A BIOCHEMICAL AND BEHAVIOURAL STUDY OF THE TOXIC EFFECTS OF 3-NITROPROPIONIC ACID AND METHAMPHETAMINE IN THE RAT.

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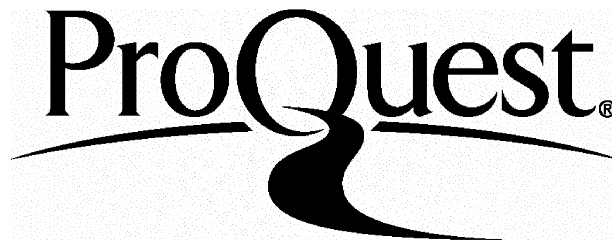

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Abstract

The effective treatment of Parkinson's disease (PD) has been hindered by a variety of factors including the lack of a precise understanding of the aetiology of the disease, as well as the loss of efficacy of the mainstay therapeutic agent, L-3,4 dihydroxyphenylalanine (L-DOPA). This work was embarked upon with the aim of producing parkinsonian conditions in a rat by harnessing various theories proposed for the aetiology of the disorder. In addition, it was intended to take a closer look at the mechanism(s) of action of L-DOPA, to determine if there are additional ways of enhancing and prolonging its effectiveness.

An over-view of the theories put forward for the cause and progression of PD reveals two schools of thought: Metabolic Stress and Oxidative Stress. While these groups of factors are independent in their own rights, they are often cyclical and inter-related. The Metabolic Stress theories suggest that PD is a consequence of an acquired or inherent metabolic defect that triggers off a variety of reactions that ultimately lead to neuron death. Proponents of the Oxidative Stress theories tend to suggest that an internal or external trigger factor impinges on and overwhelms the body's anti-oxidative mechanisms, causing metabolic failure and, ultimately, cell death. However, the specific reason(s) why the dopaminergic cell bodies of the substantia nigra pars compacta (SNpc) begin to die in PD patients remain(s) cryptic.

Methamphetamine (METH), a common drug of abuse, has been shown to induce the release of dopamine and glutamate in the basal ganglia. Its ability to reverse the dopamine (DA) transporter localises its effects to catecholaminergic neurones, while its glutamate-releasing characteristic gives it an excitotoxic potential. 3-nitropropionic acid (3-NP) is a food contaminant commonly found in mildewed sugar-cane. Its ability to inhibit succinate dehydrogenase in the electron transport chain makes it a good inducer of metabolic stress. By combining both agents, we created a metabolically impaired environment in the basal ganglia with 3-NP, and localized this to dopaminergic neurones with METH. The incorporation of METH introduced the excitotoxic effects of glutamate on these metabolically impaired DA neurones, causing further dopaminergic dysfunction.

The mutual potentiation of 3-NP and METH exploited in this regimen (which we have termed the 3-NPM model), yielded animals with alterations in tissue levels of DA and 5-hydroxytryptamine (5-HT), but not those of aspartate and glutamate in the nucleus accumbens septi (NAcc), prefrontal cortex (PFC), substantia nigra (SN) and the corpus striatum (ST). The activity of the enzyme L-DOPA decarboxylase (DDC) was not significantly altered in the four brain regions analysed, whereas the enzyme 5-hydroxytryptophan decarboxylase (5-HTPDC) was significantly reduced in the nigrostriatal tract. The results suggest that 3-NPM treatment compromises nigrostriatal dopamine function in a way that could mimic the preclinical stages of parkinsonism.

The spontaneous motility of 3-NPM-treated rats did not differ significantly from unlesioned animals, but they exhibited an enhanced sensitivity to the motor stimulant effects of direct and indirect DA agonists. There was an enhanced response to the D_2 agonist RU 24213, but not to the D_1 agonist, SKF 38393. A biphasic response to the D_1/D_2 agonist, apomorphine was observed, while the indirect DA receptor agonist, MK 801, significantly enhanced and prolonged locomotor activity in these rats.

Administration of L-DOPA in conjunction with the peripheral aromatic l-amino acid decarboxylase (AADC) inhibitor, benserazide, has been the mainstay of PD therapy. It was possible to render a threshold dose of L-DOPA (for reversing rigidity in reserpinised rats, 100 mg/kg) effective by combining it with the antiparkinson drug budipine. This drug has been shown to raise DA levels by stimulating AADC. Addition of the central AADC inhibitor, NSD 1015, to this combination further enhanced the locomotor stimulating effects of L-DOPA. This indication of a DA-independent role for L-DOPA was found to be D_2 rather than D_1 sensitive, in the three animal paradigms used. Furthermore, NSD 1015 was shown to reverse amphetamine-induced stereotypy, while enhancing its locomotor stimulant effects. These experiments were conducted in saline-treated control animals and 3-NPM-treated rats. In the third paradigm, we used rats that had their brain monoamine content depleted, as a model of parkinsonism. The choice of reserpine for this purpose was made, to present a uniform basis for comparing the results of parallel studies in our laboratory.

This work suggests the use of the 3-NPM model as a paradigm for the early stages of PD, and emphasizes the importance of the D_2 receptor pathways in the treatment of the disease. In addition, it indicates a DA-independent motor stimulant effect of L-DOPA, that can be manipulated by NMDA antagonists like budipine, as well as by various DA receptor antagonists.

Publications

Biggs, C.S., Fisher, A., Eradiri, O. and Starr, M.S. (1999) Lack of correlation between neurochemical changes and motor activity following L-Dopa administration to rats with drug-induced parkinsonism. J. Pharm. Pharmacol., 51 (Suppl.): 19.

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Abbreviations

AADC	Aromatic l-amino acid decarboxylase
Ach	Acetylcholine
αKGH	Alpha ketoglutate dehydrogenase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AMPT	α-methyl-p-tyrosine
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CCK-8	Cholecystokinin-8
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
DA	Dopamine
DDC	DOPA decarboxylase
DOPAC	3,4-Dihydroxyphenylacetic acid
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
EAA	Excitatory amino acid
GABA	γ-aminobutyric acid
GTP	Guanosine triphosphate
5-HT	5-Hydroxytryptamine
5-HTP	5-Hydroxytryptophan
5-HTPDC	5-Hydroxytryptophan decarboxylase
i.p.	Intraperitoneal
L-DOPA	L-3,4-dihydroxyphenylalanine
LDME	L-DOPA methyl ester
MAO	Monoamine oxidase
METH	Methamphetamine
MK 801	(+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)-cyclohepten-5,10-imine maleate
MPDP	1-methyl-4-phenyldihydropyridine
MPP^+	1-methyl-4-phenylpyridinium ion

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NAcc	Nucleus accumbens
NADH	Nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
3-NP	3-nitropropionic acid
3-NPM	3-nitropropionic acid/ methaphetamine
3-NPMsd.	3-nitropropionic acid/ methamphetamine single-dose schedule
3-NP3M	3-nitropropionic acid (30 mg/kg)/ methamphetamine
NSD 1015	m-hydroxybenzylhydrazine dihydrochloride
6-OHDA	6-hydroxydopamine
PCP	Phencyclidine
PD	Parkinson's disease
PFC	Prefrontal cortex
RU 24213	N-n-propyl-N-phenylethyl-p-[3-hydroxyphenyl] ethylamine hydrochloride
s.c.	Subcutaneous
SCH 23390	[R]-[+]-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol hemimaleate
SDH	Succinate dehydrogenase
SKF 38393	(±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
ST	Corpus striatum
STN	Subthalamic nucleus
TCA	Tricarboxylic acid cycle
TH	Tyrosine hydroxylase

CHAPTER ONE

GENERAL INTRODUCTION

1.1 The aetiology of Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized by akinesia, resting tremor and muscle rigidity. It is primarily a disease of the basal ganglia in which cell bodies of dopaminergic neurones in the zona compacta of the substantia nigra undergo progressive degeneration, although other neuronal systems in the brain are also affected. Cell death is followed by the appearance of eosinophilic inclusions (Lewy bodies) which are the characteristic pathological feature of PD (Jenner, 1992). The disease affects one in four hundred of the population, occurring at about fifty to sixty-five years of age (Burnham, 1989). At the moment, there is no cure for the disorder, although replacement therapy with L-DOPA and other dopamine agonists has given some relief to patients. However, prognosis after five years is poor, with the condition returning to pre-treatment status (Burnham, 1989). This stems from the fact that there is still no means of preventing the progression of the pathology. Studies are still on to identify the cause(s) of neuronal death.

The cause of idiopathic Parkinson's disease is still unknown, despite the avalanche of theories. However, two broad schools of thought exist. These are the Genetic Theories and the Environmental Theories. The proposition of a genetic or familial trend in the occurrence gained ground with the evidence that 10% of patients have an affected relative (Duvoisin, 1986). Although inheritance may play a part in some cases of the disease, it can not be said to be the sole cause. The role of the environment in the pathogenesis of parkinsonism has been the focus of interest in recent years. This perhaps stems from vistas provided by the discovery of the selective nigral toxicity of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), found as a contaminant in a cocktail of designer drugs abused by drug addicts (Langston et al, 1983). As a direct consequence of this, various epidemiological surveys of risk factors for the disease have been conducted. Factors associated with increased risk for PD include environmental exposure to toxins such as MPTP and MPTP-like compounds, herbicides and pesticides (e.g. dieldrin), and metals such as manganese and iron. Life experiences involving trauma, emotional stress and a shy or depressive personality also predispose an individual to PD (Tanner et al., 1997). In contrast, cigarette smoking, drinking alcohol, and eating a diet rich in vitamin E have been identified as factors which decrease the risk for PD (Morens et al, 1995; Tanner et al.,

1997). All these findings have generated various concepts for the cause of the disorder. Of these, the one currently growing in popularity is that which suggests that the number of dopamine neurones in the substantia nigra is slowly eroded by some continuous toxic process involving the excessive formation of free radical species that induce a state of oxidative stress. This oxidative stress is thought to be caused by either a neurotoxin or through the altered metabolism of dopamine itself (Jenner, 1992; Sonsalla et al, 1989). On the whole, the emerging view is that of a multifactorial etiology with environmental factors acting on genetically susceptible individuals with normal aging (Takakubo et al, 1996).

1.2 The neurochemistry of PD

Neurochemically, Parkinson's disease has generally been regarded as a condition arising from an imbalance between the inhibitory dopamine and excitatory acetylcholine neurotransmitters in the basal ganglia. However, various investigations have revealed that there are alterations in the noradrenergic, 5-HTergic, GABAergic, glutamatergic and neuropeptide systems as well (Avid et al, 1987). This is not the least unusual as neurotransmitters in the brain have been known to act in concert to maintain the essential delicate balance. This they do via various compensatory and modulatory mechanisms. For instance, Shimizu et al (1990) have shown that glutamate applied to the striatum evokes a release of dopamine from dopaminergic terminals. Furthermore, noradrenaline depletion in the striatum has been shown to exacerbate the loss of dopamine from its neurones induced by the administration of the neurotoxin, methamphetamine (Fornai et al, 1995). An additional example of this complex interplay can be seen in the historical use of anticholinergic agents as first-choice drugs in the treatment of parkinsonism, and their current use as adjuncts to L-DOPA therapy. In summary, the degeneration of dopaminergic neurones leads to a fall in the levels of DA in the striatum as well as in other brain regions. This causes a preponderance of excitatory activity mediated by cholinergic and glutamatergic systems (Albin et al., 1989; Starr, 1995b).

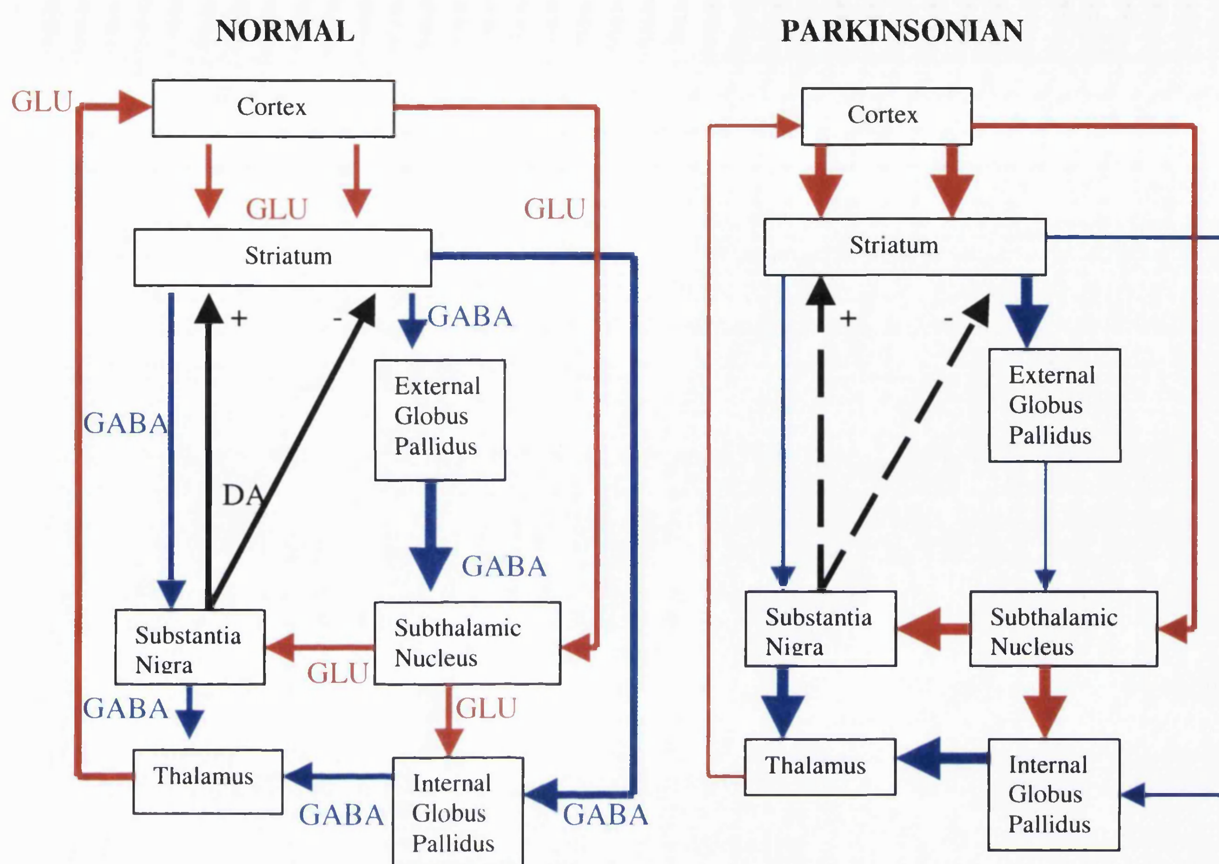


Fig. 1.1 – A schematic presentation of the basal ganglia in normal and parkinsonian brains. The width of the lines indicates the extent of neuronal firing, with the wider lines denoting an increase in activity, and the thinner lines representing a decrease in activity. The hatched lines indicate a reduction in nigrostriatal dopaminergic activity common in PD. DA, dopamine; GABA, γ-amnobutyric acid; GLU, glutamate.

Glutamatergic impulses from the cortex are processed in the basal ganglia via two major neuronal circuits. These are: the Direct and the Indirect striatonigral output pathways. These circuits differ in terms of synaptic connectivity, the type of neuropeptide they co-express with GABA, and in the DA receptors they express (Albin et al., 1989). The direct striatonigral pathway carries inhibitory impulses from the ST to the internal globus pallidus (entopenduncular nucleus) and substantia nigra pars reticulata (SNr), via GABA neurones co-expressing dynorphin and substance P. These neurones facilitate glutamate-mediated excitation via D_1 receptors. In the Indirect pathway, DA released from the nigrostriatal tract exerts an inhibitory influence on striatonigral activity via D_2 receptors. GABA neurones (co-expressing enkephalin) bearing inhibitory impulses, serve the external globus pallidus. Gabaergic output neurones from this structure in turn inhibit glutamatergic

firing in the subthalamic nucleus, allowing thalamocortical impulses to flow unhindered. The overall effect of DA is to facilitate smooth control of motor impulses sent to the basal ganglia from centres in the cerebral cortex.

Following degeneration of dopaminergic neurones in the zona compacta of the SN, there is a marked reduction in the amount of DA available from the nigrostriatal pathway. The removal of this DA "brake" in the ST leads to heightened stimulation of cholinergic and glutamatergic pathways in the basal ganglia, leading to the rigidity and akinesia observed in PD (Starr, 1995b). As shown in the figure, loss of DA in the ST disinhibits the internal globus pallidus and the subthalamic nucleus (STN), ultimately inhibiting thalamocortical feedback necessary for fluid movement (Albin et al.,1989). A heightened glutamatergic input to the SN from the subthalamic nucleus is supported by the fact that MPTP-induced parkinsonism can be reversed when this nucleus is lesioned, and decreased NMDA radioligand binding in the internal globus pallidus (Lange et al., 1997). The STN appears to be a crucial site for neurotransmission in parkinsonian brain, as lesions in this nucleus have also been reported to reverse the cardinal signs of PD, and induce wild and erratic movements in primates (Crossman et al., 1984). The excitotoxic consequences of STN hyperactivity on the DA neurones in the SN and internal globus pallidus holds potential for antiparkinsonian intervention with glutamate antagonists (Starr, 1995b).

1.2.1 Dopamine in PD

Dopamine is formed when L-DOPA is decarboxylated by the enzyme AADC. The amino acid L-DOPA can be administered exogenously, or formed by the activity of tyrosine hydoxylase (TH) on endogenous stores of tyrosine. It is then stored in vesiclce of dopaminergic neurones, and is subject to release by reversal of the dopamine transporter (Fleckenstein et al., 1997). Central stores of DA are known to be depleted by the alkaloid reserpine (Carlsson et al., 1957). These authors were able to induce the clinical and pathophysiological picture of PD (i.e. rigidity and striatal DA depletion) by administering reserpine to rodents. Administering L-DOPA to these reserpine-treated animals led to a reversal of the observed rigidity, and a rise in brain levels of DA.

The implication of DA in the reversal of rigidity in reserpinised rodents led to studies in postmortem brains that revealed massive reductions in the concentration of DA and homovanillic acid (HVA) in the caudate, putamen, nucleus accumbens and substantia nigra (Kish et al., 1988; Price et al., 1978). The tissue DA content was found to drop to as low as 3 % of control values in the putamen, 5 % in the caudate nucleus, 15 % in the SN and 40 % in the NAcc. Other studies have found nearly complete depletion of DA in all segments of the putamen, with the greatest reductions (more than 99 %) occurring in the caudal portions of the putamen (Kish et al., 1988). In all these studies, there was a greater degree of resistance to depletions in HVA, suggesting a compensatory increase in the activity of the surviving neurones. This could be through a reduced capacity for reuptake and storage of released DA (Zigmond and Stricker, 1984).

1.2.2 5-HT in PD

Tissue levels of 5-HT are markedly reduced in the ST, SN and hippocampus, but not in the raphe nucleus of PD patients (Scatton et al., 1983). The neurochemical significance of this is not clear, but this finding may explain the depression commonly observed in patients suffering from PD. In idiopathic PD, 5-HT efferents from the raphe nucleus do not degenerate to the same extent as nigrostriatal dopaminergic neurones (Zhou et al., 1991). This is important because the surviving 5-HT neurones in the ST become an additional source of DA, as 5-HT neurones are known to decarboxylate L-DOPA (Arai et al., 1994; 1995a; 1996a,b). Furthermore, 5-HT neurones impinging on DA neurones have a facilitatory effect on DA release in the ST and NAcc (Benloucif et al., 1993; Nomikos et al., 1996). This facilitation remains to be exploited in the treatment of PD.

1.2.3 Noradrenaline in PD

Noradrenaline (NA) is formed from DA by the sequential action of dopamine-β-hydroxylase and phenylethanolamine-N-methyltransferase. The locus cerulus, the principal source of NA in the brain, is known to show depigmentation and loss of of neurones, with Lewy bodies in the brains of parkinsonian patients (Farley and Hornykiewicz, 1976; Scatton et al., 1983). Although there is no generalized loss of catecholamines in PD, the loss of NA may sensitize

catecholaminergic neurones to the effects of toxins such as methamphetamine (Fornai et al., 1995). The fact that NA is involved in motor and cognitive functions (Moore and Bloom, 1979) suggests a role for this compound in the clinical presentations of PD. The ability of L-DOPA to replace central stores of both DA and NA may explain its additional clinical benefit in the treatment of patients with the disease.

1.2.4 Acetylcholine in PD

Tissue levels of acetylcholine (Ach), and the activity of its synthesizing enzyme, choline acetyl transferase (ChAT), are markedly reduced in the caudate, putamen, globus pallidus, SN, cerebral cortex and hippocampus of parkinsonian patients (Lloyd et al., 1975b; Ruberg et al., 1982). These effects may be a regulatory consequence, rather than a primary factor in PD. The loss of Ach in the cerbral cortex is thought to be responsible for the dementia observed in PD sufferers (Ruberg et al., 1982). Despite the reduction in ChAT activity, Ach tone is heightened in PD, as a result of a decrease in the D2-mediated opposition to the cholinergic drive (Starr, 1995b). This effect, coupled with the direct stimulation of cholinergic interneurones by the hyperactive corticostriatal glutamate projection, results in excessive cholinergic transmission that is thought to be responsible for the rigidity and tremor observed in PD patients (Starr, 1995a).

1.2.5 Gamma-aminobutyric acid in PD

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the CNS. There are no indications for a primary involvement of this neurotransmitter in the pathology of PD, but it is reasonable to speculate that its acivity can be up- or down-regulated as a result of DA depletion in the ST. Tissue levels of GABA are significantly elevated in the putamen (Perry et al., 1983), while the activity of glutamic acid decarboxylase (GAD, the enzyme that converts glutamic acid to GABA) is reduced by ~50 % in the ST, globus pallidus and SN of PD patients (Lloyd and Hornykiewicz, 1973). Gabaergic inhibitory tone is heightened in the striatopallidal (external globus pallidus) and nigrothalamic pathways, giving rise to a paucity of thalamocortical impulses required for a smooth flow of movement (Albin et al, 1989).

1.2.6 Glutamate in PD

Glutamate is the principal excitatory neurotransmitter in the CNS. Glutamate receptors are located on virtually all neurones in the CNS, giving glutamate the capacity to excite almost all cells of the central nervous system (Ciliax et al., 1997). There exists a delicate balance between glutamate and DA in the basal ganglia. Following the loss of the inhibitory influence of nigrostriatal DA in parkinsonian brains, striatopallidal GABAergic tone is increased, disinhibiting the subthalamic nucleus. Disinhibition of the STN results in heightened glutamatergic impulses to the SNr and internal globus pallidus, and decreased thalamocortical and thalamostriatal feedback (Parent and Hazrati, 1995a,b). The consequence of this is a further increase in corticostriatal glutamate activity (Albin et al., 1989). The overall picture is one of excessive glutamatergic tone in the basal ganglia, that offers various sites for antiparkinsonian intervention with glutamate antagonists (Starr, 1995a,b).

1.2.7 Neuropeptides in PD

The changes observed in the concentrations of various neuropeptides in the basal ganglia of PD patients appears to be secondary to the incidence of degeneration of dopaminergic neurones in the zona compacta of the SN. Using radioimunoassay techniques, the levels of substance P in the the SN, putamen and external globus pallidus were found to be reduced (Mauborgne et al., 1983), while methionine-enkephalin levels in the SN, putamen, pallidum and ventral tegmented area of parkinsonian patients (Taquet et al., 1983). Furthermore, the levels of cholecystokinin-8 (CCK-8) were found to be reduced in the SN, but not in the striatal and corticolimbic areas innervated by dopamineric neurones (Studler et al., 1982). In addition, somatostatin levels in the basal ganglia of nondemented patients with PD did not differ from controls, but were markedly reduced in the frontal cortex, hippocampus and entorhinal cortex (Epelbaum et al., 1983).

1.3 Dopamine receptors and PD

Five different dopamine receptors have been cloned and sequenced. These have been classified into two major families - D1-like and D2-like - based on pharmacologic, anatomic and biochemical considerations (Andersen et al., 1990). While the DA receptors in the D1

subfamily (D1 and D5) are positively coupled to adenylate cyclase, those in the D2 subfamily (D2, D3 and D4) are negatively coupled to this enzyme (Stoof and Kebabian, 1984). Whereas the D1 and D2 subtypes are expressed predominantly in the basal ganglia, D3, D4 and D5 have low expressions among these nuclei.

1.3.1 D1-like dopamine receptors

All dopamine receptors are members of the family of G protein-coupled receptors and have seven transmembrane regions. The D1 subfamily have long carboxy termini, while the D2 subfamily have long intracellular loops (see Sibley et al., 1993 for review). The D1 subclass consists of D1, $D1_B$ and D5 gene products which bind D1-selective ligands with high affinity, and activate adenylyl cyclase (Sunahara et al., 1990; 1991). These receptors are highly conserved, with approximately 80 % homology in the transmembrane region (Sunahara et al, 1991; Tiberi et al., 1991). The $D1_B$ receptor, cloned from rats (Tiberi et al., 1991), and the D5 receptor, cloned from humans (Sunahara et al., 1991), may be differential expressions of the same gene.

D1 and D2 receptors are immunolocalized in the striatum (Levey et al., 1993), with the D1 receptors predominantly regulating the striatonigral output pathway, and the D2 receptors regulating the striatopallidal output pathway (Gerfen et al., 1990; Wilson, 1990). The striatonigral output pathway (predominantly D1 receptors) is more susceptible than the striatopallidal output pathway (predominantly D2 receptors) to energy impairments, as would normally occur with aging (Araujo and Hilt, 1998). In addition, D1 populations in rodents can be reduced by treatment with methamphetamine (Cadet et al., 1998) or 3-nitropropionic acid (Koutouzis et al., 1994).

1.3.2 D2-like dopamine receptors

The D2 subclass of dopamine receptor consists of D2, D3 and D4 receptors, having a high affinity for D2-selective ligands, as well as a marked sequence homology (approximately 75%) in their transmembrane regions (Bunzow et al., 1988; Sokoloff et al., 1990; Van Tol et al., 1991). D2 receptors are expressed primarily by dopaminergic neurones in the SN

(autoreceptors), cholinergic interneurones in the ST, and striatopallidal neurones to the lateral globus pallidus (Wooten, 1997). Furthermore, D2 receptors are negatively coupled to adenylyl cyclase, and are tonically inhibited by the action of dopamine (Stoof and Kebabian, 1984). Striatal D2 receptors are upregulated when dopaminergic neurones are damaged by lesions with 6-OHDA in rats (Gagnon et al., 1991), or MPTP in primates (Elsworth et al., 1998) and mice (Tanji et al., 1999). In addition, D2 receptors work interdependently with D1 receptors to produce locomotion in the basal ganglia (see Clarke and white, 1987 for review).

1.4 Glutamate receptors and PD

Glutamate is the most abundant free amino acid in the CNS. It is often referred to as a "mixed agonist", as it has the ability to stimulate several classes of receptors. Glutamate receptors are broadly classified as metabotropic or ionotropic. The ionotropic glutamate receptors are further divided into AMPA, Kainate and NMDA, named after the specific agonist compound eliciting a given physiological response.

1.4.1 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are coupled to cellular effectors via GTP-binding proteins. At least eight mGluRs have been cloned (termed mGluR1-8), six of which (mGluR1-5 and mGluR7) are expressed in the brain (see Pin and Duvoisin, 1995 for review). Metabotropic glutamate receptors mediate inositol phosphate metabolism, release of arachidonic acid, and changes in cAMP levels, depending on the type of cell and the receptor subtype (Schoepp and Conn, 1993).

Metabotropic glutamate receptors have distinct localizations in the basal ganglia (Testa et al., 1994). Almost all striatal neurones express low levels of mRNA for mGluR1 and mGluR4, and moderate levels for mGluR3. The signal for mGluR5 is intense in 75 % of striatal neurrones, while the mRNA for mGluR2 is present in only 2 % of striatal neurones. Activation of mGluRs in the ST or STN induces DA-dependent rotational behaviour in rats (Kaatz and Albin, 1995; Sacaan et al., 1992). Activation of striatal mGluRs has also been shown to modulate the function of the NMDA receptor (Colwell and Levine, 1994).

1.4.2 Ionotropic glutamate receptors

Unlike mGluRs, ionotropic glutamate receptors (iGluR) are not coupled to G-proteins, but are associated with ligand-gated ion channels. Activation of these receptors leads to a rapid opening of the channels to which they are associated (Greenamyre and Porter, 1994). Subtypes of this receptor include AMPA, Kainate and NMDA receptors.

1.4.2.1 AMPA receptors

Glutamate or AMPA bind to this receptor, and gives rise to NA^+ (but not Ca^{2+}) influx from the extracellular to the intracellular compartments, mediating fast excitatory neurotransmission in the CNS (Greenamyre and Porter, 1994). AMPA receptor subunits were the first of the glutamate receptors to be cloned (Hollmann et al., 1989). Four of these units exist, GluR1-GluR4, having an ability to assemble in various combinations to form functional receptors whose characteristics depend on the constituent subunits (Ciliax et al., 1997). For instance, combinations involving GluR2 prevent, while GluR1 and GluR3 permit Ca^{2+} permeability (Hollmann et al., 1991). An additional complication in the study of the characteristics of particular AMPA receptors is the occurrence of splice variants (termed "flip" and "flop") for each of the four subunits (Sommer et al., 1990). GluR1 is found colocalized with DA D1 receptor patches in the human ST (Ciliax et al., 1997). It is also found in the globus pallidus (internal and external segments), the STN and in the SN (pars reticulata and pars compacta). Neurones rich in GluR2/3 have been found in the globus pallidus (internal segment), and SN (pars copacta and pars reticulata), whereas GluR4 containing neurones have been found in the substantia nigra pars reticulata.

1.4.2.2 NMDA receptors

NMDA receptors are multimeric heteromers, having two families of subunits: NMDAR 1 and NMDAR 2A-D (Kutsuwada et al., 1992; Meguro et al., 1992). The NMDAR 1 subunit has eight splice variants, and is widely distributed in the CNS. On the other hand, the NMDAR 2A-D subunits show a restricted anatomic distribution, and an ability to combine with the NMDAR 1 subunit to form NMDA receptors which have large current fluxes, as well as distinct pharmacological and physiological characteristics.

NMDA receptors are found throughout the basal ganglia. They are most abundant in the ST, but also occur in the globus pallidus, STN and SN (Albin et al., 1992). Activation of NMDA receptors, unlike AMPA receptors, is associated with increased Ca^{2+} permeability (alongside increased Na^{+} permeability) in the neurones. This increased Ca^{2+} permeability has been implicated for the toxic effects of the NMDA receptor (Beal et al., 1993).

The NMDA receptor has five distinct recognition sites, for glutamate, glycine, polyamines, Mg^{2+} and Zn^{2+}. There is also a recognition site in the ion channel for drugs like phencyclidine (PCP) and MK 801. The simultaneous occupation of the glycine and glutamate sites is a prerequisite for NMDA receptor activation (Kleckner and Dingledine, 1988). Agonists at the polyamine site potentiate NMDA receptor function by reducing the tonic inhibition by protons under physiological pH (Traynelis et al., 1995). Polyamine site antagonists like ifenprodil and eliprodil are useful antiparkinsonian drugs (Starr et al., 1997). Ambient levels of Mg^{2+} voltage-dependently block the flow of current through the receptor, even when the glycine and glutamate sites are occupied (Nowak et al., 1984). This voltage-dependent bolckade of the receptor ion channel by Mg^{2+} is a unique feature of the NMDA receptor. The PCP site is the locus for the effects of the ion channel antagonists, with the prototype being MK 801 (Kemp et al., 1987). Drugs binding to this site prevent the opening of the ion channel, and the influx of calcium. The parkinsonian drug budipine is weak antagonist at this site (Klockgether et al., 1993).

1.5 Dopamine/Glutamate interactions in the treatment of PD

Following dopaminergic denervation in the nigrostriatal region of parkinsonian patients, there is a paucity of DA to interact with excitatory D1 receptors of the direct (striatonigral) pathway and inhibitory D2 receptors of the indirect (striatoplallidal) pathway. The consequence of this is an underactivity of the striatonigral and an hyperactivity of the striatopallidal pathways, resulting in increased corticostriatal, subthalamo-nigral and pallido-thalamic glutamatergic activity (Albin et al., 1989; see Fig. 1.1). The heightened tone of glutamatergic and cholinergic neurones in various parts of the basal ganglia has fueled the drive for the use of glutamate antagonists in antiparkinsonian therapy (see Starr, 1995a,b for reviews).

In 1957, Carlsson et al. observed that the parkinsonian rigidity and akinesia induced by the depletion of central DA stores with reserpine was reversed when these rats were treated with L-DOPA, but not 5-HTP. Papavasiliou et al. (1972) showed that this antiparkinsonian effect of L-DOPA could be potentiated by concomitant administration of peripheral DOPA decarboxylase inhibitors. At present, DA replacement with L-DOPA remains the mainstay for treatment of PD, despite the introduction of direct-acting DA agonists, MAO-B inhibitors, L-DOPA slow-release formulations, glutamate antagonists, cholinergic antagonists, and the experimental use of COMT inhibitors (see Poewe and Granata, 1997 for review).

Exogenously administered L-DOPA is rapidly taken up into the brain, following peripheral DDC inhibition, and is rapidly converted to DA by AADC in dopaminergic and 5-HT neurones (Arai et al., 1996; Hadjiconstantinou et al., 1993). The DA so formed is then released into the extracellular space, under glutamatergic control (Biggs and Starr, 1997). DA also modulates its synthesis and release via presynaptic D_2 autoreceptors (Sokoloff et al., 1990). The released DA then restores locomotion by the simultaneous activation of postsynaptic DA D_1 and D_2 receptors (see Clarke and White, 1987 for review). This DA-dependent mechanism by which exogenous L-DOPA reverses the parkinsonian conditions in experimental animals, has come under intense scrutiny in recent times for a variety of reasons. A major reason for this is the observation that doses of L-DOPA which induce a large efflux of DA into the extracellular space (25-50 mg/kg), do not correspond to doses which activate locomotion in DA-depleted animals (Biggs et al., 1999b). Furthermore, L-DOPA can increase DA synthesis, as indicated by increased DOPAC output, without a corresponding increase in DA levels in dialysates from microdialysis experiments (Fisher et al., 2000; Wachtel and Abercrombie, 1994). In addition, L-DOPA inhibits AADC, the enzyme necessary for its conversion to DA, an effect that can be overcome by the use of glutamate antagonists (Fisher et al., 1998). The growing body of evidence suggests that L-DOPA can reverse drug-induced parkinsonism by mechanisms independent of dopamine (Fisher et al., 2000; present study).

Dopamine and glutamate have a delicate seesaw balance in the basal ganglia, which can lead to an exaggeration of the effects of one, in the absence of the other. Whereas glutamate regulates

the synthesis of DA via AADC (Fisher et al., 1998b; Hadjiconstantinou et al., 1995), both compounds regulate each other's release in the SN and entopeduncular nucleus (Biggs and Starr, 1997). NMDA antagonists have been shown to potentiate the antiakinetic properties of L-DOPA in monoamine-depleted rodents (Klockgether and Turski, 1990), suggesting the use of these compounds as adjuncts to L-DOPA in the treatment of PD (see Starr, 1995a,b for reviews).

Until recently, the exact mechanism by which glutamate antagonists facilitated L-DOPA-induced reversal of parkinsonism was not known. Biggs et al. (1998) showed an increase in the amount (by approximately 300 %) and duration of DA released from a bolus dose of L-DOPA, when the NMDA ion channel antagonist, budipine, was administered an hour before, but not after, L-DOPA. This priming of AADC by glutamate antagonists was confirmed in further experiments, where even the L-DOPA-induced inhibition of AADC was reversed by prior exposure to glutamate antagonists (Fisher et al., 1998a,b). Thus, the glutamate antagonists will not only depress the heightened glutamatergic tone in PD (see Fig. 1.1; Albin et al., 1989), but have the added benefit of stimulating the enzyme involved in maintaining endogenous DA levels.

1.6 Regulation of AADC by DA and glutamate

Aromatic l-amino acid decarboxylase is often associated with its traditional role of catalyzing the second step in the synthesis of indoleamines and catecholamines in which 5-HTP, formed from tryptophan by tryptophan hydroxylase, and L-DOPA, formed from tyrosine by tyrosine hydroxylase, are decarboxylated by AADC to yield 5-HT and DA respectively. AADC is well distributed in the CNS as well as in the periphery. Immunoreactivity for the enzyme overlaps with sites which use catecholamines or indoleamines as neurotransmitters. High levels of AADC activity have been detected in the ST, SN and locus coerulus (Hadjiconstantinou et al., 1995), with nigrostriatal dopaminergic terminals being the main site of localized AADC activity in the rat striatum (McGreer et al., 1972).

When nigrostriatal DA is reduced by 95-98 % with 6-OHDA lesions, approximately 15 % of AADC activity remained (Opacka-Juffry and Brooks, 1995). This is similar to PD patients, where AADC activity has been found to be between 5 and 15 % in the basal ganglia (Lloyd et al., 1975). These effects can be adduced to the loss of dopaminergic neurones, which are the principal carriers of AADC. Alternatively, when DA levels are depleted by reserpine (Hadjiconstantinou et al., 1993; present study), or dopaminergic tone removed by DA receptor antagonists (Zhu et al., 1992; 1993), AADC activity is known to increase. This suggests an inhibitory role for endogenous DA, that is mediated by presynaptic D_2 receptors (Sokoloff et al., 1990; Zhu et al., 1992). DA receptor agonists and L-DOPA, on the other hand, have been shown to reduce AADC activity (Rosetti et al., 1990; Biggs et al., 1998; present study).

The glutamatergic control of AADC activity was demonstrated by Hadjiconstantinou et al. (1995), when they showed an increase in AADC activity after treatment with the glutamate antagonist, MK 801. Research in our laboratory has confirmed these findings (Biggs et al., 1998; Fisher et al., 1998a,b). Our results also show that the inhibitory effect of L-DOPA on AADC activity, can be reversed by the use of NMDA and non-NMDA antagonists. The effect of glutamate antagonists on AADC activity provides a mechanism for the actions for the adjunctive use of the glutamate antagonists, amantadine and budipine, in antiparkinsonian therapy.

5-HTergic neurones can form DA from exogenous L-DOPA (Arai et al., 1994; 1995a), and 5-HTP can be decarboxylated on dopaminergic neurones (Arai et al., 1995b). These findings, and the occurrence of unique protein isoforms of the AADC protein tend to suggest that more than one AADC enzyme exists (O'Malley et al., 1995; Zhong et al., 1995). Further grounds for the proposed two-enzyme structure for the decarboxylation of L-DOPA to DA and 5-HTP to 5-HT are covered by the differential mechanisms of phosphorylation of AADC by dopamine and 5-HT receptor activation (Zhu et al., 1994). Whereas DA receptor activation involves regulates AADC activity via protein kinase A, activation of 5-HT receptors employs protein kinase C. The present study shows differential effects of 3-NP and METH on the ability of "AADC" to decarboxylate 5-HTP and L-DOPA. For these reasons, the effects on "AADC" in

this study will be presented as differential effects on 5-HTP decarboxylase and DOPA decarboxylase.

1.7 Models of PD

A variety of animal models have been developed over the years to facilitate the study of Parkinson's disease. The object of such models has been to (1) enable the reliable screening for potential new drugs; (2) elucidate the mechanism of action of these agents and (3) to clarify the pathophysiology of the corresponding human disease. The existing models have made remarkable success on the first two counts as they were instrumental to the development of anti-parkinsonian drugs and are beneficial in predicting possible anti-parkinsonian activity of new agents. In addition, they have proved useful in identifying the mechanism of action of dopaminergic agonists (Kaakkola and Teravarinen, 1990). Apart from the discovery of the profound DA depletion in the basal ganglia of PD patients, not much progress has been made on the third count. It will be the thrust of an accomplished model to incorporate the virtues of the existing models, provide satisfactory explanation for the cause of neuronal cell death, and present mechanisms for the progression of the disease. It is hoped that when this is achieved, the now elusive cure for the disease will be in sight.

In the last half of the twentieth century, giant leaps were made in the understanding and therapy of PD. The ability of L-DOPA to reverse the akinesia following DA depletion with reserpine (Carlsson et al., 1957), was a major step in this process. A survey of the older models reveals three broad classes: the cholinergic agonists, the neuronal lesion models and the brain monoamine depleters. While some of these models have undergone significant and positive metamorphosis over the years, others have become either limited in use or completely obsolete.

1.7.1 Cholinergic agonists

Various cholinergic agents such as arecoline, oxytremorine, physostigmine and nicotine induce tremor in rodents (Brimblecombe and Pinder, 1972). This investigation was triggered by the historic use of anticholinergics as first-line drugs in therapy of PD, and the identification of

tremor as one of the classical clinical features of the disease. However, it was found that the tremor induced by cholinergics resembled more a condition of cholinergic intoxication than parkinsonian tremor (Duvoisin, 1976). It was also found that large doses of cholinergic drugs given to rodents induced catalepsy, including akinesia, an ability to maintain an abnormal posture, and often, rigidity. The intensity of catalepsy was quantified, and its inhibition by muscarinic antagonists (e.g. atropine) was used as an indication of their central activity (Zetler, 1968). The benefits offered by this group of models in current research in PD are grossly limited.

1.7.2 Neuronal lesions

The neuronal lesion model was advocated by Poire et al (1966), when they caused resting tremor and hypokinesia in the monkey by electrocoagulative lesion of the ventromedial tegmentum (as a lesion limited to the substantia nigra of the monkey caused hypokinesia, but not tremor). The method was limited by the fact that it destroyed dopaminergic cells along with other nerve cells and tracts located in the lesioned area. A similar lesion of the rat striatum produced an ipsilateral turning tendency in the animal and allowed evaluation of drug effect on the intact contralateral nigrostriatal dopaminergic system (Ungerstedt, 1973). Nonetheless, it remained non-selective and did not produce the specific neuronal changes observed in the human condition.

1.7.2.1 6-hydroxydopamine lesions

Further progress was made with the neuronal lesion models when 6-OHDA was injected intracerebrally or intraventricularly to produce a degeneration of brain dopaminergic or noradrenergic neurones (Ungerstedt, 1968). Injecting 6-OHDA intothe SN produced a selective, stable and permanent destruction of dopaminergic neurones, leading to hypokinesia (Ungerstedt, 1971a). Because the animals died of aphagia and adipsia when the toxin was bilaterally administered to both substantia nigra (SN), the procedure was modified to the use of a unilateral administration (Ungerstedt, 1971b). Following the administration of apomorphine to the latter group of animals, a contralateral circling behaviour was observed. Conversely, an ipsilateral rotation was observed when (+)-amphetamine was administered

(Ungerstedt, 1971c). The explanation for this is that an imbalance in the activity between the dopaminergic systems of the left and right sides results in circling: the animal circles away from the side of higher dopaminergic activity. Consequently, the Ungerstedt model has become the appropriate method for assessing the anti-parkinsonian activity of new compounds, as well as for predicting the potential dopaminergic feature of a new agent; as a compound acting directly (postsynaptically) causes contralateral circling behaviour whereas a compound acting indirectly causes ipsilateral rotations (Kaakkola and Teravainen, 1990). In addition, the significant pitfall of a lack of selectivity in the original model of Ungerstedt was overcome by a recent modification in which only the nigrostriatal dopaminergic neurones are destroyed by 6-OHDA, using an accurate stereotactic technique (Perese et al, 1989). However, the lesions produced by the 6-OHDA models do not mimick the widespread pathology and biochemistry of idiopathic PD and the animals do not exhibit the spectrum of motor disorders associated with the disease (Jenner and Marsden, 1988).

Quite recently, a novel genetic animal model based on the circling behaviour observed in the Ungerstedt rat has been developed (Loscher et al, 1996). While this *ci* mutant rat presents a useful tool for the study of endogenous processes that lead to motor dysfunctions (i.e. without induction by exogenously administered neurotoxins), the observed locomotor hyperactivity raises question as to whether it represents a model of hyperkinetic or hypokinetic movement disorder, such as PD.

1.7.2.2 MPTP lesions

So far, the most significant contribution to the search for the ideal animal model for parkinsonism over the last two decades was the discovery of the selective nigral toxicity of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al, 1983). Until recently, the MPTP-induced parkinsonian monkey was thought to be the best available model of PD (Kaakkola and Teravainen, 1990). When administered peripherally, the lipophilic MPTP molecule enters the brain, where it is selectively metabolized by MAO-B located in mitochondria extraneuronally, possibly in glia. The $MPDP^+$ formed from this process is then converted to MPP^+ extraneuronally, and is then taken up into dopaminergic neurones via the same re-uptake mechanism as for DA (Jenner and Marsden, 1986). How the MPP^+ acts to

release DA from storage sites and how the released DA exerts its effects is unclear, although mechanisms involving inhibition of mitochondrial respiration (by blocking NADH dehydrogenase activity) and oxidative stress have been put forward (Jenner and Marsden, 1986; 1988).

MPTP has been shown to selectively damage nigrostriatal DA neurones, although some damage to the mesolimbic dopaminergic system may also occur (Jenner and Marsden, 1986). Furthermore, biochemical changes indicate that the toxin affects primarily, the brain dopaminergic system with no direct evidence of neurotoxic actions on other neuronal systems. The use of the MPTP model has enabled tremendous progress to be made in various aspects of the study of PD. Some significant outcomes include:
a) The model responds positively to L-DOPA, DOPA decarboxylase inhibitors and DA agonists (e.g. bromocriptine), suggesting it as a viable test bed for the evaluation of novel anti-parkinsonian compounds;
b) The observation that MAO-B inhibitors (e.g. pargyline and deprenyl) protected animals against the effect of MPTP (Heikkila et al, 1984) not only advanced the mechanism of action of the agent (i.e. conversion of MPTP to $MPDP^+$ by MAO-B) (Langston et al, 1984b), but also created new vistas for the clinical trials of this group of agents to delay the onset of L-DOPA therapy (Tetrud and Langston, 1989), as well as to increase the latency for the development of the debilitating effects in the patients (Birkmayer et al.,1985);
c) The use of the MPTP-treated animal has contributed significantly to the evaluation of the roles played by D_1 and D_2 receptors in PD (Braun et al, 1987; Nomoto et al, 1985);
d) Finally, the model is amenable to the induction of L-DOPA dyskinesias (Bernard et al, 1986) and so may be relevant in the study of mechanisms involved in the long-term effects of drugs in the disease.

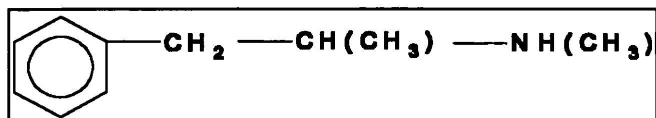
At its best, the MPTP model mimicks idiopathic parkinsonism closely but not completely, as biochemical and pathological data suggest a more selective and limited brain damage than occurs in the human patient (Jenner and Marsden, 1988). In addition, although the behavioural deficits resemble those of the disease, motor functions do eventually return. These have led to a resumption of the search for the ideal model.

1.7.3 Monoamine depleters

Reserpine is the prototype of the monoamine depleters, reducing central stores of DA, NA and 5-HT. The use of this model in experimental induction of parkinsonism in rodents gained ground with the observation that the akinesia produced by this alkaloid was reversed by the administration of L-DOPA (Carlsson et al., 1957). When parenterally injected, reserpine non-selectively depletes brain amines, giving rise to sedation, hypokinesia, rigidity (catalepsy), tremor and upregulation of DA receptors, 18-24 h later (Hornykiewicz, 1966). The loss of DA in the CNS removes the regulatory function of this amine at the hypothalamus, and leads to hypothermia. Consequently, the rats have to be kept warm by raising ambient temperatures. Reserpinization depletes the vesicular stores of DA by at least 90 %, but does not interfere with its synthesis and release (Parker and Cubeddu, 1986). Unlike PD, the significant loss of DA is accompanied by an increase in AADC activity in the nigrostriatal tract (Fisher et al., 1998a,b; Hadjiconstantinou et al., 1995; present experiments). The observed hypokinesia and its reversal by L-DOPA can be reliably measured with automatic motor activity apparatus (Cooper et al, 1987). Dopamine levels return to almost normal values 36-48 h after the administration of reserpine, and ptosis and akinesia are reversed. Due to the reversibility of its effects, the exhibition of peripheral actions and the generalized amine depletions, the induced condition can not be said to mimick the human disease in every respect (Kaakkola and Terravainen, 1990). As such the use of this model is currently on the decline. Currently, much attention is focused on another monoamine depleter, the sympathomimetic drug methamphetamine.

1.8 Methamphetamine as a model of PD

Methamphetamine is a substance widely abused for its psychostimulant effects. Although its chemistry reveals a lack of the characteristic catechol nucleus, the drug is pharmacologically classified as an indirect-acting sympathomimetic agent. Its ability to cause a release of stored neurotransmitter is not confined to the noradrenergic system, as its effects have been demonstrated in the dopaminergic, glutamatergic, and serotonergic systems as well. In fact, serotonergic neurones have been shown to be more sensitive than dopaminergic neurones to the toxic actions of METH (Ricaurte et al., 1980). The chemical structure of METH is shown overleaf:



The N-methyl substitution in its structure makes METH more potent than amphetamine as a monoamine depleter, while the α-methyl substitution makes the compound more resistant than the catecholamines to the degradative effects of monoamine oxidase. This α-methyl substitution is also necessary for its monoamine depleting activity (Hoffman and Lefkowitz, 1990). Furthermore, the (+)-isomer of the compound is more centrally active and less active peripherally than the (-) form. In addition, the drug has more pronounced central actions and, unlike reserpine and amphetamine, less prominent peripheral effects (Hoffman and Lefkowitz, 1990).

1.8.1 Toxic effects of METH

The neurotoxic effects of METH vary extremely and depend on the species, strain, and age of the animals (Seiden and Ricaurte, 1987). It is believed that mice are more susceptible than rats, while monkeys appear to be the most resistant of the three to the effects of the toxin. In contrast with the topographically specific pattern (in terms of selective nigrostriatal toxicity) of the neuronal damage induced by MPTP (Jenner and Marsden, 1986; Marshall and Navarette, 1990), METH produces a widespread array of neurodegenerative effects (Walsh and Wagner, 1992). Since the growing body of evidence suggests that neural degeneration in PD is not restricted to the nigrostriatal region, and that degenerative changes in the parkinsonian brain are much more widespread than originally believed (Jellinger, 1986), the neurotoxic profile exhibited by METH may reflect the neurochemical alterations of the disease more accurately than other available neurotoxic probes. However, METH neurotoxicity still exhibits specificity in that it damages neurones in defined anatomical regions (e.g. it affects DA in terminals in the striatum, amygdala, frontal cortex, and nucleus accumbens septi while having little effect on hypothalamic DA terminals) and neurochemical pathways (e.g. it injures DA and 5-HT terminals while sparing noradrenergic, cholinergic and GABAergic neurones) (Seiden et al., 1988). In addition, METH administration *in vivo* has been shown to selectively damage DA terminals while sparing the dendrites and cell bodies (Ricaurte et al., 1982, 1984; Dawirs et al.,

1991). However, recent experiments with this compound now suggest that dopaminergic cell bodies in the SN and ST are also damaged (Sonsalla et al., 1997). In contrast with other models of the disease METH, at its best, only produces a partial lesion (Cass, 1996) and as such may be well suited for studying the preclinical stages of PD.

1.8.2 Behavioural effects of METH

Akinesia, bradykinesia, disturbances of balance and gait, resting tremor and rigidity have long been established as the cardinal clinical features of PD in humans. It has become possible to mimic these effects in laboratory animals using a variety of neurotoxins. Historically, it was difficult to produce nigrostriatal damage in the rodent which resulted in overt motor disturbances, as impairments usually manifest when striatal DA is reduced by 90% or more; and under such conditions, the subjects were usually adipsic, aphagic and akinetic (Zigmond and Stricker, 1972). Although the rodent has proved to be behaviourally resistant to the effects of MPTP and 6-OHDA (two potent nigrostriatal toxins), treatment with METH has been shown to produce long-lasting impairment in balance beam perfomance as well as a significant increase in active avoidance response latency (Walsh and Wagner, 1992). These effects correlate well with gait disturbances and bradykinesia respectively in the human patient. This observation, coupled with the profile of METH in affecting neurotransmitters beyond the nigrostriatal tract, suggests that the compound may be the most useful tool available for modelling the disease in the rodent.

1.8.3 Mechanisms of METH toxicity

Similar to the obscurity of the precise pathway involved in the pathogenesis of idiopathic parkinsonism, the exact mechanism by which METH induces neuronal damage is not known. However, it is known that following the systemic administration of the drug, injury to DA terminals occurs in certain areas of the CNS. This is evidenced by a loss of tyrosine hydroxylase activity (Kogan et al., 1976), decreased number of high-affinity DA uptake sites (Wagner et al., 1980), lower striatal DA content (Sonsalla et al., 1989), and histological evidence of terminal degeneration (Ricaurte et al, 1982). Furthermore, the loss of DA terminals is long-lasting, with striatal DA depletions still evident at least 6 months in the rat

(Wagner et al., 1980) to 4.5 years in the monkey (Woolverton et al., 1989) after the last injection of a high-dose regimen of METH.

It is generally accepted that METH induces a significant overflow of striatal DA into the extracellular fluid by reversing the directional polarity of the high-affinity membrane DA transporter (Raiteri et al., 1979; Schmidt and Gibb, 1985). The fact that METH exerts its effects primarily by inducing DA overflow was substantiated by the findings that treatment with either DA uptake blockers (e.g. mazindol, Marek et al., 1990) or amphonelic acid (Schmidt and Gibb, 1985)), or catecholamine synthesis inhibitors (e.g. AMPT, Wagner et al, 1983), diminished METH-induced DA release and subsequently reduced the neurotoxicity. Further evidence to this fact could be adduced from the observation that METH-induced injury to striatal dopaminergic terminals can be blocked by various DA receptor antagonists, including haloperidol and chlorpromazine (Buening and Gibb, 1974), or the more selective D_1 (SCH 23390) or D_2 (sulpiride or eticlopride) antagonists (O'Dell et al., 1993; Sonsalla et al., 1986a,b). This finding further suggests that excessive stimulation of post-synaptic receptors may be necessary for the resulting neurotoxicity.

A parallel to the fundamental role of DA in mediating METH neuronal damage is the hypothesis that excitatory amino acids contribute directly to the neurotoxicity. This claim is based on the findings of Sonsalla et al. (1989, 1991), who observed that administration of competitive or non-competitive antagonists of the NMDA receptor-ion channel complex prevented METH-induced depletions of DA content. A boost to this hypothesis was the finding by Ohmori et al. (1996) that METH increased glutamate release in the ST. However, *in vivo* microdialysis studies have shown that pre-treatment with MK 801 (a non-competitive NMDA antagonist) significantly decreased the METH-induced DA over-flow in rats, under both neurotoxic (Weihmuller et al., 1992) and non-neurotoxic (Weihmuller et al., 1991) METH treatment regimens. This infers that the neuroprotective influence of MK 801 is exerted indirectly, by the attenuation of the induced DA over-flow, or its reversal of MET-induced hyperthermia (Moy et al., 1998). Interestingly, it is known that a major glutamatergic pathway projects from the cerebral cortex to the neostriatum (Fagg and Foster, 1983), and a population of these glutamatergic neurones have pre-synaptic DA receptors which, when

activated, may decrease the high-affinity uptake of glutamate (Nieoullon et al., 1983). Furthermore, NMDA receptors have been shown to be at least partly located on striatal DA nerve terminals (Krebs et al., 1991; Ohmori et al., 1991), where they have been proposed as the mediators of the DA release following administration of NMDA to neostriatal slices (Snell and Johnson, 1986). Taken together, it may well be that enhanced release in glutamate and DA work synergistically on pre-synaptic DA terminals to cause the METH-induced striatal dopaminergic neurotoxicity.

At the moment, the precise cellular and molecular mechanisms involved in the induction of neuronal damage by METH are yet to be determined. Although DA and glutamate are known to be involved, the exact sequence of events between the release of DA and neuronal death is still unclear. An over-view of the theories put forward so far reveals that either oxidative stress or metabolic stress can be implicated in the mechanism leading to terminal degeneration. According to the Oxidative Stress theories, amphetamines, like METH, disrupt the electrochemical gradient that provides energy for monoamine accumulation in synaptic vesicles, leading to a redistribution of DA to the cytosol (Sulzer and Rayport, 1990). It is known that catecholamines more readily oxidize under cytoplasmic conditions (neutral pH, low ascorbate levels, and availability to MAO) than under intravesicular conditions (low pH, high ascorbate levels and no exposure to MAO). Since DA undergoes auto-oxidation leading to the formation of reactive quinone derivatives, hydrogen peroxide, and free radicals (Graham et al., 1978; Silvka and Cohen, 1985), it may well be that local METH-induced increases in extracellular DA may overwhelm extracellular superoxide dismutase and catalase, which attenuate peroxide toxicity (Rosenberg, 1988). Alternatively, reactive DA or DOPA derivatives such as 6-OHDA, 2,4,5,-trihydroxy-phenylalanine, or quinones and semiquinones might be released from cells or produced extracellularly (Olney et al., 1990; Seiden and Ricaurte, 1987). Consequently, Cubells et al. (1994) have suggested that redistribution of DA from synaptic vesicles to the cytoplasm may be the initiating step in METH neurotoxicity, and that DA-dependent oxidative stress may be sufficient to explain the selectivity of METH toxicity for DA terminals.

On the other hand, the proponents of the Metabolic Stress theories have shown simultaneous depletion of striatal ATP and DA levels following METH treatment (Chan et al., 1994) and an enhancement of METH toxicity when animals were treated with malonate; a reversible inhibitor of succinate dehydrogenase, that acutely causes a depletion of ATP and accumulation of lactate (Albers et al., 1996). A summary of their findings suggests the possibility that METH directly inhibits the mitochondrial respiratory chain, thereby reducing cellular energy production. Alternatively, they suggest that the observed ATP depletion arose from "metabolic stress" caused by the action of METH on dopaminergic neurones: the increased energy consumption being the result of the increase in the activity of the DA transporter, as well as the METH-induced hyperthermia (Albers and Sonsalla, 1995). They also propose that the energy impairment could secondarily lead to slow excitotoxic neuronal death by an increase in the sensitivity of the DA receptors to excitatory amino acid receptor activation (Beal, 1992; Sonsalla et al., 1991).

1.8.4 Dosing schedules with METH

Following the observation that a multiple-dose regimen of METH induces a long-lasting selective damage to the striatal dopaminergic system whereas a single dose does not (Sonsalla and Heikkila, 1986; Wagner et al., 1980), it is now typical to administer a four-dose regimen of METH i.p., at 2 h intervals to induce the parkinsonian state. The need for repeated dosing perhaps stems from the observed variability of METH toxicity (Seiden and Ricaurte, 1987). Furthermore, it has been demonstrated that the amount of DA released by the fourth injection is by far more pronounced than for the preceding three injections, and is therefore critical for the production of METH-induced neuronal injury (O'Dell et al., 1993). A regimen of 4 x 12.5 mg/kg METH yields approximately 82 % striatal DA reduction in mice (Johnson et al., 1992). In contrast with the above, Fukumura et al. (1998) have recently shown a 70 % reduction of striatal DA content three days after a single dose regimen of METH. These findings are assessed in detail in Chapter four of this thesis.

It has been possible to combine METH treatment with a variety of agents with a view to identifying the mechanism(s) of toxicity of the toxin, as well as to gain additional insight to the pathogenesis of PD. Whereas some of these combination modes have enhanced its effects,

implying the use of smaller quantities of the toxin to produce the same or greater effect, others have given insight to neuroprotective mechanisms. Pre-treatment of rats with DSP-4, a selective noradrenergic neurone depleter, led to a five-fold increase in METH potency (Fornai et al., 1995), suggesting a role for the noradrenergic system in METH toxicity as well as in the pathogenesis of the disease. In addition, simultaneous treatment with 2-Deoxyglucose (an inhibitor of glucose uptake and utilization, Chan et al., 1994) and pre-treatment with malonate (a reversible inhibitor of succinate dehydrogenase, Albers et al., 1996) led to an enhancement of the METH-induced striatal ATP loss, and the subsequent neurotoxicity. This underscored neuronal death by energy impairment and led to the theory of Metabolic Stress as a mechanism for METH neurotoxicity.

The neuroprotective effects of D_1 and D_2 antagonists on the toxin was demonstrated by O'Dell et al. (1993) using SCH 23390 and eticlopride respectively. Although these agents themselves induce DA release, they completely attenuated DA over-flow when combined with the toxin, suggesting a role for these receptors in the mechanism of action of the toxin as well as in the neurodegenerative changes observed in the disease itself. Sonsalla et al. (1989, 1991) proposed a role for EAAs in the pathogenesis of the disease as well as in METH toxicity when they showed that competitive and non-competitive NMDA antagonists prevented METH-induced damage by attenuating the DA over-flow induced by it. Furthermore, by concurrent treatment with cyclopentyladenosine, an adenosine A_1 receptor antagonist, Donne and Sonsalla (1994) attenuated the METH-induced changes, indicating that activating this receptor could protect against the effects of the toxin. In addition, chlormethiazole and pentobarbital have exhibited neuroprotective features when administered prior to the METH doses (Green et al., 1992). Both drugs, being $GABA_A$ receptor agonists, are thought to exert their effects indirectly by decreasing NMDA function through an increase in GABA function; mimicking the effect of MK 801 (Tricklebank et al., 1989).

Surprisingly, pre-treatment with METH blunted the efflux of DA, 5-HT and glutamate in the ST when the same animals were challenged with a neurotoxic dose of the toxin (Stephanas and Yamamoto, 1996). Furthermore, pre-treatment with deprenyl, a MAO-B inhibitor, did not offer neuroprotection, indicating that the clinical use of this compound in adjunctive therapy is

limited since it offers protection to the toxic effects of only those toxins that are metabolized by MAO-B (e.g. MPTP).

1.8.4.1 METH and DSP-4

The noradrenergic neurotoxin, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), was combined with METH to test the theory that destruction of associated neurosystems in the nigrostriatal region rendered the dopaminergic system more vulnerable to neurotoxic insults (Fornai et al., 1995). The results of our experiments with both toxins, though inconclusive at this stage, show a trend of increased DA levels in the SN and ST following treatment with METH, after prior exposure to DSP-4. The reasons for this are not yet clear, but it is possible that compensatory mechanisms resulting from the minor NA loss may have overshadowed any effect of the DSP-4.

Although an underlying reason for this combination was to eliminate the NA system so as to allow for adequate concentration of the administered METH on the DA system (fornai et al., 1995), it appears that the low density of the NA neurons in this region was insufficient to reduce the effective amount of METH going to the DA system. The exact mechanism by which the loss of NA neurons would render the DA neurons more vulnerable to toxic insults remains to be elucidated, even though loss of NA and DA neurons occur in PD (Hornykiewicz and Kish, 1986). Further work is required to demonstrate the sensitizing role (if any) played by the noradrenergic neurotransmitter system in enhancing the loss of nigrostriatal dopaminergic neurons.

1.8.4.2 METH and 3-NP

3-nitropropionic acid, an irreversible inhibitor of Complex II (succinate dehydrogenase, SDH) in the mitochondrial respiratory chain, has been proposed as a model for Huntington's disease (Ludolph et al., 1991). Recently however, it has been reported that the dose regimen could be adjusted to produce a hypo- or hyper-kinetic state in the rodent (Borlongan et al., 1997). Treating the animal with 3-NP 30 mg/kg s.c. or 10 mg/kg/day for 1-4 days, produces three stereotypic, clinically distinct stages (Ludolph et al., 1991):

Stage 1: Somnolence;

Stage 2: Hyperactivity with uncoordinated "wobbly" gait, a tendency to fall to one side, stereotyped paddling movements, and axial "roll-over";

Stage 3: Ventral or lateral recumbency with hind limbs rigidly extended, and short periods of paddling movements.

It has been observed that only animals which show stage 3 recumbency display the characteristic pattern of neurochemical change (Hamilton and Gould, 1987). The morphological lesions in these recumbent animals were usually bilateral and symmetrical, affecting the caudate-putamen, the globus pallidus, nucleus accumbens, claustrum, pre-optic nuclei, interstitial nucleus of the striae terminalis, amygdaloid nuclei, lateral and medial septal nuclei, anterior commissure and corpus callosum. Also affected are the hippocampus, including the dentate gyrus, the thalamus and the roof of the fourth ventricle (Hamilton and Gould, 1987).

Electron microscopy reveals that neurons affected by 3-NP usually exhibit dendrosomatic swelling (Gould and Gustine, 1982), suggesting some form of excitatory damage. The involvement of excitotoxins in the mechanism of toxicity with 3-NP is further confirmed by the fact that the toxin itself has no direct depolarizing effect on neurones (Riepe et al., 1992) and its neurotoxic effects are blocked by removal of excitatory inputs *in vivo* or by EAA antagonists *in vitro* (Ludolph et al., 1992b).

The rationale for combining both agents stems from the fact that 3-NP impairs energy metabolism by inhibiting ATP synthesis, activating voltage-dependent NMDA receptors, and thereby sensitizing the dopaminergic neurones to excitotoxic damage by ambient EAAs (Brouillet et al., 1995). METH, on the other hand, has been shown to cause decrease in ATP (Chan et al., 1994), and increased glutamate release directly, or indirectly (Sonsalla et al., 1989; 1991). Furthermore, the generation of free radicals following DA and glutamate release is accelerated by inhibition of the electron transport chain (Boveris and Chance, 1973). In a cyclic manner, these generated free radicals in turn enhance the release and decrease the uptake of EAAs (Bowling and Beal, 1995).

Although the inhibition of SDH activity in the brain lacks regional specificity (Gould and Gustine, 1982; Gould et al., 1985), the geographical specificity of the lesion distribution induced by combining both toxins could be accounted for by the fact that postsynaptic neurones bearing neuroexcitatory receptors and having an impaired ability to maintain energy-dependent homeostatic mechanisms, will draw heavily on the already compromised cellular energy stores when exposed to EAAs (Gould and Gustine, 1982). Thus, METH will accentuate this metabolic specificity when combined with 3-NP. In addition, 3-NP has been shown to competitively inhibit fumarase (Porter and Bright, 1980), thereby affecting the controlling role of fumarate in the oxidation of DA to NA. The implication of this action on the build up of DA remains cryptic, but it can be expected that the large amounts of DA will contribute to its auto-oxidative processes that result in increased amounts of free radicals. Similarly, METH causes DA release from cytosolic stores and sustains extracellular levels for an extended period by inhibiting COMT, MAO-B and the DA re-uptake carrier mechanism.

1.9 Mechanisms of nigral cell death in PD

Two groups of factors have been thought to impinge on the genetic susceptibility of certain individuals, to give rise to PD. Consequently, there are two broad theories for the aetiology of PD: Metabolic stress and Oxidative Stress. Both theories have clear cut components in the mechanisms of neuronal death so far proposed for METH.

1.9.1 Metabolic Stress

The Metabolic Stress theory of PD suggests that patients suffering from the disorder have an underlying metabolic defect (possibly genetic) which is exacerbated by other factors such as ageing, and may become accelerated by certain neurotoxic insults (Linnane et al., 1989). Sonsalla et al. (1989, 1991) and Albers et al.(1996) have demonstrated the vulnerability of the ST to EAA insults, especially after prior exposure to metabolic inhibitors like malonate; an agent known to reversibly affect Complex II of the mitochondrial respiratory chain. On its own, METH has also been proposed to act by metabolic inhibition as it has been shown to cause significant depletions in ATP levels (Chan et al., 1994).

In support of the Metabolic Stress theories, mitochondrial function has been shown to be compromised in PD patients (see Mizuno et al., 1997 for review). Schapira et al. (1990a) were the first to report decreased Complex I activity in the SN of parkinsonian patients, following reports that the active moiety of MPTP, MPP+, inhibits NADH-linked mitochondrial respiration (Mizuno et al., 1987; Nicklas et al., 1985). Hattori et al.(1991) confirmed this Complex I reduction in PD, and went on to show that immunostaining for Complexes II, III and IV were well retained, despite marked degenerative changes. The loss of Complex I activity was limited to the SN of PD sufferers, and was not observed in other neurodegenerative disorders affecting the SN (Schapira et al., 1990b). Paradoxically, treatment with L-DOPA has been reported to induce a reversible loss of Complex I activity (Przedborski et al., 1995). Although this loss of Complex I activity was thought to be secondary to L-DOPA treatment, this view not be directly relevant to the parkinsonian condition, as Complex I is not normally exposed to high concentrations of DA in PD patients (Cooper et al., 1995).

Situated close to Complex I in the mitochondria, is the tricaboxylic acid cycle (TCA) enzyme complex alpha-ketoglutarate dehydrogenase (αKGDH). This complex is believed to be the rate-limiting enzyme of the TCA cycle, providing NADH for Complex I and succinate for Complex II (Lai et al., 1977). In PD, it has been found to be markedly reduced in the lateral SN, the part most severely affected in PD (Mizuno et al., 1990). The consequence of a loss of Complex I and αKGDH activity is mitochondrial respiratory failure, resulting in an imbalance in energy homeostasis. Precisely, there is a loss of ATP supply required to maintain the integrity of the Na^+-K^+ ATPase that regulates the influx of NA^+. The consequence of this is the fact that intracellular NA^+ would then have to be exchanged for Ca^{2+} in the extracellular space, leading to a rise in the intracellular levels of Ca^{2+} (Stys et al., 1990). The rise in intracellular Ca^{2+} concentration induces phospholipases, proteases and endonucleases, with toxic consequences (Nicoreta et al., 1990; Orrenius et al., 1991). Furthermore, the build up of Ca^{2+} also exerts deleterious effects on the mitochondria themselves, impeding ATP synthesis, and inducing apoptosis (Hartley et al., 1994; Wyllie, 1980). The ultimate effect of these processes is cell death.

1.9.2 Oxidative Stress

The Oxidative Stress pathways, on the other hand, suggest that PD is caused by an oxidative imbalance which results in a preponderance of free radicals, overwhelming cellular neutralizing mechanisms, impairing cellular processes and causing cell death (Jenner, 1992; 1995). The human body employs several mechanisms for neutralizing toxins that induce the formation of free radicals. With respect to PD, recent interest has been focused on the reactions catalyzed by the enzyme superoxide dismutase (SOD). Superoxide formed in respiring cells, is normally oxidized to oxygen and reduced to hydrogen peroxide by SOD. Although hydrogen peroxide is relatively stable, it could be converted to the more reactive hydroxyl radical in the presence of iron. In PD, one of the isozymes of SOD, the copper-zinc superoxide dismutase (Cu-Zn SOD) is increased in the SN (Martilla et al., 1988). Since SOD increases hydroxyl radical formation, this increase in Cu-Zn SOD will imply an increase in the formation of hydroxyl radicals, with the attendant effect of increased lipid peroxidation. Not surprisingly, Dexter et al.(1989) have reported an increase in lipid peroxidation in the SN of PD patients. It is interesting that METH toxicity has been shown to involve lipid peroxidation and increased SOD activity in the ST (Acikgoz et al., 1998), as well as increased generation of peroxynitrite (Imam et al., 1999). Furthermore, it has not been possible to induce damage to dopaminergic neurones in Cu-Zn SOD transgenic mice treated with METH (Cadet et al., 1994; Hirata et al., 1996; 1998).

Additional support for the role of Oxidative Stress in the aetiology of PD comes from the observation of a reduction in the levels of reduced glutathione in the SN of PD by 30-60 % (Perry et al., 1986; Sofic et al., 1992). Glutathione is a natural antioxidant, and serves as a substrate for glutathione peroxidase. A further boost for Oxidative Stress is the observed increase in iron and aluminium content of the SN in PD patients (Good et al., 1992). Whereas iron catalyzes the formation of hydroxyl radicals from hydrogen peroxide, its accumulation with aluminium accelerates membrane lipid peroxidation (Gutteridge et al., 1985).

Taken together, it is certain that both Metabolic Stress and Oxidative stress occur in PD. However, the exact sequence of events is still a matter of debate. Jenner et al. (1992) have suggested that Oxidative stress occurs first, judging from reduced Complex I activity and reduced glutathione levels in patients with incidental Lewy body disease. In this state, nigrostriatal dopamine neurones were not degenerated. This does not completely reflect the state of affairs in PD, where there is also a decrease in αKGDH activity. Loss of αKGDH activity by itself can itself lead to mitochondrial respiratory failure, as this enzyme supplies NADH required for complex I activity. Our experiments with 3_NP suggest that energy impairment is a primary event in lossof dopaminergic activity in the nigrostriatal pathway.

On the whole, it is reasonable to believe that both processes occur simultaneously or interchangeably. It is known that a decrease in ATP (from metabolic abnormality) would cause activation of NMDA receptors by impeding the efficiency of the ATP-dependent ionic carriers which maintain the ionic gradients across cell membranes (Di Monte et al., 1996). Such imbalance will normally lead to increased intracellular levels of Na^{+}, Ca^{2+} and Cl^{-}. The excessive amounts of Ca^{2+} in the cell would normally arrest mitochondrial respiration (Beal et al., 1993), accentuating the Metabolic Stress. Furthermore, increased Ca^{2+} in the cell would give rise to stimulation of nitric oxide synthase with the resultant production of nitric oxide. This nitric oxide will itself react with superoxide to produce the neurotoxic radical, peroxynitrite (Bowling and Beal, 1995). The large amounts of these free radicals will normally overwhelm the cellular neutralizing mechanisms leading to a state of Oxidative Stress, resulting in cell death. A summary of the possible mechanisms involved in nigral cell death are shown in Fig. 1.2 overleaf.

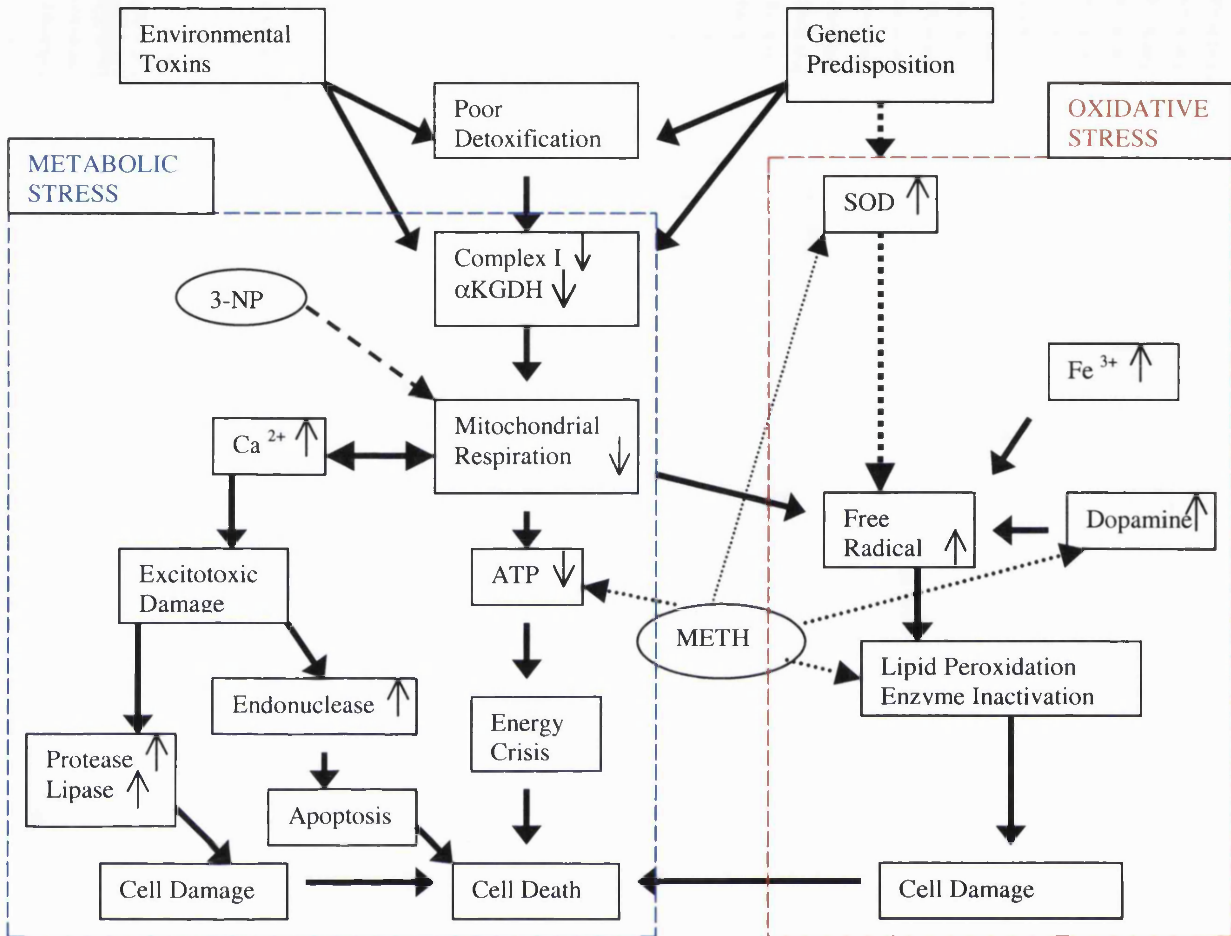


Fig. 1.2 - Mechanisms of nigral cell death.

Aims and objectives

1. To identify a suitable combination of 3-nitropropionic acid and methamphetamine that will achieve significant lesioning of the nigrostriatal dopaminergic pathway in the rat, as determined by high performance liquid chromatographic analyses of the tissue levels of monoamines, excitatory amino acids and AADC activity.

2. To assess the effects of the lesion thereby induced (i.e. by 3-NPM) on the spontaneous and drug-induced behaviour of the rat.

3. To determine the progressive neurochemical effects consequent upon exposure to the toxic effects of 3-nitropropionic acid and methamphetamine.

4. To identify additional mechanisms of locomotor induction by L-DOPA, in the apparent absence of its conversion to dopamine, in reserpine-treated, normal and 3-NPM-treated male Wistar rats.

5. To assess the roles of NMDA antagonism and dopamine receptors in the proposed dopamine-independent mechanism of action of L-DOPA.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animals

All experimental procedures were conducted in accordance with the Animal (Scientific Procedures) U.K. Act, 1986. Male Wistar rats (A.R. Tuck, U.K.) weighing 180-240g, at the start of the experiments, were used. The animals were initially group-housed at 22 $\pm$1 °C under fluorescent lighting from 0700 to 1900h, with rat diet and water available ad libitum. Experiments were conducted between 0800 and 1800 h. The gross behaviour and body weight of the rats were noted daily until the animals were sacrificed. Great care was taken to meet the temperature requirements of the hypothermic reserpinised rats and the hyperthermic, methamphetamine-treated rats. Fatality of the 3-NP and METH-treated rats was reduced by leaving the animals at 14°C, after the METH injection. Conversely, hypothermia after reserpine administration was overcome by maintaining the rats in a heated room (at least 26°C).

2.2 Induction of parkinsonism

2.2.1 Preliminary experiments

3-Nitropropionic acid hydrochloride (Sigma, USA) and methamphetamine hydrochloride (Sigma, USA) were made up in normal saline (0.9 % sodium chloride;Fluka, Switzerland). 3-NP was administered s.c. while METH was administered i.p. The injections were given between 0900 and 1800 h. The various dosing schedules for the 3-NP and METH combinations are as shown Table 2.1 below.

Table 2.1 - Index of treatment modes for 3-NP and METH

MODE	EXPERIMENTAL PARADIGM
P1	3-NP (2 x 15mg/kg,s.c., q6 h) for 1 day, followed by METH (4 x 8mg/kg,i.p., q2h) or saline (for 3-NP controls) three days after. -Five days after, apomorphine challenge. -Six days after, neurochemical assay.
P2	3-NP (2 x 15mg/kg,s.c., q6h) for two consecutive days, followed by METH (4 x 8mg/kg, i.p., q2h) or saline three days after. -Five days after, apomorphine challenge. -Six days after, neurochemical assay.
P3	3-NP (2 x 15mg/kg,s.c., q6h) for three consecutive days, followed by METH (4 x 8mg/kg,i.p., q2h) or saline three days after. -Five days after, apomorphine probe. -Five days after, neurochemical assay.
P4	3-NP (2 x 40mg/kg,s.c., q6h) for 1 day, followed by METH (4 x 8mg/kg, i.p., q2h) two days after. -Fourteen days after, the injection procedure for both drugs was repeated. -Five days after the second METH injection, apomorphine challenge. -Three days after, neurochemical assay.
P5	3-NP(2 x 40mg/kg,s.c, q6h) for 1 day, followed by METH (4 x 8mg/kg,i.p., q2h) the next day. -Five days after, apomorphine challenge. -Six days after, neurochemical assay.
P6	METH (4 x 8mg/kg,i.p., q2h), followed by 3-NP (10mg/kg,s.c.) for the next four days. -Seven days after the METH injection, the dosing schedule was repeated. -Five days after the last 3-NP injection, apomorphine challenge. -Five days after, neurochemical assay.
P7	3-NP (20mg/kg,s.c.) for four consecutive days, followed by METH (4 x 8mg/kg i.p., q2h). -Five days after, apomorphine challenge. -Four days after, neurochemical assay.

2.2.2 Model experiments

With the confirmation that a dosing schedule of 3-NP (20 mg/kg, s.c. per day) for four days, followed by METH (4 x 5mg/kg i.p., q 2 h) on the fifth day and a second course of 3-NP for the succeeding four days, gave a 70% depletion of DA in the ST, further experiments were then carried out based on this so-called 3-NPM model. METH and 3-NP were made up in normal saline, but the pH of the latter was adjusted to 7.0 using a concentrated solution of sodium hydroxide (Fluka, Switzerland).

The injections of 3-NP were typically administered at 1800 h, while the first METH injection was usually administered at 1000 h. Saline controls (monoamine intact rats) received injections of normal saline (1 ml/kg) once daily on days 1-4, four times on day 5 and once daily for the remaining four days. METH controls received saline on all days except day 5, when they received four injections of METH (5mg/kg,ip, q2h). Similarly, the 3-NP controls received daily doses of the compound on all days except day 5, when they received four injections of normal saline at 2 h intervals. The dosing schedules are illustrated in Table 2.2.

Table 2.2 Neurotoxin injection schedule

Time	3-NP	METH	3-NPM*	Control
Day 1	20 mg/kg	saline	20 mg/kg	saline
Day 2	20 mg/kg	saline	20 mg/kg	saline
Day 3	20 mg/kg	saline	20 mg/kg	saline
Day 4	20 mg/kg	saline	20 mg/kg	saline
Day 5	saline 4 x 1 ml/kg	4 x 5 mg/kg	METH 4 x 5 mg/kg	saline 4 x 1 ml/kg
Day 6	20 mg/kg	saline	20 mg/kg	saline
Day 7	20 mg/kg	saline	20 mg/kg	saline
Day 8	20 mg/kg	saline	20 mg/kg	saline
Day 9	20 mg/kg	saline	20 mg/kg	saline

* Doses refer to 3-NP, except on day 5.

Behavioural experiments were conducted on the 5th day while neurochemical assays were conducted on the 7th day after the last injection, except when the animals were used for the weekly experiments. In that case, they were sacrificed on the 7th, 14th, 21st and 28th days after the last 3-NP injection.

2.3 Behavioural experiments

2.3.1 Control rats

The rats were allowed to settle down for at least one week prior to use. Behavioural experiments were conducted in an isolated room with the humming of the ventilator serving as white noise. Four animals were observed per session. On the day of the experiment, the rats were weighed and placed individually in Perspex observation cages (2ft x 2ft x 1ft high) and the lids placed over, leaving enough room for ventilation.

Spontaneous locomotor activity was recorded using Radiospares 8960 Microwave Doppler Module units connected to an amplifier, timer and LED display unit. The units were designed and constructed in our laboratory and calibrated to detect gross movements. Locomotor activity scores and rearing counts were recorded at ten-minute intervals for two hours. The animals were then injected and observed for another two hours. The following is a checklist used for manual scoring of the presence or absence of the following behaviours:

Back-pedalling
Forward locomotion
Grooming – Facial/Whole body
Licking – Fore-paw/Hind paw
Chewing – Vacuous/Purposeful
Sniffing – Floor/Wall/Air
Rearing – Cage side/Cage centre
Head weaving
Circling
Ataxia
Jumping
Vocalisation

2.3.2 3-NPM-treated rats

3-NPM-treated rats were used for behavioural experiments 5-8 days after the eighth 3-NP injection, but typically on the 5th day; except the 3-NP 30mg/kg group that was used 18-24 h after the eighth 3-NP injection. Reserpinized rats were used 20-24 h after treatment.
The procedure of 2 h habituation prior to drug treatment was maintained for 3-NPM rats, while a 30 min habituation period was observed for reserpinized rats. Following drug treatment, the animals were again placed in their respective cages and observed for a further 3 h, using the checklist and the 10 min recordings.

2.3.3 Drug treatment in the behaviour experiments

All drugs were administered at a dose volume of 1 ml/kg body weight. In experiments were dose response curves were not determined, the choice of dose and dosing schedule was made on the basis of previous experiments in our laboratory.

2.3.3.1 d-Amphetamine

Dextroamphetamine sulphate (Sigma, USA) was administered in an aqueous solution at a dose of 4 mg/kg s.c., 30 min after NSD 1015, AMPT or L-DOPA.

2.3.3.2 AMPT

The TH inhibitor, α-methyl-DL-p-tyrosine methyl ester hydrochloride (AMPT; Sigma, USA), was administered as an aqueous solution at a dose of 200 mg/kg i.p., 30 min before the administration of d-amphetamine.

2.3.3.3 Apomorphine

An aqueous solution of apomorphine hydrochloride (Sigma, USA) was stabilized with 0.1mg/ml ascorbic acid (Sigma, USA), kept cool and away from direct light prior to injection. The drug was administered s.c. at a dose of 0.5 mg/kg body weight as a probe for DA receptor denervation supersensitivity. In the experiments to determine the selectivity of sensitization, the drug was given 30 min after administration of a selective DA D_1 or D_2 receptor antagonist.

2.3.3.4 Benserazide

Benserazide hydrochloride (Sigma, USA) was administered as an aqueous solution at a dose of 50 mg/kg body weight, i.p., 30 min before the administration of L-DOPA or LDME.

2.3.3.5 Budipine

1-t-butyl-4,4-diphenylpiperidine hydrochloride (budipine; gift from Byk Gulden, Germany) was administered at a dose of 10 mg/kg,i.p in a warm, aqueous solution. It was administered alone or in combination with L-DOPA. In the latter instance, it was administered 30 min before the AADC inhibitor (i.e. 1 h before L-DOPA).

2.3.3.6 L-DOPA

L-DOPA base (Sigma, USA) was dissolved in a slightly alkalinized aqueous solution and kept away from light just prior to injection. The drug was given i.p. at doses of 25, 50,100 and 200 mg/kg body weight, 30 min after NSD 1015 and/or benserazide, or 1 h after budipine.

2.3.3.7 L-DOPA methyl ester

L-DOPA methyl ester (LDME; Sigma, USA) was dissolved in double-deionized water and administered intraperitoneally at doses of 25, 50 100 and 200mg/kg, half an hour after NSD 1015 and/or benserazide.

2.3.3.8 (+)-MK-801

The non-competitive NMDA receptor-channel blocker, (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)-cyclohepten-5,10-imine maleate ((+)-MK-801; Tocris, UK), was administered as an aqueous solution to both mono-amine intact and depleted rats at a dose of 0.3 mg/kg, i.p.

2.3.3.9 NSD 1015

m-hydroxybenzylhydrazine dihydrochloride (NSD 1015; Sigma, USA), a central and peripheral AADC inhibitor, was administered as an aqueous solution at a dose of 100mg/kg body weight i.p., alone or in combination with benserazide 30 min prior to the L-DOPA dose.

2.3.3.10 RU 24213

This D_2 agonist was administered subcutaneously at a dose of 5 mg/kg in an aqueous solution. When combined with a DA D_1 or D_2 receptor antagonist, N-n-propyl-N-phenylethyl-p-[3-hydroxyphenyl] ethylamine hydrochloride (RU 24213; Roussel Ucclaf, Switzerland) was given 30 min after the antagonist.

2.3.3.11 SCH 23390

In order to exert its DA D_1 antagonism, [R]-[+]-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol hemimaleate (SCH 23390; Schering Corporation, NJ, USA) was administered i.p., 30 min before the DA receptor agonist, at a dose of 0.3 or 1 mg/kg.

2.3.3.12 SKF 38393

($\pm$)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF 38393; Research Biochemicals International, Natick, USA) was administered i.p. at a dose of 30 mg/kg body weight.

2.3.3.13 ($\pm$) Sulpiride

A potent DA D_2 receptor antagonist, ($\pm$) sulpiride hydrochloride (Sigma, USA) was administered in an aqueous medium (containing a few drops of concentrated acetic acid) at a dose of 100 mg/kg s.c.

2.4 Neurochemistry

All apomorphine-screened rats were sacrificed at least two days after the behavioural test (i.e seven days after the eighth injection), except the 3-NP 30 mg/kg groups (3-NP3 and 3-NP3M) which were assayed about 4 h after the apomorphine test.

2.4.1 Tissue levels of catecholamines

This procedure was designed to determine tissue levels of DA and 5-HT in the desired brain regions. Briefly, the animals were killed by decapitation, with prior stunning, and their brains promptly removed and placed in ice-chilled normal saline. The brain was then placed on a

metal dissecting board chilled on ice, and the NAcc, PFC, SN and ST were dissected out. The tissues from the individual brain regions were homogenized separately by placing each in a glass homogenizing tube containing ice-chilled 0.5 M perchloric acid (Fisons Ltd. England) and 0.5 nM isoprenaline as the internal standard. Three ml of this solution was used for PFC and ST while 1 ml each was used for the NAcc and SN. 1 ml of the homogenate was placed in an Eppendorf centrifuge tube from which 100 μl was saved in a mini-Eppendorf tube and frozen at –80°C until required for the protein assay. The homogenate was then centrifuged at approximately 3,000 x g for 10 min. Thereafter, 20 μl of the supernatant was collected into a mini-Eppendorf tube and frozen at –80°C until required for High Performance Liquid Chromatography (HPLC) analysis.

2.4.2 AADC enzyme assay

The AADC enzyme assay procedure was adapted from that used by Nagatsu and Okuno (Nagatsu et al., 1979; Okuno and Fujisawa, 1983). The assay is based on the enzymatic conversion of L-DOPA to DA, with measurement of DA by HPLC, with electrochemical detection.

The rats were killed by decapitation and their brains quickly removed and placed in ice-chilled normal saline. The brains were then quickly placed on a metal dissecting board, sliced coronally with a razor blade, and the four regions of interest – NAcc, PFC, SN and ST – were dissected out. The individual brain regions were then placed in a glass homogenizer containing 0.25 M sucrose (BDH, England); 3 ml for the PFC and ST, and 1 ml each for the NAcc and SN. Homogenization was achieved manually by the vertical motion of the glass tube against a rotating plastic rod. 1 ml of the homogenate was transferred to an Eppendorf centrifuge tube, from which 100 μl was withdrawn and placed in the corresponding mini-Eppendorf tube and stored at –80°C for future use in the protein assay. The remaining portion of the homogenate in the centrifuge tube was centrifuged at ~ 3,000 x g for 10 min. A 20 μl quantity of the resultant supernatant was then used for the enzymic incubation.

The incubation mixture consisted of the following in a total volume of 380 μl: ascorbic acid, 0.18 mM; EDTA, 0.11 mM; hepes hemisodium salt buffer (pH 7.2), 50 mM; 2-

mercaptoethanol, 1 mM; pargyline, 0.11 mM; pyridoxal-5-phosphate, 0.01 mM; L-DOPA (0.53 mM) or 5-hydroxytryptophan (5-HTP) or double de-ionized water (for blanks). To this mixture, 20 µl of the tissue supernatant (containing the AADC enzyme) was added. The incubation was done for 10 min in a water bath set at 37°C. the reaction was stopped by the addition of 80 µl of ice-chilled 0.5 M perchloric acid containing the internal standard, isoprenaline (0.5 nM). The mixture (in Eppendorf tubes) was then transferred to a centrifuge and centrifuged at ~3,000 x g for 10 min. Thereafter, aliquots of the supernatant were stored in mini-Eppendorf tubes for the HPLC analysis. The activity of the enzyme was expressed as nanomoles DA or 5-HT per milligram of protein per hour.

2.4.3 HPLC analysis

2.4.3.1 Analysis of catecholamines

Samples from both the tissue level and AADC experiments were analysed by HPLC with electrochemical detection. In outline, the HPLC system consisted of a reservoir of recirculated mobile phase with the following constituents (in mM): citric acid, 35; sodium acetate anhydrous, 90; octanesulphonic acid, 0.06; EDTA, 0.34; all dissolved in 5.5 % methanol (Rathburn, Scotland) and set at a pH of 4.2. All the constituents of the mobile phase and incubation mixture were of analytical grade and supplied by Fluka, Switzerland. The mobile phase reservoir was connected to a de-gassing unit (X-act 4-channel, Jour Research,) and to a pump (model SA-6410B, Servern analytical, USA) set at a flow rate of 1 ml/min and at a pressure of approximately 200 bar. The pump was connected to a Rheodyne (model 7125, Cotati Ltd., California, USA) with manual injection. This was linked to a guard cell (model 5020, ESA, USA) and to a C18 (80-200-C3) reverse-phase column (Rainin Corporation, Woburn, USA). The column was connected to a porous carbon electrode analytical cell (model 5011, ESA, USA), which was in turn linked to an electrochemical detector (Coulochem model 5100A, ESA, USA), and then to a personal computer (equipped with ChromPerfect software). The settings for the electrochemical detector were: guard cell +0.5V, detector 1 -0.17V and detector 2 (analytical cell) +0.40V, with the range set at 20nA/volt for full scale deflection.

The samples were assayed for levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), L-DOPA and 5-hydroxytrptophan (5-HTP). The tissue levels were determined from the "blank" incubation mix tubes (i.e. tubes containing neither 5-HTP nor L-DOPA). All samples were stored at-80°C and thawed just prior to use. The HPLC was routinely calibrated with a cocktail of standards, containing each of the desired metabolites, before each batch of experiments.

2.4.3.2 Analysis of excitatory amino acids

Tissue levels of the excitatory amino acids aspartate and glutamate were determined from the "blank" samples using HPLC with fluorometric detection. In outline, the set up consisted of two reservoirs of buffers, A and B. Buffer A contained 250 mM sodium dihydrogen phosphate (pH 5.5) with 20% v/v methanol while buffer B consisted of pure, HPLC grade methanol (Rathburn, Scotland). The buffers were pumped at a flow rate of 0.6 ml/min via a solvent delivery system model pm-80 (Biotech, UK). The solvent delivery system provided a gradient separation with the following parameters:

Time (min)	Buffer A	Buffer B
0	90 %	10 %
4	90 %	10%
5	0 %	100 %
6	0 %	100 %

The pump was connected to a CMA/200 refrigerated microsampler (Biotech, UK). To facilitate fluorescence detection, the amino acids were derivatized with an o-phthaldialdehyde-thiol (O.P.T.) complex. Briefly, 0.04 M of the reagent was prepared by dissolving 27 mg of 0-phthaldialdehyde (O.P.A.) in 500 µl of absolute ethanol, and making up to 5 ml with borate buffer (pH 9.0). A 50 µl quantity of 2-mercaptoethanol was then added and the solution kept out of light and refrigerated at 4°C overnight, prior to use. The derivatization process was carried out in the autosampler and the resultant mix was injected onto a 3 µm microbore C18 (80-215-C5) reverse phase ODS column (Rainin Corporation, USA) interfaced with a CMA

280 fluorescence detector (Biotech, UK) set at an amplification factor of 50. The temperature of the column was controlled by a model LC-22C temperature controller (Biotech, UK). The system was calibrated prior to each analysis, with a standard solution containing 25 μM of the amino acids of interest.

2.4.4 Protein assay

The method of Bradford (1976) was employed in determining the protein concentration within a tissue sample, using Bovine Serum Albumin as the standard. Briefly, 780 μl of double de-ionized water was aliquoted into clean, dry test-tubes and 200 μl of the Bio-Rad protein assay reagent (Bio-Rad laboratories, Germany) was added to each. To this solution was added a 20 μl quantity of the protein sample (brain homogenate), followed by vortexing. The solutions were analysed using an ultra-violet spectrophotometer set at a wavelength of 595 nm. The mean absorbance reading of a triplicate assay of each brain region, was read off a standard curve to give the amount of protein (in milligrams) in that region. The concentration of mono-amines and excitatory amino acids was then expressed as a function of the amount of protein in the tissue sample analysed.

2.5 Statistical analysis

The results are expressed as mean $\pm$ S.E.M. for a minimum of n=6 separate determinations. The neurochemical and behavioural data were analysed by one or two-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. Statistical analysis was done on a computer equipped with InStat, with statistical significance set at $p<0.05$.

RESULTS

CHAPTER THREE

NEUROCHEMICAL BASIS OF THE 3-NPM MODEL

3.1 Introduction

Methamphetamine is a common drug of abuse known to release DA and glutamate from the respective neurones (Sonsalla et al., 1989). The release of DA from the cytosolic pool (Wagner et al., 1983) or the vesicular stores (Anderson et al., 1998; Sabol and Seiden, 1998) occurs via the reversal of the DA transporter (Albers et al., 1996; Fleckenstein et al., 1997; Wagner et al., 1983). In energy-deficiency states, this ATP-dependent mechanism has been shown to be sufficient to trigger mechanisms that result in neuronal death via metabolic stress (Albers et al 1996; Chan, et al., 1994), and to render the neurones susceptible to the excitotoxic insults of endogenous glutamate (Albers et al., 1996; Nash et al., 1992; Sonsalla et al., 1998). Simultaneously, the release of DA in such large amounts accelerates the build-up of free radicals associated with DA auto-oxidation (O'Dell et al., 1993). This overwhelms the neutralizing mechanisms, producing a state of oxidative stress (Jenner, 1992; Zeevalk et al., 1998). Furthermore, the DA build-up has been shown to facilitate the condensation reaction of DA with acetaldehyde to yield the potent, endogenous neurotoxin, salsolinol (Maruyama et al., 1998). The ultimate effect is the death of the terminals (Albers et al., 1996) and/or cell bodies (Sonsalla et al., 1996, 1997) of DA, 5-HT and other neurones. The susceptibility of the neurones to energy impairment or METH toxicity varies from one region of the CNS to another, and from one transmitter system to the next, with DA neurones showing greater vulnerability than GABA and 5-HT neurones to lesioning (Araujo and Hilt, 1998; Hotchkiss et al., 1979; Zeevalk et al., 1998). Furthermore, the direct stratonigral output pathway is more susceptible than the indirect striatopallidal output pathway to 3-NP toxicity (Araujo and Hilt, 1998).

The ability of METH to incorporate both these fundamental theories – Metabolic and Oxidative Stress – sets the stage for its potentiation by a variety of neuroactive substances. PD is known to occur more commonly in the later stages of life, stages that are synonymous with impairment in energy metabolism or energy deficiency. An allusion to a causal role for energy impairment in the pathogenesis of PD can be seen in the deficiency of Complexes I, II and III of the mitochondrial energy cycle in patients with the early, untreated form of the disorder (Haas et al., 1995; Jenner, 1992). Examination of the substantia nigra of these patients reveals decreased levels of NADH cytochrome c reductase, a marker of Complexes I, II and III

(Schapira et al, 1990). The leading primate model of the disease, the MPTP model, is based on the ability of its active ion (MPP^+) to also inhibit the said Complex I (Adams and Odunze, 1991).

3-NP is an irreversible inhibitor of succinate dehydrogenase (Complex II) of the mitochondrial electron transport chain (Zeevalk et al., 1995). Our preference for 3-NP over malonate (another Complex II inhibitor) stemmed from the irreversible inhibition of Complex II with the former (Ludolph et al., 1991), its being ten times more potent than malonate (Zeevalk et al., 1995), its efficacy via parenteral administration, and its natural occurrence, being present as a contaminant in sugar-cane (Gould and Gustine, 1987). Furthermore, 3-NP is not known to cause release of DA or glutamate (Zeevalk and Nicklas, 1991; Zeevalk et al., 1995).

In establishing a dose for 3-NP, care was taken to achieve a balance between maximal inhibition or neuronal damage (as was demonstrated by the stage 3 phenomenon (Ludolph et al., 1991)) and minimum paralysis or death. Although the higher doses tended to produce the said phenomenon rapidly, they were more likely to cause paralysis, or even death. The lower dose of 20 mg/kg produced the phenomenon by the second or third day of injection, and yielded no hind-limb paralysis or fatality until combined with METH.

The rationale behind the different dosing schedules in the combination of both agents stemmed from the keenness to produce a simple and effective means of lesioning the nigrostriatal pathway in the rat, as determined by selective reductions in nigrostriatal DA synthesis and content, and from corresponding alterations in the spontaneous and drug-induced motor behaviour of the animals.

3.2 Results

3.2.1 Preliminary investigations

The preliminary experiments were geared towards the determination of optimum doses and treatment schedules for combining METH and 3-NP, so as to yield maximum nigrostriatal DA depletion and behavioural signs of increased sensitivity to DA receptor agonists, while limiting the toxic effects of both agents. The findings formed the basis for more elaborate investigation in the "3-NPM" treatment schedule.

3.2.1.1 Effects of METH on tissue DA levels

The brains of rats treated with four, two-hourly doses of METH (4, 8 and 12.5 mg/kg i.p.) were assayed for DA seven days after treatment. None of the doses significantly ($p>0.05$) altered tissue dopamine levels in the NAcc, PFC and SN (Fig. 3.1). However, treatment with 4x8 and 4x12.5 mg/kg METH gave rise to significant decreases in striatal DA ($p<0.01$). A dose of 4x8 mg/kg, q2 h METH was selected for future combination with 3-NP, as this gave a significant depletion of DA in the ST (43 % of control levels). Although a dose of 4x12.5 mg/kg produced a greater percentage of depletion, its high fatality rate of 80 % (compared to the 33% fatality for the 4x8 mg/kg dose (Fig. 3.27)) made it a second-choice dose. The 50 % striatal DA depletion observed with 4 x 12.5 mg/kg METH differs from the 82 % depletion reported for mice (Johnson et al., 1992).

Tissue dopamine levels following treatment with METH

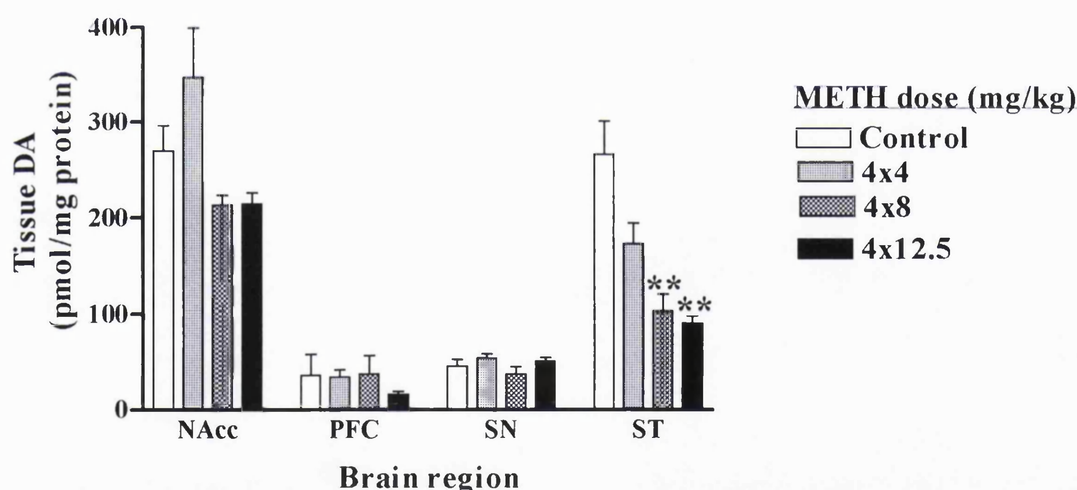


Fig. 3.1 - Tissue DA levels in the NAcc, PFC, SN and ST of rats seven days after treatment with four two-hourly doses of 4, 8 and 12.5 mg/kg METH (i.p.). The results are the means of six replicates + S.E.M. ($p<0.01$ versus control)**

3.2.1.2 Effects of different combinations of 3-NP and METH on tissue DA levels

Tissue levels of DA remained normal in the NAcc in all but one of the seven treatment paradigms (Fig.3.2). In paradigm P6, DA was reduced by 46 % compared to control values ($p<0.05$). In this dosing schedule, METH (4 x 8 mg/kg) was administered a day before the start of four daily doses of 3-NP (10 mg/kg). Two days later, the treatment was repeated, and the rats were sacrificed ten days after the last injection (see table 2.1). There were no significant changes in all four brain regions with the 3-NP controls. Dopamine levels were unaltered in the PFC ($p>0.05$) following treatment with all seven schedules (Fig.3.3). In the SN, paradigm P1 caused a paradoxical increase in DA levels ($p<0.05$; Fig. 3.4), although actual DA levels were more variable in these experiments.

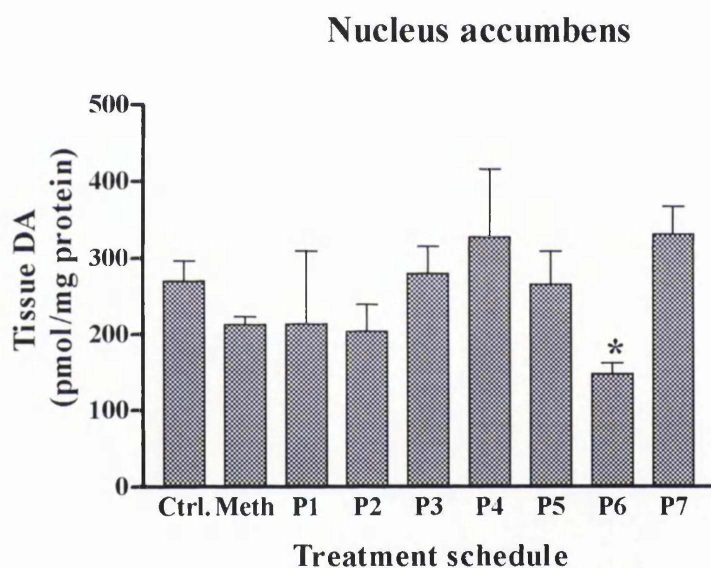


Fig. 3.2 - The effects of 3-NP and METH combinations on DA levels in the NAcc. (*p<0.05 versus control)

Prefrontal cortex

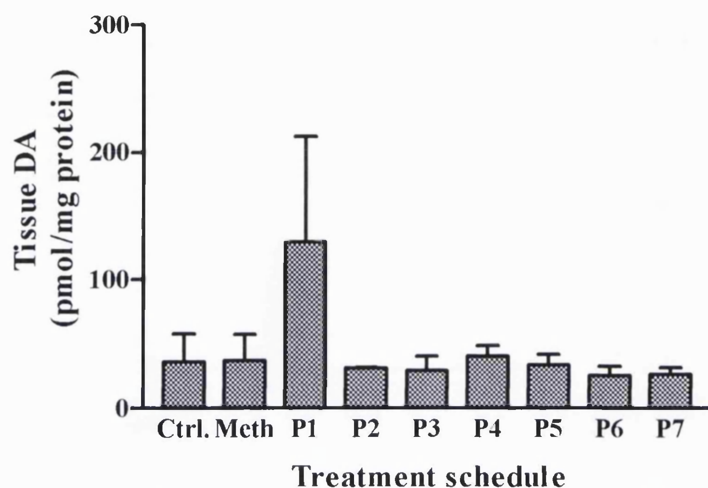


Fig. 3.3 - The effects of 3-NP and METH combinations on DA levels in the PFC. ($p>0.05$ versus control).

Substantia nigra

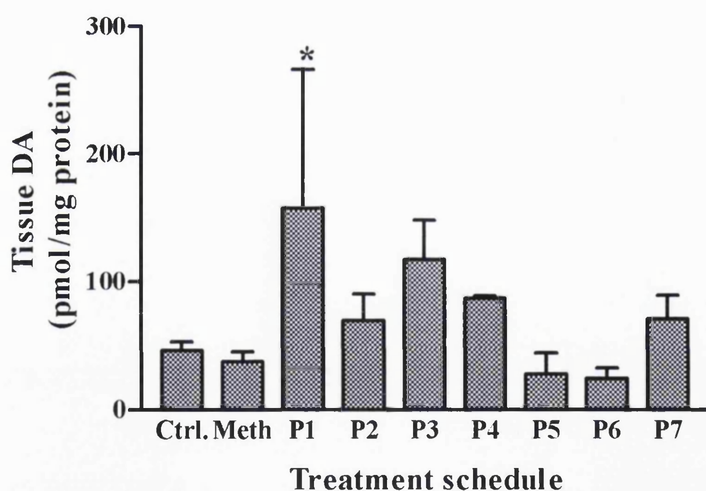


Fig. 3.4 - The effects of 3-NP and METH combinations on DA levels in the SN. (*$p<0.05$ versus control).

Tissue DA levels in the ST were significantly lowered in all paradigms ($p<0.05$, $p<0.01$), except in P1 and P5 (Fig.3.5). These marked reductions in dopamine were often accompanied by hind-limb paralysis with high fatalities among the groups (see fig. 3.26).

Corpus striatum

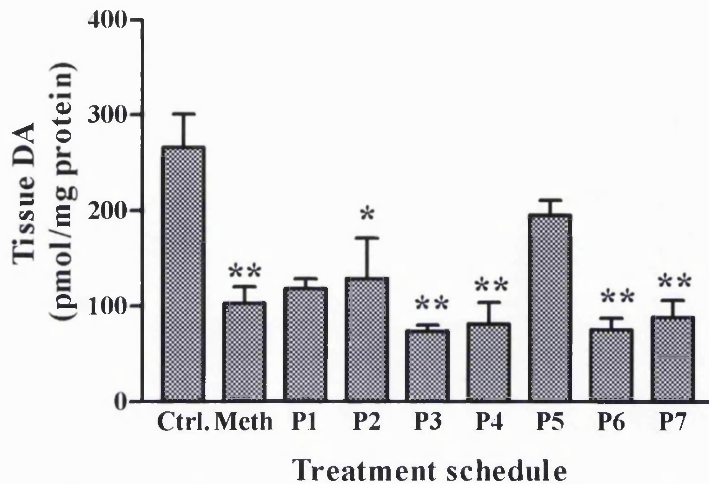


Fig. 3.5 - The effects of various combinations of 3-NP and METH on tissue DA levels in the ST. (*p<0.05, **p<0.01 versus control).

3.2.1.3 Effects of different combinations of 3-NP and METH on tissue 5-HT levels

Tissue levels of 5-HT in the NAcc showed great susceptibility to 3-NP and METH treatment, attaining 95 % and 100 % depletion with paradigms P6 and P4 respectively (Fig. 3.6). METH by itself had no significant effect on 5-HT in this region. The dosing schedule in paradigm P4 also resulted in a 100 % depletion of 5-HT in the PFC, while P6 gave rise to an 87 % reduction in 5-HT in this region (Fig. 3.7). In the SN, the P4 paradigm also caused a 100 % depletion of 5-HT (Fig.3.8). The dosing schedules in paradigms P5, P6, P7 and P3 also gave rise to significant reductions in 5-HT tissue values (p<0.01 and p<0.05 respectively). A 100 % loss of 5-HT was observed in the ST, after paradigms P4 and P5 (Fig.3.9).

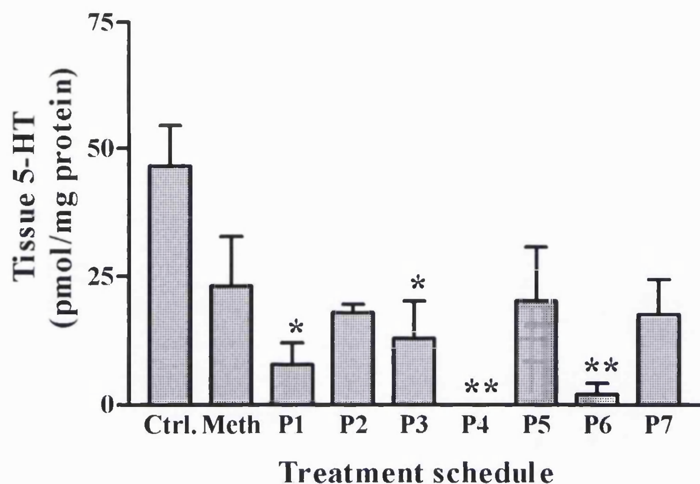


Fig. 3.6 - The effects of different combinations of 3-NP and METH on tissue 5-HT levels in the NAcc. (*p<0.05, **p<0.01 versus control)

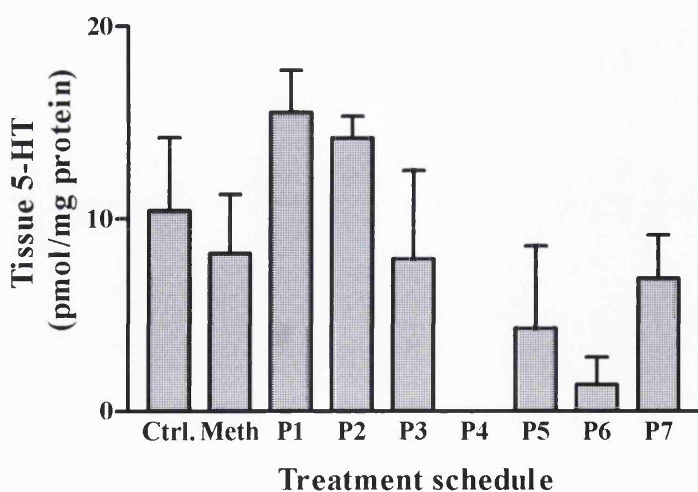


Fig. 3.7 -The effects of 3-NP and METH combinations on the tissue levels of 5-HT in the PFC. (p>0.05 versus control).

Substantia nigra

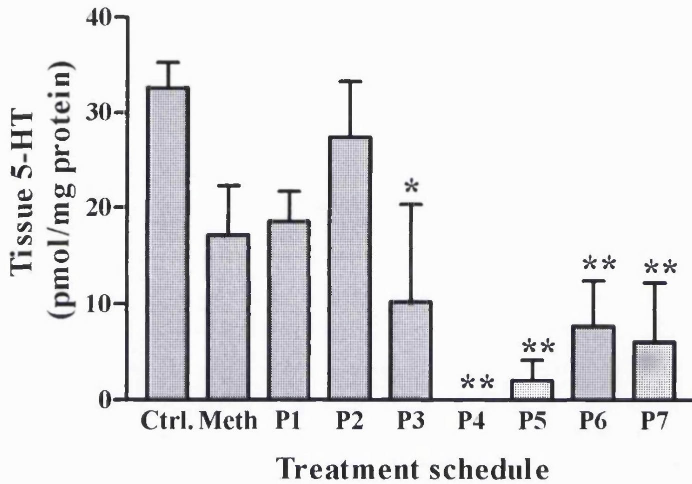


Fig. 3.8 - The effects of 3-NP and METH combinations on 5-HT levels in the SN. (*p<0.05,**p<0.01 versus control).

Corpus striatum

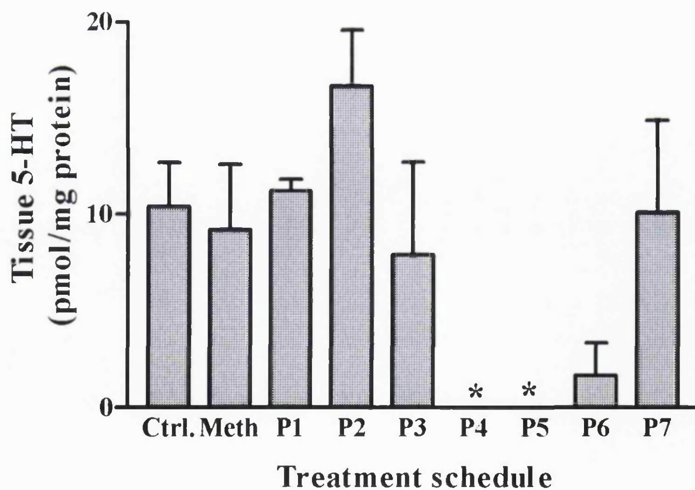


Fig. 3.9 - The effects of 3-NP and METH treatment on the tissue levels of 5-HT in the ST. (*p<0.05 versus control).

3.2.1.4 Locomotor response to a challenge with apomorphine

A behavioural assessment of the extent of lesioning was made five days after the last injection of toxin, using apomorphine (0.5 mg/kg s.c.). There were no differences in the responses of rats from paradigms P1 to P4 and P6, when compared with the locomotor response of habituated control rats (Fig. 3.10 (a) and (b)). Paradigms P5 and P7 showed significant ($p<0.001$) increases in locomotion.

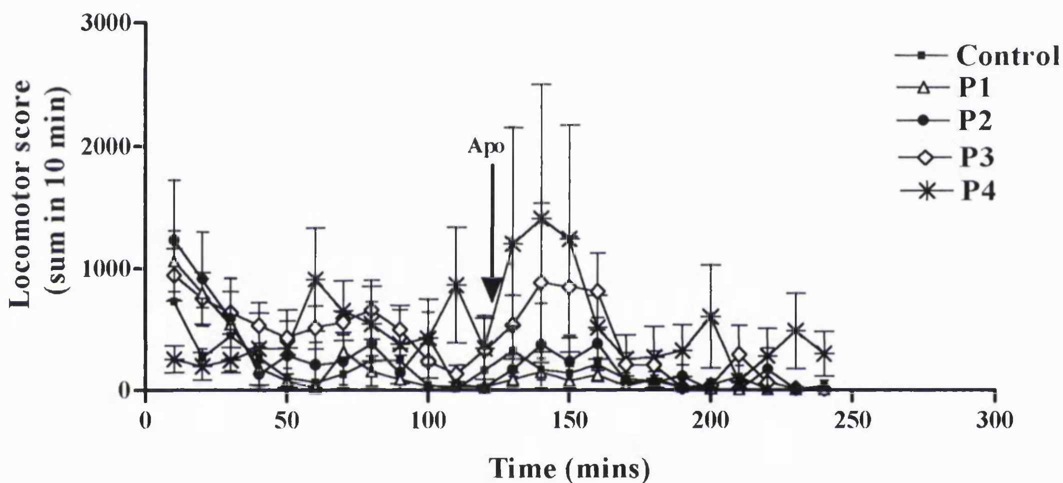


Fig. 3.10a - Responses of 3-NP and METH-treated rats to apomorphine (Apo, 0.5 mg/kg s.c.)

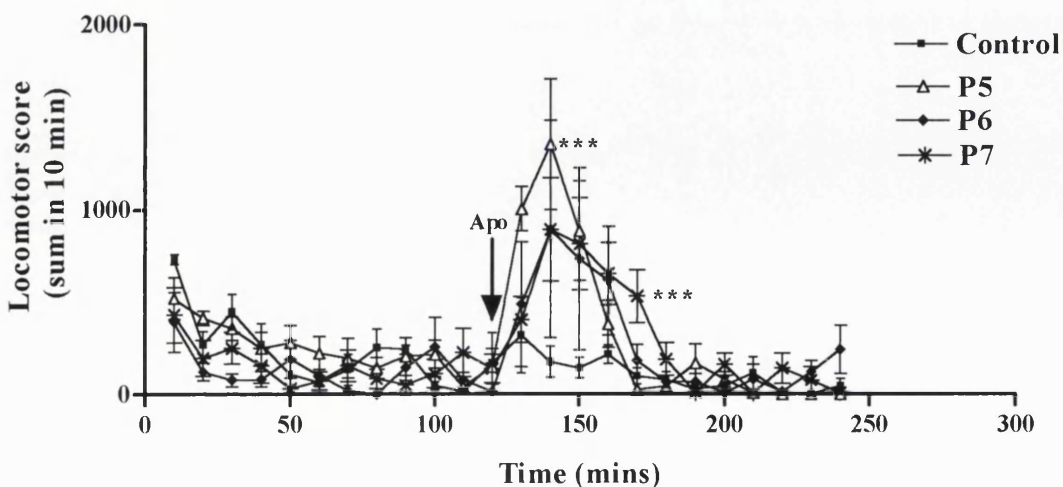


Fig. 3.10b - Responses of 3-NP and METH treated rats to apomorphine (Apo, 0.5 mg/kg, s.c.). (*$p<0.001$ versus control)**

3.2.1.5 Indications from the preliminary studies

3-NP is a common neurological toxin that prevents myelination of nerve fibres (Gould and Gustine, 1987) and induces cell death by inhibiting mitochondrial respiratory processes (Hamilton and Gould, 1987). When small doses are administered in isolated instances, the body appears to be quite capable of coping, as seen by the apparently normal levels of DA and 5-HT in all four brain regions of the 3-NP controls. In paradigms P1, P2 and P3, 3-NP was administered at a dose of 2x15 mg/kg, at 6 h intervals for one, two and three days respectively. As would be expected, when the titre of the toxin in the body built up (following repeated administration), the detoxifying mechanisms became overwhelmed and neurodegeneration escalated. Whereas striatal DA depletion was at 55 % in P1, it rose to 72 % in P3. The same trend is observed with 5-HT. It was therefore of benefit, in subsequent experiments, to increase the dose and frequency of administering the toxin, as well as to reduce the number of days between the administration of 3-NP and METH.

While it is generally accepted that an underlying energy deficiency exists in the elderly and that such a deficiency could accentuate any neurotoxic insult, there is no consensus on whether the impaired energy state is the consequence of a previous traumatic experience. This is because there is no evidence as yet to limit the cause of idiopathic PD to an exogenous toxin. If the culprit trigger factor for the disorder is some genetic deficiency or age-induced impairment in energy metabolism, then that would fit in quite well with the endogenous toxin view. This is because a reduction in ATP level has been shown to render the DA neurones susceptible to physiological levels of endogenous excitatory amino acids (Zeevalk et al, 1995, Sonsalla et al., 1998). On the other hand, the Exogenous Toxin theory will be more applicable if it can be demonstrated that administration of the said toxin would give rise to a cascade of events that will culminate in the inhibition of mitochondrial Complex I, and the ultimate death of DA cell bodies in the SN pars compacta of the nigrostriatal tract. This appears to be the case with the exogenous, synthetic toxin, MPTP. METH is not a known inhibitor of mitochondrial respiration.

Going by the Endogenous Toxin theory, we hypothesized that administering an appropriate dose of 3-NP at a suitable time prior to the administration of an ineffective dose of METH would achieve a synergism that would ultimately yield DA cell death in the SN and terminal loss in the ST.
Two doses of 40 mg/kg 3-NP were administered s.c. at 6 h interval on the same day, followed by

the METH dose of 4 x 8 mg/kg, every 2 h the next day (P5 on the index of modes, table 2.1). This yielded only 26 % DA depletion in the ST, with no apparent change in the other brain regions assayed. However, this dosing schedule was sufficient to accomplish 100 % 5-HT depletion in the ST and 93.5 % depletion in the SN. Conversely, when METH was administered prior to a lower dose of 3-NP (10 mg/kg, given daily for four days), P6, a 72 % depletion in striatal DA and 46 % depletion in NAcc DA levels was observed (Fig.3.5 and 3.2 respectively). Bearing in mind that P6 involved repeating the dosing schedule, the level of striatal DA loss is comparable to the 69 % striatal DA depletion in P4, which was a repetition of the P5 dose schedule after two weeks. The main difference between administering METH before or after 3-NP, is the greater damage to 5-HT neurones when they are rendered vulnerable to METH by pre-treatment with 3-NP.

In the behavioural experiments (section 3.2.1.4), the 3-NP control rats showed high spontaneous activity with a greater resistance to habituation. This may correspond to the hyperactive state suggested by Borlongan et al., 1997. However, these rats showed no DA receptor supersensitivity as revealed by the low scores in response to the apomorphine challenge. The spontaneous locomotor activity of rats from P4 and P6 was markedly low, but nowhere near the rigidity observed in reserpine-treated rats. All the rats showed no apparent limitation in locomotor activity, and no gross changes in behavior were observed across the paradigms. The only apparent change was seen in the extent of response to apomorphine. The rats from P5 and P7 showed significant increase in locomotion, suggesting DA receptor supersensitivity arising from DA loss.

In a bid to reduce the length of time involved in producing a functional model, while minimizing fatalities, the dose of 3-NP in P4 was reduced from 40 to 20 mg/kg, and administered for four consecutive days prior to METH (i.e. P7). This yielded a 67 % reduction in striatal DA (Fig.3.5) and a significant depletion of 5-HT only in the SN (Fig.3.8). In an attempt to further reduce nigrostriatal tissue DA levels, the inherent benefits of P6 and P7 were harnessed. This gave rise to a dosing schedule where 3-NP (20 mg/kg, s.c.) was administered once daily for four consecutive days, followed by METH (4 x 5 mg/kg, q2h, i.p.) on day five, and the 3-NP daily dose for the succeeding four days. The dose of METH was reduced to 4 x 5 mg/kg to reduce fatality. Behaviour experiments were conducted five to seven days later (but typically on day five), and the rats were sacrificed on the seventh day after the last injection of 3-NP. This is the "3-NPM" model.

3.2.2 Induction of the 3-NPM model

The impact of the 3-NPM dosing schedule on tissue monoamines, excitatory amino acids and AADC activity was determined seven days after the last injection. The assay procedure was different from that used in the preliminary investigations, and was based on a modification of the AADC assay methods of Nagatsu and Okuno (Nagatsu et al., 1979; Okuno and Fujisawa, 1983). In order to enhance the nigrostriatal DA loss induced by 3-NPM, we employed a higher dose of 3-NP (30 mg/kg, 3-NP3), and assayed the rats 24 h after the last dose of 3-NP (i.e. 3-NP3M). At this time, the rats were humped and akinetic, lacked a supersensitive response to a challenge of apomorphine. A choice of 3-NPM was made over 3-NP3M because of the more acceptable toxicity profile of the former.

3.2.2.1 Differential effects of 3-NPM and 3NP3M on tissue DA levels in the mesolimbic system

The PFC showed resistance to DA loss ($p>0.05$) following 3-NPM (Fig.3.11). The level of DA in the NAcc was reduced by about 50 % after 3-NPM, an effect that is likely due to 3-NP, as the higher dose of 30 mg/kg (3-NP3) gave rise to a significant ($p<0.05$) loss of DA in this region.

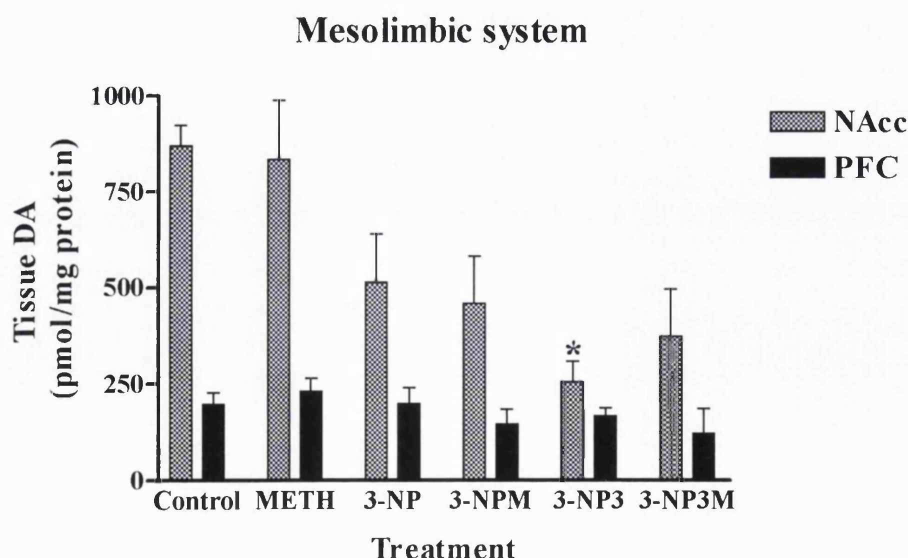


Fig. 3.11 - The effects of 3-NP and METH treatments on tissue DA levels in the NAcc and PFC. The results are the means + S.E.M. of a minimum of six determinations. (*$p<0.05$ versus control).

3.2.2.2 Differential effects of 3-NPM and 3-NP3M on tissue DA levels in the nigrostriatal tract

Following treatment with 3-NPM, DA levels in the SN are relatively unaltered (Fig. 3.12). However, a significant fall is observed in the ST ($p<0.01$). The loss of DA with the higher dose of 3-NP (i.e. 30 mg/kg) was significant ($p<0.05$) in the ST of rats treated with only 3-NP or with both toxins.

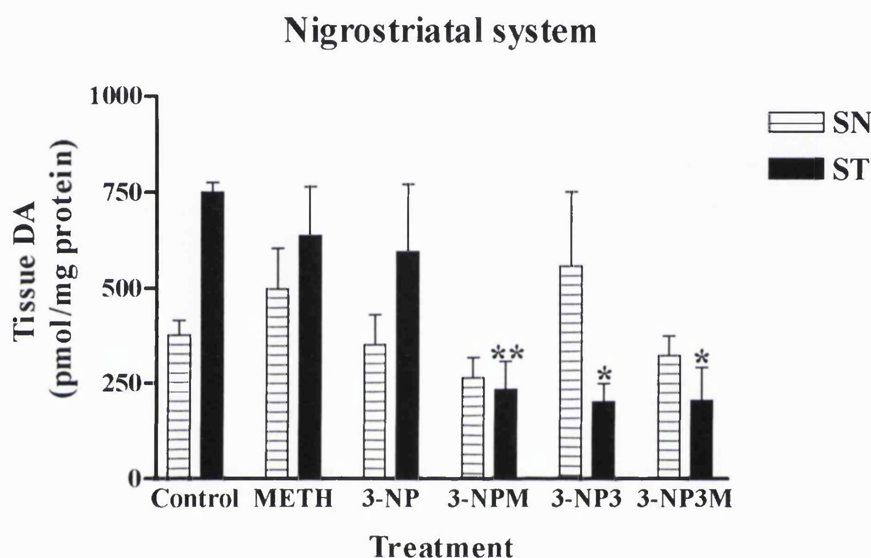


Fig. 3.12 - The effects of 3-NP and METH treatments on tissue DA levels in the SN and ST. The results are the mean + S.E.M. of at least six determinations. (*p<0.05, **p<0.01 versus control)

3.2.2.3 Comparative effects of 3-NPM and 3-NP3M on tissue 5-HT levels in the mesolimbic system

The serotonergic system in the NAcc showed resistance to the effects of 3-NPM ($p>0.05$) whereas the PFC was significantly vulnerable, showing fifty to seventy percent losses in 5-HT levels (Fig. 3.13).

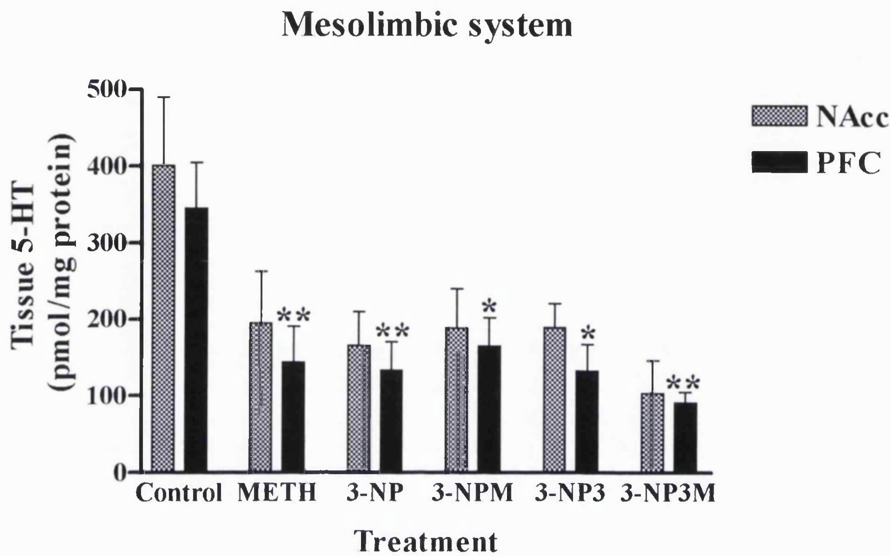


Fig. 3.13 - The effects of 3-NP and METH on tissue 5-HT levels in the NAcc and PFC. Results are mean + S.E.M. for a minimum of six determinations. (*$p<0.05$, **$p<0.01$ versus control).

3.2.2.4 Comparative effects of 3-NPM and 3-NP3M on tissue 5-HT levels in the nigrostriatal system

In the SN, METH exerted protective effects over the effects of 3-NP on 5-HT neurones (Fig. 3.14). The reverse occurred in the ST, as the significant ($p<0.05$) fall in 5-HT induced by METH was attenuated by 3-NP. This effect is common to 3-NPM and 3-NP3M.

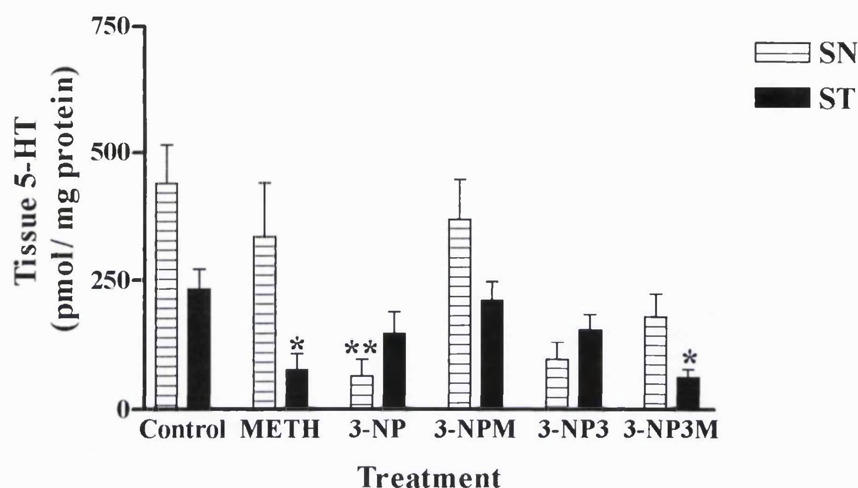


Fig. 3.14 - The effects of treatment with 3-NP and METH on tissue 5-HT levels in the SN and ST. Results are the mean + S.E.M. of a minimum of six determinations. (*$p<0.05$, **$p<0.01$ versus control).

3.2.2.5 Differential effects of 3-NPM and 3-NP3M on DDC activity in the mesolimbic system

The activity of DDC in the NAcc was unaffected by 3-NPM ($p>0.05$). A 20 mg/kg dose of 3-NP caused an increase in DDC activity in the PFC ($p<0.05$), whereas the higher dose of 30 mg/kg caused a decrease in DDC activity in both NAcc and PFC ($p<0.05$). These findings are shown in Fig.3.15.

3.2.2.6 Differential effects of 3-NPM and 3-NP3M on DDC activity in the nigrostriatal system

The 20 mg/kg dose of 3-NP showed a tendency to increase DDC activity as it did in the mesolimbic system, although this effect was not significant ($p>0.05$). Otherwise, DDC activity remained unchanged by 3-NP and METH. At a dose of 30 mg/kg of 3-NP, DDC activity was significantly reduced ($p<0.05$). A graph of these effects is shown in Fig. 3.16.

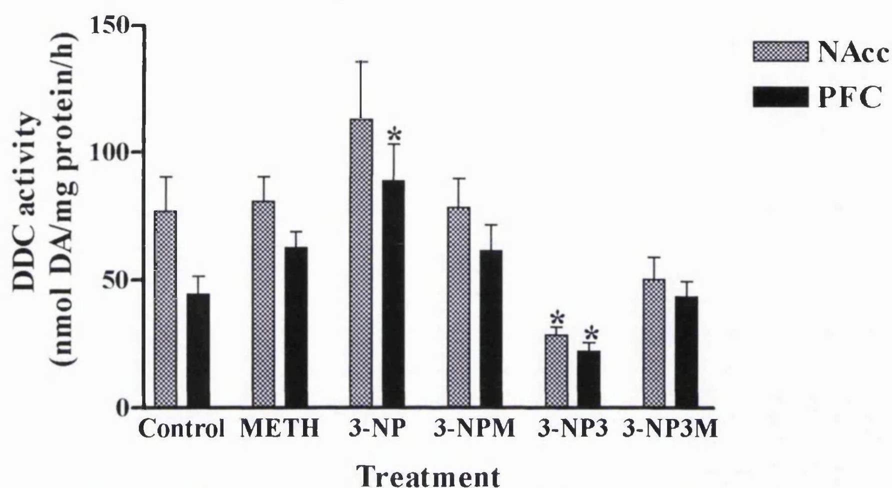


Fig. 3.15 - The effects of 3-NP and METH treatment on DDC activity in the NAcc and PFC. Results are the mean + S.E.M. of a minimum of six determinations. (*p<0.05 versus control).

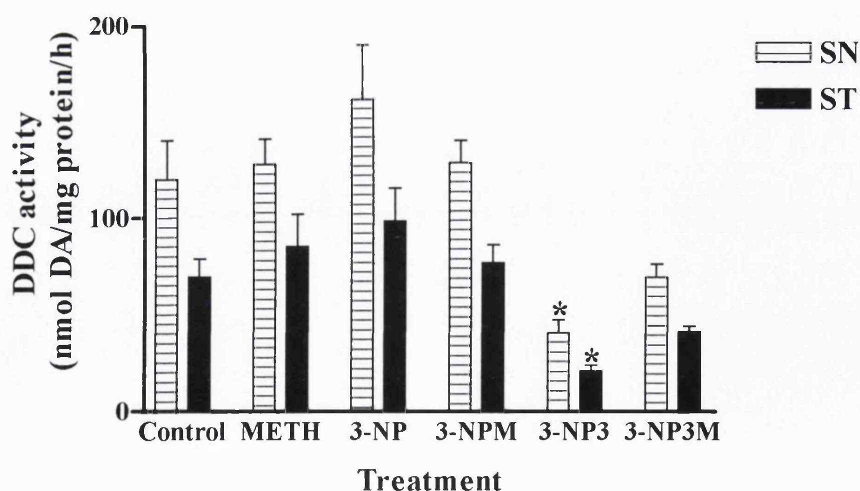


Fig. 3.16 - The effects of 3-NP and METH on DDC activity in the SN and ST. Results are the means + S.E.M. of a minimum of six determinations. (*p<0.05 versus control).

3.2.2.7 Differential effects of 3-NPM and 3-NP3M on 5-HTPDC activity in the mesolimbic system

There are no significant changes in 5-HTPDC activity in the NAcc following treatment with high and low doses of 3-NP in combination with METH. However, the 30 mg/kg dose of 3-NP resulted in a significant ($p<0.01$) depression of 5-HTPDC activity in the PFC. A chart of these results is shown in Fig. 3.17.

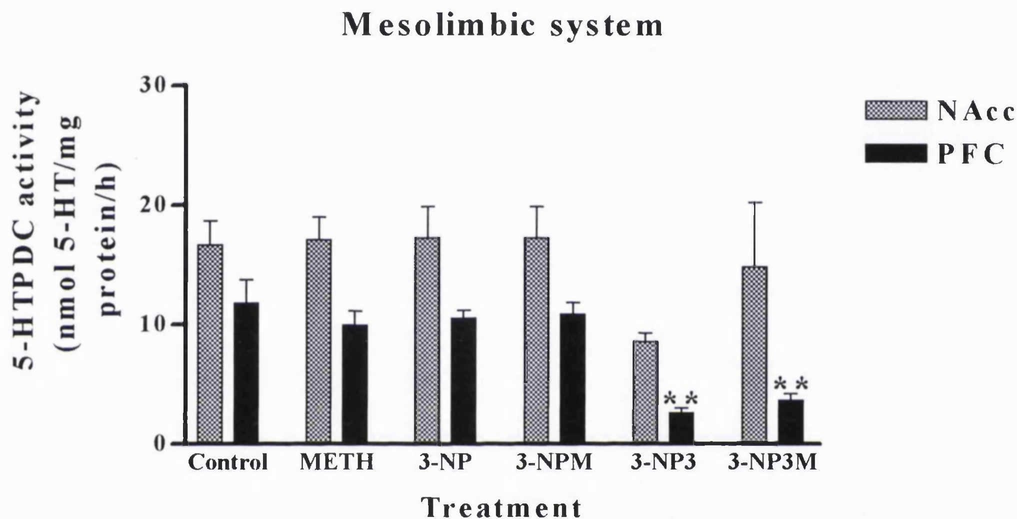


Fig. 3.17 - The effects of 3-NP and METH on 5-HTPDC activity in the NAcc and PFC. The results are the mean + S.E.M. of a minimum of six determinations. (**p<0.01 versus control).

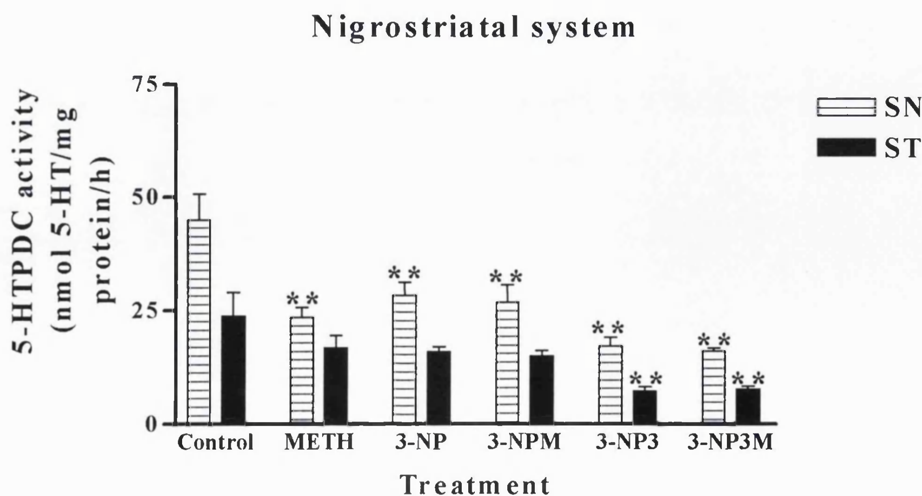


Fig. 3.18 - The effects of 3-NP and METH on 5-HTPDC activity in the SN and ST. The results are the mean + S.E.M. of a minimum of six determinations. (**p<0.01 versus control).

3.2.2.8 Differential effects of 3-NPM and 3-NP3M on 5-HTPDC activity in the nigrostriatal system

The 5-HT neurones in the SN showed significant vulnerability to 3-NPM-induced reductions in 5-HTPDC activity, causing a marked decrease in 5-HT formation ($p<0.01$). The ST, on the other hand, was resistant to these effects, except at the higher dose of 30 mg/kg 3-NP. A graph of these effects is shown in Fig. 3.18.

3.2.2.9 Comparative effects of 3-NPM and 3-NP3M on tissue levels of aspartate in the mesolimbic system

Tissue levels of the excitatory amino acid, aspartate, remain unaltered in the NAcc and PFC of rats treated with 3-NP and METH. A chart of these results is shown in Fig. 3.19.

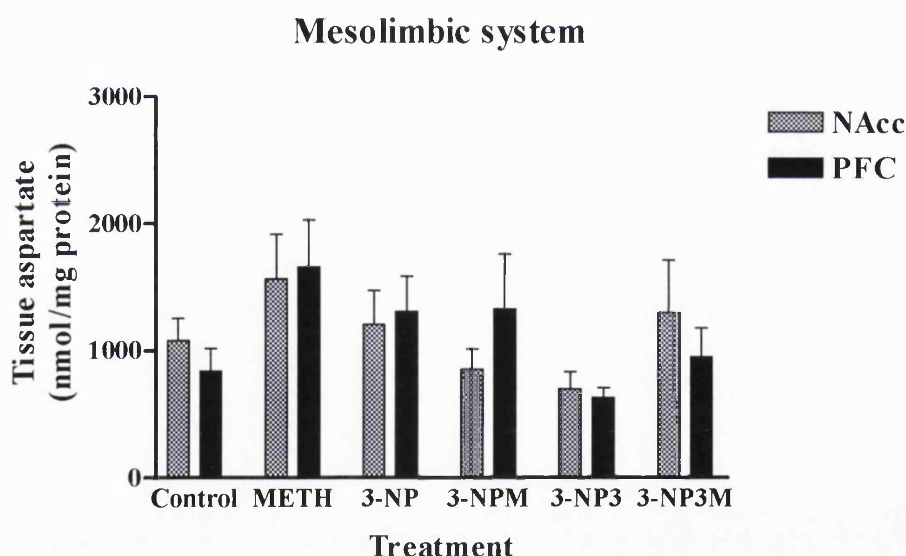


Fig. 3.19 - The effects of 3-NP and METH treatment on tissue levels of aspartate in the NAcc and PFC. The results are the means + S.E.M. of a minimum of six determinations. ($p>0.05$ versus control).

3.2.2.10 Comparative effects of 3-NPM and 3-NP3M on tissue levels of aspartate in the nigrostriatal system

There were no changes in aspartate levels in the ST with 3-NP and METH treatment. Dosing with 3-NP at 20 mg/kg resulted in a significant ($p<0.05$) elevation of aspartate levels, as shown in Fig.3.20.

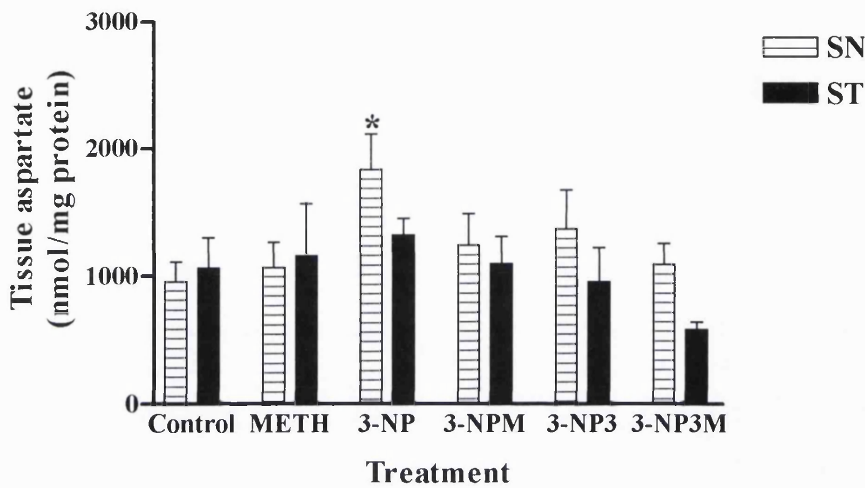


Fig. 3.20 - The effects of 3-NP and METH treatments on tissue levels of aspartate in the SN and ST. Results are the mean + S.E.M. of a minimum of six determinations. (*p<0.05 versus control).

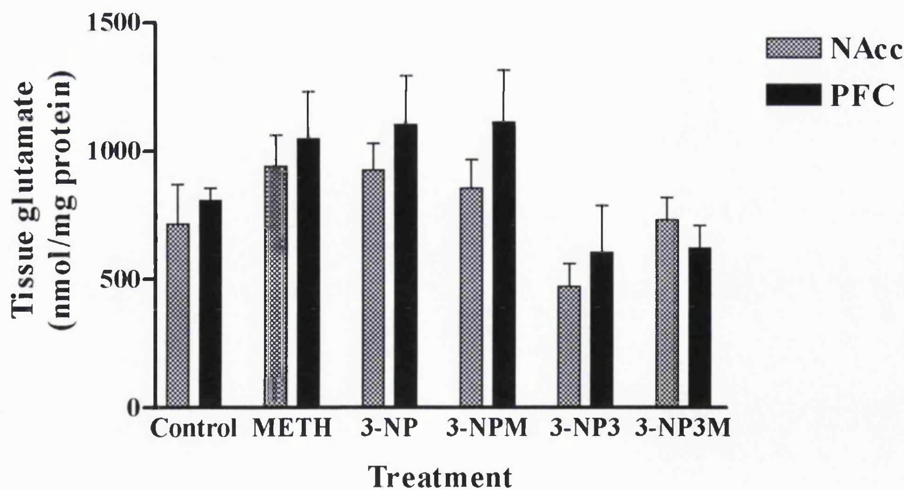


Fig. 3.21 - The effects of 3-NP and METH on glutamate levels in the NAcc and PFC. Results are the mean + S.E.M. of a minimum of six determinations. (p>0.05 versus control).

3.2.2.11 Differential effects of 3-NPM and 3-NP3M on tissue glutamate levels in the mesolimbic system

There were slight but insignificant elevations in tissue levels of glutamate in the NAcc and PFC following treatment with 3-NP and METH. A graph of these results is shown in Fig. 3.21.

3.2.2.12 Differential effects of 3-NPM and 3-NP3M on tissue glutamate levels in the nigrostriatal system

Control rats for 3-NP showed a significant increase in glutamate levels in the SN ($p<0.05$). There were slight elevations in the METH and 3-NPM groups, but these were insignificant ($p>0.05$). These findings are presented in Fig.3.22.

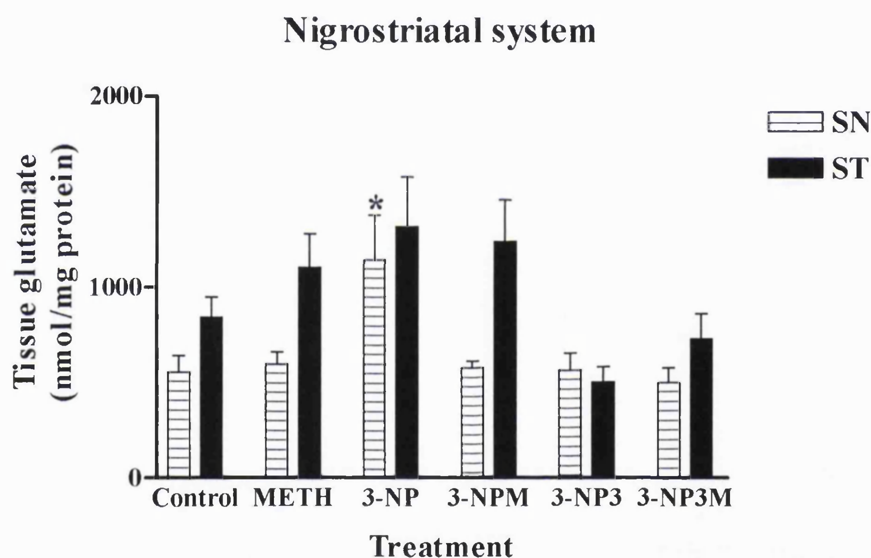


Fig. 3.22 - The effects of 3-NP and METH on glutamate levels in the SN and ST. The results are the mean + S.E.M. of a minimum of six determinations. (*$p<0.05$ versus control).

3.2.3 Toxicity profile of the toxins

Rats treated with 3-NP and METH were weighed daily, and assessed for changes in gross appearance and locomotion. Lethality was expressed as the percentage of the number of dead rats out of the total number treated. Hind-limb paralysis is a common feature of 3-NP intoxication (Gould and Gustine, 1987), and was expressed as a percentage of the ratio between the number of paralyzed rats and the total number of rats treated.

3.2.3.1 Toxic outcomes with 3-NP

Treatment with the recommended two injections of 3-NP at 40 to 60 mg/kg, at 6 h interval resulted in severe hind-limb paralysis, weight loss and eventual death within 48 h after treatment. The lower doses of 2 x 15 and 2 x 20 mg/kg did not induce paralysis or fatality. This was important in choosing a dose for 3-NP in the preliminary experiments. A picturesque account of these results is presented in Fig. 3.23.

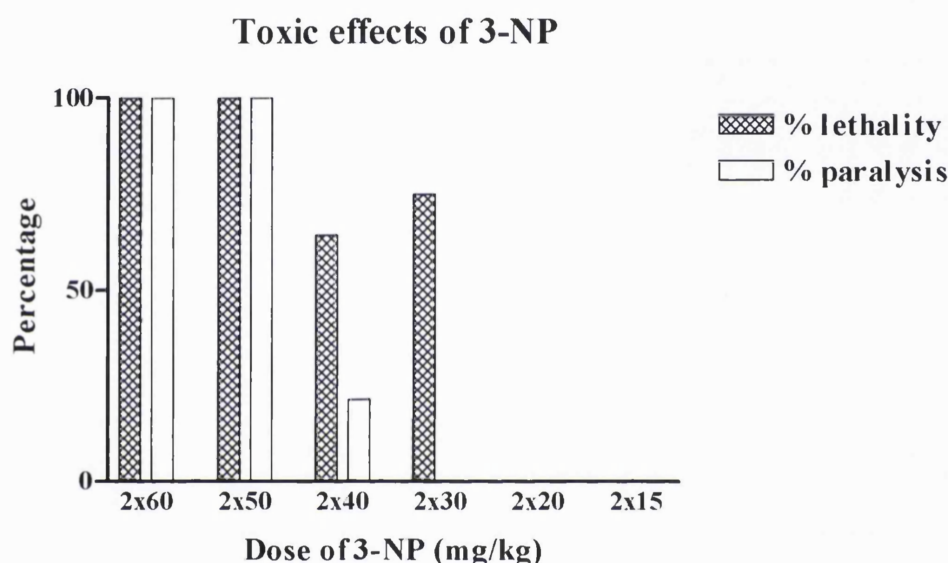


Fig. 3.23 - Toxic effects induced by 3-nitropropionic acid.

3.2.3.2 Toxic effects of METH

There was no hind-limb paralysis in any of the rats treated with METH. However, percentage lethality increased with an increase in the dose of the toxin. A graph of this is shown in Fig. 3.24.

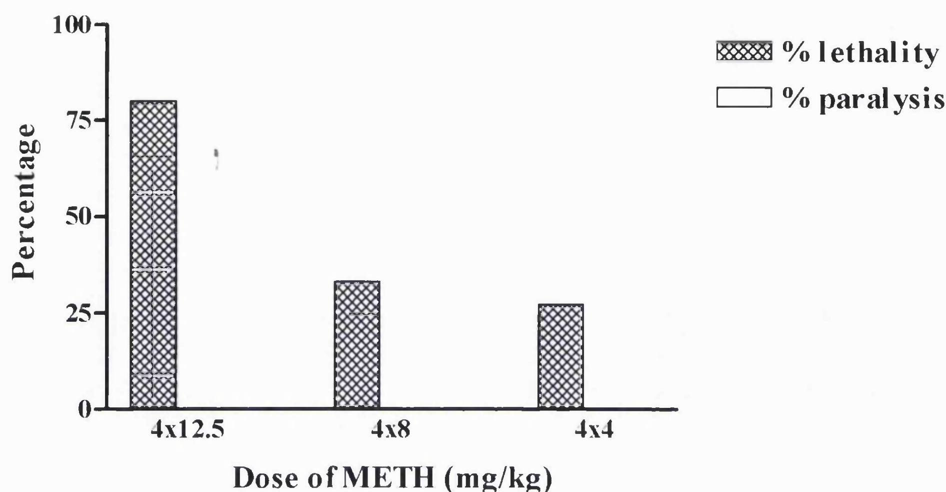


Fig. 3.24 - Toxic effects induced by METH.

3.2.3.3 Toxicity with 3-NPM

There were no toxic incidents with 3-NP (20 mg/kg) in the 3-NPM dosing schedule. Treatment with METH resulted in 8 % lethality but no paralysis (Fig.3.25). This figure was doubled by treatment with 3-NPM, also yielding approximately 8 % paralysis. The toxic outcomes with 3-NPM usually occurred within 24 h after the METH injection. Although rats treated with the higher dose of 3-NP (30 mg/kg) were more akinetic, the high incidence of toxicity with this dose discouraged its selection as a viable model.

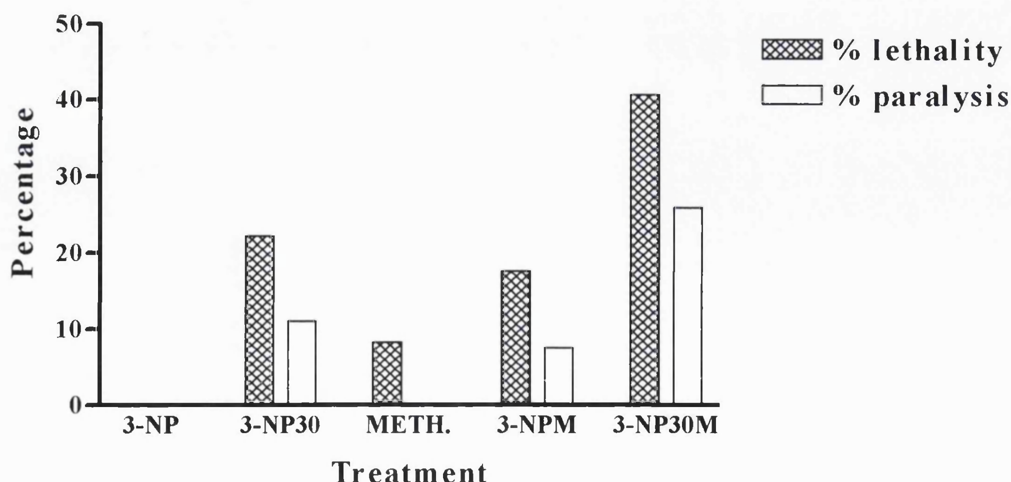


Fig. 3.25 - Toxic effects of 3-NP and METH with the 3-NPM dosing schedule.

3.2.3.4 Effects of 3-NP and METH on body weight

Rats in the 3-NP control and 3-NPM groups showed significantly impaired weight gain ($p<0.05$; $p<0.01$ respectively) during the dosing period (Fig.3.26). METH-treated rats were not different from controls.

Weight changes during 3-NP and METH administration

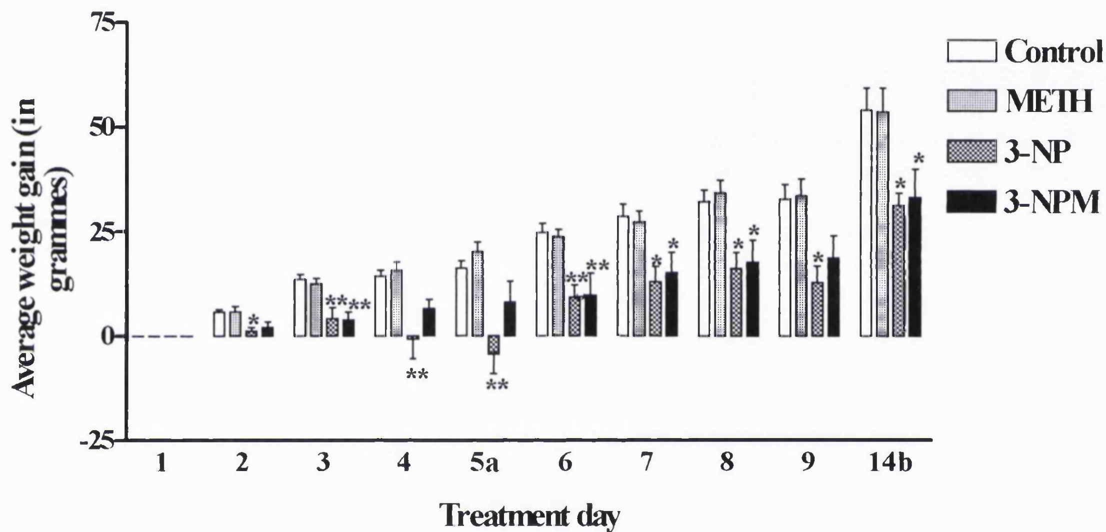


Fig. 3.26 - Average weight change from the weights at the start of dosing on day one. (*p<0.05, **p<0.001 versus control; a = METH administration, b = behaviour analysis). The results are the means + S.E.M. of a minimum of six determinations.

3.3 Discussion

Using the AADC enzyme assay procedure, a synergism was observed in the DA-depleting ability of both 3-NP and METH in all the four brain regions. There was no additional benefit in DA depletion by increasing the dose of 3-NP to 30 mg/kg (i.e. 3-NP3M), especially as the rats were more likely to show signs of intoxication, and did not respond to the apomorphine challenge. Consequently, a preference was made for 3-NPM, and the results hereafter discussed are the findings with the 3-NPM schedule.

Treatment with 3-NPM resulted in a 47 % drop in DA tissue levels in the NAcc, 26 % in the PFC, 30 % in the SN and 69 % in the ST, seven days after the last injection of 3-NP. This is in line with previous reports that the NAcc is less sensitive than the ST to METH (Cass, 1996b), an effect which may be the result of differential distribution of the DA transporter (DAT) in both regions (Cass et al., 1992). This dose combination yielded only 18 % fatality, with an 8 % chance of hind-limb paralysis (Fig. 3.25). The 3-NPM-depleted rats took a longer time to habituate, but produced a significant, three-fold increase in locomotor activity in response to apomorphine (see Chapter Five). By decreasing ATP levels therefore (Pang and Geddes, 1997), 3-NP was able to accentuate METH-induced targeting of DA neurones, as METH depletes energy stores by its effects on the ATP-dependent DAT (Fleckenstein et al., 1997).

In clinical presentations of PD, there is over 90 % loss of cell bodies in the SN, with concomitant loss of terminals in the ST. This is responsible for the reduction in tissue DA levels in both regions. The results above follow the same trend, with a 30 % DA reduction in the SN giving rise to a 70 % loss of DA in the ST. This suggests that the 3-NPM model also produces death of both cell bodies and nerve terminals. These neurochemical changes, although insufficient to produce apparent rigidity, were able to effect enough denervation to induce supersensitivity of DA receptors in the nigrostriatal tract, as revealed by the significant increase in response to apomorphine. In addition, the 47 % depletion in the NAcc affirms the involvement of the mesolimbic pathway in the symptomatology of PD. These findings depict clearly, the potential of this model as an experimental tool for the initial stages of the disease.

The 3-NPM model exerted little effect on 5-HT levels in the nigrostriatal tract, while exhibiting a greater potency in the mesolimbic pathway. This may stem from the greater density of serotonergic

neurones along this pathway. There was a 52 % drop in 5-HT levels in the PFC and NAcc, being significant in the former but not in the latter (Fig. 3.13). This indicates an effect on the serotonin transporter and therefore suggests a role for 3-NPM in the pathophysiology of drug abuse (Wilson et al, 1996). The combination of METH and 3-NP tends to exert a protective effect on the degenerative effects of METH on 5-HT in the PFC and ST.

The levels of the EAAs glutamate and aspartate were slightly but insignificantly raised in all four brain regions following treatment with 3-NPM (Fig. 3.19 - 3.22). This is not incongruent with previous theories that suggest raised levels of excitatory neurotransmitters in PD (Albin et al., 1989; Sonsalla et al., 1989, 1998). It is interesting that 3-NP by itself caused a significant elevation of the tissue levels of glutamate and aspartate in the substantia nigra (Fig. 3.22 and 3.20 respectively). This is in contrast with the previous notion that 3-NP does not alter nigrostriatal gluatmate levels (Tsai et al., 1997; Zeevalk and Nicklas, 1991). The results suggest that it does actually cause an elevation of glutamate, and that, at a critical part of the nigrostriatal tract with great potential for excitotoxin-induced neurodegeneration. This is especially so, considering recent evidence that the substantia nigra is more susceptible than the striatum to the neurodegenerative effects of the glutamate (NMDA) receptor (Sonsalla et al., 1998).

These findings bring to question the sequence of events that ultimately result in cell death. It is widely accepted that energy impairment renders striatal DA neurones susceptible to excitotoxic insults (Bazette et al., 1996; Greene et al., 1998). Without significantly altering DA levels, 3-NP increased glutamate levels in the SN (Fig. 3.12 and Araujo and Hilt, 1998). The query therefore arises: **is the accepted glutamate hyperactivity in PD a cause or a consequence of DA depletion?** If it is a cause, then the precipitating factor (or toxin) is likely to be a metabolic inhibitor. If, on the other hand, it is a consequence, then the culprit for the aetiology of idiopathic PD may be found among the barrage of endogenous and exogenous free radicals that are toxic to DA neurones. The results suggest that an underlying energy deficiency renders the DA neurones susceptible to excitotoxic and free-radical induced damage. The enhancement of 3-NP toxicity, as would occur with METH-induced glutamate release, would change the mechanism of cell death from apoptosis to necrosis (Pang and Geddes, 1997). These findings therefore lend further credence to the use of glutamate antagonists in the treatment of both the early and late stages of the disease.

In the face of 3-NPM-induced depletion, the conversion of L-DOPA to DA by DDC in the four brain regions analyzed, remained indistinct from the respective saline controls (Fig. 3.15 and 3.16). This is a slight departure from the expected, as reductions in tissue levels of DA (Fig. 3.11- 3.12) would suggest destruction of the nerve terminals which bare the DDC enzyme; as is the case in the brains of PD patients. However, the apparently normal levels of DDC activity could be part of a compensatory mechanism that also leads to up-regulation of the DA receptors.

The capacity of 5-HTPDC to decarboxylate 5-HTP to 5-HT is very significantly impeded in the SN with either agent, or a combination of both (Fig. 3.17-3.18), with no apparent changes in the other three regions. This observation would support the growing hypothesis that AADC would preferentially decarboxylate L-DOPA instead of 5-HTP in certain brain regions, under conditions such as neurotoxin-induced depletion, which compromise the activity of the enzyme. The overall picture conforms to the suggested impermanence of degeneration by METH (Wilson et al., 1996).

3-nitropropionic acid produces weight loss as part of its toxicological profile following accidental ingestion by humans (Ludolph et al., 1991). This was observed to be the case in rats, as the toxin significantly depressed the weight gain of the animals (Fig. 3.26) by decreasing their feeding and drinking tendency. Methamphetamine, though an appetite suppressant, did not induce additional weight loss.

An overview of the results indicates that it was possible to produce progressive neurodegeneration of dopaminergic and serotonergic cell bodies and terminals in both the nigrostriatal and mesolimbic pathways using the dose schedule of the proposed “3-NPM” model.
By decreasing ATP and elevating glutamate levels in the substantia nigra, 3-nitropropionic acid provided the conducive environment for methamphetamine to specifically destroy dopaminergic neurones by increasing the energy demand on the dopamine transporter, as well as by causing additional glutamate release. The pattern of behavioural and neurochemical changes thereby induced, bear relevance to Parkinson’s disease and hold potential for the use of this model as an investigational tool for the disorder.

Contrary to previous reports, it was found that 3-NP elevated tissue levels of glutamate and aspartate in the substantia nigra. This finding raises the question of the onset of glutamate hyperactivity in PD, but lends additional credence to the use of glutamate antagonists in the treatment of PD in its early and late stages.

Finally, the ability to induce parkinsonian conditions with 3-NPM (a combination of a naturally occurring food contaminant with a commonly abused substance) presents a functional model for an addition theory of the aetiology of Parkinson's disease. In addition, it inclines the search for the hypothetical trigger factor in the direction of metabolic inhibitors.

CHAPTER FOUR

PROGRESSIVE NEUROCHEMICAL CHANGES WITH 3-NPM
AND
THE EFFECTS OF BOLUS METHAMPHETAMINE DOSING

4.1 Introduction

The loss of dopamine terminals in the basal ganglia following treatment with methamphetamine has been claimed to last from six months in the rat (Wagner et al., 1980) to four-and-a-half years in the monkey (Woolverton et al., 1989), after the last injection of a high-dose regimen. Treatment with a combination of 3-NP and METH in the rat yielded significant neurochemical changes in both the mesolimbic and nigrostriatal structures (see Chapter Three). It was therefore the intention of the following groups of experiments to monitor the progressive changes, if any, at 7, 14, 21 and 28 days after the last injection of 3-NP.

In order to induce parkinsonian states, METH has traditionally been administered as two to four two-hourly doses (Albers et al. 1996; Fornai et al., 1995; Sonsalla and Heikkila, 1986, Sonsalla et al., 1989,1991; Wagner et al., 1980), with the fourth injection being the most critical for neuronal injury (O'Dell et al., 1993). However, recent findings suggest that a large single dose of METH would achieve the same objective as the multiple-dose regimen (Fukumura et al., 1998). In a bid to clarify this point, and to further simplify the 3-NPM model, rats were treated with 3-NP (20 mg/kg, s.c.) daily for four days, and given a bolus injection of METH (20 mg/kg i.p). on the fifth day. This was then followed by four additional dose of 3-NP. An assessment of DA neurone function was made five days later with apomorphine, and neurochemical assays were conducted on the seventh day after the last injection of 3-NP, as for the 3-NPM model.

4.2 Results

4.2.1 Time-dependent changes in 3-NPM-induced toxicity

4.2.1.1 Progressive changes in tissue DA levels in the mesolimbic system of 3-NPM-treated rats

The level of dopamine in the NAcc is significantly reduced ($p<0.05$) 7 days after 3-NPM treatment, but returns to normal values from 14 to 28 days after the last 3-NP injection. In the PFC, 3-NPM treatment showed a trend of decrease in DA levels seven and fourteen days after treatment, followed by a progressive rise to significant levels by 28 days. A chart of these findings is shown in Fig. 4.1.

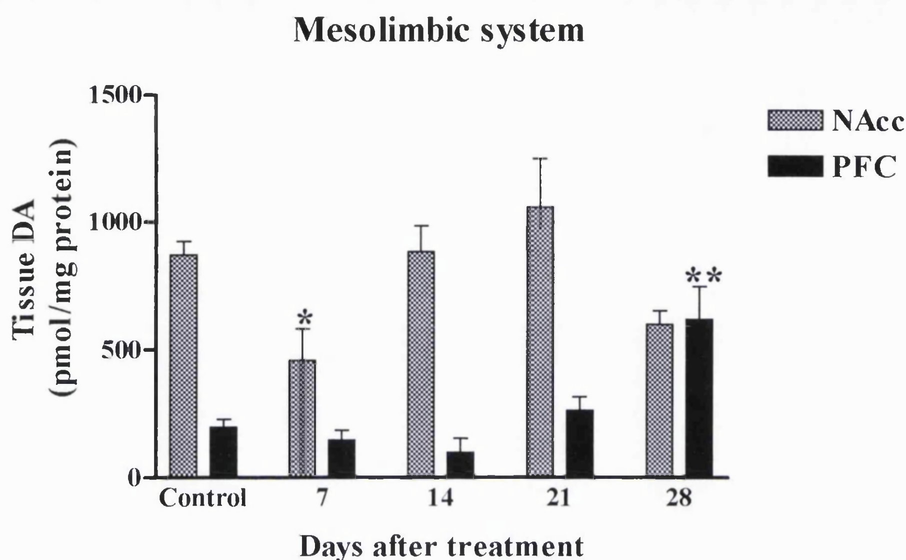


Fig. 4.1 - The effect of time on 3-NPM-induced changes in tissue DA levels in the NAcc and PFC. Results are the mean + S.E.M. of at least six determinations. (*p<0.05, **p<0.01 versus control).

4.2.1.2 Progressive changes in tissue DA levels in the nigrostriatal system of 3-NPM-treated rats

The trend of progressive fall in DA was also observed in the nigrostriatal tract. Dopamine levels in the SN are reduced by 30 % at the 7 day point, 70 % at the 14 day point ($p<0.01$), and progressively rise to 200 % of control values by 28 days ($p<0.05$). In the ST, the DA level is 30 % of control values ($p<0.01$), but progressively rises to 140 % of control values by 28 days ($p>0.05$). A graph depicting these effects is shown in Fig. 4.2.

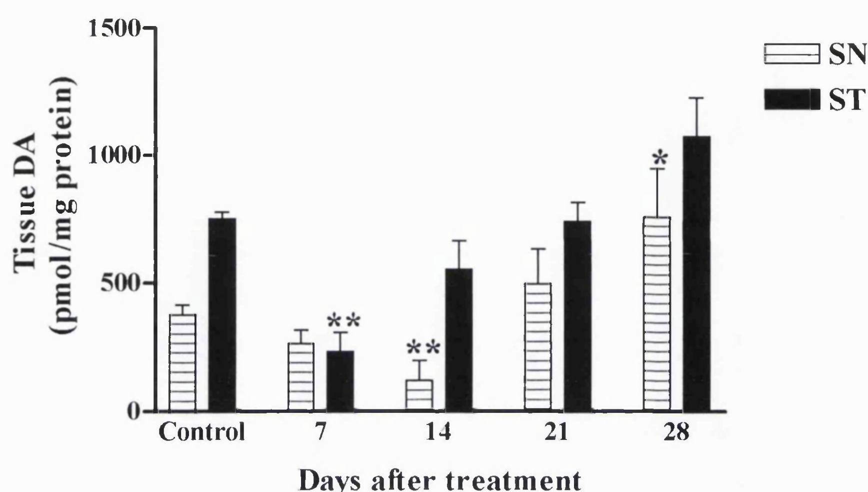


Fig. 4.2 - The effects of time on 3-NPM-induced changes in tissue DA levels in the SN and ST. Results are the mean + S.E.M. of a minimum of six experiments (*p<0.05; **p<0.01 versus control).

4.2.1.3 Progressive changes in tissue 5-HT levels in the mesolimbic system of 3-NPM-treated rats

A graphic representation of the progressive effects of 3-NPM on 5-HT in the mesolimbic system is shown in Fig.4.3. The levels of 5-HT in the NAcc and PFC are markedly reduced 7 days after 3-NPM, and fall to levels below detection 14 and 21 days after the last injection. At the 28 day point, there was a recovery of 5-HT in both regions, to the same levels as at the 7-day point.

4.2.1.4 Progressive changes in tissue 5-HT levels in the nigrostriatal system of 3-NPM-treated rats

The nigrostriatal system was relatively resistant to depletion in 5-HT at day 7. However, this resistance was lost at the 14- and 21-day time points ($p<0.01$). The SN recovered by day 28, but the 5-HT levels in the ST were still significantly below control values ($p<0.01$). These results are summarized in Fig. 4.4.

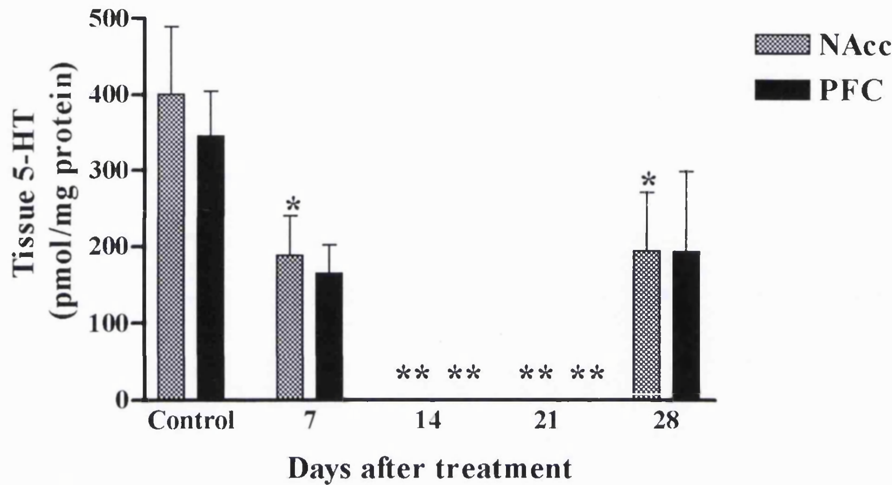


Fig. 4.3 - The effects of time on 3-NPM-induced changes in tissue 5-HT levels in the NAcc and PFC. Results are the mean + S.E.M. of a minimum of six determinations. (*p<0.05; **p<0.01 versus control).

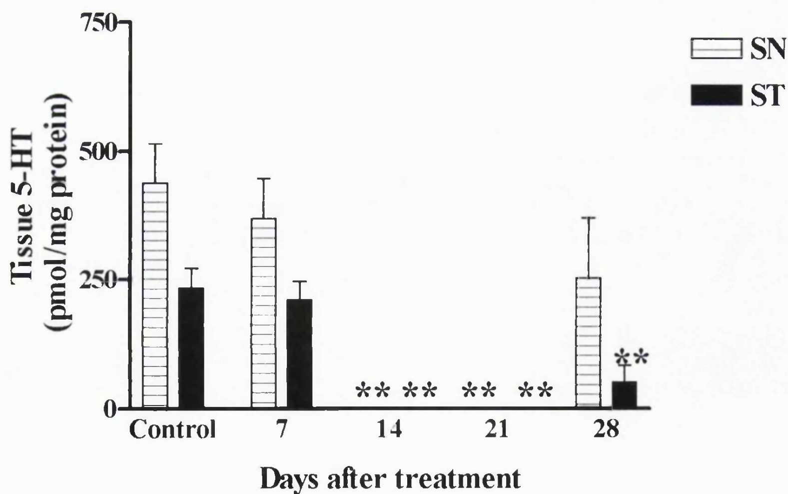


Fig. 4.4 - The effects of time on 3-NPM-induced changes in tissue 5-HT levels in the SN and ST. Results are the mean + S.E.M. of at least six determinations (**p<0.01 versus control).

4.2.1.5 Progressive changes in DDC activity in the mesolimbic system of 3-NPM rats

DOPA decarboxylase activity remained unchanged in the NAcc and PFC at the 7-day point. However, it was significantly reduced in the NAcc at 14 through to 28 days after the last injection of 3-NP. The same trend of reduction at the 14 and 21 day points was also observed in the PFC, although DDC activity was restored by 28 days. Figure 4.5 is a chart of these results.

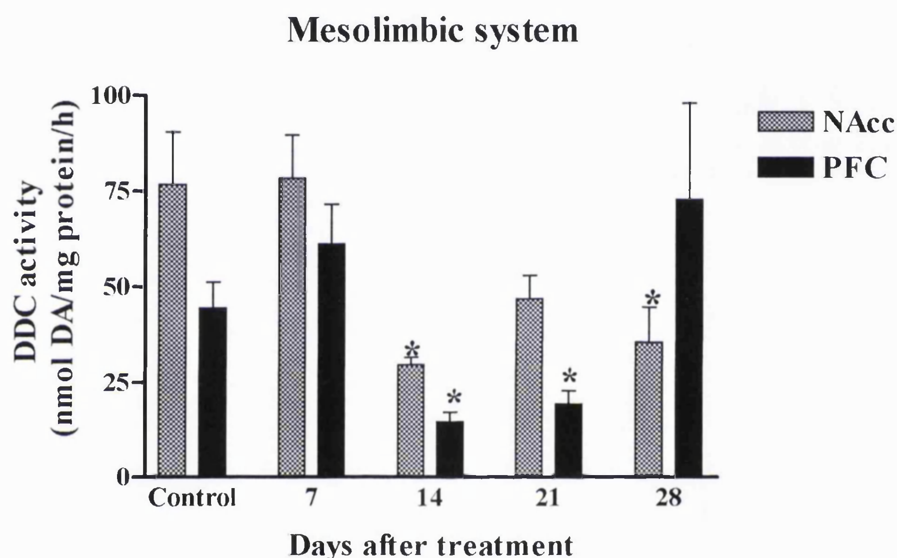


Fig. 4.5 - The effects of time on 3-NPM-induced changes in DDC activity in the NAcc and PFC. The results are the means + S.E.M. of a minimum of six determinations. (*$p<0.05$ versus control).

4.2.1.6 Progressive changes in DDC activity in the nigrostriatal system of 3-NPM-treated rats

DOPA decarboxylase activity was unchanged at 7 days in the SN and ST, but fell to between 30 and 70 % of control values in the SN, during the 14 to 28 day period. DDC activity dropped by similar proportions in the ST at the 14 and 21 day points, but recovered by the 28-day time point. A picturesque representation of these is shown in Fig. 4.6.

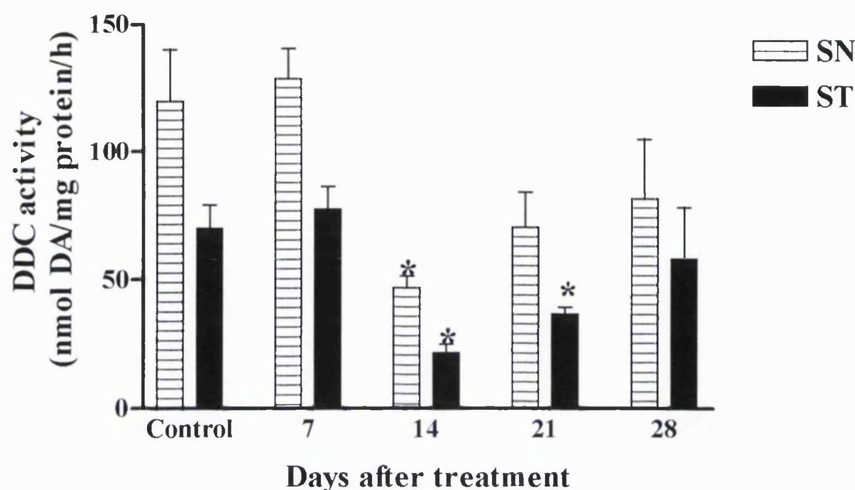


Fig. 4.6 - The effects of time on 3-NPM-induced changes in DDC activity in the SN and ST. Results are the means of a minimum of six determinations + S.E.M. (*p<0.05 versus control).

4.2.1.7 Progressive changes in 5-HTPDC activity in the mesolimbic system of 3-NPM-treated rats

5-HTP decarboxylase activity in the NAcc and PFC seven days after 3-NPM, were similar to control levels (Fig.4.7). By the 14th day after 3-NPM treatment, 5-HTPDC activity was abolished in both regions, only showing a slight activity at 28 days ($p<0.01$).

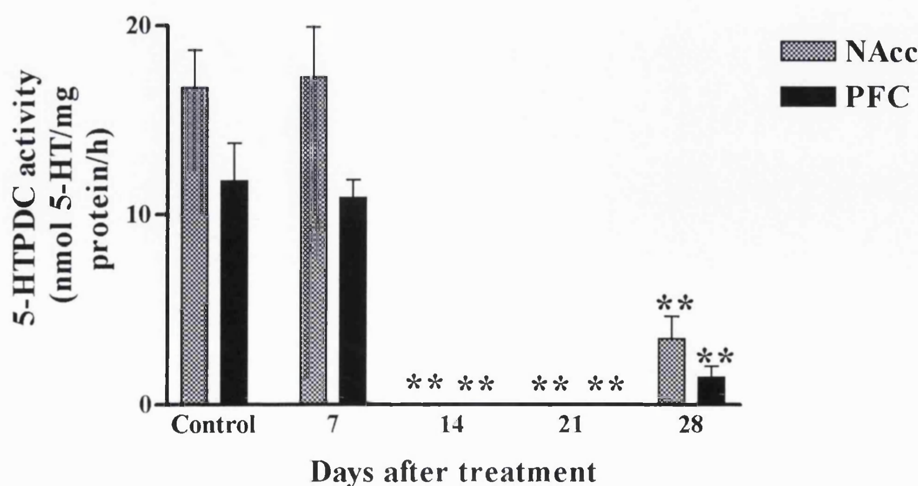


Fig. 4.7 - The effects of time on 3-NPM-induced changes in 5-HTPDC activity in the NAcc and PFC. Results are the mean + S.E.M. of a minimum of six determinations. (p<0.01 versus control).**

4.2.1.8 Progressive changes in 5-HTPDC activity in the nigrostriatal system of 3-NPM-treated rats

The activity of 5-HTPDC falls progressively and significantly in the SN from the 7th day after 3-NPM ($p<0.01$), and remains until the 28th day. 5-HTPDC activity in the ST is 60 % of control on the 7th day, undetected on the 14th and 21st days, and 20 % of control on the 28th day after 3-NPM. Figure 4.8 is a graph depicting these effects.

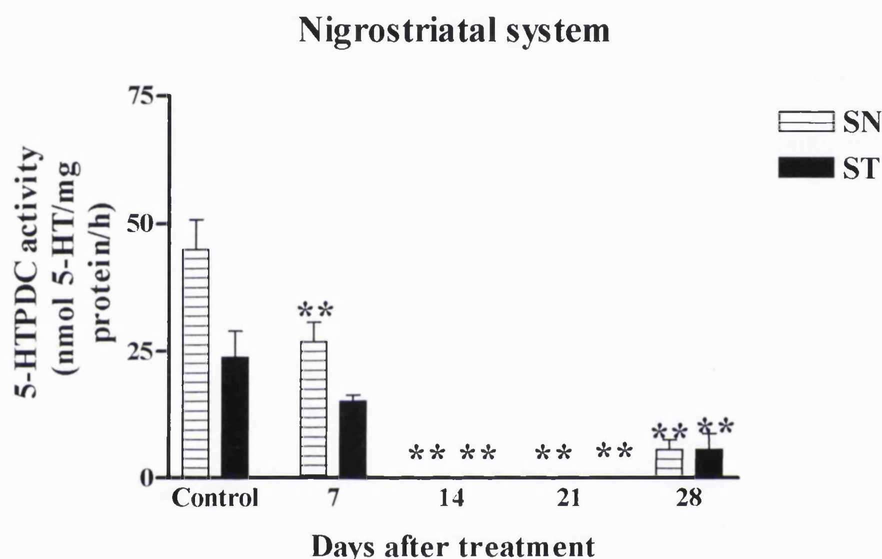


Fig. 4.8 - The effects of time on 3-NPM-induced changes in 5-HTPDC activity in the SN and ST. Results represent the mean of at least six determinations + S.E.M. ($p<0.01$ versus control).**

4.2.1.9 Progressive changes in tissue aspartate levels in the mesolimbic system of 3-NPM-treated rats

Tissue aspartate levels in the NAcc rise progressively, reaching a peak ($p<0.01$) on the 21st day after the last injection of 3-NP, and falling to 20 % of control value by the 28th day. The same trend is observed in the PFC, with a peak at day 14 ($p>0.05$), and a progressive fall thereafter to 55 % of control level by day 28. A chart of these results is shown in Fig.4.9.

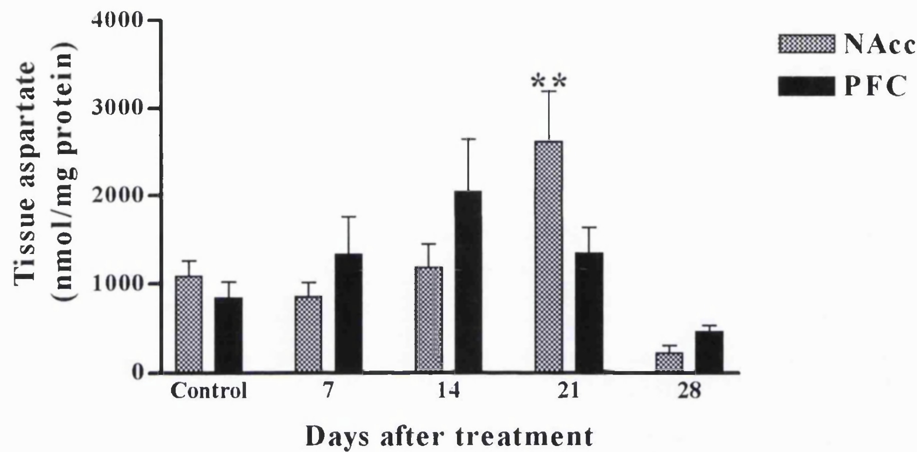


Fig. 4.9 - The effects of time 3-NPM-induced changes in tissue aspartate levels in the NAcc and PFC. The results are the mean + S.E.M. of a minimum of six determinations. (p<0.05 versus control).**

4.2.1.10 Progressive changes in tissue aspartate levels in the nigrostriatal system of 3-NPM-treated rats

In the SN, aspartate levels are 370 % of control (p<0.01) at day 14, 250 % of control at day 21, and return to normal levels by day 28. Aspartate levels are 165 % of control on day 14, 220 % of control on day 21 (p<0.05) and 70 % of control by day 28. The results are presented on the graph in Fig.4.10.

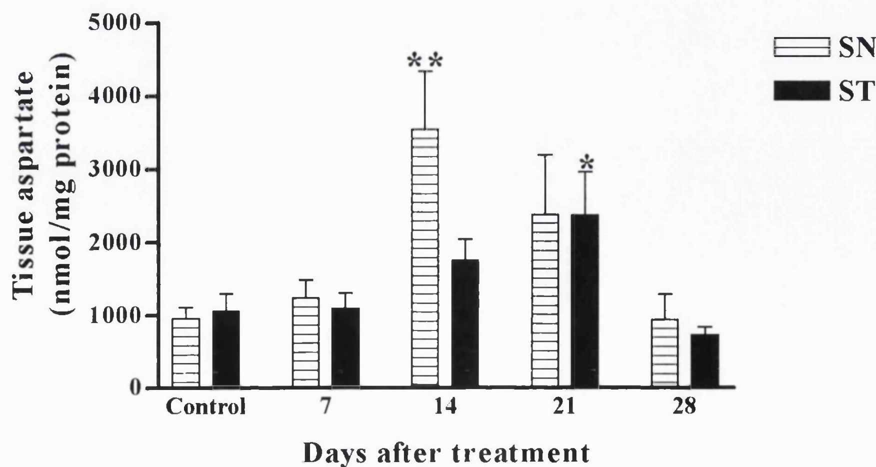


Fig. 4.10 - The effects of time on 3-NPM-induced changes in tissue aspartate levels in the SN and ST. Results are the mean + S.E.M. of a minimum of six determinations. (*p<0.05; **p<0.01 versus control).

4.2.1.11 Progressive changes in tissue glutamate levels in the mesolimbic system of 3-NPM-treated rats

There were no apparent changes in glutamate levels in the NAcc and PFC in the first 21 days after 3-NPM (Fig.4.11). On the 28th day, there was a 90 % fall in the NAcc ($p<0.01$), and a 60% fall in the PFC, when compared to control levels.

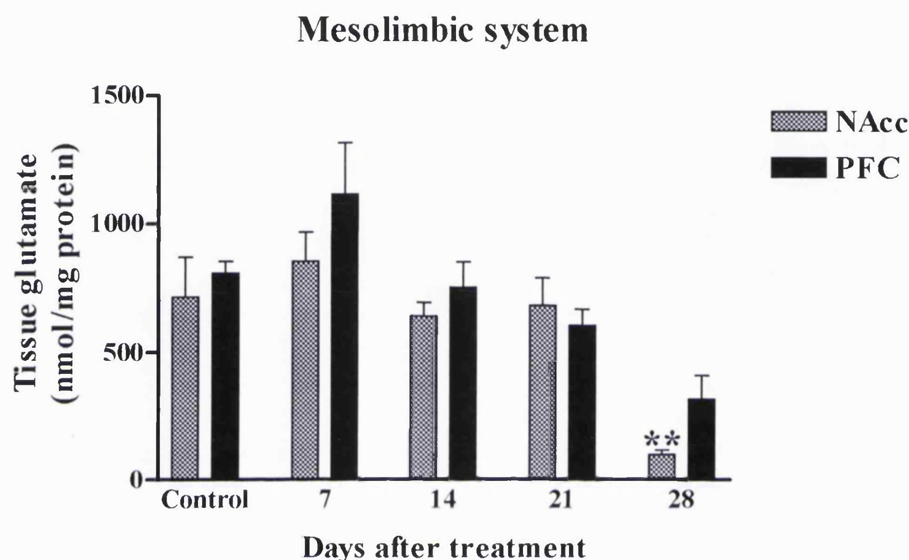


Fig. 4.11 - The effects of time on 3-NPM-induced changes in tissue glutamate levels in the NAcc and PFC. Results are the mean + S.E.M. of at least six experiments. ($p<0.01$versus control).**

4.2.1.12 Progressive changes in tissue glutamate levels in the nigrostriatal system of 3-NPM-treated rats

A 50 % fall in the concentration of glutamate was observed in the SN 28 days after 3-NPM. A similar decrease (~70 %) was observed in the ST at this time point ($p<0.05$). The amount of glutamate in both regions was not different from control in the first 21 days after 3-NPM. A chart of these findings is shown in Fig. 4.12.

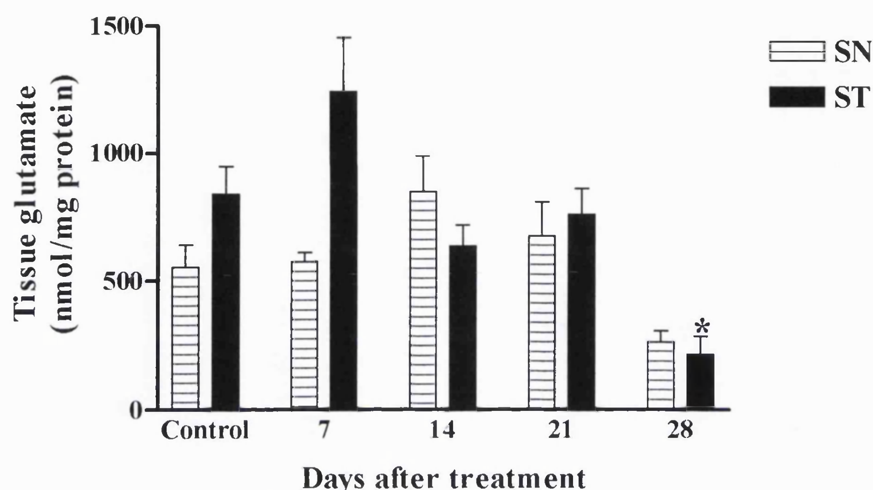


Fig. 4.12 - The effects of time on 3-NPM-induced changes in tissue glutamate levels in the SN and ST. Results are the mean + S.E.M. of at least six experiments. (*p<0.05 versus control).

4.2.2 Experiments with single-dose METH injection in the 3-NPM model

In the following experiments, METH was administered as a bolus injection of 20 mg/kg i.p. on day five in the 3-NPM schedule, to replace the multiple-dose regimen of 4 x 5 mg/kg ip. used in the previous experiments. This we termed "3-NPMsd." The animals were tested with apomorphine five days after the last injection of 3-NP, and sacrificed two days later, as for 3-NPM.

4.2.2.1 Differential effects of 3-NPM and 3-NPMsd on tissue DA levels in the mesolimbic system

Tissue DA levels in the NAcc were reduced by about 50 % after 3-NPM ($p<0.05$) whereas the single-dose METH treatment (3-NPMsd.) caused a rise to 120 % of control. Similarly, while 3-NPM induced a slight reduction of DA in the PFC, 3-NPMsd. yielded a five-fold increase in DA, as shown in Fig.4.13.

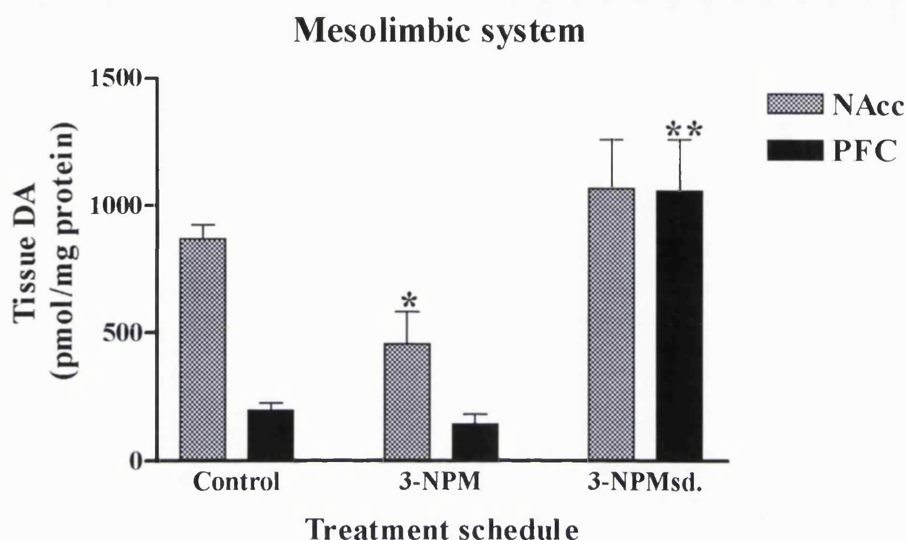


Fig. 4.13 - Differential effects of 3-NPM and 3-NPMsd. on tissue DA levels in the NAcc and PFC. Results are the mean +S.E.M. of at least six individual determinations.(*p<0.05;p<0.01 versus control).**

4.2.2.2 Differential effects of 3-NPM and 3-NPMsd. on tissue DA levels in the nigrostriatal system

In contrast with the DA-depleting effects of 3-NPM in the nigrostriatal tract, 3-NPMsd. caused a significant rise in the DA content of the SN ($p<0.01$), and a 30 % increase above the control values of DA in the ST. Figure 4.14 gives a vivid picture of these findings.

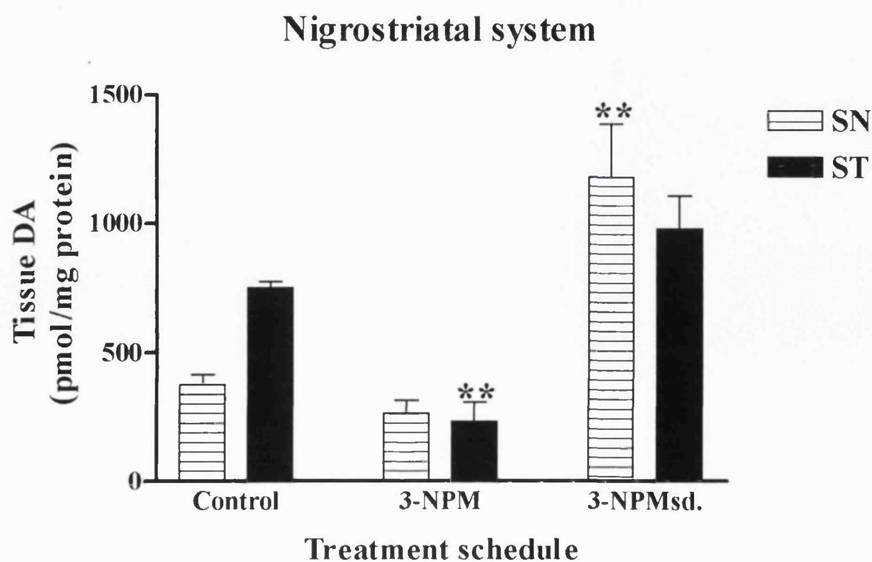


Fig. 4.14 - Differential effects of 3-NPM and 3-NPMsd. on tissue DA levels in the SN and ST. Results are the mean + S.E.M. of a minimum of six separate determinations. (p<0.01 versus control).**

4.2.2.3 Differential effects of 3-NPM and 3-NPMsd. on tissue 5-HT levels in the mesolimbic system

The effects of a bolus injection of METH were not significantly different from the loss of 5-HT in the mesolimbic system induced by 3-NPM. 5-HT levels in the NAcc and PFC are typically half the amounts in control rats (Fig. 4.15).

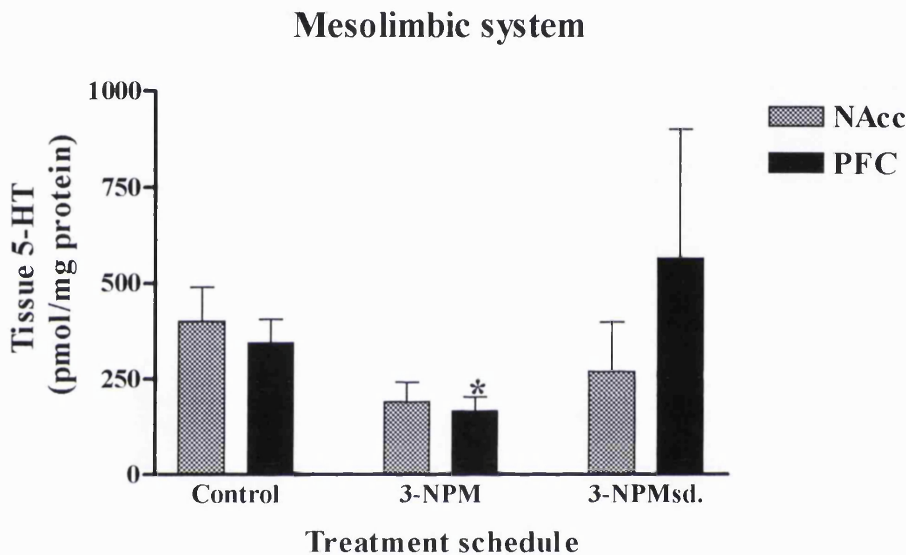


Fig. 4.15 - Differential effects of 3-NPM and 3-NPMsd. on tissue 5-HT levels in the NAcc and PFC. Results are the mean + S.E.M. of a minimum of six determinations. (*$p<0.01$ versus control).

4.2.2.4 Comparative effects of 3-NPM and 3-NPMsd. on tissue 5-HT levels in the nigrostriatal system

The amount of 5-HT in the nigrostriatal tract remained largely unchanged following treatment with either 3-NPM or 3-NPMsd. A graph showing this is found in Fig.4.16.

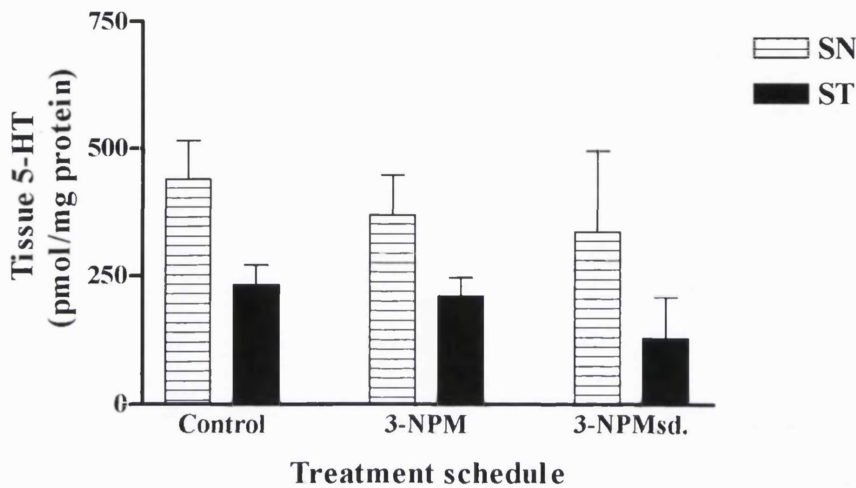


Fig. 4.16 - Comparative effects of 3-NPM and 3-NPMsd. on tissue 5-HT levels in the SN and ST. Results are the means of at least six experiments + S.E.M. ($p>0.05$ versus control).

4.2.2.5 Comparative effects of 3-NPM and 3-NPMsd. on DDC activity in the mesolimbic system

There was no significant change in DDC activity between control rats and 3-NPM or 3-NPMsd. rats in both the NAcc and PFC. Figure 4.17 is a graphic representation of these findings.

4.2.2.6 Differential effects of 3-NPM and 3-NPMsd. on DDC activity in the nigrostriatal system

The bolus injection of METH resulted in a 50 % drop in nigral DDC activity without a comparable effect in the ST. In 3-NPM-treated rats, the activity of DDC was not different from control values in the SN or ST. The results are shown in Fig. 4.18.

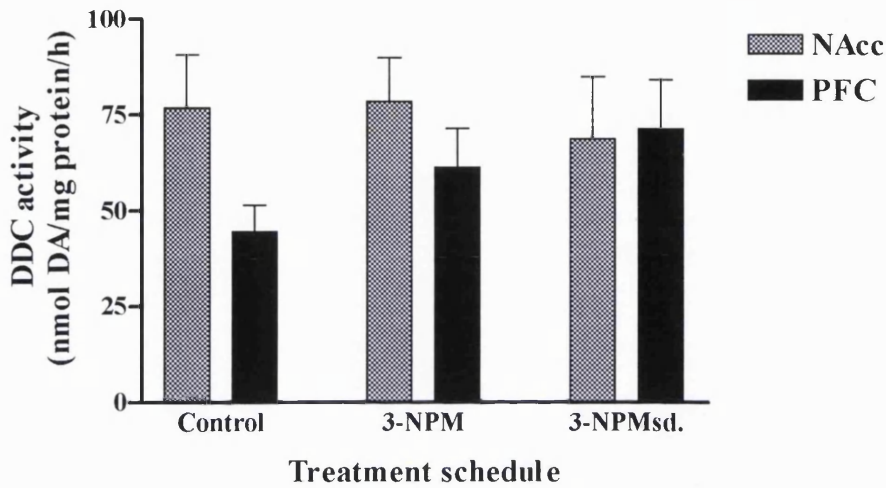


Fig. 4.17 - Comparative effects of 3-NPM and 3-NPMsd. on DDC activity in the NAcc and PFC. Results are the mean + S.E.M. ($p > 0.05$ versus control).

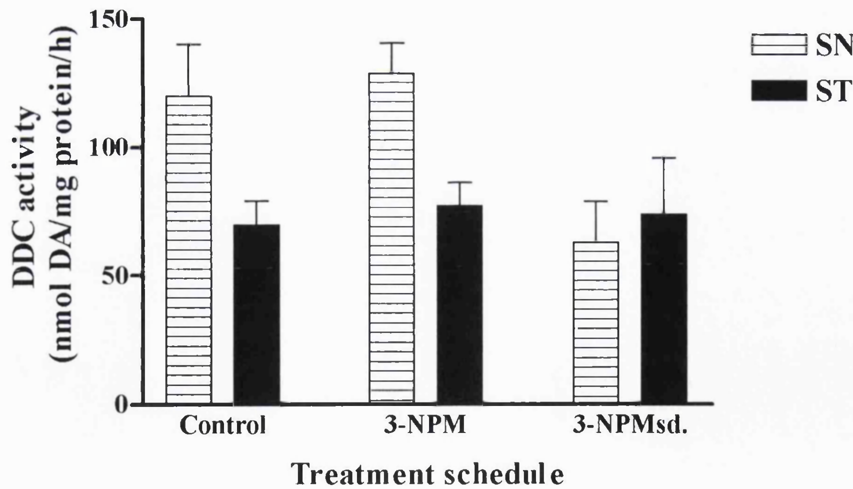


Fig. 4.18 - Differential effects of 3-NPM and 3-NPMsd. on DDC activity in the SN and ST. Results are the means of at least six determinations + S.E.M. ($p > 0.05$ versus control).

4.2.2.7 Differential effects of 3-NPM and 3-NPMsd. on 5-HTPDC activity in the mesolimbic system

Treatment with 3-NPMsd. resulted in a marked (50 %) fall in 5-HTPDC activity in the NAcc without a corresponding effect in the PFC. On the other hand, treatment with 3-NPM did not alter 5-HTPDC activity in both regions (Fig. 4.19).

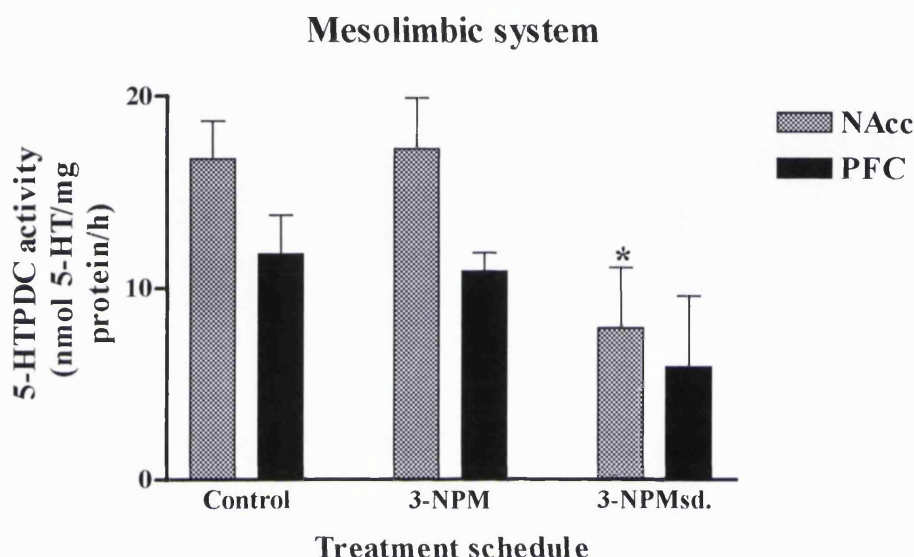


Fig. 4.19 - Differential effects of 3-NPM and 3-NPMsd. on 5-HTPDC activity in the NAcc and PFC. Results are the means of at least six determinations + S.E.M. (*$p<0.05$ versus control)

4.2.2.8 Differential effects of 3-NPM and 3-NPMsd. on 5-HTPDC activity in the nigrostriatal system

Treatment with either 3-NPM or 3-NPMsd. resulted in a marked depression of 5-HTPDC activity in the SN ($p<0.05$). In the ST however, the 40 % depression instituted by 3-NPM was accentuated to 80 % by 3-NPMsd. ($p<0.01$). These findings are depicted in Fig. 4.20.

4.2.2.9 Differential effects of 3-NPM and 3-NPMsd. on tissue aspartate levels in the mesolimbic system

There were no detectable changes in aspartate concentration in the PFC of rats treated with 3-NPM or 3-NPMsd. (Fig.4.21). Treatment with 3-NPMsd. resulted in a 60 % loss of aspartate in the NAcc ($p<0.05$).

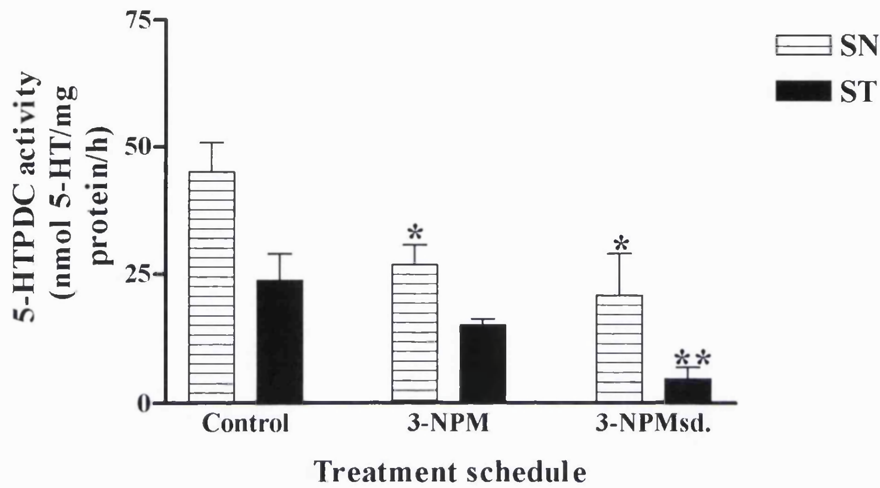


Fig. 4.20 - Differential effects of 3-NPM and 3-NPMsd. on 5-HTPDC activity in the SN and ST. Results are the means of at least six experiments + S.E.M. (*p<0.05, **p<0.01 versus control).

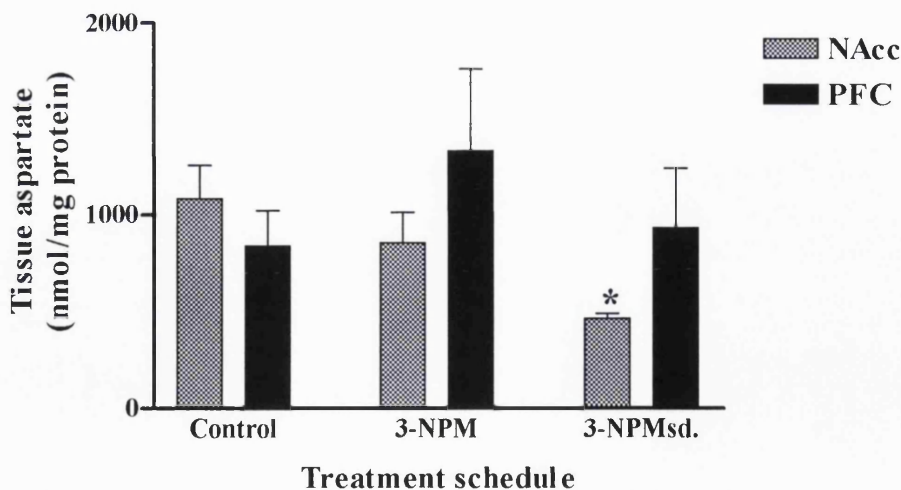


Fig. 4.21 - Differential effects of 3-NPM and 3-NPMsd. on tissue aspartate levels in the NAcc and PFC. Results are mean + S.E.M. for a minimum of six determinations. (*p<0.05 versus control).

4.2.2.10 Differential effects of 3-NPM and 3-NPMsd. on tissue aspartate levels in the nigrostriatal system

Treatment with 3-NPM did not induce changes in aspartate concentration in the nigrostriatal tract. 3-NPMsd., on the other hand, caused a significant fall in the SN ($p<0.01$), and a halving of control levels in the ST. A chart of these results is shown in Fig.4.22.

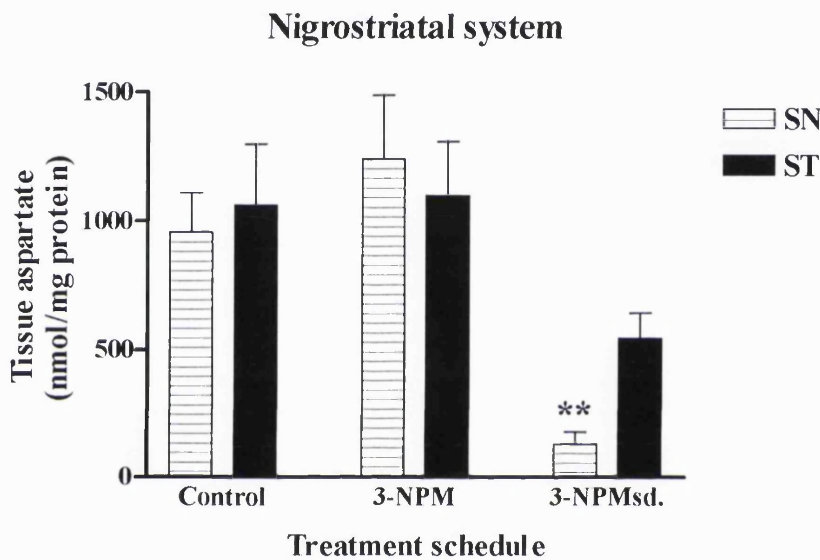


Fig. 4.22 - Differential effects of 3-NPM and 3-NPMsd. on tissue aspartate levels in the SN and ST. Results are the means + S.E.M. (P<0.01 compared to control).**

4.2.2.11 Differential effects of 3-NPM and 3-NPMsd. on tissue glutamate levels in the mesolimbic system

Tissue levels of glutamate were indistinct from controls in the NAcc and PFC of rats treated with 3-NPM. With 3-NPMsd. however, glutamate concentration dropped by 65 % in the NAcc, and by 70 % in the PFC ($p<0.05$). A graph of these results is shown in Fig. 4.23.

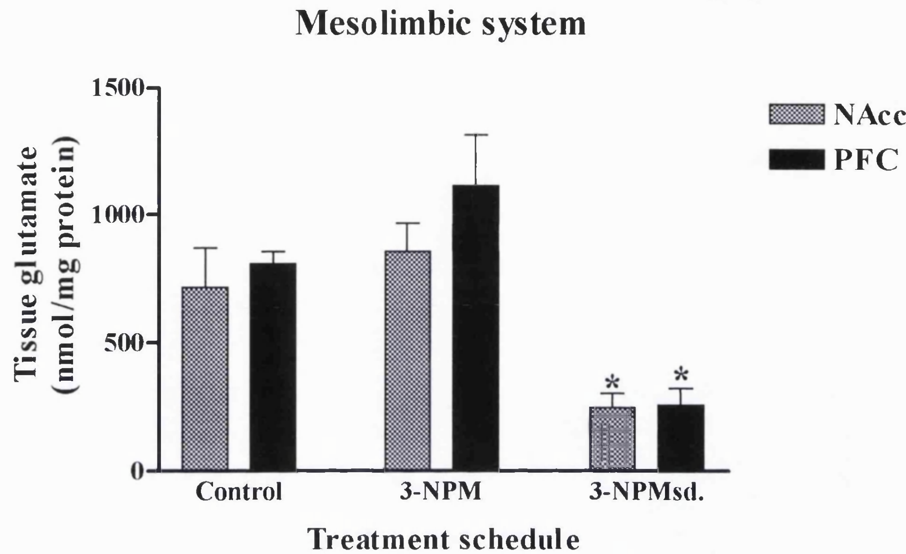


Fig. 4.23 - Differential effects of 3-NPM and 3-NPMsd. on tissue glutamate levels in the NAcc and PFC. Results are the mean + S.E.M. (*p<0.05 versus control).

4.2.2.12 Differential effects of 3-NPM and 3-NPMsd. on tissue glutamate levels in the nigrostriatal system

Similar to its effects on the mesolimbic system, 3-NPMsd. resulted in a 50 % fall in glutamate concentration in the SN ($p<0.05$) and an 80 % fall in the ST ($p<0.05$). Treatment with 3-NPM on the other hand, did not significantly alter the amount of glutamate in both tissues (Fig.4.24).

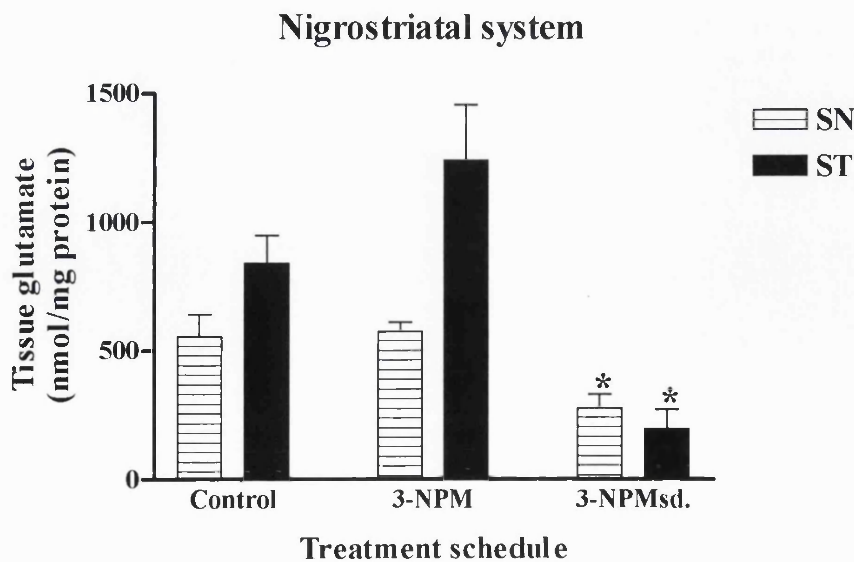


Fig. 4.24 - Differential effects of 3-NPM and 3-NPMsd. on tissue glutamate levels in the SN and ST. Results are the means + S.E.M. (*p<0.05 versus control).

4.3 Discussion

Following treatment with 3-NPM, tissue DA levels in the mesolimbic and nigrostiatal structures are significantly depressed seven days after treatment, but return to approximately normal values in the NAcc, PFC and ST, but not in the SN, 14 d after treatment. The disparity in DA levels in the SN and ST upholds the theory of differential DA system regulatory mechanisms in both regions (Melega et al., 1997). The serotonergic system showed less resistance to 3-NPM, as there were no detectable levels of 5-HT in all regions 14 and 21 d after treatment. Partial recovery of 5-HT was observed in all regions on day 28. The selective vulnerability of 5-HT neurones may be the result of their greater susceptibility to METH-induced degeneration (Cass, 1996). The reasons for these observations are not immediately apparent. However, the overall indication is that the neurotoxic damage induced by 3-NPM is impermanent, since recovery of monoaminergic function is evident 28 days after treatment.

METH-induced neurotoxic damage has been known to last as long as 4 years in rhesus monkeys (Woolverton et al., 1989), involving damage to both terminals (Albers et al., 1996) and cell bodies of DA neurones (Sonsalla et al., 1996). In rats, recovery from an ~70 % loss of DA 48 d after METH to a 20 % depletion at 237 d after treatment has been reported (Friedman et al., 1998). Quite recently, Cass and Manning (1999) reported that tissue DA levels were 54% of control 1 week after 4 x 5 mg/kg METH, 48 % at 1 month, 20 % at 6 months and similar to control at 12 months. The basal levels of extracellular DA returned to normal values by 1 month, but DOPAC and HVA levels were significantly below control, suggesting that basal extracellular levels of DOPAC and HVA are not caused solely by DA released from terminals, but may be the sum of synthetic and metabolic activities in surrounding DA terminals. This hypothesis is further strengthened by our findings that DDC and 5-HTPDC activities are significantly depressed in all regions 14 d after treatment, but undergo progressive recovery. The time course for recovery of AADC activity overlaps with the recovery of DA and 5-HT levels in the respective brain structures.

The relatively short recovery time in our experiments may be the result of specie differences, and presynaptic adaptations in DA neurones following lesioning (Robinson et al., 1994). As with 6-hydroxydopamine lesions, 3-NPM-induced lesions may result in compensatory upregulation of the surviving DA terminals. Compensatory increases in DA synthesizing

capacity are known to develop in the partially lesioned striatum (Melega et al., 1997). Furthermore, the partial recovery of 5-HT function 28 d after 3-NPM may be suggestive of the sprouting and reinervation of damaged neurones (Axt et al., 1994), as is the case with dopaminergic neurones in the substantia nigra after 6-hydroxydopamine lesions (Blanchard et al., 1996). In addition, the recovery of DA function after 3-NPM may be the result of induction of tyrosine hydroxylase cells (Bowyer et al., 1998), as well as the novel synthesis of succinate dehydrogenase to replace those inactivated by 3-nitropropionic acid.

Parkinson's disease has been shown to involve hyperactivity of the glutamate system, particularly in the subthalamo-nigral tract (Albin et al, 1989; Starr, 1995). Additional evidence can be adduced from the finding that the NMDA antagonist, MK 801, protects DA neurones against damage by MPTP or METH, indicating a role for glutamate in the mechanism of toxicity by METH (Sonsalla et al., 1989). Consequently, we determined the tissue levels of the EAAs aspartate and glutamate in both the mesolimbic and nigrostriatal systems. Aspartate levels rose appreciably, as would be expected following prolonged impairment of energy metabolism (Zeevalk et al., 1991), reaching peak values 14 and 21 d after 3-NPM. These time points correspond to the greatest decrements in DA and 5-HT levels, as well as DDC and 5-HTPDC activities. Similarly, glutamate levels were at their lowest levels at the 28 d time point (also Tsai et al., 1997), which was conducive for recovery of DA synthesis and release, and possible sprouting of 5-HT neurones (Axt et al., 1994). Although inconclusive, these results suggest a role for EAA in the mechanism of toxicity due to treatment with METH and 3-NP.

The traditional dosing schedule for inducing neurotoxicity with METH is the administration of 2-4 multiple injections every 2 h, with the fourth injection thought to be critical for inducing neuronal damage as it results in a marked overflow of DA (O'Dell et al., 1993). Recent findings suggest that a single high-dose injection of METH will accomplish the same effect (Fukumura et al., 1998). Our results show that rather than decrease DA and 5-HT levels in the mesolimbic and nigrostriatal structures, 3-NPMsd. provoked an increase in the tissue concentrations of these neurotransmitters over values for 3-NPM and for controls. This may be the consequence of a rapid induction of mRNA for TH-positive cells (Bowyer et al., 1998). DDC activity in the mesolimbic and nigrostriatal systems are not different between 3-NPM and 3-NPMsd. The

activity of 5-HTPDC is reduced in the nucleus accumbens and nigrostriatal tract of 3-NPMsd-treated rats, whereas 3-NPM treatment alters 5-HTPDC only in the substantia nigra.

The tissue levels of aspartate and glutamate in the mesolimbic and nigrostriatal systems of rats treated with 3-NPMsd. are markedly less than controls, and the values for 3-NPM rats. This may be a contributing factor to the observed elevation in monoamine content.

Taken together, the effects of a single METH injection differ appreciably from the effects of 3-NPM. Whereas tissue monoamine content is reduced with the latter, the bolus treatment results in an elevation of tissue content of DA and 5-HT. Both treatments could only be used inter-changeably when studying DDC or 5-HTPDC activity.

CHAPTER FIVE

BEHAVIOURAL ASSESSMENT OF THE 3-NPM MODEL

5.1 Introduction

The cardinal features of Parkinson's disease are akinesia or bradykinesia, resting tremor and rigidity. It has been the criterion for an effective animal model of the disorder, to incorporate one or more of these features. However, rodents offer a limited scope in this area. Reserpinized rats show bradykinesia, but 6-hydroxydopamine lesioned rats will only circle after a challenge with a dopamine receptor agonist.

3-nitropropionic acid intoxication yields rats characterized by somnolence, uncoordinated gait with stereotypical paddling movements, and ventral or lateral recumbency (Hamilton and Gould, 1987), vacuous chewing movements reminiscent of tardive dyskinesia (Andreassen and Jorgensen, 1995), as well as other hyperactive and hypokinetic states (Borlongan et al., 1997). Consequently, 3-NP treatment has become a candidate model for the induction of Huntington's chorea in rodents (Borlongan et al., 1995, 1997; Brouillet et al., 1993). Once the right dose is identified, 3-NP administration could result in hypoactive/bradykinetic states such as occur in parkinsonism.

Methamphetamine treatment results in hyperkinesis and stereotypic ambulation within minutes of administration, a consequence of massive releases of monoamines in the brain (Robinson and Becker, 1986). Judging by its ability to reduce DA necessary for inducing movement in the nigrostriatal tract, it is expected that METH will contribute to the overall picture of hypoactivity expected in a model of Parkinson's disease. The neurochemical changes in the 3-NPM-treated rats (see Chapter Three), indicate the presence of sufficient lesioning as to cause supersensitive responses of these animals to various dopaminergic drugs. The following experiments were designed to assess the extent of lesioning, with the use of various direct and indirect dopamine agonists. All animals were allowed to habituate for 2 h before drug injection.

5.2 Results

5.2.1 Locomotor responses to apomorphine

5.2.1.1 Evolution of DA receptor supersensitivity in 3-NPM-treated rats

About 18-24 h after the eighth injection of 3-NP, the rats were humped, bradykinetic and showed only a mild response to apomorphine (0.5 mg/kg s.c.). Four days later, the locomotor

counts of these rats was increased four-fold following the apomorphine challenge ($p<0.001$). A graph of these findings is shown in Fig. 5.1a. Similarly, rats treated with the higher dose of 3-NP (30 mg/kg, 3-NP3M), did not respond to apomorphine 18-24 h after the last injection, even though these rats were ~70 % striatal DA-depleted (Fig.3.12). However, there was a doubling of the cumulative locomotor score in rats tested with apomorphine three days later (Fig. 5.1b).

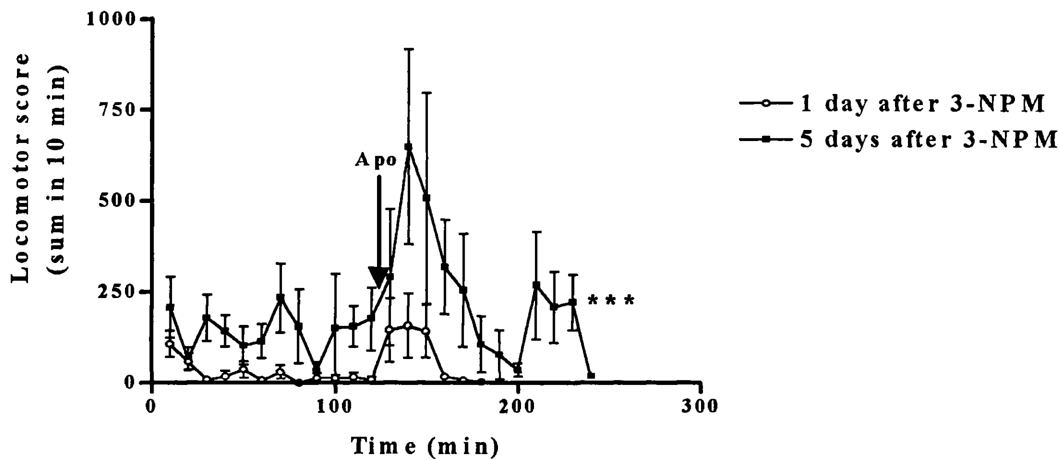


Fig. 5.1a - Development of dopamine receptor supersensitivity to apomorphine (Apo, 0.5 mg/kg s.c.) in 3-NPM rats. Results are the means ± S.E.M. of four replicates at each time point. (***$p<0.001$ compared to 1 day after 3-NPM)

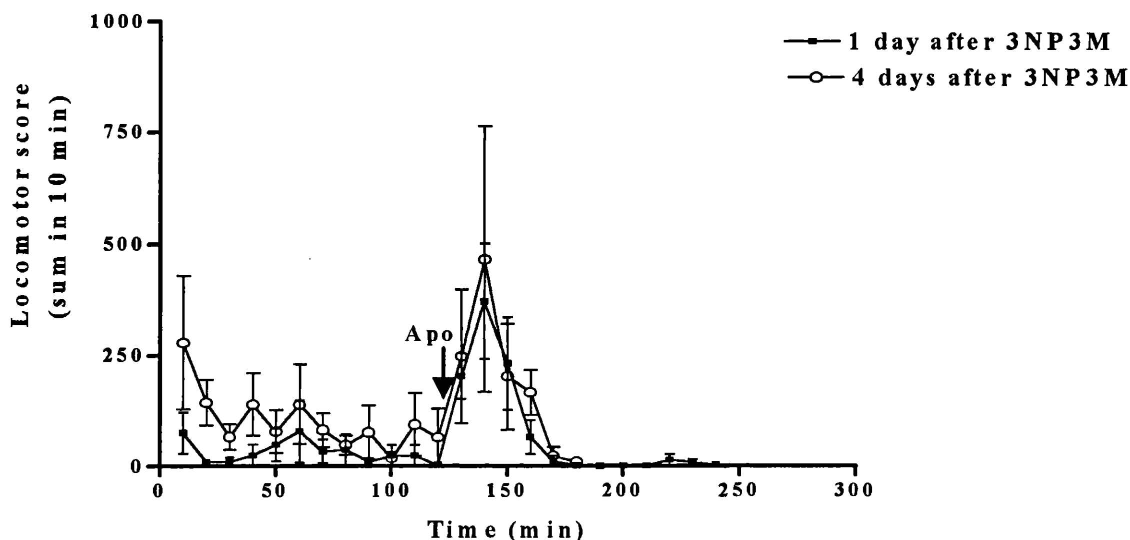


Fig. 5.1b - Response to apomorphine (Apo, 0.5 mg/kg s.c.) one and four days after 3-NP3M. The results are the means of four replicates ± S.E.M. ($p>0.05$ versus day 1).

5.2.1.2 Locomotor responses of the 3-NPM groups to apomorphine

The spontaneous locomotor activity of 3-NPM rats was not different from that of saline-treated controls. There was also no significant difference between the locomotor responses of the METH, 3-NP and saline control groups to the apomorphine challenge, five days after the last injection. However, the 3-NPM rats showed a four-fold increase over control values ($p<0.01$). This increased locomotion had a biphasic pattern, reaching two peaks, 20 and 90 mins after exposure to apomorphine (Fig. 5.2).

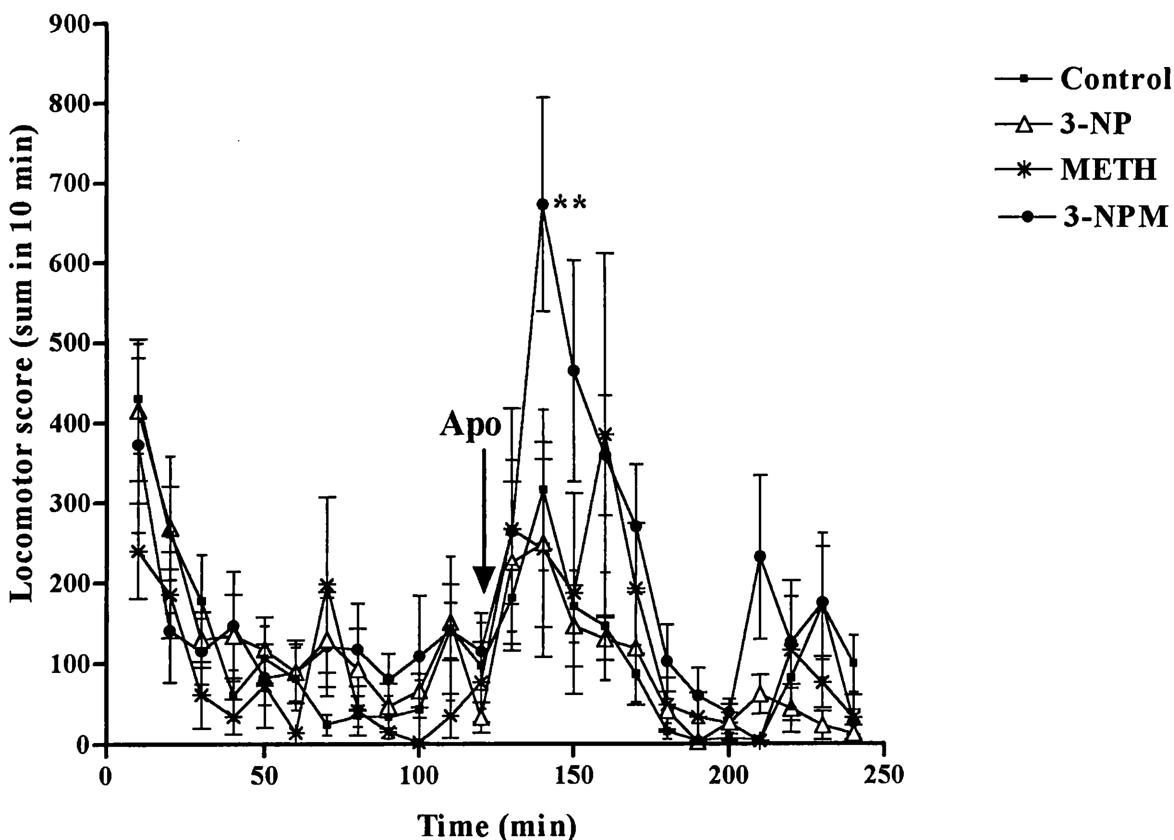


Fig. 5.2 - Group locomotor responses to apomorphine (0.5 mg/kg s.c.) five days after treatment. The results represent the means of 10 minute sums of locomotor scores from at least six experiments ± S.E.M. (p<0.01 versus control).**

5.2.1.3 Effects of DA D_1 and D_2 antagonists on the locomotor properties of apomorphine in 3-NPM-treated rats

The dopamine D_1 antagonist, SCH 23390 (0.3 mg/kg i.p.), and the selective D_2 antagonist, sulpiride (100 mg/kg s.c.), attenuated the locomotor response of the 3-NPM rats to apomorphine ($p<0.01$), indicating an involvement of both receptors. Figure 5.3 depicts these effects.

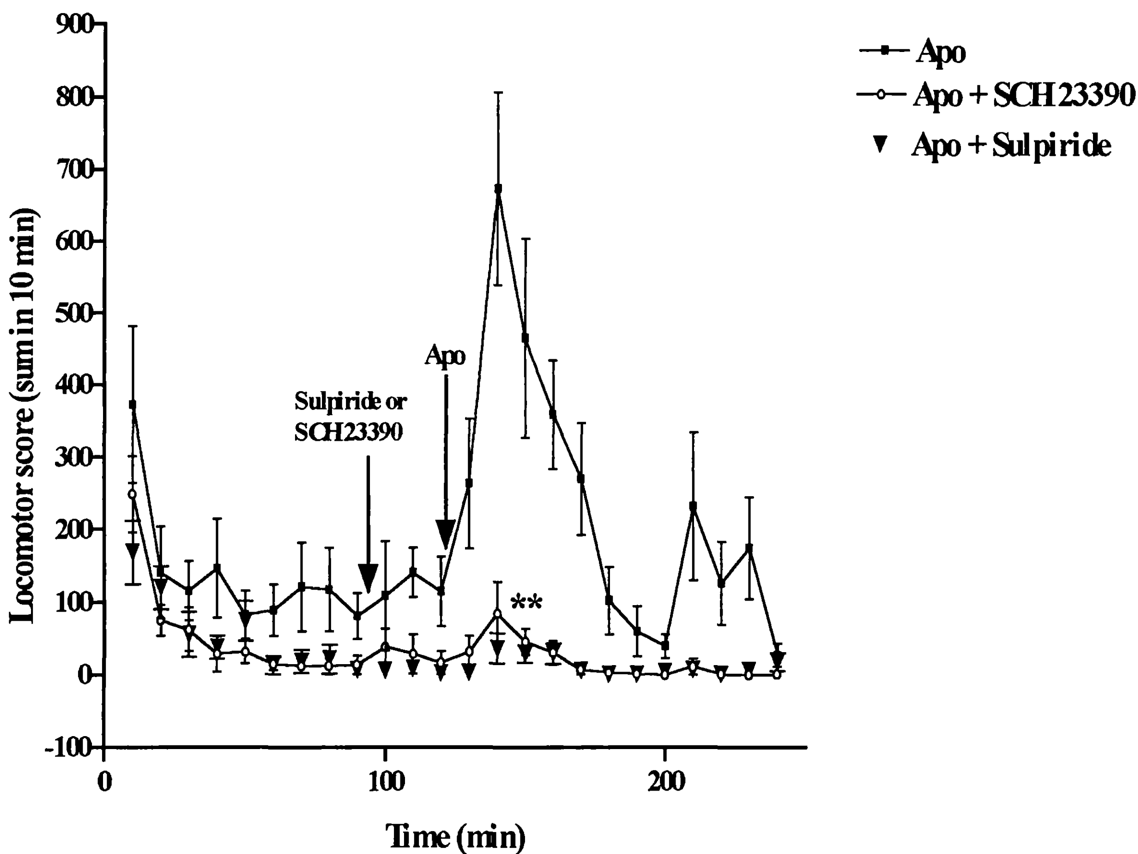


Fig. 5.3 - The effects of Sulpiride (100mg/kg sc.) and SCH23390 (0.3 mg/kg i.p.) on the locomotor response to apomorphine (0.5 mg/kg s.c.), five days after 3-NPM treatment. Results are the mean of at least five experiments ± S.E.M. (p<0.01 compared to Apomorphine only).**

5.2.1.4 Differential effects of apomorphine in 3-NPM- and 3-NPMsd.-treated rats

There was a marked reduction ($p<0.01$) in locomotion following the administration of apomorphine to rats treated with 3-NP and a bolus injection of METH (20 mg/kg i.p.) i.e. 3-NPMsd. In contrast, 3-NPM-treated rats were significantly more mobile than unlesioned rats, after a challenge with apomorphine (Fig. 5.4). Whereas the pattern of locomotion with 3-NPM showed two peaks, 3-NPMsd. had a single peak at 40 min post apomorphine.

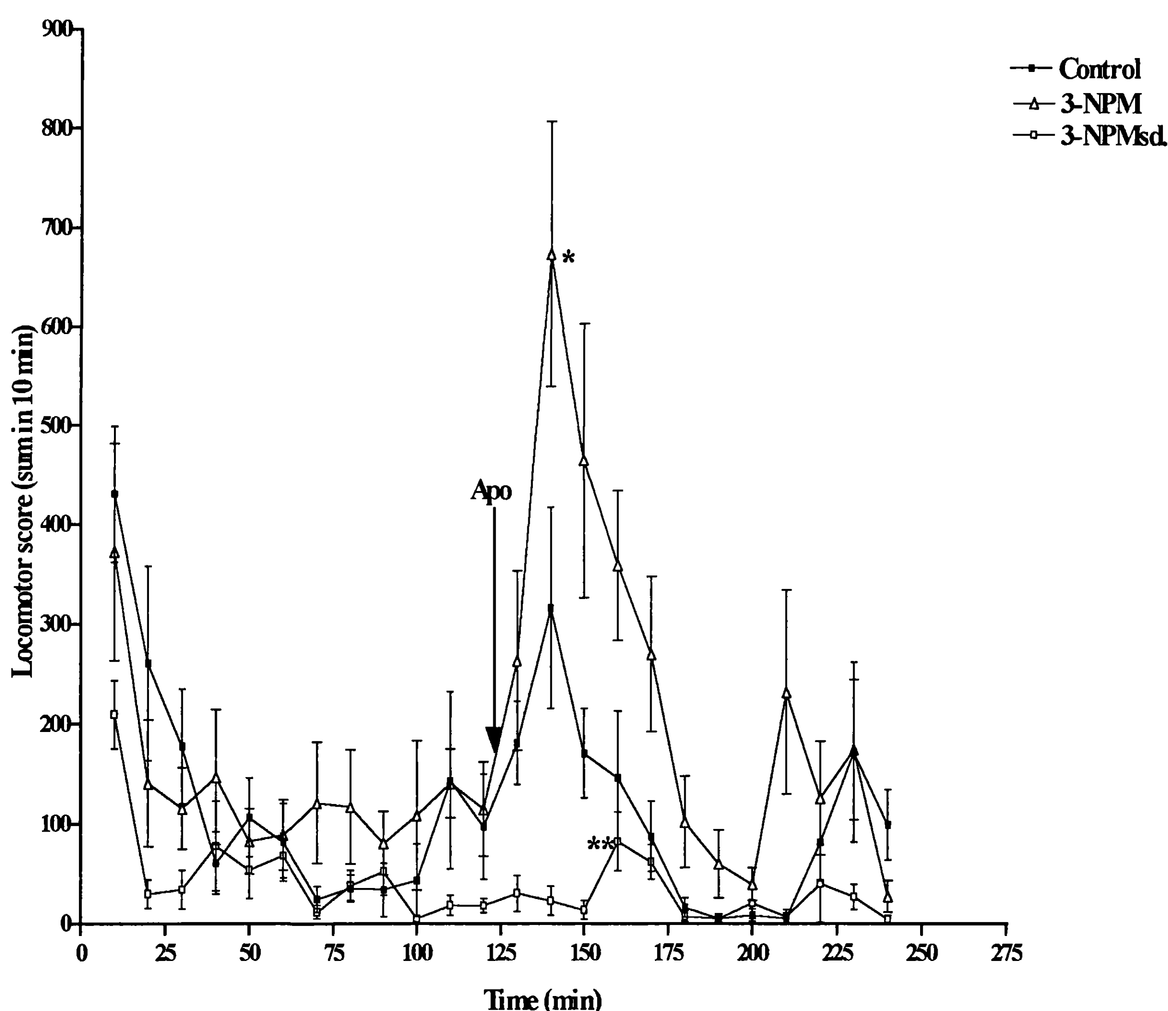


Fig. 5.4 - A comparison of the locomotor responses to apomorphine (0.5 mg/kg s.c.) in rats treated with 3-NPM or 3-NPMsd. (single dose METH at 20 mg/kg i.p., rather than 4x5 mg/kg). The results are the means ± S.E.M of at least six separate determinations. (*p<0.05, **p<0.01 compared to effect in control rats).

5.2.2 Locomotor responses to MK 801

5.2.2.1 Comparative effects of MK 801 in unlesioned and 3-NPM-treated rats

In figure 5.5, we find a marked increase ($p<0.01$) in locomotion a few minutes after the administration of the DA-releasing agent MK 801 (0.3 mg/kg i.p.), when compared to the effect of the same drug in unlesioned, monoamine intact rats. The rats would run, sniff the floor, and engage in bouts of rearing.

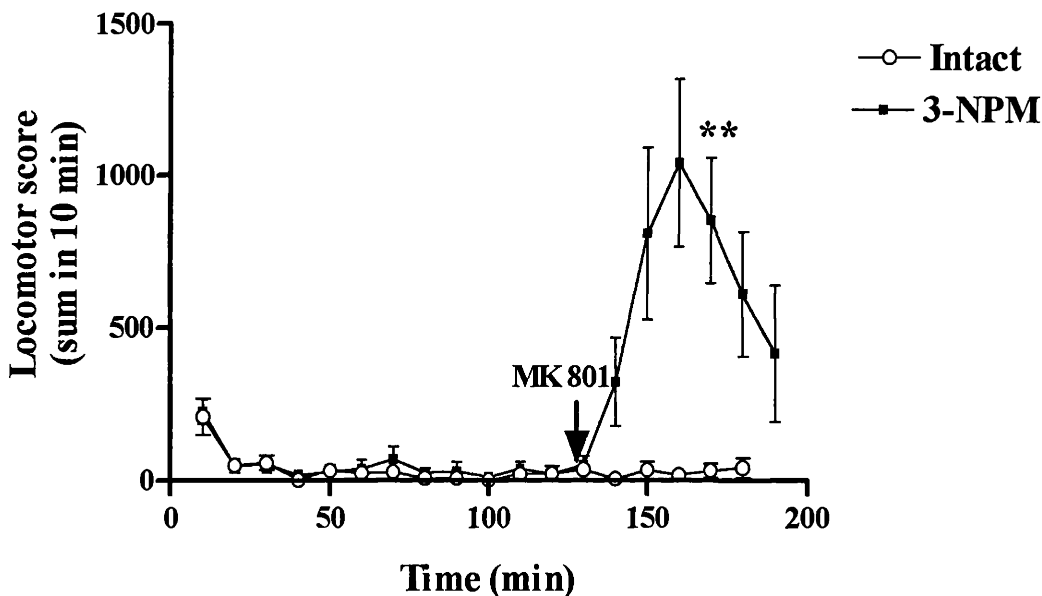


Fig. 5.5 - Locomotor effects of MK 801 (0.3 mg/kg i.p.) in intact and 3-NPM-treated rats. Results are the means ± S.E.M of six determinations (n=4 for monoamine Intact). (p<0.01 compared to intact rats).**

5.2.2.2 Effects of DA D_1 and D_2 antagonists on the locomotor properties of MK 801 in 3-NPM-treated rats

The DA D_1 antagonist, SCH 23390 (0.3 mg/kg i.p.), as well as the D_2 antagonist, sulpiride (100 mg/kg s.c.), significantly reduced ($p<0.01$) the locomotion induced by MK 801 (0.3 mg/kg i.p.). Treatment with sulpiride reduced the tendency to rear, while SCH 23390 reduced total mobility. A graph of these effects is shown in Fig. 5.6.

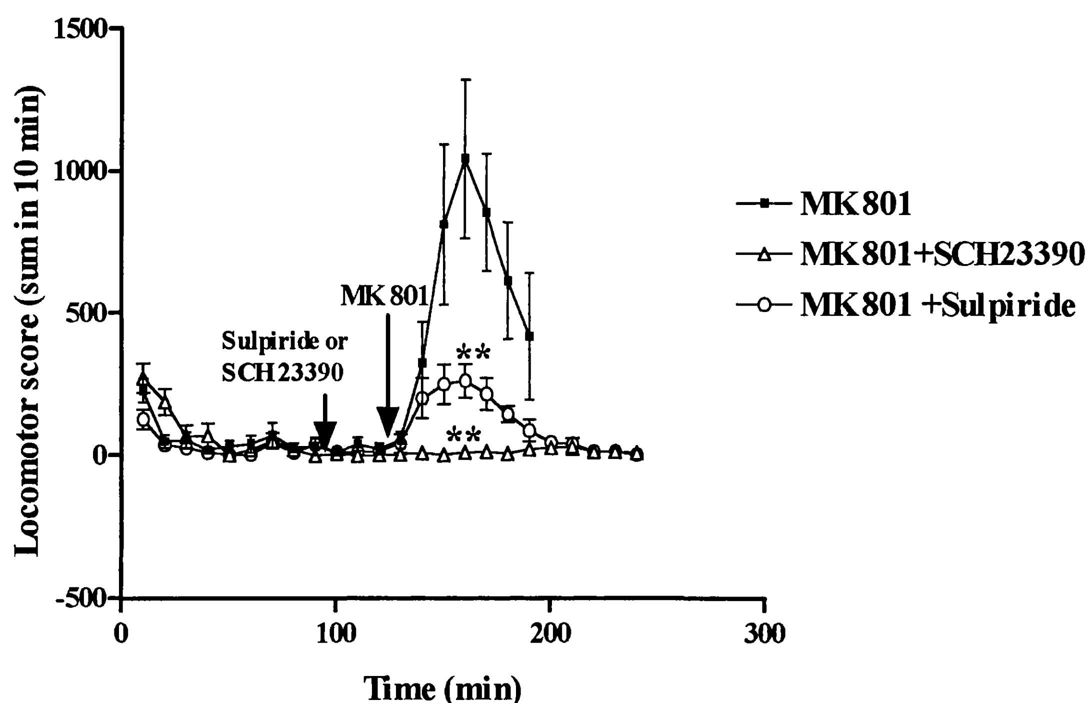


Fig. 5.6 - The effects of Sulpiride (100 mg/kg s.c.) and SCH 23390 (0.3 mg/kg i.p.) on the locomotor properties of MK801 (0.3 mg/kg i.p.) in 3-NPM rats. Results are the mean ± S.E.M. of at least five replicates. ($p<0.01$ compared to the effects of MK801 only).**

5.2.3 Locomotor responses to SKF 38393

5.2.3.1 Comparative effects of SKF 38393 in unlesioned and 3-NPM-treated rats

There was no significant difference in the locomotor responses of intact and 3-NPM-treated rats ($p>0.05$) to treatment with the D_1 agonist SKF 38393 (30 mg/kg i.p.). A comparison of the grooming time in response to SKF 38393 between both groups, showed that there were equal increments in grooming time. A graph of the locomotor responses to SKF 38393 is shown in Fig. 5.7 below. In addition, total grooming time over a 2-h period increased significantly from 3.3 $\pm$ 0.9 min to 17.5 $\pm$ 3.2 min ($p<0.001$) after SKF 38393, in control rats, and from 3.8 $\pm$ 1.0 min to 15.3 $\pm$ 2.0 min in 3-NPM rats ($p<0.001$). Although SKF 38393 increased grooming in both sets of animals, there was no additional effect with 3-NPM ($p>0.05$).

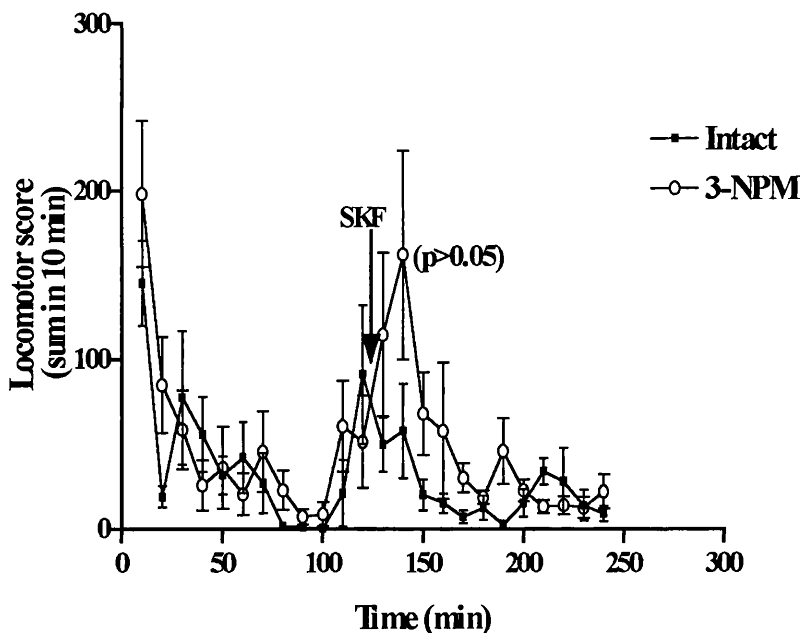


Fig. 5.7 - The locomotor effects of SKF 38393 (30 mg/kg i.p.) in intact and 3-NPM-treated rats. Results are the mean ± S.E.M. of at least six replicates. (p>0.05 versus intacts).

5.2.3.2 Effects of DA D_2 antagonism on the locomotor properties of SKF 38393 in 3-NPM-treated rats

The D_2 antagonist, sulpiride (100 mg/k s.c.), reduced the locomotor responses of 3-NPM-treated rats to SKF 38393, as shown in Figure 5.8.

The effects of D_2 antagonism on the motor properties of SKF 38393

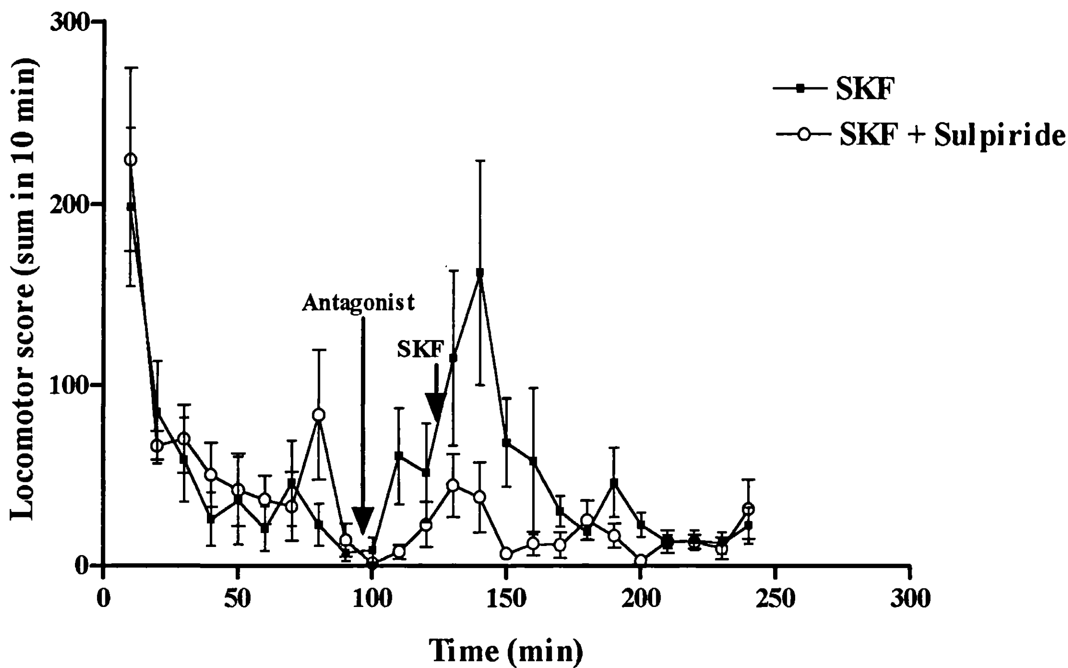


Fig. 5.8 - The effects of Sulpiride (100 mg/kg s.c.) on the locomotor activity of SKF 38393 (30 mg/kg i.p.) in 3-NPM-treated rats. The results are the means ± S.E.M for a minimum of six replicates at each time point.

5.2.4 Locomotor responses to RU 24213

5.2.4.1 Differential effects of RU 24213 in unlesioned and 3-NPM-treated rats

There is a marked increase ($p<0.01$) in locomotion in 3-NPM-treated rats within 10 min after exposure to the D_2 agonist, RU 24213 (5 mg/kg s.c.), attaining a peak 50 min after injection (Fig. 5.9). The rats moved about in trots, with bouts of rearing, sniffing and jumping. Rearing counts were significantly increased from 33 $\pm$ 8 in unlesioned rats to 219 $\pm$ 64 in 3-NPM rats.

Locomotor effects of RU24213 in intact and 3-NPM rats

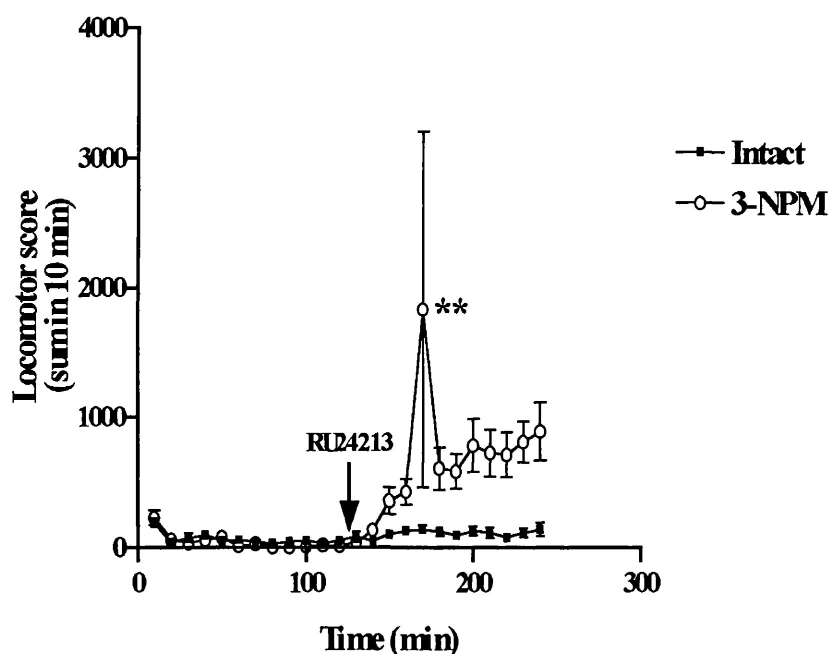


Fig. 5.9 - Locomotor effects of RU24213 (5 mg/kg s.c.) in intact and 3-NPM-treated rats. The results represent the means ± S.E.M. of seven replicates. (p<0.01 compared with intact rats).**

5.2.4.2 Effects of DA D_1 and D_2 antagonists on the locomotor properties of RU 24213 in 3-NPM-treated rats

The DA D_1 antagonist, SCH 23390 (0.3 mg/kg i.p.), and the D_2 antagonist, sulpiride (100 mg/kg s.c.) attenuated the locomotion induced by RU 24213 (5 mg/kg s.c.) in 3-NPM-treated rats. The extent of reduction was higher with sulpiride ($p<0.01$) than with SCH 23390 ($p<0.05$). A depiction of these effects can be seen in Fig. 5.10 below.

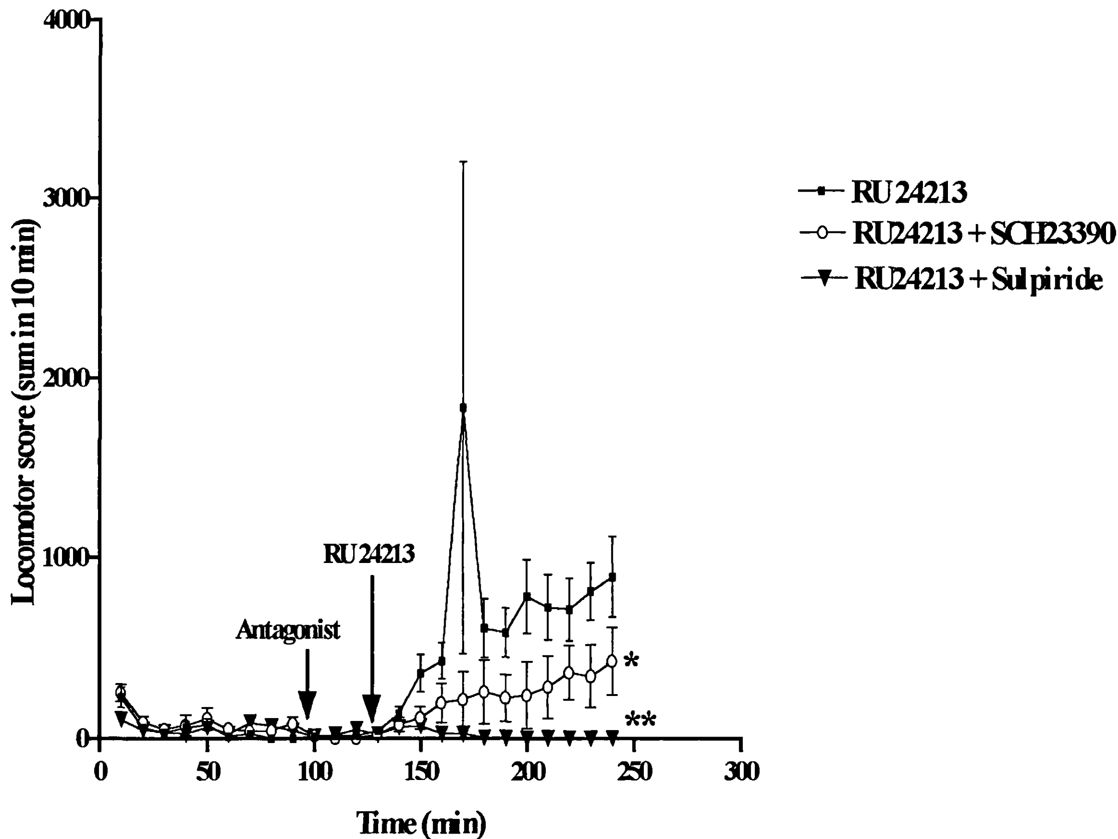


Fig. 5.10 - The effects of Sulpiride (100 mg/kg s.c.) and SCH23390 (0.3 mg/kg i.p.) on the locomotor properties of RU24213 (5 mg/kg s.c.) in 3-NPM rats. Results are the means ± S.E.M. of a minimum of five experiments. (*$p<0.05$, **$p<0.01$ compared to RU24213 only).

5.3 Discussion

Following destruction of nerve terminals or cell bodies, DA receptors become supersensitive to the effects of dopamine or dopaminergic agonists like apomorphine (Anden et al., 1967; Costall et al, 1977). The destruction of DA cells and nerve endings by 3-NPM, as reflected by the reduction of tissue DA levels, resulted in the supersensitivity of DA receptors in the denervated tissues. This was responsible for the four-fold increase in response to apomorphine (Fig. 5.2).

The development of receptor supersensitivity has not been known to be an immediate process, as it involves an up-regulation of mRNA and subsequent protein synthesis. The trigger factor for mRNA synthesis is, of course, the sustained decrease in the availability of dopamine to interact with its receptor (Rots et al., 1996). However, the fact that there is a significant decrease in tissue DA does not immediately equate with receptor supersensitivity, as is the case with reserpine and 6-OHDA-treated rats (Rubinstein et al., 1988; Rouillard et al., 1988). This was also found to be the case with rats treated with 30 mg/kg 3-NP (Fig. 3.11-3.12). At 15-19 h after the last injection, the rats were significantly rigid, with an appearance similar to that of reserpinized rats. This was reflected by their depressed spontaneous locomotor activity scores (Fig. 5.1b), an effect suggestive of hypokinesis (Araujo and Hilt, 1998). Although these rats showed a six-fold increase in locomotor score in response to apomorphine, the level of locomotion and its fluidity was different from the spontaneous locomotor activity of saline controls or 3-NPM rats. The rats also showed a 1 in 4 chance of developing paralysis, and 41% lethality (Fig. 3.25).

Neurochemical analysis of these '3-NP3M' rats showed that they had 57 % DA depletion in the NAcc and 73% striatal DA loss (Fig. 3.11-3.12). 5-HT levels in the PFC and ST were significantly reduced (Fig. 3.13-3.14), while no apparent change was detected in the tissue levels of glutamate (Fig. 3.21-3.22). The levels of aspartate in the NAcc, PFC and SN were not different from controls, but the level in the ST was reduced to half of control values (Fig. 3.19-3.20). In addition, the conversion of L-DOPA to DA in the SN and ST was markedly reduced, while all regions (except the NAcc) showed a significant impairment in their ability to decarboxylate 5-HTP (Fig. 3.16-3.17). When assessed on the fourth day post treatment, the spontaneous locomotion of these rats increased to levels comparable to those of control rats,

but their response to apomorphine was reduced.

In the 3-NPM rats, a slowing of movement was observed 24 h after the eighth injection of 3-NP. This was responsible for the low spontaneous activity counts of 239 $\pm$ 71, a third of the score in control rats or 3-NPM model rats (i.e. five days after the eighth 3-NP injection) (Fig. 5.1a). The response to apomorphine was slight, compared to that observed at five days post treatment. Spontaneous activity improved by day 5, and the 3-NPM rats were not distinct from control rats in appearance.

In normal rats, there is a cooperative/synergistic (Braun and Chase, 1986; Clarke and White, 1987) or oppositional (Eilam et al., 1992; Murray and Waddington, 1989) interaction between DA D_1 and D_2 receptors, in the production of behavioural responses to DA agonists. Following depletion of striatal DA by treatment with reserpine (Arnt, 1985) or 6-OHDA (Arnt and Hyttel, 1984), it is believed that there is a breakdown of this synergism. However, some other authors uphold the view that behaviour induced by dopamine receptor stimulation requires simultaneous stimulation of D_1 and D_2 receptors, even under supersensitive conditions (Braun and Chase, 1986; Dziewczapolski et al., 1997; LaHoste and Marshall, 1993; Rubinstein et al., 1988; Starr and Starr, 1989). The protocol employed by LaHoste and Marshall resulted in striatal DA depletions of 96%, 92%, and 71%, following treatment with reserpine, 6-OHDA and AMPT respectively. The 3-NPM dosing schedule resulted in ~70% loss of striatal DA, as well as supersensitive DA receptors, providing a useful paradigm for comparative studies.

SKF 38393, a partial D_1 agonist (Setler et al., 1978), is known to induce grooming behaviour in mice (Starr and Starr, 1986) and rats (Eilam et al., 1992; Wachtel et al., 1992), via stimulation of DA D_1 receptors in the central nervous system. This effect is abolished by pretreatment with AMPT or reserpine in mice (Starr et al., 1987) or rats (Braun and Chase, 1986). Although SKF 38393 has been reported to induce locomotion in reserpinized mice (Arnt, 1985) and developing rats (Shieh and Walters, 1996), locomotion and stereotyped behaviour (repetitive sniffing, rearing, licking and gnawing) are generally regarded as D_2-dependent behaviours (Clarke and White, 1987). Unlike AMPT in rats (Braun and Chase, 1986) and reserpine in mice (Starr et al., 1987), 3-NPM pretreatment in rats did not abolish SKF 38393-induced grooming.

Whereas SKF 38393 induced locomotion in reserpine-treated mice (Starr and Starr, 1989), prior exposure to 3-NPM did not reveal a supersensitive response to this compound. This may not be unconnected with the fact that the lateral striatum, essential for the expression of the effects of SKF 38393 (Neisewander et al., 1991), is most vulnerable to 3-nitropropionic acid (Guyot et al., 1997). Furthermore, both grooming and locomotion were attenuated in the 3-NPM rats by the D_2 antagonist sulpiride, indicating a lack of D_1-independence in the expression of the effects of SKF 38393. The latter outcome is also suggestive of an involvement of D_2 receptors in the underlying mechanism, as would be expected under conditions of supersensitivity (Braun and Chase, 1986; Dziewczapolski et al., 1997). In addition, the observed involvement of D_2 receptors in the behaviour profile of SKF 38393-treated rats may be an indication of the possible reciprocity of interaction, in which D_1 agonists require D_2 activation for the expression of their effects (Clarke and White, 1987; Wachtel et al., 1992; Waddington and O'Boyle, 1989).

Treatment with multiple doses of methamphetamine results in decreases in D_1 and D_2 receptor populations in the brains of rats (McCabe et al., 1987), and D_1 populations in mice (Cadet et al., 1998). The preferential reduction of striatal D_1 receptors may not be unconnected with the fact that the striatonigral output pathway (predominantly D_1) is more susceptible than the striatopallidal output pathway (predominantly D_2) to energy impairments, as would normally occur with aging (Araujo and Hilt, 1998). A further indication of the involvement of D_1 receptors in METH toxicity is the finding that the neurotoxic effects of METH are potentiated by treatment with the D_2 blocker, sulpiride, but prevented by the D_1 antagonist SCH 23390 (Bronstein and Hong, 1995).Whereas there is an observed decrease in the population of D_1 DA receptors, there is an upregulation of striatal D_2 receptors in 6-OHDA-treated rats (Gagnon et al., 1991) or MPTP-treated primates (Elsworth et al., 1998; Gnanalingham et al., 1993; Graham et al., 1990) and mice (Tanji et al., 1999). 3-nitropropionic acid has also been shown to induce a down-regulation of striatal D_1 receptors by interacting with striatonigral GABA output neurones (Koutouzis et al., 1994), neurones known to be endowed with D_1 dopamine receptors (Gerfen et al., 1990).

The selective D_2 agonist, RU 24213 (Euvrad et al., 1980), induces fast locomotor movements and rearing in naive (Starr and Starr, 1986) and reserpine-treated mice (Starr and Starr, 1994), and contra-lateral circling in 6-OHDA-treated rats (Rouillard and Bedard, 1988). In our experiments, RU 24213 produced weak locomotor activation in naive rats, but a pronounced increase in running and rearing following treatment with 3-NPM. This supersensitive response to RU 24213 is indicative of an upregulation of D_2 receptors, as stated above (Tanji et al., 1999). This finding is important in the light of recent reports that the D_2/D_3 agonists Pramipexole (Hall et al., 1996) and Talipexole (Kondo et al., 1998), protect dopaminergic neurones from methamphetamine-induced degeneration, holding potential for their use as antiparkinsonian agents.

Whereas D_2 blockade with sulpiride resulted in a complete inhibition of the characteristic locomotor induction of RU 24213 in our 3-NPM rats, the D_1 antagonist, SCH 23390, only partially reduced the ambulation, while markedly increasing the frequency of and time spent in sniffing the floor of the cage, and in cage-side rearing. In normal rats, SCH 23390 has been known to completely block the stereotypy and locomotor activity induced by the selective D_2 agonist, quinpirole (Breese and Mueller, 1985). Although D_1 activation is required for the expression of locomotion by selective D_2 agonists in naive and DA-depleted rodents (Arnt, 1985; Clarke and White, 1987), the contradiction in the present results may be the consequence of incomplete blockade of D_1 receptors (LaHoste and Marshall, 1993; Rouillard and Bedard, 1988), or the reported functional independence of the D_2 receptors (LaHoste and Marshall, 1993).

Apomorphine induces slow, perseverative movement, grooming and stereotyped rearing and sniffing by direct stimulation of D_1 and D_2 receptors (Seeman, 1980; Starr and Starr, 1986), as well as by reduction of GABA activity in the pallidum (Mele et al., 1998). Normal rats, and those treated with either 3-nitropropionic acid or methamphetamine, or a combination of both (3-NPM) showed behavioural activation (stereotyped locomotion and sniffing) in response to apomorphine. The biphasic effect in 3-NPM-treated rats is noteworthy as a similar effect has been reported with 6-OHDA treatment (Oberlander, et al., 1980). In the 6-OHDA model, apomorphine induces monophasic contraversive circling, but repeated challenge results in a

biphasic pattern of circling, with peak effects at about 10 and 40 min. These peak effects have been shown to correspond to the differential activation of dopamine D_1 and D_2 receptors (Coward et al., 1983). However, the observed effects in our 3-NPM model are different in that no pharmacological kindling was required, and the peaks are temporally displaced (20 and 90 minutes).

An involvement of D_1 and D_2 dopamine receptors is indicated by the fact that SCH 23390 and sulpiride attenuated the effects of apomorphine with equal potency, even though apomorphine shows higher affinity for D_2 than D_1 receptors (Murray and Waddington, 1989). The observed attenuation of apomorphine-induced locomotion and stereotypy by both D_1 and D_2 antagonists in 3-NPM rats, is in contrast with the loss of antagonism by both agents to this compound in 6-OHDA-treated rats (Rouillard and Bedard, 1988), but indicates a lack of breakdown of D_1/D_2 synergism, as would otherwise have been predicted (LaHoste and Marshall, 1993).

The non-competitive NMDA receptor antagonist, MK 801, evokes an increase in the release (Biggs et al., 1996; Whitton, 1997) and synthesis (Fisher et al., 1998; Loscher et al., 1991) of DA in various parts of the brain. Its locomotor properties are therefore thought to be the consequence of the effects of the released DA on D_1 and D_2 receptors (Hu and White, 1997; Starr, 1995; Starr and Starr, 1994), although its ability to initiate locomotion in DA-depleted rodents suggests a DA receptor-independent mechanism (Mele et al., 1998; Starr, 1995). With 3-NPM treatment, rats loose ~70 % of their striatal DA content, and become supersensitive to dopaminergic agonists. A dose of 0.3 mg/kg MK-801, resulted in a marked induction of forward locomotion and rearing in these rats, effects not observed at this dose in naive rats. These effects were completely attenuated by the D_1 antagonist, SCH 23390, and to a lesser extent by the D_2 antagonist, sulpiride, suggesting a $D_1 > D_2$ mechanism, as for reserpine-treated rats (LaHoste and Marshall, 1993).

In summary, the overall effect of lesioning with 3-NPM was an increased sensitivity of dopamine receptors ($D_2 > D_1$) in the basal ganglia, resulting in supersensitive responses to dopaminergic agonist. In addition, 3-NPM-treated rats did not show an independence, but rather an inter-dependence in the effects of D_1 and D_2 receptor behaviours.

CHAPTER SIX

EVIDENCE THAT L-DOPA-INDUCED LOCOMOTION MAY BE BOTH DOPAMINE-DEPENDENT AND DOPAMINE-INDEPENDENT

6.1 Introduction

L-DOPA, in combination with a peripheral DOPA decarboxylase inhibitor, has been the mainstay of therapy for Parkinson's disease. The underlying reason for this is the presumption that the exogenous L-DOPA will be taken up and converted to dopamine by the surviving dopaminergic (and serotonergic) neurones. This assumption was based on early experiments in which the akinetic features of reserpine-treated rats were reversed by administering large doses of L-DOPA (Carlsson et al., 1957). The interpretation of the results of these early experiments may have overlooked the intrinsic ability of L-DOPA to induce locomotion by dopamine-independent mechanisms. A primary reason for this is the fact that large doses of L-DOPA were administered in the absence of peripheral DOPA decarboxylase inhibitors. Granted that L-DOPA has poor CNS penetrability, but the large doses (up to 1 g/kg!) may have resulted in an accumulation of L-DOPA in such quantities as would be sufficient to stimulate locomotion by direct interaction with postsynaptic receptors, quite apart from its conversion to dopamine.

In order to test this theory, we administered L-DOPA, with centrally and/or peripherally acting DOPA decarboxylase inhibitors, to reserpinized, monoamine intact and 3-NPM-treated rats. Various locomotor parameters were assessed, and the animals were subsequently sacrificed for biochemical measurements of tissue monoamines, excitatory amino acids, and AADC activity in the NAcc, PFC, SN and ST. Furthermore, we examined the receptor mechanism(s) for the postsynaptic effects of L-DOPA using selective dopamine D_1 and D_2 receptor antagonists, and the sensitivity of L-DOPA-induced behavioural changes to NMDA receptor antagonism.

6.2 Methods

On-going experiments in our laboratory have shown an inverse relationship between increasing doses of L-DOPA and DA synthesis and release in reserpinized rats. The following experiments provide the behavioural and biochemical correlates to the microdialysis experiments in reserepinized rats. To determine whether these observations were a peculiarity of the reserpine model of parkinsonism, we conducted similar studies in monoamine intact and 3-NPM-treated rats, using the findings in the reserpine-treated rats as reference.

Male Wistar rats were acclimatized for at least one weak after arrival, and thereafter divided into three groups. The first group of rats were treated with reserpine (5 mg/kg i.p.). These rats

were kept artificially warm by heating the room to ~26^{0}C, and were used for the behaviour experiments 18-20 h later. The second group of rats were used as saline controls, while the rats in the third group were treated with the 3-NPM schedule. The 3-NPM rats were used 5-7 days after the eight injection of 3-NP.

On the day of the experiment, the rats were weighed and placed individually on the floor of Perspex observation boxes. Reserpinized rats were left to settle for 30 min, while monoamine intact and 3-NPM rats were habituated for 1 h, prior to the start of drug administration. The injection schedules for the various treatments are shown in tables 6.1 and 6.2 below. The locomotor scores of the rats were taken via Radiospares Doppler Module units constructed in our laboratory, and set to detect gross movements. The scores were noted in 10-min periods, for a total of 180 min. Counts for rearing and jumping were noted manually for the entire duration of the experiment. Approximately three and a half hours after the administration of L-DOPA, these rats were sacrificed for biochemical measurements of tissue monoamines, excitatory amino acids and AADC activity (results are shown in Chapter Seven).

Table 6.1 - Dosing schedule for L-DOPA and LDME combinations with AADC inhibitors, DA receptor antagonists and budipine.

Time (min)	Treatment
0	Saline (1 ml/kg) / budipine (10 mg/kg i.p.)
30	Benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.) **and** SCH 23390 (1 mg/kg i.p.) or sulpiride (100 mg/kg s.c.)
60	L-DOPA (25, 50, 100 or 200 mg/kg i.p.) or LDME (100 mg/kg i.p.)

Table 6.2 - Dosing schedule for L-DOPA and d-amphetamine combinations

Time (min)	Treatment
0	Saline (1 ml/kg i.p.) / NSD 1015 (100 mg/kg i.p.) / AMPT (200 mg/kg i.p.) / benserazide*
30	d-amphetamine (4 mg/kg s.c.) / L-DOPA (100 mg/kg i.p.)*
60*	d-amphetamine (4 mg/kg s.c.)*

* - Experiments involving prior treatment with L-DOPA.

6.3 Results

6.3.1 Experiments in reserpine-treated rats

The choice of reserpine as a model for depicting the parallels of dopamine release and L-DOPA-induced locomotion was based on the fact that this alkaloid alters the cytoplasmic storage of dopamine without interfering with its cytoplasmic synthesis and release (Parker and Cubeddu, 1986). This would then allow us to assess the effects of various inhibitors of AADC activity.

6.3.1.1 Effects of peripheral AADC inhibition on L-DOPA-induced behaviour in reserpine-treated rats

Reserpinized rats were still, humped and ptotic 18-20 h after treatment. These rats were then treated with various doses of L-DOPA (25, 50, 100 and 200 mg/kg i.p.), 30 min after the peripheral AADC inhibitor benserazide (50 mg/kg, i.p.). The rats remained still after the 25 mg/kg dose, but showed a flurry of slow, perseverative movement (apomorphine-like march) and sniffing (but not rearing) with the 50 and 100 mg/kg doses. About 10 min after the 200 mg/kg dose however, the rats began to run, jump and rear. This effect lasted approximately 100 min, with a peak effect at 30 min. A similar onset and duration of effects was observed with the 50 and 100 mg/kg doses. These effects are depicted in Fig. 6.1 overleaf.

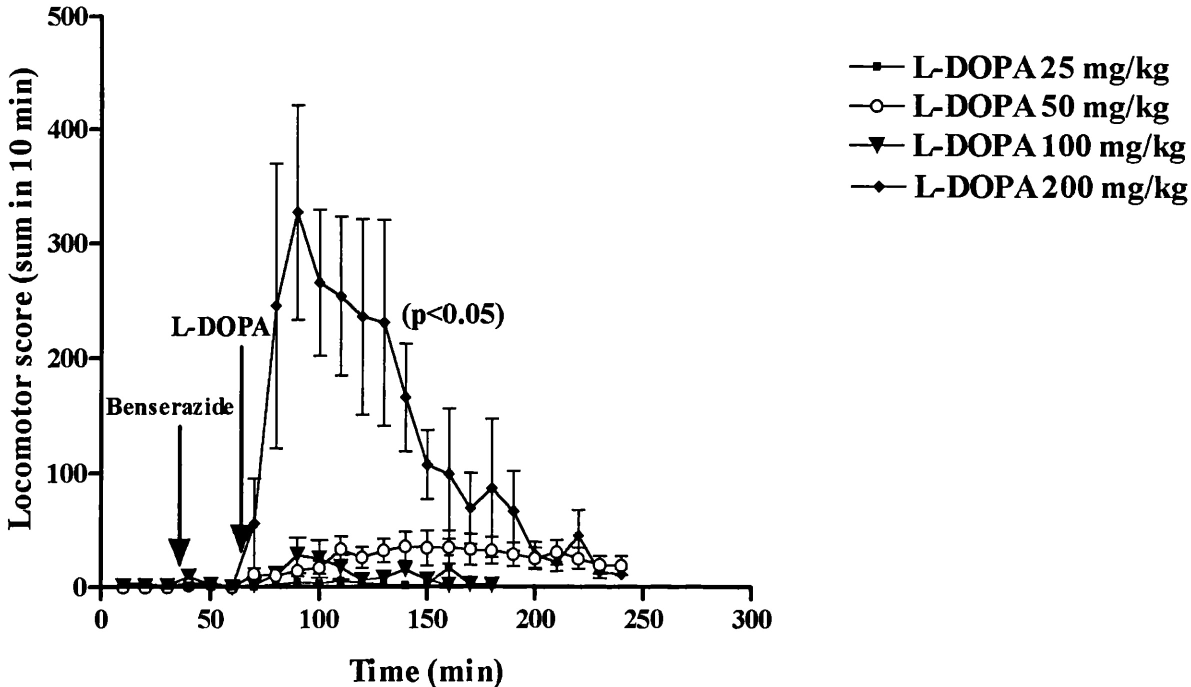


Fig. 6.1 - Locomotor effects of L-DOPA (25, 50, 100 and 200 mg/kg i.p.) administered 30 min after benserazide (50 mg/kg i.p.). The results are the means of six or more replicates ± S.E.M. ($p<0.05$ compared to saline treatment).

6.3.1.2 Effects of NMDA antagonism on L-DOPA-induced behaviour in reserpine-treated rats

The weak non-competitive NMDA ion channel blocker, Budipine at a dose of 10 mg/kg, i.p., administered 1 h before L-DOPA, did not have any additional effect on the low dose of L-DOPA (25 mg/kg), but significantly reduced the locomotion by the 50 mg/kg dose ($p<0.001$), and accentuated the locomotor effects of the 100 mg/kg dose ($p<0.01$), as shown in Figs. 6.2a,b,c.

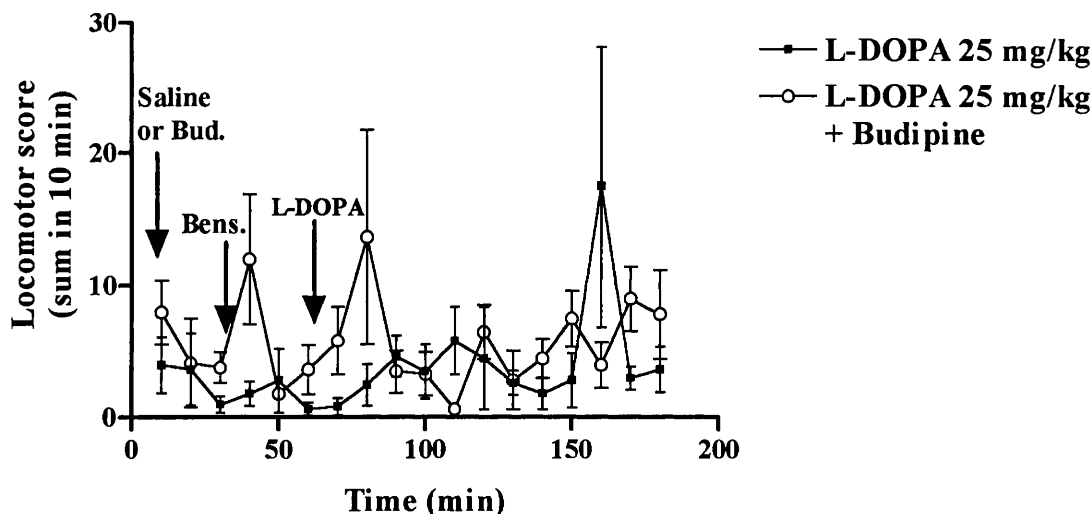


Fig. 6.2a - Effects of Budipine (Bud.; 10 mg/kg i.p.) on the locomotor properties of L-DOPA (25 mg/kg i.p.), in the presence of benserazide (Bens.; 50 mg/kg i.p.). The results represent a mean of six replicates ± S.E.M. ($p>0.05$ versus L-DOPA 25 mg/kg).

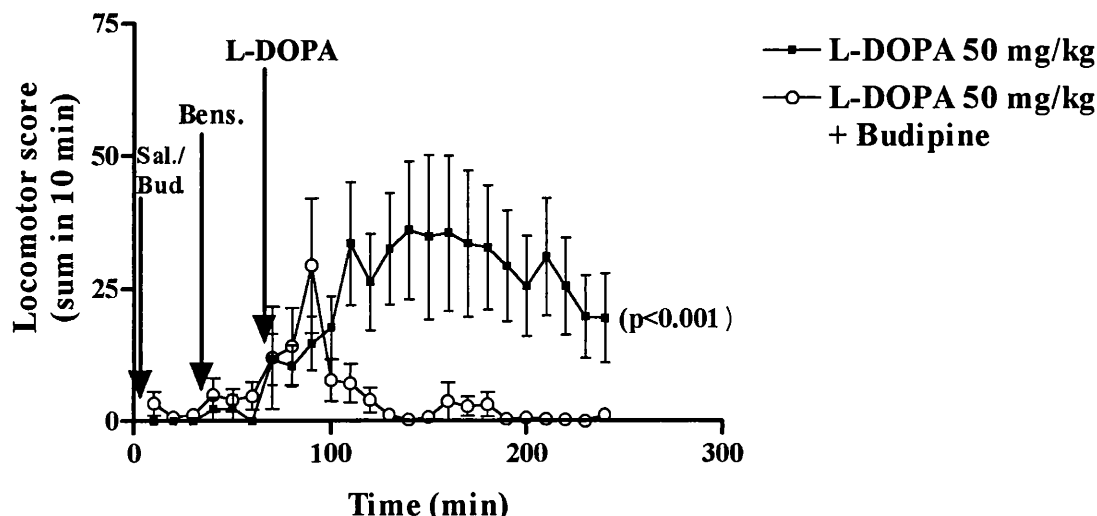


Fig. 6.2b - The effects of Budipine (Bud.; 10 mg/kg i.p.) on the locomotor effects of medium-dose L-DOPA (50 mg/kg i.p.) and benserazide (Bens.; 50 mg/kg i.p.), in reserpine-treated rats. The results are the means ± S.E.M. ($p<0.001$ versus L-DOPA 50 mg/kg).

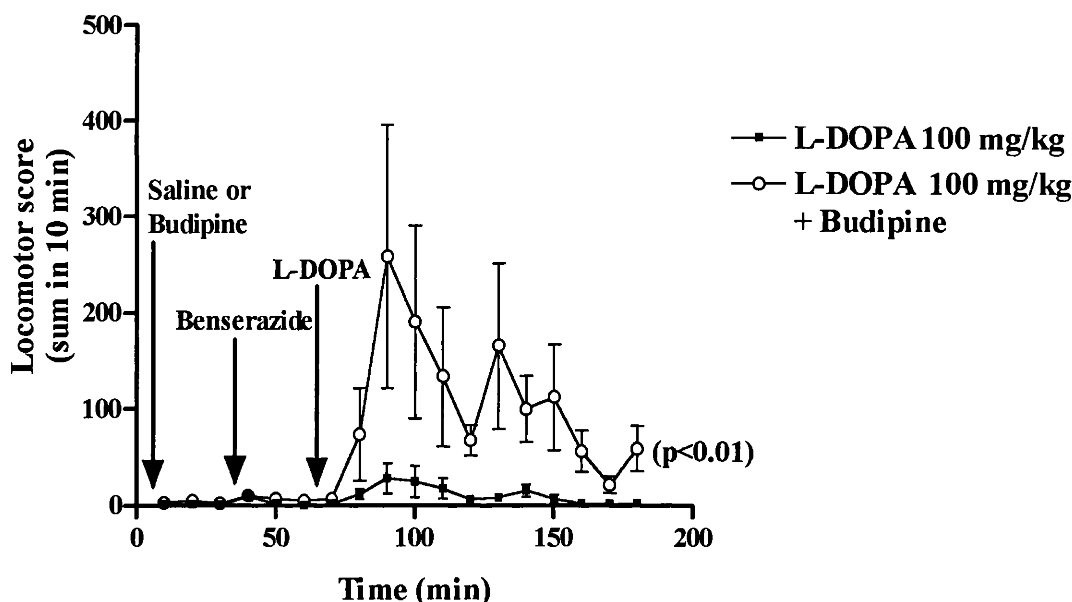


Fig. 6.2c - The effects of Budipine (10 mg/kg i.p.) on the locomotor properties of L-DOPA (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.), in reserpine-treated rats. The results are the mean of at least six replicates ± S.E.M. ($p<0.01$ versus L-DOPA 100 mg/kg).

6.3.1.3 Effects of NMDA antagonism and central AADC inhibition on L-DOPA-induced behaviour in reserpine-treated rats

The central AADC inhibitor, NSD 1015 (100 mg/kg i.p.), was administered alone or in combination with benserazide, 30 min prior to the injection of L-DOPA. Where budipine was administered, it was given 1 h before L-DOPA. Combining NSD 1015 alone with a threshold dose of L-DOPA, 100 mg/kg, resulted in locomotor activation characterized by a delayed onset of 70 min, and a duration of more than 6 h (Fig. 6.3). These rats trotted round the cages and stopped to rear at the sides of the cages. The pace was much faster than the apormorphine-like march with beserazide and L-DOPA (100 mg/kg), and the speed and fluidity of the rats treated with budipine, benserazide and L-DOPA (100 mg/kg). There was full oro-facial activity (including biting the floor and sides of the cages), jumping and a straab tail. With the addition of benserazide to this mix, the continuous trots, sporadic runs and intermittent rears and jumps became intensified, and the locomotor counts were significantly greater ($p<0.01$). A peak effect

was seen 130 min after L-DOPA, and the duration was >6 h. The addition of budipine to this combination of L-DOPA, NSD 1015 and benserazide, did not increase locomotor scores, but quickened the onset of locomotion, increased the tendency for stereotyped fixation in the rearing position, and increased the duration of locomotion to >8 h.

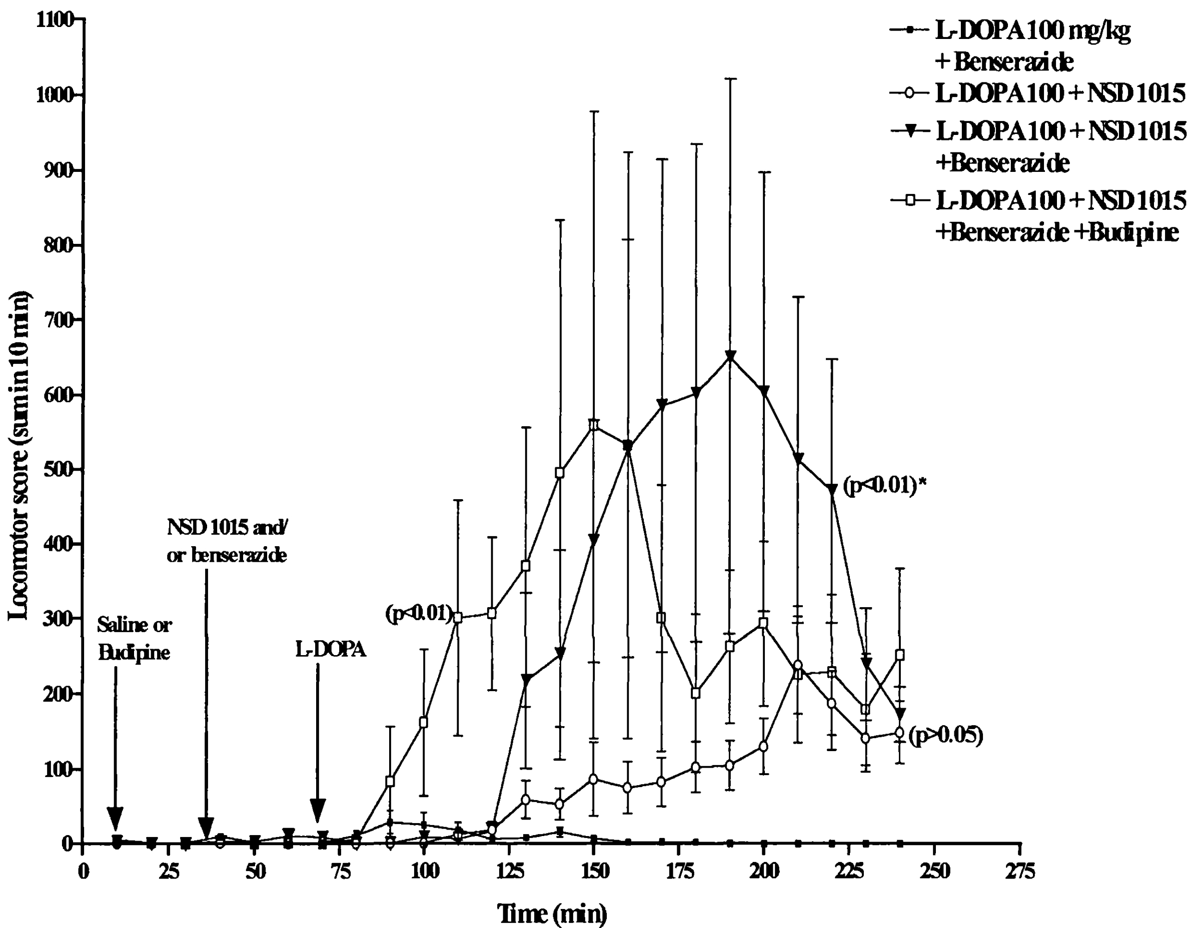


Fig. 6.3 - The effects of Budipine (10 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.) on the locomotor-inducing properties of L-DOPA (100 mg/kg i.p.), in reserpine-treated rats. The results are the means of at least six replicates ± S.E.M. (p values are compared with L-DOPA 100 + Benserazide; *= p>0.05 compared to L-DOPA 100 + NSD 1015 + Benserazide + Budipine).

The rapid attainment of a stereotyped fixation in the rearing posture in the L-DOPA 100 mg/kg combination with NSD 1015 and budipine, led to a halving of the dose of L-DOPA to 50 mg/kg (Fig. 6.4). At this dose, NSD 1015 increased L-DOPA-induced locomotion alone or with benserazide, with locomotor scores being higher in the former instance ($p<0.01$). The locomotor pattern was similar to that at the 100 mg/kg dose, but the rats showed greater

fluidity of movement and less running. Grooming was also absent, and the rats did not develop fixation in the rearing posture. Budipine neither reversed the NSD 1015-induced delay in the onset of locomotion nor increased the locomotor score above that for the combined AADC inhibition. Generally, the onset of locomotion at this dose of 50 mg/kg was delayed to about 90 min, and the peak effects to 150-170 min post L-DOPA.

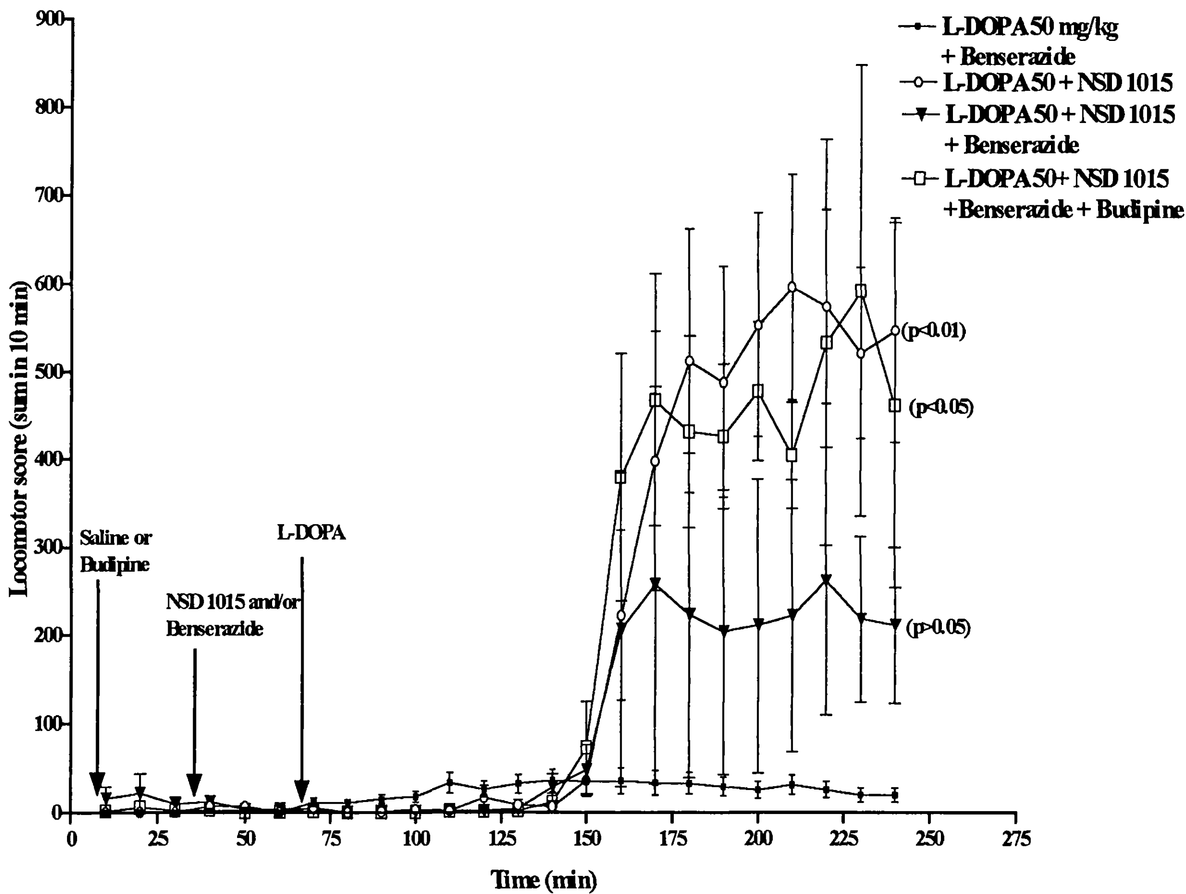


Fig. 6.4 - Locomotor effects of L-DOPA (50 mg/kg i.p.) in combination with budipine (10 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and/or benserazide (50 mg/kg i.p.). The results are the means of at least five replicates ± S.E.M. (p values are compared with L-DOPA50 + Benserazide).

At a lower dose of L-DOPA (25 mg/kg), it was impossible to reproduce the locomotor activation of budipine, NSD 1015 or the combination of these. The rats remained still, and occasionally altered their posture, as seen by the low scores in Fig. 6.5. Table 6.3 shows the rearing counts for this set of experiments.

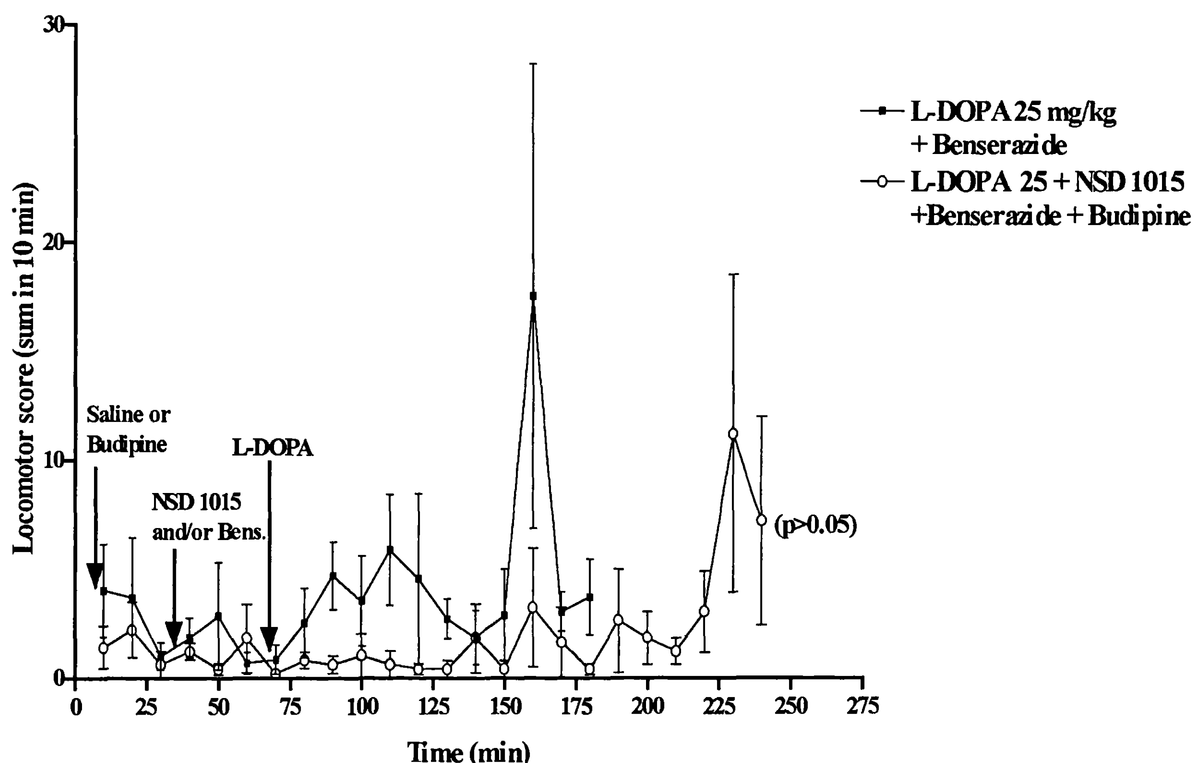


Fig. 6.5 - The effects of Budipine (10 mg/kg i.p.), NSD 1015 (100 mg/kg ip.) and Benserazide (Bens.; 50 mg/kg i.p.) on the locomotor activity of L-DOPA (25 mg/kg i.p.), in reserpine-treated rats. The results are the means of a minimum of five replicates ± S.E.M. (p values are compared with L-DOPA 25 mg/kg + Benserazide).

Table 6.3 - Counts for rearing and jumping with L-DOPA treatment schedules in reserpine-treated rats.

Treatment schedule	Total no. of rears (in 3 h)	Total no. of jumps (in 3 h)
L-DOPA 25 + Bens.	0	0
L-DOPA 25 + Bens. + Bud.	0	0
L-DOPA 100 + Bens.	14 ± 11	0
L-DOPA 100 + NSD 1015	117 ± 55	4 ± 3
L-DOPA + Bens. + NSD 1015	18 ± 15	4 ± 3
L-DOPA 100 + Bens. + Bud.	20 ± 6	0
L-DOPA 100 + Bens. + Bud. + NSD 1015	***	***
L-DOPA 50 + NSD 1015	289 ± 59	2 ± 2
L-DOPA 50 + Bens.+ NSD1015	53 ± 25	131 ± 131
L-DOPA 50 + Bens. + Bud. + NSD 1015	205 ± 73	227 ± 145
L-DOPA 200 + Bens.	149 ± 52	333 ± 129

*** - excessive rearing, >85 rears/10 min. (Bens., benserazide; Bud., budipine)

6.3.1.4 Differential involvement of D_1 and D_2 receptors in L-DOPA-induced locomotion in reserpine-treated rats

In order to establish the receptor mechanism involved in the observed locomotor activation with L-DOPA, the selective D_1 antagonist, SCH 23390 (1 mg/kg), or the selective D_2 antagonist, sulpiride (100 mg/kg), were administered 30 min prior to the dose of L-DOPA (100 mg/kg). The L-DOPA schedules involving NSD 1015 showed a selective abolition of locomotion, rearing and jumping by the D_2 antagonist sulpiride ($p<0.01$), but not by the D_1 antagonist, SCH 23390 (Figs. 6.6, 6.7; table 6.4), even in the presence of budipine (Fig.6.7). In fact, SCH 23390, in the L-DOPA (100 mg/kg)/ NSD 1015/ benserazide combination, led to higher peaks of locomotion, and greater duration of effects (lasting over 8 h) (Fig. 6.6).

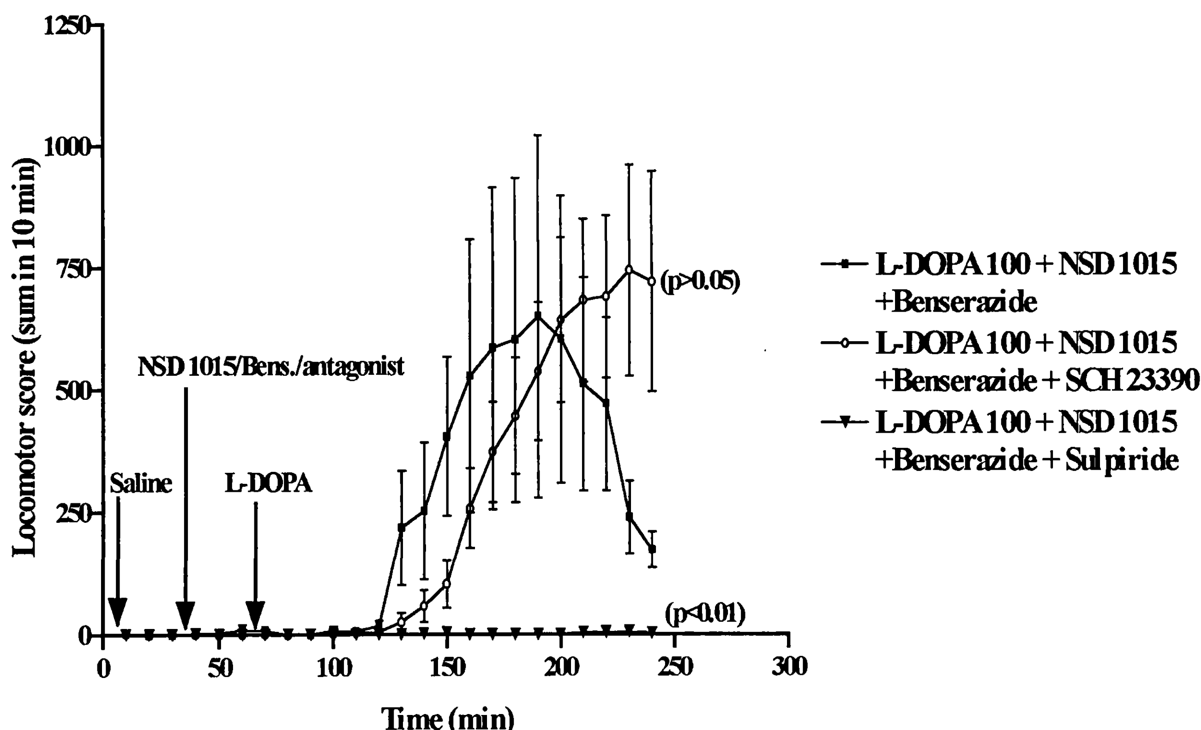


Fig. 6.6 - Effects of sulpiride (100 mg/kg s.c.) and SCH23390 (1 mg/kg i.p.) on the locomotor activity of L-DOPA (100 mg/kg i.p., in combination with NSD 1015 (100 mg/kg i.p.) and benserazide (Bens.; 50 mg/kg i.p.)). Results are the means of six replicates ± S.E.M. (p values are compared to L-DOPA 100 + NSD 1015 + Benserazide).

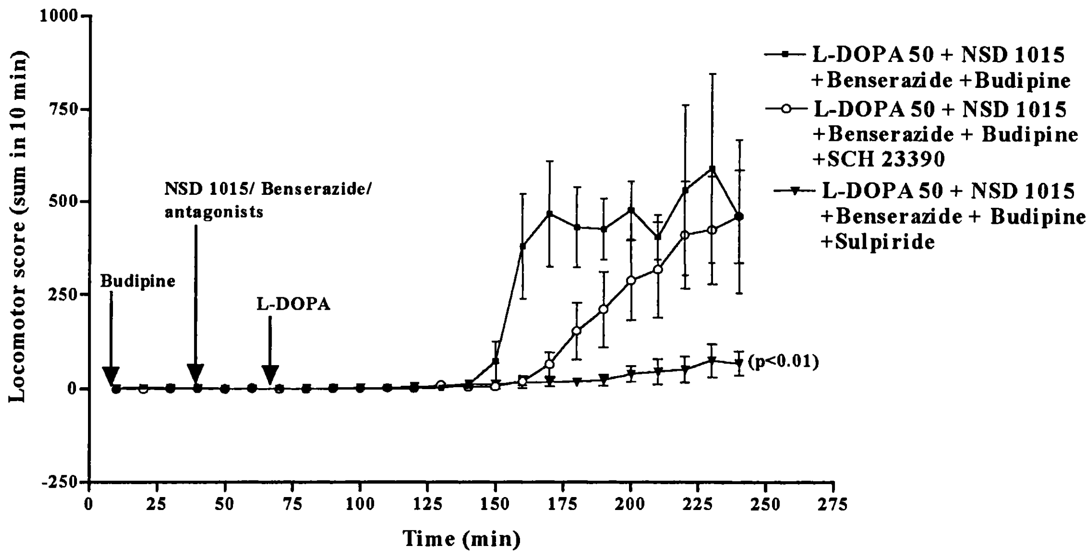


Fig. 6.7 - Effects of sulpiride (100 mg/kg s.c.) and SCH 23390 (1 mg/kg i.p.) on the potentiation of L-DOPA-induced (L-DOPA 50 mg/kg i.p.) locomotion by budipine (10 mg/kg i.p.). The results represent the means of six replicates ± S.E.M. (p values are compared with L-DOPA 50 + NSD 1015 + Benserazide + Budipine).

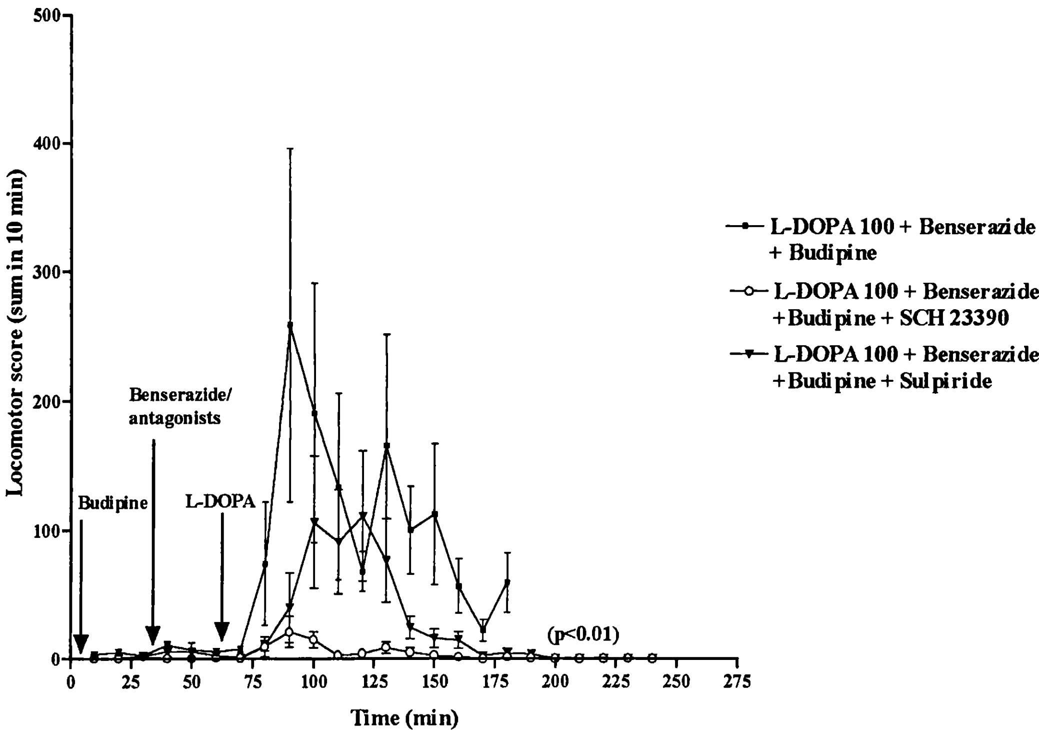


Fig. 6.8 - Differential effects of D_1 (SCH 23390 1mg/kg, i.p.) and D_2 (sulpiride 100mg/kg, s.c.) antagonists on the potentiation of L-DOPA-induced locomotion by budipine (10 mg/kg i.p.). The results are the means of six replicates ± S.E.M. ($p<0.01$ for both antagonists, versus L-DOPA 100 + Benserazide + Budipine).

When the threshold locomotor dose of L-DOPA (100 mg/kg, with benserazide) is combined with budipine, the locomotor activation induced is significantly, though not totally reduced by sulpiride ($p<0.01$), and completely abolished by SCH 23390, suggesting a DA $D_1>D_2$-dependent mechanism of action of L-DOPA (Fig. 6.8). Administering a 200 mg/kg dose of L-DOPA to reserpinized rats resulted in spontaneous reversal of akinesia within15 min of administration. The effect lasted about 3 h, and was insensitive to blockade with either sulpiride or SCH 23390 (Fig. 6.9).

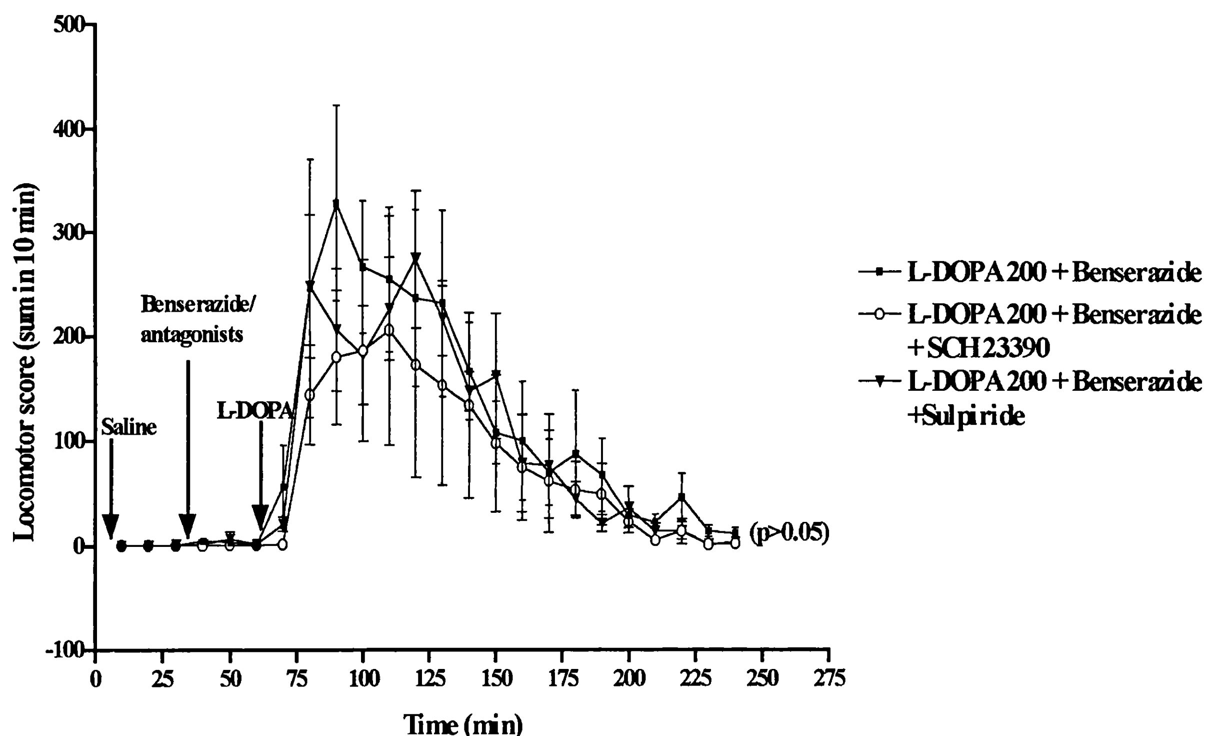


Fig. 6.9 - Differential involvement of D_1 and D_2 receptors in L-DOPA-induced locomotion. Effects of sulpiride (100 mg/kg s.c.) and SCH23390 (1 mg/kg i.p.) on L-DOPA (200 mg/kg i.p.) in reserpine-treated rats. The results are the means ± S.E.M. of at least five replicates. ($p>0.05$ for both antagonists, compared with L-DOPA 200 + Benserazide).

Table 6.4- Counts for rearing and jumping with L-DOPA treatment schedules and DA receptor antagonists in reserpine-treated rats

Treatment schedule	Total no. of rears (in 3 h)	Total no. of jumps (in 3 h)
L-DOPA 100 + Bens. + Bud.	20 ± 6	0
L-DOPA 100 + Bens. + Bud. + NSD 1015	***	***
L-DOPA 100 + Bens. + NSD 1015 + SCH 23390	197 ± 67	19 ± 12
L-DOPA 100 + Bens. + NSD 1015 + sulpiride	0	0
L-DOPA 100 + Bens. + Bud. + SCH 23390	0	0
L-DOPA 100 + Bens. + Bud. + sulpiride	14 ± 7	0
L-DOPA 50 + Bens. + Bud. + NSD 1015	205 ± 73	227 ± 145
L-DOPA 50 + Bens. + Bud. + NSD 1015 + SCH 23390	186 ± 59	2 ± 1
L-DOPA 50 + Bens. + Bud. + NSD 1015 + sulpiride	1 ± 1	0
L-DOPA 200 + Bens.	149 ± 52	333 ± 129
L-DOPA 200 + Bens. + SCH 23390	107 ± 45	34 ± 21
L-DOPA 200 + Bens. + sulpiride	92 ± 28	28 ± 13

*** - excessive rearing, >85 rears/10 min. (Bud., budipine; Bens., benserazide).

6.3.1.5 Effects of tyrosine hydroxylase inhibition on d-amphetamine-induced locomotion in reserpine-treated rats

In the quest to determine the role of endogenous L-DOPA in the proposed DA-independent induction of locomotion in monoamine-depleted rats, we treated reserpinized rats with d-amphetamine. Treatment with d-amphetamine resulted in ambulation, rearing and floor-sniffing within 5 min after injection, and lasting approximately 70 min, after which the animals became fixated on floor-sniffing for the rest of the experiment. Rats pre-treated with the tyrosine hydroxylase inhibitor, AMPT (200 mg/kg i.p.), abolished the d-amphetamine-induced locomotion, while pre-treatment with NSD 1015 significantly increased locomotion and rearing, as well as the duration of ambulation from (70 to 130 min), and prevented stereotyped floor-sniffing. These effects are depicted in Fig. 6.10 over-leaf. Whereas the effects of d-amphetamine were blocked by sulpiride and not by SCH 23390, the addition of NSD 1015 led to an involvement of D_1 rather than D_2 receptors, as the locomotor effects of this compound were blocked by SCH 23390, and not by sulpiride.

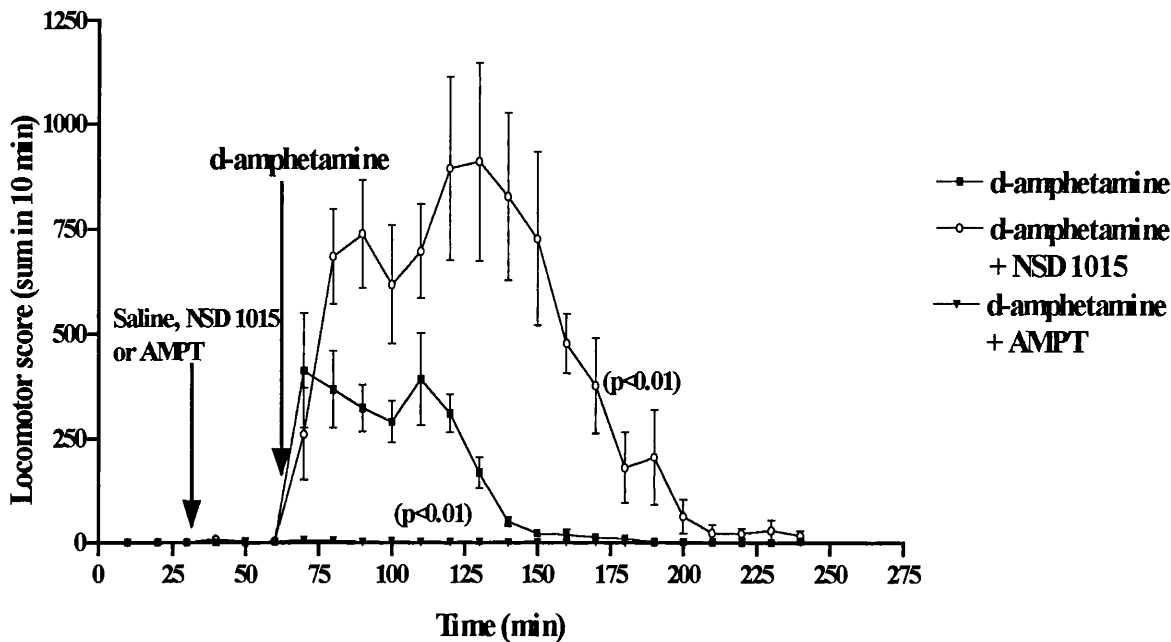


Fig. 6.10 - The effects of AMPT (200mg/kg i.p.) and NSD 1015 (100 mg/kg i.p.) on the locomotor activity of d-amphetamine (4 mg/kg i.p.) in reserpine-treated rats. The results are the means ± S.E.M. of five replicates. (p values are compared with the effects of only d-amphetamine).

We attempted to increase the duration of locomotion induced by d-amphetamine by introducing an exogenous pool of L-DOPA (100 mg/kg, with benserazide). This resulted in a rapid increase in locomotion, including rearing and jumping (table 6.5). However, these effects were short-lived as the experiments had to be terminated after 1 h, when the animals became fixated on biting the cages, and themselves. By adding NSD 1015 along with L-DOPA, this stereotyped self-mutilation in response to a challenge with d-amphetamine was reversed, and the rats were mobile for over 3 h (Fig. 6.11). There was also a ten-fold increase in counts for rearing, while jumping, hitherto absent, was present in these reserpinized rats (table 6.5).

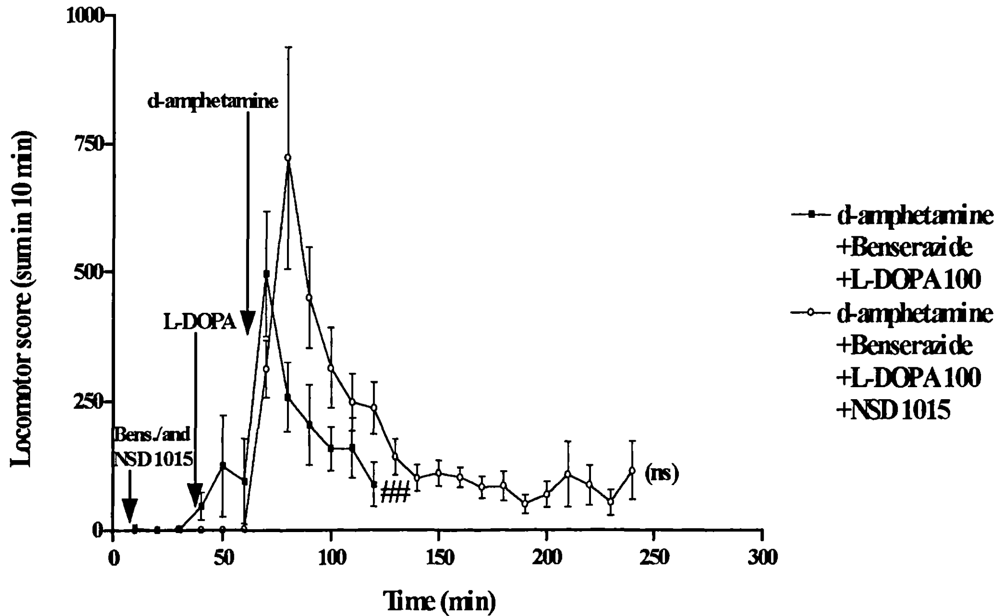


Fig. 6.11 - Reversal of stereotype behaviour induced by combined L-DOPA (100 mg/kg i.p., with benserazide (Bens.) 50 mg/kg i.p.) and d-amphetamine (4 mg/kg i.p.) treatment, by prior dosing with NSD 1015 (100 mg/kg i.p.), in reserpinized rats. The results are the means ± S.E.M. of five replicates. (## - onset of self-mutilation).

Table 6.5 - Counts for rearing and jumping with d-amphetamine treatment schedules in reserpine-treated rats

Treatment schedule	Total no. of rears (in 3 h)	Total no. of jumps (in 3 h)
D-amphetamine	24 ± 8	0
D-amphetamine + NSD 1015	93 ± 63	0
D-amphetamine + AMPT	25 ± 4	0
D-amphetamine + L-DOPA 100 + Benserzide	43 ± 8	36 ± 17
D-amphetamine + L-DOPA 100 + Benserazide + NSD 1015	225 ± 88*	193 ± 56

* $p<0.05$ compared with corresponding counts for d-amphetamine only.

6.3.1.6 Locomotor effects of L-DOPA methyl ester in reserpine-treated rats

A threshold dose of LDME (100 mg/kg, equivalent to 80 mg L-DOPA), in combination with NSD 1015 (100 mg/kg), resulted in locomotor activation in less than 70 min after injection. The rats showed a fluid pattern of running, rearing and jumping with a greater intensity (five times the total counts) than for L-DOPA. The addition of benserazide made the rats less active than for LDME/NSD 1015, as seen in Fig.6.12. The rats continued to show greater locomotor activation than the rats treated with the same drug combinations, but using L-DOPA, even at 6 h after LDME administration.

6.3.1.7 Differential involvement of D_1 and D_2 receptors in LDME-induced locomotion in reserpine-treated rats

Unlike the D_2-dependence of the locomotion induced by L-DOPA and NSD 1015, that induced by combining LDME (100 mg/kg) with benserazide and NSD 1015 could not be blocked by either the D_1 or D_2 antagonist (SCH 23390 and sulpiride, respectively). In fact, both antagonists showed a tendency to increase the locomotor scores and counts for rearing and jumping when either was employed, with this enhancement of locomotor activity being more evident with the D_1 antagonist (Fig. 6.13 and table 6.6).

Table 6.6- Counts for rearing and jumping with L-DOPA methyl ester treatment schedules in reserpine-treated rats

Treatment schedule	Total no. of rears (in 3 h)	Total no. of jumps (in 3 h)
LDME 100 + Benserazide	0	0
LDME 100 + NSD 1015	60 ± 17	17 ± 15
LDME 100 + Benserazide + Budipine	0	0
LDME 100 + Benserazide + NSD 1015	98 ± 29	76 ± 38
LDME 100 + Benserazide + NSD 1015 + SCH 23390	136 ± 38	18 ± 11
LDME 100 + Benserazide + NSD 1015 + sulpiride	40 ± 20	61 ± 42

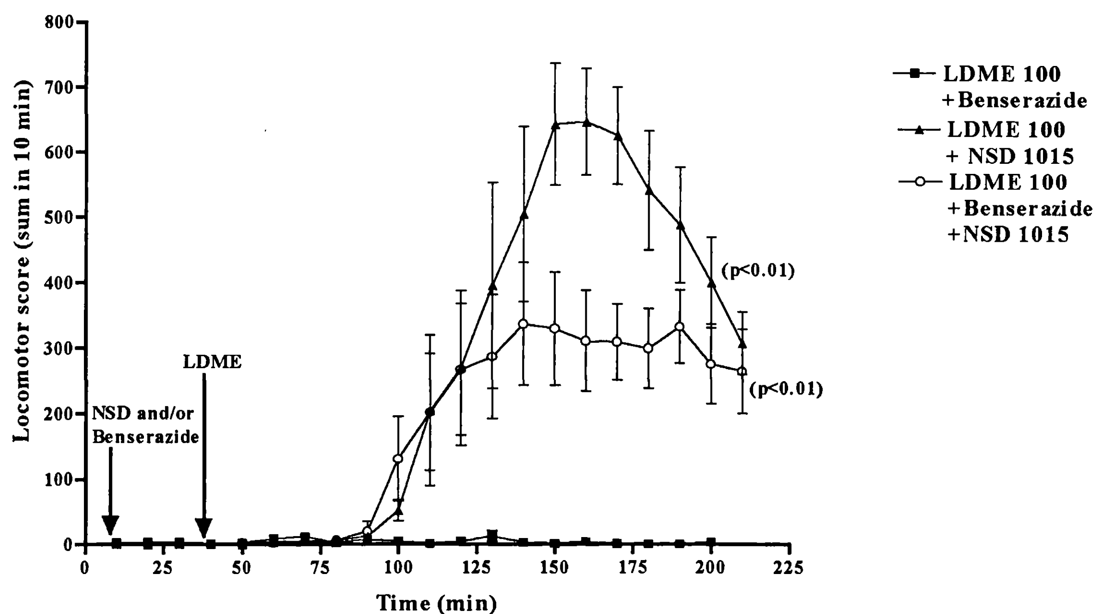


Fig. 6.12 - Locomotor effects of L-DOPA methyl ester (100 mg/kg i.p., equivalent to 80mg L-DOPA base) in combination with NSD 1015 (NSD, 100 mg/kg i.p.) and/or benserazide (50 mg/kg i.p.). The results are the means ± S.E.M of five replicates. p values are for comparisons with LDME 100 + Benserazide.

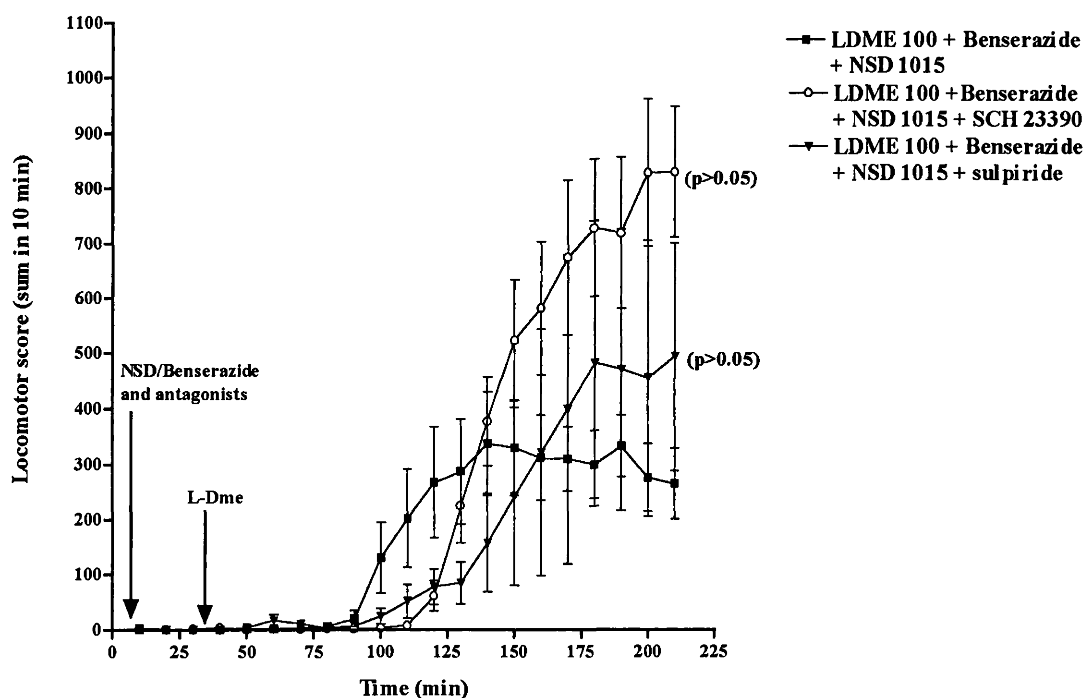


Fig. 6.13 - Differential effects of sulpiride (100 mg/kg s.c.) and SCH 23390 (1 mg/kg i.p.) on locomotion due to LDME (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and NSD 1015 (NSD, 100 mg/kg i.p.). The results are the means ± S.E.M. of a minimum of five replicates. (p values are for comparisons with LDME 100 + Benserazide + NSD 1015).

6.3.1.8 Effects of budipine on L-DOPA methyl ester-induced locomotion in reserpine-treated rats

Unlike L-DOPA, the locomotor effects of a threshold dose of LDME were not enhanced by the AADC activator, budipine (p>0.05). If any thing, the rats were less likely to initiate any kind of activity. Figure 6.14 shows the effects on locomotor score.

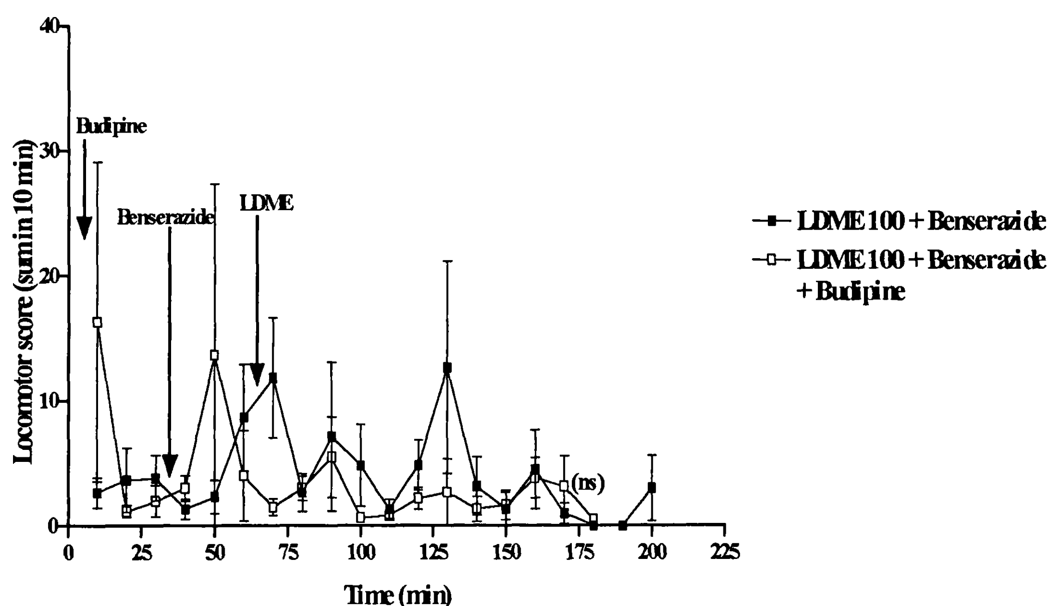


Fig. 6.14 - Non-potentiation of L-DOPA methyl-ester-induced locomotion by budipine (10 mg/kg i.p.) in reserpinized rats. Benserazide (50 mg/kg i.p.) was administered half an hour after budipine, 30 min before L-DOPA methyl ester (100 mg/kg i.p., equivalent to 80 mg base). The results are the mean ± S.E.M. of six replicates.

6.3.2 Experiments in monoamine intact rats

Naive male Wistar rats were habituated for 2 h prior to the injection of L-DOPA, and monitored for 3 h thereafter.

6.3.2.1 Effects of low- and high-dose L-DOPA on locomotion in intact rats

A low dose of L-DOPA, 25 mg/kg, in combination with benserazide, was insufficient to induce locomotion in habituated rats. The rats preferred to sit still and stare, rather than move around. At the higher dose, 100 mg/kg, the rats were even more quiescent than for the lower dose. These effects are depicted in figure 6.15.

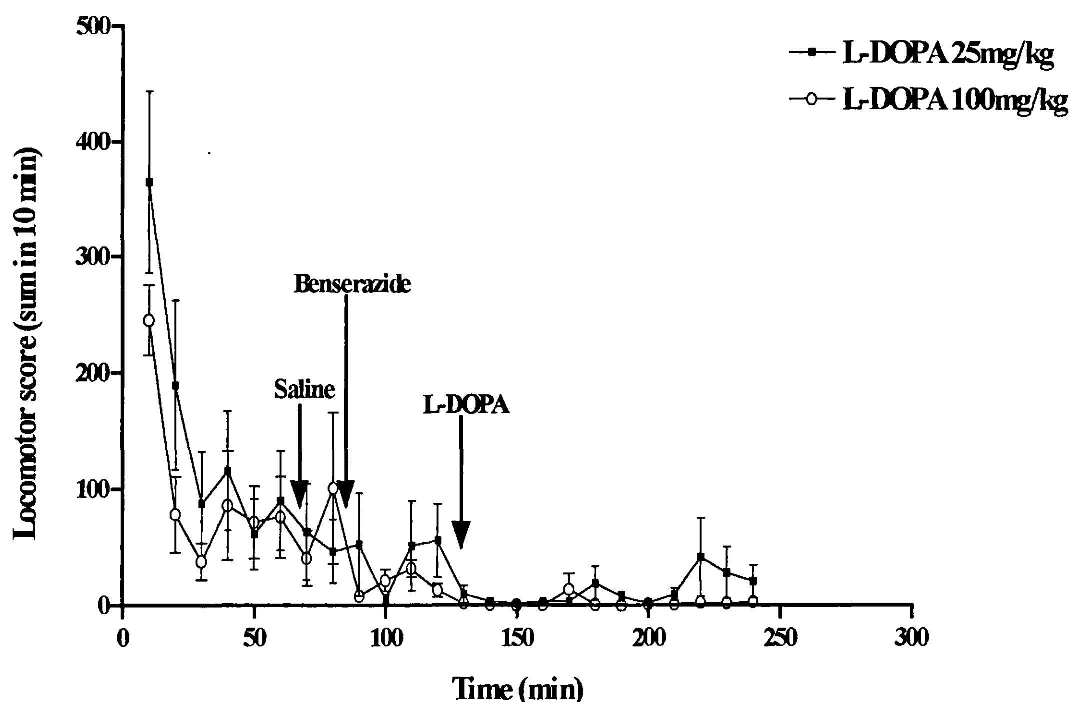


Fig. 6.15 - Locomotor effects of L-DOPA (25 and 100 mg/kg i.p.) and Benserazide (50 mg/kg i.p.) in monoamine intact rats. Results are the means of six replicates ± S.E.M.

6.3.2.2 Effects of budipine on L-DOPA-induced locomotion in intact rats

Unlike with reserpine-treated rats, budipine was unable to potentiate the locomotor effects of the low or high doses of L-DOPA in naive rats. The rats remained quiet, and moved only occasionally. Figures 6.16 and 6.17 depict this.

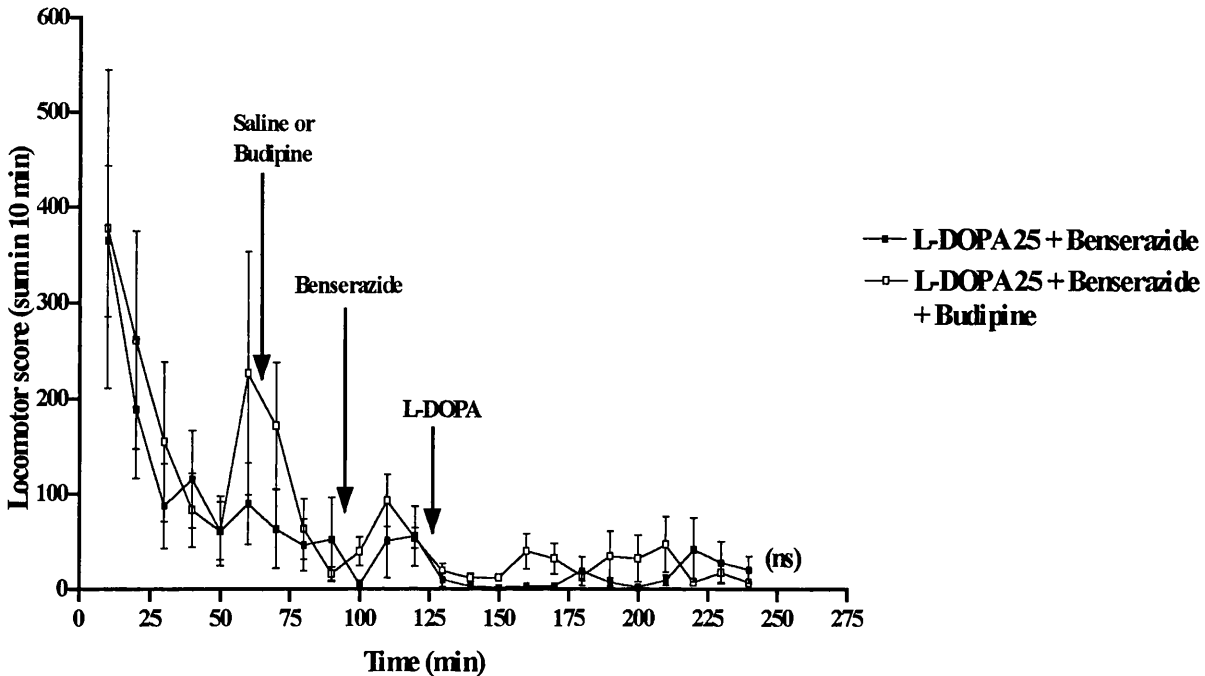


Fig. 6.16 - Effects of budipine (10 mg/kg i.p.) on the locomotor activity of L-DOPA (25 mg/kg i.p.). The results are the means ± S.E.M. of six replicates.

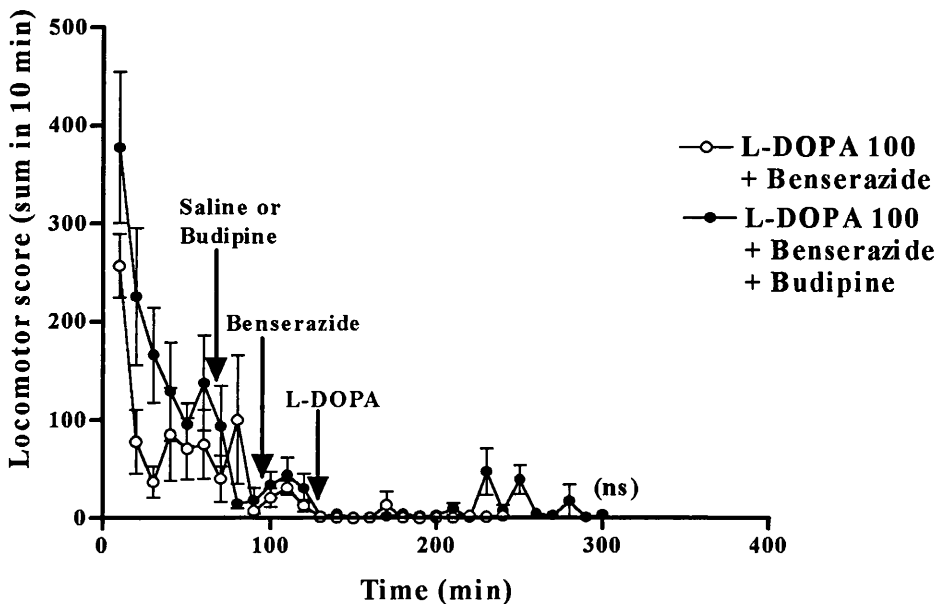


Fig. 6.17 - Effects of budipine (10 mg/kg i.p.) on the locomotor properties of L-DOPA (100 mg/kg i.p.) in naive rats. The results are the means ± S.E.M. of six replicates.

6.3.2.3 Effects of central AADC inhibition on L-DOPA-induced locomotion in intact rats

The central AADC inhibitor, NSD 1015, alone or in combination with benserazide, potentiated the locomotion induced by a threshold dose of L-DOPA (100 mg/kg). As was the case with reserpine-treated rats, there was a delayed onset of 70 min, and a duration of >6 h. The addition of benserazide reduced the trotting, running, jumping and rearing produced by the use of NSD 1015 alone (Fig. 6.18 and table 6.7).

The effects of DOPA decarboxylase inhibitors on L-DOPA-induced locomotion in monoamine intact rats

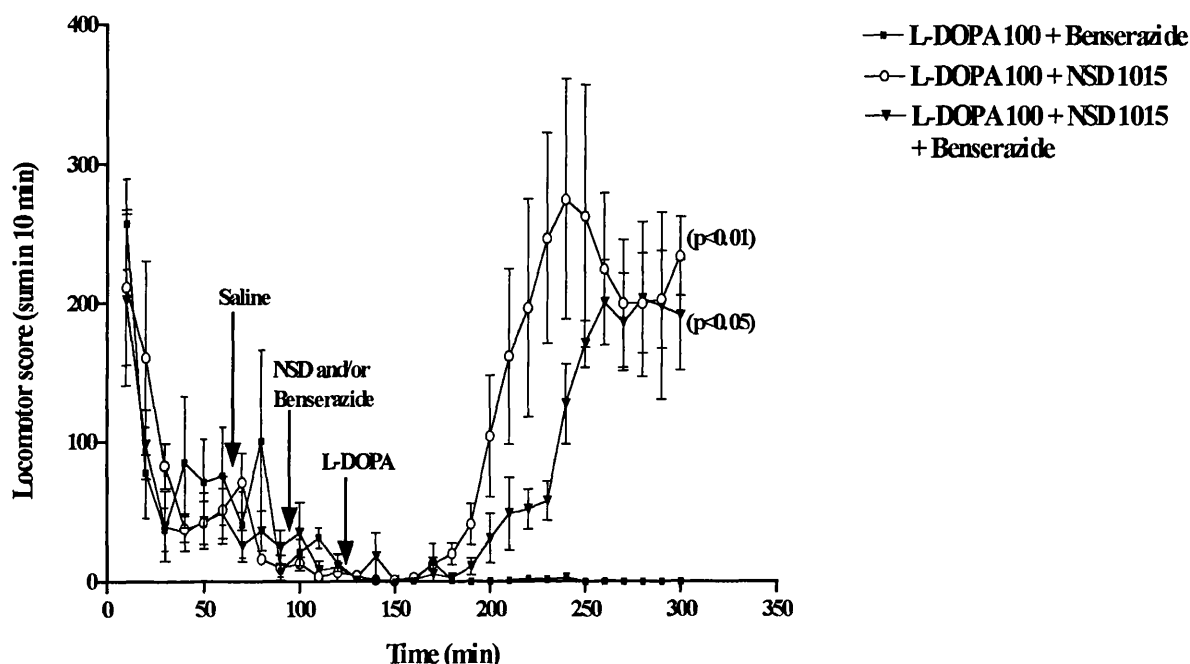


Fig. 6.18 - The effects of Benserazide (50 mg/kg i.p.) and/or NSD 1015 (NSD, 100 mg/kg i.p.) on the locomotor activity of L-DOPA (100 mg/kg i.p.) in monoamine intact rats. The results represent a mean of at least six replicates ± S.E.M. The p values are in comparison with L-DOPA 100 + Benserazide.

Table 6.7 – Counts for rearing and jumping with L-DOPA treatment schedules in monoamine intact rats

Treatment schedule	Total no. of rears (in 3 h)	Total no. of jumps (in 3 h)
Saline	11 ± 2	0
L-DOPA 25 + Benserazide	12 ± 3	0
L-DOPA 25 + Bens. + Bud.	10 ± 2	0
L-DOPA 100 + Benserazide	2 ± 1	0
L-DOPA 100 + Bens. + Bud.	7 ± 3	0
L-DOPA 100 + Bens. + NSD	151 ± 32	18 ± 10

Bens., benserazide; Bud., budipine; NSD, NSD 1015.

6.3.3 Experiments in 3-NPM-treated rats

6.3.3.1 Effects of central AADC inhibition on L-DOPA-induced locomotion in 3-NPM-treated rats

As in reserpine-treated rats, the combination of the central and peripheral AADC inhibitors resulted in a marked potentiation of the locomotion induced by a threshold dose of L-DOPA (100 mg/kg) in 3-NPM-treated rats. This effect was greater than when either NSD 1015 or benserazide were used alone (Fig. 6.19 and table 6.8), although the NSD 1015 combination resulted in a ten-fold increase in cumulative locomotor counts. The onset of locomotion following central AADC inhibition, was about 80 min (after L-DOPA), and the duration of running, rearing and jumping was >6 h. There were significantly more rears when L-DOPA was combined with NSD 1015, than for its combination with benserazide ($p<0.05$; table 6.8). The combination of both AADC inhibitors with L-DOPA resulted in less rearing ($p>0.05$) than for L-DOPA/NSD 1015. Peak effects were observed at 130 min, as for reserpine-treated rats.

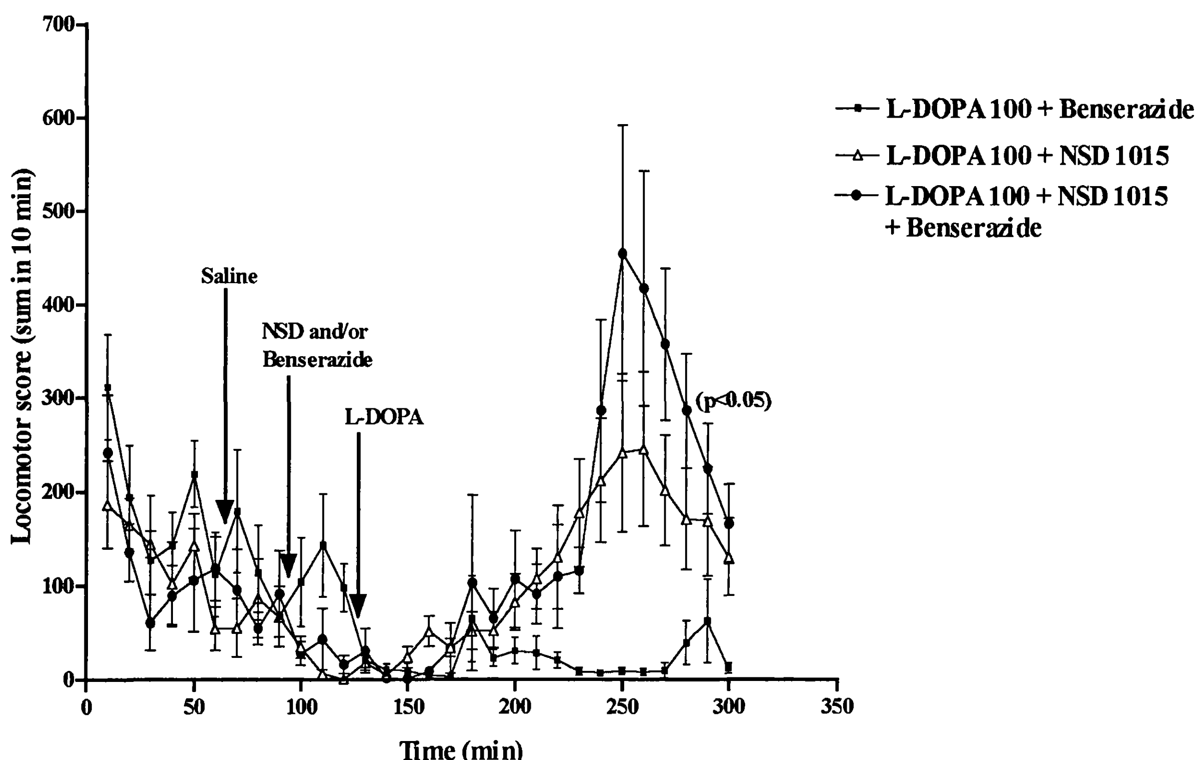


Fig. 6.19 - Locomotor activity of L-DOPA (100 mg/kg i.p.) in combination with NSD 1015 (NSD, 100 mg/kg i.p.) and/or benserazide (50 mg/kg i.p.), in 3-NPM-treated rats. The results are the mean ± S.E.M. of a minimum of six replicates. The p values are for comparisons with L-DOPA 100 + Benserazide.

Table 6.8 – Counts for rearing and jumping with L-DOPA treatment schedules in 3-NPM-treated rats

Treatment schedule	Total no. of rears (in 3 h)	Total no. of jumps (in 3 h)
L-DOPA 100 + Benserazide	3 ± 2	0
L-DOPA 100 + Benserazide + Budipine	0	0
L-DOPA 100 + NSD 1015	110 ± 39*	12 ± 10
L-DOPA 100 + Benserazide + NSD 1015	68 ± 27*	14 ± 7

*p<0.05 versus L-DOPA + Benserazide.

6.3.3.2 Effects of budipine on L-DOPA-induced locomotion in 3-NPM-treated rats

As in reserpine-treated rats, budipine gave rise to a ten-fold potentiation of the locomotion induced by a threshold dose of L-DOPA. These rats did not rear or jump, but moved swiftly round the sides of the cages. There was a rapid onset of locomotion (25 min) and a short duration of 2 h (Fig. 6.20; table 6.8).

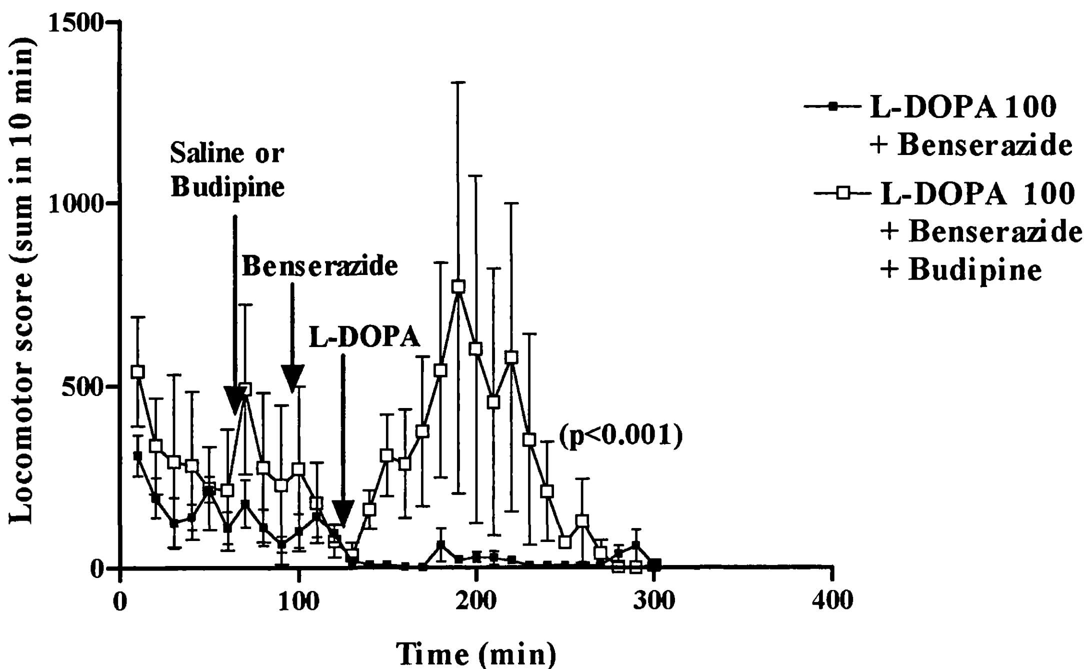


Fig. 6.20 - Potentiation of L-DOPA-induced locomotion by budipine (10 mg/kg i.p.) in 3-NPM-treated rats. Results are the means ± S.E.M. p<0.001 versus L-DOPA 100 + Benserazide.

6.4 Discussion

L-DOPA is assumed to reverse akinesia in monoamine-depleted rodents by providing a pool of freshly synthesised DA in the synaptic cleft, which stimulates postsynaptic receptors in the CNS (Butcher and Engel, 1969; Lloyd et al., 1975). At low to moderate doses (25-50 mg/kg), and in the presence of a peripheral AADC inhibitor, L-DOPA stimulates the synthesis and release of DA in the SN and ST of reserpinised (Biggs et al., 1998; Fisher et al., 1998) and 6-OHDA-treated (Sarre et al., 1994), and normal (Sarre et al., 1998) rats. However, at higher doses (100-200 mg/kg) in reserpine-treated rats, L-DOPA inhibits the release of DA by stimulating presynaptic D_2 receptors in the nigrostriatal tract (Fisher et al., 2000). Paradoxically, L-DOPA is known to inhibit its synthesis via AADC in reserpinised rats, an effect that is overcome by the use of NMDA antagonists (Biggs et al., 1998; Fisher et al., 1998). At a dose of 100 mg/kg, L-DOPA decreases AADC activity by >75% (Fisher et al., 2000). Thus, at the 100 mg/kg dose, we find a significant impairment of DA synthesis and release, whereas the rats show only a brief flurry of movement, being still under the bradykinetic effects of the DA-depletion induced by reserpine.

Budipine, a weak non-competitive NMDA ion channel antagonist (Klockgether et al., 1993; Kornhuber et al., 1995), stimulates AADC and reverses the inhibitory effects of L-DOPA on this enzyme (Fisher et al., 1998). This compound has also been shown to cause a 400 % increase in DA release when combined with 25 mg/kg, but not 50 mg/kg L-DOPA (Biggs et al., 1998). Although microdialysis detects an abundant amount of DA in the extracellular space (and hence, presumably, in the synaptic cleft), the reserpine-induced akinesia still persists. In our hands, a 100 mg/kg dose of L-DOPA was found to be the threshold dose for initiating movement in reserpine-treated rats. The apomorphine-like slow march induced by this dose was amplified ten-fold when budipine was administered an hour previously, with the rats moving around with rapid pace, but neither rearing nor jumping. A similar effect was observed with 3-NPM-treated rats, but not with monoamine intact rats. This enhancement of L-DOPA-induced locomotion by budipine was attenuated by the D_1 antagonist, SCH 23390, but not by the D_2 antagonist sulpiride, suggesting a mediation by D_1 rather than D_2 DA receptors.

The precise mechanism by which budipine potentiates locomotion by a threshold dose of L-DOPA in the apparent absence of DA release, remains cryptic. Budipine simulates AADC,

leading to an increase in the amount of DA formed from a bolus dose of L-DOPA (Fisher et al., 1998). However, even though DA synthesis is known to increase by as much as 300 %, we were unable to reverse the inhibition of DA release by L-DOPA 100 mg/kg, with budipine (Fisher et al., 2000). The levels of DOPAC in the dialysates were unchanged between the L-DOPA 50, 100 and 200 mg/kg doses, suggesting that there was on-going DA synthesis, but that the DA so formed was not released into the extracellular space with the higher doses (100-200 mg/kg). The enhancement of L-DOPA-induced locomotion by budipine was blocked by the D_1 antagonist, but not by sulpiride, suggesting the involvement of dopamine at the postsynaptic D_1 site. The absence of the potentiating effect budipine on L-DOPA activity in monoamine intact rats suggests that it is an effect uncovered in supersensitive states of the dopamine receptor. It may well be that using much higher doses than those employed in supersensitive rats, may be sufficient to reproduce the effect in normal rats. Although tyrosine hydroxylase is the rate limiting enzyme for DA synthesis in normal rats, the ability of budipine to potentiate AADC in supersensitive (and not in normosensitive) states of the DA receptor, also supports the notion that AADC may become rate-limiting the formation of DA from exogenous L-DOPA, under parkinsonian conditions.

The conversion of L-DOPA to dopamine by peripheral AADC has attendant side-effects in parkinsonian patients undergoing dopamine-replacement therapy. For this reason, L-DOPA has traditionally been administered with a peripheral AADC inhibitor, so as to increase the carrier-mediated uptake of this amino acid into the brain (Butcher and Engel, 1969; Sarre et al., 1994). The locomotion we found at doses of L-DOPA which markedly impaired DA synthesis and release led us to believe that there might be non-dopamine dependent mechanisms by which L-DOPA induces locomotion. To test this theory, we prevented the conversion of L-DOPA to DA in the CNS by the use of the central AADC and MAO inhibitor, NSD 1015 (Hunter et al., 1993; Nissbrandt et al., 1988), and attempted to determine the effects of L-DOPA itself under a variety of conditions. A dose of 100 mg/kg NSD 1015 was chosen as this was the concentration required to completely block AADC activity in the SN, whereas only 10 mg/kg was required to do so in the ST (Fisher et al., 2000).

Combining a threshold dose of L-DOPA with NSD 1015, instead of benserazide, resulted in a complete reversal of akinesia in reserpinised rats, and locomotor activation in monoamine intact

and 3-NPM-treated rats. The rats ran, reared and jumped for over six hours after the administration of L-DOPA. The addition of benserazide to this mix resulted in greater ambulation than for either AADC inhibitor alone, in reserpine- and 3-NPM-treated rats, but not in monoamine intact rats. This effect could be attributed to a greater amount of unchanged L-DOPA being made available in the CNS, following peripheral and central AADC blockade. In all instances involving NSD 1015, the ambulation induced by L-DOPA was sensitive to D_2 receptor blockade, but not to D_1 blockade, suggesting that the L-DOPA may be acting as a direct agonist at D_2 receptors. This finding is supported by the fact that the D_2 antagonist haloperidol, induces accumulation of L-DOPA, while the D_1/D_2 agonist, apomorphine, decreases L-DOPA accumulation following administration of L-DOPA and NSD 1015 (Nissbrandt et al., 1988).

The pattern and characteristics of the locomotion induced by L-DOPA in the presence of NSD 1015, are markedly distinct from those found in the absence of central AADC blockade. Whereas the direct effects of L-DOPA (following administration of NSD 1015) have a delayed onset of about seventy minutes and a duration of more than six hours, the locomotion induced by L-DOPA in the absence of central AADC blockade has a much quicker onset of approximately twenty minutes, and a duration of seventy minutes. Furthermore, DA-dependent locomotion induced by L-DOPA (in the absence of central AADC blockade) is not characterized by running and jumping, as is the case with L-DOPA. The prolonged duration of the effects of L-DOPA when combined with NSD 1015, could be attributed to the inhibition of its breakdown by the inhibitory activity of NSD 1015 on the enzyme monoamine oxidase (Hunter et al., 1993).

The addition of budipine to the combination of L-DOPA and both AADC inhibitors significantly enhanced the locomotion produced in reserpine-treated rats, with the rats showing a greater tendency for stereotyped fixation in the rearing position. The NMDA antagonist maintained the D_2 receptor sensitivity of L-DOPA's effects, increased the duration of locomotion (over 8 h), and reversed the NSD 1015-induced delay in the onset of locomotion. This delay is thought to be necessary to build up L-DOPA in sufficiently high concentrations as to stimulate D_2 DA receptors. The large amount of L-DOPA required to stimulate this receptor suggests that this may not be a primary receptor for L-DOPA, lending additional weight to the search for a

putative L-DOPA receptor (Misu et al., 1996).

The rapid attainment of a ceiling effect (indicated by fixated rearing) led us to repeat the combination with a sub-threshold dose of L-DOPA (50 mg/kg). At this dose, there was a further increase in the onset of locomotion following the combination of L-DOPA with both AADC inhibitors. Budipine was unable to reverse this NSD 1015-delay, but potentiated the locomotion due to the combination of L-DOPA with both AADC inhibitors. Unlike the rats treated with the higher dose, the rats here showed a greater fluidity of movement, with less running and jumping, and the absence of fixated rearing. In addition, these effects remained sensitive to D_2 receptor blockade, but not to D_1 blockade. A further reduction of the dose of L-DOPA led to a loss of the potentiating effects of budipine. The fact that budipine could enhance the locomotion induced by L-DOPA, as it did for DA, suggests a facilitatory role for NMDA antagonists in the mechanism of action of the proposed L-DOPA receptor. These results also show the ability of budipine to potentiate L-DOPA by both DA-dependent and DA-independent mechanisms.

To further clarify the mechanism(s) by which L-DOPA induces locomotion in monoamine intact and depleted rats, we went a step backwards in the dopamine synthesis pathway, and inhibited the conversion of tyrosine to L-DOPA with the use of the TH inhibitor, AMPT. It was possible to induce locomotion in reserpine-treated rats with the catecholamine releaser, d-amphetamine. The rats began to rear and sniff within five minutes of injection, and continued to walk around the cages for approximately seventy minutes, before they became fixated on sniffing the floor for the remaining 110 min. Prior treatment with AMPT obliterated this effect, emphasizing the need for L-DOPA stores. Combining d-amphetamine with NSD 1015 reversed the fixation, leading to a significant increase in ambulation, lasting approximately 130 min. Whereas the direct effects of d-amphetamine are D_2-dependent, as would be expected for stereotyped behaviour (Clarke and White, 1987), the addition of NSD 1015 led to a D_1 receptor dependence of the locomotor effects of d-amphetamine. The reversal of reserpine-induced akinesia by d-amphetamine, in the apparent absence of DA, suggests that d-amphetamine may be releasing L-DOPA, similar to its DA-releasing effects. L-DOPA is thought to be released as a co-transmitter with DA (Misu and Goshima, 1993). However, the DA-releasing effect of d-amphetamine may be via a different carrier mechanism, as L-DOPA is presumed to be

released from the cytoplasmic compartment of non-DA neurones, as opposed to DA that is released by exocytosis from vesicles of catecholaminergic neurones (Koshimura et al., 1992; Misu and Goshima, 1993; Sarre et al., 1994).

Extending the role for L-DOPA stores in the effects of d-amphetamine, we combined the threshold L-DOPA dose with d-amphetamine in the presence and absence of NSD 1015. In the absence of NSD 1015, locomotor counts for the d-amphetamine and L-DOPA combination were higher than for d-amphetamine alone, but the rats rapidly attained stereotyped biting (within 70 min). This stereotyped biting was reversed by the addition of NSD 1015. The greater degree of locomotion with NSD 1015, d-amphetamine and L-DOPA also supports the argument for L-DOPA stores. The locomotion here is similar to the potentiation by budipine, showing a rapid onset, as well as a similar duration of locomotion. However, biochemical experiments will be required to confirm the extent of L-DOPA release in these reserpine-treated rats, as we have been unable to induce DA release with this dose of d-amphetamine (4 mg/kg i.p.) in microdialysis experiments (Biggs, personal communication).

L-DOPA methyl ester, the more soluble form of L-DOPA, has been known to increase DA synthesis and release in the nigrostriatal tract (Koshimura et al., 1992). In our hands, LDME has been found to activate AADC, unlike its native form, and release freshly synthesized DA in microdialysis experiments. LDME has also been shown to competitively antagonise the effects of L-DOPA on catecholamine release (Misu and Goshima, 1993). To determine whether there were differences in the abilities of both compounds to induce locomotion independently of DA, we repeated the earlier experiments by combining the central and peripherally-acting AADC inhibitors with a threshold dose of LDME (100 mg/kg, equivalent to 80 mg/kg L-DOPA), in reserpine-treated rats. The combination of LDME and NSD 1015 resulted in marked reversal of akinesia approximately 70 min after injection of LDME, with the rats showing a well-coordinated pattern of running, rearing and jumping, with five times the overall locomotor counts of a similar treatment with L-DOPA. The addition of benserazide to this mix reduced the intensity of running, rearing and sniffing, as was the case with L-DOPA. The total number of rears and jumps with LDME were less than for the equivalent treatment with L-DOPA.

Similar to the effects of L-DOPA 200 mg/kg (with benserazide), the effects of LDME were insensitive to either D_1 or D_2 receptor blockade with SCH 23390 or sulpiride, respectively. Furthermore, unlike L-DOPA, budipine did not potentiate the locomotor effects of the threshold dose of LDME in reserpine-treated rats. The reasons for this are unclear, but an overview of the results indicates that L-DOPA and its methyl ester act differently with regard to potentiation by NMDA antagonism, and that the methyl ester is a more potent inducer of locomotion. In addition, the apparent non-involvement of DA receptors in the locomotor activation by LDME is indicative of non-dopaminergic mechanisms, while its greater potency suggests that it may be a more selective agonist for a putative L-DOPA receptor.

In summary, it was possible to induce locomotion with L-DOPA, in the apparent absence of dopamine (with reserpine treatment), and under conditions of DA receptor supersensitivity (with 3-NPM treatment). This effect was potentiated by budipine, and involved the stimulation of D_2 receptors. Budipine also potentiated locomotion due to DA, but via D_1 receptor activation. Furthermore, L-DOPA and its methyl ester exhibit different mechanisms of direct induction of locomotion.

CHAPTER SEVEN

COMPARATIVE STUDY OF THE BIOCHEMICAL EFFECTS OF L-DOPA IN RESERPINE AND 3-NPM-TREATED RATS

7.1 Introduction

In DA deficient conditions which induce akinesia, it is customary to stimulate DA receptors indirectly by DA replacement, or directly by the use of DA receptor agonists. In the former instance, the precursor amino acid for DA, L-DOPA, is administered along with a peripheral DOPA decarboxylase inhibitor. It is presumed that the injected L-DOPA is taken up into cathecolaminergic neurones, where it is converted to DA by the enzyme AADC. Formed DA is stored in vesicles, and is subject to release into the extracellular space by exocytosis. The release of DA is modulated by presynaptic D_2 receptors. The released DA produces movement by simultaneous activation of postsynaptic DA D_1 and D_2 receptors.

L-DOPA may not be an inert amino acid as previously thought, judging from the results from recent experiments which suggest that the afore-stated pathway may not be the only mechanism by which the exogenous administration of L-DOPA can produce movement. L-DOPA paradoxically inhibits the enzyme responsible for its conversion to AADC (Fisher et al., 1998). At high doses (100-200 mg/kg), it also inhibits the release of DA by interacting with presynaptic D_2 receptors (Fisher et al., 2000). When DA synthesis from administered L-DOPA is blocked by the use of a centrally-acting AADC inhibitor such as NSD 1015, it is expected that the tissue levels of L-DOPA will rise by as much as a hundred-fold (Nissbrandt et al, 1988). The L-DOPA so accumulated in the cytoplasm is subject to release as a co-transmitter with DA (Misu and Goshima, 1993). Furthermore, NSD 1015 is known to release endogenous L-DOPA rather than DA, in a ratio of 150:1 (Nakamura et al., 1992). The exact mechanism for L-DOPA release is yet to be determined. The tissue levels of DA, 5-HT and 5-HTP will be expected to rise alongside the levels of L-DOPA, as NSD 1015 is also an inhibitor of MAO (Hunter et al.,1993).

7.2 Methods

In the previous chapter, we showed the effects of L-DOPA on locomotor behaviour of reserpine-treated, monoamine intact and 3-NPM-treated rats. It was deemed necessary to assess the corresponding neurochemical status of these rats. The activity of L-DOPA was prolonged in the presence of NSD 1015, lasting at least six hours after the injection of L-DOPA. Consequently, the locomotor scores were taken in the first 3 h after L-DOPA administration, and the animals were sacrificed approximately 30 min after. The neurochemistry herein presented reflects the state of the rats three and a half hours after treatment with L-DOPA.

7.3 Results

7.3.1 Experiments in reserpine-treated rats

7.3.1.1 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on tissue DA levels in reserpine-treated rats

The tissue levels of DA were significantly decreased in the PFC ($p<0.05$), and increased in the ST ($p<0.001$) of reserpinized rats after treatment with L-DOPA (100 mg/kg), NSD 1015, and benserazide, when compared with saline treatment (Fig. 7.1 and 7.2).

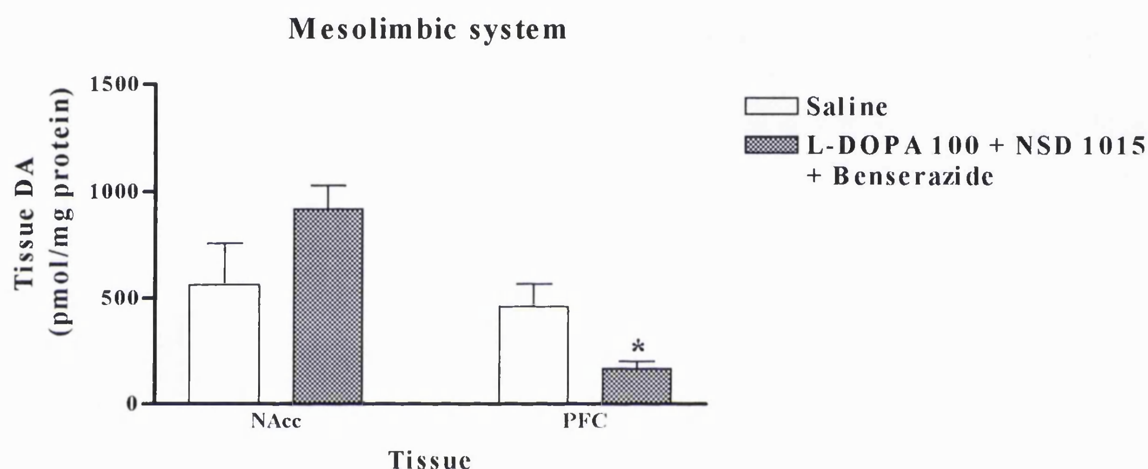


Fig. 7.1 - Tissue dopamine levels in the NAcc and PFC of reserpinized rats treated with saline or L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). The results are the means + S.E.M. of six replicates. (*p<0.05 versus saline treatment).

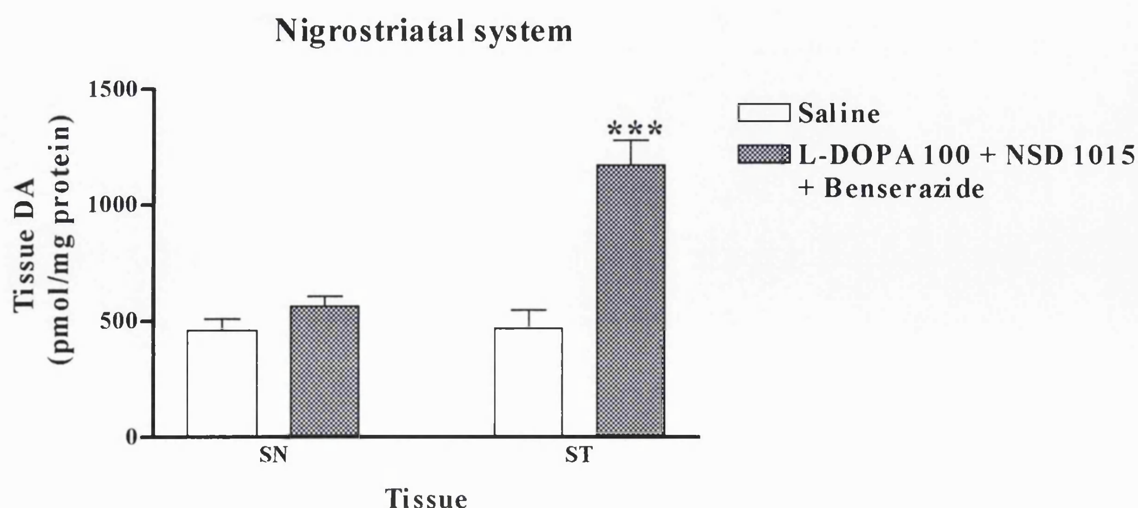


Fig.7.2 - Tissue dopamine levels in the SN and ST of reserpinized rats after treatment with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50mg/kg i.p.). The results are the means + S.E.M of six replicates. (***p<0.001 versus saline treatment).

7.3.1.2 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on tissue L-DOPA levels in reserpine-treated rats

In both the mesolimbic and nigrostriatal systems, treatment with a combination of L-DOPA (100 mg/kg), NSD 1015 and benserazide resulted in a marked fall ($p<0.001$) in the tissue levels of L-DOPA, as shown in figures 7.3 and 7.4 below.

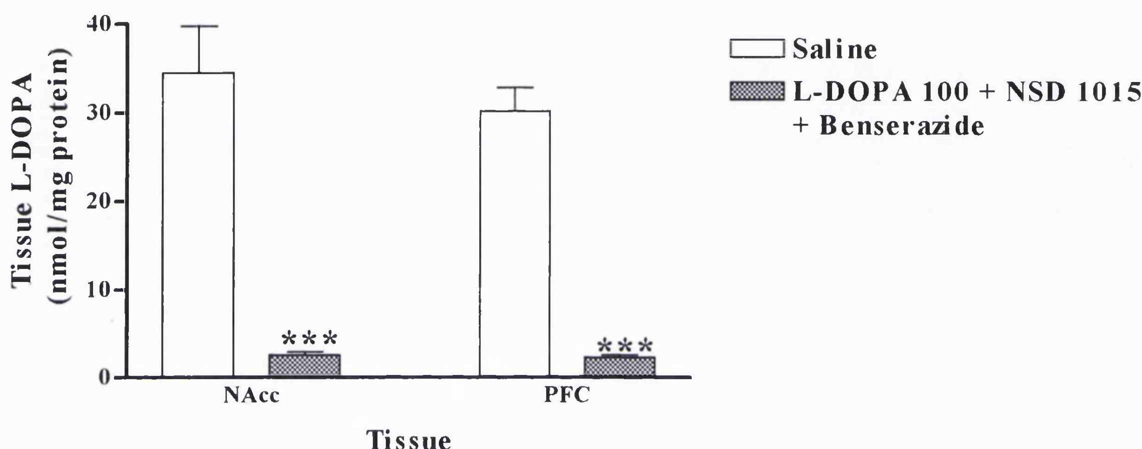


Fig. 7.3 - Tissue levels of L-DOPA in the NAcc and PFC of reserpinized rats after treatment with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). The results are the means + S.E.M. of a minimum of five replicates. (*p<0.001 versus saline treatment).**

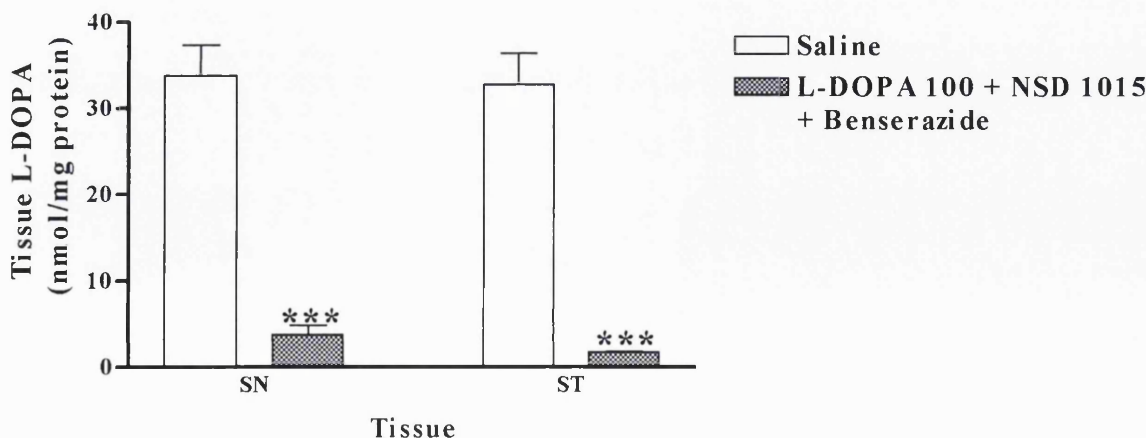


Fig. 7.4 - Tissue levels of L-DOPA in the SN and ST of reserpinized rats following treatment with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.) . The results are the means + S.E.M. of a minimum of five replicates. (***p<0.001 versus saline treatment).

7.3.1.3 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on tissue 5-HT levels in reserpine-treated rats

Reserpine treatment resulted in a marked depletion of tissue 5-HT, to levels below the detection of our system. Tissue 5-HT levels did not recover after treatment with the combination of L-DOPA, NSD 1015 and benserazide. Tissue levels of 5-HT in control animals are usually 400.9 ± 89.4, 345.7 ± 59.7, 439.3 ± 76.8 and 233.4 ± 38.9 pmol/mg protein in the NAcc, PFC, SN and ST respectively.

7.3.1.4 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on DA formation in reserpine-treated rats

There was a marked inhibition in DA formation in the mesolimbic and nigrostriatal structures analysed, following treatment of reserpinised rats with L-DOPA, NSD 1015 and benserazide. DOPA decarboxylase activity was significantly inhibited ($p<0.01$) in the NAcc, PFC, SN and ST (Figures 7.5 and 7.6).

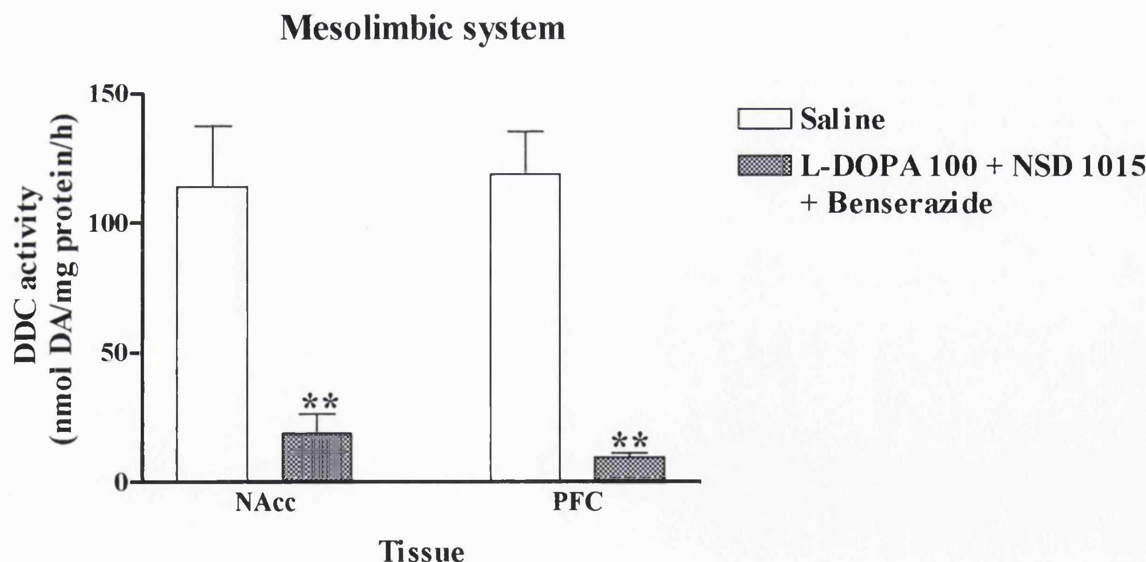


Fig. 7.5 - Dopa decarboxylase activity in the NAcc and PFC of reserpinized rats treated with saline or a combination of L-DOPA (100mgkg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). The results are the means + S.E.M. of six replicates. (p<0.01 versus saline treatment).**

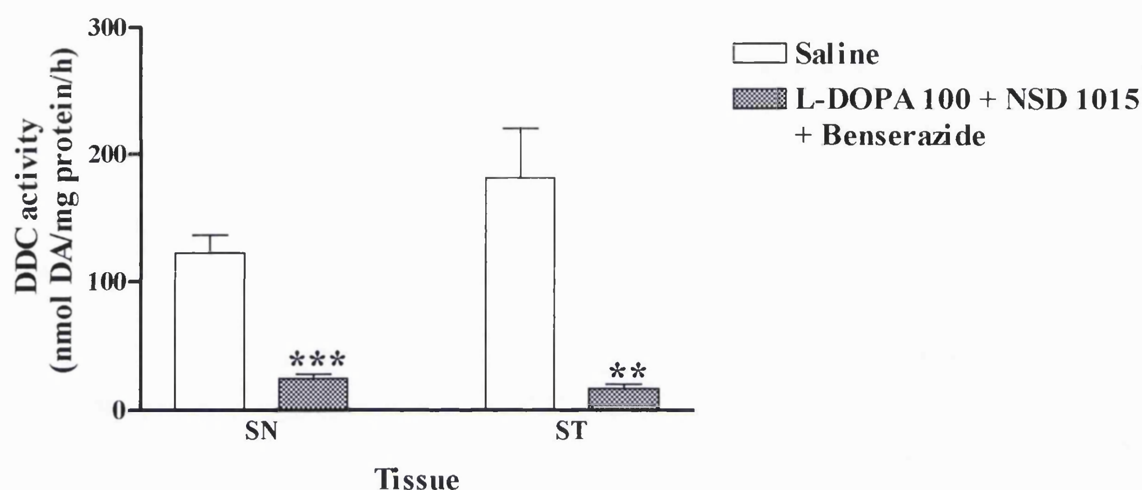


Fig. 7.6 - Dopa decarboxylase activity in the SN and ST of reserpinized rats after treatment with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). Results are the means + S.E.M. of six replicates. (p<0.01, ***p<0.001 versus saline treatment).**

7.3.1.5 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on 5-HT formation in reserpine-treated rats

Treatment with L-DOPA, NSD 1015 and benserazide completely inhibited the formation of 5-HT by 5-HTPDC, in the 5-HTP incubation mix (Fig. 7.7 and 7.8; see methods in sections 2.4.2 and 2.4.3). 5-HT can also be formed from endogenous 5-HTP by DDC in L-DOPA incubation mixes, as seen in saline-treated reserpinised rats (Fig. 7.9 and 7.10). However, when these rats were treated with L-DOPA, NSD 1015 and benserazide, this synthetic pathway is completely abolished in both the mesolimbic and nigrostriatal structures.

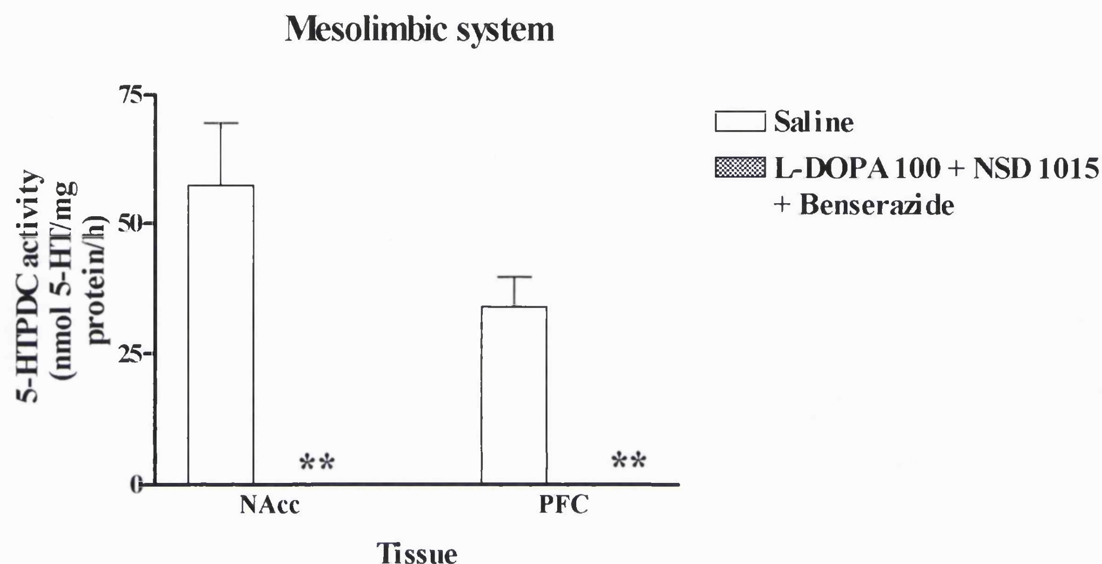


Fig. 7.7 - 5-HTP decarboxylase activity in the NAcc and PFC of reserpinized rats treated with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). The results are the means + S.E.M. of six separate determinations. (**$p<0.01$ versus saline treatment).

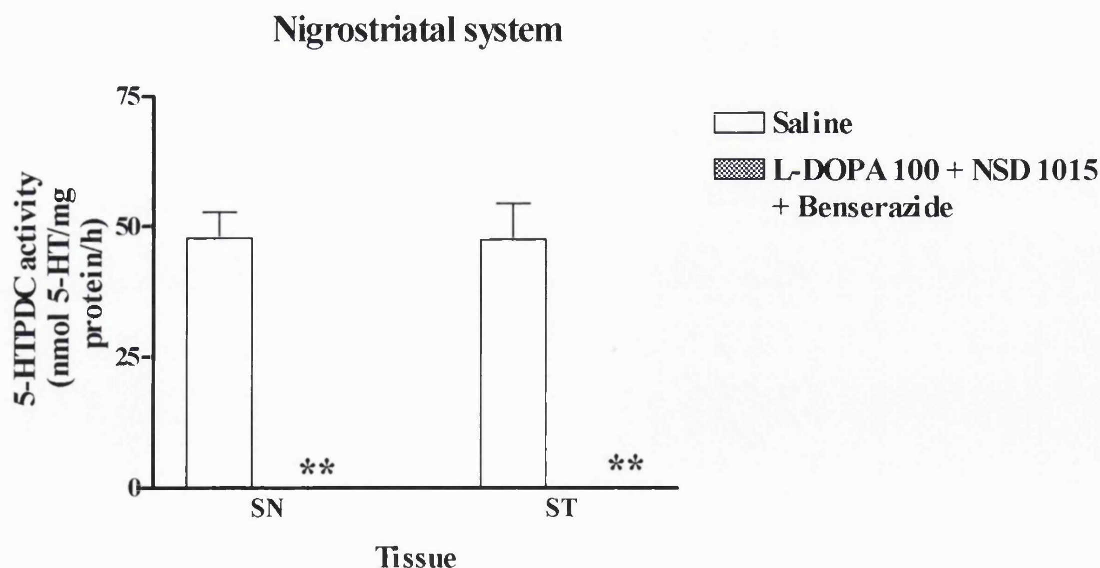


Fig. 7.8 - 5-HTP decarboxylase activity in the SN and ST of reserpinized rats after treatment with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). Results are the mean + S.E.M of six determinations. (**$p<0.01$ versus saline treatment).

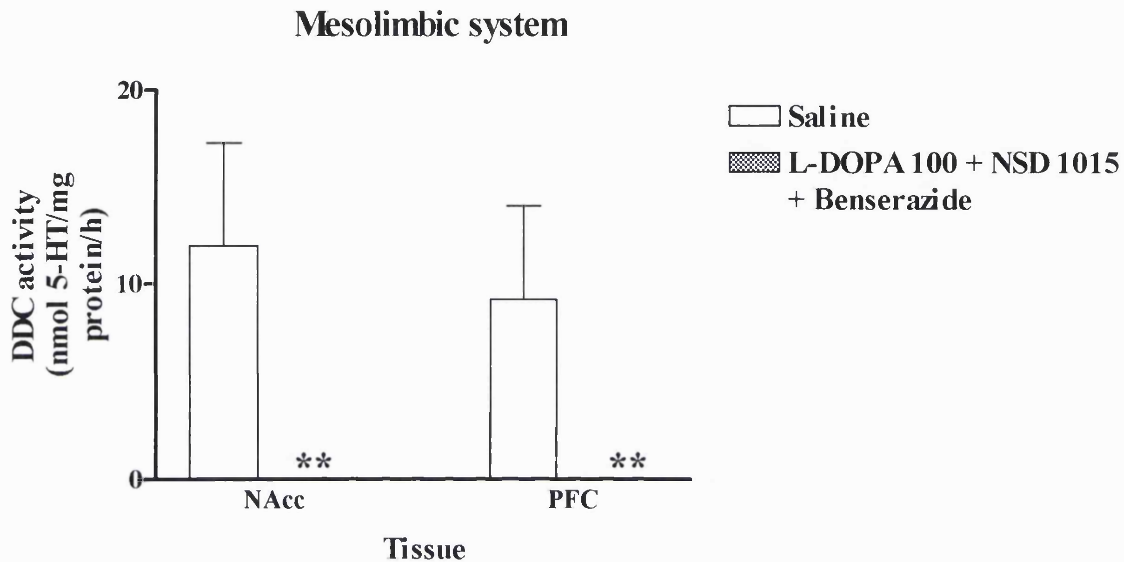


Fig. 7.9 -Formation of 5-HT in the NAcc and PFC of reserpinized rats treated with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). Results are the means + S.E.M. of six replicates. (**$p<0.01$ versus saline treatment).

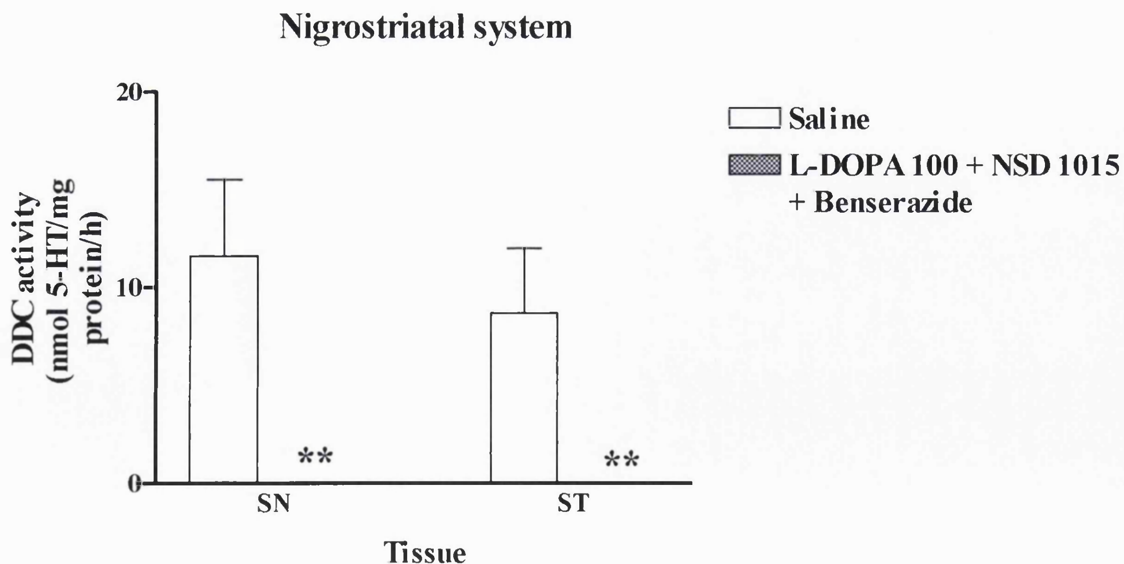


Fig. 7.10 - Formation of 5-HT in the SN and ST of reserpinized rats treated with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). The results represent the means of six determinations + S.E.M. (**$p<0.01$ versus saline treatment).

7.3.1.6 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on tissue levels of excitatory amino acids in reserpine-treated rats

Tissue levels of the excitatory amino acids, aspartate and glutamate, are significantly elevated in the mesolimbic structures ($p<0.001$ for aspartate, and $p<0.01$ for glutamate), as seen in figures 7.11 and 7.13. In the nigrostriatal system, the levels of both amino acids are markedly elevated, but do not attain levels of significance ($p>0.05$; Fig. 7.12 and 7.14).

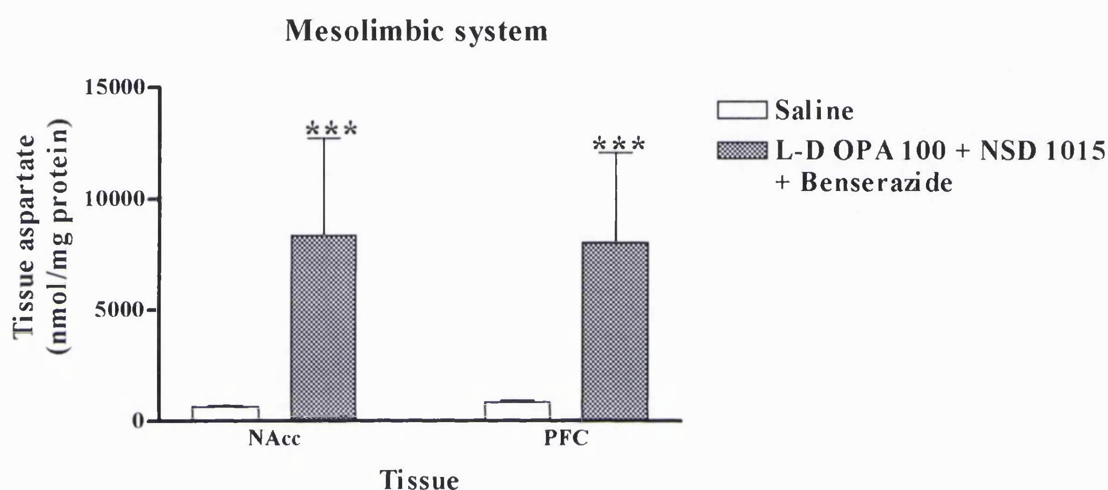


Fig. 7.11 - Tissue aspartate levels in the NAcc and PFC of reserpinized rats after treatment with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). The results are the mean + S.E.M. (*p<0.001 versus saline treatment).**

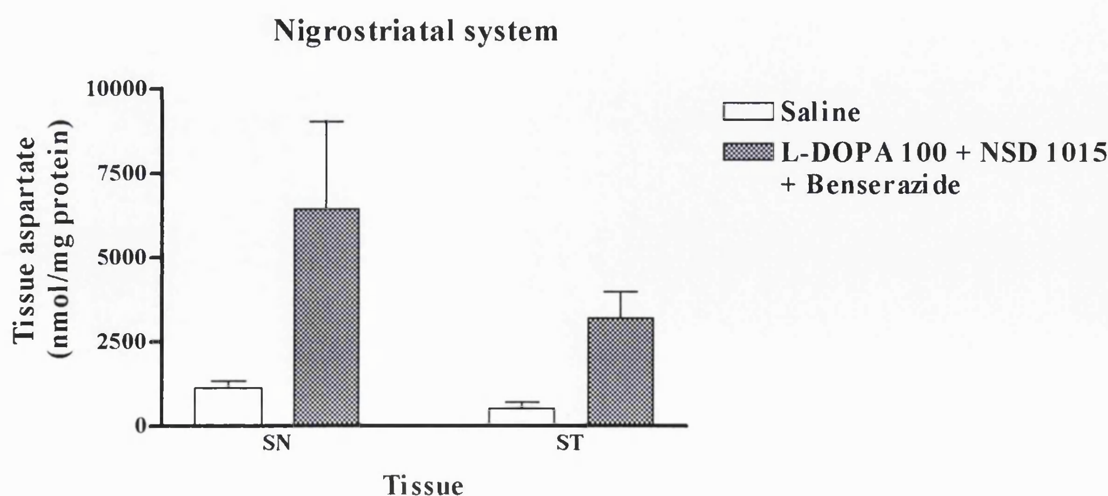


Fig. 7.12 - Tissue aspartate levels in the SN and ST of reserpinized rats after treatment with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). The results are the means + S.E.M. (p>0.05 versus saline treatment).

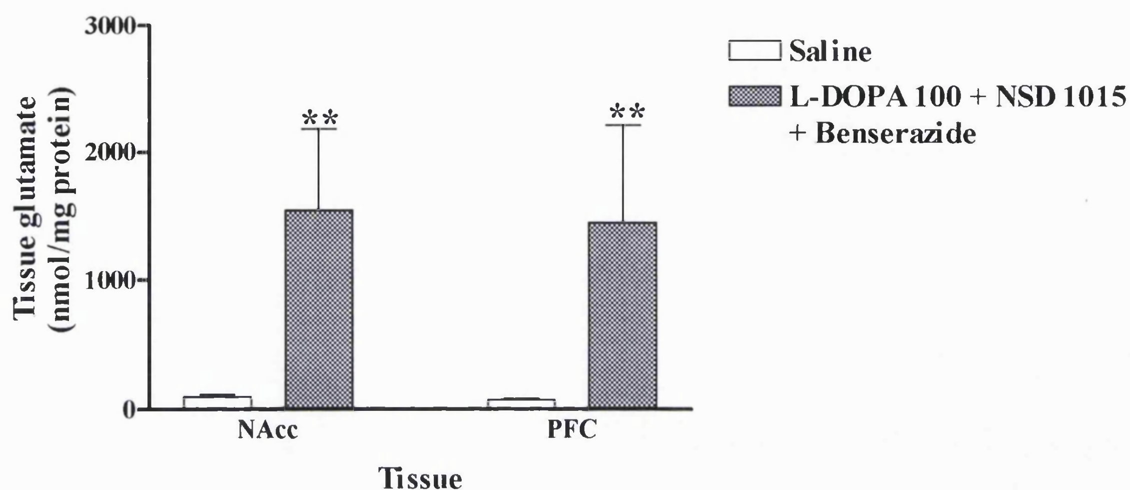


Fig. 7.13 - Tissue glutamate levels in the NAcc and PFC of reserpinized rats after treatment with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). Results are the means +S.E.M. (p<0.01 versus saline treatment).**

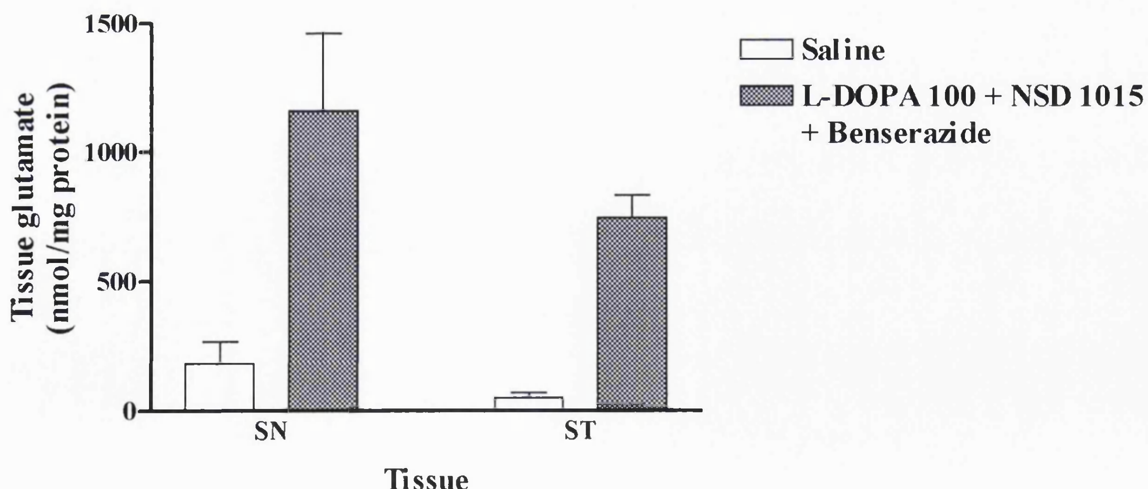


Fig. 7.14 - Tissue glutamate levels in the SN and ST of reserpinized rats after treatment with saline or a combination of L-DOPA (100 mg/kg i.p.), NSD 1015 (100 mg/kg i.p.) and benserazide (50 mg/kg i.p.). Results are the means + S.E.M. (p>0.05 versus saline treatment).

7.3.2 Experiments in 3-NPM-treated rats

7.3.2.1 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on tissue DA levels in 3-NPM-treated rats

Tissue DA levels are unchanged in all four structures after treatment with L-DOPA (100 mg/kg) and benserazide. However, the combinationof L-DOPA with the central AADC inhibitor, NSD 1015, alone or with benserazide, resulted in significant elevation of tissue DA in the NAcc and ST, but not in the PFC (Fig. 7.15 and 7.16).

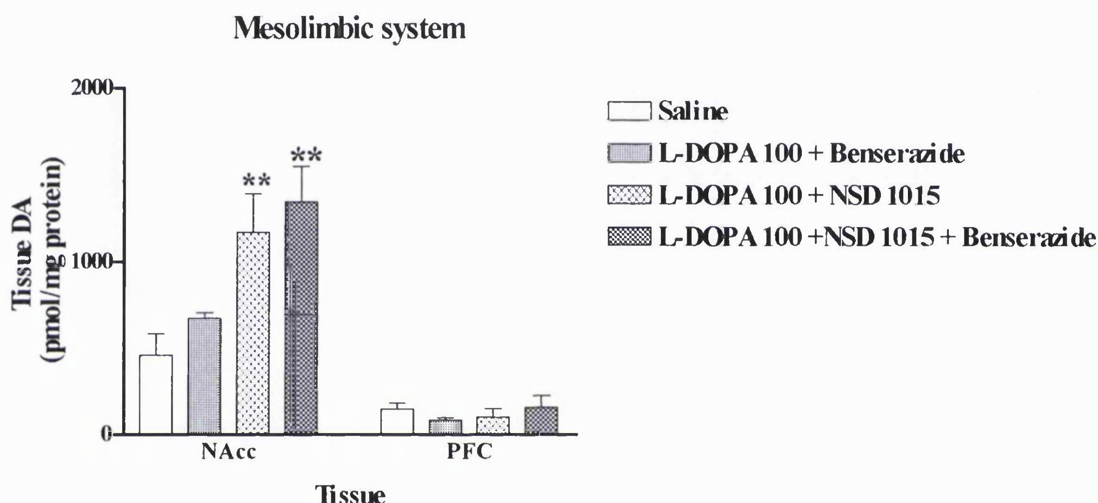


Fig. 7.15 - Tissue DA levels in the NAcc and PFC of 3-NPM rats treated with saline or L-DOPA (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.). The results are the means + S.E.M. of a minimum of six determinations. (p<0.01 versus saline treatment).**

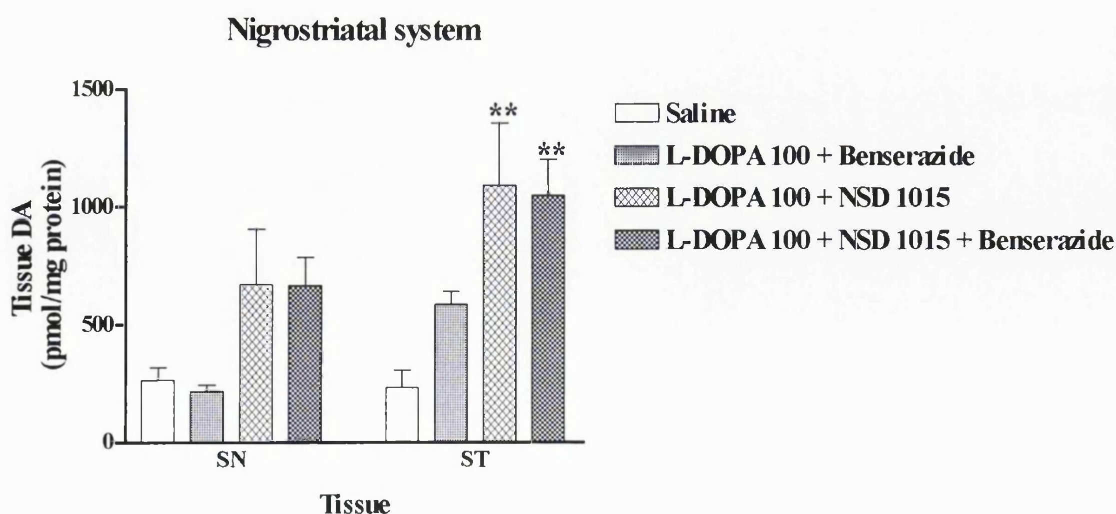


Fig. 7.16 - Tissue DA levels in the SN and ST of 3-NPM rats after treatment with saline or L-DOPA (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.). The results are the means + S.E.M. of a minimum of six determinations. (p<0.01 versus saline treatment).**

7.3.2.2 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on tissue L-DOPA levels in 3-NPM-treated rats

The L-DOPA content of the NAcc was increased with the use of NSD 1015, but unchanged when benserazide alone was combined with L-DOPA. L-DOPA levels were unchanged in the PFC with all combinations (Fig. 7.17). On the contrary, the tissue levels of L-DOPA in the nigrostriatal structures were markedly reduced ($p<0.01$) with all treatment combinations (Fig. 7.18).

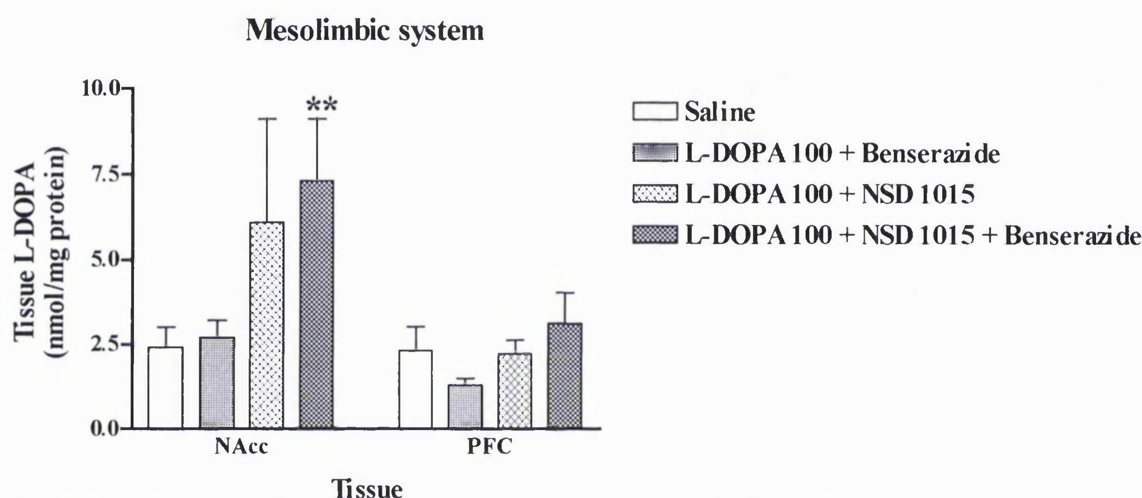


Fig. 7.17 - Tissue L-DOPA levels in the NAcc and PFC of 3-NPM rats after treatment with saline or L-DOPA (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.). Results are the means + S.E.M. of six determinations. (p<0.01 versus saline treatment).**

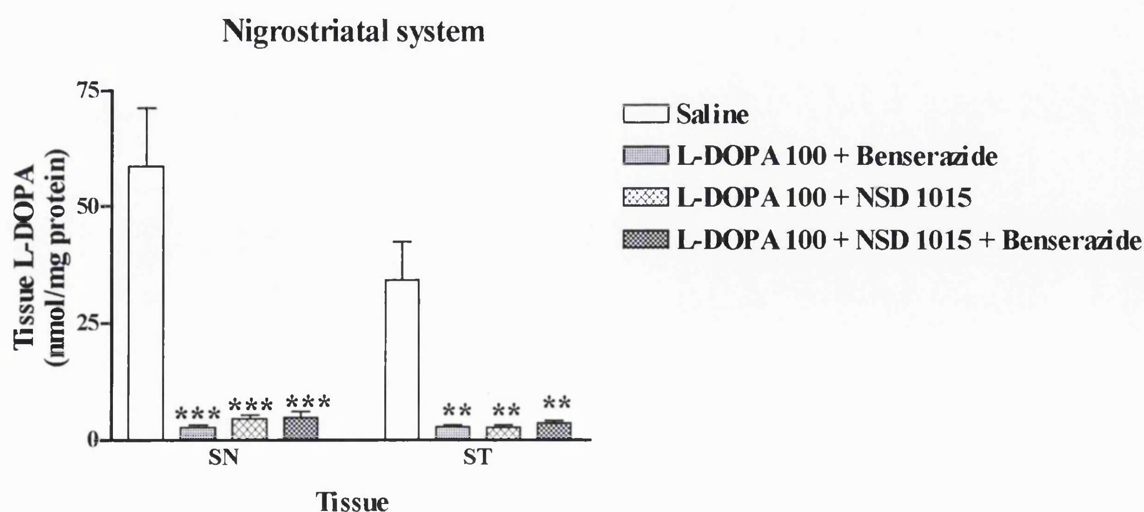


Fig. 7.18 - Tissue L-DOPA levels in the SN and ST of 3-NPM rats after treatment with saline or L-DOPA (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.). The results are the means + S.E.M. of six determinations. (p<0.01, ***p<0.001 versus saline treatment).**

7.3.2.3 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on tissue 5-HT levels in 3-NPM-treated rats

The tissue levels of 5-HT were undetectable in 3-NPM rats treated with L-DOPA, benserazide and/or NSD 1015. In saline controls tissue 5-HT levels were: 189.1 $\pm$ 51.4, 164.9 $\pm$ 37.8, 369.3 $\pm$ 78.4 and 211.0 $\pm$ 36.3 pmol/mg protein in the NAcc, PFC, SN and ST respectively.

7.2.2.4 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on DA formation in 3-NPM-treated rats

The formation of DA from L-DOPA incubation mix by L-DDC (see section 2.4.2) was greatly impeded in the mesolimbic and nigro-striatal structures by all combinations of L-DOPA and AADC inhibitors (Fig. 7.19 and 7.20). In the SN, the extent of AADC inhibition with NSD 1015 alone was significantly greater than for benserazide ($p<0.05$).

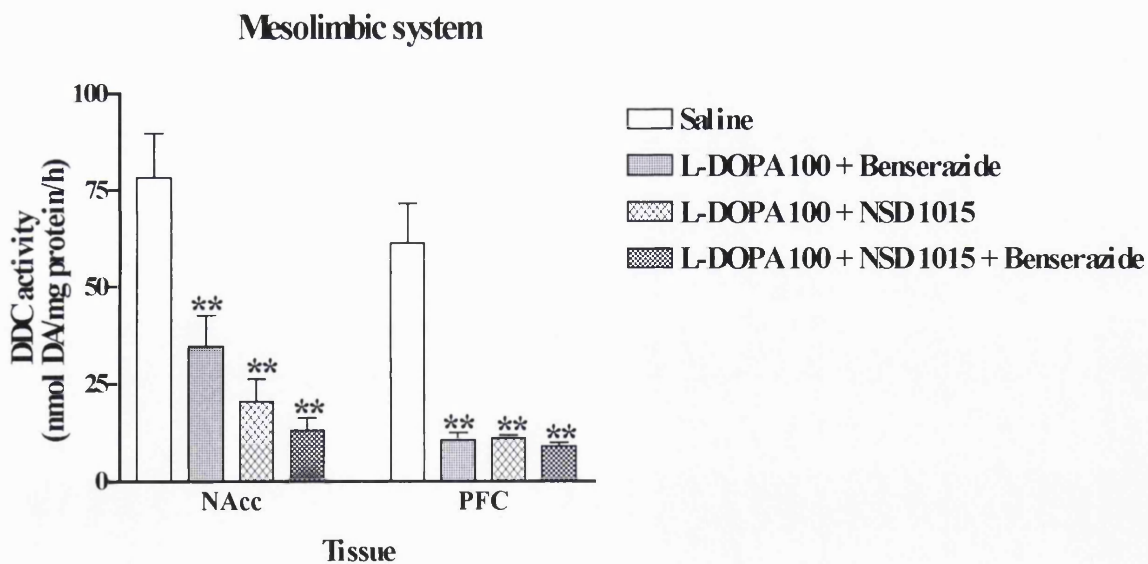


Fig. 7.19 - DA formation by DDC in the NAcc and PFC of 3-NPM rats following treatment with saline or L-DOPA (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.). The results are the means + S.E.M. of six replicates. ($p<0.01$ versus saline treatment).**

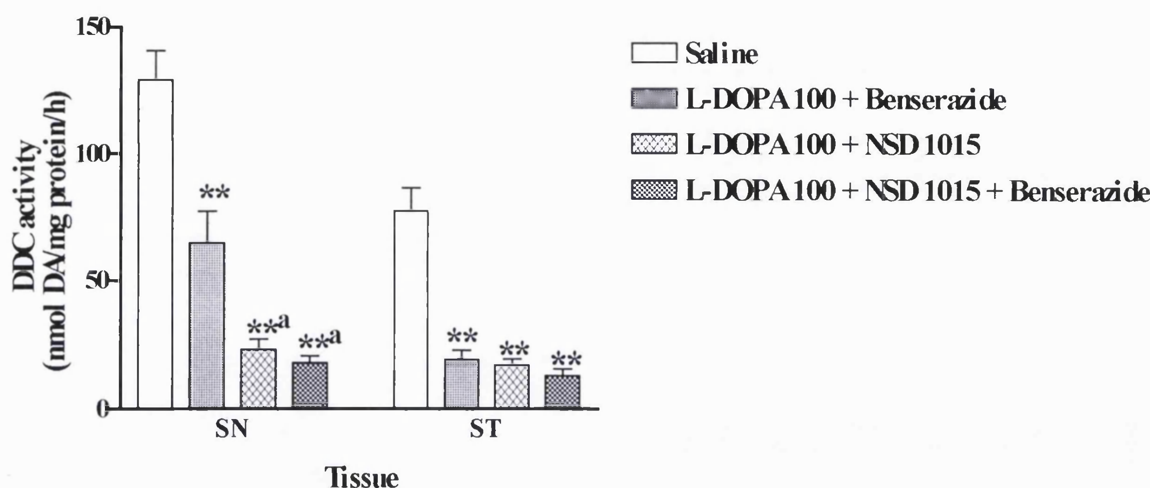


Fig. 7.20 - DA formation by DDC in the SN and ST of 3-NPM rats following treatment with saline or L-DOPA (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.). The results are the means + S.E.M. of six replicates. (p<0.01 versus saline treatment; a=**p<0.01 compared to L-DOPA 100 + Benserazide).**

7.3.2.5 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on 5-HT formation in 3-NPM-treated rats

Following treatment with L-DOPA and AADC inhibitors, the formation of 5-HT by 5-HTPDC was completely blocked. In saline treated 3-NPM rats, 5-HT formation by 5-HTPDC was at the rates of 17.3 $\pm$ 2.6, 10.9 $\pm$ 1.0, 26.8 $\pm$ 3.9 and 15.1 $\pm$ 1.1 nmol/mg protein/h in the NAcc, PFC, SN and ST respectively.

7.3.2.6 Effects of L-DOPA in the presence and absence of central and peripheral AADC inhibitors on tissue levels of excitatory amino acids in 3-NPM-treated rats

Tissue levels of aspartate were generally higher following treatment with L-DOPA and an AADC inhibitor, in both the nigrostriatal and mesolimbic structures. However, the large individual variations among the rats meant that this effect was not statistically significant ($p>0.05$; Fig. 7.21 and 7.22). A similar trend was also observed for glutamate, when either AADC inhibitor was used in combination with L-DOPA. In the nigrostriatal tract, the combination of both AADC inhibitors with L-DOPA lowered (in the ST) or maintained (in the SN) the tissue levels of glutamate (Fig. 7.23 and 7.24).

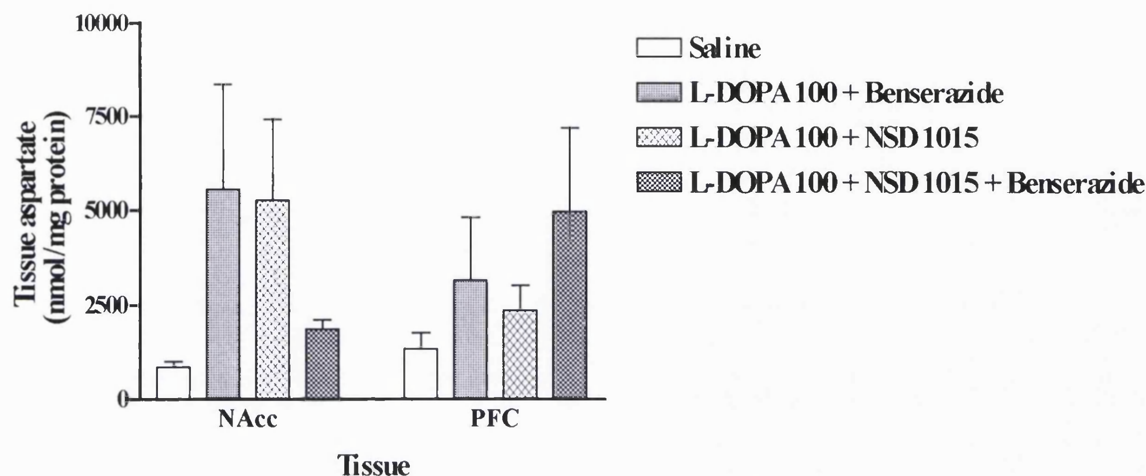


Fig. 7.21 - Tissue aspartate levels in NAcc and PFC of 3-NPM rats after treatment with saline or L-DOPA (100 mg/kg i.p.), benserazide (50mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.). Results are the means + S.E.M of six replicates. ($p>0.05$ versus saline treatment).

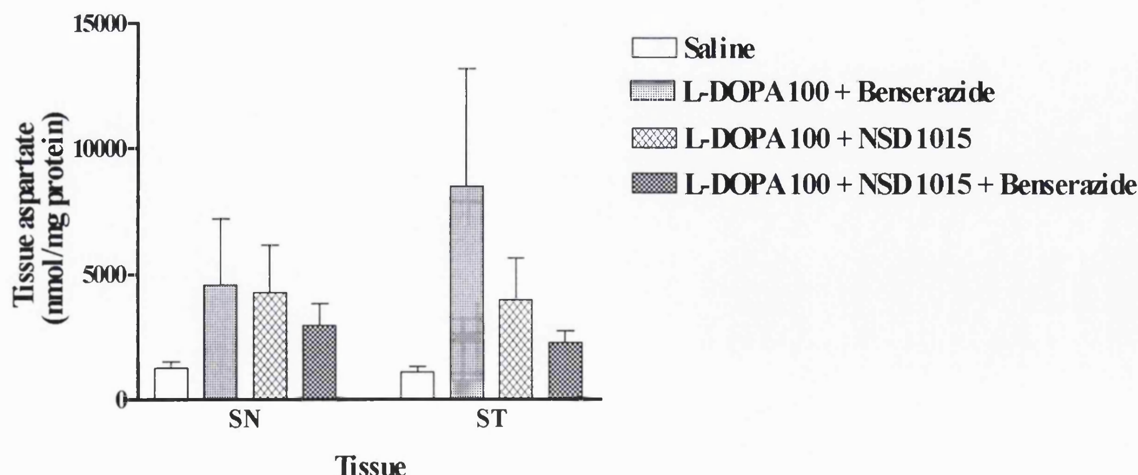


Fig. 7.22 - Tissue aspartate levels in the SN and ST of 3-NPM rats following treatment with saline or L-DOPA (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.). The results are the means + S.E.M. of six replicates. ($p>0.05$ versus saline treatment).

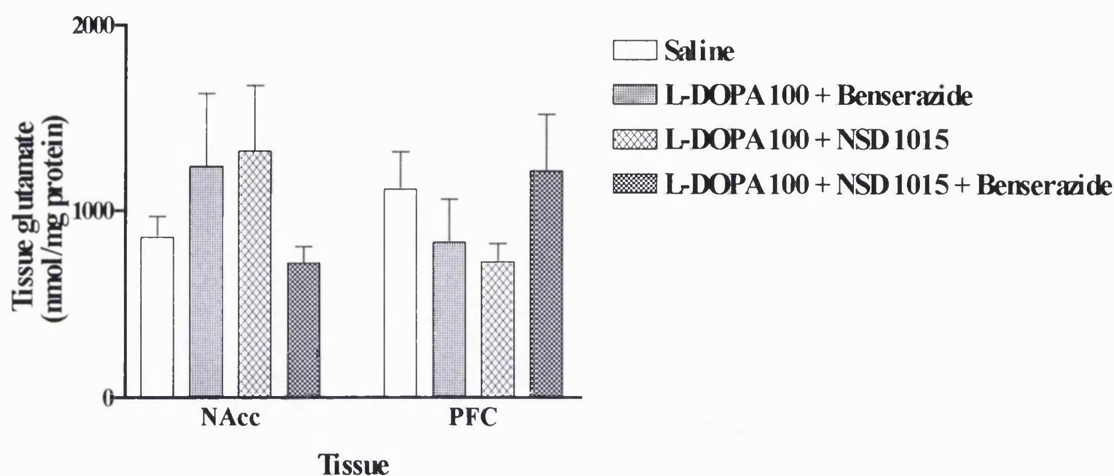


Fig. 7.23 - Tissue glutamate levels in the NAcc and PFC of 3-NPM rats following treatment with saline or L-DOPA (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.).The results are the means + S.E.M. of six replicates. ($p>0.05$ versus saline treatment).

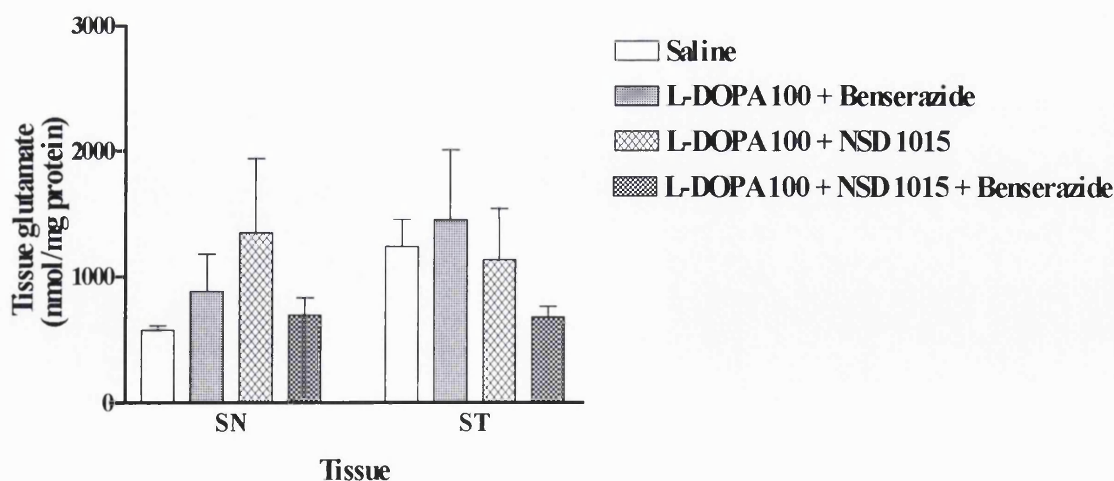


Fig. 7.24 - Tissue glutamate levels in the SN and ST of 3-NPM rats following treatment with saline or L-DOPA (100 mg/kg i.p.), benserazide (50 mg/kg i.p.) and/or NSD 1015 (100 mg/kg i.p.). The results are the means + S.E.M. of six replicates. ($p>0.05$ versus saline treatment).

7.4 Discussion

Reserpine depletes central and peripheral stores of catecholamines, including DA and 5-HT (Carlsson et al., 1957). As such, our reserpine-treated rats showed a significant loss of tissue content of DA, L-DOPA and 5-HT in the NAcc, PFC, SN and ST 24 h after treatment with the alkaloid. There was an increase in the tissue content of DA in the ST, but not in the Nacc, PFC or SN after treatment with L-DOPA and the combination of both the central and peripheral AADC inhibitors. This is in agreement with similar experiments conducted by Nissbrandt et al. (1988), as inhibition of AADC should prevent DA formation by this enzyme. The observed increase in DA in the ST, though significant, does not necessarily equate with DA available in the synaptic cleft, as we have shown that this dose of L-DOPA (100 mg/kg) does not release DA in reserpine-treated rats (Fisher et al., 2000). In addition, Wachtel and Abercrombie (1994) have shown that this dose of L-DOPA induces an increase in DOPAC recovery in dialysates without a similar elevation in DA, suggesting that the newly synthesized DA is immediately converted to DOPAC by monoamine oxidase, and is therefore not available in the extracellular space. Consequently, the locomotor activity observed following treatment of reserpinised rats with a combination of L-DOPA and central and peripherally-acting AADC inhibitors could not have been induced by DA, but rather by a direct effect of L-DOPA at postsynaptic D_2 (and other) receptor sites.

The tissue levels of L-DOPA were a hundred times higher than those of DA in all the four brain regions following reserpine treatment, suggesting that L-DOPA may be more resistant than DA to the depleting effects of this alkaloid. Saline treatment did not alter these values, but the combination of L-DOPA and the AADC inhibitors significantly lowered the tissue levels of L-DOPA in all regions, by as much as ten fold. We are at a loss for an adequate explanation for this finding, as the brain levels of L-DOPA are known to rise after exogenous administration of L-DOPA, especially in the presence of central AADC inhibition (Hunter et al., 1993; Nissbrandt et al., 1988). Nakamura et al.(1992) have shown that under conditions of AADC inhibition with NSD 1015, there is a preferential increase in the release of L-DOPA to a 150-fold higher level, with decrease in DA release into the extracellular space. It is known that NSD 1015 induces "a substantial efflux of DOPA from the brain" (Nissbrandt et al., 1988). In the previous chapter, we showed the ability of d-amphetamine to induce locomotion in the apparent absence of DA. Although we did not measure the relative amounts of L-DOPA and DA released in dialysates

from the SN and ST, our low tissue levels of L-DOPA may suggest that this compound is released (Misu and Goshima, 1993) to act at postsynaptic sites within these brain structures. Considering that these levels of L-DOPA were determined 3.5 h after L-DOPA injection, it is plausible that substantial amounts of L-DOPA could have been lost to the general circulation.

Treatment with reserpine increased DDC activity above the rates for naive rats, in line with earlier reports (Hadjiconstantinou et al., 1993; Zhu et al., 1992). This implies that DA exerts an inhibitory control on DDC that can be removed by DA depletion or the use of DA receptor antagonists (Hadjiconstantinou et al., 1993; Zhu et al., 1992). In advanced stages of PD, AADC activity is known to be 5-15 % of control levels (Lloyd et al., 1975). Although AADC is not normally saturated, under conditions of extensive damage to neurones containing this enzyme, AADC could become rate-limiting in the formation of DA from exogenously applied L-DOPA. L-DOPA itself inhibits AADC (Fisher et al, 1998). Combining it with the peripherally-acting AADC inhibitor benserazide, and the centrally-acting AADC inhibitor NSD 1015, would be expected to markedly reduce AADC activity. This was the case in our experiments, where DDC activity was lowered to less than 20 % in both the mesolimbic and nigrostriatal structures, following treatment with L-DOPA and both types of AADC inhibitors. This percentage of AADC activity is sufficient to maintain DA formation from exogenous L-DOPA, as indicated by high levels of DOPAC (Fisher et al., 2000). However, this DA is not available extracellularly. Whereas our rats had extensive DA losses following reserpine treatment, and a marked reduction in DA synthesis,

, they showed complete reversal of akinesia, and locomotor activation via stimulation of D_2 receptors, after the administration of L-DOPA and NSD 1015 /and benserazide. These rats were able to run, rear and jump for more than six hours. From these results therefore, we can conclude that the locomotion here was dopamine-independent.

Whereas DDC activity was reduced by as much as 80 % with L-DOPA (100 mg/kg)/NSD 1015 and benserazide, there was a total loss of 5-HTPDC activity in all four regions. This suggests a differential sensitivity of both enzymes, and supports the theory that they may actually be two separate but related enzymes (Fisher and Starr, in press). In addition, it was possible to form 5-HT from L-DOPA incubation mixture, via DDC in saline-treated reserpinized rats. This ability was completely abolished when the rats were treated with L-DOPA and the AADC inhibitors.

The overall implication is an apparent non-involvement of 5-HT in the direct locomotor-inducing properties of L-DOPA.

A significant rise in the levels of aspartate and glutamate in the mesolimbic, but not the nigrostriatal structures, was observed. This supports the earlier suggestion of a role for glutamatergic activation in the locomotor stimulation by L-DOPA. It was possible to potentiate the locomotor effects of L-DOPA by prior treatment with the NMDA antagonist, budipine. The rise in glutamate levels observed here and elsewhere (Misu et al., 1996), may accelerate the neurodegenerative process in PD patients receiving chronic L-DOPA, especially when AADC activity has been reduced to less than 20 % (present experiments). AADC activity is typically between 5 and 15 % in PD patients (Lloyd et al., 1975).

Similar findings, as for reserpine-treated rats, were made with 3-NPM-treated rats exposed to L-DOPA and NSD 1015 /and benserazide. Tissue levels of DA were significantly elevated in the NAcc and ST whenever NSD 1015 was administered. This is understandable because 3-NPM depletes DA by only 70 % (Chapter 3), allowing NSD 1015 to induce an accumulation of DA by inhibiting its breakdown by MAO. The tissue levels of L-DOPA were increased in the NAcc, unchanged in the PFC, and decreased in the SN and ST, following central AADC inhibition.e sufficient L-DOPA to interact with postsynaptic dopaminergic or L-DOPAergic receptors. Taken together, these results suggest a preferential storage of DA in the tissues, and a release of L-DOPA. An alternative interpretation of the results is the possibility of a conversion of L-DOPA to DA. This can not be supported by microdiaysis data which suggest that any DA so formed is not available at the synaptic cleft. The inability of the elevated tissue DA stores to metamorphose into synaptic DA supports the argument for a DA-independent mechanism of locomotor activation following the administration of L-DOPA (100 mg/kg), NSD 1015/ and benserazide.

As for reserpine treatment, treatment of 3-NPM rats with L-DOPA and the AADC inhibitors led to a massive fall in DDC and 5-HTPDC activities. Nigrostriatal DDC activity was below 18%, and 5-HTPDC was not detected. Similarly, tissue levels of excitatory amino acids were unaltered in all four brain regions. Again, there was significant locomotor activation in the face of an apparent non-involvement of DA. It would have been proper to determine the relative

amounts of DA and L-DOPA in the extracellular spacet by dual probe microdialysis, but this was impossible because of the spate of running and jumping in which the rats were involved.

In summary, the neurochemical state of dopamine receptor supersensitive (by treatment with reserpine or 3-NPM) rats treated with L-DOPA and a central AADC inhibitor, presents a scenario of an accumulation of DA and excitatory amino acids, a loss of 5-HT and L-DOPA in mesolimbic and nigrostriatal structures at the time of locomotion. Furthermore, the fact that the synthesizing mechanisms for DA and 5-HT were inactivated suggests that the exogenously administered L-DOPA did not induce locomotion as a result of its conversion to dopamine. How L-DOPA goes about eliciting its locomotor effects remains cryptic, but appears to involve D_2 dopamine receptors, non-dopamine receptors (possibly L-DOPA receptors), as well as NMDA receptors.

CHAPTER EIGHT

FINAL COMMENTS

8.1 The 3-NPM model and Parkinson's disease

By treating rats with a threshold dose of the mitochonrial toxin, 3-nitropropionic acid, it was possible to produce marked striatal deletions in dopamine by a sub-threshold dose of the common drug of abuse, methamphetamine. Treating rats with either 3-NP or METH alone did not alter DA levels in the nigrostriatal or mesolimbic systems, but resulted in 70 % reduction of DA in the ST and 50 % reduction in the Nacc when both toxins were combined. The mutual potentiation induced by combining these two toxins (i.e. 3-NPM) suggests that a similar pattern of toxicological synergism may be involved in dopamine depletion in parkinsonian primates or humans suffering from Parkinson's disease.

Behavioural assessment of these 3-NPM-treated rats revealed a progressive development of supersensitive locomotor response to the direct and indirect D_1/D_2 DA receptor agonists, apomorphine and MK 801 respectively. However, when the selective D_1 or D_2 agonists were used, a supersensitive response was found to only the D_2 agonist, suggesting a greater damage to D_1 receptors, as would be predicted by their selective susceptibility to energy depletion (Araujo and Hilt, 1998). This D_2 selectivity with 3-NPM is important when one considers the fact that the dopaminergic agonists commonly used in treating PD (e.g. bromocriptine, pergolide) possess strong D_2 receptor agonist activity. Furthermore, the extent of the striatal lesion produced did not result in a complete independence of the effects of D_1 receptors from those of D_2, as would otherwise have been predicted for supersensitive, DA-depleted rats (Rubinstein et al., 1988). In addition, the rise in nigrostriatal tissue levels of glutamate and aspartate following energy inhibition by 3-NP, lends further credence to the use of NMDA antagonists as part of the treatment regimen in the early stages of neurodegenerative disorders like PD. This is because progressive energy loss, as would normally occur with aging, renders existing DA neurones susceptible to excitotoxic damage by endogenous levels of excitatory amino acids (Albers et al., 1996; Sonsalla et al., 1991).

8.2 L-DOPA-induced locomotion: a myth or a reality?

The ability of L-DOPA to induce locomotion in the apparent absence of DA release was assessed in normosensitive and DA receptor supersensitive rats. We found this direct locomotor effects of L-DOPA to have a bias for the D_2 receptor, a delayed onset of locomotion, an ability to completely reverse reserpine-induced akinesia, and a duration of ambulation of at least six hours. The implications of these findings for PD sufferers is the elimination of the On/Off phenomenon by the sustained stimulation of postsynaptic receptors. The non-selectivity of NSD 1015 on AADC precludes its use in humans, but the use of this compound has opened vistas in the search for a possible L-DOPA receptor, using selective agonists, that will by-pass the limited benefits of replacing DA at dopamine receptor sites. Further experiments are awaited to confirm that L-DOPA-induced, DA-independent locomotion is a reality, rather than a myth.

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