1992;

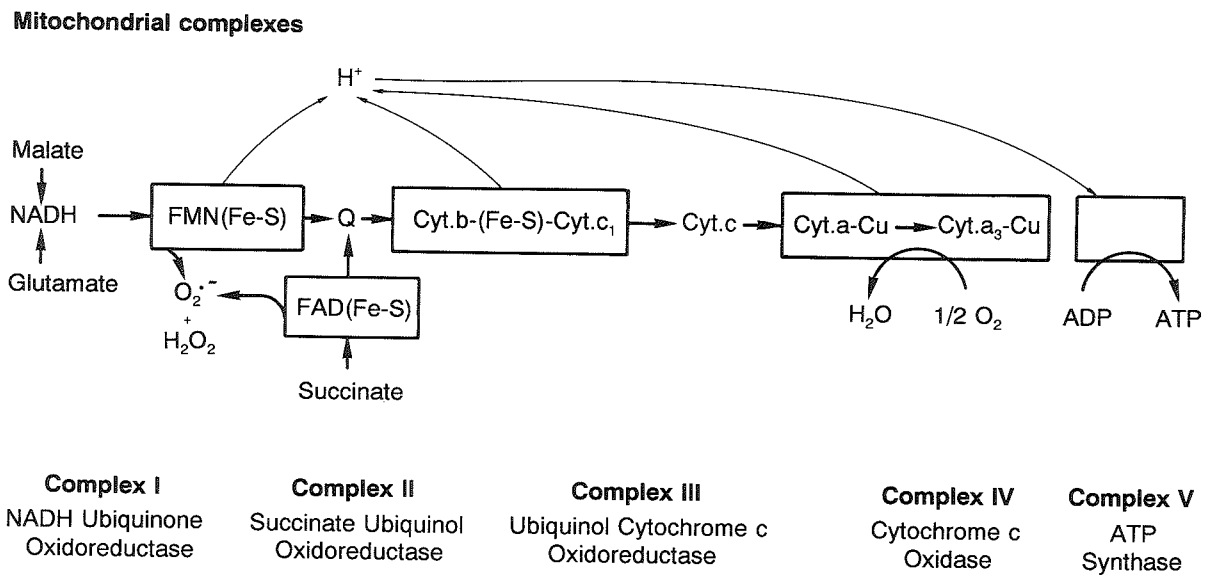


Fig. 1: *Mitochondrial respiratory chain complexes and $O_2^{\cdot-}$ leakage (adapted from Freeman and Crapo, 1982 and Beal, 1992)*

Simpson and Isacson, 1993). In addition, in recent years, specificdefects in complexes of the mitochondrial respiratory chain have been discovered in PD, Huntington's disease (HD) and Alzheimer's disease, which have been hypothetically linked to mitochondrial DNA mutations and oxidative stress (Schapira et al., 1992; Wallace, 1992; Coyle and Puttfarcken, 1993).

Impairments in GSH metabolism and mitochondrial energy metabolism may induce considerable oxidative stress and may account for (slow) selective neurodegeneration as will be discussed in sections 1.3.2.3 and 1.3.2.4.

1.1.3.1. Cytochrome P-450 enzymes

It has been demonstrated that P-450 oxidative enzyme activity induced by xenobiotics results in increased $O_2^{\cdot-}$ generation and utilizes NADH or $NADPH^+$ both in liver (Kappus, 1986) and in the brain (Ghersi-Egea et al., 1991). Despite the low P-450 content and activity of the brain as compared to liver microsomes (Ravindranath et al., 1989; Ghersi-Egea et al., 1993; Anandatheerthavarada et al., 1993), drug metabolism by the cytochrome P-450 mono-oxygenase system in the CNS may have specific consequences for the expression of toxic effects in this organ (reviewed by Mesnil et al., 1984; Minn et al., 1991). In particular, brain mitochondria may be susceptible, since they are 10 times more enriched in total P-450 content than brain microsomes (Ghersi-Egea et al., 1993).

For example, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity can be increased and decreased by P-450 manipulation (Pai and Ravindranath, 1991). In the latter study, the cytochrome P-450 inhibitors piperonyl butoxide and SKF 525A were found to offer protection against MPTP-induced neurotoxicity in brain slices without affecting monoamine oxidase (MAO), whereas pretreatment with phenobarbital, an inducer of cytochrome P-450, potentiates MPTP toxicity. In addition, the MAO blockers pargyline and deprenyl inhibit cytochrome P-450 activity in brain slices, whereas pargyline decreases these levels *in vivo*.

This relatively new area needs further investigations to prove its significance in neurotoxicology, including effects associated with oxidative stress.

PART II

1.2. The basal ganglia

1.2.1. Neurobiology of the basal ganglia

The basal ganglia circuitry (Fig. 2A) consists of interconnected subcortical nuclei, including caudate, putamen (or neostriatum in rodents), globus pallidus interna (GPi, or entopeduncular nucleus in rodents) and externa (GPe), substantia nigra (SN), subthalamic nucleus (STN), and some thalamic subnuclei (ventral lateral, ventral anterior, mediodorsal, and centromedian-parafascicular complex, of which the latter projects to the striatum) that regulate complex extrapyramidal or automatic voluntary movements.

The basal ganglia receive excitatory, glutamatergic input from large parts of the neocortex, and project predominantly back to the cortex (excitatory, Glu) via thalamic nuclei, thereby closing the cortico-basal ganglia-thalamic loop. Functionally the intrinsic basal ganglia motor circuits can be distinguished into a direct and an indirect pathway. The direct pathway involves a direct inhibitory (GABA/substance P) striatal output to the GPi and SN pars reticulata, which sends inhibitory projections (GABA) to target nuclei in the thalamus. The indirect pathway consists of an inhibitory striatal output (GABA/enkephaline) to the GPe that projects (GABA) to the STN, which in turn projects to (excitatory, Glu) the Gpi and SN pars reticulata (Alexander and Crutcher, 1990). The role of the SN pars compacta, which sends dopaminergic (DA) input to the striatum is ambivalent and complex. This DA input exerts contrasting effects on the direct and indirect pathways, a net excitatory and inhibitory effect respectively, thereby reinforcing any cortically initiated activation of a particular selected circuit (=action) by both facilitating conduction through the direct pathway (net excitatory effect on thalamus) and suppressing conduction through the indirect pathway (net inhibitory effect on thalamus). The mosaic-like patch-matrix organization (or striosomes-modules) of the striatum, which will not be discussed here, may represent a functional compartmentalization related to functional columns and layers in the cortex (Gerfen, 1989 and 1992; Graybiel, 1990).

It has been suggested that the direct pathway selects specific motor synergies to carry out a desired action, and that the indirect pathway

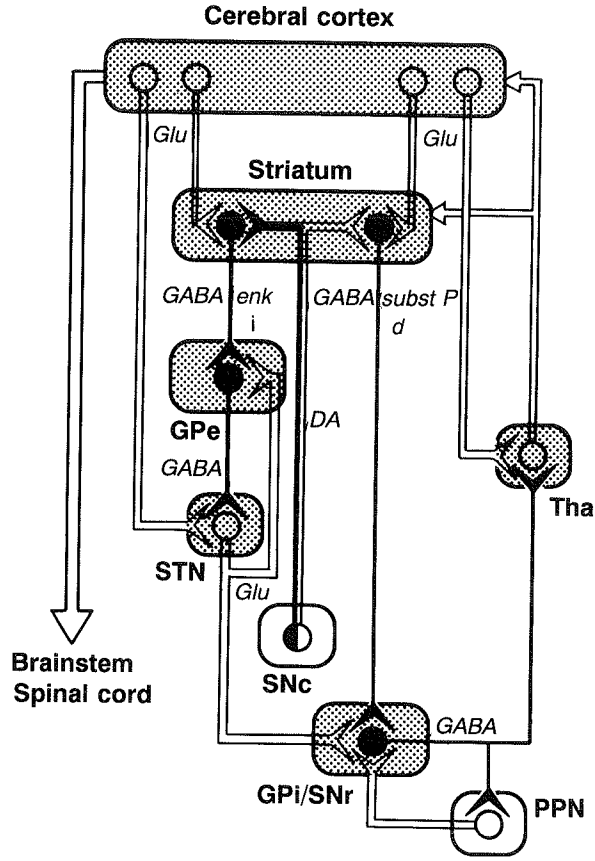


Fig. 2A: *Schematic diagram of the circuitry and inhibitory (filled symbols) and excitatory (open symbols) neurotransmitters of the basal ganglia, indicating the 'direct' (d) and 'indirect'(i) pathways from the striatum to its output nuclei (GPe, GPi/SNr). Abbreviations: DA, dopamine; enk, enkephalin; GABA, γ-aminobutyric acid; GPe, external segment of globus pallidus; GPi, internal segment of globus pallidus; glu, glutamate; PPN, pedunculopontine nucleus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; subst P, substance P; STN, subthalamic nucleus; Thal, thalamus.*

inhibits these synergies (Hallett, 1993). In addition, the pathway to the thalamus is meant for specific movements, while the pathway to the pedunculopontine nucleus (PPN), which receives input from the basal ganglia via the GPi/SN pars reticulata, is meant for postural and reflex influences. These pathways are supposed to work antagonistically to each other. In other words, when voluntary movements are facilitated, reflexes are inhibited and vice versa (Hallett, 1993).

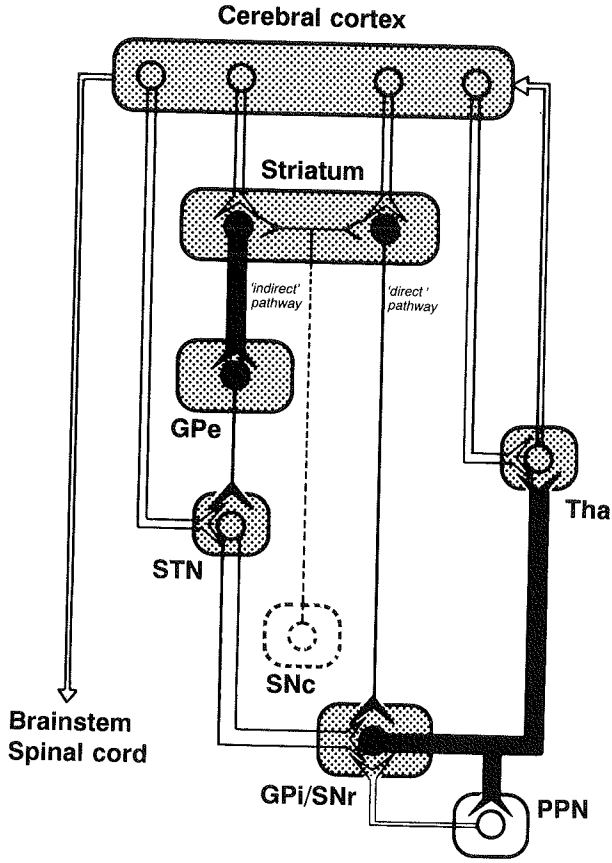


Fig. 2B: *Schematic diagram representing neuronal activity in the basal ganglia-thalamocortical circuitry in hypokinetic disorders after neurodegeneration of the dopaminergic nigrostriatal pathway (dashed). Excessive inhibition of GPe within the indirect pathway leads to disinhibition of the STN, which in turn provides excessive excitatory drive to the basal ganglia output nuclei (Gpi/SNr), thus leading to excessive thalamic inhibition. This is reinforced by reduced inhibitory input to GPi/SNr through the direct pathway. Overall, these effects are postulated to result in a reduction in the usual reinforcing influence of the motor circuit upon cortically initiated movements. For abbreviations see Fig. 2A.*

1.2.2. Clinical features of basal ganglia disorders

A disbalance between the direct and indirect pathways may account for hypo- or hyperkinetic features of *basal ganglia disorders* (see Fig. 2B; Albin et al., 1989; DeLong, 1990). In addition, depending on expression of these pathways, the influence on the PPN gives rise to an accessory state of reflex responsiveness (Hallett, 1993). Basal ganglia or extrapyramidal

disorders are a heterogeneous group of abnormal movements with a common anatomic locus within the basal ganglia. Based on clinical appearance, pharmacological and post-mortem (human and experimental/animal) data, these abnormal movements can be classified into two major groups (Albin et al., 1989). The first group, hyperkinetic movement disorders, are typified by an excess of movement with uncontrollable and relatively rapid motor acts (chorea) intruding into the normal flow of motor activity, and are represented by Huntington's disease (HD), ballism, and tics (Gilles de la Tourette's syndrome). The second group, hypokinetic disorders, are characterized by akinesia, bradykinesia and rigidity, which involves slowness of movements, increased muscular tone, a paucity of spontaneous movements, and tremor, and is represented by parkinson's disease (PD). Dystonia may be catagorized separately, since it consists of the spontaneous assumption of unvoluntary, unusual fixed postures lasting seconds to minutes, or prolonged voluntary movements, which are slow and clumsy, due to excessive activity in muscles not needed for the task. Similarities and differences exist between dystonia and PD, but are sometimes difficult to distinguish.

In general, it appears that the complex clinical features of movement disorders result from selective loss or altered "behavior" of (sub)populations of neurons intrinsic to the basal ganglia or, in other words, cause the disbalance between the direct and indirect pathways. In approximation, the abnormal movements of hyperkinetic disorders result from impairment of subthalamic nucleus (STN) function, either as a result of destruction of the STN itself or, more commonly, as a consequence of the selective impairment of output from the striatum (subpopulation of GABAergic neurons) to the lateral globus pallidus. Parkinsonism seems to be associated with an increase in basal ganglia output due to complex changes in the activity of striatal neuron subpopulations as a consequence of dopamine loss. Although post-mortem data are scanty, dystonia (symptomatic hemidystonia) seems to be associated with lesions in the putamen, pallidum, or thalamus, which can be the end result of HD or other neurodegenerative diseases such as cases of cerebral palsy and progressive supranuclear palsy (PSP)(Marsden, 1988). The latter are called secondary or symptomatic dystonia. It has been suggested that dystonia results from a gross loss of basal ganglia output rather than a specific alteration in any striatal neuron subpopulation (Albin et al., 1989).

While the role of the basal ganglia in automatic execution of learned motor movements and movement disorders is established, the involvement of these brain regions in cognitive functions and dysfunctions is

increasingly studied (Brown and Marsden, 1990).

1.2.3. Neuropathology and intoxications of the basal ganglia

The cause(s) of neurodegenerative diseases of the basal ganglia of endogenous origin, including idiopatic PD (iPD), are largely unknown. However, a wide variety of acute and chronic conditions of exogenous origin, including MPTP, Mn and other intoxications, cause selective lesions of the basal ganglia. Some drug-induced lesions of the basal ganglia, including those produced by 6-hydroxy-DA (Heikkila et al., 1989) and excitotoxins (Jackson et al., 1989) in experimental animals, have been used as models of PD and HD, and together with the other chemicals and conditions here discussed, are speculative for some common mechanism of neurodegeneration in the basal ganglia.

Striatal and pallidal lesions have been observed under various conditions, including cerebrovascular diseases such as compromised blood flow conditions and atherosclerosis, head injuries, intoxications, increased intracranial pressure of different origin, and all types of hypoxia-ischemia or hypoxic-hypotensive insults both in immature and adult brain, including cardio-respiratory failure, hanging, and hypoglycemia, and occur as late sequelae of bilirubin encephalopathy/kernicterus, and perinatal brain damage referred to as infantile bilateral striatal necrosis (reviewed by Jellinger, 1986a, b). In some of these disorders or individual cases the pathological lesions are more or less confined to striatum or pallidum, but most often both are involved and are associated with lesions in the reticular zone of substantia nigra, or may show additional multi-focal or diffuse non-selective lesions in the cerebral gray and white matter, or extensions to the subthalamic nucleus. Acute and chronic intoxications reported to affect the basal ganglia are listed in Table IV.

The pathogenesis of striatal and pallidal necrosis in most exogenous conditions remains to be elucidated. However, the frequent occurrence in a wide range of hypoxic/ischemic-hypotensive and toxic conditions as well as metabolic disorders affecting the basal ganglia suggests a preferentially vulnerability of these nuclei to oxidative stress. Indeed, oxidative stress of the basal ganglia is demonstrated after hypoxia or ischemia-reperfusion (Cao et al., 1988; Zhang and Piantadosi, 1992; Althaus et al., 1993; Hall et al., 1993a), head injuries (Hall and Braughler, 1993; Hall et al., 1993b), and multiple intoxications, in particular after exposure to mitochondrial toxins (see Table IV). Recent studies of Beal et al., using local or systemic

administration of toxins acting at various sites of the mitochondrial chain, have shown selective lesions of the basal ganglia often associated with additional excitotoxicty (Beal et al., 1992; Brouillet et al., 1994). However, the selectivity within the basal ganglia differs, which may depend on the degree of energy impairment or the particular complex of the mitochondrial respiratory chain involved (Brouillet et al., 1994). For instance, 3-nitropropionic acid and azide selectively deplete striatal interneurons and irreversibly inhibit complex II and IV, respectively. Aminooxyacetic acid, 1-methyl-4-phenylpyridinium (MPP^+) (Complex I), and malonate spare striatal interneurons and effects on mitochondrial respiratory chain complexes are reversible or in the case of MPP^+ slowly irreversible (Cleeter et al., 1992).

Table IV: *Acute and chronic intoxications (M = mitochondrial toxins) producing selective basal ganglia lesions accompanied with oxidative stress (OxS)*

Acute	**Chronic**
methanol	alcohol
carbon monoxide[F] (M, OxS)	carbon monoxide (M)
cyanide[E] (M, OxS)	cyanide (M)
carbondisulfide	carbondisulfide
morphine and other drugs	neuroleptic drugs (tardive dyskinesia)
barbiturates	lead
aminooxyacetic acid[K]	
3-nitropropionic acid[A, H, L] (M, OxS)	3-nitropropionic acid[G] (M)
salvarsan	
methylchloride/bromide[D] (OxS)	methylchloride
MPTP (M) and analogues[C, I] (OxS)	MPTP (M) and analogues
phosphorus	
manganese[M] (M, OxS)	manganese (M)
malonate[A]	
rotenone[J] (M)	
sodium nitrite	
sodium azide[L] (M)	

References: Jellinger, 1986a & b, unless indicated otherwise:
A, Beal, 1992; B, Brouillet et al., 1993; C, Wu et al., 1993; Chiueh et al., 1994; D Davenport et al., 1992; E, Johnson et al., 1987; F, Zhang and Piantadosi, 1992; G Hamilton and Gould, 1987; H, Beal et al., 1993; I, Adams and Odunze, 1991a; J, Heikkila et al., 1985; K, Beal et al., 1991; L, Brouillet et al., 1994; M, Liccione and Maines, 1988 Brouillet et al., 1993b.

1.2.4. Intrinsic vulnerability of the basal ganglia

Already in 1922, Vogt and Vogt postulated the so-called pathoclisis theory for striato-pallidal lesions (Jellinger, 1986a), representing an inherent selective vulnerability based upon that region's intrinsic metabolic and general physicochemical properties, including the pattern of oxidative metabolism and high iron content. Another possible cause for the pathogenesis of the pallidum was based on the different aspects of regional vascular supply. It has also been suggested that multiple factors are responsible for the development of basal ganglia lesions.

In the brain, the basal ganglia contain the highest levels of iron (Hill and Schwitzer, 1984; Hill, 1990; Youdim et al., 1990) and DA (Westerink, 1979 and 1985) and highest DA turnover (Riederer and Youdim, 1986), and possess abundant glutamatergic (Glu) receptors (Albin et al., 1992). Under non-physiological conditions these endogenous substances are potentially harmful, and may enhance each other's toxicity leading to local oxidative stress via different mechanisms.

1.2.4.1. Iron homeostasis

The basal ganglia are frequently the foci of major damage in iron storage disorders, including Hallervorden-Spatz disease (Swaiman, 1991), Pick's disease (Swaiman, 1991) and Fahr's syndrome (Beall et al., 1989) due to unknown, but aberrant (brain) iron metabolism. In postmortem Hallervorden-Spatz tissue, the GP and SN pars reticulata are rust-brown pigmented by strong iron accumulation, and clinically patients demonstrate dystonia, rigidity and choreoathetosis, which are distinguishable from other extrapyramidal diseases. In addition, iron metabolism alterations have been shown in PD and other extrapyramidal disorders (see section 1.3.2.1). Visualizing these iron stores by MRI can be used to study these movement disorders (Rutledge et al., 1987).

In particular the GP and SN pars reticulata contain iron levels, which are similar to or may even exceed liver iron stores. The basal ganglia and other Fe-containing brain areas seem to be associated with GABAergic projections (Hill, 1990). Microscopic examinations revealed that iron in the extrapyramidal system is predominantly associated with glial cells, i.e. oligodendrocytes and their myelin sheaths (Francois et al., 1981). The function(s) of iron (stores) within the basal ganglia are largely unknown (Youdim et al., 1990). Besides the requirement of iron as a co-factor in the

functioning of tyrosine hydroxylase, a cytosolic rate-limiting enzyme in the synthesis of catecholamines (Nagatsu et al., 1964, Rausch et al., 1988), a function for Fe has been suggested in binding of DA to the D2-receptor (Ben-Shachar et al., 1986; Youdim et al., 1980, 1983, 1989 and 1990) and utilization of GABA (Hill et al., 1985a; Hill, 1990).

1.2.4.2. Dopamine and oxidative stress

Recently, direct injections of DA into striatum have demonstrated dose-dependent tissue damage pre- and postsynaptic to the nigrostriatal pathway and no damage after NaCl or GABA injections (Filloux and Townsend, 1993). At present, this finding offers the most direct evidence for the *in vivo* low-potenty toxicity of DA under non-physiological circumstances. For instance, DA is released in large amounts during hypoxia/ischemia (up to 700-fold increase)(Phebus et al., 1986; Akiyama et al., 1991) even greatly exceeding the release of the excitotoxic neurotransmitter glutamate under these conditions (Baker et al., 1991 Filloux and Townsend, 1993). In addition, hypoxic-ischemic damage can be attenuated by pre-destruction of the DAergic nigrostriatal pathway (Globus et al., 1987a,b). Likewise, (indirect) DA release by drugs of abuse such as (3,4-methylenedioxy)metamphetamine and cocaine (Schmidt et al., 1985 and 1991) may contribute to their neurotoxicity.

Explanations for the neurotoxicity of DA have predominantly focussed on excessive DA oxidation processes accompanied by oxidative stress including non-enzymatic DA autoxidation (Graham, 1984) and increased enzymatic DA catabolism by MAO (see Fig. 3)(Cohen, 1983 and 1988; Cohen and Spina, 1989; Spina and Cohen, 1989; Fahn and Cohen, 1992).

The mechanism of cytotoxicity of DA and related compounds as studied *in vitro* has been suggested to be due to the non-enzymatic (and metal-catalyzed) formation of toxic (semi)quinones as well as formation of the toxic triad superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) (Graham et al., 1978; Graham, 1978 and 1984 Kalyanaraman et al., 1987). Quinones are very toxic compounds that can readily react with almost every molecule in the cell, in particular strong nucleophilic ones such as neuromelanin, cysteines (incl. GSH), thio proteins, and DNA (Moldéus et al., 1983; reviewed by Monks et al., 1992 Swartz et al., 1992). The model neurotoxins 6-OHDA (6-hydroxydopamine and 6-OH-DOPA (2,4,5-trihydroxyphenylalanine) oxidize to quinones less reactive than DA and DOPA. Therefore, it has been suggested that the relative importance in killing neuroblastome cells (as measured by

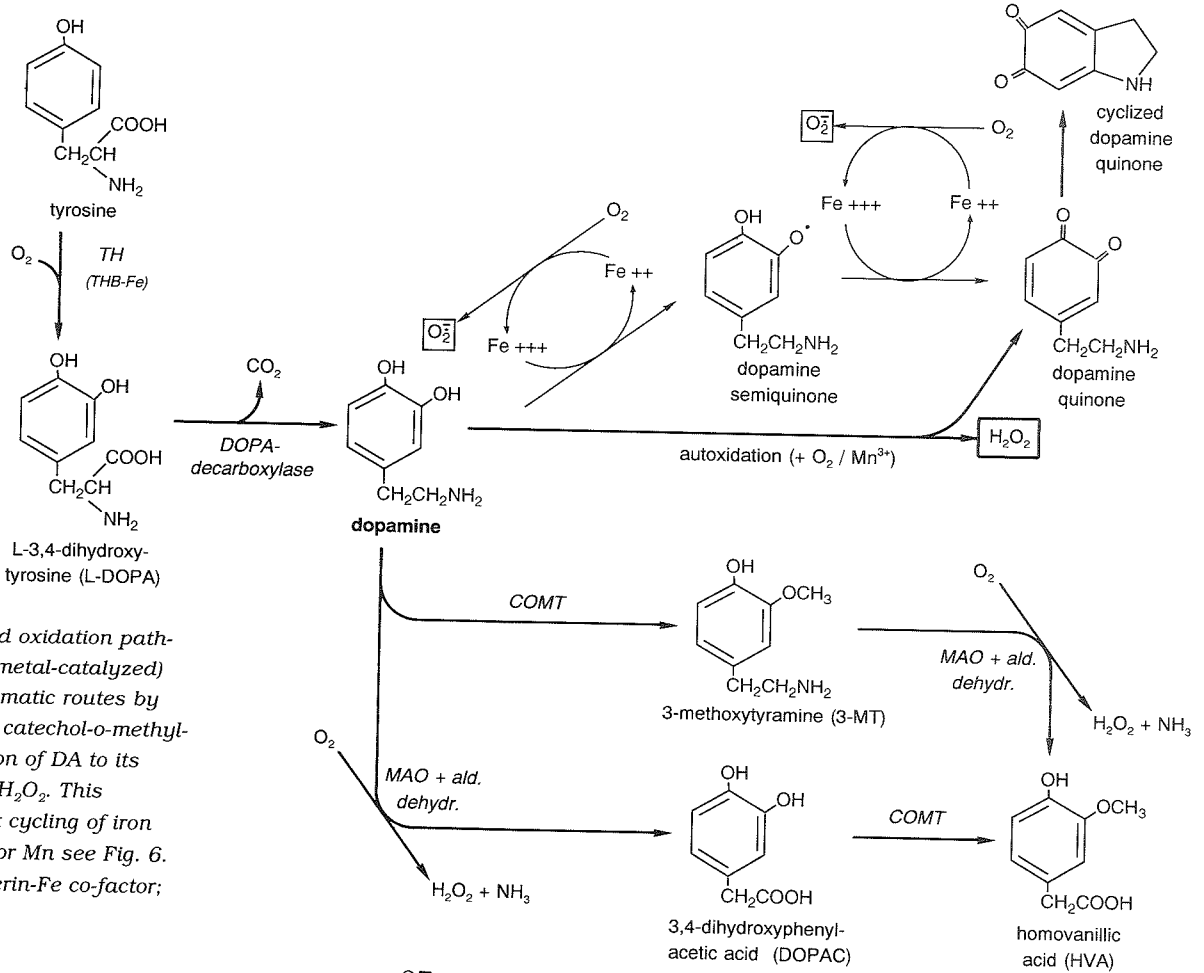


Fig. 3: *Dopamine synthesis and oxidation pathways, including the proposed (metal-catalyzed) non-enzymatic routes and enzymatic routes by monoamine oxidase (MAO) and catechol-o-methyltransferase (COMT). Autoxidation of DA to its quinone results in formation of H_2O_2. This reaction is accelerated by redox cycling of iron with formation of superoxide. For Mn see Fig. 6. Abbr.: THB-Fe, tetrahydrobiopterin-Fe co-factor; TH, tyrosine hydroxylase.*

inhibition of [^{3}H]thymidine incorporation in DNA) through production o reactive oxygen species by 6-OHDA and 6-DOPA is greater than through their respective quinones, while for DA and DOPA the reverse holds true (Graham et al., 1978; Graham 1978; Monks et al., 1992).

Polymerization of quinones from autoxidized catecholamines has been proposed in the genesis of neuromelanin during life (apart from enzymatic formation through tyrosinase), which may be accelerated after chronic Mn neurotoxicity and somehow in iPD patients (Graham, 1978 and 1984). (For Mn and DA autoxidation, see section 1.3.3.1.) Hence, true autoxidation reactions probably do not occur *in vivo* unless metals are present (Millar et al., 1990).

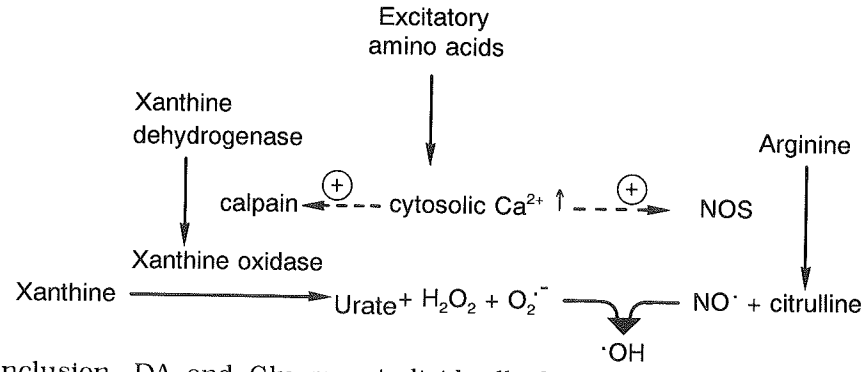
No *in vivo* evidence was offered for Graham's DA autoxidation hypotheses until Rosengren et al. (1985) demonstrated the formation of 5-S-cysteinyl-DA adducts. 5-S-cysteinyl-DA and related adducts have been suggested to reflect *in vivo* DA autoxidation and represents the reaction product between DA quinones and cysteines, probably L-cysteine or GSH (Rosengren et al., 1985; Fornstedt et al., 1986; Fornstedt and Carlson, 1989 and 1991). The concentrations of these adducts have been reported to be low in man (about 5% of the striatal DA contents) and very low in the guinea pig (0.025 - 0.06%) but had doubled in the 3-year old guinea pig (Rosengren et al., 1985; Fornstedt et al., 1990; Fornstedt and Carlsson 1989 and 1991). 5-S-cysteinyl-DA/DA ratios are elevated in substantia nigra, but not in putamen of PD patients (Fornstedt et al., 1989). In addition, such ratios are also increased after reserpine treatment suggesting a cytosolic process, but not after challenge with pargyline (Fornstedt and Carlsson, 1989 and 1991). In the latter reports, striatum and limbic areas, which are less vulnerable in PD, show similar 5-S-cysteinyl-DA/DA ratios after drug challenge. In conclusion, the significance of these adducts in DA autoxidation, oxidative stress, and as a possible detoxifying pathway remains to be elucidated.

The concept of oxidative stress through an enhanced DA turnover causing increased H_2O_2 production (and ammonia) by MAO, which exists in the remaining cells in PD, was tested with reserpine, clorgyline or deprenyl (Cohen, 1983 and 1988; Cohen and Spina, 1989; Spina and Cohen, 1989; Fahn and Cohen, 1992). Reserpine evoked an increased DA turnover associated with increases of oxidized GSH (GSSG) levels in striatum (but not cortex) that could be blocked by the MAO inhibitors clorgyline or deprenyl suggesting oxidative stress through enhanced MAO activity (Cohen, 1988; Spina and Cohen, 1989). The latter hypothesis seems supported by a recent study with transgenic mice that aberrantly overexpress MAO-B in a

neurons that showed a specific decrease (-20%) in perikaryon size of catecholaminergic neurons in substantia nigra and locus ceruleus (Andersen et al., 1994a). However, these transgenic mice demonstrated no greater sensitivity to MPTP neurotoxicity (Andersen et al., 1994b).

1.2.4.3. Glutamate and oxidative stress

The excitatory neurotransmitter glutamate is thought to increase oxidative stress in nerve cells primarily by activation of its ionotropic receptors (Coyle and Puttfarcken, 1993), including voltage-dependent Ca^{2+} and $Na^+.K^+$ permeable channels linked to N-methyl-D-aspartate (NMDA) receptors and Na^+ permeable channels linked to non-NMDA receptors (i.e. AMPA (α-amino-3-hydroxy-5-methyl-4-isoxasole-proprionic acid) or kainate sensitive receptors). Glu-induced elevated intracellular calcium levels (reviewed by Siesjö and Bengtsson, 1989; Orrenius et al., 1989; Choi, 1990) may cause oxidative stress (reviewed by Coyle and Puttfarcken, 1993) through increased $O_2^{\cdot-}$ levels via stimulation of phospholipase A_2 and xanthine oxidase, or nitric oxide (NO) generation via stimulation of NO-synthase (Lafon-Cazal et al., 1993). The $NO^{\cdot}$ can react with $O_2^{\cdot-}$ to form the peroxynitrite anion, which is toxic in itself and partially decomposes to $^{\cdot}OH$ and NO_2 (Beckman et al., 1990, see also section 1.1.1.1).



In conclusion, DA and Glu may individually lead to oxidative stress via different routes, but may also enhance each other's action in this respect.

The toxic interaction of DA and Glu systems can be illustrated by several studies. Glu-receptor (NMDA) antagonists can partially block the neurotoxic effect of metamphetamine (Sonsalla et al., 1991) and MPTP (Carboni et al., 1990; Turski et al., 1991), and striatal DA destruction by 6-hydroxy-DA injections into SN ameloriated the neurotoxic effects of NMDA into striatum (Chapman et al., 1989; Filloux and Wamsley, 1991) as well as

hypoxic/ischemic damage (Globus et al., 1987a,b). These and other studies (Turski et al., 1991) indicated that critical links between DA- and Glu-ergic systems exist within the basal ganglia with regard to neurotoxicity. In addition, awareness of this close Glu-DA interaction had also led to speculations about supplement treatment with Glu-antagonists in PD which has been suggested to act synergistically with DA agonists (or α-adrenergic agonists) with regard to motor activity (Carlsson and Carlsson 1990; Klockgether and Turski, 1990; see also section 1.3.1.1).

1.2.4.4. Neuromelanin

Neuromelanin has been proposed to contribute significantly to DAergic neurodegeneration in PD, which is supported by the relative sparing of non-pigmented DA neurons in PD and the estimated number of dying cells relatively rich in melanine (Hirsch et al., 1988; Hirsch 1992). However, other studies have reported that the most affected ventrolateral part of the SN in PD appeared to contain less neuromelanin than other parts of the SN (Gibb, 1992; Kastner et al., 1992). Another controversial point is the role of iron and neuromelanin in basal ganglia disorders (Youdim et al., 1993). Neuromelanin, a nucleophilic non-protein, can bind several organic compounds and metals, including MPTP, its metabolite (D Amato et al., 1987; Hirsch 1992) and Fe^{3+}. The latter may participate in free oxyradical-induced tissue damage (Ben-Shachar et al., 1991 and 1992 Jellinger et al., 1992; Swartz et al., 1992; Youdim et al., 1993; Aime et al. 1994). *In vitro* spin trapping techniques have revealed that melanin generates $O_2^{\cdot-}$ and H_2O_2 probably through autoxidation of melanin (Swartz et al., 1992). Notably, the highest levels of Cu/Zn-SOD have been demonstrated in the neuromelanin-containing neurons of the SN pars compacta from aged subjects, suggesting a compensatory effect for the presence of an increased $O_2^{\cdot-}$ level (Ceballos et al., 1990). The percentage of melanized cells among catecholaminergic areas of the midbrain in controls varies considerably, and those devoid of neuromelanin seem less sensitive to damage as observed in the same regions in PD (Hirsch 1992). Whether neuromelanin may participate in nerve cell death in PD through enhancing oxidative stress, or whether the accumulation of neuromelanin may be indicative of a pathological process, for example oxidative stress, need further clarification.

Part III

1.3. Oxidant stress and basal ganglia pathology: Idiopatic Parkinson's disease and related models

1.3.1. Idiopathic Parkinson's disease: General aspects

Idiopathic PD (iPD) is often described as a multi-factorial neurodegenerative disease with a clinical manifestation usually beyond the age of 40, affecting more than 1% of the population over the age of 65 (Standaert and Stern, 1993). The etiology of iPD comprises probably multiple partly overlapping causes, including genetic predisposition and/or defects, environmental agents, and oxidative stress (Schapira et al., 1992).

1.3.1.1. Clinical (mis)diagnosis and pathology

The most prominant clinical features of iPD include tremor (at rest), rigidity, bradykinesia, akinesia and postural instability, and emerge gradually after depletion of 70 to 90% of the pigmented neurons (Shoulson, 1993) and over 80% of striatal DA contents. In postmortem tissue of PD patients, Lewy bodies in the substantia nigra are considered to be the pathological hallmark of iPD as well as depigmentation of the SN pars compacta through the loss of melanine-containing cell bodies (Bernheimer et al., 1973), resulting in depletion of dopaminergic terminals in striatum (caudate nucleus and putamen). Occasionally, Lewy bodies are observed in the cortex, and, if more abundant, are regarded as an accompaniment of the Parkinsonism-dementia syndrome. Experimental data have demonstrated a remarkable adaptational potential of the nigrostriatal system under DA-lesioning conditions of up to 80 - 90% by maintaining normal extracellular DA levels (Zigmond et al., 1990; Calne and Zigmond, 1991). However, the neuropathology of iPD is not entirely restricted to the nigrostriatal system, and may, to a lesser extent, also affect noradrenergic, serotonergic and peptidergic neurons of the brainstem (e.g. locus ceruleus) (Halliday et al., 1990), and cholinergic neurons of the nucleus basalis of Meynert and hippocampus (reviewed by Adams and Odunze, 1991).

Since there are many clinical disorders resulting from abnormalities in the basal ganglia, the accuracy of diagnosing iPD in life is poor and amounted to 76% in a study of 100 cases (Hughes et al., 1992; Calne et al.,

1992). Misdiagnoses as shown by necropsy findings often appear to be progressive supranuclear palsy (PSP) or multisystem atrophies (MSA) such as the Shy-Drager syndrome and olivopontocerebellar atrophy, but also basal ganglia vascular disease, or even Alzheimer's disease may be mis-evaluated as iPD (Table V).

Future examinations with positron emission tomography (PET; incl. ^{18}F-DOPA) may improve diagnosis (Brooks et al., 1990; Sedvall, 1990; Calne, 1992). Under the age of 40 Wilson's disease, a copper storage disease, should be excluded (Standaert and Stern, 1993).

Table V: *The differential diagnosis of Parkinsonism*

Parkinson's disease
Secondary (symptomatic) parkinsonism
Infectious (postencephalitic Parkinsonism, luetic aneurysm, Creutzfeldt-Jakob disease)
Vascular (multi-infarct, lacunar state)
Drug-induced (phenothiazines, metoclopramide, reserpine)
Toxic (MPTP, manganese, carbon disulfide, cyanide, carbon monoxide)
Metabolic
Wilson's disease
Hallervorden-Spatz syndrome
Hypoparathyroidism
Structural
Brain tumors
Hydrocephalus
Trauma
Degenerative
Progressive supranuclear palsy
Multiple system atrophy
Stratonigral degeneration
Olivopontocerebellar atrophy
Shy-Drager syndrome
Spinocerebellar-nigral degeneration
Corticonigral degeneration with neuronal achromasia
Parkinson-dementia complex of Guam
Parkinsonism with amytrophy
Senile gait apraxia
Alzheimer's disease

Adapted from: Stern, M.B. The clinical characteristics of Parkinson's disease and parkinsonian syndromes: Diagnosis and assessment, in *The Comprehensive Management of Parkinson's Disease* (Stern M.B. and Hurtig H.H., eds), pp 3-50. PMA, New York.

1.3.1.2. Therapy of Parkinson's disease

In almost all cases of parkinsonism irrespective of its exact nature, a trial of the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) is indicated with varying success. L-DOPA is usually combined with peripheral metabolic blockers such as aromatic amino acid decarboxylase inhibitors (i.e. blocking conversion to DA: carbidopa and benserazide) and catechol-O-methyltransferase inhibitors (i.e. blocking degradation: nitecapone) to increase the bioavailability of L-DOPA and to diminish sympathomimetic effects. For the latter also peripheral DA antagonists (domperidone) have been used (Kopin, 1993).

The initial success of L-DOPA, whose's rationale is based on DA replacement, is confronted with the "wearing-off" and "on and off" phenomena most patients eventually encounter if they respond to the drug (Nutt, 1994). The former side-effect is a shortening of duration of efficacy between (increasing) doses, the latter are unpredictable fluctuations in motor function, dyskinesia, and choreoathetosis. Why L-DOPA fails after some years is not clear, but both pre- synaptically induced (shortening of respons to L-DOPA through inevitable degeneration of DA neurons) and post-synaptically induced chamges (shortening of respons to apomorphine) are thought to be responsible for the worsening of the disease. Post-synaptic changes seem reversible and can be pharmacologically modified (Nutt, 1994). On the other hand, in some patient populations, a slower rise and longer plateau of L-DOPA plasma levels, which are reached by continuous intravenous or oral administration of L-DOPA or by using special formulations of L-DOPA + carbidopa with an erodible matrix, reduce dose-related fluctuations and side-effects (Standaert and Stern, 1993).

Other therapies also derive their benefit largely from restoration of depleted nigrostriatal DA (MAO inhibitors) or stimulation of DA receptors (aporphines, ergot derivatives). DA receptor agonists have the theoretical advantage of acting independently of the degenerating DA neurons, but the mode of pharmacological action on the different DA receptor populations (D1 through D5) is variable and will only mimic authentic (= regulated) DA release to some degree. In particular, side-effects such as motor fluctuations and abnormal movements can be attenuated by DA agonists in adjunction with L-DOPA. Clozapine, an atypical neuroleptic with high affinity to the D4 receptor, is of interest because it does not produce Parkinsonian side-effects such as as psychosis and tardive dyskinesia (Standaert and Stern, 1993; Kopin, 1993).

Special attention has been focused on deprenyl (or selegiline), an

irreversible MAO-B inhibitor, which does not potentiate sympathomimetic amines, diminishes motor fluctuations, prolongs L-DOPA efficacy and, most of all, results in a longer life expectancy as determined in a clinical study with 800 patients by the principal end point: reaching initiation of L-DOPA therapy (Parkinson's Study Group, 1989; Shoulson 1993). Whether this effect could be attributed to a neuroprotective effect or improved DA availability or functioning is not clear. It is known that deprenyl also forms two DA-active metabolites, L-amphetamine and L-metamphetamine (Shoulson, 1993). A neuroprotective effect might be concluded from other features of deprenyl. It appears that the drug induces soluble SOD (Clow et al., 1991) and suppresses the oxidant stress associated with increased DA turnover as measured by the accumulation of GSSG in mice (Cohen and Spina, 1989). The latter is, however, also observed with the MAO-A blocker clorgyline (Spina and Cohen, 1989). Other highly selective and reversible MAO-B inhibitors that are not metabolized to active amphetamines are under investigation (Shoulson, 1993). The antioxidant vitamin E, which was also used in the above mentioned clinical study of 800 patients, did not retard the progression of PD (DATATOP studies; Shoulson, 1993). However, in another study it has been shown, that a combined treatment of high doses of vitamins E and C slowed down the progression of iPD (Fahn, 1992).

The excitatory input of Glu and its close interactions with DA in the basal ganglia has led to speculations about a combined treatment with DA agonists and Glu antagonists in PD, which has been suggested to produce a synergistic effect with regard to motor functions (Carlsson and Carlsson, 1990; Klockgether and Turski, 1990). This also places some older anti-Parkinsonian drugs in another perspective by showing additional NMDA-antagonistic properties (Olney et al., 1987). However, the known psychotic side-effects of Glu antagonists, in particular of NMDA-receptor antagonists (e.g. MK-801), have been troublesome (Carlsson and Carlsson, 1990). Nevertheless, based on improved understanding of the (patho)physiology of basal ganglia circuitries (see section 1.2.1, Albin et al., 1989), anti-Parkinsonian effects have been experimentally demonstrated through elimination of an overactive STN via inhibition of AMPA receptors (Klockgether and Turski, 1990) or lesioning this structure (Bergman et al., 1990). Recently, similar conclusions were drawn after the use of novel Glu anatagonist (with different profiles) in different primate models of PD (Greenamyre et al., 1994).

Whether improved versions of drugs mentioned, in particular drugs with (combined) antioxidant features including iron-chelating, NO-synthase-inhibiting, P-450-blocking, GSH metabolism-enhancing, antioxidant-

mimicking and/or free radical-scavenging properties, will be successful in the treatment or improvement of basal ganglia disorders is still being studied (Kopin, 1993). To establish the value of these treatments in the different forms of Parkinsonism (or hyperkinetic movement disorders) will depend as much on exact clinical diagnosis as on molecular science. Non-pharmaceutical therapeutic strategies for PD such as implanting (autologous) DA-producing cells, fetal DA cells or genetically modified cells producing DA (and other factors) have also been explored with marginal success, but may be improved in the future.

1.3.1.3. Environmental, occupational and genetic factors

Environmental or occupational exposure to toxic chemicals have been suggested in the etiology of iPD or Parkinsonism, which has been strengthened by the discovery of the PD drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a synthetic heroin and industrial compound (Langston, 1988) that produces clinical, biochemical and neuropathological changes highly reminiscent of those observed in iPD (Bloem et al., 1990; Jenner et al., 1992; Tipton and Singer, 1993). The prevalence of PD in highly industrialized countries (North America, about 350:100,000, and Europe) is found to be higher than elsewhere, whereas living in China (on average 59:100,000) in industrialized urban areas increases the risk of developing PD (Poirier et al., 1991; Tanner, 1992). Vegetable farming, well water drinking, wood pulp, paper and steel industries are factors that appear to be associated with PD. Environmental chemicals may be related to the development of PD, but specific chemicals and their specific mechanism(s) have not been identified (Tanner, 1992). Recently a family history of PD was found to be the strongest predictor of PD risk, followed by head trauma and occupational herbicide use (Semschuk et al., 1992 and 1993). The hypothesis of a multifactorial etiology of PD was confirmed in the latter study.

An impairment in metabolizing toxins, for instance enzymes metabolizing sulfur-containing compounds, may render an individual susceptibility for developing PD or, in other words, genetic predisposition may contribute to the occurrence of PD (Jenner et al., 1992). Heredity itself cannot account for the totality of the illness as is believed now (Jenner, 1992). The recently discovered genetic defect in mitochondrial Complex I in iPD (Schapira et al., 1992) and the relation to oxidative stress will be discussed in section 1.3.2.4.

1.3.2. Parkinson's disease, experimental parkinsonism and oxidative stress

Parkinsonism may be defined as a syndrom resembling iPD and includes experimental models of PD chemically induced by MPTP or 6-OHDA.

1.3.2.1. Parkinsonism and iron

Idiopathic PD and other extrapyramidal disorders have shown alterations in iron metabolism (Sofic et al., 1988; Riederer et al., 1992; Dexter et al., 1991, 1992 and 1993; Youdim et al., 1993).

Specific changes in the SN (total and pars compacta) of PD postmortem tissues, i.e. increased total iron and zinc levels and decreased ferritin and copper content as well as shifts in ferrous and ferric iron, suggest a state of oxidative stress induced by alterations in local iron handling (Sofic et al., 1988; Dexter et al., 1991, 1992, 1993). However, reduced ferritin levels are more widespread in the brain, whereas one report showed increased ferritin immunoreactivity in SN (Riederer et al., 1989). Whether changes in iron metabolism are a cause or consequence of iPD is a matter of debate, but concentrations of metals were not changed in mildly affected PD patients (Sofic et al., 1988; Riederer et al., 1989; Dexter et al., 1994). On the other hand, more detailed micro-analyses within the parkinsonian SN revealed intracellular iron spots, and accumulation of iron and aluminum to Lewy bodies in an early stage of the disease, emphazising a more primary role of iron (Hirsch et al., 1991, Hirsch 1992). Notably, aluminum stimulates iron-induced lipid peroxidation (Gutteridge et al., 1985).

Other extrapyramidal disorders examined also demonstrated changes in iron metabolism specific to the regional pathology of the disease. For example, increased total nigral iron in progressive supranuclear palsy (PSP) and multisystem atrophy (MSA) was, in contrast to PD, associated with normal or increased immunoreactive ferritin levels, and total striatal iron was increased in PSP, MSA and Huntington disease, but not in PD, while total pallidal iron was reduced in PD (Dexter et al., 1991, 1992, 1993).

In MPTP-induced hemi-parkinsonian monkeys, a unilateral increase of iron is observed not only in SN pars compacta, but also in the surrounding matrix and glial cells (Temlett et al., 1994). The cerebral transferrin-receptor distribution in MPTP-treated mice and PD patients were

similar, showing significant reductions only in striatum and putamen respectively (Mash et al., 1991). The putamen is known to suffer the most severe DA losses.

6-Hydroxy-DA and 6-hydroxy-DOPA release iron from ferritin *in vitro* in contrast to related benzenediols (Monteiro and Winterbourn, 1989; Lode et al., 1990), whereas the neurotoxicity of 6-hydroxy-DA is attenuated by the iron chelator deferoxamine (Ben-Shachar et al., 1991).

Experimental injection of ferrous and ferric iron into SN induces degeneration of nigrostriatal neurons (Ben-Shachar and Youdim, 1991; Sengstock et al., 1992). A single infusate of iron into the substantia nigra produces progressive local neurodegeneration, i.e. neuronal loss and gliosis restricted to the pars compacta and dorsal-most pars reticulata, accompanied with modest striatal DA depletions through at least 6 months (Sengstock et al., 1994). Therefore, low amounts of iron (1.25 nmol) injected into rat substantia nigra may serve as a model of progressive PD.

1.3.2.2. Parkinsonism and lipid peroxidation

Increased basal lipid peroxidation in the SN in PD has been demonstrated in regional postmortem studies (Dexter et al., 1986 and 1989), which has not been shown in MPTP-treated mice (Corongiu et al., 1987; Adams et al., 1990; Adams and Odunze, 1991). In *in vitro* studies, MPP^+ was found to stimulate lipid peroxidation, whereas MPTP inhibited this process (Rios and Tapia, 1987; Lambert and Bondy, 1989.

1.3.2.3. Parkinsonism and antioxidant enzymes

In iPD, a selective increase of particulate SOD activity (= MnSOD), normal Cu/ZnSOD activity (Saggu et al., 1989), and selective decrease of GSH levels in SN have been reported (Perry et al., 1982; Riederer et al., 1989; Sofic et al., 1992; Sian et al., 1994a). In addition, GSH contents were not decreased in SN of MSA or PSP patients. Since postmortem delay also reduces GSH levels, the complete absence of GSH found by Perry et al. is probably an artifact (Di Monte et al., 1992; Jenner, 1993). Both an increase of Mn-SOD activity and GSH depletion have been interpreted as signs of oxidative stress. Marttila et al. (1988) found an increase in cytosolic SOD (Cu/Zn), but this may be due to an artificial solubilization of mitochondrial enzyme (Jenner, 1991). Notably, MPTP and rotenone both increase SOD (Hasegawa et al., 1990) and inhibit mitochondrial Complex I (see also section 1.3.2.4).

At present the depletion of GSH appears the earliest marker of cellular pathology in PD (Jenner, 1993). The exact cause of GSH depletion is unknown. That is, the levels of GSH peroxidase and reductase as well as the rate-limiting enzyme of GSH synthesis are unaltered, whereas GSH catabolism via γ-glutamyltranspeptidase (one route) and GSSG (mildly) are increased, suggesting an increase in its elimination (Jenner, 1993; Sian et al., 1994a, b).

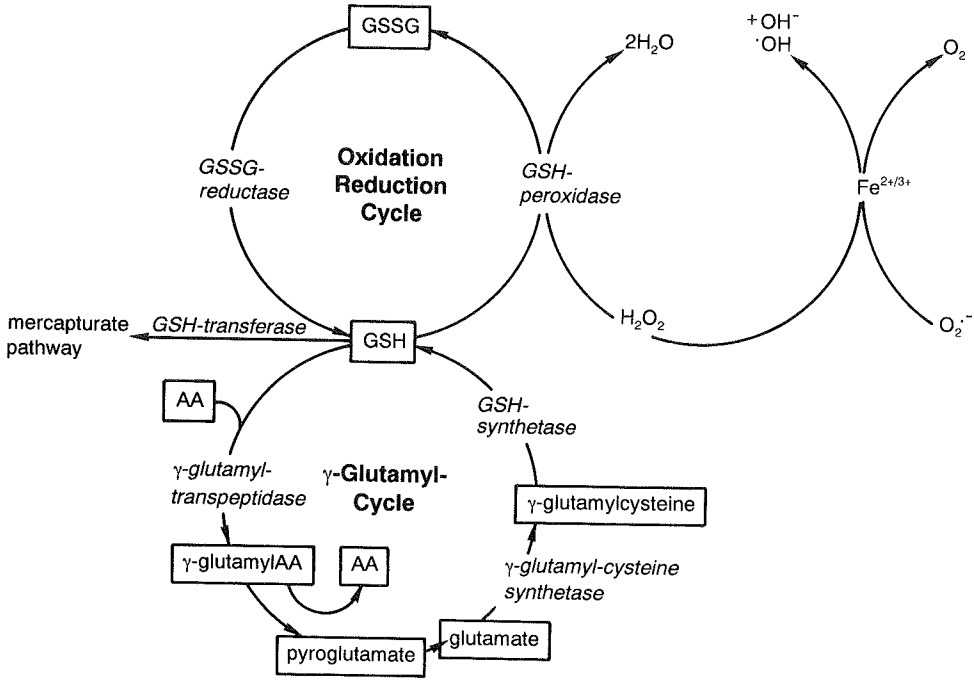


Fig. 4: *Schematic illustration of the synthesis, degradation, and utilization of reduced gluthatione (GSH)*

Specific changes in GSH metabolism have also recently been found in MSA and PSP (Sian et al., 1994a, b). In MSA, GSH increases and GSSG decreases are observed only in the globus pallidus together with a rise in Gpx levels in globus pallidus and caudate nucleus, whereas in PSP the only change is a reduction of GSH in caudate nucleus. HD only shows a GSSG reduction in caudate nucleus (Sian et al., 1994a).

It has been proposed that GSH depletion in the cytosol, which is the origin of GSH synthesis, results in mitochondrial GSH depletion and subsequently mitochondrial damage (through excessive local H_2O_2 production), which has been experimentally supported by inducing GSH

depletion (without a GSSG rise) by buthionine sulfoximine, a GSH synthesis inhibitor. Alternatively, it has been suggested that mitochondrial damage as initial event (see section 1.3.2.4) can reduce GSH levels by impairing GSH synthesis (GSH synthetase is ATP-dependent). Such a suggestion has been supported by effects of mitochondrial toxins, including KCN, antimycine A and MPP^+, which decreased GSH but did not lead to a rise in GSSG and could be counteracted by addition of substrates for glycolytic production of ATP (Di Monte et al., 1992). Both events mentioned are highly interlinked, as indicated in the Figure 3, and need further studies to make it possible to identify a specific cause in this respect (Monte et al., 1992; Jenner, 1993).

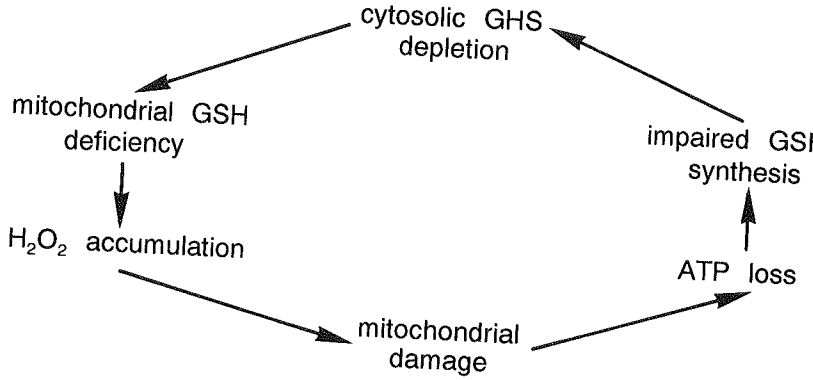


Fig. 5: *The interlink between GSH reduction and mitochondrial damage*

MPTP reduces glutathione in the midbrain and not in striatum, which may be caused by local ATP decreases induced by MPTP or free radicals, since large amounts of α-tocopherol or β-carotene can prevent GSH loss (reviewed by Adams and Odunze, 1991: Ferraro et al., 1986; Yong et al., 1986). Effects of MPTP in mice were not changed by increasing or decreasing DA turnover suggesting free-radical formation independent of DA (Fuller and Hemrick-Luecke, 1985; Yong et al., 1986). Carmustine, an irreversible GSSG reductase inhibitor, potentiates MPTP toxicity as measured by DA depletion in SN (Adams and Odunze, 1991).

Intracerebroventricular injections of 6-OHDA produces decreases in the activity of SOD and catalase, and a reduction of GSH in striatum and brain stem (Perumal et al., 1989). In addition, Gpx activity is only slightly reduced in the brain stem. The DA-depleting effect of 6-OHDA is potentiated upon inhibition of GSH synthesis by L-buthionine-sulfoxime (Pileblad et al., 1989). This suggests that GSH is needed to detoxify 6-OHDA, which may be depleted through binding to its autoxidation product *p*-quinone (Fornstedt

et al., 1990).

1.3.2.4. Parkinsonism and mitochondrial dysfunction

In iPD, an increasing body of evidence has implicated a defect in mitochondrial Complex I (NADH CoQ_1 reductase), of which the defect in the brain seems confined to the SN and is specific for Complex I (Schapira et al., 1990 and 1992; Mann et al., 1992; Jenner, 1993). Since measurements have been conducted in substantia nigra homogenates containing ca. 95% glial cells, the effect should be attributed to glia. On the other hand, a 37% decrease in complex I activity might be an underestimation of the effect in neurons, which is masked by normal activity of glial cells (Schapira et al., 1992). A Complex I defect is not present in a related basal ganglia disorder, MSA, which is usually more severe than PD.

The specificity for Complex I as observed in the CNS can also be detected in thrombocytes (Parker et al., 1989), but is at variance with skeletal tissues in PD. In muscle biopsies of PD patients effects on other mitochondrial complexes (II and IV) have been reported (Shoffner et al., 1991; Bindoff et al., 1989; Mann et al., 1992; reviewed by Schapira et al., 1992). The discrepancies in these studies are difficult to explain and may be due to such factors as heterogeneity, etiology, treatment, and age matching (reviewed by Schapira et al., 1992). Complex I defects are also reported in Leber's disease (a hereditary optic neuropathy) with dystonia (Parker et al., 1989), and in thrombocytes of HD patients (Parker et al., 1990), but not in caudate-putamen of such patients (Mann et al., 1990; Jenner, 1993). In addition, in the latter study a decreased Complex II/III activity was noticed exclusively in the caudate. Others found also reductions in Complex IV activity and normal Complex III activity in caudate nucleus of HD patients (Brennan et al., 1985).

Since age-related degenerative diseases, including PD, and normal aging are associated with defects in oxidative phosphorylation (Benzi et al., 1992; Wallace, 1992; Bowling et al., 1993; Di Monte et al., 1993), these issues have been hypothetically linked to defects in mitochondrial genes (Wallace, 1992; Mecocci et al., 1992; Coyle and Puttfarcken, 1993). The predominant maternal (= non-Mendelian) hereditary nature of mitochondrial genes and the existence of a 10 times greater mitochondrial DNA mutation rate as compared to nuclear DNA caused, for instance, by free oxygen radicals are compatable with the idea of PD as a heterogenous biochemical and genetic disease (Wallace, 1992; Schapira et al., 1992).

Selective uptake of MPP^+, the active metabolite of MPTP, into mitochondria of DAergic cells irreversibly inhibits Complex I of the mitochondrial respiratory chain in a slow fashion (Pai and Ravindranath, 1991; Cleeter et al., 1992), resulting in ATP depletion and finally cell death (Heikkila et al., 1985; Niklas et al., 1985; Mizuno et al., 1988; Schapira et al., 1992; Storey et al., 1992). This feature of MPTP toxicity is compatible with the theory that iPD may be caused by some environmental toxin that inhibits Complex I activity (Jenner et al., 1992). Inhibition of Complex I activity in beef heart mitochondria by MPP^+ can be prevented by GSH, ascorbate and catalase (Cleeter et al., 1992), whereas the cytotoxicity of MPTP using neuronal cell lines can be decreased in the presence of SOD and catalase (Lai et al., 1993), suggesting a role of free oxygen radicals in precipitating MPTP or MPP^+ toxicity. This notion is supported by the reported NADH-dependent increase of $O_2^{\cdot-}$ formation in bovine heart mitochondrial particles induced by MPP^+ (Hasegawa et al., 1990).

Likewise, inhibition of Complex I by rotenone or Complex III by antimycin A enhances the production of free radicals by the respiratory chain (Cleeter et al., 1992). Furthermore, MPP^+ and $MPDP^+$ (a metabolic intermediary of MPTP) may produce $O_2^{\cdot-}$ and $^{\cdot}OH$ through interactions with melanin, iron, oxygen and NADPH cytochrome P450 reductase (Adams and Odunze, 1991), whereas Chiueh et al. reported production of $^{\cdot}OH$ after intrastriatal infusion of MPTP metabolites as measured by salicylate hydroxylation (Obata and Chiueh, 1992; Wu et al., 1993: Chiueh et al., 1994).

The relative importance of factors associated with oxidative stress in the etiology of iPD as discussed (i.e. increased iron and lipid peroxidation, impaired GSH system, impaired mitochondrial respiration) do not point to one specific cause (Schapira et al., 1992; Jenner, 1993). However, studies using Lewy body-positive (incidental) control groups without significant DA loss and symptoms of PD have revealed unchanged total iron and ferritin levels, reduced GSH levels as strong as in more advanced PD, and intermediate levels of Complex I activity (between controls and PD patients) in substantia nigra (Jenner et al., 1992; Jenner, 1993; Dexter et al., 1994). This suggests that decreased GSH levels, whatever the cause, is at present the earliest marker of cellular pathology in iPD (Jenner, 1993; Dexter et al., 1994). In addition, recent studies employing sensitive methods have demonstrated Lewy bodies distributed throughout the brain in virtually every PD patient, suggesting that PD is perhaps a general brain insult that happens to be concentrated on the substantia nigra because of its local

metabolic vulnerability (Schapira et al., 1992).

Furthermore, MPTP lesions have previously been regarded as stable over time. However, short-term exposure to MPTP in man can cause progressive nigral pathology as demonstrated for the first time using fluorodopa PET scans with intervals of 7 years (Vingerhoets et al., 1994). The latter supports the hypothesis that an environmental agent/factor can cause selective and progressive neurodegeneration.

1.3.3. Manganese neurotoxicity: General aspects

1.3.3.1. Clinical diagnosis and pathology

Parkinsonian-like features after Mn intoxication such as (action) tremors, rigidity and other abnormal hypokinetic movements are often accompanied with dystonia as seen in dystonia musculorum deformans, which is defined as postural instability of complementary muscle groups (Barbeau et al., 1976). Nowadays it is recognized that the combination of dystonia and parkinsonism occurs in several conditions, and that dystonia is sometimes an integral part of juvenile parkinsonism (Nygaard and Duvoisin, 1986). Furthermore, extrapyramidal symptoms of Mn poisoning which develop over months are preceded by a phase of so-called Mn psychosis or Mn madness (locura manganica). This first phase include schizophrenia-like symptoms such as violent behavior, compulsive acts, emotional instability and hallucinations (Barbeau et al., 1976; Donaldson, 1987).

Autopsy of patients suffering from chronic Mn poisoning or manganism showed neuropathological lesions in caudate nucleus and putamen, more severe lesions in pallidum and subthalamic nucleus, and less frequently depigmentation and cell damage in the SN (Bernheimer et al., 1973; reviewed by Barbeau, 1984; Yamaha et al., 1986). Outside the basal ganglia diffuse lesions in the cortex and lesions of the hypothalamus may also occur. Experimental studies using chronic Mn administration (independent of the route) in rodents (Chandra and Shukla, 1981; Autissier et al., 1982), and non-human primates, including inhalation of Mn-dust, essentially affirmed the above described neuropathology and showed DA depletions in caudate nucleus, putamen (or striatum) and pallidum (reviewed by Barbeau, 1984; Bird et al., 1984; Eriksson et al., 1987). Recent PET and magnetic resonance imaging (MRI) studies in primates support the occurrence of pre- and postsynaptic damage to the DAergic nigrostriatal

pathway (Wolters et al., 1989, Eriksson et al., 1992a,b). In addition, MRI has also been used to visualize Mn accumulation in the basal ganglia of rodents and primates (London et al., 1989; Newland et al., 1989; Nelson et al., 1993). Interestingly, in some experimental Mn studies, in which data were collected over a long period, an initial increase of DA levels and tyrosine hydroxylase activity has been shown with a gradual decline thereafter (Bonilla, 1980; Chandra and Shukla, 1981; Chandra, 1983). This might reflect the clinical phases of schizophrenia and extrapyramidal symptoms, respectively.

In summary, chronic Mn poisoning comprises an initial phase reminiscent of schizophrenia and a second phase with a more extended pattern of basal ganglia lesions than observed in iPD, which is consistent with the (subtle) differences in clinical extrapyramidal features.

1.3.3.2. Therapy of manganese poisoning

The discovery of both L-DOPA with all its initial complications in the treatment of parkinsonism and the link of manganism with catecholamines and extrapyramidal disorders in the late 1960s have led to the use of this DA precursor in treating Mn neurotoxicity (Cotzias et al., 1971; Barbeau et al., 1976; Barbeau 1984). L-DOPA has been found to attenuate symptoms of Mn poisoning in man such as reduction or disappearance of rigidity and hypokinesia, and improvement of postural reflexes, without inducing involuntary movements (choreoid-athetoid) as occasionally seen with PD patients after L-DOPA administration (Mena et al., 1970; Barbeau et al., 1976; Rosenstock et al., 1971; Cook et al., 1974; Huang et al., 1993). However, the success was partial, because L-DOPA may also be ineffective or even worsen the situation in some cases of human Mn intoxication. One such case of an aggravated neurological status caused by L-DOPA could be improved by changing to 5-hydroxytryptophane treatment (Mena et al., 1970). Similarly, L-DOPA has been found variably useful in the treatment of dystonia musculorum deformans (Barbeau et al., 1976).

Other features of L-DOPA are, that it interacts with the status of trace metals, including Mn. Prolonged L-DOPA administration increases concentrations of Mn and DA in control tissues, whereas reserpine has the reverse effect (Papavasiliou et al., 1986 from Donaldson et al., 1974; Cotzias et al., 1971; Bonilla et al., 1974; Donaldson et al., 1974). In rats (sub)chronically exposed to Mn, L-DOPA lowered liver Mn levels (Bonilla et al., 1974), and increased striatal DA, the latter being not correlated with changes in local Mn concentrations (Bonilla and Diez-Ewald, 1974; Shukla

and Chandra, 1981). L-DOPA potentiated striatal DA depletions after experimental injection of Mn^{2+} into substantia nigra (Parenti et al., 1986).

The successful treatment of Wilson's disease with metal-binding agents seemed to provide a precedent for treating chronic Mn poisoning (Mena et al., 1970). Both tissue burdens of Mn and the turnover of radioactive Mn have been found significantly higher in "healthy" miners than in miners removed from the scene because of extrapyramidal signs, and also higher than in normal control subjects. Thus, these observations demonstrated that Mn overload was present only during exposure and did not parallel irreversible symptoms, suggesting that chelation therapy would be of no value (Barbeau, 1984). Nevertheless, on occasion, chelation therapy with ethylenediaminetetraacetic acid (EDTA) has been attempted in cases of Mn poisoning in man, again with variable success (Emara et al., 1971; Cook et al., 1974; Scheuhammer and Cherian, 1982). Additional experiments in rats subchronically exposed to Mn^{2+} revealed increased urinary excretion of metals (Mn, Zn, and Fe, but not Cu) after multiple EDTA injections but, apart from muscle, showed no elimination from several other tissues, including the brain (Scheuhammer and Cherian, 1982). In the same study, Mn treatment was accompanied by decreased hepatic Fe and increased Cu levels in the brain, the latter of which remained after discontinuation of Mn treatment.

1.3.3.3. Occupational, environmental, and iatrogenic hazards

Historically, the neurotoxicity of Mn is linked to the chronic occupational exposure in manganese mines (Barbeau et al., 1976; Barbeau 1984). Although Mn neurotoxicity or manganism is not considered a great risk any more in industrialized countries such as Europe and North America, the occupational work in Scandinavian countries on railroad tracks made of Mn steel alloy is considered a health risk (Nelson et al., 1993; Wennberg et al., 1991 and 1992). In addition, a very recent case of Mn intoxication has been reported in the Netherlands, notably because of the use of an unregistered alternative herbal pill against arthritis (de Krom et al., 1994). Several cases of Mn intoxication from ferro-manganese plants, which are the largest consumers of Mn (Bencko and Cikrt, 1984), have been reported recently in Taiwan (identical cases described by Wang et al., 1989; Huang et al., 1989 and 1993 and Wolters et al., 1989). In particular, effects of chronic low-level occupational exposure to Mn are of concern. Results of two of such studies have been interpreted as preclinical signs of intoxication (Roels et al., 1987) and even as early (subclinical) signs of parkinsonism

(Wennberg et al., 1992). Other sources of concern are the use of organic Mn compounds such as the widely used pesticide manganese ethylenebis(dithiocarbamate) (MANEB) (Ferraz et al., 1988: Verberk et al., 1991) and anti-knock agent methylcyclopentadienyl manganese tricarbonyl (MMT) in unleaded gasoline (Gianutsos and Murray, 1982; Gianutsos et al., 1985; Yong et al., 1986).

Cases of a form of amyotrophic motor neuron disease (Kihira et al., 1990) and other neurological signs in certain populations have been linked with environmental exposure to Mn (Kilburn, 1987). Mn has also been associated with ALS (Kihira et al., 1990) and the Guam ALS/Parkinson-dementia complex (Kilburn, 1987).

1.3.4. Manganese neurotoxicity and oxidative stress

The first observation by Borg and Cotzias (1962a,b) that Mn may generate free radicals came from studying *in vitro* interactions between phenothiazines and Mn^{3+}, a reaction shown to be caused by the formation of a semiquinone free-radical ion in the chromophore (reviewed by Barbeau, 1984). The red-colored product could also be formed by the action of Fe^{3+}, Co^{3+} and Mn^{2+} with oxygen, and could be prevented by reducing agents such as Fe^{2+}, ascorbic acid, glutathione and cysteine. However, until today, the exact mechanism of by which Mn produces free radicals and brain pathology remains to be elucidated.

1.3.4.1. Manganese and dopamine

DA depletion by Mn through enhanced non-enzymatic oxidation of DA resulting in the production of several toxic (semi)quinones as well as free oxygen radicals and H_2O_2 has been suggested to be the underlying mechanism of cytotoxicity of DA and related compounds (see also section 1.2.4.2; Graham et al., 1978a and 1978b; Graham, 1984, and others). However, one must take into account that autoxidation of catechol compounds as measured spectrophotometrically by oxygen electrodes (Graham et al., 1978b), or in presence of sodium periodate (Graham et al., 1978a), only occurred for DA, DOPA and norepinephrine at alkaline pH (> 9.0). In contrast, 6-OHDA and 6-OH-DOPA, which already autoxidize at pH 6.2 (or higher), consume much more oxygen than DA, DOPA or norepinephrine (at pH 10.5). Therefore, *in vivo* autoxidation of DA (and formation of oxyradicals) is unlikely unless metals are present to catalyze

such reactions (Millar et al., 1990).

Indeed, *in vitro* studies on the mechanism of Mn-induced oxidation of DA (Archibald and Tyree, 1987; Segura-Aguilar and Lind, 1989), have revealed that DA is rapidly oxidized by Mn^{3+}-pyrophosphate complex (at physiological pH) to its cyclized *o*-quinone, an irreversible reaction resulting in a decreased level of DA. However, the latter reaction does not generate reactive oxygen species since oxygen is neither consumed nor required in this reaction. Mn^{3+} was far more efficient in oxidizing catechol compounds (including DOPA, epinephrine and norepinephrine) than Mn^{2+}, Mn^{4+} (MnO_2), $O_2^{\cdot-}$, or H_2O_2, whereas this reaction could be prevented (not reversed) by NADH, GSH or ascorbic acid (Halliwell, 1984; Archibald and Tyree, 1987; Segura-Aguilar and Lind, 1989). In addition, it has been suggested by these authors that Mn^{2+} can be easily oxidized to Mn^{3+} (e.g. by $O_2^{\cdot-}$) and bound to pyrophosphate *in vivo*. Furthermore, the combination of DT diaphorase (which performs a 2-electron transfer) and SOD (which reduces Mn^{3+}) is an efficient system for maintaining cyclized *o*-DAquinone in its fully reduced state (the hydroquinone), a prerequisite for detoxification of the quinone by conjugation with sulfate or glucuronic acid (see Fig. 4). However, if one of the two enzymes is absent or present at insufficient levels, reactive free oxygen radicals may arise resulting eventually in cell death (Segura-Aguilar and Lind, 1989). DT diaphorase is found in most animal tissues, including various brain regions, where it is localized in neurons, especially DAergic ones in striatum and substantia nigra, as well as in various glial cells (Segura-Aguilar et al., 1987; Schultzberg et al., 1988).

1.3.4.2. Manganese and iron

Like Fe, Mn is an essential nutrient for normal function as a constituent of some metalloproteins such as SOD (Mn^{3+}) and pyruvate carboxylase (Keen et al., 1984). In the brain the glial cytoplasmic enzyme glutamine synthetase, which converts glutamate into glutamine, accounts for 80% of all available brain Mn^{2+}, and is not regionally distributed (Wedler and Denman, 1984). Deficiency of Mn may cause seizures and convulsions, probably due to decreased glutamine synthetase and Mn-SOD activity (Keen et al., 1984; Wedler and Denman, 1984; Carl et al., 1993).

It has been suggested that there exists a relationship between iron and Mn homeostasis on the basis of Mn incorporation into hemoglobin (Diez-Ewald et al., 1968; Barbeau, 1984), its increased gastrointestinal uptake in iron-deficient rats and vica versa (Chandra and Shukla, 1976; Kostial et al., 1980; review by Keen et al., 1984 and Aschner and Aschner,

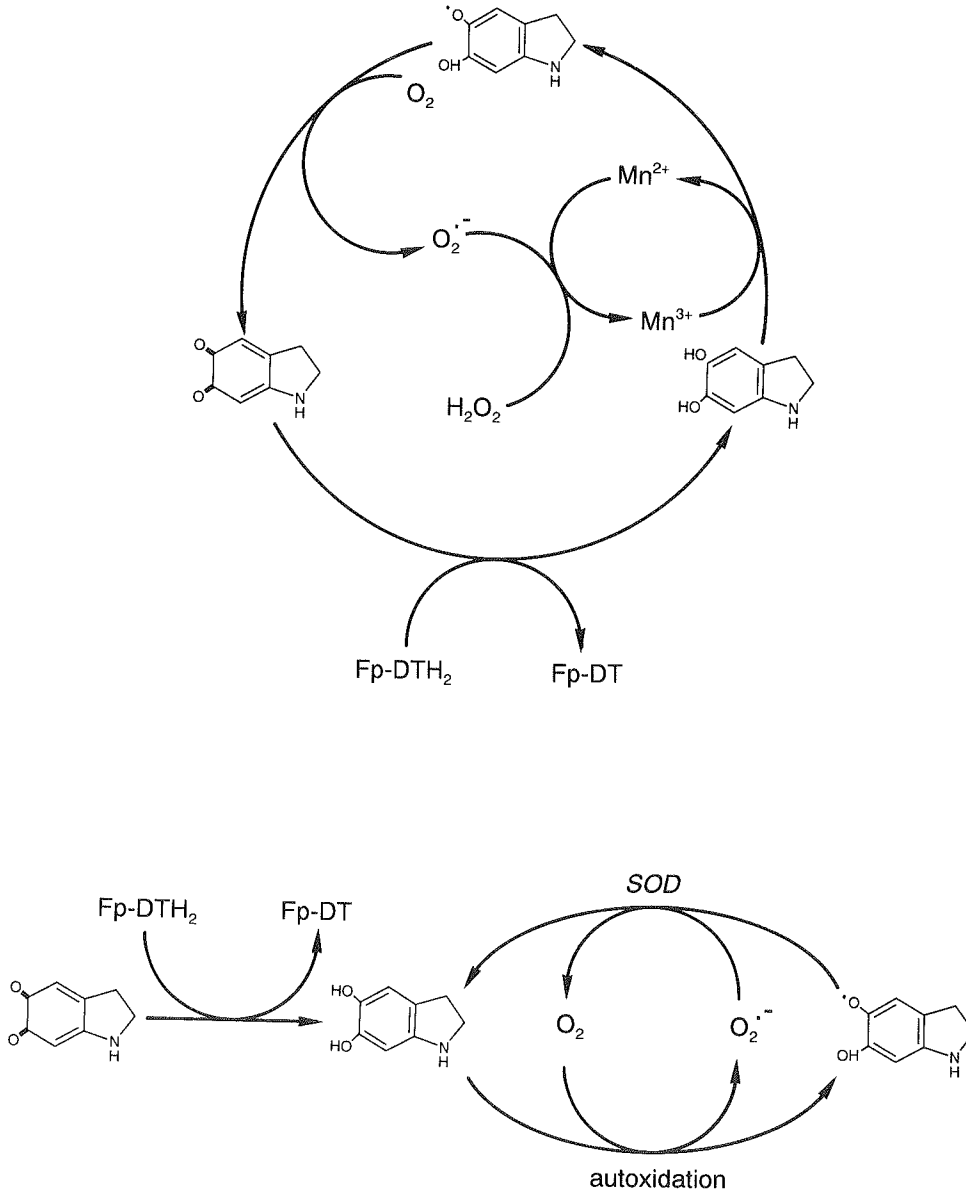


Fig. 6: *Proposed mechanisms for reduction of cyclized dopamine o-quinone by flavoproteine DT diaphorase (Fp-DT, NAD(P)H:quinone oxidoreductase) and autoxidation of cyclized dopamine hydroquinone by Mn^{3+} regenerated from Mn^{2+} by $O_2^{\cdot-}$ (upper panel), and SOD-mediated inhibition of autoxidation coupled to DT diaphorase (Fp-DT) reduction of cyclized dopamine o-quinone (lower panel; adapted from Segura-Aguira and Lind, 1989)*

1991), and the binding of Mn to transferrin followed by sequestration of Mn into ferritin (Suarez and Eriksson, 1993). In addition, uptake and retention of iron and Mn into the brain seem highly dependent on the maturity of the blood-brain barrier (Dallman et al., 1975; Mena et al., 1974; Valois and Webster, 1989; Aschner and Aschner, 1991). Uptake of these metals is probably facilitated through transferrin receptor-mediated processes at the mature BBB (Aschner and Aschner, 1990; Murphy et al., 1991; Taylor et al., 1991; Roberts et al., 1992; Rabin et al., 1993). The iron-transferrin binding in CSF is often at or near iron saturation, which suggests that there exists no overcapacity in metal binding in CSF (Gutteridge, 1992). The further transport of Mn and Fe (Dwork et al., 1990) within the brain is largely unkown.

1.3.4.3. Manganese and lipid peroxidation

While iron is the key metal to initiate lipid peroxidation (Auroma et al., 1989), Mn^{2+} has been reported to inhibit lipid peroxidation both *in vitro* in a variety of systems (Cavallini et al., 1984; Tampo and Yonaha, 1992; Tsujimoto et al., 1988) and *in vivo* in tissues of Mn^{2+}-exposed rats (Shukla and Chandra, 1981; Donaldson et al., 1982). This is probably due to the superoxide radical-scavinging or SOD-mimicking properties of Mn^{2+} or simple Mn complexes which, for example, has been observed in some bacteria (Archibald and Fridovich, 1982; Halliwell, 1984; Archibald and Tyree, 1987; Bruce et al., 1992; Tampo and Yonaha, 1992).

1.3.4.4. Manganese and antioxidant enzymes

Mn produces GSH reduction in neuroblastoma cells (Heilbronn et al., 1982) and in striatum of subchronically exposed rats (Liccione and Maines, 1988). In the latter study glutathione metabolism (see Fig. 6, page 38) was significantly compromised with a particular susceptibility for mitochondria in striatum concomitantly with a mild DA reduction, including marked GSH depletions, decreased GSH-peroxidase and GSSG-reductase activities, as well as an increased GSH catabolic activity and unaltered GSH synthetic activity.

It has been suggested that various other drugs, including anti-cancer drugs (adriamycine, doxorubicine and bleomycine), paraquat, and quinones, exert their toxicity by interfering with redox-cycling processes resulting in excessive formation of $O_2^{\cdot-}$ (Kappus and Sies, 1981; Kappus, 1986).

In conclusion, specific defects in cellular defense mechanisms may

cause or at least contribute to oxidative stress and selective neurodegeneration.

1.3.4.5. Manganese and mitochondrial dysfunctions

In 1955, Maynard and Cotzias (reviewed by Barbeau, 1984) were the first to describe that Mn preferentially accumulates in mitochondria. The slow entrance via the Ca^{2+} uniporter into mitochondria (Chance, 1965; Vainio et al., 1970; Lehninger, 1972; Gavin et al., 1990) is accompanied with an even slower efflux of Mn^{2+} itself, but also of Ca^{2+}, resulting in accumulation of both cations (Gavin et al., 1990). Once inside, Mn^{2+} inhibits oxidative phosphorylation in a direct way, most likely by binding to the F_1ATPase site (Complex V), but perhaps also to Complex I (Gavin et al., 1992). This notion is supported by ATP depletion and lactate increases demonstrated after intrastriatal injection of (relatively high concentrations of) Mn^{2+} (Brouillet et al., 1993). The latter study also suggests an additional *excitotoxic process* by Mn^{2+} because the lesions were attenuated after prior removal of the glutaminergic cortical input or by treatment with MK-801, a non-competitive NMDA antagonist.

Furthermore, subchronic Mn^{2+} exposure produced a stronger elevation in *cytochrome P-450 activity* and concentration in brain mitochondria than in microsomes, which is accompanied by alterations in heme metabolism and a slight but significant decrease in the concentration of several respiratory cytochromes (Qato and Maines, 1985; Liccione and Maines, 1989).

Conclusion

Although links exist between Mn-induced damage to the basal ganglia and oxidative stress, the exact mechanism(s) of Mn neurotoxicity and the role of free (oxy)radicals therein, in particular in the *in vivo* situation, is still unclear.

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Selective lesions by manganese and extensive damage by iron after injection into rat striatum or hippocampus

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2.0. Summary

Regional ^{45}Ca accumulation and analysis of monoamines and metabolites in dissected tissues were used to localize, quantify and characterize brain damage after intracerebral injections of manganese (Mn^{2+}) into striatum and hippocampus. The specificity of Mn^{2+} induced lesions is described in relation to brain damage produced by local iron (Fe^{2+})- or 6-hydroxydopamine (6-OHDA) injections. In striatum Fe^{2+} and Mn^{2+} produced dose-dependent (0.05-0.8 µmol) dopamine (DA) depletion, with Fe^{2+} being 3.4x more potent than Mn^{2+}.

Studies examining the time course of monoamine changes in striatum following local application of 0.4 µmol Mn^{2+} revealed maximum depletion of all investigated substances (except 5-hydroxyindoleacetic acid) after 3 days. The effects on DA (87 % depletion at day 3) and its major metabolites were most pronounced and lasted until at least 90 days (40% depletion), whereas serotonin and noradrenaline levels recovered within 21 and 42 days respectively. In addition, 3-methoxytyramine (3-MT), which is used as an index of DA-release, also recovered within 42 days, indicating a functional restoration of DA neurotransmission despite substantial loss of DA content.

Intrastriatal Mn^{2+} (0.4 µmol) produced time-dependent ^{45}Ca accumulation in striatum, globus pallidus, entopeduncular nucleus, several thalamic nuclei and SN pars reticulata ipsilateral to the injection site. In contrast, 6-OHDA injected at a dose equipotent in depleting DA, produced significantly less ^{45}Ca accumulation in striatum and GP and no labeling of other brain areas, whereas Fe^{2+} (0.4 µmol) produced extensive ^{45}Ca accumulation throughout basal ganglia, accumbens and cerebral cortex. In hippocampus high Mn^{2+} (0.4 µmol) produced limited ^{45}Ca accumulation in subiculum and dentate gyrus, whereas low Fe^{2+} (0.1 µmol) produced wide-spread ^{45}Ca accumulation throughout hippocampus, thalamus and cerebral cortex. It is concluded that (1) Mn^{2+} is selectively neurotoxic to pathways intrinsic to the basal ganglia, (2) intrastriatal injections can be used as a model for systemic Mn^{2+} intoxications and (3) high endogenous Fe^{3+} and/or catecholamine levels potentiate the neurotoxicity of Mn^{2+}.

2.1. Introduction

Cases of acute and chronic intoxications (Jellinger, 1986a,b) and some epidemiological studies (Tanner, 1989; Semchuk et al., 1992) suggest that environmental or occupational exposure to toxic chemicals may play a role in the etiology of some basal ganglia disorders, including Parkinsonism and dystonia. In addition, oxidative stress in the basal ganglia, as a result of oxidation of catecholamines, the presence of high levels of iron, and mitochondrial dysfunction, has been hypothetically linked to the etiology of these neurodegenerative diseases (Olanow et al., 1992; Jenner et al., 1992; Youdim et al., 1993). Autooxidation of dopamine (Donaldson et al., 1982; Graham, 1984; Halliwell, 1984), iron binding sites (Scheuhammer and Cherian, 1982; Aschner and Aschner, 1990; Murphy et al., 1992) and mitochondrial enzymes (Liccione and Maines, 1988) have also been implicated in manganese (Mn) neurotoxicity.

For more then a century Mn has been recognized as a neurotoxic agent causing psychiatric (Donaldson, 1987) and permanent extra-pyramidal or dystonia-like symptoms after chronic exposure in mining or industry (Barbeau et al., 1976). In addition to adverse effects of inorganic Mn dust or vapor among steel manufacturing workers or welders (Roels et al.,1987; Wang et al., 1989), health risks of exposure to organic Mn compounds, including the widely-used pesticide manganese ethylene-bis-dithiocarbamate (MANEB) (Ferraz et al., 1988) and the anti-knock agent methylcyclopentadienyl manganese tricarbonyl (MMT) (Gianutsos and Murray, 1982) in unleaded gasoline have raised concern.

Postmortem studies in humans (Yamada et al., 1986), and chronic studies in non-human primates (Bird et al., 1984; Eriksson et al., 1987; Eriksson et al., 1992a,b) and rodents (Bonilla, 1978; Chandra and Shukla, 1981; Autissier et al., 1982; Gianutsos and Murray, 1982) revealed that Mn^{2+} intoxication produces neuropathological changes in the basal ganglia, especially globus pallidus, caudate and putamen, with lesions being localized both pre- and postsynaptically to the dopaminergic nigrostriatal pathway. Under physiological conditions brain Mn is uniformly distributed (Wedler and Denman, 1984), but after overexposure the metal accumulates in the basal ganglia (Eriksson et al., 1987; Newland et al., 1989), which have high iron binding capacity (Hill and Schwitzer, 1984).

Since both entry and clearance of Mn^{2+} from the central nervous system is slow (London et al., 1989; Newland et al., 1989; Murphy et al., 1991), this may explain the relatively late onset of clinical signs after chronic Mn^{2+} exposure. In rats, intracerebral injections of Mn^{2+} into striatum (Lista et al.,

1986) or substantia nigra (Parenti et al., 1986; Parenti et al., 1988; Daniels and Abarca, 1991) produce rapid dose-dependent depletions of striatal dopamine (DA) and may thus provide an easy and cheap animal model to study effects and mechanisms of Mn^{2+} neurotoxicity.

The present study was done to further characterize the dose-dependency, time-course, regional distribution and selectivity of brain injury following intrastriatal Mn^{2+} administration in rats using ^{45}Ca-autoradiography (Dienel, 1984; Gramsbergen et al., 1988; Gramsbergen and Van der Sluijs-Gelling, in press) and analysis of monoamines and catabolites in dissected striatum and substantia nigra, including not only DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) - as in most other studies -, but also noradrenaline (NE) and the DA-metabolite 3-methoxytyramine (3-MT), which reflects DA-transmitter release (Westerink, 1979; Wood and Altar, 1988). The selectivity of Mn^{2+}-induced lesions was studied using two approaches. First, lesions produced by Mn^{2+} injection into striatum - a brain area with high iron and catecholamine content - were compared with 6-hydroxydopamine and Fe^{2+} lesions. Like Mn, Fe is a transition metal and intracerebral $Fe^{2+/3+}$ administration is known to cause lipid peroxidation (Triggs and Willmore, 1984; Willmore et al., 1986) and DA depletion in vivo (Ben-Shachar and Youdim, 1991; Sengstock et al., 1992). Second, cerebral ^{45}Ca accumulation following Mn^{2+} or Fe^{2+} injections into hippocampus, - a brain area with low iron and catecholamine content - was studied and compared with effects of intrastriatal injections.

2.2. Materials and methods

2.2.1. Animals and materials

Experimental protocols regarding animal experimentation were approved by the "Ethical committee for laboratory animal experiments" TNO/Regio West (Woudenberg, The Netherlands) as registered under DEC No.56-3A. Locally bred, Wistar-derived male rats (WAG-MBL, TNO Rijswijk, The Netherlands) were kept on a 12:12h light-dark cycle and housed in a room with a humidity of 50-70% and a temperature of 24° C with free access to water and chow food.

Manganese ($MnCl_2$; >96% pure), iron ($FeCl_2.4H_2O$; >99% pure) and dopamine (3-hydroxy-phenylethylamine.HCl) were obtained from Merck, Darmstadt, Germany. All other HPLC standards and 6-hydroxydopamine.HBr (6-OHDA) were purchased from Sigma, St.Louis, USA. Radioactive calcium ($^{45}CaCl_2$) with a specific activity of 10-40 mCi/mg Ca was obtained from Amersham Int., Amersham, England.

2.2.2. Surgery

Chloral hydrate anaesthetized (400 mg/kg i.p.) rats were placed in a stereotactic frame with the nose-bar at -2.5 mm and injected into the left striatum (A 1.0, L -2.5 from bregma and V -6.0 mm from the skull) or hippocampus (A -3.3, L -2.0 from bregma and V -4.6 mm from the skull) (Paxinos and Watson, 1986) using a 5 μl syringe (SGE, Ringwood, Victoria, Australia). Rats of 180-200 g received a unilateral (left) intrastriatal or intrahippocampal injection of 0.1, 0.2, 0.4 and 0.8 μmol $MnCl_2$ (12.5-100 μg), 0.05, 0.1, 0.2 and 0.4 μmol $FeCl_2.4H_2O$ (9.9-79 μg), or 0.4 μmol NaCl (23 μg) in 1 μl Milli-Q water (Millipore, Molsheim, France), or 0.1 μmol 6-OHDA.HBr (24 μg) in 3 μl physiological saline containing 0.1% ascorbic acid. The volumes were infused at a rate of 1 μl per minute and the needle was kept in situ for an additional minute before being slowly withdrawn. Only freshly made solutions were used. Since the Mn^{2+} solution was not clear, all solutions were filtered through a 0.2 μm disc-filter (Schleicher & Schuell, Dassel, Germany). The concentration of the filtered Mn^{2+} solution was checked using Atomic Absorption Spectrometry and contained more than 92% of the originally weighed Mn.

2.2.3. Experimental groups

Four sets of experiments have been carried out to study the dose-dependency, time course and selectivity of Mn^{2+} neurotoxicity.

Dose-dependency in striatum.

Dose-dependent DA depletion and ^{45}Ca-accumulation in the same dissected striata were studied 3 days after unilateral intrastriatal NaCl, Mn^{2+} or Fe^{2+} injections using 4 rats per dose (total n = 36).

Time course.

For the time course study rats received intrastriatal injections of 0.4 μmol Mn^{2+} and were used either for HPLC analysis of dissected striatum and substantia nigra (n = 35; 5 per time point) or for ^{45}Ca-autoradiography and histology (n = 21; 3 per time point). The following time points were studied: 1, 3, 7, 10, 21, 42 or 90 days after surgery.

Selectivity in basal ganglia

The selectivity of Mn^{2+} neurotoxicity in the basal ganglia was studied 10 days after intrastriatal injection by comparing lesions produced by 0.4 μmol Mn^{2+} with those produced by 0.4 μmol NaCl, 0.4 μmol Fe^{2+}, or 0.1 μmol 6-OHDA. For each treatment 5 rats were used for HPLC analysis of monoamines in dissected tissues (total n = 20) and 3 rats were used for ^{45}Ca-autoradiography and histology (total n = 12). In addition, some autoradiograms were made at day 3 and 21 following 6-OHDA injections and compared with autoradiograms of Mn^{2+} lesions at those time points (see Time course).

Selectivity in hippocampus

The selectivity of Mn^{2+} neurotoxicity in the hippocampus was studied using unilateral injections of a low (0.1 μmol) or high (0.4 μmol) dose of $MnCl_2$ and compared with equimolar $FeCl_2$ injections into that brain area. Three rats per dose were used (total n = 12) and the resultant lesions were analyzed using ^{45}Ca-autoradiography 10 days after surgery.

2.2.4. Determination of biogenic amines

For the determination of biogenic amines, rats were sacrificed by decapitation and their brains were subsequently removed from the skull and rapidly dissected on ice. Dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT), serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and noradrenaline (NE) were determined in ipsi- and contralateral striatum or SN using a slightly modified HPLC-procedure with electrochemical detection (Santiago and Westerink, 1990) in one chromatographic run. Tissues were sonicated in at least 20 volumes ice-cold 0.1 M $HClO_4$, centrifuged for 15 min at 10.000g, diluted in the case of striatal samples (SN samples were undiluted), and directly injected into the HPLC-system.

A Gynkotec model 300 pump (flow: 0.8ml/min) was used in conjunction with a glassy carbon working electrode set to -780 mV with respect to an Ag/AgCl reference electrode (ANTEC, Leiden, The Netherlands). A spherisorb ODS2 cartridge analytical column (100x4.6 mm) with reverse phase C18 3 μm pore size together with a ODS1 precolumn (30x4.6 mm, 5μm) was used for separation (Phase Separation Ltd, Deeside, England). The mobile phase consisted of a mixture of 0.1 M sodium acetate adjusted to pH 4.1 with acetic acid, 10-15 mg/L Na_2EDTA, 0.43-0.45 g/L sodiumoctylsulfonic acid and 140-150 ml/L methanol.

2.2.5. ^{45}Calcium autoradiography and histology

Rats used for ^{45}Ca autoradiography and histology received 100 μCi radioactive calcium in 1 ml saline i.p. 20-24hr before decapitation. Brains were quickly removed from the skull, frozen in isobutanol chilled with dry-ice (-40°C), and stored at -70°C until further use. ^{45}Ca-autoradiography was conducted with 30 μm sections cut from the prefrontal cortex up to the substantia nigra on a crystat at -20°C. The heat-dryed sections were exposed to β-sensitive film (Hyperfilm-βmax, Amersham Int., Amersham, England) for 7 days and developed in Kodak D-19 (Kodak-Pathé, Chalon-sur-Saône, France). The same sections were used for thionine staining.

Autoradiograms of each 5th section (distance between analyzed sections: 120 μm) were analyzed in a semi-quantitative way using relative optical density (ROD) and area measurements [mm^2] of computerized images (MCID software: Imaging Research Inc., Brock University, St.Catharines, Canada). In addition, the anatomical localization of the radiolabel was assessed using video overlay images of corresponding thionine-stained sections. ^{45}Ca accumulation was expressed as a cumulative dose, that is the sum of relative optical densities above background (usually the relative optical density of brain areas contralateral to the injection site) multiplied by the area of hot spots: $\Sigma(ROD_{hs}-ROD_{bkg})$*area [$mm^2$]. In a previous paper (Gramsbergen and Van der Sluijs-Gelling, 1993) it was shown that the ROD obtained by our autoradiographic procedure is a reliable index to quantify ^{45}Ca accumulation.

2.2.6. ^{45}Calcium determination in dissected tissue

^{45}Calcium accumulation was determined in dissected ipsi- and contralateral brain tissues using liquid scintillation counting of β-irridiation as described previously (Gramsbergen and Van der Sluijs-Gelling, 1993). Briefly, rats received 10 μCi radioactive calcium in 1 ml saline i.p. 20-24 hr before sacrifice. Tissues were weighed and dissolved overnight in tissue solubilizer (Soluene-350, Packard, Groningen, The Netherlands) at a temperature of 50 °C. The next day scintillation liquid (UltimaGold, Packard) was added to the samples and analyzed for

^{45}Ca in a liquid scintillation counter (CA2000, Packard). Results are expressed as ratios of ipsi- : contralateral ^{45}Ca contents (cpm/mg wet weight).

In addition to ^{45}Ca counting, biogenic amines were determined in the same striatal tissues. In these samples, perchloric acid was added as described above ("Determination of biogenic amines"). Of the acidic samples, 1/8 of the supernatant was used for HPLC-analysis, and the remaining supernatant and tissue pellet was used for ^{45}Ca-counting. Tissue solubilizer was added after adjusting pH (>7), sonicating and freeze drying of the samples.

2.3. Results

2.3.1. Dose-dependency in striatum

A. Dopamine depletion by Mn^{2+} or Fe^{2+} (Fig.1A)

Both Mn^{2+} and Fe^{2+} produced a dose-dependent DA depletion 3 days after intrastriatal injection ($F = 36.17$, $p < 0.001$) with Fe^{2+} being the most potent neurotoxin. At this time point DA depletion by Mn^{2+} reached a maximum (Fig.2). Plotted on a semi-logarithmic scale the dose-response relationships are linear with correlation coefficients of 0.86 for Mn^{2+} and 0.90 for Fe^{2+} ($p < 0.001$). Statistical analysis of regression (Snedecor and Cochran, 1980) of the Mn^{2+} and Fe^{2+} dose-response curves using $[DA] = -35.6 * \log [Mn^{2+}] + 7.56$ or $[DA] = -28.8 * \log [Fe^{2+}] - 3.09$, revealed significantly different elevations ($p < 0.001$, $F = 36.2$) with no statistically difference in the slopes. The EC_{50}'s (50% DA-depletion) calculated from the above equations are 0.22 μmol for Mn^{2+} and 0.06 μmol for Fe^{2+}. In addition, ANOVA and subsequent analysis by Fisher test demonstrated that all Mn^{2+} and Fe^{2+} doses were different from controls (62.5 ± 2.1 pmol DA/mg wet weight; $p < 0.05$). Saline injected rats were not different from untreated controls.

B. Correlation between ^{45}Ca-accumulation and DA-depletion (Fig.1B)

^{45}Ca accumulation induced by Fe^{2+} reached far higher levels (^{45}Ca-ratios increased 3-13 times) then Mn^{2+} (0.7-1.7 times). The Fe^{2+} treated group showed a high correlation ($r = 0.86$, $p < 0.001$) between DA-depletion and increase of ^{45}Ca-ratios with a slope significantly different from zero ($p < 0.001$). In contrast, the Mn^{2+} group demonstrated a low but significant correlation ($r = 0.58$), $p < 0.02$) with a very flat slope. The slopes of the Fe^{2+} and Mn^{2+} curves were significantly different ($p < 0.001$).

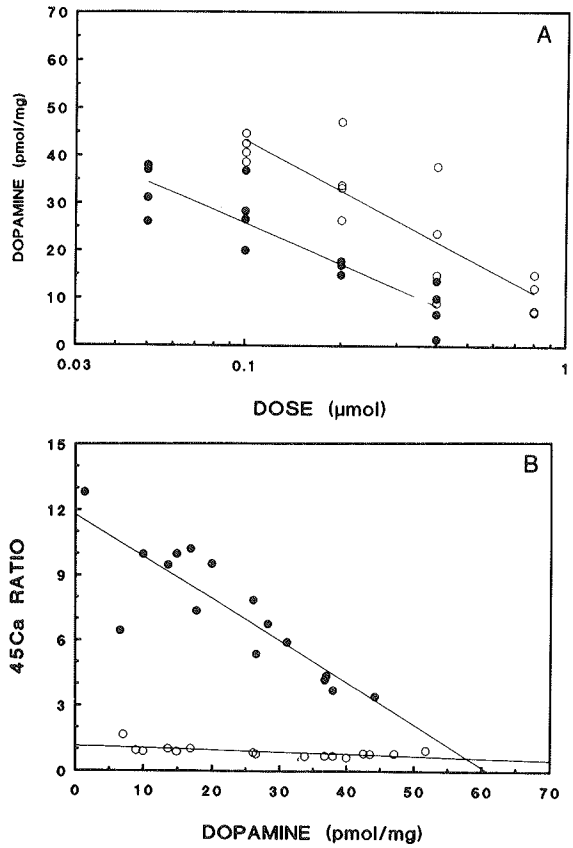


Fig. 1A. *Dose-dependent DA depletion by Mn^{2+} (open circles) or Fe^{2+} (closed circles) three days after injection into striatum.* For Mn^{2+} correlation coefficient (r) is 0.86 ($p < 0.001$) and EC_{50} is 0.22 μmol; for Fe^{2+} r is 0.90 ($p < 0.001$) and EC_{50} is 0.06 μmol. Statistical analysis of regression of the Mn^{2+} and Fe^{2+} dose-response curves revealed significantly different elevations ($F = 36$, $p < 0.001$), with no statistically different slopes. Control striatum contained 62.5 ± 2.1 pmol of DA/mg wet weight (mean ± SEM).

Fig. 1B. *Relationship between ^{45}Ca accumulation as determined by liquid scintillation counting and DA depletion in striatum three days after Mn^{2+} (open circles) or Fe^{2+} (closed circles) administration (0.05-0.8 µmol).*
Control striatum contained 62.5 ± 2.1 pmol DA/mg wet weight (mean ± SEM). ^{45}Ca accumulation is expressed as the ^{45}Ca ratio of ipsilateral striatum (in dpm/mg)/contralateral striatum (in dpm/mg). The r values for Fe^{2+} and Mn^{2+} are 0.86 ($p < 0.001$) and 0.58 ($p < 0.02$) respectively, with significantly different slopes ($p < 0.001$).

2.3.2. Time course

Injection of 0.4 μmol Mn^{2+} produced maximum depletion of DA (87%) and 3-MT (91%) in the ipsilateral striatum within 3 days. Three months afterinjection, striatal DA content was still substantially reduced (40%; Fig.2). HVA and DOPAC were also persistently reduced (Table 1). However, 3-MT levels returned to almost normal limits within 6 weeks as compared to control values (Table 1). In addition, NE and 5-HT levels were significantly reduced within 3 days, but reached normal levels within 3-6 weeks (Fig.2). The time

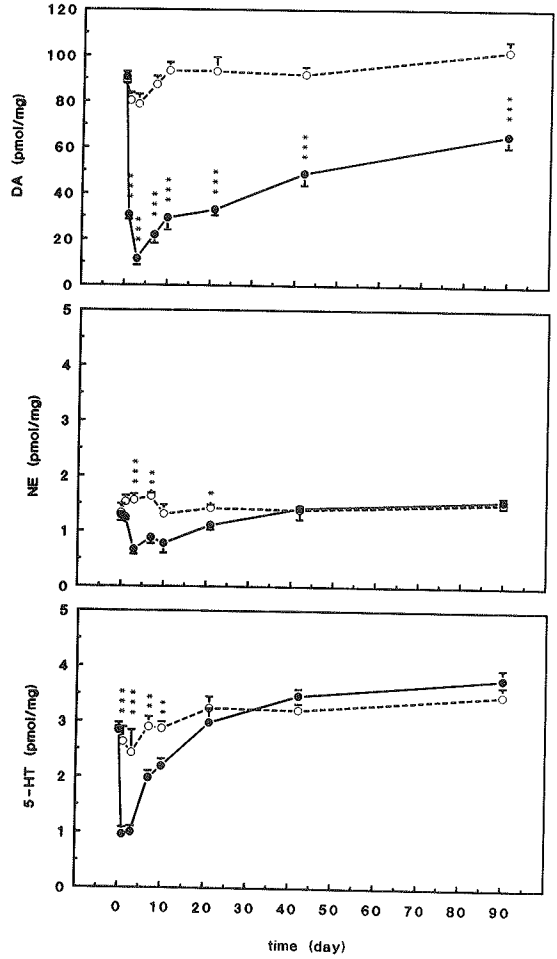


Fig. 2. *Time course of dopamine (DA), norepinephrine (NE) and serotonin (5-HT) contents in ipsi- (closed circles) and contralateral (open circles) striatum after a unilateral intrastriatal injection of $MnCl_2$ (0.4 µmol).* Data are mean ± SEM (bars) values from four to six animals per time point. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ as compared to ipsi- or contralateral striatum by Student's *t* test. Note the different scale for DA on the Y-axis.

course of the 3-MT/DA ratio revealed large reductions (~10x) at days 1-3, normal ratios at days 7-10, an overshoot (~2x) after 3-6 weeks, and a return to normal ratios at day 90 after Mn^{2+} (Fig.3). All other metabolite/transmitter ratios were not reduced as compared to control at any time point, and were significantlyincreased from day 1 or 3 through day 10 (5-HIAA/5-HT; ~3.0x), day 21 (HVA/DA; 4.6x) or day 42 (DOPAC/DA; 2.3x) (Fig.3).

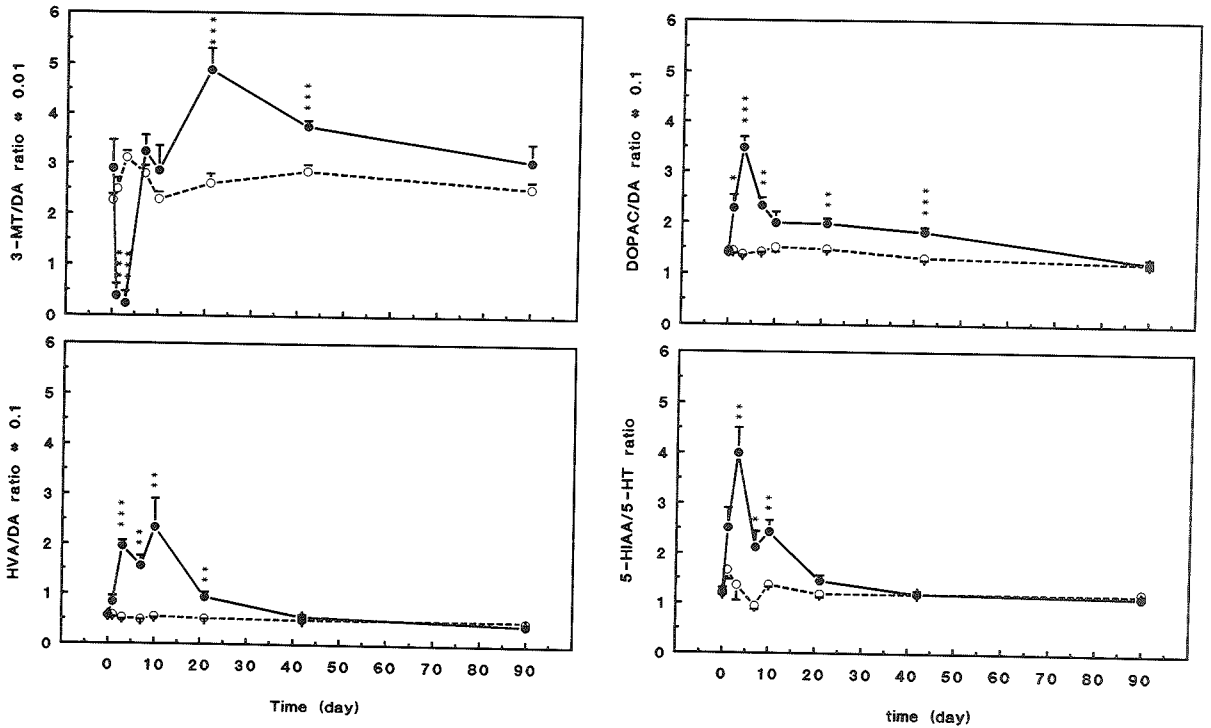
Untreated control rats did not show any regional accumulation of ^{45}Ca in the brain. Following an unilateral intrastriatal injection of Mn^{2+}, ^{45}Ca accumulated in the ipsilateral striatum, GP, nucleus entopeduncularis (EP), thalamic subnuclei (ventrolateral and ventromedial nucleus), and SN pars reticulata. In addition, in the cerebral cortex overlying the injected striatum

Table 1. *Time course of DA and 5-HT metabolites in striatum after unilateral intrastriatal injection of $MnCl_2$ (0.4 μmol)*

days	DA and 5-HT metabolites (pmol/mg)							
	3-MT		DOPAC		HVA		5-HIAA	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
C	2.31 ± 0.40	1.90 ± 0.09	12.81 ± 0.49	12.54 ± 0.65	4.55 ± 0.19	4.73 ± 0.06	3.63 ± 0.13	4.07 ± 0.28
1	0.23 ± 0.03[a]	2.01 ± 0.22	6.87 ± 0.68[a]	11.52 ± 0.51	2.52 ± 0.31[b]	4.64 ± 0.38	2.34 ± 0.10[c]	4.51 ± 0.89
3	0.21 ± 0.01[a]	2.52 ± 0.16	3.76 ± 0.60[a]	10.77 ± 0.51	2.17 ± 0.44[b]	4.07 ± 0.15	4.09 ± 0.77	2.86 ± 0.18
7	0.73 ± 0.19[a]	2.44 ± 0.04	5.04 ± 0.69[a]	12.42 ± 0.41	3.25 ± 0.08[b]	4.26 ± 0.20	4.22 ± 0.76	2.74 ± 0.23
10	0.94 ± 0.22[a]	2.13 ± 0.14	6.38 ± 1.09[b]	12.24 ± 0.41	5.11 ± 0.62	5.11 ± 0.24	5.17 ± 0.26[b]	3.89 ± 0.14
21	1.59 ± 0.13[b]	2.45 ± 0.23	6.44 ± 0.24[a]	13.76 ± 0.58	3.04 ± 0.20[a]	4.79 ± 0.21	4.23 ± 0.18	3.80 ± 0.10
42	1.87 ± 0.25[c,d]	2.60 ± 0.19	8.62 ± 1.05[b]	12.01 ± 0.24	2.63 ± 0.12[a]	4.53 ± 0.17	4.13 ± 0.18	3.83 ± 0.16
90	1.87 ± 0.21[b,d]	2.56 ± 0.10	7.99 ± 0.57[b]	12.42 ± 0.78	2.62 ± 0.11[a]	4.83 ± 0.21	4.25 ± 0.08	4.27 ± 0.24

Data are mean ± SEM values from four to six rats. Rats were killed on the given days after intrastriatal injections. Levels in controls (C) were measured 10 days after intrastriatal injection of saline. [a] $p \leq 0.001$; [b] $p \leq 0.01$; [c] $p \leq 0.05$, as compared to contralateral striatum by Student's *t* test. [d] Not significantly different from saline-injected striatum by Student's *t* test.

Fig. 3. *Time course of metabolite/transmitter ratios in ipsi- (closed circles) and contralateral (open circles) striatum after a unilateral intrastriatal injection of $MnCl_2$ (0.4 µmol).*



Data are mean ± SEM (bars) values from four to six rats. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, as compared to ipsi- or contralateral striatum, Student's *t* test. Note the different scales on the Y-axis.

some label was present in some rats at day 3 or 10. ^{45}Ca accumulated in a time-dependent fashion: different regions were labeled at different time points following the insult. From day 1 through day 21 ^{45}Ca accumulated in striatum; the thalamic subnuclei accumulated ^{45}Ca from day 10 until day 90, whereas GP, EP and SN pars reticulata were labeled at day 10, but not at earlier or later time points after intrastriatal Mn^{2+}. Since all vulnerable brain areas

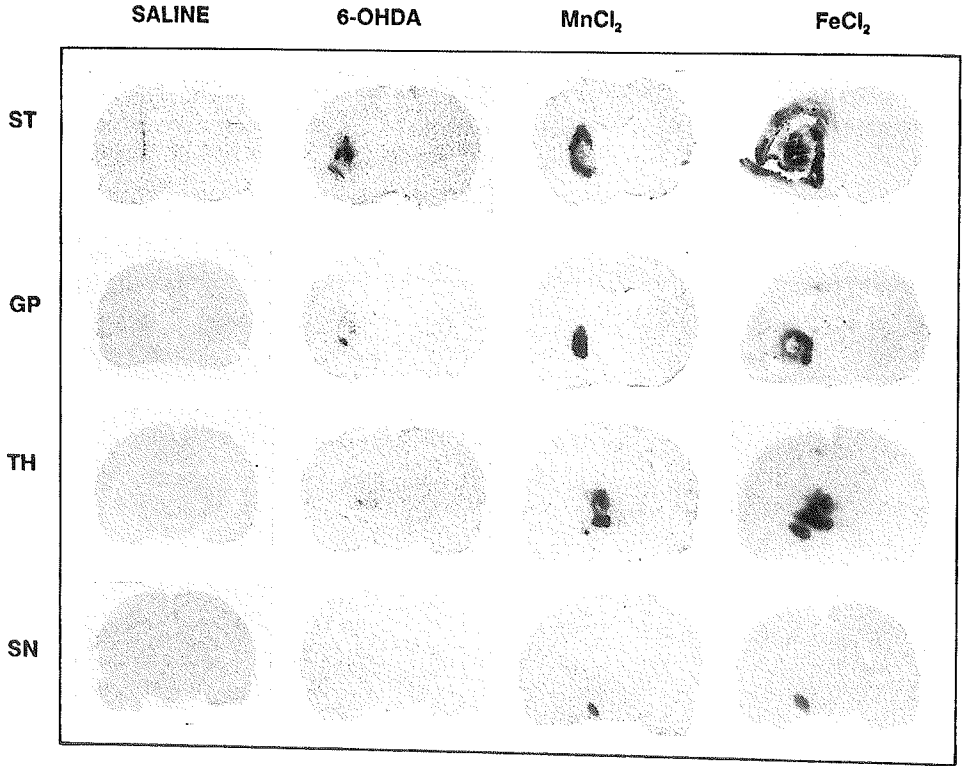


Fig. 4. *Representative $^{45}Calcium$ autoradiograms of coronal brain sections (30μm) at the level of the striatum (ST), globus pallidus (GP), thalamus (TH) and substantia nigra (SN) illustrate regional ^{45}Ca accumulation 10 days after administration of 0.1 μmol 6-OHDA or 0.4 μmol NaCl, $MnCl_2$ or $FeCl_2$ into the left striatum.*

$^{45}CaCl_2$ (100 μCi) was injected intraperitoneally 20-24 h before decapitation. Quantitative data are given in Table 3. Abbreviations: CX, cerebral cortex; HP, hippocampus.

accumulated the label at day 10, autoradiograms of this time point were used for quantification of ^{45}Ca accumulation (see section 3c). Representative autoradiograms of this time point are shown (Fig.4).

Gross histological examination of thionine-stained sections revealed neuronal loss and gliosis in structures with high ^{45}Ca-labeling and also in brain areas which had accumulated the label at earlier time points. Histological changes as observed on thionine-stained sections were most pronounced in the GP, and to a lesser extent in striatum, thalamus and SN pars reticulata.

2.3.3. Selectivity in basal ganglia

A. Monoamines in striatum (Table 2)

Unilateral injection of 0.1 μmol 6-OHDA into striatum produced a DA depletion (62%) not significantly different from that produced by 0.4 μmol Mn^{2+} (67%) as examined at day 10 (Table 2). Reductions of other monoamine levels

Table 2. *Dopamine contents (pmol/mg) and DOPAC/DA ratios in striatum and substantia nigra 10 days after unilateral intrastriatal injection of 0.4 μmol NaCl, $MnCl_2$ or $FeCl_2$, or 0.1 μmol 6-OHDA.*

Treatment	Striatum Ipsilateral	Contralateral	Substantia Nigra Ipsilateral	Contralateral
Saline				
DA	80.9 ± 3.03	84.1 ± 2.20	4.63 ± 0.33	4.40 ± 0.40
DOPAC/DA	0.10 ± 0.01	0.11 ± 0.01	0.29 ± 0.02	0.29 ± 0.02
6-OHDA				
DA	34.5 ± 5.89[a,d]	88.4 ± 2.71	4.35 ± 0.54	5.87 ± 0.48
DOPAC/DA	0.14 ± 0.01[b,c]	0.11 ± 0.003	0.19 ± 0.01[c]	0.21 ± 0.01[c]
$MnCl_2$				
DA	29.3 ± 5.25[a,d]	93.3 ± 3.67	4.47 ± 0.22	5.54 ± 0.43
DOPAC/DA	0.15 ± 0.02[c]	0.11 ± 0.01	0.20 ± 0.004[c]	0.23 ± 0.01[f]
FeCl2				
DA	6.3 ± 1.10[a,d,h]	83.4 ± 2.78	5.43 ± 0.31[c]	7.32 ± 0.68[f]
DOPAC/DA	0.27 ± 0.01[a,d,g]	0.13 ± 0.01	0.15 ± 0.014[d,g]	0.17 ± 0.01[d,g]

Data represent mean ± SEM values from four to six rats. [a] $p \leq 0.001$, [b] $p \leq 0.01$, [c] $p \leq 0.05$ for ipsilateral versus contralateral levels by Students's *t* test; [d] $p \leq 0.001$, [e] $p \leq 0.01$, [f] $p \leq 0.05$ for treated versus control levels by Student's *t* test with Bonferroni correction for multiple comparisons; [g] $p \leq 0.001$, [h] $p \leq 0.05$ for 6-OHDA- or Fe-treated group versus Mn-treated group by Student's *t* test with Bonferroni correction for multiple comparisons.

in the 6-OHDA injected striatum at this time point were: 3-MT (39%); DOPAC (53%); HVA (46%) and NE (59%), which is essentially similar as those produced by Mn^{2+} (see section 2). In contrast to Mn^{2+}, 6-OHDA had no significant effect on 5-HIAA and 5-HT levels.

Unilateral injection of 0.4 μmol Fe^{2+} into striatum produced far larger reductions of monoamines than equimolar Mn^{2+} or 0.1 μmol 6-OHDA: DA (92%); NE (>95%); 3-MT (>81%); DOPAC (84%); HVA (73%); and 5-HT (66%). 5-HIAA levels were not significantly changed, whereas 5-HT levels of the contralateral striatum were also reduced (27%) as compared to control levels.

B. Monoamines in substantia nigra (Table 2)

Monoamines were determined in ipsi- and contralateral substantia nigra 10 days after intrastriatal injection of Mn^{2+}, Fe^{2+}, 6-OHDA or saline; DA levels and DOPAC/DA ratios are shown in Table 2. In substantia nigra of saline-injected rats the levels of the DA metabolites DOPAC and HVA were 1.34 ± 0.07 and 0.41 ± 0.03 pmol/mg ± SEM. The 3-MT level was below detection limit. Levels of NE, 5-HT and 5-HIAA were 1.45 ± 0.11, 5.94 ± 0.29 and 4.70 ± 0.1 pmol/mg ± SEM respectively.

Striatal Mn^{2+} lesions produced in the ipsilateral SN no change of DA, HVA and HVA/DA, but significant DOPAC (73% of control) and DOPAC/DA reductions. In addition, NE (146% of control) and 5-HT (118% of control) were increased. In the contralateral SN, DA tended to be elevated, HVA and DOPAC were normal, and the DOPAC/DA ratio was significantly reduced, whereas NE and 5-HT levels were normal.

Striatal 6-OHDA lesions produced in the ipsilateral SN similar changes as Mn^{2+}: no change of DA, HVA or HVA/DA, but significant DOPAC (61% of control) and DOPAC/DA reductions. In addition, NE and 5-HT were normal. In the contralateral SN, DA tended to be elevated, HVA (129% of control) was significantly increased, DOPAC normal, and DOPAC/DA was significantly reduced, whereas NE (137% of control) was significantly increased, and 5-HT was normal.

Striatal Fe^{2+} lesions produced in the ipsilateral SN not significantly elevated DA, normal HVA levels, and significant HVA/DA, DOPAC (59% of control) and DOPAC/DA reductions. In addition, NE (240% of control) and 5-HT (145% of control) were significantly increased. In the contralateral SN, DA and HVA (144% of control) were significantly increased, DOPAC normal, and DOPAC/DA was significantly decreased, whereas NE (161% of control) was significantly increased, and 5-HT was normal.

C. ^{45}Ca-autoradiography (Fig.4 and Table 3)

Representative ^{45}Ca-autoradiograms obtained 10 days after unilateral injections of saline, 6-OHDA, Mn^{2+} or Fe^{2+} into striatum are shown in Fig.4.

Table 3. *Quantification of ^{45}Ca-accumulation in whole brain from autoradiograms obtained 10 days after injection of 6-OHDA, Mn^{2+} or Fe^{2+} into striatum or hippocampus*

Toxin	Dose (μmol)	Injection site	Whole brain ^{45}Ca accumulation
6-OHDA	0.1	Striatum	2.3 ± 0.8[a]
$MnCl_2$	0.1	Hippocampus	0.2 ± 0.06[b]
	0.4	Hippocampus	4.3 ± 0.8
	0.4	Striatum	11.1 ± 3.7
$FeCl_2$	0.1	Hippocampus	23.7 ± 5.0[b]
	0.4	Hippocampus	22.8 ± 4.3[b]
	0.4	Striatum	44.1 ± 24.5

Data are mean ± SEM values from three rats and are expressed as $\Sigma(ROD_{hs}-ROD_{bg}*area)[mm^2]$, which represents the cumulative ^{45}Ca dose above background (ROD = relative optical density of hot spots (hs) minus ROD of background (bg) of non-labeled tissue) in each 5th section of defined brain regions (see Materials and Methods). Whole brain represents the total of labeled regions from fore- and midbrain structures up to the level of SN. For detailed qualitative description of regional ^{45}Ca accumulation, see Results. [a] Significantly different from Mn^{2+}- or Fe^{2+}-treated rats (striatum) by Mann-Whitney *U* test, p = 0.05. [b] Significantly different from 0.4 μmol Mn^{2+}- treated rats (hippocampus) by Mann-Whitney *U* test, p = 0.05.

Saline injection produced only ^{45}Ca accumulation at the site of the needle tract.

As compared to Mn^{2+} lesions, 6-OHDA produced less ^{45}Ca accumulation in the ipsilateral striatum and GP, while only neglectable or no label was present in the thalamus, and no label was observed in the SN at any time point studied. As compared to both Mn^{2+} and Fe^{2+} rats, 6-OHDA injections produced significantly less ^{45}Ca accumulation in whole brain (Table 3).

In contrast to Mn^{2+}, Fe^{2+} produced not only ^{45}Ca accumulation in the brain areas vulnerable for Mn^{2+}, but also in other brain areas, includingipsilateral prefrontal, frontal and parietal cortex, corpus callosum, nucleus accumbens, and subthalamic nucleus. Histologic examination of striatal sections revealed severely damaged and shrunken striatum and cerebral cortex and disappearance of the corpus callosum ipsilateral to the injection site. Since ^{45}Ca accumulation was assessed in only three rats per toxin with large variation in the severity of the lesions, quantitative differences between Mn^{2+} and Fe^{2+} rats did not reach significance in the analyzed ipsilateral brain areas or whole cerebral hemisphere. However, in Fe^{2+} treated rats significantly more brain areas accumulated ^{45}Ca then in Mn^{2+} treated ones (Mann-Whitney *U* test, p = 0.05).

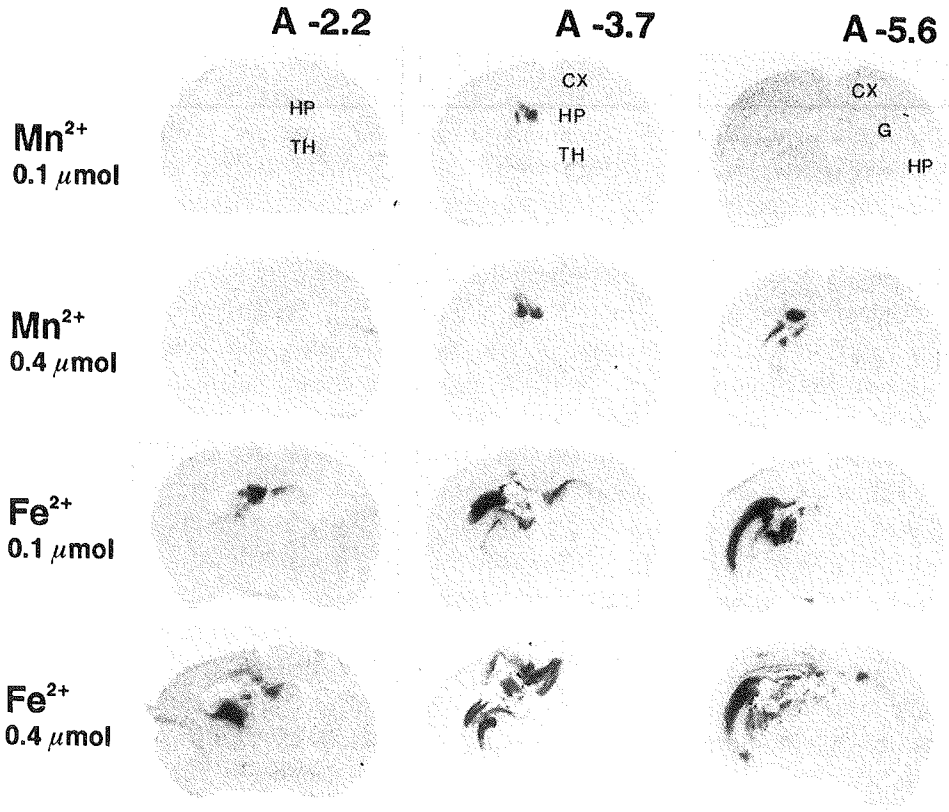


Fig. 5. *Representative ^{45}Calcium autoradiograms of coronal brain sections (30 μm) at the level of the dorsal (A-2.2 and A-3.7) and ventral hippocampus (HP, A-5.6) illustrate regional ^{45}Ca accumulation 10 days after administration of 0.1 or 0.4 μmol $MnCl_2$ or $FeCl_2$ into the left hippocampus [A-3.3 (stereotaxic coordinates according to Paxinos and Watson (1986)].* $^{45}CaCl_2$ (100 μCi) was injected intraperitoneally 20-24 h before decapitation. Quantitative data are given in Table 3. Abbreviations used: CX, cerebral cortex; G, geniculate nucleus; TH, thalamus.

2.3.4. Selectivity in hippocampus (Fig.5 and Table 3)

Unilateral injection of 0.1 μmol Mn^{2+} into the hippocampus resulted in both qualitatively (Fig.5) and quantitatively limited ^{45}Ca accumulation in subiculum and dentate gyrus as studied 10 days after Mn^{2+} administration. In addition, we observed some histological damage in parts of the dentate gyrus

not labeled with ^{45}Ca (since ^{45}Ca accumulation is associated with neurodegeneration this suggests that the degenerative process has stopped in that region; see section 2."Time course"). Injection of 0.4 μmol Mn^{2+} producedmore ^{45}Ca accumulation in the ipsilateral dorsal hippocampus including dentate gyrus, subiculum, CA4 and partially CA3. In addition, some label was present in the underlying dorsolateral thalamus in the vicinity of the injection site. More remote from the injection site, the dentate gyrus subiculum, and geniculate nucleus were labeled. In whole brain, the higher Mn^{2+} dose produced significantly more ^{45}Ca accumulation than the lower dose. In addition, injection of Mn^{2+} into hippocampus produced substantially less ^{45}Ca accumulation in whole brain than injection into striatum (2.6x), although the difference did not reach statistical significance.

In contrast to Mn^{2+} injections, 0.1 μmol Fe^{2+} produced extensive ^{45}Ca accumulation. In the vicinity of the injection site the complete dorsal hippocampus, including CA 1-4, dentate gyrus and subiculum, the underlying dorsolateral thalamus and partly the overlying cerebral cortex were labeled. Brain areas more rostral and caudal to the injection site, including corpus callosum, parts of the anterior thalamus, dorsolateral and ventrolateral hippocampus, and dorsolateral midbrain, including the geniculate nucleus were also labeled. In addition, ^{45}Ca accumulation occured in the contralateral dorsal hippocampus, especially subiculum and CA3. Rats injected with 0.4 μmol Fe^{2+}, showed qualitatively similar, but quantitatively more severe lesions than the lower Fe^{2+} dose in all labeled regions, except in the ipsilateral hippocampus, where ^{45}Ca accumulation was actually lower than in the low dose group. Differences in time course of neurodegeneration and massive desintegration of tissue as observed on thionine-stained sections of the high dose group may explain the lack of a significant dose-dependent ^{45}Ca accumulation in the ipsilateral hippocampus of Fe^{2+} treated rats.

2.4. Discussion

The present results indicate that Mn^{2+} is selectively neurotoxic to pathways intrinsic to the basal ganglia, and suggest that endogenous iron and/or dopamine contribute to the neurotoxicity of Mn^{2+}.

Injections of Mn^{2+} into striatum produced dose-and time-dependent monoamine depletions, with peak effects at day 3 with DA and its metabolites being more affected than NE or 5-HT. The stronger effect on DA terminals may be explained by in vitro data indicating that DA autoxidizes more readily than

NE (Graham, 1984). At a dose of 0.4 μmol Mn^{2+} depletions of DA, DOPAC and HVA are persistent until at least 90 days, whereas Lista et al. (1986) reported that a dose of 0.2 μmol produced reversible DA depletion within two weeks. In addition, others using injections into rat substantia nigra reported also striatal DA depletions by transition metals, including Mn^{2+} (Parenti et al., 1986; Dabiels and Abarca, 1991), Cu^{2+} (Javoy et al., 1976), Ni^{2+} (Parenti et al., 1988) or Fe^{3+} (Ben-Shachar and Youdim, 1991; Sengstock et al., 1992), but not by other metals, including Mg^{2+} and Li^{+} (Parenti et al., 1988). From these studies it is not clear, whether Mn^{2+} produces more or less specific lesions than the other transition metals. The present results using ^{45}Ca accumulation as a general and quantitative index of brain injury (Gramsbergen and Van der Sluijs-Gelling, in press), revealed that Mn^{2+} produced more selective lesions than Fe^{2+} in both striatum and hippocampus. For instance, doses of Fe^{2+} and Mn^{2+} producing similar DA depletions 3 days after striatal injections, produced strong ^{45}Ca accumulation in Fe^{2+} rats, but little, if any in Mn^{2+} rats (Fig.1B).

An interesting new finding is the effect of Mn^{2+} on striatal levels of the DA-metabolite 3-MT, which is an index of extracellular or released DA (Westerink, 1979; Wood and Altar, 1988). The observed rapid decline of 3-MT, which preceded depletion of DA as reflected by the decreased 3-MT/DA ratio, suggests a direct effect of Mn^{2+} on DA synapses. Despite persistent reductions of DA, DOPAC and HVA, after longer survival times DA transmission seems to be normalized, since 3-MT levels and all metabolite/transmitter ratios recover. Similar adaptive phenomena have also been observed following 6-OHDA lesions (Altar et al., 1987; Zigmond et al., 1990).

In contrast to the elevated DOPAC/DA ratio in the Mn^{2+}, Fe^{2+} or 6-OHDA lesioned striatum, this ratio was reduced in the SN both ipsi- and contralateral to the lesion at day 10. This phenomenon that both sides adapt to the loss of DA terminals in striatum, has been reported previously by Berger et al. (1991) using assays for DA receptors and DA re-uptake sites after unilateral intrastriatal 6-OHDA injections. The lack of ^{45}Ca accumulation in the SN pars compacta ipsilateral to the 6-OHDA or Mn^{2+} injection suggests that the used dose did not kill DA cell bodies at the examined time points.

Using autoradiography we observed time- and region-dependent ^{45}Ca accumulation in the basal ganglia, including striatum, GP, EP, thalamus and SN pars reticulata following intrastriatal Mn^{2+}. In contrast to ^{45}Ca autoradiograms obtained at different time points following intrastriatal Mn^{2+} injection, we observed no significant ^{45}Ca accumulation in dissected striata of 3 day old Mn^{2+} lesions. Since autoradiography is more sensitive than liquid scintillation counting of tissue to detect anatomically restricted and quantitatively small changes, this may provide an explanation. In 6-OHDA

lesioned rats with similar DA depletions as Mn^{2+} lesions less ^{45}Ca accumulation was present in striatum and GP with no labeling in other areas. This indicates that not only the DAergic nigrostriatal/pallidal pathway but also other circuits localized postsynaptically to this pathway are targets for Mn^{2+} toxicity. The heavy ^{45}Ca-labeling of GP, SN reticulata and thalamus suggests that γ aminobutyric acid (GABA)ergic nerve terminals arising from striatum, GP and SN or cell bodies in those areas (Alexander and Crutcher, 1990; Albin et al. 1989) are damaged by Mn^{2+}. Using the present Mn^{2+} model we observed modest reductions of glutamic acid decarboxylase (GAD) activity in striatal and pallidal tissues (unpublished results) suggesting that Mn^{2+} may kill GABAergic neurons. It is, however, difficult to draw conclusions from GAD data after Mn^{2+} lesions, since destruction of the nigrostriatal pathway with 6-OHDA causes significant increases of GAD activity and GAD mRNA expression in striatum (Segovia et al., 1990). Recently, in Mn^{2+} intoxicated monkeys both pre- and postsynaptic DA changes have been demonstrated in striatum using in vitro radioligand binding assays of DA up-take sites (75% reduction) and D1 receptors (45% reduction) (Eriksson et al., 1992a). Postsynaptic changes have also been suggested in Mn-patients using PET with ^{18}F-deoxyglucose, whereas ^{18}F-DOPA scans were normal (Wolters et al., 1989). In addition, Eriksson et al (1992b) using PET scans of Mn^{2+} intoxicated monkeys showed that [^{11}C]-L-DOPA uptake was normal too, while DA-uptake sites were decreased (-60%) indicating that L-DOPA uptake is not a very sensitive presynaptic DA-marker.

As discussed above, locally applied Mn^{2+} produces extensive (selective) damage in the basal ganglia, but limited lesions in the hippocampus. Fe^{2+}, in contrast, produces wide spread damage after local injection into both striatum and hippocampus. This suggests that the mechanism of Fe^{2+} and Mn^{2+} neurotoxicity are not identical. Free $Fe^{2+/3+}$ is known to cause lipid peroxidation *in vivo* in cerebral cortex (Triggs and Willmore, 1984) or hippocampus (Willmore et al., 1986) by initiating and catalyzing free radical formation via the Haber-Weiss reaction (reviewed by Halliwell and Gutteridge, 1986; Halliwell 1992). In contrast to previous reports using Fe^{3+} injections into substantia nigra (Ben-Sachar and Youdim, 1991; Sengstock et al., 1992).), our results obtained after Fe^{2+} injections into striatum do not support the hypothesis that Fe^{2+} is selectively neurotoxic to DA neurons.

Hypotheses on the mechanism of Mn^{2+} toxicity have emphasized the formation of free radicals in the presence of catecholamines (Donaldson et al. 1982; Halliwell, 1984; Graham, 1984). Therefore brain areas with high levels of catecholamines (e.g. DA in striatum and GP, and NE in subiculum and dentate gyrus) may be selectively vulnerable for Mn^{2+}. Since Mn^{2+} appears to be preferentially toxic to iron containing brain areas (e.g. basal ganglia) o

subregions (e.g. dentate gyrus), iron binding sites/proteins or endogenous iron itself (Hill et al., 1985; Dwork et al., 1988) may also be important contributing factors in Mn^{2+} neurotoxicity. Especially the physiological distribution of ferric iron (Hill and Switzer, 1984) mostly bound to ferritin strongly resembles that of Mn^{2+} after overexposure. This relationship is particularly striking for the SN reticulata and GP, of which the latter is most vulnerable for Mn^{2+} (Yamada et al., 1986; Jellinger, 1986a; Newland and Weiss, 1992). Ferric iron is predominantly localized in glial cells and the myeline envelop of axons of the GABAergic striato-pallido-nigral system (Francois et al., 1981). Since ferritin can exchange and interact with several (toxic) transition metals both in vitro and in vivo (Joshi and Zimmerman, 1988), the presence of ferritin and iron could make these neurons more vulnerable under pathological conditions, including cellular energy depletion causing acidosis during which bound iron may be liberated (Siesjö et al., 1989). The pathological significance of endogenous iron for DA cells is illustrated by the mechanism of 6-OHDA neurotoxicity, which is thought to involve free radical formation (Cohen and Heikkila, 1974) and release of iron from ferritin (Monteiro and Winterbourn, 1989). In addition, 6-OHDA neurotoxicity can be attenuated by the iron chelator desferrioxamine (Ben-Sachar et al., 1991).

In conclusion, striatal injection of Mn^{2+} into rat brain produces selective basal ganglia pathology which is very similar to that observed after chronic systemic exposure. Using this model the role of both exogenous and endogenous transition metals in basal ganglia disorders can be studied. Brain areas with high levels of endogenous iron, iron binding sites and/or catecholamines were most vulnerable for Mn^{2+} neurotoxicity. The relative importance of these factors for precipitating toxic cell death, however, remains to be clarified.

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Axonal transport of manganese and its relevance to selective neurotoxicity in the rat basal ganglia

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3.0. Summary

The present study provides evidence for anterograde axonal transport of manganese (Mn) in the basal ganglia. Microinjections of ^{54}Mn into rat substantia nigra or striatum revealed region-specific accumulation and retention of the isotope in globus pallidus, striatum, thalamus and substantia nigra for up to at least 48 or 72 hours respectively. Within 4 hours after intra-striatal injection of ^{54}Mn, radioactivity accumulated in the substantia nigra, suggesting axonal transport of the metal. Subsequent studies using bilateral ^{54}Mn injections into striatum or substantia nigra and unilateral colchicine injections into or transection of the medial forebrain bundle confirmed axonal transport of Mn through these fibres. Selective destruction of the striatonigral or nigrostriatal pathways using quinolinic acid or 6-hydroxydopamine two weeks before injection of the isotope, revealed uptake of ^{54}Mn by cell bodies of both γ-aminobutyric acidergic striatal and dopaminergic nigral neurons and subsequent anterograde transport through striatonigral or nigrostriatal fibres. In addition, the quinolinic acid-lesioned striatum retained three times more radioactivity than the intact striatum. In conclusion, the present data suggest that both glial cells and striatonigral and nigrostriatal neurons are potential targets for Mn toxicity. These results and the selective neurotoxicity of Mn are discussed with respect to the iron transport protein transferrin, transferrin receptors, the iron storage protein ferritin, and mitochondrial dysfunction.

3.1. Introduction

The brain is susceptible to both manganese (Mn) deficiency and Mn toxicity. The metal is essential for normal brain function as a necessary constituent of some metalloproteins, including the mitochondrial enzymes superoxide dismutase (Mn^{3+}-SOD) and pyruvate carboxylase (Mn^{2+}), as well as the glial cytoplasmic enzyme glutamine synthetase (Keen et al., 1984; Prohaska, 1987), which converts glutamate into glutamine. Under physiological conditions, glutamine synthetase accounts for 80% of all available Mn^{2+} in the brain and is not regionally distributed (Wedler and Denman, 1984). Deficiency of Mn may cause seizure activity and convulsions, probably due to decreased glutamine synthetase and Mn-SOD activity (Keen et al., 1984; Wedler and Denman, 1984; Carl et al., 1993), whereas overexposure to Mn produces extrapyramidal symptoms and dopamine deficits in the basal ganglia (Barbeau et al., 1976; Donaldson, 1987).

Although it has been established that the distribution of Mn after overexposure is predominantly confined to brain areas containing high levels of non-haem iron (Fe)(Hill and Switzer, 1984), including the caudate-putamen, globus pallidus, substantia nigra and subthalamic nucleus (Bird et al., 1984; Yamada et al., 1986; Eriksson et al., 1987; Newland et al., 1989), its cellular localization and pathophysiological mechanism are still not well understood. Recently, we have demonstrated that intrastriatal injections of Mn produce nerve cell death and injury not only to the -dopaminergic (DA) nigrostriatal pathway, but also to γ-aminobutyric acid (GABA)-ergic nerve cells intrinsic to the basal ganglia (Sloot et al., 1994). The mechanism by which Mn destroys both DA- and GABA-ergic pathways in the basal ganglia could involve both direct or indirect formation of free radicals by glutathione depletion (Liccione and Maines, 1988), reduction of glutathione peroxidase activity (Liccione and Maines, 1988), auto-oxidation of dopamine (Graham, 1984; Halliwell, 1984), abnormal carbohydrate metabolism (Hurley et al., 1984; Keen et al., 1984) and inhibition of mitochondrial respiration (Liccione and Maines, 1989; Gavin et al., 1992; Brouillet et al., 1993). Recently, it has been suggested that impaired oxidative energy metabolism by Mn causes additional excitotoxicity (Brouillet et al., 1993).

In vitro experiments have shown uptake of Mn by astrocytes (Aschner et al., 1992) and catecholamine-containing neuroblastoma cells (Suárez and Eriksson, 1993) through a high affinity uptake system and internalization of

a Mn-transferrin complex, respectively. To further explore the mechanism by which Mn produces selective neurotoxicity, the present study was aimed at investigating the clearance, regional distribution, cellular localization, and axonal transport of Mn in the rat basal ganglia. Since striatum and substantia nigra have reciprocal projections, intracellular transport can occur from striatum to substantia nigra or vice versa, in anterograde or retrograde direction or both. The experimental approach in this study is similar to that previously used to study axonal transport of monoamine oxidase using [^{3}H]-pargyline (Gramsbergen et al., 1986). Here we used intrastriatal or intranigral tracer injections of ^{54}Mn in conjunction with different means of blocking axonal transport or with selective lesions.

3.2. Materials and methods

3.2.1. Animals and surgery

For all experiments locally bred, Wistar-derived rats (WAG/Rij/MBL, MBL-TNO, The Netherlands) were used. The animals were kept on a 12:12h light dark cycle in a climate-controlled room with free access to water and chow food. Male rats weighing 180-200 g were anaesthetized with chloral hydrate (400 mg/kg, ip.) and placed in a stereotaxic frame with the nose-bar set on -2.5 mm. A 5 μl syringe (SGE, Australia) was used for microinjections in the striatum (1.0 A, 2.5 L from bregma; 6.0 V from the skull), medial forebrain bundle (2.8 P, 1.8 L from bregma; 8.5 V from the skull) or substantia nigra (5.2 P, 2.0 L from bregma; 8.5 V from the skull) based on coordinates from the atlas of Paxinos and Watson (1986). The volume was infused at a rate of 1 μl per minute and the needle was kept *in situ* for an additional minute before being slowly withdrawn.

3.2.2. Materials

$^{54}MnCl_2$ was obtained from DuPont de Nemours (s'Hertogenbosch, The Netherlands; specific activity of 2.369 TBq/mg). 6-Hydroxydopamine.HBr (6-OHDA), quinolinic acid (QUIN) and colchicine (COL) were purchased from Sigma (St. Louis, Mo, USA).

3.2.3. Experimental groups

Experiment 1: Time course and saturability of ^{54}Mn binding after intrastriatal injection.

Rats received a unilateral tracer injection of carrier-free $^{54}MnCl_2$ (80,000 cpm/2 μl) into striatum and were decapitated 1, 4, 24 or 72 hours after injection. Some rats (3 vs 3) received together with the radioactive tracer dose (< 10 ng Mn^{2+}) a carrier dose of cold $MnCl_2$ (50 μg) and were killed 24 hours after injection.

Experiment 2: Blockade of axonal transport from striatum to substantia nigra.

Rats received a unilateral injection of colchicine (15 μg/1 μl) into the medial forebrain

bundle (MFB) to block axonal transport (Karlsson et al., 1971). Ten minutes or 24 hours later a tracer amount of ^{54}Mn (180,000 cpm/2 μl) was injected bilaterally into striatum. In another group of rats the MFB was transected on one side of the brain by lowering a 3 mm spatula to the bottom of the skull at the same coordinates from bregma as used for colchicine injections. Twenty-four hours after ^{54}Mn the animals were decapitated.

Experiment 3: Axonal transport from striatum to substantia nigra in rats with a nigro-striatal (6-OHDA) or striato-nigral (QUIN) lesion.

One group of rats received a unilateral injection of 6-hydroxydopamine (6-OHDA, 8 μg/1 μl phosphate buffered saline containing 0.1% ascorbate) into substantia nigra (SN) to selectively damage dopaminergic nigrostriatal cells (Javoy et al., 1976). Another group received a unilateral injection of quinolinic acid (QUIN, 50 μg/1 μl was dissolved in a few drops of 1 N NaOH and then phosphate buffered saline was added) into striatum to destroy striatonigral cells, while sparing dopamine terminals (Schwarcz et al., 1983). Two weeks after the selective lesions ^{54}Mn (180,000 cpm/2 μl) was administered bilaterally into striatum, and twenty-four hours after injection of label animals were decapitated.

Experiment 4: Axonal transport from substantia nigra to striatum.

Two groups of rats received an unilateral injection of colchicine[34] (7.5 μg/0.5 μl) and a contralateral injection of saline (1μl) into the MFB 24 hours prior to bilateral injection of ^{54}Mn (80,000 cpm/1 μl) into substantia nigra (SN). The groups of animals were decapitated 24 or 48 hours after ^{54}Mn.

Experiment 5: Axonal transport from substantia nigra to striatum in rats with a nigro-striatal 6-OHDA lesion.

Rats received a unilateral injection of 6-OHDA (8 μg/1 μl) and a contralateral injection of vehicle (1μl) into SN two weeks prior to bilateral injection of ^{54}Mn (80,000 cpm/1 μl) into SN. Animals were decapitated 24 hr after injection of label.

3.2.4. Dissection and γ-counting of brain tissues

After decapitation, the brain was quickly removed from the skull and dissected within 15 minutes. Tissues were immediately frozen on dry ice. In experiments using striatal ^{54}Mn injections, dissection was done as previously described (Gramsbergen et al., 1986) and included frontal cortex (FC), striatum (ST), globus pallidus (GP), medial forebrain bundle (MFB) I (rostral to colchicine injection or transection), hippocampus (HP), thalamus (TH), MFB II (caudal to or including colchicine injection or site of transection), substantia nigra (SN), area dorsal to the SN (DSN) and cerebellum (CB). In experiments using bilateral nigral ^{54}Mn injections dissected tissues included FC, nucleus accumbens (NA), ST, GP, HP, TH, MFB II, SN, ventral tegmental area (VTA) and CB. Dissected tissues were weighed in pre-weighed plastic tubes, and subsequently counted for 5 minutes using a Cobra γ-counter with a NaI well-type crystal (Packard, Groningen, The Netherlands). Total counting efficiency was 20-25%.

3.2.5. Evaluation of 6-hydroxydopamine (6-OHDA) and quinolinic acid (QUIN) lesions

The success of the 6-OHDA lesion was determined in three randomly chosen rats by analysing striatal dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) contents using HPLC with electrochemical detection (ECD) as described previously (Sloot et al.,

1994). Analyses revealed depletions of 89 ± 2% and 80 ± 3% respectively. QUIN lesions were not checked in this study, but in our hands this procedure causes reductions of glutamic acid decarboxylase (GAD) activity of more than 70% (Gramsbergen and Van der Sluijs-Gelling, 1993).

3.2.6. Presentation of data and statistics

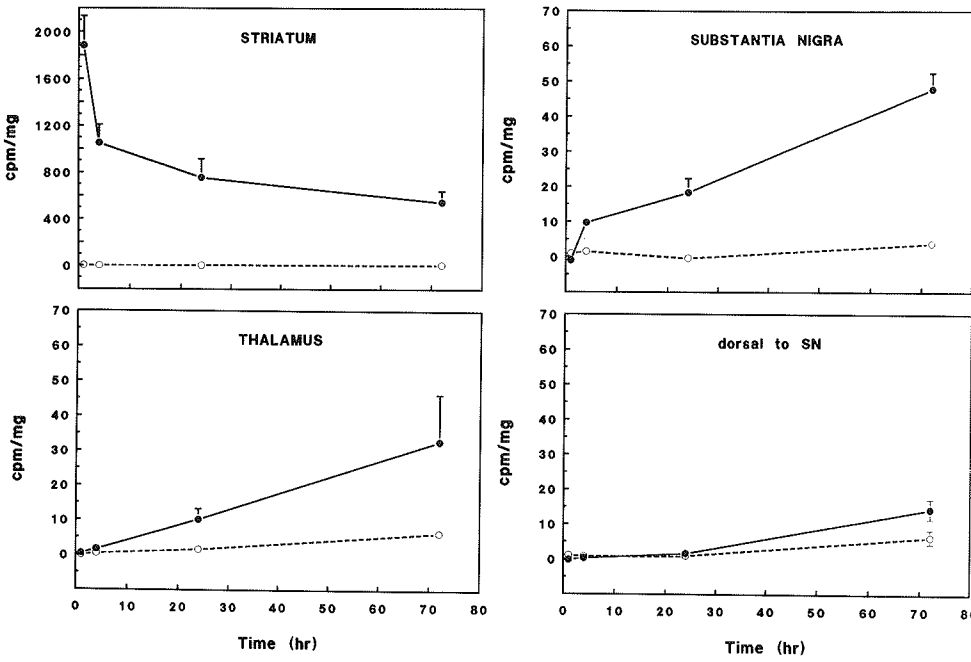
Radioactivity contents are expressed as cpm/mg wet weight tissue and as normalized values. The latter are a percentage of radioactivity at the injected site corrected for background activity (i.e., mean radioactivity in cerebellum) using the formula: [(tissue (cpm/mg) - average CB (cpm/mg)) : (injection site (cpm/mg) - average CB (cpm/mg))] * 100%. Differences between ipsi- and contralateral tissues in time (Exp.1) were statistically evaluated using ANOVA followed by Fisher's multiple comparisons test. In other experiments differences between tissues from treated (ipsilateral) and control (contralateral or not blocked side), or between tissues of the same hemisphere (SN and DSN) were evaluated using Mann-Whitney *U* test ($n \leq 4$) or Student's *t* test.

3.3. Results

3.3.1. Experiment 1: Time course and saturability of ^{54}Mn binding after intrastriatal injection (Fig. 1).

A unilateral intrastriatal injection of ^{54}Mn produced region-specific accumulation of the radiolabel in frontal cortex, medial forebrain bundle II, thalamus (Fig. 1) and substantia nigra (SN, Fig. 1) ipsilateral to the injection site up to at least 72 hours after treatment ($p < 0.005$, ANOVA). In addition, in SN, but not in thalamus, significant accumulation was already present after 4 hours ($p < 0.05$, Mann-Withney *U* test), despite the more distant position of the SN to the injected striatum. In ipsilateral globus pallidus there was a trend of accumulation of ^{54}Mn in time, but because of the large variation of the separate data points, probably due to contamination with striatal tissue, the time related change did not reach significance. In MFB I and tissue dorsal to SN ipsi- versus contralateral differences in time just reached significance ($0.01 < p < 0.05$). In other investigated areas, including hippocampus and cerebellum, radioactivity remained low at all time points, and differences between ipsi- and contralateral tissues were not significantly changed in time. The clearance of ^{54}Mn from striatum ($p < 0.001$, ANOVA) was very slow as demonstrated by the asymptotic decline: both at 24 and 72 hours approximately one third of the radioactivity measured after 1 hour is still present (Fig.1). Radioactivity contents in brain tissues of tracer-treated (799 ± 273 cpm/mg) and tracer+carrier dose-treated (459 ± 29) rats were

Fig. 1. *Time-course of ^{54}Mn binding in striatum, substantia nigra (SN), thalamus and tissue dorsal to SN after an unilateral tracer injection (closed circles) into striatum.*



Data are mean ± SEM (bars) values (cpm/mg wet weight) from three to four animals per time point (SEM is not shown if smaller than symbol). Statistics: Differences between ipsi- and contralateral tissues (open circles) in time were highly significant in striatum ($p < 0.001$), substantia nigra ($p < 0.001$) and thalamus ($p < 0.005$) and reached just significance in tissue dorsal to SN ($p = 0.041$) by ANOVA.

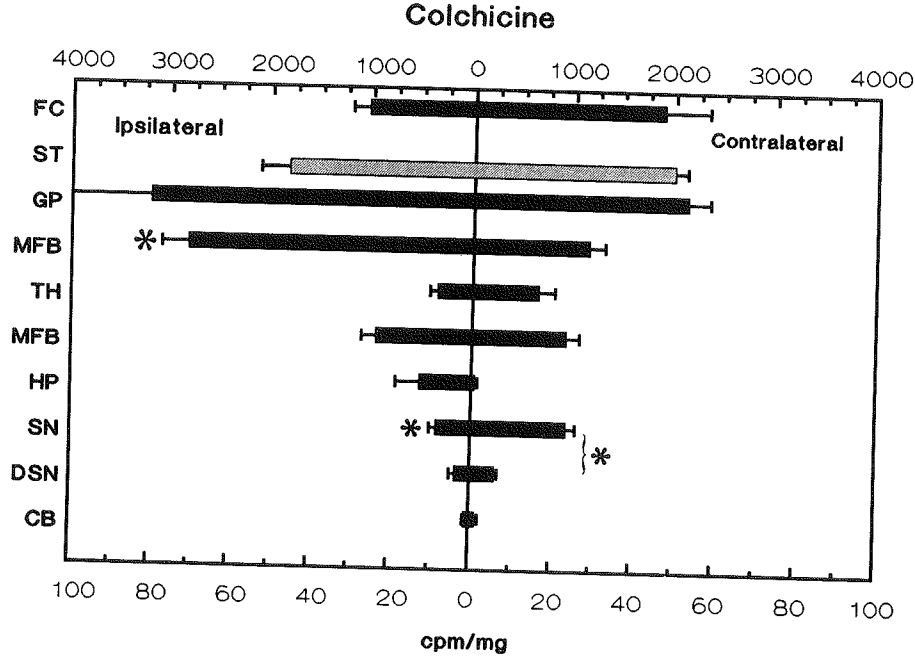
not significantly different after 24 hr.

In experiments 2 to 5 as described below using bilateral ^{54}Mn injections, no significant differences in radioactivity content between the two injection sites were observed, except in the QUIN-lesion of experiment 3 (Fig. 3B) and the 6-OHDA lesion of experiment 5 (Fig. 5). Thus, from experiments 2, 3A and 4 conclusions can be drawn by directly comparing radioactivity contents in ipsi- and contralateral brain areas (Fig. 2, 3A, 5). However, in some individual rats asymmetric ^{54}Mn-injections had occurred. Therefore, also normalized data are shown in Table 1 and 2 (for details see "Presentation of data and statistics").

3.3.2. Experiment 2: Blockade of axonal transport from striatum to substantia nigra (Fig. 2).

Pretreatment with colchicine in the MFB 10 minutes prior to bilateral ^{54}Mn injection, had no effect on the regional ^{54}Mn distribution (Table 1). A 24 hours pretreatment with colchicine, however, blocked the accumulation of radioactivity in the SN by 65% (Table 1), while the contralateral SN accumulated significantly more radioactivity than surrounding tissue (DSN, Fig. 2). In comparison, transection of the MFB produced a similar reduction of radioactivity in the SN (57%, Table 1), and also showed a similar regional distribution with respect to other investigated structures.

Fig.2. *Effect of a unilateral colchicine injection into the medial forebrain bundle (MFB; 24 hours before ^{54}Mn) on regional ^{54}Mn distribution 24 hours after bilateral tracer injections into striatum.*



Top axis indicates amount of ^{54}Mn (cpm/mg wet weight) in the injected striatum (hatched bar), bottom axis indicates radioactivity content (cpm/mg wet weight) in other structures (black bars). Abbreviations used: FC = frontal cortex; ST = striatum; GP = globus pallidus; MFB = medial forebrain bundle; TH = thalamus; HP = hippocampus; SN = substantia nigra; DSN = area dorsal to SN in ventral midbrain; CB = cerebellum. Bars are mean ± SEM (n=3). Statistics: *) $p < 0.05$, Mann-Whitney *U* test between ipsi- or contralateral tissues, or adjacent tissues of the same hemisphere (indicated with a brace).

3.3.3. Experiment 3: Axonal transport from striatum to substantia nigra in rats with a nigro-striatal (6-OHDA) or striato-nigral (QUIN) lesion (Fig 3A and B).

6-OHDA lesions had no effect on axonal transport from striatum to SN (Table 1, Fig. 3A). The QUIN lesioned striatum retained significantly more label (303%) than contralateral control striata, whereas the SN ipsilateral to the QUIN lesion contained significantly less label (4% of control) as compared to the contralateral SN (Table 1, Fig. 3B). Furthermore, low radioactivity content in the ipsilateral SN was not

Table 1: *Transport of ^{54}Mn from striatum to substantia nigra after unilateral injection of 15 µg colchicine (COL) into the medial forebrain bundle (MFB), or transection (CUT) of the MFB 24 hours prior to bilateral injection of the radiolabel, and after unilateral striatonigral quinolinic acid (QUIN) or nigrostriatal 6-hydroxydopamine (6-OHDA) lesions two weeks prior to bilateral injection of ^{54}Mn.*

	^{54}Mn injected site			normalized % transport		
	striatum (cpm/mg ± SEM)			substantia nigra (% ± SEM)		
	ipsi	contra	ratio	ipsi %	contra %	ratio
COL 10 min before ^{54}Mn (n=5)	2171 ± 267	1991 ± 299	1.14 ± 0.13	2.17 ± 0.39	2.16 ± 0.48	1.18 ± 0.30
COL 24 hr before ^{54}Mn (n=3)	1826 ± 282	2001 ± 120	0.94 ± 0.15	0.40 ± 0.09	1.12 ± 0.15	0.35# ± 0.04
CUT 24 hr before ^{54}Mn (n=4)	1875 ± 392	1958 ± 317	1.02 ± 0.24	1.11 ± 0.52	2.78 ± 0.48	0.43# ± 0.19
6-OHDA 14 d before ^{54}Mn (n=5)	1883 ± 442	1679 ± 319	1.24 ± 0.35	2.23 ± 0.08	2.20 ± 0.12	1.03 ± 0.08
QUIN 14 d before ^{54}Mn (n=5)	3376 ± 311	1168 ± 178	3.03 ± 0.24	0.13 ± 0.04	3.57 ± 0.23	0.04* ± 0.01

Rats were decapitated 24 hours after administration of radioactivity into striatum. Radioactivity data in SN represent normalized values (for details see Materials and Methods). Data are mean ± SEM values from three to five rats (n). Statistics: Ipsi- versus contralateral side #) $p < 0.05$ by Mann-Whitney *U* test; *) $p < 0.001$ by Student's *t* test.

significantly different fromsurrounding tissue (DSN). In addition, other ipsilateral structures, including FC, MFB, and TH had very low radioactivity levels.

Fig. 3A: *Effect of a unilateral 6-hydroxydopamine lesion (intranigral 6-OHDA; two weeks before ^{54}Mn) on regional ^{54}Mn distribution 24 hours after bilateral tracer injection into striatum.*

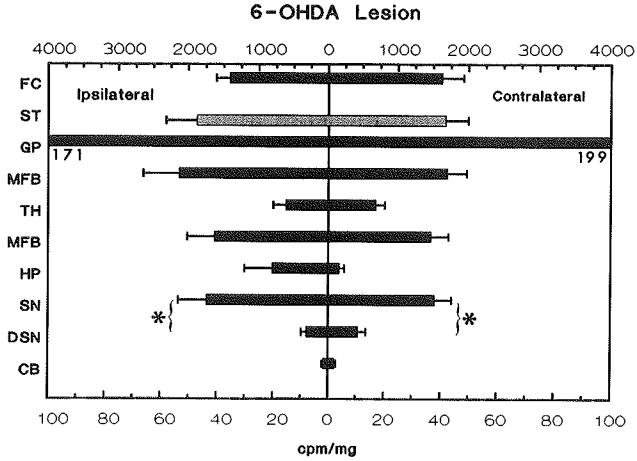
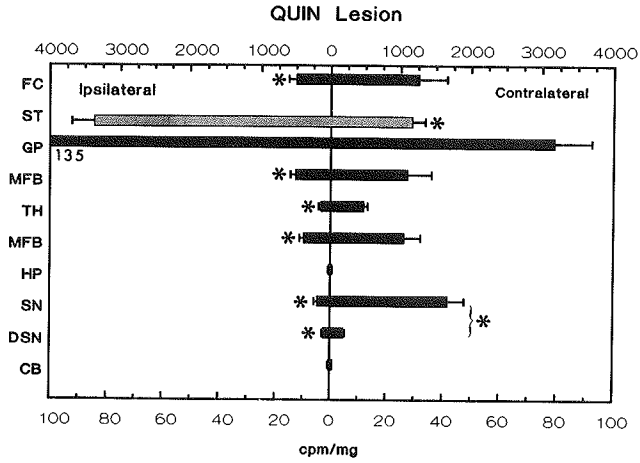


Fig. 3B: *Effect of a unilateral quinolinic acid lesion (intrastriatal QUIN: two weeks before ^{54}Mn) on regional ^{54}Mn distribution 24 h after bilateral tracer injection into striatum*



Top axis indicates radioactivity contents (cpm/mg wet weight) in the injected striatum (hatched bar), bottom axis indicates amount of label (cpm/mg wet weight) in other structures (black bars). Abbreviations used, see legend to Fig. 2. Bars are mean ± SEM (n = 5). Statistics: *) $p < 0.05$ by Student's *t* test between ipsi- and contralateral tissues, or adjacent structures of the same hemisphere (indicated with braces).

In experiments 4 and 5 as described below using injections of ^{54}Mn into the ventral midbrain, 73-93% of the injected amount of radioactivity was found in the SN, the rest in the VTA (7-27%).

Fig. 4. *Effect of a unilateral colchicine injection into the medial forebrain bundle (MFB; 24 hours before ^{54}Mn) on regional ^{54}Mn distribution 24 hours after bilateral tracer injection into substantia nigra (SN).*

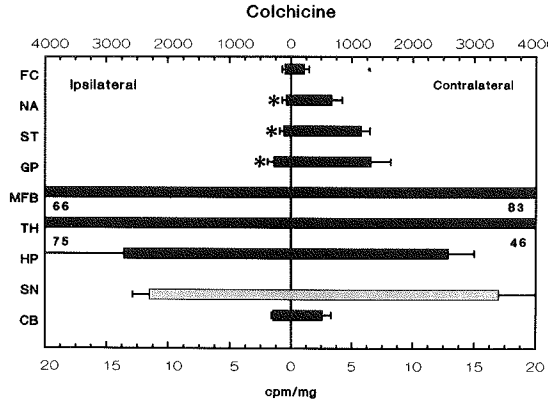
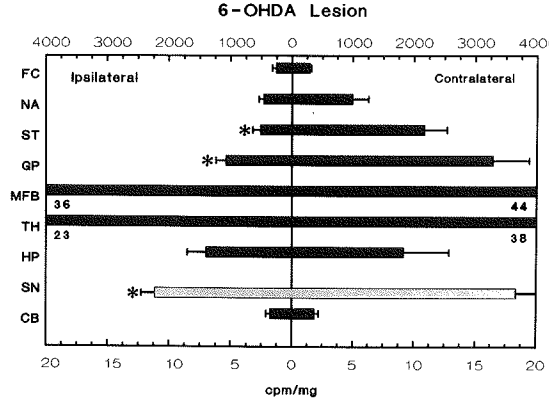


Fig. 5. *Effect of a unilateral 6-hydroxydopamine lesion (intranigral 6-OHDA: two weeks before ^{54}Mn) on regional ^{54}Mn distribution 24 hours after bilateral tracer injection into substantia nigra (SN).*



Top axis indicates radioactivity content (cpm/mg wet weight) in the injected SN (hatched bars), bottom axis indicates amount of label (cpm/mg wet weight) in other structures (black bars). Abbreviations used: NA = nucleus accumbens; for others see legend to Fig. 2. Bars are mean ± SEM (n = 4-5). Statistics: *) $p < 0.05$ by Student's *t* test between ipsi- and contralateral tissues.

3.3.4. Experiment 4: Blockade of axonal transport from substantia nigra to striatum (Fig. 4).

Twenty-four hours after bilateral administration of ^{54}Mn into the SN significantly less label was present in the striatum ipsilateral to the colchicine injection (9% of control, Fig. 4) and was not higher than background activity (Table 2). In addition, transport of ^{54}Mn was also significantly blocked by colchicine to other ipsilateral forebrain structures, including NA and GP. After 48 hours axonal transport of ^{54}Mn from SN to striatum was still significantly blocked by 93%. In addition, the regional ^{54}Mn distribution at both time points studied were very similar to each other with respect to the other investigated structures.

Table 2. *Transport of ^{54}Mn from substantia nigra (SN) to striatum after unilateral injection of 7.5 µg colchicine (COL) into the medial forebrain bundle (MFB) 24 hours prior to bilateral administration of the radiolabel into SN, or after an unilateral nigrostriatal lesion (6-OHDA) two weeks prior to bilateral injection of the radiolabel into SN.*

Ipsilateral blockade or lesion	^{54}Mn injected site			normalized % transport		
	subst. nigra (cpm/mg ± SEM)			striatum (% ± SEM)		
	ipsi	contra	ratio	ipsi %	contra %	ratio
6-OHDA	2235 ± 214	3673 ± 337	0.63* ± 0.11	0.041 ± 0.035	0.26 ± 0.05	0.14* ± 0.08
COL24	2307 ± 267	3403 ± 732	0.85 ± 0.34	-0.042 ± 0.004	0.14 ± 0.05	0.00* ± 0.10
COL48	2130 ± 519	1582 ± 364	1.71 ± 0.58	0.015 ± 0.015	0.27 ± 0.07	0.07** ± 0.07

Rats were decapitated 24 (COL24, 6-OHDA) or 48 hours (COL48) after administration of ^{54}Mn. Radioactivity data in striatum represent normalized values (for details see Materials and Methods). Data are mean ± SEM values from four to five rats. Statistics: Ipsi- versus contralateral side *) $p < 0.05$, **) $p < 0.01$ by Student's *t* test.

3.3.5. Experiment 5: Axonal transport from substantia nigra to striatum in rats with a nigro-striatal 6-OHDA lesion (Fig. 5).

Twenty-four hours after bilateral administration of ^{54}Mn into SN, transport to the striatum ipsilateral to the 6-OHDA lesion was blocked by 86% (Table 2). In addition, transport to the GP was also significantly blocked at the lesioned side, whereas transport to the FC and NA was not affected.

3.4. Discussion

Following a tracer injection of ^{54}Mn into rat striatum or substantia nigra, we observed accumulation of Mn in specific, anatomically related brain areas, and a very slow clearance from the basal ganglia. In addition, Mn is localized in both (reactive) glial and neuronal cells, and is transported in anterograde direction through both GABAergic striato-nigral and DAergic nigro-striatal fibres.

Although the above conclusions indicate some specificity of in vivo ^{54}Mn binding, this binding was unsaturable, suggesting ubiquitous and probably multiple binding sites for ^{54}Mn. Others have also reported a lack of saturability of Mn binding in brain tissue (Daniels et al., 19881). In addition, the slow clearance of Mn from the striatum is very similar to that observed by Lista et al. (1986) using atomic absorption spectrophotometry. The here reported regional distribution of ^{54}Mn following intrastriatal injection of the radiolabel in rats bears strong resemblance to the regional accumulation of Mn in caudate, putamen, globus pallidus, and substantia nigra after chronic systemic exposure to Mn in rodents (Bonilla, 1980; Chandra and Shukla, 1981), monkeys (Bird et al., 1984; Eriksson et al., 1987; Newland et al., 1989) and man (Yamada et al., 1986). These brain regions show also neuropathological and neurochemical changes after chronic systemic or acute intracerebral exposure to Mn (Bird et al., 1984; Yamada et al., 1986; Eriksson et al., 1987 and 1992; Newland and Weiss, 1992; Sloot et al., 1994).

Blockade of axonal transport in both anterograde and retrograde directions in both striatonigral and nigrostriatal cells using transection of the medial forebrain bundle (MFB) or colchicine injection into the MFB, demonstrated that Mn is taken up by nerve cells in striatum or SN and transported to SN or striatum respectively. Subsequent experiments using 6-OHDA lesion of nigrostriatal cells revealed that these DAergic neurons take up and transport Mn in anterograde direction, but not in retrograde direction. Using QUIN lesion of striatonigral cells, we showed that these GABAergic neurons take up and transport Mn in anterograde direction. Transport in retrograde direction by these cells (from SN to striatum) was not directly investigated by this approach, because the QUIN-lesioned striatum strongly accumulate ^{54}Mn, which may complicate interpretation in terms of axonal transport. However, since 6-OHDA lesions and colchicine injections revealed similar reductions of radioactivity in striatum after

intranigral administration of ^{54}Mn, we conclude that there is no significant retrograde transport through GABAergic striatonigral fibres.

As compared to the total amount of injected radioactivity, a relatively small proportion of ^{54}Mn is transported intraneuronally. The larger proportion of ^{54}Mn has probably been accumulated by glial cells. The predominant glial localisation of ^{54}Mn became particularly apparent in the QUIN-lesioned striatum, which is depleted of most intrinsic nerve cells (Schwarcz et al., 1983) and contains abundant activated microglial cells, recruited macrophages from the periphery (Marty et al., 1991) and reactive astrocytes (Björklund et al., 1986). Thus, the three times increase of ^{54}Mn accumulation observed in the 14 days old QUIN-lesioned striatum suggests uptake of Mn in these glial cells. However, accumulation of ^{54}Mn in brain areas remote from the injection site, is predominantly due to axonal transport.

The question then arises as to which intracellular constituents in both glial and neuronal cells bind ^{54}Mn, and which of those constituents are transported from cell bodies to nerve terminals. Several lines of evidence suggest that the transport and accumulation of Mn occur in a manner similar to that of Fe (Keen et al., 1984; Aschner and Aschner, 1990 and 1991). The uptake of iron from the blood into the brain and transport of Fe within the brain seem to be mediated by transferrin and transferrin receptors (Aschner and Aschner, 1990; Murphy et al., 1991; Rabin et al., 1993). High levels of non-haem Fe, mainly in the form of ferritin are found in the basal ganglia and seem to be closely linked to GABAergic circuits (Francois et al., 1984; Hill and Switzer, 1984). The principle cells of iron regulation in the brain are oligodendrocytes and probably also microglial cells (Francois et al., 1984; Morris et al., 1992; Benkovic and Connor, 1993). The Fe^{3+}-transferrin complex is internalized by its receptor, released intracellularly, utilized, and subsequently stored into ferritin (Conner et al., 1990; Hill, 1990; Morris et al., 1992). The regional distribution of brain iron and transferrin-receptors is uneven (Hill et al., 1985; Dwork et al., 1988). Most frequently iron accumulating areas, including the basal ganglia, are efferent to areas of high transferrin-receptor density, suggesting that the basal ganglia receive iron through neuronal transport (Hill et al., 1985; Mash et al., 1990). This is consistent with changes in regional brain ^{59}Fe distribution over time after systemic administration (Dwork et al., 1990). In addition, *in vitro* studies have shown that astrocytes take up Mn by a specific high affinity transport system (Aschner et al., 1992), and that in catecholamine-containing neuroblastoma cells Mn is internalized as a Mn-transferrin complex and subsequently bound to ferritin (Suárez and

Eriksson, 1993). Thus, in the present study, ^{54}Mn at the sites of injection is probably mainly bound to ferritin associated with glial cells, whereas the axonally transported ^{54}Mn could be bound to transferrin. Iron transport and storage pathways may therefore determine the selective accumulation of Mn in and within the basal ganglia.

In addition, in GABA-ergic fibres Fe seems to be important for GABA metabolism, since inhibition of the mitochondrial enzyme GABA-transaminase reduces iron levels in the basal ganglia (Hill, 1985). At present, however, it is not clear which cellular constituents in GABA-ergic neurons could use Fe and be affected by Mn. Furthermore, in DA-ergic fibres ^{54}Mn could be bound to ATP-catecholamine complexes (Rajan et al., 1971 and 1972), which are stored in synaptic vesicles. However, typical synaptic vesicles are probably not subjected to axonal transport (Grafstein and Forman, 1980). Tyrosine hydroxylase the rate-limiting enzyme of catecholamine synthesis is iron-dependent (Youdim et al., 1990) and is mainly transported in anterograde direction (Grafstein and Forman, 1980) and may bind Mn as well. Since we found only anterograde axonal transport of ^{54}Mn, it is conceivable that constituents with Mn or Fe binding sites (Youdim et al., 1980 and 1990) are important for neurotransmission in both nigrostriatal and striatonigral neurons. For instance, dopamine D_2-receptors are iron-dependent (Youdim et al., 1983), and K^+ depolarization causes release of ^{54}Mn together with tritiated DA in striatal slices (Daniels et al., 1981).

Other important intracellular sites of Mn accumulation are mitochondria, which are transported bidirectionally, resulting in a zero net displacement (Grafstein and Forman, 1980), and thus can not explain the here reported anterograde transport of Mn. However, the preferential but slow accumulation of Mn in mitochondria via the Ca^{2+} uniporter (Maynard and Cotzias, 1955; Gavin et al., 1990) may disturb the function of a variety of metal dependent enzymes (Keen et al., 1984; Prohaska, 1987; Liccione and Maines, 1989) (cytochromes, MAO, SOD, pyruvate carboxylase) and cause cellular energy depletion. Under toxic conditions Mn has been shown to inhibit oxidative phosphorylation (Gavin et al., 1992), raise lactate and decrease ATP levels (Brouillet et al., 1993), alter gluconeogenesis (Hurley et al., 1984; Keen et al., 1984), decrease gluthatione-peroxidase activity and gluthatione content (Liccione and Maines, 1988), and decrease cerebral glucose utilization (Wolters et al., 1989).

It seems that neurons of the basal ganglia are particularly vulnerable for mitochondrial defects (Beal, 1992). Together with the local accumulation of Mn by iron transport and storage proteins this may provide an explana-

tion for the selective neurotoxicity of Mn. The exact nature of transport and storage of Mn and its relationship to disturbance of iron homeostasis and mitochondrial function, which may cause free radical production, dopamine depletion and excitotoxicity, however, needs further clarification.

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3.5. References

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Detection of salicylate and its hydroxylated adducts 2,3- and 2,5-dihydroxybenzoic acids as possible indices for *in vivo* hydroxyl radical formation in combination with catechol- and indoleamines and their metabolites in cerebrospinal fluid and brain tissue

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4.0. Summary

It has been suggested that salicylate (SA) hydroxylation can be used to detect hydroxyl radical formation in vivo. Here we describe a rapid and sensitive HPLC method using ultraviolet absorbance (UV) and electrochemical detection (EC) to detect SA (UV), its hydroxylated adducts 2,3- and 2,5-dihydroxybenzoic acids (DHBA) and catechol in combination with catechol- and indoleamines and related metabolites (EC) in one isocratic run. These compounds were measured in acidified cerebrospinal fluid (CSF) and perchlorate extracts of striatal tissues of untreated and SA-loaded rats (300 mg/kg SA i.p.). Peaks were identified by comparing retention times of samples and standards, by adding standards to biological samples, by voltamograms, and by comparing chromatograms of manganese (Mn^{2+})-injected striata of SA-loaded rats with several control conditions. Six hours after unilateral injection of 0.4 μmol Mn^{2+} into striatum, 2,3- and 2,5-DHBA levels in striatum were respectively 4- and 7-fold increased as compared to not-injected (contralateral) striata, suggesting in vivo hydroxyl radical formation. In addition, dopamine and serotonin levels were depleted in Mn^{2+}-injected striata by 46% and 64% respectively. In CSF of Mn^{2+}-injected rats, [DHBA/SA] ratios were not significantly changed as compared to those of control rats. In conclusion, the here described technique can be applied to study in vivo hydroxyl radical formation in direct relation with dopaminergic and serotonergic neurotransmitter changes during neurotoxic processes.

4.1. Introduction

Oxygen-derived free radicals may play a role in a wide variety of pathological conditions affecting the central nervous system (CNS)(Halliwell, 1992). These conditions include neurodegenerative diseases (Olanow et al., 1992; Coyle and Puttfarcken, 1993), brain trauma and ischemia (Siesjö et al., 1989; Hall and Braughler, 1993), and intoxications affecting the basal ganglia (Jellinger, 1986). In particular hydroxyl radicals (.OH), which are generated via the iron-catalyzed Haber-Weiss reaction (Floyd and Lewis, 1983; Halliwell and Gutteridge, 1985 and 1990; Aust et al., 1993) or alternatively via NO-related mechanisms (Beckman et al., 1990; Hammer et al. 1993), are extremely toxic in biological systems causing (per)oxidations of lipids, proteins and nucleic acids.

Electron spin resonance (ESR) trapping (Pou et al., 1989) and aromatic hydroxylation assays (Radzik et al., 1983; Halliwell et al., 1989) belong to the most specific and direct measurements of free oxygen radicals today, but are often of limited use in living systems. Using biologically meaningful hydroxyl radical (·OH) generating *in vitro* systems it was demonstrated (Richmond et al., 1981; Floyd et al., 1984; Maskos et al., 1990), that salicylate (SA) can be used to trap ·OH by producing the two hydroxylated adducts 2,3- (~49%) and 2,5-dihydroxybenzoic acid (DHBA)(~40%) out of four theoretically possible isomers, and catechol (~11%) (percentages by Grootveld and Halliwell, 1986). *In vivo* studies using (acetyl)-SA preloading in adriamycine-treated rats (Floyd et al., 1986) or rheumatoid arthritis patients (Grootveld and Halliwell, 1986) seemed to confirm the *in vitro* results by showing increased DHBA levels in several tissues and body fluids, suggesting *in vivo* ·OH production .

Since these studies have been published, ·OH formation has been investigated in various brain pathologies using either intracerebral or systemic administration of SA as an ·OH-trapping agent, including neurotoxic insults by MPTP or its analogues (Chiueh et al., 1992 a&b and 1993; Obata and Chiueh 1992; Wu et al., 1993), by methamphetamine (Zigmond et al., 1993), or by N-methyl-D-aspartate (Hammer et al., 1993), as well as cerebral ischaemia/hypoxia (Cao et al., 1988; Zhang and Piantadosi, 1992; Althaus et al., 1993; Delbarre et al., 1993; Hall et al., 1993a), head trauma (Hall et al., 1993b), and aging (Zhang et al., 1993). The presentation of results in many of those studies as relative units, or by total DHBA or only 2,5-DHBA levels, however, calls for cautious interpretation in terms of *in vivo* ·OH formation. It has been suggested that 2,5-DHBA levels can also be formed enzymatically via P450-systems, while 2,3-DHBA cannot both *in vitro* (Halliwell et al., 1991;

Ingelman-Sundberg et al., 1991) and *in vivo* (Grootveld and Halliwell, 1988).

In the present paper, a rapid and sensitive HPLC-EC/UV method for the combined determination of SA, 2,3- and 2,5-DHBA, and catechol as well as catechol- and indoleamines and their metabolites in cerebrospinal fluid (CSF) and striatal tissue is described. The usefulness of the method is illustrated by the effect of intrastriatal Mn^{2+}-injection, which produces dopamine depletion and irreversible lesions in the basal ganglia (Sloot et al., 1994). It has been hypothesized that Mn, a redox-cycling multivalent metal, damages dopaminergic nigrostriatal neurons via free radical formation either directly or indirectly (Graham, 1984; Halliwell, 1984). With respect to possible clinical application of the SA-hydroxylation method, the DHBA effects of intracerebral Mn^{2+} were also assessed in CSF.

4.2. Materials and Methods

4.2.1. Chemicals and HPLC-standards

$MnCl_2$ (>96% pure), $MgCl_2.6H_2O$ and dopamine (DA, 3-hydroxytyramine.HCl) were obtained from Merck (Darmstadt, Germany). Salicylic acid (SA) sodium salt, 2,3- and 2,5-dihydroxybenzoic acid (DHBA), catechol, homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine creatinine sulfate complex (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) were purchased from Sigma (Brunschwig Chemie, Amsterdam, The Netherlands) and stored according to the description of the manufacturer. All other chemicals used were of the highest available purity.

External HPLC-standard solutions of approximately 1 mmol/L were dissolved in Milli-Q water (Millipore, Molsheim, France) containing 50 mmol/L HCl (4 ml 37% HCl/L), 0.20 g/L $Na_2S_2O_5$ and 0.050 g/L Na_2EDTA (pH ~2). In order to dissolve 2,3-DHBA properly, first a few drops of methanol were used. Prepared this way, all standards were stable for at least 2 months (less then 5% decline) if kept stored in the dark at 4°C. Working dilutions for calibration were prepared daily in mobile phase buffer (MPB) in the concentration range of the samples.

4.2.2. Animals, surgery and experimental design

Chloral anaesthetized (400 mg/kg i.p.) Wistar derived male rats (WAG/Rij Harlan) received 0.4 μmol $MnCl_2$ or $MgCl_2$ in 1 μl of Milli-Q water (Millipore, Molsheim, France) into the left striatum using stereotaxic procedures according to the atlas of Paxinos and Watson (A 1.0 and L 2.75 mm from bregma; V 6.0 mm from the skull) as described previously (Sloot et al, 1994). Six hours after intrastriatal injection, cerebrospinal fluid (CSF) was collected under light diethylether (Merck, Darmstadt, Germany) anaesthesia, and immediately thereafter, animals were decapitated. Their brains were rapidly dissected on ice to collect ipsi- and contralateral striata as described previously (Sloot and Gramsbergen, 1994). In addition, two hours prior to sacrifice, experimental rats (7 Mn^{2+} versus 7 Mg^{2+}) were injected i.p. with 300 mg/kg SA,

whereas other groups did not receive SA to serve as negative controls (8 Mn^{2+} and 8 Mg^{2+}).

4.2.3. Collection, preparation and analysis of samples

Cerebrospinal fluid. Clear CSF (50-120 μl) was collected in 1.5 ml brown test tubes (Eppendorf, Merck, Amsterdam, The Netherlands) by holding the head of the animal in a 90° position and puncturing a 23G needle attached to a piece of silicone tubing (OD 0.50, ID 0.25) through the cisterna magna. The CSF was immediately acidified with 1/10 volumes of ice-cold 1 M perchloric acid (PCA) and put on ice (end pH 2-3, universal indicator paper), and subsequently stored in the dark at 4°C. The next day, CSF was diluted and adjusted with MPB (usually 1/5 for analysis of SA and 1/25 for 5-HIAA and 2,5-DHBA levels) and injected directly into the HPLC-system.

Striatal tissues. Dissected tissues were immediately frozen on dry ice, weighed (25-40 mg) and stored at -70°C until further use. At the day of analysis, frozen tissues were sonicated for 30 seconds in 200 μl ice-cold 0.1 M PCA containing 0.2 g/L $Na_2S_2O_5$ as antioxidant, and centrifuged at 10,000 g for 20 minutes at 4 °C (Eppendorf 5145, Merck, Germany). Subsequently, two different dilutions of the supernatant (usually 1/3 and 1/50 by adding MPB) were kept on ice until injection into the HPLC-system.

4.2.4. Chromatography: HPLC-system with electrochemical and ultraviolet absorbance detection

Levels of SA, 2,3- and 2,5-DHBA, and catechol as well as catechol- and indoleamines and their metabolites were determined in CSF and striatal tissue using slightly modified HPLC procedures according to Floyd et al. (1984) and Sloot et al. (1994).

The HPLC-system contained a Gynkotec model 300 pump (ANTEC Leyden BV.) equipped with an in line 0.2 μm degassing filter (Aqueous IFD, Whatman, Kent, England), which was used to pump mobile phase at a flow rate of 0.8 ml/min through a Spherisorb ODS1 guard column (30 x 4.6 mm; particle size 5μm) together with an ODS2 cartridge analytical column (100 x 4.6 mm; particle size 3 μm) both packed with reverse-phase C18 material (Phase Separation Ltd., Deeside, U.K.) in order to separate the compounds of interest in one isocratic run. The mobile phase buffer (MPB) consisted of 0.1 M (13.61 g/L) sodium acetate trihydrate, 6.0 % methanol, 19.5 mg/L (84 μM) n-octyl sodium sulphate (Merck, Darmstadt, Germany) and 10-15 mg/L disodium ethylenediaminetetraacetate (EDTA) dissolved in Milli-Q water and adjusted to pH 4.1 with glacial acetic acid. The MPB was degassed with He for 10 minutes before use. The amounts of methanol and n-octyl sodium sulphate were very critical for the separation and needed at least a 6-8 hour (or overnight) equilibration with the columns by recycling the mobile phase. n-Octyl sodium sulphate was used to increase the retention times of amino-containing compounds, including DA and 5-HT, independent from other compounds. The electrochemical detector (EC), which was placed after an UV-absorbance detector (UV) with a 313 nm filter (model 440 absorbance detector, Waters Associates BV., Etten-Leur, The Netherlands), was set at an oxidation potential of -650 mV against an Ag/AgCl reference electrode (ANTEC Leyden BV., Leiden, The Netherlands). SA was detected by UV, whereas the other compounds were detected by EC.

Samples or external standards were injected into an electronically switching Vici Valco Injector (Valco Instruments, via ANTEC Leyden BV) with a 50 μL loop. Quantification of peaks

was done using both peak height and peak area measurements by direct comparison to external standards using a Chromjet integrator (Spectra-Physics, via ANTEC Leyden BV.)

4.2.5. Statistics

Significant differences between groups of 7 rats were analyzed using Student's *t* test ($p < 0.05$).

4.3. Results

4.3.1. Characterization of the HPLC-UV/EC method

Standards. As shown in Fig. 1, nine external standards were practically baseline separated using the present HPLC-conditions with simultaneous

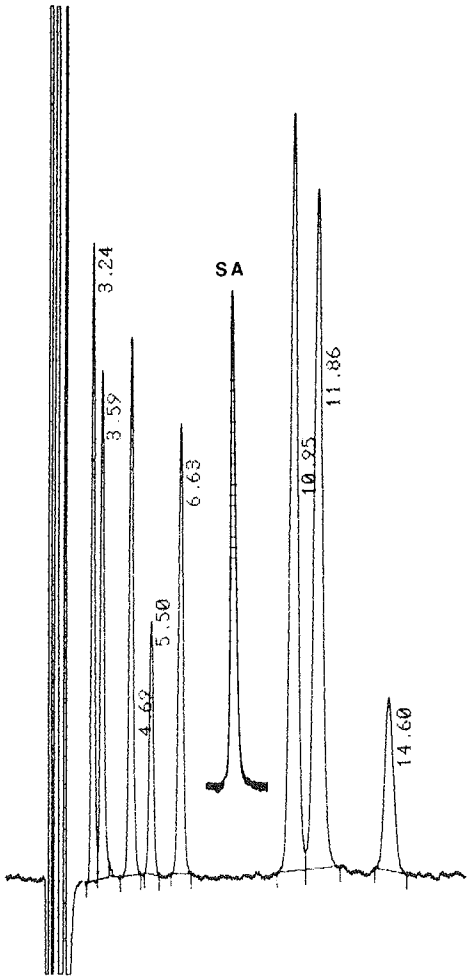

Fig. 1. *Representative HPLC separation in one single run of 1.5 to 2 pmoles of external standards, including 2,5-DHBA (3.24), 2,3-DHBA (3.59), DA (4.69), DOPAC (5.50), catechol (6.63), 5-HIAA (10.95), 5-HT (11.86) and HVA (14.60) using EC detection with the potential set at 650 mV or of 1.5 nmol SA (~8.0) using UV absorbance detection with a 313 nm filter (inset).*
Retention times (minutes per hundreds of seconds) are denoted between brackets.

4A: intrastriatal Mn^{2+} injection; no SA i.p.

4B: no intrastriatal injection; SA i.p.

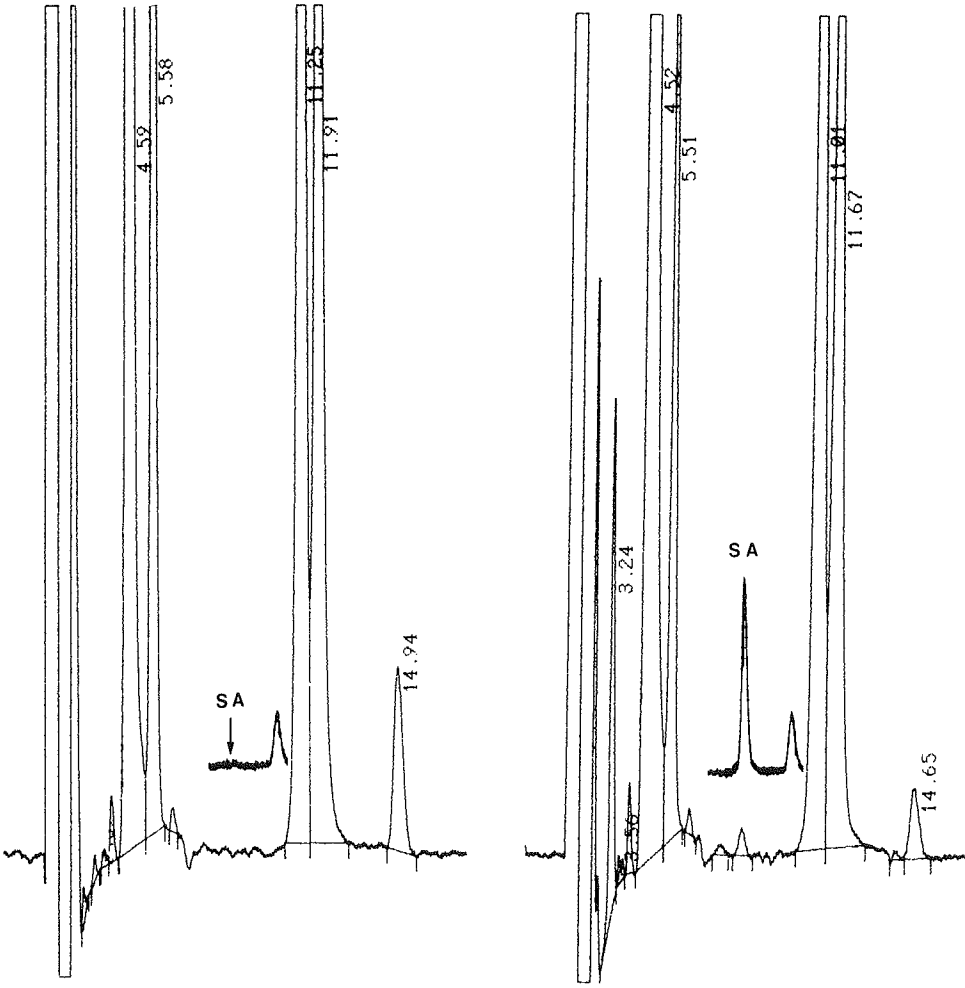

Fig. 4. *Representative chromatograms of PCA extracts of striatal tissues (n=7-8/group; dilutions 1/3) 6 hours after an in situ Mn^{2+}-injection (**A and C**), no injection (**B**, contralateral side of Mn^{2+} injected rats), or Mg^{2+}-injection (**D**) of SA-untreated (**A**) or SA-loaded rats (**B, C, D**).*
SA (300 mg/kg i.p.) was given two hours prior to sacrifice. UV- and EC-settings are the same as in Fig. 1; retention times (RT) depicted in Fig. A, B, C and D correspond to the following compounds (in the given order): 2,5-DHBA (no RT; 3.24; 3.18; 3.27); 2,3-DHBA (no RT; 3.56 3.57; 3.59); DA (4.59; 4.52; 4.54; 4.63); DOPAC (5.58; 5.51; 5.50; 5.61); 5-HIAA (11.25; 11.01 11.00; 11.48); 5-HT (11.91; 11.67; 11.75; 12.37) and HVA (14.94; 14.65; 14.65; 15.08). Inset depicts UV detection of SA; the arrow in Fig. A depicts the position of SA.

4C: intrastriatal Mn^{2+} injection; SA i.p.

4D: intrastriatal Mg^{2+} injection; SA i.p.

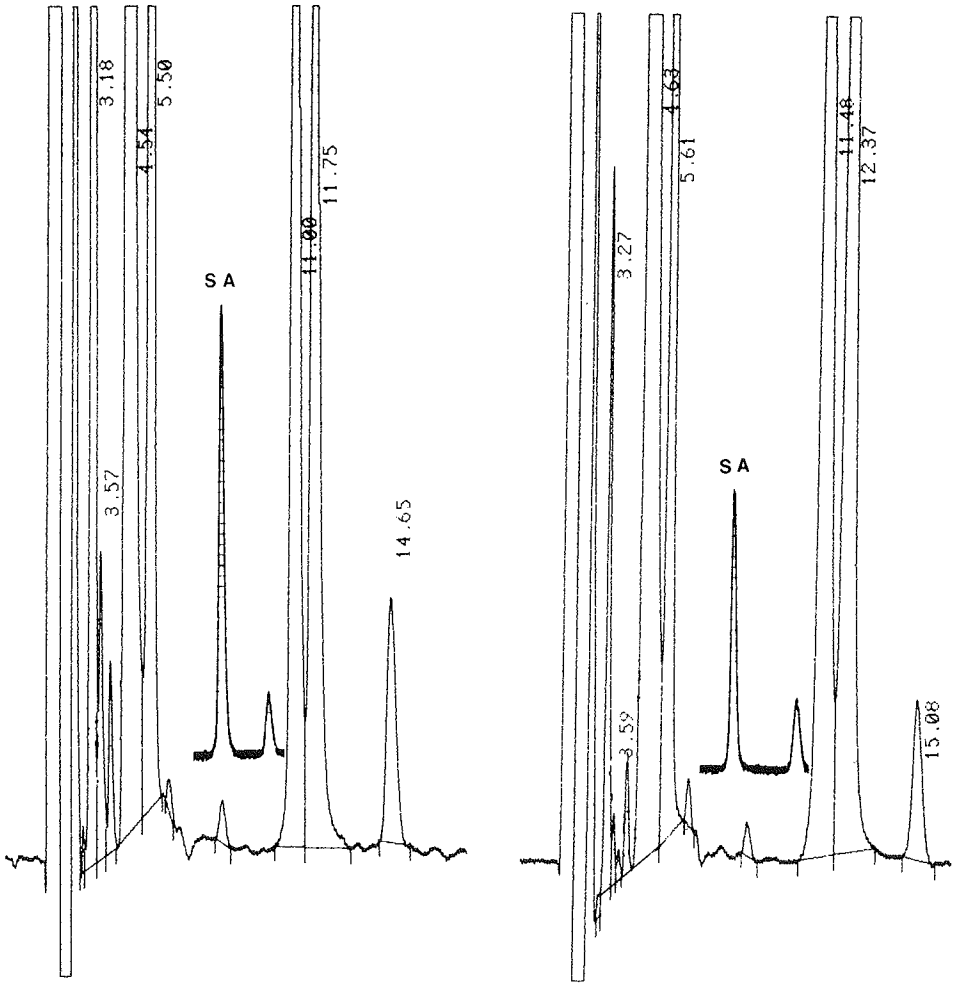

Fig. 4 (continued).

present in striatal tissues of any condition. Furthermore, a peak at the position of SA was detected by UV in striatal tissues of SA treated rats (inset Fig. 4B, C, D), but not in SA-untreated rats (inset Fig. 4A).

In striatal PCA extracts of all experimental conditions the DHBA isomers and other compounds of interest were stable on ice for at least 8 hours. In addition, adding Mn^{2+} *in vitro* to SA-containing tissues had no effect on DHBA levels.

Table 3: *Levels of salicylate (SA) and dihydroxybenzoic acids (DHBA) and [DHBA/SA] ratios in rat cerebrospinal fluid six hours after a unilateral injection of 0.4 µmol $MnCl_2$ or $MgCl_2$ into striatum*

	Levels and ratios in cerebrospinal fluid				
injection into striatum	SA (µM)	2,3-DHBA (µM)	2,5-DHBA (µM)	2,3-DHBA/SA (mmol/mol SA)	2,5-DHBA/SA (mmol/mol SA)
$MgCl_2$	789 ± 35	0.28 ± 0.034	1.93 ± 0.053	0.35 ± 0.037	2.47 ± 0.078
$MnCl_2$	787 ± 36	0.31 ± 0.020	2.13 ± 0.066*	0.39 ± 0.022	2.74 ± 0.158

Rats were loaded with SA (300 mg/kg i.p.) two hours before sacrifice. Data are expressed as means ± SEM of seven rats (n). * $p < 0.05$, significant difference between Mg^{2+}- and Mn^{2+}-treatment by Student's *t* test.

4.4. Discussion

This paper describes the methodology of a rapid and sensitive HPLC-EC/UV procedure to detect salicylate (UV) and its hydroxylated adducts 2,3- and 2,5-DHBA and catechol in combination with catechol- and indoleamines and their metabolites (EC) in CSF and striatum based on previously described assays of Floyd et al. (1984) and Sloot et al. (1994). Using this technique it is shown, that Mn^{2+} induces ·OH formation in rat striatum *in vivo*.

One group (Chiueh et al., see quoted references) has reported combined measurements of DHBAs and catecholamines in brain dialysates, but they did not describe the used methodology in detail. In the present study, the separation of 2,3- and 2,5-DHBA and the sensitivity of DHBA detection (fmolrange) is improved as compared to the assay of Floyd et al. (1984) and it is combined with detection of biogenic amines and related compounds. Peakidentification by comparing electronically registered retention times of standards and biological samples, by adding standards to biological samples, and by comparing chromatograms of Mn^{2+}-injected striata with several control conditions (Fig. 4A-D), has demonstrated the usefulness of the described method. SA-loaded rats revealed clear 2,5-DHBA, 2,3-DHBA and SA levels in CSF and striatum as compared to SA-untreated controls, and also showed a consistently low but detectable content of 2,3-DHBA and about 6-7 fold higher 2,5-DHBA levels in CSF and in control striatum (i.e. contralateral side; Table 1 and 3). In CSF, levels of SA and 2,3-DHBA were approximately 2-fold and of 2,5-DHBA 3-fold higher than in striatum. Since the extracellular space compromises about 15% of the total striatal volume, this suggests that intracellular concentrations of SA and DHBAs are lower than extracellular levels.

In the present study, injection of 0.4 μmol Mn^{2+} into striatum produced pronounced 2,3- and 2,5-DHBA increases in striatum at six hours (Tabel 1). The increased [2,3-DHBA/SA] ratio suggests, that Mn^{2+} induces .OH formation in striatum *in vivo*. As outlined in the introduction, the source of 2,5-DHBA formation remains unclear (Ingelman-Sundberg, 1991), and may partially be derived from conversion of SA by brain cytochrome P-450 enzymes (Chand and Clausen, 1982; Ravindranath et al., 1990), which may be enhanced by the glial response to the injury. The significant increase of SA by Mn^{2+} may be explained by leakage through damaged membranes as a result of the lesion, which seems consistent with the pronounced DA and serotonin depletions after Mn^{2+} (Table 2). Whether .OH formation is cause or consequence of these neurotransmitter depletions, for instance due to autoxidation of DA (Graham 1984; Halliwell 1984; Chiueh et al., 1992b; Obata and Chiueh, 1992; Chiueh et al., 1993a&b),

can not be concluded from this study.

In CSF, levels of DHBA/SA (Table 3), but also the levels of biogeni amines and related metabolites were not significantly changed by intrastriata Mn^{2+} injection. Although local DHBA-effects are considerably diluted in CSF DHBA changes in CSF could occur at later stages when the Mn^{2+} lesion develops progressively, involving not only striatum, but also globus pallidus thalamus and substantia nigra (Sloot et al., 1994).

In conclusion, the present methodology of simultaneous determinatio of dopamine changes and ·OH formation in the living brain is especiall suitable to study neurotoxic effects in the highly vulnerable basal gangli (Jellinger, 1986), which are rich in dopamine, iron and H_2O_2 (Olanow et al. 1992). Further experiments on Fe2+- and Mn^{2+}-induced ·OH formation in th brain are in progress.

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In vivo hydroxyl radical formation by iron as determined by salicylate hydroxylation proceeds dopamine and serotonin depletion in rat striatum

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5.0. Summary

The purpose of the studies was to investigate in vivo hydroxyl radical formation following iron intoxication of the brain. Generation of hydroxyl radicals (·OH) was assessed in rat striatum and cerebrospinal fluid (CSF) after intrastriatal Fe^{2+}-injection (0.4 μmol) and systemic salicylate (SA) loading (2 hours; 300 mg/kg) by measuring the SA hydroxylation adducts 2,3- and 2,5-dihydroxybenzoates (DHBA). 2,3-DHBA levels (·OH) in striatum peaked (10-fold increase) at thirty minutes after Fe^{2+}-injection and gradually declined until a non-significant elevation (1.5-fold) at 31 days. During the first two hours, no or relatively small effects on dopamine (DA), serotonin (5-HT) and related metabolite levels were observed in striatum. Specific alterations of DA turnover after 2 hours, suggest an early effect of Fe^{2+} on both ana- and catabolism of DA, thereby (over)producing H_2O_2 and possibly driving the Fenton reaction. Our results suggest, that ·OH initiate damage to DA- and 5-HTergic nerve terminals, as determined by irreversible DA (> 90%) and 5-HT depletion (about -50%) after 1 month. In addition, ·OH are continuously formed during the process of neurodegeneration. Differences in SA dose-dependency and time-courses between 2,5- and 2,3-DHBA formation after Fe^{2+} suggest, that only 2,3-DHBA may serve as an index for in vivo ·OH formation. In CSF of Fe^{2+}-treated rats, DHBA levels were significantly increased at 30 minutes and 3 days (+50-80%), indicating that oxidative stress in the brain can be monitored in vivo by this approach.

5.1. Introduction

Cellular levels of superoxide anion radicals ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are normally strictly controlled by Mn- or Cu/Zn-superoxide dismutases, catalase and gluthatione peroxidase (Del Maestro and McDonald, 1987), whereas iron is safely handled and stored by transferrin, transferrin receptors and ferritin (Hill, 1990; Morris et al., 1992; Benkovic and Connor, 1993). However, under pathological conditions, hydroxyl radicals ($^{\cdot}OH$) may be generated via the iron catalyzed Haber-Weiss reaction: $O_2^{\cdot -} + H_2O_2 \longrightarrow {}^{\cdot}OH + OH^- + O_2$ (Halliwell and Gutteridge, 1990; Halliwell, 1992; Hall and Braughler, 1993; Gutteridge, 1994), or alternatively via $O_2^{\cdot -}$ and NO-dependent peroxynitrite formation (Beckman et al., 1990; Hammer et al., 1993; Chiueh et al., 1994). Of the free oxygen radicals, $^{\cdot}OH$ are most toxic, causing oxidative degradation of membrane lipids, proteins and nucleic acids, and finally cell death. Therefore, the presence and cellular source(s) of $O_2^{\cdot -}$, H_2O_2, free $Fe^{2/3+}$, and the oxidant defense system are important determinants of severity and pattern of oxidative tissue damage.

Recently, alterations in brain iron levels, and iron transport and storage proteins have been reported in Parkinsons disease (PD) and other chronic disorders of the basal ganglia (Youdim et al., 1993; Olanow et al., 1992; Rutledge et al., 1987; Dexter et al., 1991 and 1993). The latter brain regions are rich in Fe^{3+}- ferritin and have moderate transferrin-receptor densities (Hill and Switzer, 1984; Hill, 1990; Morris et al., 1992; Benkovic and Connor, 1993). It has been hypothesized that during both acute and chronic pathological and toxic conditions iron may be liberated from proteins such as haemoglobin (Puppo and Halliwell, 1988), transferrin, ferritin and melanin (Halliwell and Gutteridge, 1990; Olanow et al., 1992; Bralet et al., 1992; Youdim et al., 1993). Experimental injections of iron salts into cerebral cortex or hippocampus of rats have demonstrated increased $O_2^{\cdot -}$ formation (Willmore et al., 1983), lipid peroxidation (Triggs and Willmore, 1984; Willmore et al., 1986) and extensive regional calcium accumulation associated with neuronal death (Sloot et al., 1994). In addition, intrastriatal Fe^{2+}-injection produces dose-dependent dopamine (DA) depletion and calcium accumulation in the basal ganglia (Sloot et al., 1994). Likewise, intranigral Fe^{3+}-injection induces neurodegeneration in the zona compacta of the substantia nigra (Sengstock et al., 1992 and 1994) and striatal DA depletion (Ben-Shachar and Youdim, 1991).

In general, a role of free radicals in tissue damage has been suggested on the basis of indirect evidence obtained by determination of oxidation

products of membrane lipids, proteins and DNA, or by studying tissue protection by free radical scavengers or Fe-chelators (Halliwell and Gutteridge, 1990; Halliwell, 1989). Recently, however, salicylate (SA) hydroxylation has been used as more direct evidence of *in vivo* oxidative stress by measuring the relatively stable SA-adducts 2,3- and 2,5-dihydroxybenzoic acid (DHBA) in brain tissue, brain dialysates or cerebrospinal fluid (CSF) (for overview of studies using this approach see: Sloot and Gramsbergen, 1995). Using this technique we have demonstrated *in vivo* ·OH formation by manganese in rat striatum.

In the present investigation, we used Fe^{2+}-injections into striatum as a free radical generating *in vivo* model (Sloot et al., 1994) to study the time-course of 2,3- and 2,5-DHBA formation in striatum and CSF of SA-loaded rats. In addition, changes in catechol- and indoleamines and related metabolites were determined in the same tissues to address the question whether Fe^{2+}-induced ·OH are cause or consequence of (irreversible) damage. If ·OH derived from brain tissue can be detected in CSF, the SA trapping method may be applied in the clinic to study antioxidant treatments in human neurodegenerative diseases.

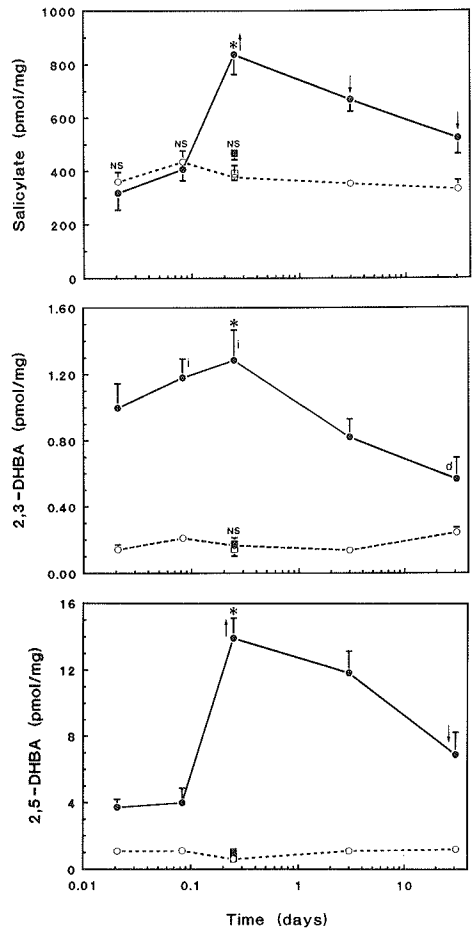
5.2. Materials and methods

5.2.1. Animals and materials

Experimental protocols regarding animal experimentation were approved by the Ethical Committee for Laboratory Animal Experiments, TNO/Regio West (Woudenberg, The Netherlands), as registered under DEC no.56-3A. Wistar derived male rats (WAG/Rij Harlan, Zeist, The Netherlands) were kept on a 12/12 h light/dark cycle and housed in a climated room with a humidity of 50-70% and a temperature of 24°C with free access to water and chow food.

$FeCl_2.4H_2O$ (> 99% pure), $MgCl_2.6H_2O$ and dopamine (DA, 3-hydroxytyramine.HCl) were obtained from Merck (Darmstadt, Germany). Solutions of Fe^{2+} and Mg^{2+} were made freshly and were filtered through a 0.2 μm pore size disc filter (Schleicher & Schuell, Dassel, Germany). Apart from DA, all other external standards used for HPLC, including salicylic acid (SA) sodium salt, 2,3- and 2,5-dihydroxybenzoic acid (DHBA), catechol, 4-hydroxy-3-methoxyphenylacetic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine creatinine sulfate complex (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) were purchased from Sigma (Brunschwig Chemie, Amsterdam, The Netherlands) and stored according to the description of the manufacturer. Stock solutions of standards (~1 mM) dissolved in 50 mM HCl containing 0.20 g/L $Na_2S_2O_5$ and 0.050 g/L Na_2EDTA were at least stable for 2 months if kept stored in the dark at 4°C.

Fig. 1: *Time-course (0.5, 2, 6, 72 hours or 31 days) of salicylate (SA), 2,3- and 2,5-DHBA levels (pmol/mg) in ipsi- (closed symbols) and contralateral (open symbols) striatum following a unilateral intrastriatal injection of 0.4 µmol Fe^{2+} (circles) or Mg^{2+} (squares) in SA loaded rats.*



SA was administered two hours prior to decapitation (300 mg/kg i.p.). Note that time on the x-axis is plotted on a log-scale. The effects of time, Fe^{2+}-injection, and interaction between the two are highly significant (ANOVA; $p < 0.001$). Changes among time points were analyzed using Newman-Keuls post-hoc test ($\alpha = 0.05$): arrows indicate significant increase (↑) or decrease (↓) relative to the preceding time point, and letters indicate such changes (resp. i and d) as compared to other time points. Values between ipsi- and contralateral striatum are significantly different (Student's *t* test; $p < 0.05$) unless indicated with NS. *: indicates significant difference between Fe^{2+}- and Mg^{2+}-injected striatum at 6 hours (Student's *t* test; $p < 0.05$).

5.3.2. Time-course of striatal SA, 2,3-DHBA and 2,5-DHBA levels (Fig. 1)

In Fe^{2+}-injected striatum of SA-loaded rats SA levels peaked significantly (2.3-fold increase) after 6 hours and gradually declined to a still significant 1.6-fold increase at day 31, with no changes in the first two hours after intrastriatal injection as compared to the contralateral or Mg^{2+}-injected striatum (Fig. 1). Already thirty minutes after Fe^{2+}-injection striatal 2,3-DHBA levels were 8.7-fold and 2,5-DHBA levels were 3.6-fold increased and remained significantly elevated until at least 31 days post-injection. In addition, 2,3-DHBA levels reached a significantly elevated plateau during the first 6 hours as compared to later time points. Like SA, 2,5-DHBA peaked at 6 hours (23.5-fold increase) and gradually declined thereafter. In contralateral striata, levels of SA, 2,3- and 2,5-DHBA were not significantly changed in time. To correct for differences in tissue levels of SA, striatal DHBA levels are expressed as [DHBA/SA] (mmol adduct/mol SA) and [ipsi-/contralateral] ratios of [DHBA/SA] have been calculated to show the factor of increase by Fe^{2+} or Mg^{2+} (Fig.2). The [ipsi-/contralateral] ratio of [2,3-DHBA/SA] peaked (10-fold increase) at thirty minutes post-lesion and gradually declined until a non-significant 1.5-fold increase at day 31, whereas the [ipsi-/contralateral] ratio of [2,5-DHBA/SA] peaked at 6 hours (10.4-fold increase) and was elevated 4- to 5-fold at earlier and later time points.

Intrastriatal Mg^{2+}-injections produced a slight, but significant increase in 2,5-DHBA/SA values (+37%) as compared to contralateral tissues, whereas 2,3-DHBA/SA was not changed by Mg^{2+}.

5.3.3. Time-course of striatal DA, 5-HT and metabolite levels

Neurotransmitters. DA levels in Fe^{2+}-injected striatum of SA-loaded rats were significantly increased (+23%) after 30 minutes, decreased significantly (-16%) after 2 hours, dropped to less than 8% thereafter, and remained depleted until at least day 31 as compared to the contralateral striatum (Fig. 2). Serotonin levels were slightly decreased (-14%) at 30 minutes, dropped significantly at 2 hours (-48%) and reached a minimum (12-14% of contralateral striatum) at 6 and 72 hours. Thereafter 5-HT levels increased until 54% of contralateral tissue at day 31 (Fig. 2). In not-injected (contralateral) striata, DA and 5-HT levels were not significantly changed in time (resp. means of 74.7 and 3.2 pmol/mg). Mg^{2+}-control injections produced small but significant changes of DA (+14%) and 5-HT levels (-14%) in striatum.

Metabolites and turnover (Table 2 & 3): In Fe^{2+}-injected striatum DOPAC levels were 70% increased at 30 minutes, peaked at 2 hours (4.5-fold), and

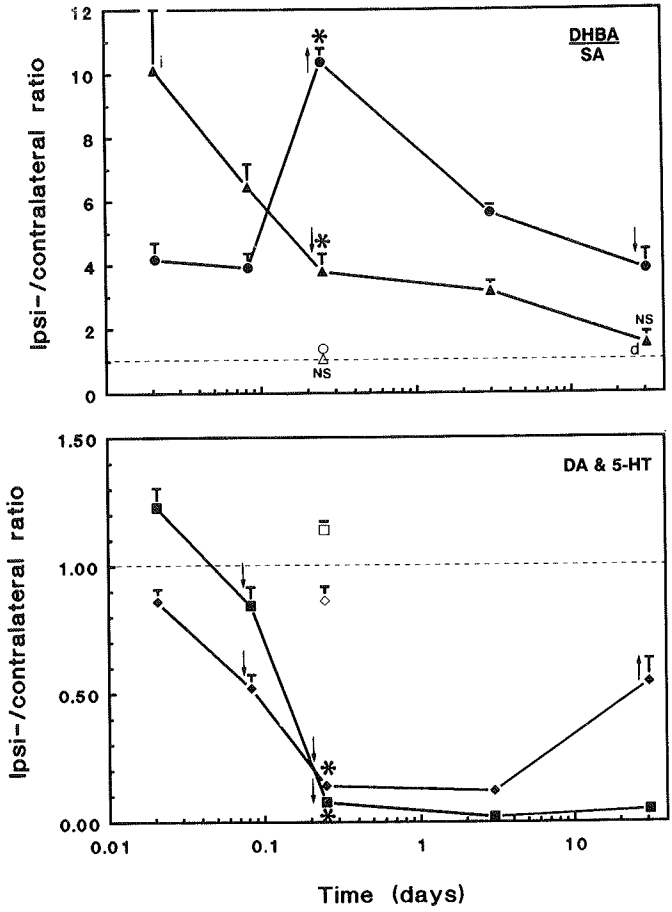


Fig. 2: *Time-course (0.5, 2, 6, 72 hours or 31 days) of in vivo 2,5-(circles) and 2,3-DHBA (triangles, i.e. ·OH formation), and DA (squares) and 5-HT (diamonds) depletions in striatum expressed as [ipsi-/contralateral] ratios following a unilateral intrastriatal injection of 0.4 µmol Fe^{2+} (closed symbols) or Mg^{2+} (at 6 hours, open symbols) in SA loaded rats.*
DHBA levels are corrected for SA levels (mmol adduct per mol SA). SA was administered two hours prior to decapitation (300 mg/kg i.p.). Note that time on the x-axis is plotted on a log-scale. For statistics and symbols see legend to Fig. 1.

dropped strongly thereafter until more than 90% depletion at day 31 as compared to contralateral tissues (Table 2). In contrast, striatal HVA levels were not changed by Fe^{2+} within the first two hours. Thereafter, HVA dropped like DOPAC. Striatal 5-HIAA levels were not changed by Fe^{2+} within the first 2 hours, decreased (-80%) after 6 hours, increased after 3 days (89% of contralateral side), and were reduced (-41%) at day 31 as compared to contralateral tissues. In non-injected striata, DOPAC, HVA and 5-HIAA levels were increased during the first two hours as compared to the later time points. The effects of Fe^{2+} or Mg^{2+} on DA and 5-HT turnover, expressed as [DOPAC/DA], [HVA/DA] and [5-HIAA/5-HT] ratios are shown in Table 3. Thirty minutes after Fe^{2+} only small, if any, changes were observed. Two hours after Fe^{2+}-injection, the [ipsi-/contralateral] ratio of [DOPAC/DA] was 5.4-fold increased, whereas these ratios of the other [metabolite/transmitter] values

Table 2: Time course of levels of DA and 5-HT metabolites (pmol/mg) in ipsi- and contralateral striatum after unilateral intrastriatal injection of 0.4 μmol $FeCl_2$ or $MgCl_2$ (control, t= 6hr) in salicylate (300 mg/kg i.p.) loaded rats.

Time	DOPAC		HVA		5-HIAA	
	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral	contralateral
0.5 hour (8)	16.6 ± 1.60 $	9.95 ± 0.58	9.72 ± 0.56	10.38 ± 0.60	4.71 ± 0.25	5.01 ± 0.23
2 hours (7)	43.8 ± 1.36↑,$	10.32 ± 0.82	13.2 ± 0.84 ↑	13.00 ± 1.17 ↑	5.59 ± 0.26 ↑	5.29 ± 0.26
6 hours (7)	5.60 ± 0.24 ↓	6.14 ± 0.28 ↓	1.11 ± 0.17↓,$	4.98 ± 0.39 ↓	0.83 ± 0.07↓,$	4.47 ± 0.20
control (7)	8.54 ± 0.35*,$	5.33 ± 0.22	8.18 ± 0.25*,$	4.43 ± 0.24	4.02 ± 0.11 *	4.22 ± 0.14
3 days (6)	2.88 ± 0.31 $	5.84 ± 0.51	0.81 ± 0.17 $	4.64 ± 0.62	3.37 ± 0.51 ↑	3.92 ± 0.41 ↓
31 days (7)	0.64 ± 0.06 $	6.72 ± 0.26	0.19 ± 0.13 $	5.87 ± 0.26	2.01 ± 0.13↓,$	3.46 ± 0.10

Data are mean ± SEM values from six to eight rats (n). The effects of time, Fe^{2+}-injection, and interaction between the two are highly significant (ANOVA; $p < 0.001$). Newman-Keuls post-hoc test ($\alpha = 0.05$) revealed significant increases (↑) or decreases (↓) as compared to the preceding time point. $: Significant difference between ipsi- and contralateral striatum, or *: between Fe^{2+}- and Mg^{2+}-injected striatum at 6 hours (Student's *t* test; $p < 0.05$).

Table 3: *Time course of turnover factor of DA and 5-HT neurotransmitters expressed as [ipsi-/contralateral] ratios of [metabolite/neurotransmitter] ratios in striatum after unilateral intrastriatal injection of 0.4 µmol $FeCl_2$ or $MgCl_2$ (control, t= 6 hr) in salicylate (300 mg/kg i.p.) loaded rats.*

Time post-Fe^{2+} injection	Turnover factor		
	$\frac{[DOPAC/DA]_{ipsi}}{[DOPAC/DA]_{contra}}$	$\frac{[HVA/DA]_{ipsi}}{[HVA/DA]_{contra}}$	$\frac{[5HIAA/5HT]_{ipsi}}{[5HIAA/5HT]_{contra}}$
0.5 hour	1.39 ± 0.14	0.77 ± 0.05	1.12 ± 0.06
2 hours	5.44 ± 0.58 ↑	1.27 ± 0.09	2.11 ± 0.11
6 hours	14.45 ± 2.47 ↑	3.23 ± 0.52	1.37 ± 0.10
Mg^{2+}-control	1.44 ± 0.05 *	1.68 ± 0.08 *	1.13 ± 0.05
3 days	27.63 ± 2.58 ↑	12.74 ± 4.48 ↑	8.28 ± 1.79 ↑
31 days	2.62 ± 0.70 ↓	2.21 ± 0.40 ↓	1.43 ± 0.38 ↓

Data are mean ± SEM values from six to eight rats. The effects of time, Fe^{2+}-injection, and interaction between the two are highly significant (ANOVA; $p < 0.001$, with Newman-Keuls post-hoc test; $\alpha = 0.05$). For symbols see legend to Table 2.

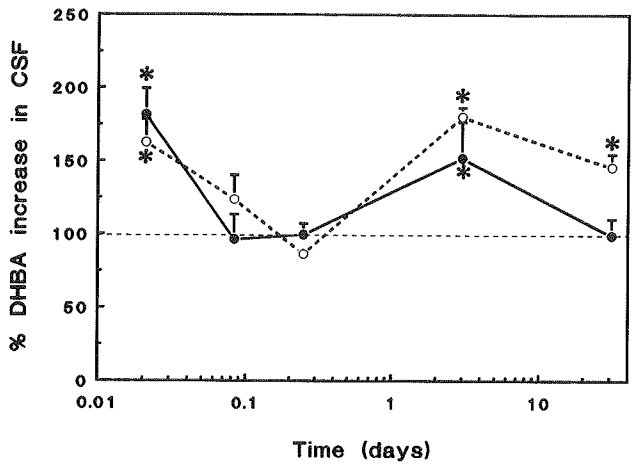
were not significantly changed at this time point. The turnovers peaked three days after Fe^{2+} (8- to 28-fold increase). At day 31, [DOPAC/DA] and [5-HIAA/5-HT] ratios were not significantly increased, whereas [HVA/DA] ratios still reached significance.

Mg^{2+}-injections induced at 6 hours small, but significant increases of DOPAC and HVA, and [DOPAC/DA] and [HVA/DA] ratios (respectively +44% and +68%), whereas 5-HIAA and [5-HIAA/5-HT] ratios were not different from contralateral striatum. However, the changes produced by Fe^{2+} at this time point are far greater than those of Mg^{2+}.

5.3.4. Time-course of DHBA levels in CSF after intrastriatal Fe^{2+}-injection (Fig. 3)

Following a unilateral striatal Fe^{2+}-injection in SA-loaded rats, significant biphasic increases of 2,3- and 2,5-DHBA levels in CSF were observed (ANOVA, $p < 0.001$). The first peaks (2,3-DHBA 182% and 2,5-DHBA 163% of Mg^{2+}-injected controls) occurred at thirty minutes, whereas the second peaks (2,3-DHBA 152% and 2,5-DHBA 180%) were observed at 3 days. After 31 days, 2,3-DHBA levels in CSF were back to normal, whereas 2,5-DHBA levels were still significantly elevated (146%).

Fig. 3: *Time-course (0.5, 2, 6, 72 hours or 31 days) of 2,3- (straight line) and 2,5-DHBA (broken line) levels in cerebrospinal fluid (CSF) corrected for SA concentrations (mmol adduct per mol SA) following a unilateral intrastriatal injection of 0.4 µmol Fe^{2+} in SA-loaded rats.*



SA (300 mg/kg i.p.) was given two hours prior to CSF sampling. 100% represents Mg^{2+} control at 6 hours: 0.35 ± 0.037 mmol 2,3-DHBA per mol SA (closed circles), or 2.47 ± 0.08 mmol 2,5-DHBA per mol SA (open circles). Note that time on the x-axis is plotted on a log-scale.
*: indicates significant peak effects in time using ANOVA ($p < 0.001$) with Newman-Keuls post-hoc test ($\alpha = 0.05$).

5.4. Discussion

The main conclusion from the present study using SA as ·OH-trapping agent is, that free Fe^{2+} triggers ·OH formation in living brain before damage to dopaminergic (irreversible) and serotonergic (partially reversible) nerve terminals occurs. In addition, these acute Fe^{2+}-induced free radical processes in brain tissue could be detected in CSF by analyzing its DHBA content at particular time points.

To address the question whether ·OH are cause or consequence of damage, it is important to discuss early (0.5 - 2h) and late events (2h - 1 month) following Fe^{2+}-injection. In non-injected, contralateral striata we observed during the first two hours relatively small effects on biogenic amines and related acids, probably due to anaesthesia with chloralhydrate, which is known to decrease the efflux of acidic metabolites from the brain (Westerink, 1985). Likewise some "aspecific" effects occured after Mg^{2+}-injections into striatum, probably due to mechanical injury by the needle. It has been taken into account, that similar effects occur in Fe^{2+}-injected striata. Importantly, both control conditions did not increase 2,3-DHBA levels c.q. ·OH formation. Within two hours after Fe^{2+}-injection, initial peak levels of 2,3-DHBA (10-fold increase) suggest immediate and abundant ·OH formation, while DA, 5-HT (27-

39% reduction) and metabolite levels (Table 2) in striatum are not or only slightly altered (Fig. 2). At two hours after Fe^{2+}-injection, the high DOPAC levels (4.5-fold), no change in HVA and moderate DA reductions suggest, that both DA anabolism and catabolism are greatly, and specifically enhanced by iron. Iron, which is a co-factor of the cytosolic enzyme tyrosine hydroxylase (Nagatsu et al., 1964), can stimulate the enzyme activity by 2- to 10-fold *in vitro* (Rausch et al., 1988); the effect may explain our *in vivo* results on DA-synthesis. Evidently, at these early time points, DA is available in the cytosol and is degraded immediately by monoamine oxidase -which is localized on the outer membrane of mitochondria- and not by catechol-*o*-methyltransferase -which is predominantly localized extra-neuronally (Westerink, 1987). In this phase, (over)production of H_2O_2 via DA-oxidation by monoamine oxidase may drive the Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$. Following intracortical Fe^{3+} injection, increased $O_2^{\cdot -}$ formation was reported within 15 min. (Willmore et al., 1983), whereas in another study using Fe^{2+} lipid peroxidation rised significantly between 1 and 2h. (Triggs and Willmore, 1984), which is in line with our conclusion, that oxygen radicals trigger tissue damage.

In the subsequent period between 2 hours and 1 month, DA, DOPAC and HVA levels decline rapidly by Fe^{2+} and remain reduced for more then 90% after 1 month, suggesting irreversible damage to the nigrostriatal DA-system. The latter is in line with a recent report showing progressive striatal DA depletion and substantia nigra atrophy following a single intranigral Fe^{3+}-infusion (Sengstock et al., 1994). In the present investigation, 5-HT levels also drop strongly (86-88% reduction), but slowly recover between 3 and 31 days (54% of contralateral), whereas 5-HIAA levels fluctuate and are still ~50% reduced after 1 month, indicating partial regeneration of 5-HT terminals in striatum. The Fe^{2+}-induced increase of DA and 5-HT turnover (i.e. turnover factor, see Table 3) peaked after three days (8-27 fold), implicating enhanced H_2O_2 production, while in the same period the ${}^{\cdot}OH$ yield (2,3-DHBA ratio) is gradually declining. This indicates that not H_2O_2, but other factors -probably free Fe^{2+}- limit the generation of ${}^{\cdot}OH$ during this period. Following intrastriatal injection of ^{54}Mn, ~1/3 of the injected label remained present in striatum for at least three days, most likely representing ^{54}Mn bound to ferritin (Sloot and Gramsbergen, 1994). In analogy with Mn, injected Fe^{2+} is presumably also still present in the tissue, but bound to ferritin or other Fe-binding constituents and thus less capable to catalyze the Haber-Weiss reaction (see also Addendum). Nevertheless our data suggest, that ${}^{\cdot}OH$ are continuously formed during the process of neurodegeneration. After 1 month 5-HT and DA turnover is (almost) back to normal, indicating no further neurodegeneration. This is

consistent with the low ·OH yield (2,3-DHBA ratio) at that time point.

The increase of SA content in the Fe^{2+}-injected striatum could be explained by progressive tissue damage after two hours as discussed above. Since SA-levels of blood and CSF (Chen et al., 1978) are respectively about 5- (unpublished result) and 2-fold higher than in control striatum, the ample 2-fold rise of SA in Fe^{2+}-injected striatum at 6 hours, suggests (local) loss of membrane integrity and/or a compromised blood-brain-barrier (Fig. 1). Differences in SA dose-dependency (Table 1) and time-course (Fig. 2) between 2,3- and 2,5-DHBA formation by Fe^{2+}, implicate that these compounds are indices of distinctive processes. The greater Fe^{2+}-induced increase of 2,3-DHBA levels at higher SA dose suggests, that in presence of high ·OH levels the local SA concentration is the limiting factor for 2,3-DHBA formation (and not for 2,5-DHBA). Hall et al. (1993) reported also that cerebral 2,5-DHBA levels increased proportional to the systemic SA dose, but they did not measure 2,3-DHBA. An explanation of the different time-course of 2,5-DHBA may be provided by enzymatic conversion of SA by cytochrome P-450, which is not the case for 2,3-DHBA (Ingelman-Sundberg et al., 1991). Although brain P-450 contents are low, their enzymatic activity can be stimulated by xenobiotics (Mesnil et al., 1984; Minn et al., 1991; Anandatheerthavarada et al., 1990 and 1993; Ghersi-Egea et al., 1993), including SA (Chand and Clausen, 1982), and might be enhanced by induction in neurons, infiltrated immunocompetent cells, and/or activated micro- and astroglia in response to the lesion. The latter processes are nearly always observed after brain injuries (Benavides et al., 1990; O'Callaghan 1993). Hence, 2,5-DHBA may (co-)monitor P-450 activity *in vivo*, while 2,3-DHBA is strictly an index for ·OH formation.

The effects of acute and severe Fe^{2+}-lesions in brain tissue could be detected in CSF (Fig. 3). The first DHBA peaks in CSF are -most likely- a reflection of the 10-fold increase of 2,3-DHBA and 4.2-fold increase of 2,5-DHBA in striatum thirty minutes post-Fe^{2+}, whereas the second 2,3-DHBA peak in CSF after 3 days is probably derived from Fe^{2+} mediated ·OH formation in areas more remote from the injection site, including ipsilateral basal ganglia, n. accumbens and cerebral cortex (Sloot et al., 1994). The second 2,5-DHBA peak in CSF may, as argued before, co-monitor P-450 activity from these injured areas, which is still significantly elevated after 1 month. Only the 10-fold rise of striatal 2,5-DHBA after 6 hours is not reflected in CSF, which might be explained by a local response in striatum. Thus, our results using an acute brain Fe^{2+}-injection demonstrate, that by measuring hydroxylated adducts of SA in CSF, oxidative stress in the CNS can be detected. Whether this technique can be applied in the clinic to study chronic conditions of oxidative stress and its treatment in for instance PD (Olanow et al., 1992) and amyotrophic lateral

sclerosis (Bowling et al., 1993), needs further investigation.

Addendum: After completion of the studies as presented above, we found that the sonication procedure itself in presence of difference amounts of added Fe^{2+} *in vitro* (20-200 nmol per sample) may cause instantaneous 2,3-DHBA (but absolutely no 2,5-DHBA) formation. This momentary "in-vitro-effect" of Fe^{2+} was completely abolished using PCA containing the iron chelator deferoxamine mesylate (10 mM final concentration), which also resulted in about 5-fold lower 2,3-DHBA levels in control tissues. Since samples of striatal tissues obtained at the earliest time points after *in vivo* Fe^{2+}-injection contain the highest low-molecular-weight (LMW) iron levels (as determined according to Voogd et al., 1992), i.e. approximately 8,000 pmol/mg at 30 minutes, 500 pmol/mg at 6 h, and 50 pmol/mg in control tissues, the time-dependent decrease of 2,3-DHBA formation could simply be due to *in vitro* differences in LMW-iron content rather than to *in vivo* effects. Assessment of 2,3-DHBA effects after *in vivo* Fe^{2+}-injection this time using deferoxamine in the homogenation mixture, resulted in ipsi-/contralateral [2,3-DHBA/SA] ratios of 24.1 ± 4.6 at 30 minutes (n=5) and 8.6 ± 2.0 (n=5) at 2 h, indicating that the presented data in Fig. 1 and 2 do indeed reflect time-dependent *in vivo* effects of Fe^{2+}. Although the magnitude of 2,3-DHBA effects as presented in Figs. 1 and 2 may be underestimated because of the relatively high 2,3-DHBA background levels of samples homogenized without deferoxamine, the stated conclusions of the presented paper remain valid.

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Manganese-induced hydroxyl radical formation in rat striatum is not attenuated by dopamine depletion or iron chelation *in vivo*.

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6.0. Summary

The present studies were aimed at investigating the possible roles of dopamine (DA) and iron in production of hydroxyl radicals (·OH) in rat striatum after Mn^{2+}-intoxication. For this purpose, DA depletions were assessed concomitant with in vivo 2,3- and 2,5-dihydroxybenzoic acid (DHBA) formation from the reaction of salicylate with ·OH, of which 2,3-DHBA is a non-enzymatic adduct. Following intrastriatal Mn^{2+}-injection, marked 2,3-DHBA increases were observed in a time- and dose-dependent fashion reaching maximum levels at 6-18 h and a plateau beyond 0.4 µmol (4-fold increase). The delayed increase of 2,3-DHBA levels suggest that Mn^{2+} induces ·OH formation in the living brain by an indirect process. The early DA depletion (2 h) and relatively late ·OH formation (6 h) indicate independent processes by Mn^{2+}. In addition, depletion of DA (about 90%) by reserpine pre-treatment did not significantly alter Mn^{2+}-induced 2,3-DHBA formation or the extent of DA depletion, suggesting that DA or DA-autoxidation are not participating in Mn^{2+}-induced ·OH formation in vivo. Furthermore, Mn^{2+}-injection did not significantly alter the low molecular weight iron pool in striatum, and co-injections of the iron-chelator deferoxamine with Mn^{2+} into striatum did not significantly attenuate Mn^{2+}-induced 2,3-DHBA formation. These findings suggest no role of chelatable iron in generation of Mn^{2+}-induced ·OH, but does not exclude a role for mitochondrial heme-iron or peroxynitrite (Fe-independent) in Mn^{2+}-induced ·OH formation.

6.1. Introduction

Chronic inhalation of manganese (Mn) dust or vapour in humans and monkeys causes Parkinsonism and dystonia (Barbeau et al., 1976; Barbeau 1984; Donaldson, 1987) and produces selective neuropathology in the basal ganglia with lesions being localized both pre- and postsynaptically to the dopaminergic (DA) nigrostriatal pathway (Bird et al., 1984; Yamada et al. 1986; Wolters et al., 1989; Eriksson et al., 1987, 1992a and 1992b). In addition, Mn^{2+}-injection into the basal ganglia of rats provides a model of chronic systemic Mn-exposure in primates (Lista et al., 1986; Parenti et al. 1986 and 1988; Brouillet et al., 1993; Sloot et al., 1994).

For more than a decennium, it has been hypothesized from *in vitro* studies, that Mn-neurotoxicity is mediated by free radicals via enhanced nonenzymatic auto-oxidation of catecholamines (Graham et al., 1978; Graham 1984; Halliwell, 1984; Archibald and Tyree, 1987; Millar et al., 1990) or production of 6-OHDA (Cohen and Heikkila, 1974; Cohen, 1984; Garner and Nachtman, 1989). Both processes would lead to production of toxic (semi)quinones, and enhanced levels of H_2O_2 and superoxide anion radicals ($O_2^{\cdot-}$) as well as hydroxyl radicals ($^{\cdot}OH$). This concept is in agreement with attenuation of Mn^{2+}-induced DA-depletion by vitamine E treatment (Parenti et al., 1988). On the other hand, it has been reported that lipid peroxidation is inhibited by Mn^{2+} both *in vitro* (Cavallini et al., 1984; Tampo and Yonaha 1992) and in postmortem brain tissues of Mn^{2+}-exposed rats (Shukla and Chandra, 1981; Donaldson et al., 1982).

Although production of (semi)-quinones or free oxygen radicals after Mn have not been reported *in vivo*, it seems likely that DA plays a role in Mn-neurotoxicity. For instance, pretreatment with the DA synthesis blockers α-methyltyrosine and lisuride attenuate the neurotoxicity of Mn^{2+} (Parenti et al. 1988), whereas the monoamine oxidase inhibitor pargyline, and L-DOPA (+carbidopa) potentiate its toxicity (Parenti et al., 1986).

Besides DA, brain iron could mediate neurotoxic effects of Mn^{2+}. The selective accumulation of Mn^{2+} in the basal ganglia (Eriksson et al., 1987; London et al., 1989; Newland et al., 1989), and anterograde axonal transport of Mn^{2+} in nigrostriatal and striatonigral neurons (Sloot and Gramsbergen 1994), may be dependent on iron transport and storage pathways (Hill and Switzer, 1984; Hill, 1990; Aschner and Aschner, 1991; Morris et al., 1992; Benkovic and Connor, 1993). *In vitro* studies have shown that Mn binds to the iron transport protein transferrin and its receptor on catecholamine-containing neuroblastoma cells, and that Mn -like Fe- is internalized followed by storage

into ferritin (Suárez and Eriksson, 1993). Therefore, Mn^{2+} may (in)directly liberate endogenous iron by disturbing iron-homeostasis, particularly in mitochondria. In these organelles, Mn^{2+} induces decreased gluthation (GSH) contents and GSH-enzyme activities (Liccione nad Maines, 1988), thereby compromising a major cellular defense mechanism against oxyradicals. In addition, Mn^{2+} has been reported to impair ATP production, decrease respiratory cytochrome contents, inhibit oxidative phosphorylation, and increase lactate production (Liccione and Maines, 1989; Gavin et al., 1992; Brouillet et al., 1993).

The aim of the present studies was dual. First, time- and dose-dependent OH formation by Mn^{2+} was assessed in relation to DA and 5-HT depletions in order to examine whether OH generation is cause or consequence of brain damage. For this purpose we used salicylate (SA) as an OH-trapping agent, which forms the stable adducts 2,3- and 2,5-dihydroxybenzoates (DHBA)(Ingelman-Sundberg et al., 1991; Sloot and Gramsbergen, 1995 and references therein), and micro-injections of Mn^{2+} into rat striatum as described previously (Sloot et al., 1994). Since it has been reported that 2,5-DHBA can also be formed via P-450 enzymes, which is not the case for 2,3-DHBA, the latter appears to be a more reliable index for OH formation (Ingelman-Sundberg et al., 1991). Second, the roles of DA and iron in generating Mn^{2+}-induced OH formation were investigated by analyzing 2,3-DHBA levels in striata of reserpine-pretreated (DA-depleted) or deferoxamine-treated (iron-chelated) rats, as well as by assessing different endogenous iron pools in Mn^{2+}-injected striata.

6.2. Materials and methods

6.2.1. Animals

Wistar-derived male rats (WAG, Harlan, Zeist, The Netherlands) were kept on a 12/12-h light/dark cycle and housed in a room with a humidity of 50-70% and a temperature of 24°C with free access to water and chow food. Animal experimentation protocols were approved by the Ethical Committee for Laboratory Animals Experiments, TNO/Regio West (Woudenberg, The Netherlands, reg.no. 56-3A).

6.2.2. Materials

$MnCl_2$ (>96% pure), $FeCl_2.4H_2O$ (> 99% pure), $MgCl_2.6H_2O$ and dopamine (DA, 3-hydroxytyramine.HCl) were obtained from Merck (Darmstadt, Germany). Solutions of metals were made as described previously (Sloot et al., 1994). All other compounds were purchased from Sigma (Brunschwig Chemie, Amsterdam, The Netherlands) and stored according to the

description of the manufacturer.

6.2.3. Experimental groups

Chloralhydrate anaesthetized (400 mg/kg i.p.) rats (190-230 g) received unilateral injections of $MnCl_2$, $FeCl_2$ or $MgCl_2$ in 1 μl of Milli-Q water (Millipore, Molsheim, France) into striatum using stereotaxic procedures as described previously (Sloot et al., 1994).

DHBA formation by Mn: Time-course and dose-dependency. Two, six or eighteen hours after intrastriatal injection of 0.4 μmol Mn^{2+}, or six hours after injection of 0.4 μmol Mg^{2+}, rats (n = 6-7 per group) were decapitated to dissect striatal tissues from the brain. Likewise, rats (n = 6-7 per dose) were injected with 0.13 or 1.20 μmol Mn^{2+} six hours before collection of their striata. In addition, two hours prior to sacrifice, rats were loaded with 300 mg/kg salicylate (SA; i.p.), whereas others (6-8 striata per group) did not receive SA to serve as negative controls.

DHBA formation by Mn after dopamine depletion. Eight rats were depleted of dopamine by 24 hours pre-treatment with reserpine (2.5 mg/kg i.p.; Callaway et al., 1989). These rats received a unilateral injection of 0.4 μmol Mn^{2+} into striatum, and were decapitated 6 hours later. In addition, SA (300 mg/kg i.p.) was given two hours before sacrifice.

Effect of Mn on total and LMW iron levels. Six hours after bilateral injection of 0.4 μmol Mn^{2+}, Mg^{2+} or Fe^{2+} into striatum, rats (n = 4-8 per treatment) were decapitated and their striata were dissected for immediate assay of total and low molecular weight (LMW) iron contents.

Effect of deferoxamine on Mn-neurotoxicity. Ten rats received an injection (1 μl total) of 0.4 μmol Mn^{2+} plus 0.2 or 2.0 nmol deferoxamine mesylate (Desferal; DFX) into the left striatum, and 0.4 μmol NaCl plus DFX into the right striatum. Six control rats received similar injections without DFX. All rats were loaded with SA (300 mg/kg i.p.) and sacrificed respectively 4 and 6 hours after stereotaxic injection.

6.2.4. Collection of samples

Collection and handling of samples for analysis of DA, 5-HT, SA, and related metabolites was done as described previously (Sloot and Gramsbergen, 1995). Briefly, on ice dissected striatal tissues (20-40 mg w.w.) were immediately frozen on dry ice and stored at -70°C. At the day of analysis, striata were sonicated in ice-cold 200 μl perchloric acid containing $Na_2S_2O_5$, centrifuged, diluted with mobile phase buffer, and kept on ice before injection into the HPLC-system.

For analysis of iron, freshly dissected striatal tissues were immediately weighed in pre-weighed potter tubes, homogenized in 100 mM Tris/HCl buffer (pH 7.4, 20% w/v), and subsequently DFX (2 mM final concentration) was added as described previously for heart tissue (Voogd et al., 1992). Depending on expected concentrations, striatal tissues of 1 or 2 rats were pooled before homogenizing.

6.2.5. Analysis of 6-hydroxydopamine, dopamine, serotonin, salicylate, and related metabolites

Levels of SA, 2,3- and 2,5-DHBA as well as 6-hydroxydopamine (6-OHDA), DA, 5-HT (serotonin) and their metabolites DOPAC (3,4-dihydroxyphenylacetic acid), HVA (4-hydroxy-3-

methoxyphenylacetic acid) and 5-HIAA (5-hydroxyindole-3-acetic acid) were determined in striatal tissues according to a previously described HPLC procedure with UV-absorbance and electrochemical (EC) detection (Sloot and Gramsbergen, 1995). Since 6-OHDA standards had a retention time between 2,3- and 2,5-DHBA standards, 6-OHDA measurements were conducted in samples without SA with a detection limit of about 50 fmol. Briefly, a Spherisorb ODS2 cartridge analytical column (100 x 4.6 mm, 3 μm) with a ODS1 precolumn (30 x 4.6 mm, 5 μm) (Phase Separation Ltd., Deeside, U.K.) were used to separate the compounds of interest at a flow rate of 0.8 ml/min. The mobile phase buffer (MPB) consisted of 0.1 M sodiumacetate, 6.0 % methanol, 19.5 mg/L n-octyl sodium sulphate (Merck, Darmstadt, Germany) and 10-15 mg/L Na_2ethylenediaminetetraacetate (EDTA) dissolved in Milli-Q water and adjusted to pH 4.1 with glacial acetic acid. Stock solutions of standards (~1 mM) dissolved in 50 mM HCl containing 0.20 g/L $Na_2S_2O_5$ and 0.050 g/L Na_2EDTA were stable for at least 2 months if kept stored in the dark at 4°C.

6.2.6. Analyses of total and low molecular weight (LMW) iron pools

After centrifuging the striatal homogenate at 10,000 G for 15 minutes, the supernatant was removed for low molecular weight (LMW) iron measurements in presence of the hexadentate iron chelator DFX (Voogd et al., 1992), which forms a strong complex with iron: ferrioxamine (FX). LMW iron assays were conducted under physiological or reducing conditions by adding ascorbic acid (20 mg/ml in solid state). Ascorbic acid was used to reduce iron from its ferric to its ferrous state, thereby liberating iron from Fe^{3+}-complexes, resulting in total LMW-iron measurements. Briefly, samples were incubated for 60 minutes at 37°C, and pre-purified by passing it through a 50 mg Extract-Clean C18 column (Alltech) on which DFX and FX are retained. After washing with 0.3 ml water and elution with 0.2 ml 90% methanol/10% water, the eluate was injected into the HPLC-system, which was equipped with an ODS2 analytical column (as mentioned above) and two variable wavelength detectors to allow simultaneous detection of FX at 430 nm and DFX at 229 nm (Waters 484). The mobile phase consisted of 88 % 20 mM Na_2HPO_4/NaH_2PO_4, 2 mM NaEDTA, 0.2 M triethylammoniumchlorid (pH 6.6) and 12% acetonitril. Recovery of DFX and FX was respectively > 80% and almost 100%. The detection limit for FX was 0.25 nmol. Iron levels were based on FX/DFX ratios from $FeSO_4$ standards incubated and extracted as the samples. Mn^{2+} (100-800 fold excess) did not affect chelation of iron by DFX in this procedure.

Total iron in the resuspended pellet (20% w/v in Tris/HCl) was determined using the iron (II) chelator Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4,-triazine monosodium salt, Sigma) essentially according to Riederer et al. (1989). Briefly, samples were added with Ferrozine (4.2 mM), ascorbic acid (20 mg/ml) to reduce iron, and pepsine (0.1%) at a final pH of 2.5 using HCl. Subsequently, samples were incubated at 37°C for 20 minutes, and absorbances were read at 560 nm. Blank and standard iron samples were treated in a similar manner. Mn^{2+} did not affect chelation of iron by Ferrozine.

6.2.7. Statistics

Changes of the differences between ipsi- and contralateral tissues over time or with increasing dose were statistically evaluated using ANOVA (BMDP Statistical Software program SOLO, Los Angeles, USA) followed by Newman-Keuls multiple comparisons test. In addition,

the effect of time or dose in contra- or ipsilateral striata were also tested. Differences between values of ipsi- and contralateral tissues at the separate time points or doses were evaluated using Student's *t* test.

6.3. Results

6.3.1. Time-dependent effects of 0.4 μmol Mn^{2+}

SA levels expressed as [ipsi-/contralateral] ratios were significantly increased by Mn^{2+} at 6 and 18 h by respectively 1.5- and 1.9-fold, but not at 2 h (1.3 fold). To correct for differences in SA tissue levels, DHBA levels are expressed as [DHBA/SA] (mmol adduct/mol SA) followed by calculations of [ipsi-/contralateral] ratios to determine the factor of increase by Mn^{2+}.

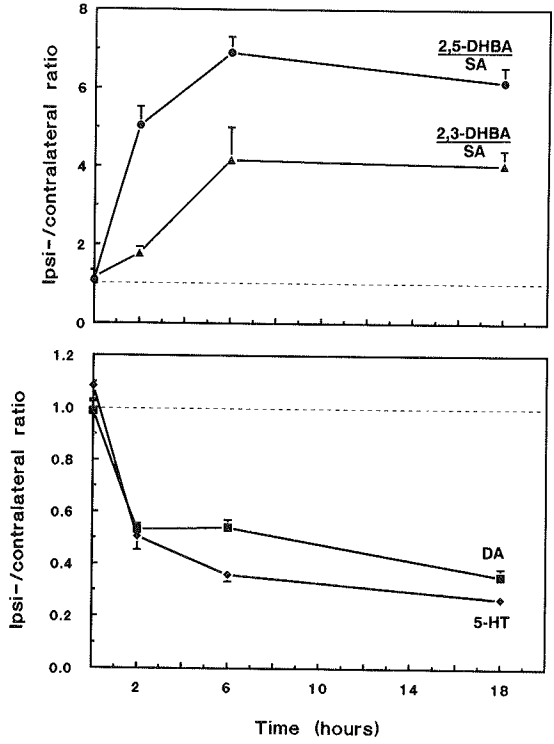


Fig. 1: *Time-course of 2,3- (triangles) and 2,5-DHBA (circles) formation corrected for SA (upper panel), and of dopamine (squares) and serotonin (diamonds) depletion (lower panel) in striatum following a unilateral injection of 0.4 µmol Mn^{2+} into striatum of salicylate (SA) loaded rats (300 mg/kg i.p.; 2 hours).*

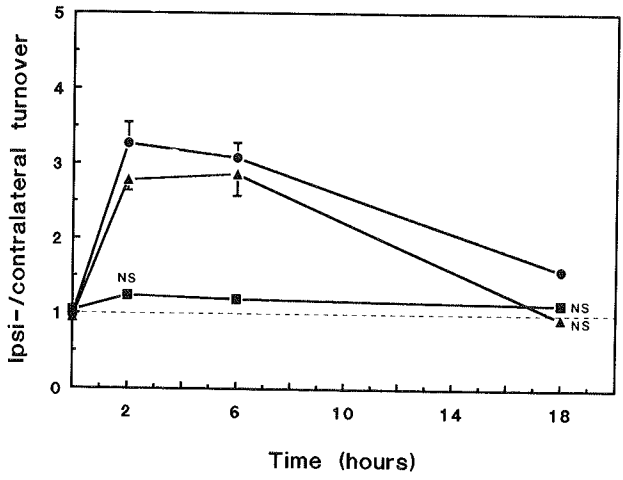
Data represent ipsi-/contralateral ratios of 6-7 rats per time point. All compounds changed time-dependently (ANOVA, $p < 0.001$). Newman-Keuls post-hoc multiple comparison tests ($\alpha = 0.05$) indicated a leveling off of DHBA increases and 5-HT decrease at 6 h, and no significant change between 0 and 2 h of 2,3-DHBA/SA and between 2 and 6 h of DA.

[Ipsi-/contralateral] ratios of [DHBA/SA] were significantly increased by Mn^{2+} over time ($p < 0.001$), reaching maximum levels at 6 h for both 2,3-DHBA (4.1-fold increase) and 2,5-DHBA (6.9-fold increase) and no significant increase between 0 and 2 h of 2,3-DHBA/SA ratios (Fig. 1).

DA levels (Fig. 1) were significantly decreased by Mn^{2+} at 2 hours (-53%), and declined further thereafter until at least 18 hours (-65%) as compared to contralateral tissues ($p < 0.001$). Serotonin levels (Fig. 1) were significantly decreased (-49%) by Mn^{2+} at 2 hours, and declined further thereafter reaching the lowest levels at 18 hours (-73% of contralateral striatum).

DOPAC (1.7-fold) and HVA (1.5- to 1.7-fold) levels were significantly increased by Mn^{2+} at 2 and 6 hours, and were significantly decreased at 18 hours (-40% and -65% of contralateral striatum respectively). The resulting DA turnover expressed as DOPAC/DA and HVA/DA ratios was significantly elevated at 2 hours (3.3- and 2.8-fold respectively), and declined thereafter until a still significant increase of DOPAC/DA ratios (1.6-fold of contralateral) and normal HVA/DA ratios at 18 hours (Fig. 2). 5-HIAA levels were significantly decreased (-39%) by Mn^{2+} at 2 hours, and declined further thereafter until -69% of contralateral tissue at 18 h. The resulting 5-HIAA/5-HT ratios were slightly,

Fig. 2: *Time-course of dopamine and serotonin turnover following unilateral intrastriatal injection of 0.4 µmol Mn^{2+} into striatum of salicylate loaded rats (300 mg/kg i.p.; 2 hours).*

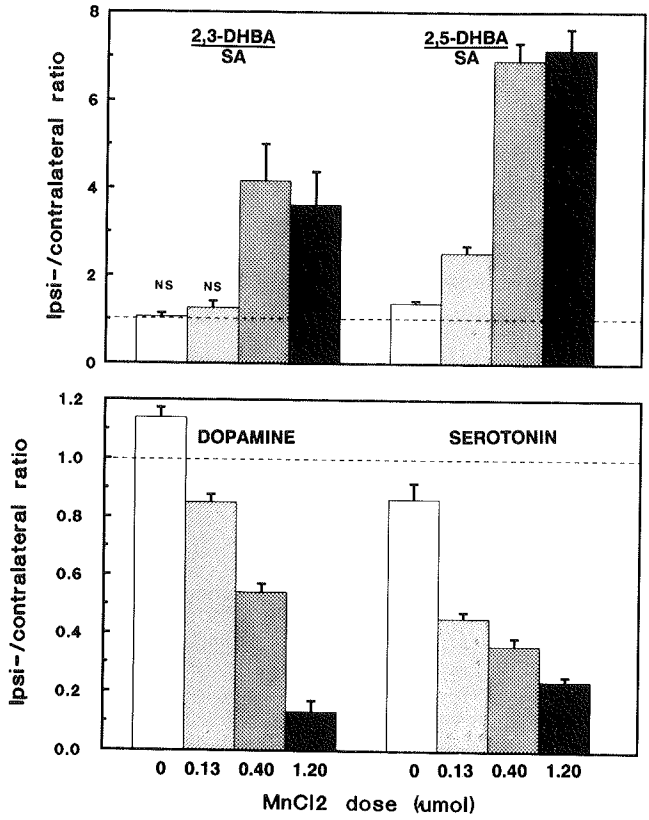


Data represent [ipsi-/contralateral] ratios of DOPAC/DA (circles), HVA/DA (triangles) and 5-HIAA/5-HT (squares) ratios of 6-7 rats per time-point. The DA and 5-HT turnover changed time-dependently (ANOVA, $p < 0.001$). Newman-Keuls post-hoc multiple comparison tests ($\alpha = 0.05$) indicated that DOPAC/DA and HVA/DA increased between 0 and 2 h and decreased between 6 and 18 h. 5-HIAA/5-HT ratios increased slightly between 0-2 hrs. NS indicates no significant difference between ipsi- and contralateral values.

but significantly elevated by Mn^{2+} ($p < 0.001$), but did not change among 2, 6 and 18 h (Fig. 2).

In non-injected (contralateral) striatum, the means ± SEM of 2,3- and 2,5-DHBA/SA values, and DA, 5-HT, DOPAC, HVA and 5-HIAA levels were respectively: 0.30 ± 0.033 and 2.03 ± 0.11 mmol/mol SA, and 68.9 ± 1.43, 3.15 ± 0.08, 5.16 ± 0.16, 4.71 ± 0.16 and 4.38 ± 0.08 pmol/mg, except at 2 h where levels of DA, DOPAC and HVA were significantly elevated ($p < 0.001$). The latter effect is most likely due to chloralhydrate anaesthesia (Westerink, 1985).

Fig. 3: *Dose-dependent 2,3- and 2,5-DHBA formation corrected for SA (upper panel), and dopamine and serotonin depletions (lower panel) in striatum six hours after a unilateral control (Mg_{2+}) or Mn^{2+}-injection (dose as indicated) into striatum of SA-loaded rats.*

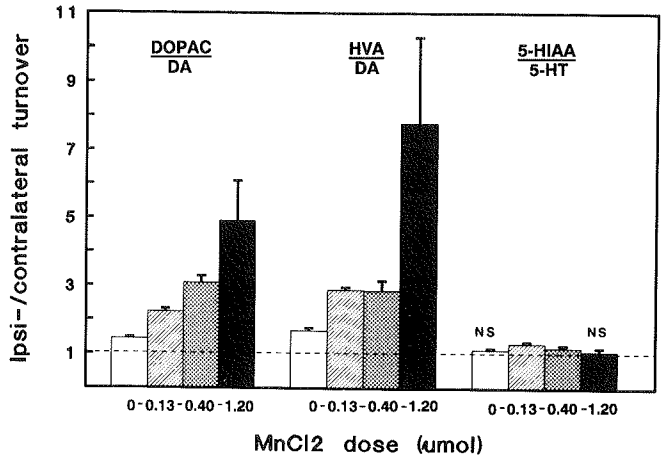


Data represent ipsi-/contralateral ratios of six to seven rats. All compounds changed dose-dependently (ANOVA; $p < 0.001$). Newman-Keuls post-hoc multiple comparison tests ($\alpha = 0.05$) revealed an increased DHBA plateau at 0.4 μmol Mn^{2+}, and no 2,3-DHBA increase between 0 and 0.13 μmol Mn^{2+}, and no 5-HT decrease between 0.13 and 0.4 μmol Mn^{2+}. NS indicates no significant difference between ipsi- and contralateral values.

6.3.2. Dose-dependent effects of Mn^{2+} at 6 hours

Six hours after intrastriatal injection of different doses of Mn^{2+} (0.13-0.4-1.2 μmol), SA levels expressed as [ipsi-/contralateral] ratios were significantly increased in a dose-dependent manner ($p < 0.001$) by respectively 1.2-, 1.5- and 2.0-fold. The [ipsi-/contralateral] ratios of DHBA levels corrected for SA are shown in Fig. 3. [2,3-DHBA/SA] values were significantly increased by 0.4 and 1.2 μmol Mn^{2+} ($p < 0.001$), but not by 0.13 μmol Mn^{2+} or 0.4 μmol Mg^{2+}. [2,5-DHBA/SA] values were significantly elevated by all doses, including the control injection with Mg^{2+} (+37%). Both 2,3- and 2,5-DHBA increases reached plateau levels by 0.4 μmol Mn^{2+} (respectively 4.2- and 6.9-fold).
DA levels (Fig. 3) were significantly reduced by 0.13 μmol Mn^{2+} (-15%), and declined dose-dependently to -87% by 1.2 μmol. Control injections with Mg^{2+} increased DA levels slightly, but significantly (+14%; $p < 0.05$). Serotonin levels (Fig. 3) were significantly reduced by 0.13 μmol Mn^{2+} (-55%) and declined dose-dependently until -76% of contralateral tissues ($p < 0.001$). Following Mg^{2+}-injections, 5-HT levels were slightly, but significantly decreased (-14%; $p < 0.05$).

Fig. 4: *Dose-dependent changes of dopamine and serotonin turnover six hours after a unilateral control injection (Mg^{2+}) or Mn^{2+} (dose as indicated) into striatum of salicylate loaded rats (300 mg/kg i.p.; 2 hours).*



Data represent [ipsi-/contralateral] ratios of [metabolite/neurotransmitter] ratios of 6-7 rats. The DA turnover changed dose-dependently (ANOVA; $p < 0.005$ for DOPAC/DA and $p < 0.05$ for HVA/DA), whereas the 5-HT turnover did not. Newman-Keuls post-hoc multiple comparison tests ($\alpha = 0.05$) revealed only significant DOPAC/DA and HVA/DA increases between 0.40 and 1.20 μmol Mn^{2+}. NS indicates no significant difference between ipsi- and contralateral values.

DOPAC and HVA levels were significantly increased by 0.13 μmol Mn^{2+} (resp. 1.9- and 2.4-fold) and declined dose-dependently reaching levels of -51% and -32% at 1.2 μmol respectively. Mg^{2+}-injections increased DOPAC (+61%) and HVA (+88%) levels significantly ($p < 0.001$). The resulting DOPAC/DA and HVA/DA ratios were significantly increased in a dose-dependent manner ($p < 0.001$), reaching 4.9- and 7.8-fold elevations by 0.4 and 1.2 μmol Mn^{2+} respectively (Fig. 4). 5-HIAA levels were significantly reduced by 0.13 μmol Mn^{2+} (-41%), and declined dose-dependently until -76% ($p < 0.001$). The 5-HIAA/5-HT ratios were significantly elevated by 0.13 (+31%) and 0.4 (+19%) μmol Mn^{2+}, but this increase was not dose-dependent (Fig. 4).

6.3.3. Detection of 6-OHDA after Mn^{2+}

In Mn^{2+}-injected striata of rats not loaded with SA, no peaks at the position of 6-OHDA standards were observed (data not shown).

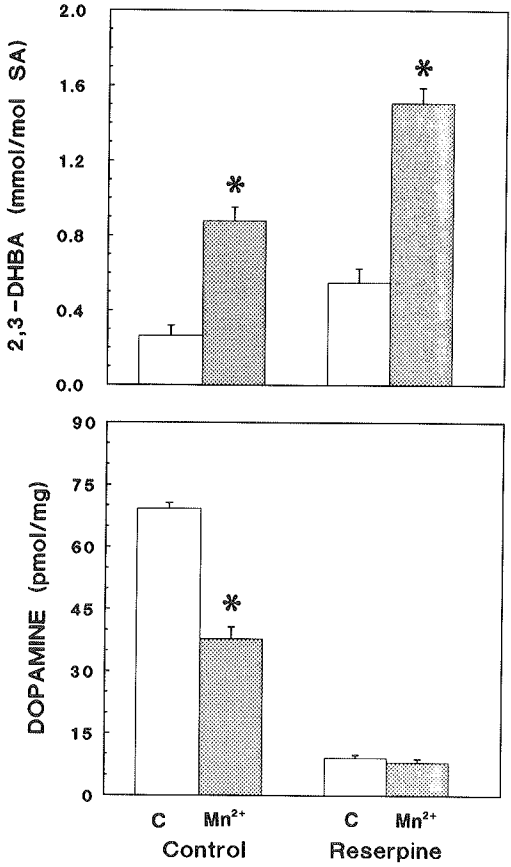


Fig. 5: *Effect of pre-treatment with reserpine (2.5 mg/kg i.p.; 24 hours) on 2,3-DHBA corrected for SA (mmol/mol SA; upper panel) and dopamine levels (pmol/mg; lower panel) in striatum 6 hours after unilateral injection of 0.4 μmol Mn^{2+} into striatum of SA-loaded rats (300 mg/kg i.p.; 2 hours).*
Significant differences between Mn^{2+}-injected (hatched bars) and contralateral striata (C, white bars) are indicated by asterisks (* Student's *t* test; $p < 0.001$). Comparison of contralateral striata revealed that basal 2,3-DHBA/SA values (upper panel) were significantly higher in reserpine-treated rats, and that DA levels (lower panel) were significantly depleted by reserpine (Student's *t* test; $p < 0.001$). In addition, the increase of 2,3-DHBA by Mn^{2+} expressed as [ipsi-/contralateral] ratios was not significantly different between reserpine-treated and control rats.

Table 1: *Effect of intrastriatal injection of 0.4 μmol Mn^{2+}, Fe^{2+} or Mg^{2+} (controls) on total low[2] or high molecular weight (LMW & HMW) iron (Fe^{2+} + Fe^{3+}), or direct chelatable LMW iron[1] (essentially Fe^{2+}) levels in striatum (pmol/mg wet weight) six hours after administration.*

Intrastriatal injection	LMW iron[1]	Total iron levels (Fe^{2+} + Fe^{3+})		
		LMW[2]	HMW	LMW+HMW
Non-injected	ND	24 ± 12 (3)	75 ± 4 (3)	100 ± 14 (3)
$MgCl_2$	53 ± 11 (4)	39 ± 11 (3)	76 ± 5 (7)	118 ± 16 (3)
$MnCl_2$	32 ± 9 (4)	26 ± 9 (4)	74 ± 11 (8)	98 ± 43 (4)
$FeCl_2$	515 ± 87 (5)	7,247 ± 1,839 (4)	4,958 ± 274 (10)	12,500 ± 290 (4)

Data are means ± SEM values from three to ten samples (n). Except for Fe^{2+}-injected striata, determinations are based on 2-4 pooled striata per sample. LMW and total iron levels in Mn^{2+}-injected striata were not significantly different from Mg^{2+}- or not-injected tissue. LMW iron determined under physiological[1] or reducing[2] conditions (for details see Materials and Methods) were not significantly different in Mg^{2+}- or Mn^{2+}-injected striata. In Fe^{2+}-injected striata, LMW and HMW iron pools were clearly increased. The difference between LMW iron levels assessed in physiological and reduced media of Fe^{2+}-injected striata, is probably due to a fine ferric hydroxide precipitate. ND = not determined.

6.3.4. Effect of reserpine pre-treatment on Mn-neurotoxicity

The striatal DA and 5-HT contents of rats pre-treated with reserpine were depleted by 89% and 56% respectively. In reserpine-treated rats, Mn^{2+}-injections did not significantly change DA levels (Fig. 5), but significantly decreased 5-HT levels (-76%) as compared to contralateral tissues. 2,3- and 2,5-DHBA levels corrected for SA (mmol/mol SA) were significantly higher in both not-injected (2.1- and 1.9-fold respectively) and Mn^{2+}-injected striata (1.7- and 1.9-fold respectively) of reserpine-treated rats as compared to striata of control rats (Fig. 5). However, both 2,3- and 2,5-DHBA formation (ipsi-/contralateral ratios of DHBA/SA) by Mn^{2+} in reserpine-treated rats (2.8- and 6.5-fold increase respectively) was not significantly different from DHBA formation by Mn^{2+} in control rats (4.2- and 6.9-fold increase respectively).

6.3.5. Effect of Mn^{2+} on total and LMW iron in striatum

Six hours after intrastriatal injection, Mn^{2+} produced no significant changes in striatal low-molecular weight (LMW) or total iron pools as compared to Mg^{2+}- or not-injected tissues (Table 1). Furthermore, striatal LMW iron levels of Mn^{2+}- or Mg^{2+}-injected rats were similar when measured under reducing or non-reducing conditions. In contrast, six hours after intrastriatal Fe^{2+}-injection, both LMW and total iron pools in striatum were markedly increased. In addition, in these striata under reducing conditions about 15-fold more iron was chelated by DFX in the LMW pool than in physiological medium. This difference can be due to a fine ferric hydroxide precipitation.

6.3.6. Effects of deferoxamine (DFX) on Mn-neurotoxicity

Co-injections of Mn^{2+} with 0.2 or 2.0 nmol DFX into striatum did not significantly alter 2,3- (Table 2) or 2,5-DHBA/SA increases, or the extent of DA-depletions (Table 2) as compared to Mn^{2+}-injections without DFX. There were no significant effects of DFX in contralateral tissues. Differences between ipsi- and contralateral levels of 2,3-DHBA/SA, 2,5-DHBA/SA and DA were highly significant in all tested groups ($p < 0.001$). The means ± SEM of 2,3- and 2,5-DHBA/SA (mmol/mol SA) and DA levels (pmol/mg) after NaCl-injection were respectively: 0.40 ± 0.03, 2.1 ± 0.24, and 72.7 ± 3.4.

Table 2: *Effect of co-injection of the iron-chelator deferoxamine (DFX) on striatal ·OH formation (2,3-DHBA/SA ratio) and dopamine depletion expressed as [ipsi/contralateral] ratios 6 hours after unilateral intrastriatal injection of 0.4 μmol Mn^{2+} (left) or NaCl (right) in SA-loaded rats (300 mg/kg i.p.; 2 hours).*

Ipsi-/contralat. ratio	Intrastriatal deferoxamine (nmol)		
	0	0.2	2.0
2,3-DHBA/SA	1.92 ± 0.12	2.14 ± 0.23	1.85 ± 0.06
Dopamine	0.46 ± 0.06	0.47 ± 0.02	0.40 ± 0.02

Data are mean ± SEM values from five to six rats. DFX co-injections did not significantly change Mn^{2+}-induced 2,3-DHBA formation (corrected for SA) or DA depletion. DFX alone did not produce any significant effects in NaCl-injected striata. Differences between ipsi- and contralateral 2,3-DHBA/SA values and DA levels were highly significant in all three dose groups using Student's *t* test ($p < 0.001$).

6.4. Discussion

The present studies using SA as a ·OH-trapping agent demonstrate time- and dose-dependent ·OH formation by Mn^{2+} in the living brain. Additional findings suggest that these ·OH are not generated by DA, or by the so-called low molecular weight (LMW) iron pool.

Pronounced increases of 2,3-DHBA (up to 4-fold) in Mn^{2+}-injected striatum (Fig. 1 and 3), suggest that Mn^{2+} induces ·OH formation. In addition, our results indicate that ·OH are not triggered by Mn itself, since maximum levels are only reached after 6-18 h. The time-course of 2,3-DHBA formation after Mn^{2+} is very different from that after equimolar injections of Fe^{2+} into striatum, where highest increases of 2,3-DHBA (10-fold) were observed after 30 minutes (Sloot et al., 1995). The present findings suggest that Mn^{2+} can not catalyze the Haber-Weiss reaction *in vivo*, provided that ·OH in the present study are formed by this reaction.

Since SA-levels in striatum are lower than those in blood and cerebrospinal fluid (resp. 5- and 2-fold), the dose- and time-dependent increases of striatal SA levels by Mn^{2+} indicate loss of local membrane integrity and/or a relatively small compromising effect at the blood-brain-barrier (appr. 6% at best). Such an effect was also observed beyond two hours after intoxication of the brain by Fe^{2+} (Sloot et al., 1995). These facts imply that the observed increases of 2,3-DHBA are largely of cerebral origin.

DA and 5-HT levels in striatum decreased by Mn^{2+} in a time- and dose-

dependent manner (Fig. 1 and 3). Already two hours after Mn^{2+}-injection, DA and 5-HT levels were significantly reduced (about -50%), whereas ·OH formation (ipsi-/contralateral ratio of 2,3-DHBA/SA) was not significantly increased at this time point as compared to later time points. This suggests that the depletion of DA and 5-HT are not due to tissue damage or loss of membrane integrity, but are probably mediated through a pharmacological effect of Mn^{2+}. Also the dose-dependent decline of DA and 5-HT levels at higher doses and leveling off of accumulation ·OH generation at the highest Mn^{2+}-dose, implicate independent processes.

An increased turnover of DA could be associated with oxidative stress derived from increased production of H_2O_2 (Cohen and Spina, 1989; Spina and Cohen, 1989). In the present study, the turnover of DA (DOPAC/DA and HVA/DA ratios) increases dose-dependently (Fig. 4), at 2 hours (3-fold increase) and declines thereafter (Fig. 2). However, the time- and dose-dependent changes in DA turnover are not parallelled by ·OH formation after Mn^{2+}. This suggests that not H_2O_2 from DA oxidation, but other factors determine ·OH generation.

The question then arises: where do these ·OH radicals come from? Since ·OH formation is not attenuated in DA-depleted rats, DA is not involved in Mn^{2+}-induced ·OH formation *in vivo* (Fig. 5). The reduced DA content after reserpine could also not be depleted any further by Mn^{2+}, in contrast to 5-HT levels (data not shown). Thus, ·OH formation via DA-autooxidation can not explain the neurotoxicity of Mn^{2+}, despite suggestions from *in vitro* studies (see Introduction). Reserpine elevated basal ·OH production in striatum by about 2-fold as compared to tissues of control rats (Fig. 5). This is in line with another study, showing oxidative stress by 90% increased levels of oxidized GSH in striatum after reserpine treatment (Spina and Cohen, 1989).

Recently, it has been demonstrated that DA itself can act as a low potency neurotoxin after direct injection of this neurotransmitter into striatum (Filloux and Townsend, 1993), and that treatment with DA-pharmaca such as pargyline or L-DOPA results in augmentation of Mn-neurotoxicity (Parenti et al., 1986). The exact mechanism, however, by which DA exerts its toxic action *in vivo* remains far from clear (Filloux and Townsend, 1993), and may not be through the proposed formation of reactive oxygen species as a result of Mn^{2+}-enhanced DA autoxidation. Also attenuation of Mn^{2+}-neurotoxicity by inhibition of DA-synthesis using an immediate and single treatment of α-methyltyrosine or lisuride (Parenti et al., 1988), -of which the latter is better known for its potent D_2-receptor agonist properties-, can be interpreted differentially. Since intrastriatally injected Mn^{2+} is substantially retained for at least several days in the basal ganglia (Lista et al., 1986; Sloot and Gramsbergen, 1994) and the

protecting effect in Parenti's studies was measured at day 14, it would be more plausible that α-methyltyrosine and lisuride ameloriate the toxicity of Mn^{2+} by chelating it, -like reported for some phenothiazines (reviewed by 4)-, rather then via inhibition of DA-synthesis.

Since Mn^{2+} itself can not trigger ·OH formation, and the likely candidates DA and 6-OHDA, -which we did not detect *in vivo*-, can not explain the increased ·OH formation by Mn^{2+}, the role of endogenous iron was examined after Mn^{2+}. However, in Mn^{2+}-injected striatum LMW iron, which is considered a relevant pool for catalyzing the Haber-Weiss reaction (Koster and Sluiter, 1994), was not different from that in Mg^{2+}- or not-injected striata (Table 1). Results obtained in Fe^{2+}-injected striata indicated that our assay procedures worked.

Although tissue levels of LMW-iron were not changed by Mn^{2+}, a role of 'free' or LMW-iron in generating ·OH after Mn-intoxication at local sites can not be ruled out. Therefore intrastriatal co-injections with the iron-chelator DFX were conducted using doses known to retard 6-OHDA neurodegeneration (Ben-Shachar et al., 1991) and sufficient to chelate the measured LMW-iron pool completely. However, different intrastriatal DFX-injections could not attenuate Mn^{2+}-induced 2,3-DHBA formation (Table 2), which is consistent with our data on LMW-iron. In addition, in the present study, iron-chelation by DFX in large excess of Mn^{2+} was not affected *in vitro*.

There are, however, other mechanisms which may be responsible for Mn^{2+}-induced ·OH. Mn^{2+} accumulates preferentially in mitochondria via the Ca^{2+} uniporter, thereby inhibiting its own and Ca^{2+}-effluxes (Gavin et al., 1990). In additon, Mn^{2+} impairs several mitochondrial energy functions and GSH-metabolism (Liccione and Maines, 1988 and 1989; Gavin et al., 1992; Brouillet et al., 1993). Under such conditions, disturbances of cytochrome heme metabolism (Qato and Maines, 1985; Liccione and Maines, 1988) and/or heme-associated electron transfers by Mn^{2+} may lead to oxoheme oxidants in the presence of H_2O_2 or the postulated heme-associated ferryl radicals (Grisham, 1985; Grisham and McCord, 1986; Puppo and Halliwell, 1988; Dykens, 1994), which may cause SA-hydroxylation in our studies (Puppo and Halliwell, 1988). DFX can probably not chelate iron from heme proteins, and this may explain the lack of an effect of DFX on Mn^{2+}-induced ·OH.

Alternatively, ·OH may be formed independent from Fe via decomposition of peroxynitrite, which is a reaction product of NO and $O_2^{\cdot-}$ (Beckman et al., 1990; Hammer et al., 1993; Lafon-Cazal et al., 1993). Indeed, protection has been shown recently by NO-synthase inhibitors after brain injury by some mitochondrial toxins (Schulz et al., 1994). Normally, heme-associated ferryl radicals and peroxynitrite cause lipid peroxidation (Grisham, 1985; Darley-

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Manganese neurotoxicity: a model for oxidative stress and selective neurodegeneration in the basal ganglia

Intoxications and other pathologies of the CNS are most often characterized by selective damage. In particular, the basal ganglia that control automatic movements are vulnerable brain regions under abnormal conditions such as idiopathic Parkinson's disease (iPD) and manganese (Mn) poisoning. At present the etiology and pathogenesis of iPD (and related disorders) as well as the mechanism of Mn-induced neurodegeneration of the basal ganglia (or most other intoxications affecting this area) are unkown. In addition, both pathologies result in irreversible hypokinesia (rigidity, tremor, bradykinesia, unstable postural reflexes, dystonia), while there is no long-lasting effective treatment available.

Specific properties, or a unique combination of basic, endogenous factors of the basal ganglia, such as the presence of dopamine (DA), glutamate (Glu), iron (Fe) and neuromelanin, may render them more susceptible to damage, in particularly oxidative injury, than other brain regions. Since all these intrinsic factors can participate in oxidative stress events via different mechanisms (see Chapter 1), which are often considered a final common pathway of cell death, this may explain the selective vulnerability of the basal ganglia under abnormal conditions. On the other hand, defects in generally occurring cell systems, including the mitochondrial respiration chain (the main natural source of reactive oxygen species) and oxidative defense mechanisms such as glutathione (GSH) metabolism, may lead to selective oxidative damage of the basal ganglia because of their intrinsic metabolic properties. Questions addressed to understand this vulnerability may provide better fundamental and therapeutic insight for the treatment of basal ganglia disorders.

One should bear in mind that there exists no 'perfect' animal model for PD as it occurs in humans. All models of Parkinsonism show differences and similarities with iPD, of which Mn intoxication may be specified as a model for dystonia. Table 1 summarizes various oxidative stress phenomena in relation to basal ganglia lesions which cause hypokinetic movements as outlined in Chapter 1, including Mn-induced lesions which are described in this thesis. In the present thesis, Mn-neurotoxicity has been chosen as a model to study *in vivo* fundamental aspects of oxidative stress and selective neurodegeneration in the rat basal ganglia. In particular, the role of iron and DA in producing

oxidative stress by Mn is emphasized as a relevant and integral part of Mn neurotoxicity and one of the main topics in current PD research.

Using systemic Mn administration to study its neurotoxicity has the practical disadvantage, that Mn accumulates (very) slowly in the brain and that the onset of lesions in the basal ganglia will not occur until several months after chronic exposure. Since some authors have reported acute depletions of DA after a single Mn injection into rat striatum or substantia nigra, we used this experimental approach to further characterize an acute Mn intoxication model in teh rat. It followed from our studies that intrastriatal Mn injection produced time- and dose-dependent DA depletions and displayed selective nerve cell death as reflected in ^{45}Ca accumulation in the basal ganglia, which virtually matched the described pathology after chronic systemic exposure to Mn. The observed regional distribution of ^{45}Ca accumulation in striatum, globus pallidus (GP), entopeduncular nucleus (external GP), substantia nigra and several thalamic nuclei involved in motor functions, suggested both pre- and post-synaptic damage to the DAergic nigrostriatal pathway. Postsynaptic damage probably includes GABAergic neurons.

Selective effects of Mn were also observed at the level of biogenic amines in striatum and substantia nigra: partially reversible DA depletions, and reversible changes of noradrenaline (NE) and serotonin neurotransmitter levels in striatum. In addition, DA neurotransmission in striatum, based on measurements of the 'synaptic' DA-metabolite 3-methoxytyramine, was completely abolished at day 1 and 3 after Mn injection, but recovered within 6 weeks. This indicated a functional restoration of DA neurotransmission, despite still substantial losses of tissue DA, and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA).

Selectivity of both Mn and Fe was further tested by comparing injections of either metal in striatum or hippocampus, two areas with a respectively high and low DA and iron content. These studies demonstrated a relatively low toxicity of Mn in the hippocampus, and a high toxicity of Fe in the brain independent of the injection site. The finding that the gyrus dentatus, a subregion of the hippocampus with the highest iron levels locally (albeit still low compared to the basal ganglia), was the preferential site for ^{45}Ca accumulation after intrahippocampal Mn-injection, was consistent with our hypothesis that Mn needs iron or iron-binding sites to exert its toxicity. In addition, the selective lesions by Mn and extensive damage by Fe after intracerebral injection suggest different mechanisms of action.

Characterized in more detail, this Mn model offered the possibility to further study the mechanism or prevention of Mn neurotoxicity with the use

of pharmaceutical intervention (antioxidants, DA/Glu (ant)agonists, etc.). With this in mind, the distribution, retention and transport of Mn was studied, which could possibly help to explain some aspects of its selective toxicity. Using trace injections of ^{54}Mn into striatum or substantia nigra, it was not only demonstrated that substantial amounts of label were retained in both regions until at least 2-3 days after injection, but also that Mn was transported by neurons. By blocking axonal transport through the medial forebrain bundle using a local colchicine injection or mechanical transection of the bundle, or by producing selective lesions of the DAergic nigrostriatal pathway (using 6-OHDA) or GABAergic striatonigral pathway (using quinolinic acid), it was shown that Mn is transported through nigro-striatal and striato-nigral nerve fibres in anterograde direction.

In addition, the striatum lesioned with quinolinic acid, which is depleted of most intrinsic nerve cells and contains reactive glial cells, accumulated three times more ^{54}Mn, suggesting a predominant micro- and/or astroglial localization of ^{54}Mn. The exact cellular entity responsible for Mn transport is not known from these or other studies, but it is discussed that iron transport pathways and storage proteins are the most likely candidates that may determine selective transport and accumulation of Mn in and within the basal ganglia.

Since it has been hypothesized from *in vitro* studies, that Mn may produce free oxygen radicals directly or indirectly via auto-oxidation of catecholamines, our next goal was to measure free oxygen radicals *in vivo* in direct relation to (biochemical) damage. Recently, salicylate (SA) has been used as an *in vivo* trapping agent for the highly deleterious hydroxyl radicals ($^{\cdot}$OH), which upon reaction with SA form the relatively stable adducts 2,3- and 2,5-dihydroxybenzoic acids (DHBA). For that purpose, we developed a HPLC method to analyze catechol- and indoleamines and related metabolites in combination with SA and its hydroxylated adducts 2,3- and 2,5-DHBA (in striatum and CSF). This enabled us to study metal-induced $^{\cdot}$OH formation and DA/5-HT changes simultaneously in the same tissue samples. Since iron is considered to catalyze the Haber-Weiss reaction *in vivo*, thereby producing $^{\cdot}$OH, it was obvious to study brain Fe intoxication using this $^{\cdot}$OH-trapping technique. In addition, the contrast between a hypothesized different mechanism of action of both transition metals (as concluded above), and their close physico-chemical interrelationship and thus possible interference of Mn with brain iron homeostasis, may shed light on aspects of Mn neurotoxicity and oxidative stress. In other words, endogenous iron released by Mn may be the crucial factor in Mn-toxicity.

Time-course studies expanding from 30 minutes to 1 month following intrastriatal Fe injection revealed that 2,3-DHBA, the non-enzymatic product from SA and ·OH, peaked at 30 minutes (10-fold increase), whereas DA and 5-HT levels were relatively unaltered during the first two hours. These data suggest, that Fe-triggered ·OH formation precedes striatal DA (which was irreversibly depleted) and 5-HT depletions which occurred at later time points (beyond 2 hours). In addition, ·OH are continuously formed during Fe-induced neurodegeneration in a gradually declining fashion. At two hours after Fe injection, the high DOPAC level, no change in HVA, and a moderately reduced DA level, indicated specific enhancement of both DA anabolism and catabolism. Based on the iron dependency of the rate-limiting DA-synthetic enzyme tyrosine hydroxylase (TH), and the specific localization of TH (cytosol) and the DA catabolic enzymes monoamine oxidase (outer membrane of mitochondria) and catechol-*o*-methyltransferase (extraneuronally), it was concluded that this Fe-effect was within the DA neuron.

Whether the SA ·OH-trapping technique could be applied in the clinic to study chronic conditions of oxidative stress, for example by measuring SA adducts in CSF of PD or amyotrophic lateral sclerosis (ALS) patients, was experimentally investigated in CSF of rats injected with Fe into striatum. Indeed, DHBA levels in CSF of these rats were significantly elevated at time-points 30 minutes and 3 days. However, less severe "lesions" produced by Mn in striatum (as examined until 18 h; see Chapter 6) did not induce significantly elevated DHBA levels in CSF. Whether chronic neurodegenerative diseases may produce increased DHBA levels in CSF remains to be resolved. Since comparable doses of SA (100 mg/kg) are used to treat juvenile arthritis, the relatively high SA dose needed for such studies in humans is still in the clinically safe range.

Subsequent studies described in Chapter 6 showed that intrastriatal Mn injections increase 2,3-DHBA formation in a time- and dose-dependent manner in striatum, suggesting that Mn induces ·OH *in vivo*. Since the 2,3-DHBA effects by Mn demonstrated a time-delayed increase (maximum at 6-18 h), in contrast to our 2,3-DHBA effect by Fe over time, it was concluded that Mn induces ·OH by an indirect mechanism, implicating that Mn can not catalyze the Haber-Weiss reaction *in vivo*. It also followed from time- and dose-related effects (of simultaneously measured DA and 2,3-DHBA) that DA depletion and ·OH formation by Mn appear to be independent processes. It has been suggested by *in vitro* observations that Mn oxidizes DA rapidly and irreversibly to its cyclized *o*-DA-quinone resulting in a decrease of DA, but not in the formation of reactive oxygen species, since oxygen is neither consumed nor required. This is in line with the rapid initial DA-depleting effect induced by Mn

in vivo without a significant rise of ·OH in the first two hours, indicating that Mn-induced DA depletion *in vivo* is similar to that observed *in vitro*. However, the reducing capacity of DA neurons will probably be exhausted by such DA (auto)oxidation processes (and together with mitochondrial energy depletion) resulting in, for example, a severely compromised GSH metabolism as has been shown by Liccione and Maines, which could make these cells potentially more susceptible (e.g. to excitotoxicity).

In an attempt to find out via what mechanism ·OH are formed by Mn we investigated the possible role of DA (or non-enzymatic DA (auto)oxidation products) and 'free' or low-molecular-weight (LMW) iron as a potential source that can catalyze the Haber-Weiss reaction. Studies in which rats were depleted of striatal DA (90% reduction) by pre-treatment with reserpine, or in which rats received co-injections of Mn and the iron chelator deferoxamine (DFX) into striatum, did not demonstrate attenuation of Mn-induced 2,3-DHBA formation, while the extent of DA reductions was similar. Furthermore, the level of chelatable LMW iron in Mn-injected striata was not different from controls. In conclusion, these findings suggest that neither DA or DA (auto)oxidation products nor chelatable iron participate in Mn-induced ·OH formation.

Other possibilities by which the observed Mn-induced ·OH formation might be explained are (1) through the formation of peroxynitrite (ONOOH), the reaction product of NO· and $O_2^{\cdot-}$, or (2) heme-associated ferryl radicals. The former implicates iron-independent ·OH formation from the decomposition of peroxynitrite. Since Mn shares aspects of (additional) excitotoxicity with several other mitochondrial toxins whose neurotoxicity could be attenuated by NO-synthase inhibitors, NO-triggered oxidative damage after Mn might be a possible mechanism. Other features of Mn toxicity, such as impairment of (mitochondrial) GSH metabolism and several mitochondrial energy supply functions by Mn, including heme-associated functions (respiratory and P-450 cytochromes) and oxidative phosphorylation, may lead to or be the result of formation of heme-associated ferryl radicals, which are similar in reactivity to ·OH or ferryl iron (FeO^{2+}). If the postulated formation of heme-associated ferryl radicals (and H_2O_2) occurs, this would imply that such radicals are capable of hydroxylating SA, which is not unlikely and could be tested *in vitro*.

In summary, it is proposed that the selective neurotoxicity of Mn within the basal ganglia is most likely exerted via iron-dependent distribution characteristics (transferrin receptors, ferritin) and subsequent transport (Ca uniporter) that determine the preferential accumulation into mitochondria. Here, high local Mn concentrations will disturb several important energy supply functions and anti-oxidant defense mechanisms (GSH), resulting in

Table I: *Oxidative stress phenomena in relation to idiopathic Parkinson's disease and three representative models of Parkinsonism induced by chemicals*

Oxidative stress phenomena	**Idiopatic Parkinson's disease**	**Manganese**	**MPTP**	**6-Hydroxydopamine**
GSH-metabolism defects	GSH ↓, γ-glutamyl-transpeptidase ↑, and mild GSSG ↑ only in SN[1,2,3,4]	GSH ↓ (mito > cyto), Gpx and GSSG-reductase activ. ↓, γ-glutamyl-transpeptidase act. ↑ in ST[5]	GSH ↓ only in midbrain[6,7,8]	GSH ↓ in ST and SN, slightly Gpx act. ↓ in SN[9]
SOD/catalase changes	MnSOD ↑ and ↔ CuZnSOD in SN[10], CuZnSOD ↑ in SN[11]	No reports	No reports	SOD and catalase ↓ in ST and SN[9]
Mitochondrial respiration (chain) defects	Complex I deficiency in SN[12,13,14,15]	Oxidative phosphory-lation[16] ↓, Complex V (or I) defect[16], or cytochromes[17] ↓, ATP ↓ and lactate[18] ↑	Irreversible Complex I inhibition in SN[19,20,21,22]	No reports
Mitochondrial P-450	No reports	Cytochrome P-450 act. and content[17] ↑ (mito > microsomes)	Protection by cyt. P-450 inhibitors, potentiation by cyt. P-450 inducers[21]	No reports

Iron accumulation/ aberrant metabolism	Total iron ↑ in SN[23,24] ↓ in GP[24], shift FeII→III[23,26], ferritin[25] ↓ and ↑[26], transferrin receptor density ↓ in putamen[27], iron in Lewy bodies[28] and neuromelanin[29]	↔ iron in ST and no protection by iron chelation[30], binding to transferrin and ferritin[31], possible axonal transport via iron pathways[32]	Total iron ↑ in SN compacta: dopamine and glial cells[33], transferrin receptor density ↓ in ST[31]	*In vitro* iron release from ferritin[34,35], attenuation by iron chelation[36]
Dopamine autoxidation	5-S-cysteinyl-dopamine/dopamine ratio ↑ in SN[37]	*In vitro* DA-quinones & oxyradicals[38a], no DA autoxidation by oxygen: no oxy-radicals[38b], *in vivo* no role of dopamine[39]	No role of dopamine[40]	*In vitro* autoxidation of 6-OHDA itself[38]
Glutamate-mediated oxidative stress	Partial protection by glutamate receptor antagonists[41]	Partial protection by glutamate receptor antagonists[41]	Partial protection by glutamate receptor antagonists[41]	Partial protection by Glu-receptor antagonists[41]
Lipid peroxidation	In SN[50]	Not *in vivo*[51], *in vitro* MPP^+ stimulates[52], MPTP inhibits[52,53]	Inhibits both *in vitro*[54,55] and *in vivo*[56,57]	*In vitro*[34]
DNA/protein/carbon-hydrate oxidations	No reports	DNA and RNA content[58] ↓	No reports	No reports

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local oxidative stress possibly through formation of site-specific heme-associated ferryl radicals, peroxynitrite, and/or peroxynitrite-derived ·OH, which eventually will lead to cell death. In this respect, a study of the role of DA oxidation products (quinones) might be of value to assess the reducing capacity of the neuron.

Mangaan neurotoxiciteit: een model voor oxidatieve stress en selektieve neurodegeneratie in de basale ganglia

Intoxikaties en andere pathologieën van het CZS worden vaa gekarakteriseerd door selektieve schade. Vooral de basale ganglia, die c automatische bewegingen coördineren, zijn kwetsbare strukturen ond abnormale kondities zoals de idiopathische vorm van de ziekte van Parkinson (iz en mangaanvergiftiging. Op dit moment zijn de etiologie (ontstaanswijze) e pathogenese (ziekteproces) van izP (en gerelateerde ziekten) alsmede h mechanisme van door Mn geïnduceerde neurodegeneratie (zenuwcelafstervin van de basale ganglia (of de meeste andere intoxicaties die dit gebied treffe onbekend. Daar komt bij, dat beide pathologieën resulteren in irreversibe hypokinesia (bewegingsarmoede) (rigiditeit, tremor, bradykinesie, instabie houdingsreflexen, dystonie), terwijl er geen langdurig effektieve behandelin beschikbaar is.

Specifieke eigenschappen, of een unieke combinatie van intrinsiek endogene faktoren van de basale ganglia, zoals de aanwezigheid van dopamin (DA), glutamaat (Glu), ijzer (Fe) en neuromelanine, zouden hier een grote gevoeligheid voor schade kunnen opleveren, vooral oxidatieve letsels, vergeleke met andere hersengebieden. Daar al deze intrinsieke faktoren kunne participeren via verschillende mechanismen bij oxidatieve stress gebeurtenisse die vaak beschouwd worden als een algemene finale weg tot celdood, zou dit d selektieve kwetsbaarheid van de basale ganglia kunnen verklaren. Aan de ande kant zouden defekten in algemeen voorkomende celsystemen, waaronder d mitochondriële ademhalingsketen (de natuurlijke hoofdbron voor reaktiev zuurstofspecies) en oxidatieve verdedigingsmechanismen zoals het glutathio (GSH)-metabolisme, kunnen leiden tot selektieve oxidatieve schade van de basal ganglia juist vanwege zijn intrinsieke metabolische eigenschappen. Vragen gerich op het begrijpen van deze kwetsbaarheid in relatie tot oxidative stress zal mogelij een beter mechanistisch en therapeutisch inzicht geven voor de behandeling va ziekten van de basale ganglia (met bv. anti-oxidantia).

Met moet zich echter bedenken dat er geen 'perfekt' diermodel bestaat voo de zP zoals die zich voordoet bij de mens. Alle modellen van Parkinsonism

vertonen verschillen en overeenkomsten met izP, waarbij Mn-intoxikatie opgevat zou kunnen worden als een model voor dystonie. Tabel 1 geeft een samenvatting van verscheidene oxidatieve phenomenen gerelateerd aan basale ganglia lesies die hypokinetische bewegingsstoornissen veroorzaken, zoals is uiteengezet in hoofdstuk 1 (inklusief de Mn-geinduceerde lesies zoals gepresenteerd in dit proefschrift). In dit proefschrift is Mn-neurotoxiciteit gekozen als een model om *in vivo* fundamentele aspekten van oxidatieve stress en selektieve neurodegeneratie in de basale ganglia van de rat te bestuderen. Vooral de rol van ijzer en dopamine bij de produktie van zuurstofstress door Mn wordt benadrukt als zijnde een relevant en integraal onderdeel van zowel Mn-neurotoxiciteit als één van de hoofdthema's in het huidige zP-onderzoek.

Het gebruik van systemische Mn-toedieningen om de neurotoxiciteit te bestuderen heeft als praktische nadeel, dat Mn (erg) langzaam akkumuleert in de hersenen en dat lesies in de basale ganglia niet eerder dan enkele maanden na chronische blootstelling zullen ontstaan. Daar sommige auteurs akute depleties van dopamine hebben vermeld na één enkele Mn-injektie in het striatum of de substantia nigra, hebben we deze rapporten als basis gebruikt om een meer gedetailleerd akuut Mn-intoxikatiemodel te ontwikkelen en te karakteriseren. Uit onze studies kwam naar voren, dat intrastriatale Mn-injektie tijds- en dosisafhankelijke DA-depleties geeft en selektieve zenuwceldood laat zien in de basale ganglia zoals aangetoond met ^{45}Ca akkumulatie, die de beschreven pathologie na chronische systemische Mn-blootstelling grotendeels weerspiegelt. De geobserveerde regionale verdeling van ^{45}Ca-akkumulatie in het striatum, de globus pallidus (GP), de entopedunculaire kern (externe GP), de substantia nigra en enkele kernen in de thalamus betrokken bij motorische funkties, suggereert zowel pre- als postsynaptisch letsel ten opzichte van het dopaminerge nigrostriatale pad. Postsynaptische schade houdt waarschijnlijk schade aan γ-aminoboterzuur (GABA) bevattende neuronen in (of reaktieve gliacellen).

Selektieve effekten werden ook waargenomen op het nivo van biogene aminen in het striatum en de substantia nigra: partieel reversibele DA-depleties, en reversibele redukties van noradrenerge (NE) en serotonerge neurotransmittergehalten in het striatum. Daar komt bij dat de DA-neurotransmissie in het striatum, zoals die gebaseerd is op de bepaling van de 'synaptische' DA metaboliet 3-methoxytyramine, volledig afwezig was op dag 1 en 3 na Mn-injektie, maar zich herstelde binnen 6 weken. Dit wijst op een funktioneel herstel van DA-neurotransmissie, ondanks blijvende substantiële

tekortkomingen van DA, en zijn metabolieten DOPAC (3,4 dihydroxyphenylalaninezuur) en HVA (homovanile? zuur) in het striatum.

De selektiviteit van zowel Mn en Fe werd verder getest door vergelijking va injekties van één der metalen in het striatum of de hippocampus, twee strukture die respektievelijk een hoog en laag DA en ijzergehalte hebben. Deze studie demonstreerden een relatief lage toxiciteit van Mn in de hippocampus, en ee hoge toxiciteit van Fe in de hersenen onafhankelijk van de plaats van injektie. D bevinding dat de gyrus dentatus, een subregio van de hippocampus die lokaal he hoogste ijzergehalte bevat (deze nivo's zijn ten opzichte van de basale ganglia no laag), de voorkeursplek was voor ^{45}Ca-akkumulatie na intrahippocampale Mn injektie, bevestigde onze hypothese dat Mn ijzer en/of ijzerbindingsplaatsen nodi heeft voor het doen gelden van zijn toxiciteit. Bovendien suggereren de selektiev lesies door Mn en wijdverbreide schade door ijzer na intracerebrale injektie ee verschillend werkingsmechanisme.

Dit in meer detail gekarakteriseerde Mn-model bood de mogelijkheden tc verdere studie van het mechanisme of de preventie van Mn-neurotoxiciteit doc middel van farmaceutische interventie (DA/GLU-(ant)agonisten, antioxidantia etc.). Met dit in het achterhoofd, werd de regionale verdeling, de retentie en he transport van Mn bestudeerd, wat tevens mogelijk zou kunnen helpen bij he verklaren van aspekten rond de selektieve toxiciteit van Mn. Door gebruik t maken van 'tracer'-injekties met ^{54}Mn in het striatum of de substantia nigra, wer niet alleen aangetoond dat substantiële hoeveelheden van het radioaktieve labe werden vastgehouden in beide regionen tot ten minste 2-3 dagen na injektie, maa dat het Mn ook werd getransporteerd. Door het blokkeren van axonaal transpor door de mediale voorbrein bundel met behulp van lokale colchicine injektie c mechanische transsektie van de bundel, of door het maken van selektieve letsel van het DAerge nigrostriatale pad (met 6-OHDA) of GABAerge striatonigrale pa (met quinolinezuur), werd aangetoond, dat Mn wordt getransporteerd doc nigrostriatale en striatonigrale zenuwvezels in anterograde richting (naar he zenuwuiteinde).

Daarnaast akkumuleerde het met quinolinezuur beschadigde striatum da reaktieve gliacellen bevat en gedepleteerd is van de meeste intrinsiek zenuwcellen, driemaal zoveel ^{54}Mn, wat suggereert dat ^{54}Mn zich vooral bevind in micro- en/of astroglia. De precieze cellulaire eenheid (struktuu verantwoordelijk voor transport van Mn is niet bekend uit deze of andere studies maar wij suggeren dat ijzertransport en opslageiwitten de meest waarschijnlijk kandidaten zijn, die het selektieve transport en de akkumulatie van Mn zoude

kunnen bepalen naar en binnen de basale ganglia.

Daar het gesuggereerd is vanuit *in vitro* onderzoek dat Mn direkt of indirekt vrije zuurstofradikalen zou genereren via (auto)-oxidatie van catecholaminen, was het volgende doel *in vivo* vrije zuurstofradikalen te meten in direkte relatie tot (biochemische) schade. Recentelijk is salicylzuur (SA) gebruikt als een *in vivo* vangstof voor zeer schadelijke hydroxylradikalen (·OH), die in reaktie met SA de relatief stabiele addukten 2,3- en 2,5-dihydroxybenzeenzuur (DHBA) vormen. Voor dat doel, ontwikkelden we een HPLC-methode om catechol- en indolaminen en gerelateerde metabolieten gecombineerd met SA en zijn gehydroxyleerde verbindingen 2,3- en 2,5-DHBA te analyseren (in het striatum en in hersenvocht). Dit maakte het ons mogelijk om door metaal geïnduceerde ·OH formatie en DA/5-HT veranderingen simultaan in weefselmonsters te bestuderen. Omdat men ervan uitgaat dat ijzer *in vivo* de Haber-Weiss reaktie katalyseert, wat leidt tot de produktie van ·OH, leek het voor de hand te liggen Fe-intoxikaties in het brein te bestuderen met behulp van deze ·OH-invangtechniek. Bovendien zou de tegenstelling tussen een gesuggereerd verschillend werkingsmechanisme van beide metalen (zie hierboven), en de nauwe fysisch-chemische interrelatie en dus de mogelijke interferentie van Mn met ijzer homeostase in de hersenen, licht kunnen werpen op aspekten van Mn-neurotoxiciteit en oxidatieve stress. Met andere woorden, door Mn vrijgemaakt endogeen ijzer zou de cruciale faktor kunnen zijn in Mn-toxiciteit.

Studies in de tijd lopend van 30 minuten tot 1 maand na intrastriatale Fe-injektie onthulde dat 2,3-DHBA, het niet-enzymatische produkt van SA en ·OH, op 30 minuten piekte (10-voudige toename), terwijl DA en 5-HT gehalten relatief onveranderd bleven gedurende de eerste twee uren. Deze data suggereren dat door Fe 'getriggerde' ·OH formatie voorafgaat aan striatale depleties van DA (dat irreversibel verlaagd was) en 5-HT, die zich voordeden op latere tijdstippen (na meer dan 2 uur). Bovendien worden ·OH continu gevormd gedurende door Fe-geinduceerde neurodegeneratie, wat op een geleidelijke manier afneemt (in de tijd). De hoge DOPAC, de onveranderde HVA en gematigde redukties van DA-gehalten 2 uur na Fe-injektie wijzen op een specifieke bevordering van zowel het DA-anabolisme als DA-katabolisme (resp. opbouw en afbraak). Gebaseerd op de ijzer-afhankelijkheid van het snelheidsbepalende, synthetiserende enzym tyrosinehydroxylase (TH), en de specifieke lokalisatie van TH (cytosol) en de DA-kataboliserende enzymen monoamine-oxidase (buitenmembraan van mitochondriën) en catechol-o-methyltransferase (extraneuronaal), is gekonkludeerd dat dit Fe-effekt zich binnen het DA-neuron afspeelt.

Of de SA ·OH-invangtechniek toegepast zou kunnen worden in de klinie
om chronische kondities van oxidatieve stress te bestuderen, bijvoorbeeld do
SA-addukten te meten in hersenvocht (CSF) van patiënten met zP of amyotro
lateraalsclerosis (ALS), is experimenteel onderzocht in CSF van ratten geïnjekteer
met Fe in het striatum. Inderdaad waren de DHBA-gehalten in het hersenvoc
van deze ratten significant verhoogd na 30 minuten en na 3 dagen. Mind
ernstige 'lesies' geproduceerd door Mn in het striatum (zoals onderzocht tot 1
uur in hoofdstuk 6) induceerde echter geen significante verhogingen in het CS
Of in het geval van chronische neurodegeneratieve aandoeningen verhoogd
DHBA-gehalten in het CSF kunnen worden aangetoond, blijft onopgehelderd. D
relatieve hoge SA-dosering die nodig is voor zulke studies, is gezien vergelijkbar
SA-doseringen (100 mg/kg) gebruikt voor de behandeling van juveniele arthrit
(acute reuma), in het klinisch veilige bereik.

De volgende studies uit hoofdstuk 6 hebben laten zien dat intrastriata
Mn-injekties 2,3-DHBA-vorming verhoogt in het striatum op een tijd- e
dosisafhankelijke manier, wat suggereert dat Mn *in vivo* ·OH induceert. Omdat d
2,3-DHBA-effekten door Mn een tijdsvertragende toename lieten zien, i
tegenstelling tot de tijdsafhankelijke bevindingen voor 2,3-DHBA-vorming door F
wordt gekonkludeerd, dat Mn ·OH induceert via een indirekt mechanisme, wa
impliceert dat Mn niet de Haber-Weiss reaktie kan katalyseren *in vivo*. Tever
volgde er uit tijd- en dosisafhankelijke effekten (van simultane metingen van D
en 2,3-DHBA) dat DA-depletie en ·OH-vorming door Mn klaarblijkelij
onafhankelijke processen zijn. *In vitro* observaties suggereren dat Mn DA snel e
irreversibel oxideert tot zijn geringde *o*-DA-quinone, resulterend in een DA
afname, maar niet in de vorming van reaktive zuurstofspecies, omdat zuurst
hierbij niet werd geconsumeerd en niet nodig was. Dit komt overeen met h
snelle depleterende effekt op DA door Mn *in vivo* zonder dat er sprake is van ee
signifikante toename van ·OH-produktie in de eerste twee uur, wat suggereert da
Mn-geinduceerde DA depletie *in vivo* op eenzelfde manier verloopt als *in vitro*
aangetoond. De reducerende kapaciteit van DA-neuronen zal echter vermoedelij
uitgeput raken door zulke DA-(auto)oxidatieprocessen (samen met mitochondriël
energiedepletie) resulterend in een ernstige aantasting van bijvoorbeeld het GSH
metabolisme zoals is aangetoond door Liccione en Maines, dat vervolgens dez
cellen potentieel gevoeliger kan maken (bijvoorbeld voor excitotoxiciteit).

In een poging om meer te weten te komen over het mechanisme achter ·OH
vorming door Mn, werd de mogelijke rol hierin onderzocht van DA (of DA
autoxidatie produkten) en 'vrij' ijzer of ijzercomplexen met een laag molekula

gewicht (LMW) als een potentiële bron die de Haber-Weiss-reaktie kan katalyzeren. Studies waarin ratten werden gedepleteerd van striataal DA (90% afname) door behandeling met reserpine, of waarin ratten een co-injektie ontvingen van de ijzerchelator deferoxamine (DFX) in het striatum, gaven geen vermindering te zien van door Mn geïnduceerde 2,3-DHBA-vorming, terwijl de DA-afnames gelijk bleven. Daarnaast waren de gehalten aan LMW ijzer in met Mn-geïnjekteerde striata niet veranderd ten opzichte van controles. De konklusies uit deze bevindingen zijn, dat noch DA of DA-(auto)oxidatieprodukten, noch cheleerbaar ijzer participeert in door Mn geïnduceerde vorming van ·OH.

Andere mogelijkheden waarmee de geobserveerde door Mn geïnduceerde ·OH-vorming verklaard zou kunnen worden zijn: (1) de vorming van peroxynitriet (ONOOH), een reaktie produkt van NO· en $O_2^{\cdot-}$, en (2) haem-geassocieerde ferrylradikalen. De eerstgenoemde impliceert de ijzer-onafhankelijke vorming van ·OH door het uiteenvallen van peroxynitriet. Omdat Mn overeenkomstige aspekten van (additionele) excitotoxiciteit deelt met enkele andere mitochondriële toxines waarvan de toxiciteit verzwakt kon worden door NO-synthase remmers, zou door NO 'getriggerde' oxidatieve schade na Mn een mogelijk mechanisme kunnen zijn. Andere kenmerken van Mn-toxiciteit, zoals een aangetast (mitochondrieel) GSH-metabolisme en verscheidene mitochondriële energie leverende funkties door Mn, waaronder haem-geassocieerde funkties (ademhalings- en P-450 cytochromen) en de oxidatieve fosforylering, zouden kunnen leiden tot of het resultaat kunnen zijn van de vorming van haem-geassocieerde ferrylradikalen, die even reaktief zijn als ·OH of ferryl-ijzer (FeO^{2+}). Als de voorgestelde vorming van haem-geassocieerde ferrylradicalen (en waterstofperoxide) zich voordoet, dan zou dit impliceren dat zulke radikalen in staat zijn om SA te hydroxyleren, wat niet onwaarschijnlijk is en getest zou kunnen worden *in vitro*.

Samenvattend wordt er voorgesteld, dat de selektieve neurotoxiciteit van Mn in de basale ganglia naar alle waarschijnlijkheid verloopt via ijzergerelateerde distributiekarakteristieken (transferrine-receptors, ferritine), en vervolgens transport (via de Ca-uniporter) dat de voorkeur voor akkumulatie in mitochondriën bepaalt. Hier zullen hoge lokale Mn-concentraties verscheidene belangrijke energie voorzienende funkties en anti-oxidatieve verdedigings-mechanismen (GSH) verstoren, resulterend in lokale oxidatieve stress door waarschijnlijk de vorming van plaatsgebonden haem-geassocieerde ferrylradikalen, peroxynitriet, en/of peroxynitriet afkomstige ·OH, die uiteindelijke zullen leiden tot celdood. Hierin is een studie van de rol van DA oxidatieprodukten (quinonen) mogelijk van belang bij het vaststellen van de reducerende kapaciteit van het neuron.

Curriculum vitae

De schrijver van dit proefschrift werd geboren op 25 december 1960 te Ermelo om 8.30 h, terwijl de kerkklokken luidden. Na het VWO te hebben doorlopen is hij in 1982 biologie gaan studeren aan de Rijksuniversiteit te Utrecht, met als specialisatierichting medische biologie. De eerste praktische onderzoekservaring werd opgedaan op het "oude complex" aan de Catharijne Singel van het Academisch Ziekenhuis Utrecht bij de vakgroep Haematologie onder leiding van Prof.Dr. J.J. Sixma, waar hij onderzoek heeft verricht aan hemostase (stollingsonderzoek: Dr. J. Koedam en Prof.Dr. J.J. Sixma) en atherosclerose (aderverkalking: Prof.Dr. J.J. Sixma). Vervolgens werkte hij bij het Instituut voor Moleculaire Neurobiologie (RUU) onder leiding van Prof.Dr. W.H. Gispen aan het thema neurodegeneratie en regeneratie (zenuwherstel; Dr. J. Verhaagen en Dr. P. de Koning). Na het behalen van zijn doctoraalexamen in januari 1988 en een kortstondige kennismaking met het Pedagogisch-Didactisch Instituut te Utrecht, is hij in februari 1989 in dienst getreden bij het toenmalige Medisch Biologisch Laboratorium van de Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO) te Rijswijk. Hier heeft hij als zelfstandig wetenschappelijk onderzoeker gewerkt aan verschillende onderzoekslijnen, waaronder de thema's neuro-endocrinologische markers en het Organo-Psychosyndroom (neurotoxiciteit van organische oplosmiddelen), en de neurotoxiciteit van polychloorbifenylen (PCBs) tijdens ontwikkeling onder leiding van Dr. B.M. Kulig. Vanaf 1990 werkte hij aan het onderhavige promotiewerk onder leiding van Dr. J-B.P. Gramsbergen (EUR) en Prof.Dr. J. Korf (RUG). Sinds 1 november 1994 is hij als commercieel medewerker bij TNO Voeding te Zeist aktief in de verkoop van produkten gericht op de farmaceutische industrie en de gezondheidszorg (onder coaching van Marketing Manager Drs. C. van Leeuwen).

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