MECHANISMS OF 1-METHYL-4-PHENYLPYRIDINIUM REGULATE THE FUNCTIONAL ACTIVITY OF DOPAMINE TRANSPORTER: POTENTIAL INSIGHTS INTO PARKINSON'S DISEASE PATHOGENESIS

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ABSTRACT

The effect of 1-methyl-4-phenylpyridinium (MPP^+), a Parkinsonism-inducing toxin, has been studied in the regulation of dopamine transporter in order to investigate cellular events underlying the mechanisms of toxicity. This study was demonstrated that MPP^+ produced a concentration- and time dependent reduction in the uptake of [^{3}H]dopamine in HEK293 cells stably expressing the human dopamine transporter. It was observed that dopamine transporters undergo increased phosphorylation upon treatment of cultured cells with MPP^+. MPP^+ doses of 1-1000 μM caused statistically significant decreases in [^{3}H]WIN 35428 binding in the intact cell after 3 h. The saturation analysis of the [^{3}H]WIN 35428 binding obtained from total membrane fractions revealed a decrease in the transporter density (B_{max}) with an increase in the dissociation equilibrium constant (K_d) after MPP^+ treatment. Furthermore, biotinylation experiments confirmed that MPP^+ reduced both plasma membrane and intracellular dopamine transporter immunoreactivity. Incubation with MPP^+ in PC12 cells caused a rapid increase of phosphorylation on tyrosine residues of several proteins, including synaptophysin, a major 38-kDa synaptic vesicle protein implicated in exocytosis. Tyrosine phosphorylation maximized between 20-40 min of treatment. A time of accelerated dopamine release by MPP^+, which correlated with synaptophysin phosphorylation, was observed. The Ca^{2+} chelating agent EGTA abolished MPP^+-enhanced dopamine release. Exposing the cells to MPP^+-triggered to inhibition of Complex I activity and overproduction of reactive oxygen species within 60 min of treatment. This overproduction was blocked by mazindol, a dopamine uptake blocker. Additionally, pretreatment with selegiline, the selective monoamine oxidase B inhibitor, significantly suppressed MPP^+-mediated reactive oxygen species generation. Lastly, the results have shown that dopamine transporter is required for MPP^+ mediated cytotoxicity.

Taken together, these findings suggest that the reduction in cell surface dopamine transporter protein expression in response to MPP^+ may be a contributing factor in the down-regulation of dopamine transporter function, while enhancing dopamine release through the Ca^{2+}-dependent vesicular mechanism and the plasmalemmal dopamine transporter. These unique events initiated by MPP^+ may indicate a process of dopaminergic neuronal degeneration in Parkinson's disease.

KEY WORDS : 1-METHYL-4-PHENYLPYRIDINIUM / DOPAMINE TRANSPORTER / REACTIVE OXYGEN SPECIES / PARKINSON'S DISEASE

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กลไกของสารเมทิลฟีนิลพิริดิเนียมในการควบคุมการทำงานของตัวขนส่งโดปามีนในการเกิดโรคพาร์คินสัน
(MECHANISMS OF 1-METHYL-4-PHENYLPYRIDINIUM REGULATE THE FUNCTIONAL ACTIVITY OF DOPAMINE TRANSPORTER: POTENTIAL INSIGHTS INTO PARKINSON'S DISEASE PATHOGENESIS)

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บทคัดย่อ

เมทิลฟีนิลพิริดิเนียม เป็นสารพิษที่ทำให้เกิดอาการคล้ายโรคพาร์คินสันได้ถูกนำมาศึกษาถึงกลไกที่มีผลต่อการทำงานของตัวขนส่งโดปามีนเพื่อที่จะเข้าใจการเป็นพิษต่อเซลล์ผลการศึกษาในเซลล์ hDAT-HEK293 พบว่า เมทิลฟีนิลพิริดิเนียมลดการทำงานของตัวขนส่งโดปามีนซึ่งแปรผันโดยตรงกับความเข้มข้นและเวลา นอกจากนี้ได้เกิดกระบวนการ phosphorylation ของตัวขนส่งโดปามีนร่วมด้วย เมทิลฟีนิลพิริดิเนียมที่ความเข้มข้น 1-1000 μM ทำให้ปริมาณของตัวขนส่งโดปามีนที่บริเวณผนังเซลล์ลดลงอย่างมีนัยสำคัญทางสถิติหลังจากใส่ลงไปในน้ำเพาะเลี้ยงเซลล์เป็นเวลา 3 ชั่วโมง โดยการศึกษานี้ได้วัดจำนวนตัวขนส่งโดปามีน (B_{max}) ในชิ้นส่วนของเซลล์ทั้งหมดหลังจากได้รับเมทิลฟีนิลพิริดิเนียมพบว่ามีค่าลดลง ในขณะที่ค่า K_d เพิ่มขึ้น ผลการศึกษาต่อมาโดยวิธี biotinylation ได้สนับสนุนว่า ปริมาณตัวขนส่งโดปามีนที่บริเวณผนังเซลล์และภายในเซลล์ลดลงหลังจากได้รับเมทิลฟีนิลพิริดิเนียม ในขณะที่การศึกษาในเซลล์ PC12 พบว่าเมทิลฟีนิลพิริดิเนียมทำให้เกิดการ phosphorylation อย่างรวดเร็วของโปรตีนหลายชนิดที่ตำแหน่ง tyrosine โดยเฉพาะ synaptophysin ซึ่งเป็น synaptic vesicle โปรตีนที่มีขนาด 38 kDa และมีบทบาทในกระบวนการหลั่งของสารแบบ exocytosis โดยจะสังเกตได้ชัดเจนในช่วง 20-40 นาที และในช่วงเวลาดังกล่าวสอดคล้องกับการหลั่งของสารโดปามีนที่เพิ่มขึ้นจากการได้รับ เมทิลฟีนิลพิริดิเนียม เมื่อใส่สาร EGTA ซึ่งมีฤทธิ์ในการจับ Ca^{2+} ร่วมด้วยพบว่าการหลั่งของโดปามีนที่เกิดจากผลของเมทิลฟีนิลพิริดิเนียมลดลง ในการทดลองต่อมาพบว่าหลังจากได้รับเมทิลฟีนิลพิริดิเนียม 60 นาที เซลล์จะมีการสร้างสารอนุมูลอิสระเพิ่มขึ้นและการทำงานของเอนไซม์ Complex I ลดลง mazindol ซึ่งเป็นสารปิดกั้นตัวขนส่งโดปามีน และ selegiline ซึ่งเป็นสารที่ยับยั้งการทำงานของเอนไซม์ monoamine oxidase B จะช่วยลดผลของเมทิลฟีนิลพิริดิเนียมต่อการสร้างสารอนุมูลอิสระ ทั้งนี้ยังพบว่า เมทิลฟีนิลพิริดิเนียมจำเป็นต้องอาศัยตัวขนส่งโดปามีนเพื่อมีผลทำให้เซลล์ตาย ผลการศึกษาทั้งหมดสรุปได้ว่าเมทิลฟีนิลพิริดิเนียมมีผลลดการทำงานของตัวขนส่งโดปามีนโดยเกิดจากการลดลงของปริมาณตัวขนส่งโดปามีน และยังเพิ่มการหลั่งของโดปามีนโดยการใช้ Ca^{2+} และผ่านทางตัวขนส่งโดปามีน ซึ่งเหตุการณ์ดังกล่าวมีผลต่อกระบวนการเสื่อมสลายของเซลล์ในโรค พาร์คินสัน

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LIST OF ABBREVIATIONS

% control	percentage of control
^0C	degree centigrade
6-OHDA	6-hydroxydopamine
B_{max}	transporter density
CaM-K II	calmodulin-dependant protein kinase II
CNS	central nervous system
COMT	catechol-*O*-methyl transferase
DA	dopamine
DA-RT	dopamine reverse transport
DAT	dopamine transporter
DBH	dopamine β-hydroxylase
$DCFH_2$-DA	dichlorofluorescein-diacetate
DMEM	Dulbecco's modified Eagle's medium
DOPA	dihydroxyphenylalanine
DOPAC	dihydroxyphenylacetic acid
GABA	gamma aminobutyric acid
h	hour
HEK-293	human embryonic kidney-293
K_d	dissociation equilibrium constant
kDa	kilodalton
LBs	Lewy bodies
M	molar
MAO-B	monoamine oxidase B
min	minute
ml	millilter
mol	mole
$MPDP^+$	1-methyl-4-phenyl-2,3-dihydropyridinium

LIST OF ABBREVIATIONS (CONT.)

$MPFP^+$	1-methyl-4-phenyl-2,3, dihydropyridinium
MPP^+	1-methyl-4-phenyl pyridium
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide reagent
ng	nanogram
NM	neuromelanin
NSF	*N*-ethylmaleimide-sensitive factor
PARP	poly(ADP-ribose)polymerase
PBS	phosphate buffer saline
PC12	rat pheochromocytoma
PD	Parkinson's disease
PET	positron emission tomography
PKC	protein kinase C
ROS	reactive oxygen species
SNpc	substantia nigra par compacta
SPECT	single photon emission computerized tomography
TH	tyrosine hydroxylase
μCi	microcuries
μl	microliter
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area

CHAPTER 1
INTRODUCTION

Methyl-4-phenylpyridium (MPP^+), the active metabolite of the neurotoxin 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP), mediates selective damage to dopaminergic neurons and has been widely used to generate a model of Parkinson's disease. However, the mechanisms of the neurotoxic action of MPP^+ are not fully understood.

It is generally accepted that MPP^+ is one of the most potent dopamine release agents. However, the mechanism of MPP^+-induced dopamine release from presynaptic nerve terminals has not been fully characterized. When MPP^+ enters into the cells, it causes the release of dopamine from secretory vesicles and subsequently generate of free radicals (Speciale et al., 1998; Chang et al., 1986; Obata et al., 1992; Lotharius et al., 2000).

The molecular events initiated by MPP^+ which lead to reactive oxygen species (ROS)-induced cell death have been widely investigated. Recent reports suggest that the production of ROS influence many aspects of cellular function. These include altered signal transduction and second messenger systems (Ebadi et al., 2001; 2003), modulated expression of transcription factors (Cassarino et al., 2000; Eberhardt et al., 2003), inhibition of receptor-coupled phosphoinositide activity (Santini et al., 1993; Park et al., 2001), and activation of protein tyrosine phosphorylation (Monteiro et al., 1996; Li et al., 1998; Di Stasi et al., 1999; Lu et al., 2002), which could lead to both necrotic and apoptotic cell death. Moreover, modulation of protein tyrosine phosphorylation is known to occur during synaptic transmission and neurotransmitter exocytotic release (Pang et al., 1988; Schweizer et al., 1995; Liu et al., 1997). Several synaptic vesicle proteins are phosphorylated during the steps of mobilization, fusion, recycling and exocytotic release of neurotransmitter (Pang et al., 1988; Schweizer et al., 1995). Synaptophysin is the presynaptic vesicular protein that plays an important role in the secretion of neurotransmitters by interaction with synaptobrevin to form a complex, which is necessary for vesicular exocytosis (Becher et al., 1999; Shibaguchi

et al., 2000). MPP^+ is transported into cells via the dopamine transporters (DAT) where it mediates cellular toxicity (Tipton and Singer, 1993, Gainetdinov et al., 1997; Javitch et al., 1985). DAT is a presynaptic plasma membrane protein responsible for the regulation of extracellular dopamine levels and termination of its action by mediating the reuptake of dopamine (Amara and Kuhar, 1993; Reith et al., 1993). Functional impairment of DAT alters many physiological and behavioural processes that are mediated by dopamine. A dysfunction of dopamine transmission could consequentially interrupt motor neural circuits, which control movement as seen in Parkinson's disease.

Recently it has been shown that cellular mRNAs encoding DAT and vesicular monoamine transporters are decreased in Parkinson's disease (Uhl et al., 1994; Harrington et al., 1996). Indeed, an alteration of dopaminergic neurotransmission by the modulation of DAT activity could have an important implication in the cellular events, which lead to PD, and could be a target for a potential therapeutic intervention.

When MPP^+ enters into the cells, it causes the release of dopamine from secretory vesicles and subsequently generate of free radicals (Speciale et al., 1998; Chang and Ramirez, 1986; Obata and Chiueh, 1992; Lotharius and O'Malley, 2000). Inside the cell, MPP^+ disrupts cellular respiration by inhibiting mitochondria complex I system (Przedborski and Jackson-Lewis, 1998; Ramsay et al., 1986), reducing the level of ATP and hence contributing to degeneration of dopaminergic neurons.

Several studies have shown that MPP^+ causes a significant decrease in the activity of DAT (Barc et al., 2001; Fonck and Baudry 2001, 2003; Storch et al., 1999). However, it remained unclear whether the decrease in DAT activity resulted from the selective uptake of MPP^+ through DAT, reduced dopaminergic neurons mediated by the toxin, or changes in the trafficking of the transporter molecules. In the present study, we have hypothesized that a reduction in cell surface expression of DAT may be an underlying mechanism for the down regulation of DAT function by MPP^+ and subsequent neurotoxicity. To test this hypothesis, we selected the human embryonic kidney-293 cells, which stably express human DAT in order to monitor specially the effects of MPP^+ on DAT function. The data indicate that MPP^+ significantly reduced cell viability, dopamine uptake, and cell surface expression of

DAT, thus correlating directly the neurotoxic effects of MPP^+ with alterations in the kinetic parameters of dopamine transporters.

The collective expertise of the studies will allow successful completion of these aims and thorough testing of these hypotheses, providing crucial data regarding the mechanism of MPP^+ induce cellular toxicity, furthering our understanding of their role in the pathogenesis of PD. Furthermore, the tools and hypothesises in this study will be invaluable to researchers studying other disorders in which monoaminergic function is perturbed, such as cocaine and amphetamine abuse, schizophrenia and Tourette syndrome.

CHAPTER 2
OBJECTIVES

Parkinson's disease (PD) is characterized by a preferential loss of nigrostriatal dopaminergic neurons, and the resultant depletion of dopamine innervation to the striatum is believed to be responsible for the hallmark symptoms of PD. The overall hypothesis is that MPP^+ disrupts dopamine transport system in the cell, and that may induce the early cell functional changes, and thus correspond to cell degeneration. The goal of this proposal is begin to identify tile role of l-methyl-4-phenylpyridine (MPP^+)-mediated toxicity in dopaminergic cells as a model of neurodegeneration in PD.

It has been proposed that the varying susceptibility observed among different dopaminergic cell groups in idiopathic PD and in MPTP-induced Parkinsonism be governed by the relative concentrations of DAT. Here, the study capitalizes on in vitro models using cell lines and transfected human DAT cell line to further understand how MPP^+ participate in cell physiological function, as well as disease susceptibility. The designed experiments will test the following hypotheses:

1. To investigate the mechanisms of MPP^+-induced down regulation of DAT function and subsequent neurotoxicity.
2. To assess DAT phosphorylation, cell surface expression, and subcellular localization in the response to MPP^+, in order to monitor specially the effects of MPP^+ thus correlating directly the DAT function.
3. To clarify the mechanisms of MPP^+-medicated dopamine release. In particular, to examine the effects of MPP^+ on protein tyrosine phosphorylation and ROS generation.

CHAPTER 3
LITERATURE REVIEW

Overall view of Parkinson's disease

Parkinson's disease (PD) is a progressive disease with a mean age at onset of 55, and the incidence increases markedly with age, from 20/100,000 overall to 120/100,000 at age 70. In about 95% of PD cases, there is no apparent genetic linkage (referred to as "sporadic" PD), but in the remaining cases, the disease is inherited. Over time, symptoms worsen, and prior to the introduction of levodopa, the mortality rate among PD patients was three times that of the normal age-matched subjects. While levodopa has dramatically improved the quality of life for PD patients, population-based surveys suggest that these patients continue to display decreased longevity compared to the general population (Hely et al., 1989; Morgante et al., 2000; Levy et al., 2002). Furthermore, most PD patients suffer considerable motor disability after 5–10 years of disease, even when expertly treated with available symptomatic medications.

Clinically, any disease that includes striatal dopamine deficiency or direct striatal damage may lead to "parkinsonism," a syndrome characterized by tremor at rest, rigidity, slowness or absence of voluntary movement, postural instability, and freezing. PD is the most common cause of Parkinsonism, accounting for ~80% of cases. PD tremor occurs at rest but decreases with voluntary movement, so typically does not impair activities of daily living. Rigidity refers to the increased resistance (stiffness) to passive movement of a patient's limbs. Bradykinesia (slowness of movement), hypokinesia (reduction in movement amplitude), and akinesia (absence of normal unconscious movements, such as arm swing in walking) manifest as a variety of symptoms, including paucity of normal facial expression (hypomimia), decreased voice volume (hypophonia), drooling (failure to swallow without thinking about it), decreased size (micrographia) and speed of handwriting, and decreased stride length during walking. Bradykinesia may significantly impair the quality of life

because it takes much longer to perform everyday tasks such as dressing or eating. PD patients also typically develop a stooped posture and may lose normal postural reflexes, leading to falls and, sometimes, confinement to a wheelchair. Freezing, the inability to begin a voluntary movement such as walking (i.e., patients remain "stuck" to the ground as they attempt to begin moving) is a common symptom of Parkinsonism. Abnormalities of affect and cognition also occur frequently; patients may become passive or withdrawn, with lack of initiative; they may sit quietly unless encouraged to participate in activities. Responses to questions are delayed, and cognitive processes are slowed ("bradyphrenia"). Depression is common, and dementia is significantly more frequent in PD, especially in older patients.

Neurochemical and neuropathological features of Parkinson's disease

The pathological hallmarks of PD are the loss of the nigrostriatal dopaminergic neurons and the presence of intraneuronal proteinacious cytoplasmic inclusions, termed "Lewy Bodies" (LBs) (Figure 3.1). The cell bodies of nigrostriatal neurons are in the SNpc, and they project primarily to the putamen. The loss of these neurons, which normally contain conspicuous amounts of neuromelanin (NM) (Marsden, 1983), produces the classic gross neuropathological finding of SNpc depigmentation (Figure 3.1B). The pattern of SNpc cell loss appears to parallel the level of expression of DAT mRNA (Uhl et al., 1994) and is consistent with the finding that depletion of DA is most pronounced in the dorsolateral putamen (Bernheimer et al., 1973), the main site of projection for these neurons. At the onset of symptoms, dopamine is depleted ~80%, and ~60% of the substantia nigra par compacta (SNpc) dopaminergic neurons have already been lost. The mesolimbic dopaminergic neurons, the cell bodies of which reside adjacent to SNpc in the ventral tegmental area (VTA), are much less affected in PD (Uhl et al., 1985). Consequently, there is significantly less depletion of DA in the caudate (Price et al., 1978), the main site of projection for these neurons. Neuropathological studies of PD-related neurodegeneration suggest possible clues to the pathogenesis of the disease. First, PD-associated loss of dopaminergic neurons has a characteristic topology, distinct from the pattern seen in normal aging. In PD, cell loss is concentrated in ventrolateral and

caudal portions of the SNpc, whereas during normal aging the dorsomedial aspect of SNpc is affected (Fearnley and Lees, 1991). Thus, even though age is an important risk factor for PD, the processes that produce age-related dopaminergic neuronal death are probably different from those in PD. Second, the degree of terminal loss in the striatum appears to be more pronounced than the magnitude of SNpc dopaminergic neuron loss (Bernheimer et al., 1973), suggesting that striatal dopaminergic nerve terminals are the primary target of the degenerative process and that neuronal death in PD may result from a "dying back" process. Experimental support for the concept of dying back includes the observations that in MPTP-treated monkeys the destruction of striatal terminals precedes that of SNpc cell bodies (Herkenham et al., 1991), and in MPTP-treated mice, protection of striatal terminals prevents the loss of SNpc dopaminergic neurons (Wu et al., 2003). Third, the mechanism of synaptic dopamine clearance in the striatum seems to be more dependent on DAT than in the prefrontal cortex, where other monoaminergic transporters and the synaptic enzyme catechol-O-methyltransferase play a greater role in terminating the actions of dopamine (Giros et al., 1996; Gogos et al., 1998; Mundorf et al., 2001). The prefrontal cortex is a primary site of projection for ventral VTA dopaminergic neurons, so this difference may be of importance in understanding the relative resistance of VTA neurons to PD-related degeneration. Differences in neuronal milieu have also been identified surrounding SNpc dopaminergic cell bodies. The neuropil of the substantia nigra, composed of axon projections from the striatum and globus pallidus, stains strongly for calbindin D28K, and most dopaminergic cell bodies reside within this calbindin-rich neuropil (Damier et al., 1999a). However, the susceptible neurons in PD tend to be in calbindin-poor areas of the substantia nigra (Damier et al., 1999b). Although it is commonly thought that the neuropathology of PD is characterized solely by dopaminergic neuron loss, the neurodegeneration extends well beyond dopaminergic neurons (Hornykiewicz and Kish, 1987). Neurodegeneration and LB formation are found in noradrenergic (locus coeruleus), serotonergic (raphe), and cholinergic (nucleus basalis of Meynert, dorsal motor nucleus of vagus) systems, as well as in the cerebral cortex (especially cingulate and entorhinal cortices), olfactory bulb, and autonomic nervous system. Degeneration of hippocampal structures and cholinergic cortical inputs contribute to

the high rate of dementia that accompanies PD, particularly in older patients. However, the clinical correlates of lesions to the serotonergic and noradrenergic pathways are not as clearly characterized, as are lesions in the dopaminergic systems. Thus, while involvement of these neurochemical systems is generally thought to occur in more severe or late-stage disease, the temporal relationship of damage to specific neurochemical systems is not well established. For example, some patients develop depression months or years prior to the onset of PD motor symptoms, which could be due to early involvement of nondopaminergic pathways.

In life, the diagnosis of PD is made on clinical grounds, but definite diagnosis requires the identification of both LB and SNpc dopaminergic neuron loss. LBs are not specific for PD, however, and are also found in Alzheimer's disease, in a condition called "dementia with LB disease," and as an incidental pathologic finding in people of advanced age at a greater frequency than the prevalence of PD (Gibb and Lees, 1988). In Parkinson's disease, the nigrostriatal pathway degenerates. There is a marked loss of dopaminergic neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (solid line). The photograph demonstrates depigmentation (i.e., loss of dark-brown pigment neuromelanin; arrows) of the SNpc due to the marked loss of dopaminergic neurons (Figure 3.1).

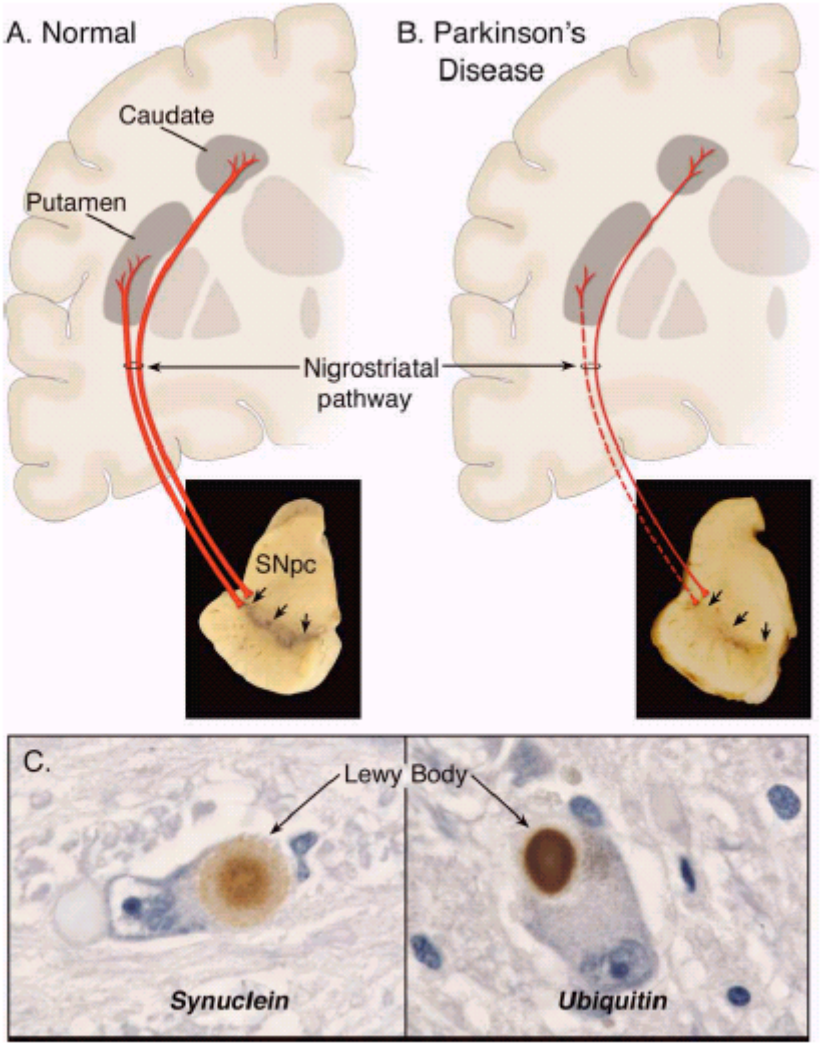

Figure 3.1 Neuropathology of Parkinson's disease (Dauer and Przedborski, 2003).

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Aetiology of Parkinson's disease

The cause of sporadic PD is unknown, with uncertainty about the role of environmental toxins and genetic factors. The environmental toxin hypothesis was dominant for much of the 20th century, especially because of the example of postencephalitic PD and the discovery of MPTP-induced Parkinsonism. However, the discovery of PD genes has renewed interest in hereditary susceptibility factors. Both probably play a role. The environmental hypothesis posits that PD-related neurodegeneration results from exposure to a dopaminergic neurotoxin. Theoretically, the progressive neurodegeneration of PD could be produced by chronic neurotoxin exposure or by limited exposure initiating a self-perpetuating cascade of deleterious events. The finding that people intoxicated with MPTP develop a syndrome nearly identical to PD (Langston et al., 1983) is a prototypic example of how an exogenous toxin can mimic the clinical and pathological features of PD. Paraquat is structurally similar to MPP^+, the active metabolite of MPTP, and has been used as herbicide. Like MPP^+, rotenone is also a mitochondrial poison present in the environment, and it is used as an insecticide and to kill unwanted lake fish. Human epidemiological studies have implicated residence in a rural environment and related exposure to herbicides and pesticides with an elevated risk of PD (Tanner, 1992). Yet, there are no convincing data to implicate any specific toxin as a cause of sporadic PD, and chronic environmental exposure to MPP^+ or rotenone is unlikely to cause PD. MPP^+'s quaternary ammonium cation prevents its passage across the blood-brain barrier, and rotenone is so unstable in solution that it only lasts a few days in lakes (Hisata, 2002). Still, cigarette smoking and coffee drinking are inversely associated with the risk for development of PD (Hernan et al., 2002), reinforcing the concept that some environmental factors do modify PD susceptibility. Another possibility, which does not fit neatly into a genetic or environmental category, is that an endogenous toxin may be responsible for PD neurodegeneration. Distortions of normal metabolism might create toxic substances because of environmental exposures or inherited differences in metabolic pathways. One source of endogenous toxins may be the normal metabolism of dopamine, which generates harmful ROS (Cohen, 1984). Consistent with the endogenous toxin hypothesis is the report that patients harbouring specific polymorphism in the gene encoding the xenobiotic detoxifying enzyme

cytochrome P450 may be at greater risk of developing young-onset PD (Sandy et al., 1996). Further, isoquinoline derivatives toxic to dopaminergic neurons have been recovered from PD brains (Nagatsu, 1997).

Pathogenesis of Parkinson's disease

Whatever insult initially provokes neurodegeneration, studies of toxic PD models and the functions of genes implicated in inherited forms of PD suggest two major hypotheses regarding the pathogenesis of the disease. One hypothesis posits that misfolding and aggregation of proteins are instrumental in the death of SNpc dopaminergic neurons, while the other proposes that the culprit is mitochondrial dysfunction and the consequent oxidative stress, including toxic oxidized dopamine species. The pathogenic factors cited above are not mutually exclusive, and one of the key aims of current PD research is to elucidate the sequence in which they act and whether points of interaction between these pathways are keys to the demise of SNpc dopaminergic neurons. Potential points of interaction are diagrammed in Figure 3.2. Another uncertain issue is whether the multiple cell death-related molecular pathways activated during PD neurodegeneration ultimately engage common downstream machinery, such as apoptosis, or remain highly divergent. Clearly, this issue is of great consequence in deciding about possible therapeutic strategies for PD.

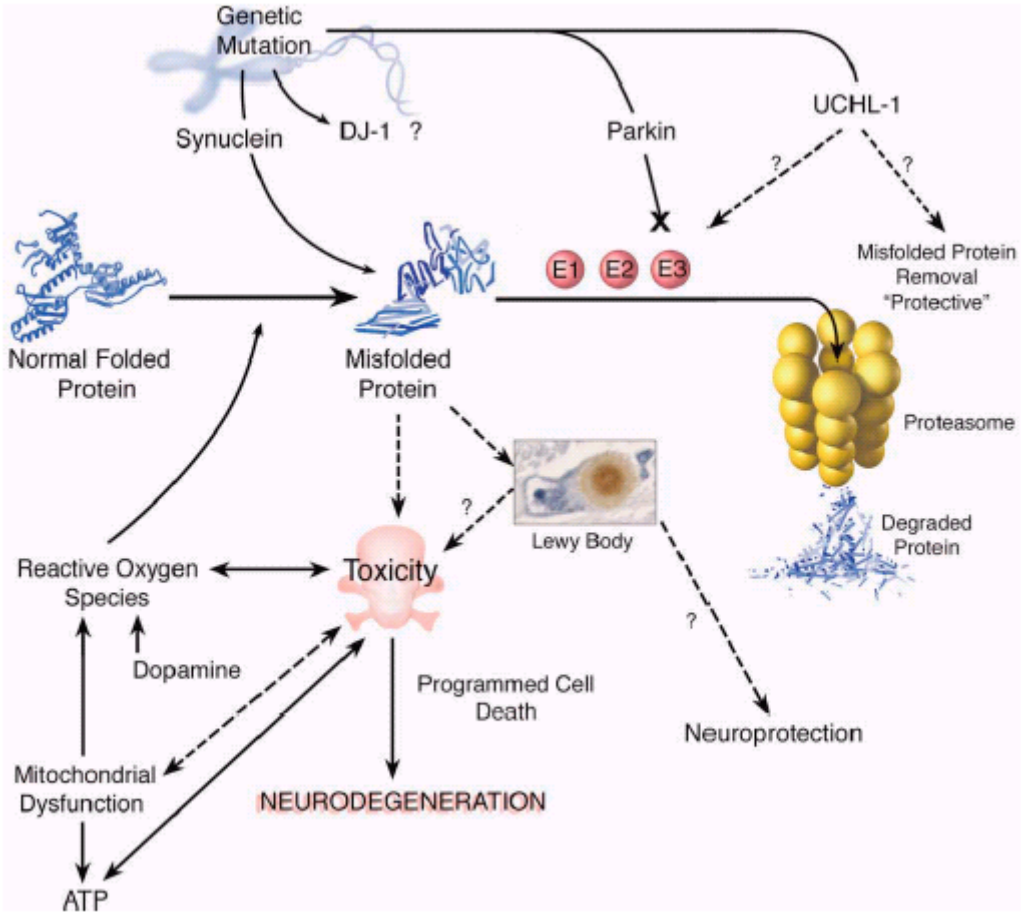


Figure 3.2 Pathogenesis hypotheses of Parkinson's disease (Giasson et al., 2000).

Mechanism of cell death in Parkinson's disease

Current dogma states that cell death processes can be divided into apoptotic pathways, which are "programmed" forms of cell death that terminate in destruction of DNA chains, and "necrotic" death, which essentially means anything else that kills cells. While these pathways were elucidated to analyse mitotically active cells in cancer and other systems, the pull to apply them to neurodegeneration has been irresistible, in part because it would seem that specific steps in the pathways might be inhibited as a clinical therapy. Much effort has been spent to observe or claim an apoptotic mode of SN death in PD, although the rate of cell death, in which tens of thousands of neurons likely die over a period of decades, would be unobservable by DNA labelling or other approaches which would show ongoing processes that last only for hours. These comments notwithstanding, there is evidence that apoptotic pathways could occur, as PD patients may express up-regulation of proteins expressed in apoptotic pathways such as p53, CD95, and caspases (Offen et al., 2000). It may be that apoptosis in PD is so far downstream from the ultimate neurotoxic insult that the cell would die in a necrotic pathway if denied the postmitotic equivalent of an apoptotic demise. In contrast to PD itself, there is good evidence for apoptotic pathways in experimental PD models. MPTP activates poly(ADP-ribose) polymerase (PARP) and Bax, enzymes linked to apoptosis, and induces DNA modifications downstream from peroxynitrite synthesis, activation of caspase-3, and release of cytochrome C from mitochondria, while up-regulation of anti-apoptotic proteins blocks MPTP-mediated cell death (Przedborksi et al., 2001).

Substantia nigra neuronal death in comparison to the ventral tegmental area

A remarkable feature of PD is that while SN DA neurons, particularly in the ventral tier, undergo selective death, the neighbouring DA neurones of VTA are generally unaffected. The reason for this selective pattern of dopaminergic neuronal death remains unclear. In addition to not exhibiting NM, and thus apparently under less long-term oxidative stress from cytosolic DA, suggestions have been centred on differential expression of a variety of proteins. The calcium binding protein, calbindin,

is more highly expressed in the VTA (Iacopino et al., 1992). VMAT2 expression may also be somewhat higher (Peter et al., 1995). Rats, which are resistant to MPTP toxicity, appear to package DA in synaptic vesicles more efficiently than do mice (Staal et al., 2000), which are susceptible to the toxin. SN neurons appear to have a preferential expression of GIRK2 (Liss et al., 1999), a potassium channel that is mutated in the *weaver* mouse, which exhibits specific SN and cerebellar degeneration. The growth factor GDNF plays a greater neuronal rescue role for VTA than SN DA neurons, although this has only been observed for early postnatal neurons (Burke et al., 1998). Whatever the reason for the difference in susceptibility of these neighbouring dopaminergic populations, it is likely an important clue for the pathway of PD pathogenesis.

MPTP: A toxin-based model of Parkinson's disease

Among the neurotoxins used to induce dopaminergic neurodegeneration, 6-hydroxydopamine (6-OHDA), MPTP, and more recently paraquate and rotenone have received the most attention. Presumably, all of these toxins provoke the formation of ROS. Rotenone and MPTP are similar in their ability to potently inhibit complex I, though they display significant differences, including, importantly, their ease of use in animals. Only MPTP is clearly linked to a form of human Parkinsonism, and it is thus the most widely studied model. In 1982, young drug users developed a rapidly progressive Parkinsonian syndrome traced to intravenous use of a street preparation of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), an analogue of the narcotic meperidine (Demerol) (Langston et al., 1983). MPTP was the responsible neurotoxic contaminant, inadvertently produced during the illicit synthesis of MPPP in a basement laboratory. In humans and monkeys, MPTP produces an irreversible and severe Parkinsonian syndrome characterized by all of the features of PD, including tremor, rigidity, slowness of movement, postural instability, and freezing. In MPTP-intoxicated humans and nonhuman primates, the beneficial response to levodopa and development of long-term motor complications to medical therapy are virtually identical to that seen in PD patients. Also similar to PD, the susceptibility to MPTP increases with age in both monkeys and mice (Rose et al., 1993; Irwin et al., 1993; Ovadia et al., 1995). The data regarding the comparison between PD- and MPTP-

related neuropathology derive largely from MPTP studies in monkeys (Forno et al., 1993), because only four human MPTP cases have come to autopsy (Davis et al., 1979; Langston et al., 1999). These studies show that, as in PD, monkeys treated with low-dose MPTP exhibit preferential degeneration of putamen versus caudate dopaminergic nerve terminals (Moratalla et al., 1992). Similarly, MPTP damages the dopaminergic pathways in a pattern similar to that seen in PD, including relatively greater cell loss in the SNpc than the VTA and a preferential loss of neurons in the ventral and lateral segments of the SNpc (Sirinathsinghji et al., 1992; Varastet et al., 1994); this regional pattern is also found in MPTP-treated mice (Seniuk et al., 1990; Muthane et al., 1994). Also reminiscent of PD (Hirsch et al., 1988), dopaminergic neurons that contain NM are more susceptible to MPTP-induced degeneration (Herrero et al., 1993). NM may contribute neurodegeneration in PD and MPTP-treated monkeys by catalysing ROS formation through an interaction with iron selectively in pigmented neurons (Zecca et al., 2001). A variety of organic molecules interact with NM, including pesticides, MPTP, and MPP^+ (D'Amato et al., 1986), so it may contribute to toxicity of pigmented neurons by acting as a depot for toxic compounds. The monkey MPTP model does not include two characteristic features of PD. First, neurons are not consistently lost from other monaminergic nuclei, such as the locus coeruleus, a typical feature of PD (Forno et al., 1986, 1993). Second, although intraneuronal inclusions resembling LBs have been described (Forno et al., 1986), classical LBs have not been demonstrated convincingly in the brains of MPTP-intoxicated patients or monkeys (Forno et al., 1993). These cases were exposed to acute regimens of MPTP, so the lack of LB-like formation in MPTP-intoxicated humans and monkeys may reflect the fact that in these cases dopaminergic neurons were rapidly injured. Chronic infusion of rotenone does produce intraneuronal α-synuclein-containing proteinacious aggregates (Betarbet et al., 2000), consistent with the possibility that the speed of intoxication may influence the subsequent neuropathologic features. Despite these neuropathologic shortcomings, the MPTP model is the gold standard for the assessment of novel strategies and agents for the treatment of PD symptoms. For example, electrophysiological studies of MPTP monkeys revealed that hyperactivity of the subthalamic nucleus is a key factor in the genesis of PD motor dysfunction (Bergman et al., 1990). This seminal discovery led

to the targeting of this structure using chronic high-frequency stimulation procedures (also called deep brain stimulation) to effectively ameliorate the motor function of PD patients whose symptoms cannot be further improved with medical therapy (Limousin et al., 1998).

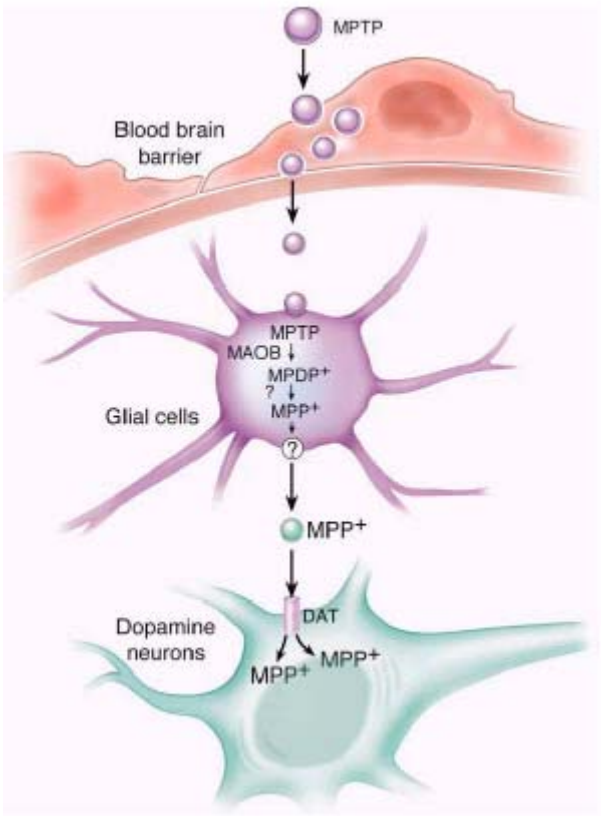


Figure 3.3 Schematic representation of MPTP metabolism after systemic administration (Dauer and Przedborski, 2003).

MPTP metabolism and Parkinson's disease neurodegeneration selectivity

Since the initial discovery of MPTP-induced Parkinsonism, much has been learned about the molecular pathway used by this toxin, as illustrated in Figure 3.3. Importantly, this knowledge enables investigators to use MPTP as a biological probe to explore the functions of PD genes and dissect the molecular events that occur during neurodegeneration of dopaminergic neurons. For example, mice mutant for PD genes (or other genes of possible relevance to dopaminergic neuronal death) can be injected with MPTP, and if these mice display markedly enhanced or suppressed dopaminergic neuronal death, one can then investigate which of the known molecular targets of MPTP are altered. After systemic administration, MPTP, which is highly lipophilic, crosses the blood-brain barrier within minutes (Markey et al., 1984). Once in the brain, the pro-toxin MPTP is oxidized to 1-methyl-4-phenyl-2, 3-dihydropyridinium ($MPDP^+$) by monoamine oxidase B (MAO-B) in glia cells and serotonergic neurons, the only cells that contain this enzyme. It is then converted to MPP^+ (probably by spontaneous oxidation), the active toxic molecule, and released by an unknown mechanism into the extracellular space.

MPTP (IV) is a by-product sometimes formed in the illicit synthesis of MPPP (III), a narcotic that differs by a single methyl group from alphaprodine (1,3-dimethyl-4-phenyl-4-propionoxypiperidine; Nisentil). Compound I (3,6-dimethyl-6-phenyltetrahydro-1, 3-oxazine), which can be produced from easily available ingredients, is treated with sulfuric or hydrochloric acid to produce II (1-methyl-4-phenyl-4- propionoxypiperidine). Compound II, when treated with propionic anhydride and sulfuric acid is converted to III (1-methyl-4-phenyl-4-propionoxypiperidine; MPPP). Under excess acidity, in the presence of "wet" propionic anhydride, or in the presence of dehydrating reagents, II can be converted to IV, (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine; MPTP). Compound I can also be directly converted to MPTP in excess acid. Following entry into cells, monoamine oxidase (particularly MAO B, which is more highly expressed in astrocytes than DA neurons) in mitochondria converts MPTP (via conversion to 1-methyl-4-phenyl-2, 3, dihydropyridinium; $MPFP^+$, not shown) to V, MPP^+ (1-methyl-4-phenyl-1, 2,5,6-

tetrahydropyridinium), which is accumulated by catecholamine uptake transporters selectively into DA and other catecholamine neurons (Figure 3.4).

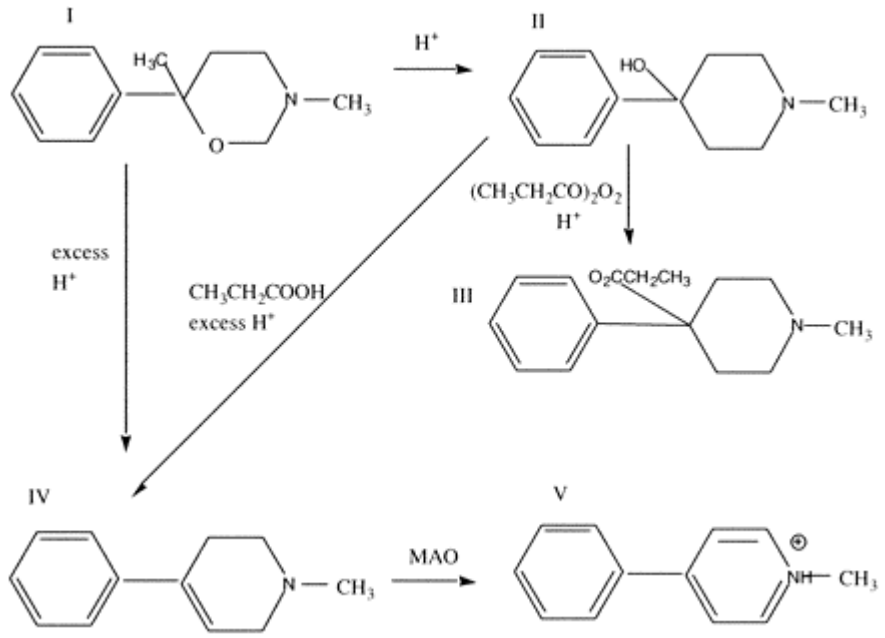


Figure 3.4 The different steps in MPP^+ synthesis (Fahn and Sulzer, 2004).

Since MPP^+ is a polar molecule, it depends on the plasma membrane carriers to enter cells. MPP^+ is a high-affinity substrate for the DAT, as well as for norepinephrine and serotonin transporters (Javitch et al., 1985; Mayer et al., 1986). Pharmacological inhibition or genetic deletion of DAT prevents MPTP-induced dopaminergic damage (Javitch et al., 1985; Bezard et al., 1999), demonstrating the obligatory character of this step in MPTP neurotoxicity. However, uptake by DAT does not entirely explain the selectivity of the nigrostriatal dopaminergic lesion caused by MPTP. While there are quantitative differences in DAT expression between more susceptible SNpc neurons and less susceptible VTA neurons in monkeys (Haber et al., 1995), differences in dopamine uptake activity of comparable magnitudes between rats and mice and among mouse strains do not correlate with differences in MPTP sensitivity (Giovanni et al., 1991, 1994). Furthermore, while MPP^+ is concentrated in (Speciale et al., 1998) and produces biochemical alterations in all monoaminergic neurons (Burns et al., 1983; Hallman et al., 1984; Wallace et al., 1984; Rose et al., 1993; Ovadia et al., 1995), degeneration is most prominent in dopaminergic neurons. In this regard, it is particularly striking that the highest levels of MPP^+ are found in the adrenal medulla without causing the loss of chromaffin cells (Reinhard et al., 1987).

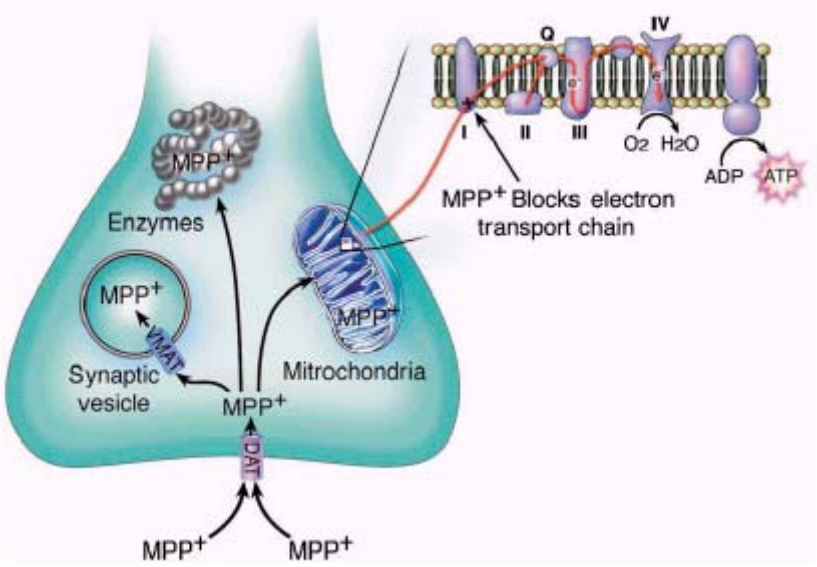


Figure 3.5 schematic representations of MPP^+ intracellular pathways inside dopaminergic neurons (Dauer and Przedborski, 2003).

Inside neurones (Figure 3.5), MPP^+ can follow at least three routes: (1) it can bind to the vesicular monoamine transporter-2 (VMAT2), which translocates MPP^+ into synaptosomal vesicles (Liu et al., 1992); (2) it can be concentrated within the mitochondria by a mechanism that relies on the mitochondrial transmembrane potential (Ramsay and Singer, 1986); and (3) it can remain in the cytosol to interact with cytosolic enzymes, especially those carrying negative charges (Klaidman et al., 1993). Vesicular sequestration of MPP^+ appears to protect cells from MPTP-induced neurodegeneration by sequestering the toxin and preventing it from accessing mitochondria, its likely site of action (see below). The importance of vesicular sequestration has been established by a number of experiments, including those showing that cells transfected to express greater density of VMAT2 are converted from MPP^+-sensitive to MPP^+-resistant cells (Liu et al., 1992) and that heterozygous VMAT2 null mice display enhanced sensitivity to MPTP-induced neurodegeneration (Takahashi et al., 1997). It appears that the ratio of DAT to VMAT2 expression predicts the likelihood of neuronal degeneration both in PD and the MPTP model. For instance, the putamen dopaminergic terminals, which are most severely affected by both MPTP and PD, have a higher DAT/VMAT2 ratio than those in the caudate, which are less affected (Miller et al., 1999).

Proposed mechanism of MPTP neurotoxicity

Once inside the mitochondria, MPP^+ impairs oxidative phosphorylation by inhibiting the multienzyme complex I of the mitochondrial electron transport chain (Nicklas et al., 1985). This blockade rapidly leads to decreases in tissue ATP content, particularly in the striatum and ventral midbrain (Chan et al., 1991; Fabre et al., 1999), the brain regions the most sensitive to MPTP. In vitro experiments in mitochondria isolated from whole brain demonstrate that complex I activity must be inhibited by ~70% to significantly impair ATP production (Davey and Clark, 1996), but data from PD post-mortem tissues demonstrate only a ~40% inhibition of complex I activity (Schapira et al., 1990). Interestingly, in vitro experiments with synaptic-derived mitochondria demonstrate that significant ATP depletion results from as little as ~25% inhibition of complex I (Davey et al., 1998), indicating a much tighter functional relationship between complex I activity and ATP production in synaptic

than in somatic mitochondria. Thus, mitochondria from phenotypically distinct neuronal populations may be differentially affected in PD, and the current approach of assessing mitochondrial function in specimens from whole tissue may not depict accurately abnormalities present in only a minority of cells. Furthermore, even the small alterations in complex I activity observed in PD may be particularly harmful to dopaminergic nerve terminals, which are rich in synaptic mitochondria.

Another early effectors of complex I inhibition due to MPP^+ may be oxidative stress. Indeed, by hampering the flow of electrons through complex I, MPP^+ can stimulate the production of ROS, especially superoxide (Hasegawa et al., 1990, 1997). MPP^+ effects on mitochondria can also indirectly stimulate the production of ROS by triggering dopamine leakage from synaptic vesicles to the cytosol, likely due to the inability of VMAT2 to maintain concentration gradients in the face of the ATP depletion (Johnson, 1988). Findings from in vivo studies provide support for the importance of ROS in MPTP-induced neurodegeneration. Mice transgenic for superoxide dismutase-1 (SOD1), a key ROS scavenging enzyme, are resistant to MPTP-induced dopaminergic neuron degeneration (Przedborski et al., 1992), and other studies in mice imply a key role for reactive species, including NO, as critical effectors in MPTP toxicity (Przedborski and Vila, 2003; Przedborski et al., 2003). Alterations in energy metabolism and generation of ROS peak within hours of MPTP administration, days before overt neuronal death have occurred (Jackson-Lewis et al., 1995). Therefore, these initial events are not likely to directly kill most cells but rather set into play downstream cellular events that ultimately kill most dopaminergic neurones (Mandir et al., 1999; Saporito et al., 2000; Vila et al., 2001).

One site susceptible for ATP loss that may be relatively particular to SN neurons is the ouabain-sensitive plasma membrane sodium/potassium exchanger, as these currents, which are responsible for maintaining normal resting potential, are exceptionally large in midbrain DA neurons (Seutin et al., 1996); the ATP-dependent transporter is said to normally use 40% of the body's ATP, but the relative level may be even higher in the SN. Another possible site sensitive to ATP loss is the ATP-driven proton pump on synaptic vesicles; inhibition of this pump leads to redistribution of DA to the cytosol in a manner very similar to methamphetamine (Pothos et al., 2002). There are of course many other potential sites, including others

within the mitochondria, such as aconitase, which is involved in iron and citric acid handling. Blockade of oxidative phosphorylation at any step would necessarily add to overall oxidative stress, as electrons would not be passed normally between the different substrates of this pathway. Perhaps most importantly, DA itself can block complex 1 activity (Przedborski et al., 1993), although it may be that the depletion of ATP is more directly due to monoamine oxidase conversion of DA to its metabolite DOPAC, which engenders production of hydrogen peroxide (Gluck et al., 2002). MPTP, via a combination of these effects, induces a range of oxidative reactions, including nitration reactions via peroxynitrite, which has been found to react with alpha-synuclein (Przedborski et al., 2001) and tyrosine hydroxylase (Blanchard-Fillion et al., 2001). While 6-OHDA model, which is selectively neurotoxic for DA neurons (Miller et al., 1981), suggests that the DA uptake transporter and oxyradical products derived from DA metabolism may initiate selective SN degeneration, the role for oxidized cytosolic DA initially came from work with methamphetamine toxicity (Cubells et al., 1994; Giovanni et al., 1995). This drug redistributes DA from synaptic vesicles, where the transmitter is held in a reduced state at nearly molar concentrations, to the cytosol, which generally manifests low micromolar concentrations and is a comparatively oxidizing environment (Sulzer et al., 1990). The resulting oxidized compound, DA-quinone (Hastings et al., 1999; Sulzer and Zecca, 2000; Graham et al., 1978), has been found to react with the DA uptake transporter (Whitehead et al., 2001), and as above, synuclein, and is likely involved in reactions with many other sites. It has been suggested that MPP^+ may similarly act to increase cytosolic DA, in addition to its effects on complex 1 (Lotharius and O'Malley et al., 2000). Native antioxidant supplements, including reduced glutathione, its precursor N-acetylcysteine, vitamin E, tetrahydrobiopterin (which is highly expressed in DA neurons as a cofactor for tyrosine hydroxylase), the enzyme superoxide dismutase, and the monoamine oxidase inhibitor selegiline (monoamine oxidase produces hydrogen peroxide) have all been neuroprotective in experimental PD models (Davidson et al., 2001), as are compounds such as S-methylthiocitrulline (Matthews et al., 1997), which inhibit nitric oxide synthesis and downstream peroxynitrite formation.

One obvious clue that even normal SN (and locus ceruleus) neurons undergo stress from reactive cytosolic DA (or norepinephrine) is the presence of NM, which is specifically expressed in these neurons. This pigment is composed of DA-quinone, DA-semiquinone, and the lipids and proteins (mostly via reaction with cysteine residues) to which these oxidizing agents have reacted (Zecca et al., 2002). NM avidly binds iron and a variety of other metals, which seems to explain the basis for high iron levels in the SN, and has been suggested to act as a pool for transition metals that could contribute to oxyradical formation by the Fenton reaction (Berg et al., 2001). A particular component of NM may be DA-glutathione, as glutathione may provide a first line of defense against cytosolic DA-quinone. NM is located within macroautophagic granules (Sulzer et al., 2000), which are normally organelles that are destined to translocate organelles and cytoplasm to lysosomes for degradation under conditions of cellular stress (Larsen and Sulzer, 2002). For both NM granules, and lipofuscin granules containing the aging pigment that is also present in NM granules, the fusion with lysosomes and breakdown is either slowed or halted so that the pigment builds up throughout human lifetime.

Another strong reason to suspect a role for cytosolic DA in PD is that neuroprotection is provided by expression of the synaptic vesicle catecholamine uptake transporter (vesicular monoamine transporter; VMAT2). Indeed, this transporter was originally identified in cell lines selected for resistance to MPP^+ toxicity (Liu et al., 2002). Overexpression of the transporter increases DA accumulation in synaptic vesicles (Pothos et al., 2000), and so may also reduce the level of DA in the cytosol, although this has not yet been directly demonstrated. DA neurons that underexpress VMAT2 are more susceptible to methamphetamine (Larsen et al., 2002; Fumagalli et al., 1999) and MPTP toxicity (Gainetdinov et al., 1998), whereas overexpression blocks the biosynthesis of NM (Sulzer et al., 2000).

Prolonged administration of low to moderate doses of MPTP to mice leads to morphologically defined apoptosis of SNpc dopaminergic neurons (Tatton and Kish, 1997). Under this regimen of MPTP intoxication, Bax, a potent PCD agonist and member of the Bcl-2 family, is unregulated in SNpc dopaminergic neurons (Vila et al., 2001). Bax upregulation coincides with its translocation to mitochondria, mitochondrial release of cytochrome instruct (an electron carrier and a mediator of

PCD), and activation of caspases 9 and 3 (Viswanath et al., 2001). At the same time, PCD antagonists such as Bcl-2 are downregulated in the SNpc (Vila et al., 2001). Consistent with these observations, Bax null and Bcl-2 transgenic mice are both resistant to MPTP neurotoxicity (Yang et al., 1998; Offen et al., 1998; Vila et al., 2001). How MPTP provokes these changes in Bcl-2 family members remains to be elucidated. MPTP causes oxidative damage to DNA (Mandir et al., 1999; Mandavilli et al., 2000), which may be important in inducing Bax via p53 activation. The tumour suppresser protein p53 is one of the few molecules known to regulate Bax expression and is activated by DNA damage. Furthermore, pharmacological inhibition of p53 attenuates MPTP-induced Bax upregulation and the subsequent SNpc dopaminergic neuron death (Duan et al., 2002), and p53 null mice are resistant to MPTP-induced neurodegeneration (Trimmer et al., 1996). Activation of the JNK pathway following DNA damage required in vitro for Bax mitochondrial translocation and the ensuing recruitment of the mitochondrial apo- pathoptotic pathway (Ghahremani et al., 2002; Lei et al., 2002).

Activation of the JNK pathway follows MPTP administration (Saporito et al., 2000; Xia et al., 2001), and pharmacological blockade of JNK (Saporito et al., 1999) or adenoviral directed expression of the JNK binding domain UCHL of JNK-interacting protein-1 (Xia et al., 2001) results in marked attenuation of MPTP-induced SNpc dopaminergic cell death. Approaches aimed at inhibiting PCD at a more downstream level, such as by interfering with activation of caspases, have yielded inconsistent results. Adenoviral gene transfer of X chromosome-linked inhibitor of apoptosis (XIAP), a protein caspase inhibitor, prevents MPTP-induced SNpc dopaminergic neuron death, and though it does not prevent the loss of striatal dopaminergic terminals (Eberhardt et al., 2000). In contrast, transmutations neuronal expression of the general caspase inhibitor protein baculoviral p35 specifically attenuates both MPTP-induced neuronal death and DA depletion (Viswanath et al., 2001). As with XIAP, some in vitro studies suggest that resistance to PCD can be induced selectively in the cell body. The broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoro methylketone and peptide inhibitors of caspases 2, 3, and 9 prevent the loss of dopaminergic cell bodies of cultured ventral midbrain neurons exposed to MPP^+, but the neurites are not spared (Bilsland et al.,

2002); the molecular pathways governing neuronal death may differ from those governing axonal destruction (Raff et al., 2002).

MPTP administration also leads to the accumulation and nitration of α-synuclein in the cytosol of SNpc dopaminergic neurons (Vila et al., 2000; Przedborski et al., 2001), and ablation of α-synuclein in mutant mice prevents MPTP-induced dopaminergic neurodegeneration (Dauer et al., 2002). While it is not clear whether α-synuclein plays any direct role in regulating PCD, the expression of mutant α-synuclein in cell cultures may promote apoptosis (Xu et al., 2002), and cytochrome c has been reported to stimulate in vitro aggregation of α-synuclein (Hashimoto et al., 1999). Collectively, these data demonstrate that the activation of PCD is instrumental in MPTP toxicity. They also suggest that PCD alterations in PD post-mortem samples are of pathological significance and that targeting specific PCD molecules may be a valuable neuroprotective strategy for the treatment of PD (Vila and Przedborski, 2003).

The classical view of the dopaminergic system

Dopamine, norepinephrine, and epinephrine belong to a class of neurotransmitters known as catecholamines, which are structurally defined by a catechol ring and an amine side chain. Catecholamines and indolamines (*i.e.*, serotonin) are referred to as monoamines. Monoamines are small, water-soluble molecules that are the decarboxylated derivatives of amino acids. Production from their respective amino acids is catalysed by several enzymes that act in sequence, the first of which serves as the rate-limiting step. Monoamines are stored at high concentrations in secretory granules. These granules provide protection against degradation by metabolic enzymes and enable a regulated release via exocytosis. Like other neurotransmitters, monoamines act very rapidly and their action can be terminated by both metabolic conversions to inactive compounds as well as by reuptake into the producing cell.

Dopamine is synthesized primarily in the central nervous system (CNS), but limited production also occurs in the adrenal medulla. Dopamine is also detectable in a few non-neuronal tissues, *e.g.*, the pancreas and the anterior pituitary. Dysfunction

of dopaminergic systems is associated with a number of diseases. For example, deficiency of dopamine in midbrain nigrostriatal neurons has long been recognized in the pathogenesis of Parkinson's disease, while overactivity of the limbic and cortical dopaminergic neurons has been implicated in schizophrenia and psychoses. These dopaminergic neurons are also affected by neurotoxins, psychostimulants, and drugs of abuse. It is not surprising, therefore, that this relatively simple molecule has been at the centre of interest of basic scientists and clinicians alike for many years.

Dopamine can have either an excitatory or inhibitory effect on the postsynaptic potential. In other words, when dopamine leaves the presynaptic neuron and goes into the synapse, it can then bind to receptors on the postsynaptic neuron. After dopamine is bound to the postsynaptic cell, it can either facilitate an action potential or inhibit it. Dopamine is present in most parts of CNS but in particular in the nigrostriatal pathway comprising the neurons of the substantia nigra (A9) and projecting to neurons of the neostriatum and the mesocorticolimbic pathway composed of neurons of the ventral tegmental area (A10) connecting with those of the limbic cortex and other limbic structures (Bjorklund et al., 1964).

The involvement of the dopaminergic nigrostriatal pathway in extrapyramidal dysfunctions was shown by the discovery that degeneration of this pathway occurs in the brains of patients afflicted with Parkinson's disease (Poirier LJ, Sourkes, 1965; Ehringer et al., 1960). The depletion of dopamine resulting from the degeneration of the nigrostriatal pathway led to the development of dopamine-replacement therapies, which are successful in alleviating Parkinson's disease (Birkmayer et al., 1962; Hornykiewicz, 1966). The hypothesis that dopamine is involved in the pathogenesis of psychosis, in particular schizophrenia, rests on the finding that most antipsychotic drugs are dopamine receptor antagonists and that agents which cause excessive release of dopamine mimic schizophrenia-like states (Carlsson. 1988; Creese et al., 1976; Selbie et al., 1989). The mesocorticolimbic pathway has been implicated as the principal dopaminergic pathway involved in the etiology of psychoses. These data explain the dilemma associated with dopamine-related drug therapies: The blockade of the dopaminergic system, desired for reducing psychoses, induces extrapyramidal dysfunctions and vice versa.

Dopamine synthesis and metabolism

Dopamine biosynthesis begins with the amino acid tyrosine (Figure 3.6). The majority of circulating tyrosine originates from dietary sources, but small amounts are derived from hydroxylation of phenylalanine by the liver enzyme phenylalanine hydroxylase (Nagatsu and Stjarne, 1998). Tyrosine enters neurons by an energy-dependent uptake process and is converted to dopamine by two enzymes that act in sequence, tyrosine hydroxylase (TH) and L-aromatic amino acid decarboxylase, also called dihydroxyphenylalanine (DOPA) decarboxylase (DDC). Neurons that contain active dopamine ß-hydroxylase (DBH) convert dopamine to norepinephrine, and those that also contain phenylethanolamine *N*-methyl transferase convert norepinephrine to epinephrine. The latter are classified as noradrenergic and adrenergic neurons, respectively, and their distribution in the brain differs considerably from that of the dopaminergic neurons. Regardless of the catecholamine being produced, TH is the rate-limiting step in their biosynthetic pathway. As shown in Figure 3.6, the processes of dopamine biosynthesis, release, and metabolism are; 1) Tyrosine is taken into the neuron by a sodium-dependent mechanism; 2) conversion of tyrosine to L-DOPA by TH is the rate-limiting step in the biosynthetic pathway; 3) L-DOPA is converted to dopamine by DDC; 4) dopamine is translocated into secretory vesicles for storage, protection, and secretion; 5) fusion of secretory vesicles with the plasma membrane results in dopamine release into the synaptic cleft or the extracellular space ((s is the case with the TIDA neurons); 6) dopamine binds to its membrane receptors and initiates multiple effects in target cells; 7) unbound dopamine is taken up by the DAT, located in the plasma membrane of the presynaptic neuron; 8) both newly synthesized dopamine and that taken up into the cell are translocated into secretory vesicles by the VMAT; 9) MAO, located in the outer mitochondrial membrane, converts dopamine to a deaminated metabolite; 10) COMT converts dopamine or its deaminated metabolite to biologically inactive products.

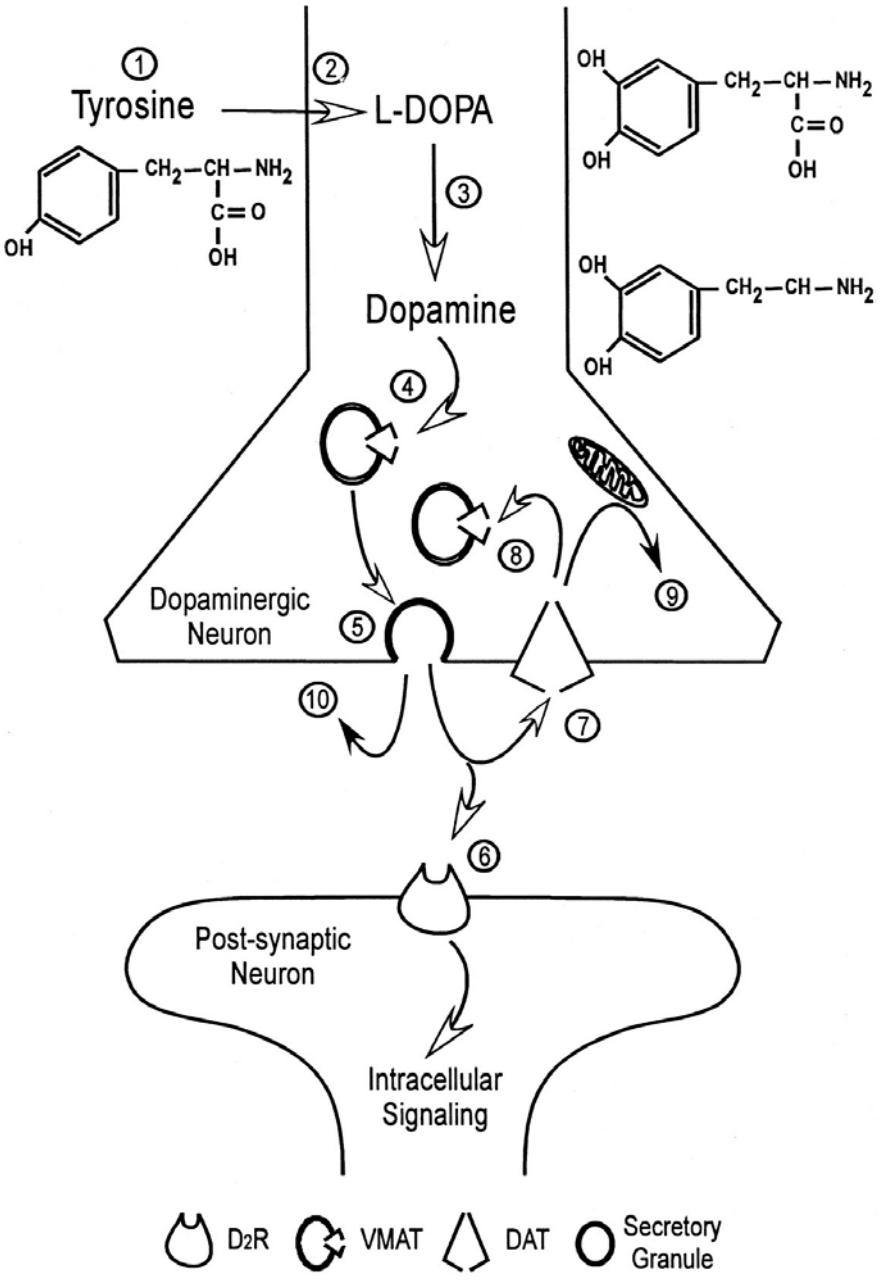


Figure 3.6 Diagram of dopamine biosynthesis, release, and metabolism (Ben-Jonathan N, Hnasko R, 2001).

Catabolism is one of the effective mechanisms for dopamine inactivation (Boulton and Eisenhofer, 1998). This involves multiple pathways that include oxidative deamination by monoamine oxidase (MAO), *O*-methylation by catechol-*O*-methyl transferase (COMT), and conjugation by sulfotransferases or glucoronidases. The preferred metabolic pathway at a given site depends on the compartmentalization of the metabolic enzymes. For example, MAO is located in the external membrane of the mitochondria and acts intracellular, whereas COMT is associated with the external cell membrane and acts only extracellularly.

MAO exists as two isoenzymes, A and B, with an apparent molecular mass of 60–63 kDa each. The two MAO genes, each comprised of 15 exons, are located on the X-chromosome and appear to have been derived from the same ancestral gene (Shih et al., 1999). They differ in substrate specificity as well as selectivity for inhibitors. MAO-A is more highly expressed in catecholaminergic neurons, whereas MAO-B is more abundant in serotonergic and histaminergic neurons and in glial cells (Luque et al., 1995). Enzyme inactivation in humans or its deletion in transgenic mice is compatible with life but result in neurochemical and behavioral abnormalities (Shih et al., 1999). Deamination of dopamine by MAO produces dihydroxyphenylacetic acid (DOPAC). Determination of the ratio of DOPAC/dopamine concentrations serves as a good method for estimating rapid changes in neuronal activity, with a major advantage being that it does not require drug pretreatment. *O*-Methylation by COMT is primarily responsible for inactivation of circulating catecholamines. Consecutive conversion of dopamine by MAO and COMT yields homovanillic acid.

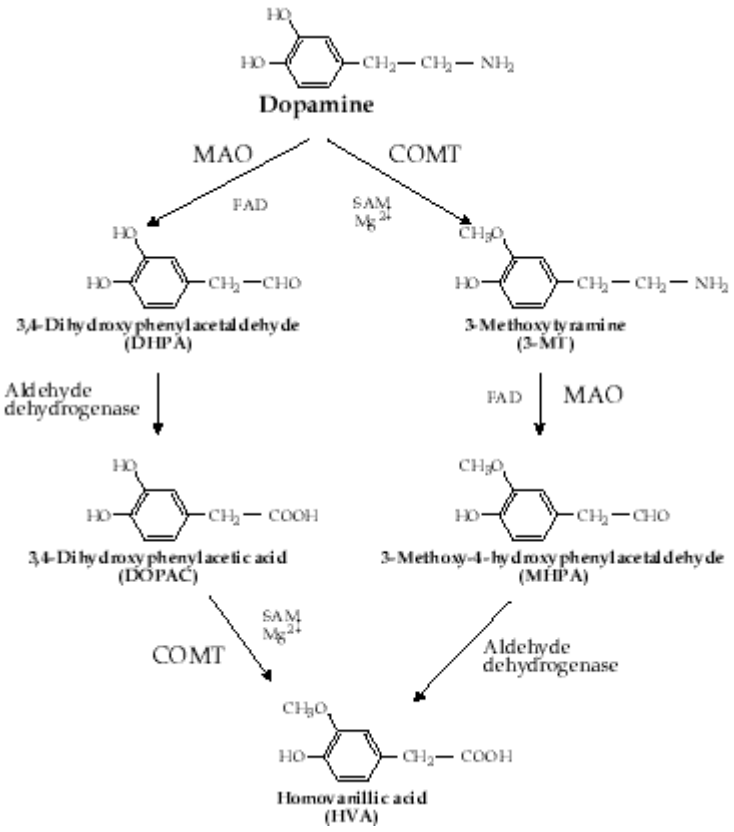


Figure 3.7 Catabolism of dopamine. Dopamine catabolism can proceed starting either with oxidative deamination by MAO (left pathway) or with O-methylation by COMT (right pathway) (Ben-Jonathan and Hnasko, 2001).

Storage and release of dopamine

Dopamine is stored in secretory vesicles at a 100- to 1000-fold higher concentration than neuropeptides. This is attributed to several distinct features of monoaminergic neurons. First, unlike neuropeptides whose synthesis occurs within the endoplasmic reticulum and Golgi apparatus, dopamine biosynthesis can take place within the terminals themselves. Second, synthesis that occurs in a close proximity to the site of release permits a much faster turnover rate than the slow axoplasmic transport that brings proteins from cell bodies to the nerve terminals. Third, a unique reuptake process replenishes most of the released dopamine back into the secretory vesicles and maintains high intragranular concentration, whereas a released neuropeptide cannot be restocked.

After synthesis, dopamine is stored in synaptic vesicles at extremely high concentrations, 0.5–0.6 M, which is near its limit of solubility. Dopamine is translocated from the cytoplasm into the vesicles by the vesicular monoamine transporter (VMAT), shown schematically in Figure 3.9, The function of the vesicles is 4-fold: 1) to protect dopamine from enzymatic degradation by MAO, 2) to minimize constitutive secretion by diffusion from the cells, 3) to facilitate regulated release, and 4) to enable rapid replenishment of depleted stores. The life cycle of the vesicles includes: 1) targeting to the active zone of the presynaptic membrane, 2) docking, 3) fusion, 4) release of the vesicular content, 5) retrieval by endocytosis, and 6) refilling with the neurotransmitter. Selected aspects of these events are discussed below. For a comprehensive coverage, please refer to several outstanding reviews (Betz and Angleson, 1998; Langley et al., 1997; De Potter et al., 1997; Brunger, 2000).

Monoamines are stored primarily in small translucent ("clear") vesicles (50–100 nm in diameter) but are also present in large dense core vesicles (up to 500 nm in diameter), often cosequestered with neuropeptides (Millhorn and Hokfelt, 1988). The relationship between large and small vesicles, their predominance in catecholaminergic *vs.* neuroendocrine cells, their membrane composition, and their precise role in quantal neurotransmitter release are not clear. Also, most information on synaptic vesicles has been obtained from chromaffin cells, which contain primarily norepinephrine and epinephrine and differ from dopaminergic neurons by the

presence of intravesicular DBH, chromogranins, and other constituents (Winkler, 1993). It remains to be determined whether the content of the vesicles and the process of exocytosis are identical in dopaminergic and noradrenergic neurons.

Storage vesicles are formed in the neuronal perikarya and are transported to the terminals by slow axoplasmic flow. Although early studies suggested formation of vesicles from the outer membrane of the terminals by pinocytosis, it was later realized that this represented retrieval by endocytosis of previously fused vesicles. In fact, to maintain adequate transmitter storage and permit a sustained response to stimuli, endocytosis must occur at a rate that parallels exocytosis. The synaptic vesicle is a highly specialized structure whose membrane is composed of a lipid bilayer with embedded integral proteins that participate in vesicular trafficking, docking, and fusion. The vesicle membrane also contains an H^+-ATPase, which maintains the proton gradient that energizes VMAT and preserves an acidic intravesicular environment. Each vesicle is filled with several thousand molecules of dopamine as well as other soluble constituents (Payne, 1989).

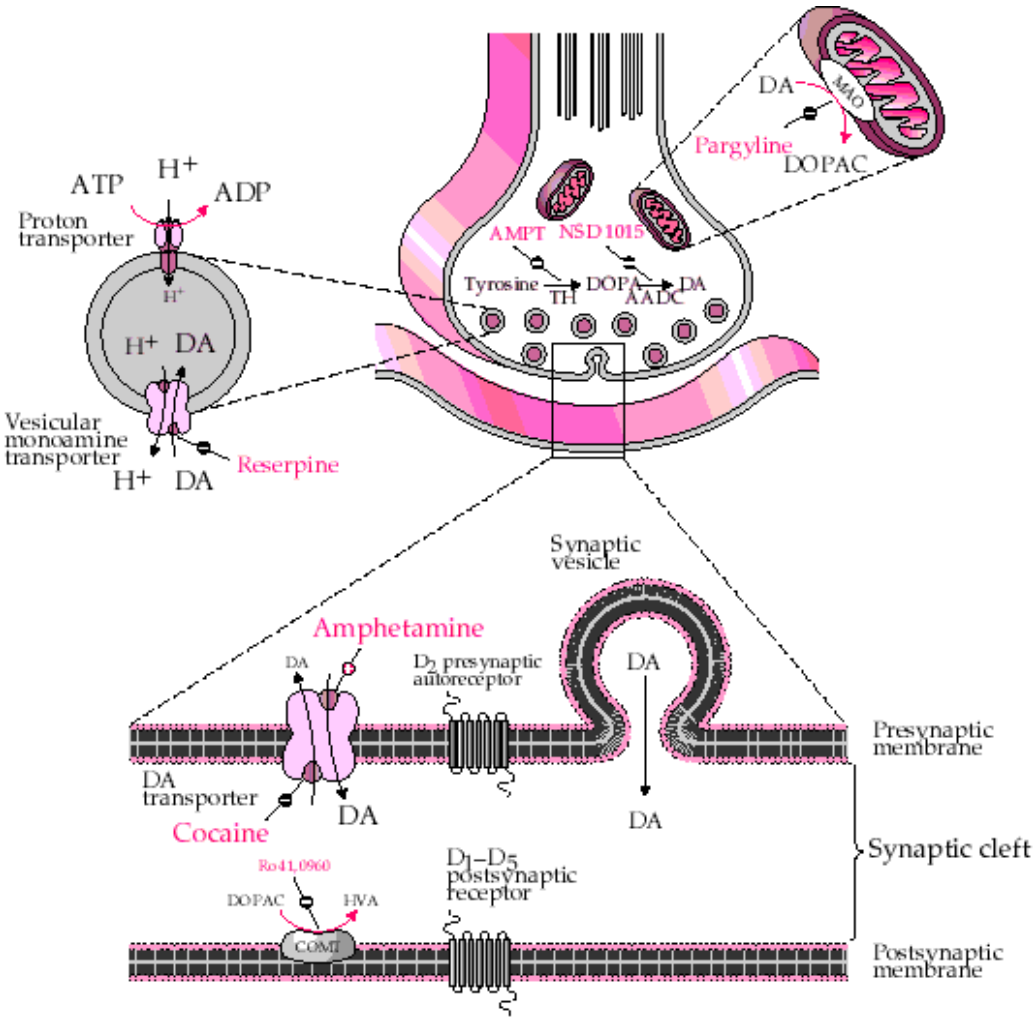


Figure 3.8 Illustrates the processes of dopamine synthesis and metabolism, presynaptic and vesicular uptake, and vesicular release. Pre- and postsynaptic DA receptors and sites of action of some dopaminergic drugs are also shown (Feldman et al., 1996).

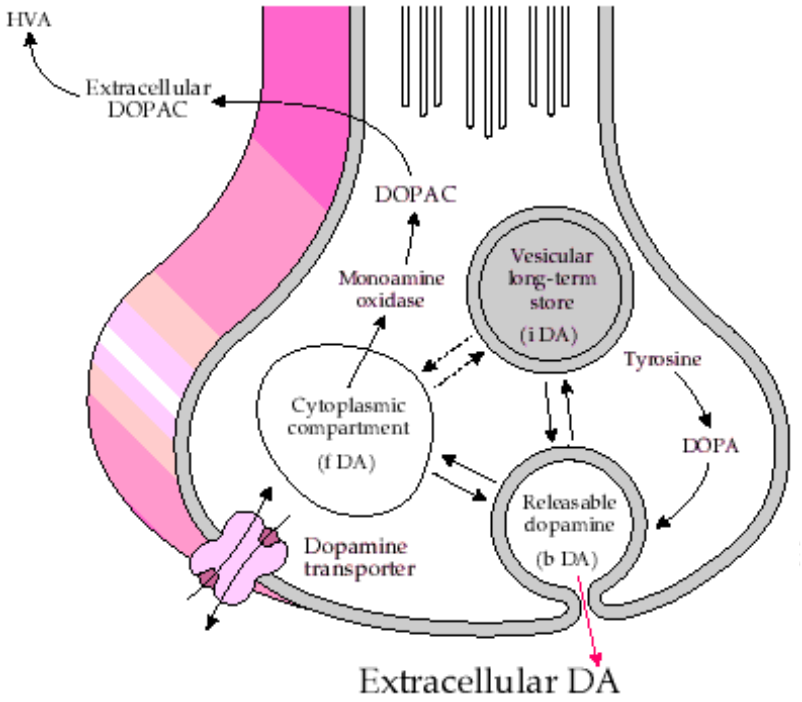


Figure 3.9 Dopamine pools within a dopaminergic nerve terminal. According to the model illustrated, DA synthesis feeds directly into a releasable bound (vesicular) pool (bDA), which is in equilibrium with a pool of free (cytoplasmic) DA (fDA) and an inactive bound pool (iDA). An alternative model involves the entry of newly synthesized DA into the cytoplasmic pool before it is taken up into vesicles (Arbuthnott et al., 1990).

Much information has been gathered in recent years on the docking mechanism (Betz and Angleson, 1998; Hilfiker et al., 1999; Marqueze et al., 2000; Klenchin and Martin, 2000). It involves a family of proteins termed αsoluble *N*-ethylmaleimide-sensitive factor (NSF) attachment proteins (αSNAP) receptors (SNARE) complexes: v-SNAREs, designating vesicular-associated proteins, and t-SNAREs, designating target (plasma membrane) cognate complexes. At least seven to eight proteins are essential for docking: vesicular synaptobrevin and synaptotagmin; SNAP-25 and syntaxin, which are located in both the vesicles and plasma membrane; and two soluble proteins, NSF and SNAP, which catalyze the disassembly of the SNAP-25-syntaxin-synaptobrevin complex during docking and fusion (Hodel, 1998). SNAP-25, in association with syntaxin, binds to and modulates voltage-gated calcium channels, thus bringing the vesicle into close proximity with a source of calcium.

The critical role of calcium in exocytosis, termed the "stimulus-secretion coupling" hypothesis, has been long recognized. Calcium is central to all aspects of exocytosis, including rapid fusion and unloading of the vesicles as well as recruitment and translocation of loaded vesicles. Resting levels of cytoplasmic calcium within the neuron are approximately 0.1 μM and can rise to 5–10 μM upon arrival of action potentials (Langley and Grant, 1997). Calcium influx occurs through voltage-gated calcium channels and leads to fusion of the synaptic vesicles with the plasma membrane and release of their content to the extracellular space. This is a much faster process than the relatively slow release of peptide or protein hormones from endocrine cells.

In most neurons, dopamine is released into the synaptic cleft and binds to postsynaptic receptors. It has been argued that the speed of neurotransmitter release is reciprocally related to the distance of its site of action, but the mechanism responsible for this feature is unclear (Langley and Grant, 1997).

Calcium influx in chromaffin cells induces an initial fast release, termed the "exocytotic burst," which occurs in milliseconds and is followed by a slower and sustained release phase that lasts several seconds (Klenchin and Martin, 2000). It is assumed that only a small fraction of docked vesicles can instantaneously release their cargo in response to calcium influx. These vesicles comprise the "fusion-ready" pool that undergoes a very rapid ATP-dependent fusion. The slower release phase is carried

out by docked vesicles that exist in a different biochemical state and require priming to promote fusion. These vesicles constitute a precursor pool that replenishes the rapid release pool. Priming is ATP dependent, involves the SNARE proteins, and is associated with production of phosphoinositides and protein phosphorylation. An even slower pool is composed of vesicles that are anchored to the cytoskeleton via actin-binding synapsins but are not docked to the membrane (Hilfiker et al., 1999). When synapsins become phosphorylated in response to an influx of calcium, the vesicles detach from the cytoskeletal elements and can translocate to the active zone of the presynaptic membrane. However, vesicular translocation is too slow to account for the immediate calcium-dependent exocytosis.

Two pathways have been proposed to explain formation of fusion pores that connect the vesicle lumen with the extracellular space. One is termed the "kiss-and-run pathway," in which a pore is formed to allow partial or full emptying of the vesicle content. The other is termed the "complete fusion pathway," in which the pore dilates and the vesicle membrane collapses into the plasma membrane (Betz and Angleson, 1998; Oberhauser and Fernandez, 1996). At least two membrane proteins, synaptophysin and synaptoporin, have been associated with pore formation. Questions that remain to be resolved include the three- dimensional structure of the putative pores, the precise mechanism of vesicular retrieval, and the dynamic forces that drive intracellular trafficking of the internalized vesicles.

Table 3.1 Proteins involved in regulation of exocytosis

Presynaptic proteins	Bind to	**Phosphorylated by**	Suggested function
Synaptophysins	Synaptobrevin	**CaMKII, tyrosine phosphorylation**	Modulatory role in exocytosis
Synapsins	Actin filaments, microtubules, SH3 domains, calmodulin, annexin VI	**CaMKI and II, PKA**	Vesicle availability
Synaptobrevins (VAMP)	SNAREs, SNAPs, synaptophysin	**CaMKII**	Essential for exocytosis
SNAP-25	SNAREs, SNAPs, Ca^{2+} channels, synaptobrevin	**PKC**	Essential for exocytosis
Munc 18s (nSec1)	Syntaxin	**PKC**	Inhibition of exocytosis
a-,b-,g-SNAPs	SNAREs, NSF,syntaxin (a-SNAP), synaptotagmin (b-SNAP)	**PKA, PKC**	NSF attachment proteins

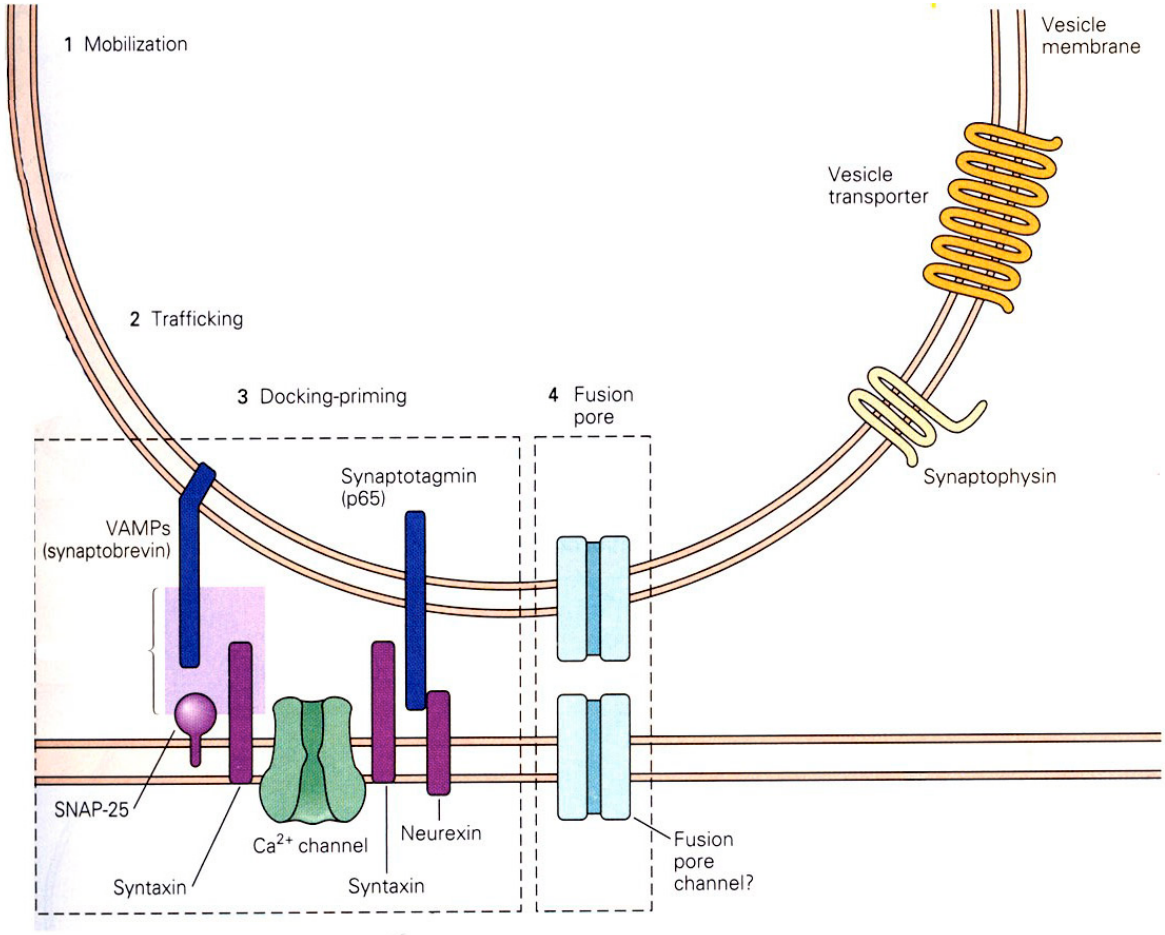


Figure 3.10 Diagram depicts characterized synaptic vesicle proteins and some of their postulated receptors and function (Kandel et al., 2000).

Dopamine transporters

Reuptake is the process by which the released transmitter is brought back into presynaptic nerve terminals or is internalized by surrounding glial cells. It is unique to monoamines and amino acid neurotransmitters and is the main mechanism by which the action of the released transmitter is rapidly terminated (Figure 3.11). As an added benefit for monoamines, reuptake permits recycling of the same molecules while saving in energy costs of their biosynthesis. In contrast, the action of the released neuropeptide is terminated either by diffusion or by proteolysis.

DAT is targeted by psychostimulants and neurotoxins such as cocaine, amphetamine, and MPP^+. By binding to the transporter and preventing dopamine reuptake, these drugs cause a prolonged increase in extracellular dopamine, resulting in augmentation of its effects. Because DAT is an excellent marker for functional extrahypothalamic dopaminergic neurons, *in vivo* imaging of cocaine analogs is used to evaluate the state of dopaminergic neurons in patients with Parkinson's disease and other neurological disorders (Laruelle, 2000). The generation of transgenic mice with DAT inactivation added significant information on the physiological role of this transporter. These mice are hyperactive, do not respond to cocaine or amphetamine, are resistant to the neurotoxic effects of MPP^+, and their dopamine receptor expression is down regulated (Giros et al., 1996).

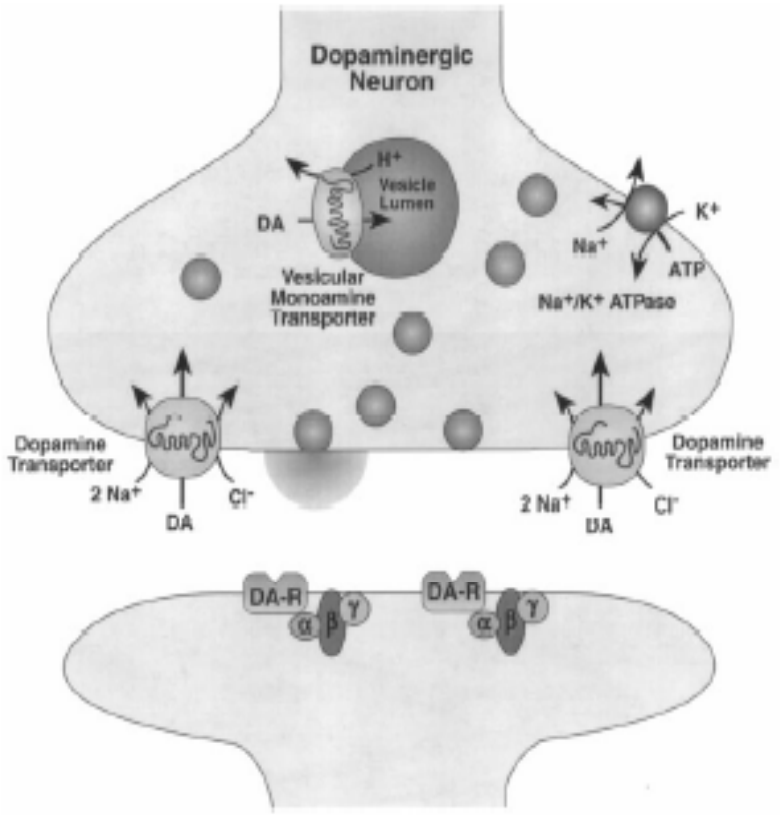


Figure 3.11 The mechanism of dopamine reuptake that mediated by two classes of transporters: dopamine transporter (DAT) and vesicular monoamine transporter (VMAT).

Reuptake of dopamine is mediated by two classes of transporters: dopamine transporter (DAT), which transports dopamine from the extracellular to the intracellular space, and VMAT, which reloads dopamine into the vesicles (Hoffman et al., 1998). The two transporters differ in structure, cellular localization, substrate specificity, antagonist selectivity, and energy requirements.

The search for membrane transporters began after observing rapid uptake of labelled catecholamines into brain slices and synaptosomes. This reuptake was Na^+ and Cl^- dependent and inhibited by cocaine and amphetamine (Blakely and Bauman, 2000; Amara and Kuhar, 1993; Uhl and Johnson, 1994). The importance of reuptake was underscored by dramatic physiological and behavioural effects of several drugs of abuse that interfere with this process in both humans and laboratory animals. These observations lead to the notion that the presynaptic membrane must contain distinct molecules that act as symporters, *i.e.*, they have the capacity for concentrating the transmitter by a concurrent movement of Na^+ down its electrochemical gradient (Uhl and Johnson, 1994). The process of uptake has an apparent stoichiometry of $2Na^+$:$1Cl^-$:1dopamine, suggesting an electrogenic process (Sonders et al., 1997).

Structure and function of dopamine transporter

DAT belongs to a subfamily that includes transporters for GABA, norepinephrine, serotonin, glycine, and proline and is distinguished by 12 TMDs and dependence on both Na^+ and Cl^-. Another subfamily is chloride independent, has 6–9 TMDs, and includes transporters for the excitatory amino acids glutamate and aspartate (Iversen, 2000). DAT encodes a 69-kDa protein of 620 residues with both the N and C termini located intracellularly (Bannon et al., 2000). The protein lacks a consensus signal sequence and has 3–4 potential N-linked glycosylation sites in the second large extracellular loop. The nature and extent of glycosylation are tissue specific and may be involved in transporter targeting, stability, or ligand binding. Residues within TMD 1–3 influence binding affinity for dopamine and cocaine, whereas those in TMD 11–12 affect the affinity for MPP^+ neurotoxin (Buck et al., 1996; Giros et al., 1994).

Other features shown include three potential glycosylation sites on the second extracellular loop (Y-shaped symbols), as well as sites for phosphorylation by protein

kinase A (PKA), protein kinase C (PKC), and Ca^{2+}-calmodulin-dependant protein kinase II (CaM-K II). The amino acid residues shown in red are those that differ in the rat DA transporter. These represent only 8% of the total amino acids, indicating a high degree of sequence homology between the human and rat proteins. The details of the mechanism of how these transporters move substrate from the outside of the membrane to the inside is unknown. The transport is known to be coupled to the sodium gradient. As is typical for all neuronal transporters, DAT has lower affinity and reduced ligand specificity than the dopamine receptor. Several potential sites can be phosphorylated by PKC and may determine the rate of uptake or serve as a signal for transporter internalisation (Vaughan et al., 1997).

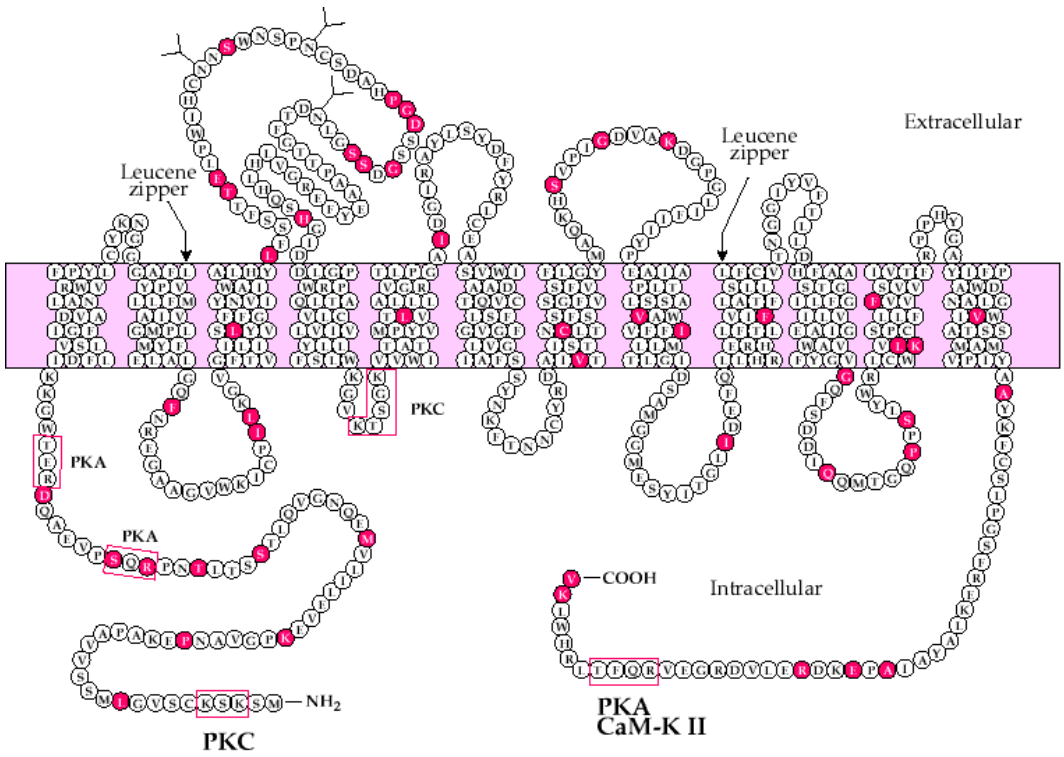


Figure 3.12 Structure of the human dopamine transporter (Feldman et al., 1996).

As revealed by combined *in situ* hybridization and immunocytochemistry, DAT has restricted localization within the brain and is not expressed outside the CNS (Hoffman et al., 1998). The transporter colocalizes with TH, and because it is limited to dopaminergic neurons, it serves as a unique marker for these neurons. The highest expression of DAT is in the substantia nigra, followed by the ventral tegmental area. DAT in these neurons is detected in perikarya, dendrites, and axonal processes. A significant presence of DAT is also seen in mesolimbic and mesocortical dopaminergic pathways (Ciliax et al., 1995), whereas the hypothalamic dopaminergic neurons exhibit moderate and restricted expression of DAT. Unexpectedly, electron microscopy reveals that the DAT protein is found primarily in the extrasynaptic area rather than in the active zone of the synapse (Nirenberg et al., 1996). This suggests that DAT may play a role in limiting diffusion of dopamine after being released. An unresolved issue is the mechanism by which dopamine (and other monoamines) is taken up by glia, because DAT is undetectable in these cells.

1. Uptake by the dopamine transporter: Ion dependence of transport

Neuronal uptake of dopamine and other biogenic amines is controlled by the transmembranal gradient of Na^+ and abolished in a medium in which Na^+ is iso-osmotically replaced with sucrose, choline or lithium (Harris and Baldessarini, 1973; Holz and Coyle, 1974). Substitution of Cl^- with any of several anions also causes a marked reduction of high-affinity uptake (Kuhar and Zarbin, 1978). A detailed evaluation of the effects of external ions on dopamine uptake shows the relationship between the initial rate of dopamine uptake and external Na^+ being sigmoidal whereas the rate of uptake versus Cl^- can be described by a rectangular hyperbola in rat striatal preparations (Krueger et al., 1990; McElvain and Schenk, 1992; Wheeler et al., 1993), and the cell transfected with DAT cDNA (Gu et al., 1994; Pifl et al., 1997; Earles and Schenk, 1999; Chen et al., 1999). A possible interpretation of these results is that DAT-mediated dopamine uptake requires the presence of two Na^+ ions and a single molecule of dopamine randomly binds first to the transporter followed by the binding of a single Cl^- ion before the movement of dopamine across the membrane (McElvain and Schenk, 1992; Povlock and Schenk, 1997). However, a fixed binding order of Na^+ binding before dopamine was observed in two independent studies on cells

transfected with the human DAT (Chen et al., 1999; Earles and Schenk, 1999). In the presence of either 36 mM or 136 mM Na^+ neuronal dopamine uptake is optimal with1-3 mM Mg^{2+}, K^+ or Ca^{2+}. An increase in K^+ concentrations from 0 to 10 mM, in an incubation medium containing a high Na^+ concentration, modifies the dopamine uptake according to a bell-shaped curve. Such an increase in uptake probably results from an activation of the K^+/Na^+ exchange through the K^+/Na^+ -ATPase (adenosine triphosphatase) and, consequently, in a more favorable transmembrane ionic gradient (Amejdki-Chab et al., 1992; Corera et al., 1996). The dependency of dopamine uptake on Na^+ is greatly affected by cations such as K^+ and $Tris^+$, present in many buffer systems (Zimany et al., 1989; Amejdke-Chab et al., 1992). Membrane depolarization by action of veratridine or elevated external K^+ reduces the rate of uptake (Holz and Coyle 1974; Krueger, 1990). Kinetic studies provide evidence for Na^+ and Cl^- being co-transported with dopamine into the cell and are consistent with a rheogenic, that is, a net ionic current carrying process (Krueger 1990; Amejdki-Chab et al., 1992).

Uptake blockers are strictly speaking drugs, which by interacting with the transporter lock it in a conformational state incapable of translocation in both directions. “Pure” uptake inhibitors such as cocaine, nomifensine and benztropine are devoid of releasing effect at uptake-inhibiting concentrations in contrast to amphetamine and its derivatives (Heikkila et al., 1975; Raiteri et al., 1978). Drugs selectivity blocking the DAT in the nanomolar range are diphenyl-substituted piperazine derivatives of the GBR series (van der Zee et al., 1980). However, this selectivity is not more than 20-fold on recombinant plasmalemmal monoamine transporters whereas drugs with a 100- to 1,000-fold selectivity for the noradrenaline or serotonin transporter are available (Buck and Amara, 1994; Eshleman et al., 1999). Competitive inhibition of uptake was reported for mazindol (Krueger, 1990; Meiergerd and Schenk, 1994), nomifensine, benztropine (Krueger, 1990; Jones et al., 1995) and amphetamine (Kruger, 1990; Chen et al., 1990) Different form of inhibition of dopamine uptake by cocaine where found competitive (Krueger, 1990; Jones et al., 1995; Chen et al., 1999; Earles and Schenk, 1999), non–competitive (Missale et al., 1985) and an uncompetitive type of inhibition (McElvain and Schenk, 1992). Allosteric interactions between cocaine and Na^+ on the DAT have been observed: its blocking action is enhanced by Na^+ (Wheeler et al., 1994; Chen et al., 1999), and

cocaine, just as mazindol, depends on a minimum of Na^+ for blockade of reverse transport (Pifl et al., 1997). Cocaine competitively inhibits the involvement of Na^+ in the uptake process in striatal preparations (McElvain and Schenk, 1992) but does not seem to have an effect at the Na^+ binding site of the human DAT (Chen et al., 1999; Earles and Schenk, 1999). On the other hand, raising Na^+ enhances the apparent affinity of substrates for the human DAT more than of inhibitors (Chen et al., 1999).

2. Reverse transport by the dopamine transporter

The mechanism by which dopamine-releasing agents involve RT follows several steps (Figure 3.13), identified during the last 20 years. First, the releasing molecules enter the terminal buttons, uptake or not by the carrier itself. Second, the concentration of neurotransmitters rises in the terminal cytosol. As discussed below, this rise results from multiple origins. Third, transmitter molecules are transported outside the cell most likely through an exchange-diffusion process. The exchange-diffusion processes have been extensively studied and various mathematical models were proposed over the course of the last three decades (Chubb et al., 1972)

The first step (accumulation)

Several dopamine-releasing substances have been shown to accumulate in the dopamine terminals. Various sympathomimetic amines (tyramine, amphetamine, parahydroxy-amphetamine, etc.) are substrates for uptake into neuron terminals and are able to competitively inhibit dopamine transport (Horn, 1973). It was also observed that when this occurs, transport of tyramine and amphetamine is Na^+-dependent (Bönisch, 1986) and that the potentials of amphetamine-like drugs to release cytoplasmic [^{3}H] dopamine is well correlated with the IC50 values of these agents in inhibiting dopamine uptake. A large set of substances can compete with dopamine for uptake, but only a few of them possess the releasing activity. Thus, benztropine, nomifensine, methyl phenidate seem to be uptake blockers only (Hunt et al., 1974; Bauman and Maitre, 1976; Hunt et al., 1979; Bonnet et al., 1984). A major problem in studying the transport and sequestration of dopamine-related substances is due to the double gate constituted by the plasma membrane and the vesicle membrane. The plasma membrane does not determine a pH gradient, while the

vesicular matrix does not exceed pH 5. Thus, internalization could involve two different mechanisms, one for cytosolic accumulation and the other for vesicular sequestration. Understanding of the mechanism by which these drugs enter the terminals remains incomplete, but at least part of them or even the whole, enter the cell terminals using the dopamine specific carrier, following the experimental conditions.

Second step (increasing cytosolic dopamine)

The origin of the amine released by the releasing agents is now better understood. Indeed, if the mechanism by which substance released dopamine involves DAT, which is located on the cytoplasmic membrane, dopamine efflux elicited by the releasing agents should derive from a presumably cytoplasmic compartment. Firstly, one must consider the nature of the terminal compartment involved in the action of the releasing agent. The cytoplasmic compartment is maintained by a continuous dopamine synthesis and replenished by the displacement from the vesicular stores constituting the endogenous dopamine. It is now well established that the main catabolic enzyme in dopamine metabolism, MAO, is located intraterminally, associated with mitochondrial crests and gives origin to DOPAC.

Until now, there was no evidence of an active mechanism for DOPAC efflux. Finally, the natural uptake of dopamine through DAT also participates in replenishing the cytosolic dopamine compartment. Endogenous dopamine, located mainly in a vesicular compartment, appears to be less sensitive to the substance than cytosolic dopamine. It can thus be concluded that dopamine is present in the terminal, separated in two different compartments (Figure 3.13), and that the releasing agent acts preferentially on the cytoplasmic pool (Arbuthnott et al., 1990). During continuous superfusion of the cat caudate nucleus with [^{3}H]tyrosine, the neosynthesized [^{3}H] dopamine was first released by amphetamine; the unlabeled stored amine being only secondarily released. As we will see below, the amount of cytosolic dopamine is likely very small and a central point of the drug effect is the process by which the substances are able to induce the intraterminal rise in cytoplasmic dopamine.

Dopamine release

Fischer and Cho (1979) proposed that releasing substances produced a chemical release of dopamine by an exchange-diffusion taking place at the binding site of the uptake carrier. They observed that this exchange is temperature-dependent, saturable, Na^+-dependent, stereoselective, and cocaine-sensitive. Tested amphetamine, octopamine and phenylethylamine as dopamine-releasing substances and observed the same blockade with another uptake inhibitor, nomifensine. Using in vivo or in vitro experiments as well, many studies conducted during the last 15 years have confirmed the role played by DAT in the releasing mechanism (Butcher et al., 1988). The finding by Giros et al. (1996) that mice lacking the dopamine carrier protein due to disruption of the transporter gene are insensitive to amphetamine, thus unable to increase extracellular dopamine, is the most recent and direct demonstration of the involvement of DAT in the amphetamine releasing effect. Thus, it may be said that a general agreement exists about the fact that amphetamine-like substances release dopamine, at least partly, by inducing dopamine reverse transport from a cytosolic DA compartment, even if the molecular mechanisms involved are not completely understood.

Role of calcium on dopamine reverse transport (DA-RT)

A particular mention must be given to the role of calcium. The DA-RT is clearly independent of Ca^{2+} fluxes, in contrast with the dopamine synthesis, clearly dependent on Ca^{2+} ions. A large number of studies have pointed out the role of Ca^{2+} in the TH activity in vitro. In a recent study realized in vivo, a set of arguments were raised, in line with a Ca^{2+} dependence of the synthesis with indirect consequences on the carrier mediated release. On one side, it was evidenced that increased Ca^{2+} influx (by the presence of a calcium ionophore) activates the TH enzyme activity, which can be visualized by increased neosynthesized dopamine in terminals. On the other side, it was proposed that this increase in the intraterminal DA pool could favor DA-RT rather than exocytosis. This led to the conclusion that DA-RT is well dependent on Ca^{2+} entry (even if indirectly) or of an intraterminal mobilization of the Ca^{2+} pools.

Since DA-RT uses the same translocating system as for uptake, virtually every factor-influencing uptake is also potentially a regulator of the DA-RT. The large

difference between extracellular and intracellular media, however, indicates that an increased uptake is not necessarily linked to an increased DA-RT. As we shall see, the two functions appear to be regulated in opposite directions.

Side by side with classical in vitro methods, in vivo regulation of dopamine uptake was often investigated by measuring the clearance of dopamine in the extracellular space. Some authors introduced a pulse of dopamine through an implanted micropipette and monitored the disappearance of this extrinsic substance. These voltametric methods allowed a detailed analysis of the consequences of alterations in dopamine uptake. However, they are sometimes hard to interpret, due to the multiple factors, which can interact with extracellular dopamine clearance including nonsynaptic uptake, glial uptake, tissue diffusion, etc. We have already mentioned the sensitivity of exchange-diffusion to temperature and Na^+ gradient. In addition, certain endogenous factors and hormones were also involved in the regulation of dopamine transport.

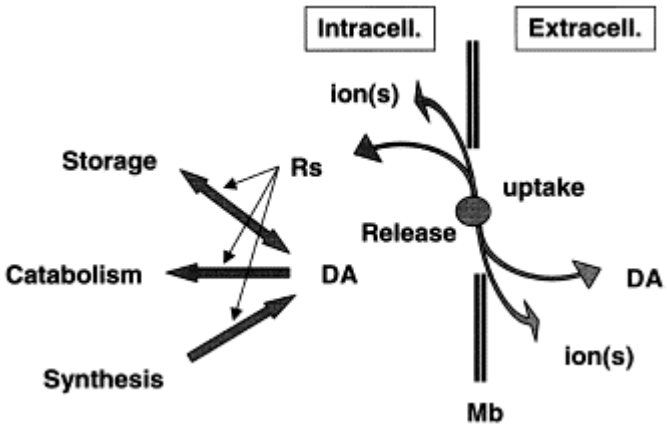


Figure 3.13 The reverse transports of dopamine (DA-RT), when evoked by a releasing substance (Rs), are based on three successive steps. First, the Rs enter the terminal by the DA-uptake carrier or not. When using the DA-carrier, ions are co-transported. Second, Rs induces a rise in cytosolic DA acting on the DA synthesis, the DA catabolism and/or the DA stores. Third, the cytosolic accumulated DA is co-transported with ions outside the terminal (Leviel, 2001).

Substrate of the dopamine transporter

Substrates are compounds, which by interacting with the DAT are translocated to the opposite side of the plasma membrane. The initial velocity of the transport can be described by the Michaelis-Menten equation. The natural substrate dopamine is translocated with an apparent K_m of 0.3-1.2 μM and turnover members of 0.3-1.5S^{-1} at 37 ^{0}C depending on conditions of equilibrium exchange or zero trans entry of dopamine into rat striatal preparations (Meiergard and Schenk, 1994). Turnover of the human DAT in stably transfected cells at 37 ^{0}C was estimated to be 14-18S^{-1} (Pifl et al., 1996; Earles and Schenk, 1999). The somehow higher K_m values (1-5 μM) found in the majority of studies on recombinant transporters may be due to differences in post-translational modifications. However, recently, cells heterologously expressing high-affinity uptake by the human DAT have been reported (Zhang et al., 1998; Pristupa et al., 1998). The DAT has considerably lower affinity for noradrenaline, K_m values 3-5 times higher than that for dopamine with V_{max} values quite similar for both catecholamines (Snyder and Coyle, 1969; Pifl et al., 1996). There is no stereoselectivity with regard to noradrenaline (Snyder and Coyle 1969; Meiergard and Schenk, 1994). The toxic metabolite MPP^+ of the Parkinson's-inducing agent MPTP is a good substrate of the DAT in rat striatal synaptosome (Chiba et al., 1985; Javitch et al., 1985). MPP^+ obeys a kinetic similar to that of noradrenaline on the human DAT (Pifl et al., 1996). Other drugs translocated by the DAT in a Na^+- and temperature-dependent and cocaine-blockable manner are amphetamine and tyramine (Petrali et al., 1979; Zaczek et al., 1991; Sittle et al., 1998). The V_{max} values are less than a fourth of that measured using dopamine as a substrate (Sitte et al., 1998); an intact catechol with a primary ethylamine side chain has been shown to be necessary for optimal uptake activity (Horn 1973; Meiergerd and Schenk, 1994). Thermodynamic analysis suggested substrate binding to the transporter to occur with a change in entropy (Bonnet et al., 1990). The cationic form of dopamine, perhaps including the zwitterions, is the most likely substrate of the transporter based on the pH-dependence of dopamine uptake and [^{3}H] cocaine-analogue displacement (Berfield et al., 1999).

Regulation of dopamine transporter

Changes in dopamine transport would be expected to impact on the temporal and spatial dynamics of dopamine neurotransmission by affecting dopamine inactivation. Alterations in dopamine transporter characteristics have been reported following chronic cocaine exposure in both animal models and in humans (Mash and Staley, 1997). The time since the last cocaine administration is an important factor in the end result. Thus, in vivo imaging in human cocaine addicts tends to show an initial increase in dopamine transporter density following acute cocaine binges followed by a normalization or decrease following longer withdrawal times (Mash and Staley, 1997). Human cocaine overdose victims who died following a syndrome of excited delirium did not show an elevation of high-affinity radioligand binding to the dopamine transporter, perhaps explaining the agitation and paranoia associated with elevated dopamine in the absence of unregulated inactivation through transport (Mash and Staley, 1997).

In other cases, observed changes in dopamine transporters are more likely due to changes in dopamine nerve terminal densities rather than regulation of transporter numbers per terminal. For instance, Parkinson's disease is an example where the observed reduction in dopamine transporters is due, at least in part, to the loss of nerve terminals carrying these transporters (Uhl and Kitayama, 1993). There is also evidence for acute regulation of dopamine transport. Activation of dopamine receptors has been shown to increase striatal dopamine transport by an elevation in the V max (Meiergerd et al., 1993). Thus, increased synaptic dopamine levels may enhance clearance of dopamine in a feedback manner. Other evidence focuses on the role of second-messenger systems. The cloned human and rat dopamine transporters contain consensus sites for phosphorylation by cAMP-dependent protein kinase and by protein kinase C in the third intracellular loop and in the N- and C-terminal domains (Giros and Caron, 1993). Indeed, cAMP has been shown to enhance uptake of dopamine into rat hypothalamic tuberoinfundibular dopamine neurons (Kadowaki et al., 1990), and activation of protein kinase C by phorbol esters decreases the dopamine transporting capability of the cloned rat (Kitayama et al., 1994) or human (Zhang et al., 1997) dopamine transporter. Recent information links phosphorylation of the dopamine transporter directly to uptake capability (Huff et al., 1997), although

it is possible that phosphorylation at steps distal from the transporter is involved as well. Such complexities may play a role in the observed effects of increasing or decreasing phosphorylation on dopamine release through reversed transport that led to the conclusion that enhanced phosphorylation enhances rather than reduces dopamine transporter function (Giambalvo, 1992; Bugnon et al., 1995).

In addition to the action of the classic second messenger diacylglycerol on protein kinase C, other membrane phospholipids take part in activating protein kinase C such as arachidonic acid originating in the phospholipase A-induced phosphatidylcholine hydrolysis pathway (Asaoka et. al., 1992). Thus, different pathways can converge upon protein kinase C to regulate dopamine transmission through changes in dopamine transporter function. In addition to the arachidonic acid-induced amplification of diacylglycerol stimulated protein kinase C, the same signals that activate protein kinase C frequently cause the release of arachidonic acid through phospholipase A activation (Nishizuka, 1995). Recent work addressed the possibility that the effect of protein kinase C activation on dopamine transporter function was mediated by the release of arachidonic acid (Zhang and Reith, 1996). Indeed, as observed with protein kinase C activation, preincubation for 45–60 min with exogenously added arachidonic acid caused a reduction in human dopamine transporter measures, characterized by a decrease in both the V_{max} of [^{3}H]dopamine uptake and the B_{max} of [^{3}H]WIN 35428 binding (Zhang and Reith, 1996). Enhancement of endogenous arachidonic acid by activating phospholipase A or interfering with lipooxygenase, which breaks down arachidonic acid, also reduced transporter function. Because staurosporine, an inhibitor of protein kinase C, did not counteract the arachidonic acid-induced decrease in dopamine uptake and ii bovine serum albumin, which binds arachidonic acid, attenuated the effect of arachidonic acid but not that of protein kinase C activation, it is likely that the inhibitory effects of arachidonic acid activators and those of protein kinase C activators on dopamine uptake are mediated by separate mechanisms. Curiously, shorter preincubations with arachidonic acid enhanced dopamine uptake by the human dopamine transporter, but this effect was not further characterized (Zhang and Reith, 1996). In comparison, L'hirondel et al. (1995) have reported inhibition of dopamine uptake by rat striatal synaptosomes by arachidonic acid; at the same concentrations, dopamine release was

stimulated, but not by transporter block because the release effect persisted in the presence of nomifensine. The releasing effect required protein kinase C activity, reminiscent of the phenomena reported in the two dopamine release studies discussed above (Giambalvo, 1992; Bugnon et al., 1995). As proposed by the group of Glowinsky (L'hirondel et al., 1995), the interesting possibility exists that glutamate, released from glutamate afferents in dopamine terminal areas in the brain, acts on glutamate receptors located on GABA cells to release arachidonic acid that in turn can act on dopamine terminals. The resulting dopamine releasing activity, presumably mediated by protein kinase C, in combination with both stimulatory and inhibitory effects of arachidonic acid on dopamine uptake, would make complex regulation possible with a role for transmitters that act on G protein-coupled receptors altering phospholipase A activity.

Potential role of dopamine transporter in Parkinson's disease

DAT in Parkinson's disease: PD is characterized by a substantial loss of midbrain DA neurons (particularly in the ventral tier of the substantia nigra) with a consequent loss of DA innervation to forebrain structures (particularly the putamen). The vulnerability of certain subgroups of DA neurons in PD and MPTP-induced Parkinsonism correlates with higher basal levels of DAT gene expression (Sanghera et al., 1994; Uhl, 1998; Bannon et al, 1995; Bannon and Whitty, 1997). It is conceivable that avid transport of neurotoxins or even endogenous DA by the DAT may play a role in idiopathic PD.

Given the extent of DA cell loss, it is not surprising that significant decreases in DAT ligand binding sites are detected in PD striatum postmortem (Bannon et al., 1995; Miller et al., 1997). DAT binding is reduced equivalently in progressive supranuclear palsy, a disease involving global degenerative changes throughout the basal ganglia and associated nuclei including the substantia nigra (Maloteaux et al., 1988; Chinaglia et al., 1992). Although initial estimates of DAT losses in PD were unexpectedly modest, this turned out to be (at least in part) an artifact of one of the DAT ligands commonly used (GBR 12935; Seeman and Niznik, 1990). The recent development of DAT-specific antisera has facilitated the direct quantification and localization of DAT protein in PD brains. Miller et al (Miller et al., 1997) reported

substantial DAT protein reductions in putamen (75%), caudate (64%) and nucleus accumbens (53%). Immunocytochemical staining reveals a similar gradient of DAT loss. Even within the most impacted regions, discrete islands of DAT immunoreactivity persist, in association with the matrix compartment.

One of the more interesting observations in PD relates to alterations in gene expression within the surviving midbrain DA neurons. In PD, the abundance of mRNAs encoding the DAT and VMAT per DA cell are decreased, while TH mRNA levels per cell are increased, as compared to control brains (Uhl et al., 1994; Harrington et al., 1996; Joyce et al., 1997). These data might be interpreted as evidence for the occurrence of compensatory mechanisms within surviving DA cells: an increased production of TH and diminished capacity to recapture and sequester DA may augment DA neurotransmission and partially offset DA cell loss. A number of cocaine-related DAT ligands of high affinity and reasonable specificity have been developed for positron emission tomography (PET) and single photon emission computerized tomography (SPECT). Recent studies using these reagents have detected regional losses of DAT binding even in relatively early PD cases (Fischman et al., 1998; Frost et al., 1993; Guttman et al., 1997; Innis et al., 1993; Tissingh et al., 1998). The ability to monitor DAT (and therefore to some extent DA terminal) density in vivo may facilitate earlier diagnosis of PD and should provide a valuable tool for assessing the efficacy of new treatments aimed at slowing or reversing the disease process.

1-Methyl-4-phenyl pyridium modulate dopamine transport function

MPP^+ is transported into cells via the dopamine transporter (DAT) where it mediates cellular toxicity (Javitch et al., 1985; Tipton et al., 1993; Gainetdinov et al., 1997) DAT is a presynaptic plasma membrane protein responsible for the regulation of extracellular dopamine levels and termination of its action by *mediating the re*uptake of dopamine (Amara and Kuhar, 1993; Reith et al., 1997). Functional impairment of DAT alters many physiological and behavioural processes that are mediated by dopamine. A dysfunction of dopamine transmission could consequentially interrupt motor neural circuits, which control movement as seen in PD.

Recently it has been shown that cellular mRNAs encoding DAT and vesicular monoamine transporters are decreased in PD (Uhl et al., 1994; Harrington et al., 1996). Indeed, an alteration of dopaminergic neurotransmission by the modulation of DAT activity could have an important implication in the cellular events, which lead to PD, and could be a target for a potential therapeutic intervention. Several studies have shown that MPP^+ causes a significant decrease in the activity of DAT (Storch et al., 1999; Barc et al., 2001; Fonck and Baudry, 2001; 2003). However, it remained unclear whether the decrease in DAT activity resulted from the selective uptake of MPP^+ through DAT, reduced dopaminergic neurons mediated by the toxin, or changes in the trafficking of the transporter molecules.

Another mechanism whereby MPP^+ may cause a reduction in DAT activity is phosphorylation. In recent observations it was proposed that dopamine transporter activity was regulated by phosphorylation of DAT (Vaughan et al., 1997; Huff et al., 1997; Zhang et al., 1997). A consequence of phosphorylation is the possible sequestration of the transporter proteins (Pristupa et al., 1998; Melikian et al., 1999; Bauman et al., 2000) and lower DAT activity.

CHAPTER 4
MATERIALS AND METHODS

Chemicals

PC12 cells were originally obtained from the American Type Culture Collection, (Manassas, Virginia, USA); Dulbecco's modified Eagle's medium (DMEM) and was purchased from Gibco Invitrogen, (Grand Island, New York, USA); penicillin-streptomycin 50 U/ml and nerve growth factor were purchased from Sigma, (St. Louis, Missouri, USA); HEK 293 cells were generous gift from Dr. Roxanne Vaughan, (Department of Biochemistry and Molecular Biology, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota, USA); hDAT cDNA was cloned in the laboratory of Dr. Eshleman Amy, (Oregon Health Sciences University, Portland, Oregon, USA); [^{3}H]dopamine was purchased from Amersham, (Piscataway, New Jersey, USA); Protease inhibitor mixture tablets were purchased from Roche Diagnostics, (Indianapolis, USA) anti-synaptophysin and phosphotyrosine monoclonal antibody were purchased from Chemicon, (Temecula, California, USA); protein A agarose beads was purchased from Calbiochem, (San Diego, California, USA); Hypersensitive ECL film was purchased from Amersham, (Arlington Heights, Illinois, USA); Quantity One Software was obtained from Bio-Rad Laboratories (Hercules, California, USA); dichlorofluorescein-diacetate, $DCFH_2$-DA (DCF) and 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MTT) were purchased from Sigma, (St. Louis, Missouri, USA); [^{3}H]WIN 35428 was purchased from Dupont-NEN, (Boston, Massachusetts, USA); Sulfo-NHS-biotin solution and immobilized neutravidin beads were purchased from Pierce Chemical Co., (Rockford, Illinois, USA); anti-dopamine transporter monoclonal antibody was purchased from Chemicon Inc., (Tamecula, California, USA); [^{32}P]orthophosphatic acid and Protein G-Agarose were obtained from Boehringer Mannheim, (Mannheim, Germany); goat-raised hDAT specific antibody was purchased from Santa Cruz Biotech Inc., (Santa Cruz, California, USA);

Bio-Imaging Analyzer BAS-1500 was obtained from Fuji film Co., (Tokyo, Japan). GraphPad Prism 3.0 Software, (Sandiego, California, USA).

Cell culture

PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum and penicillin-streptomycin 50 U/ml, in 5% CO_2 at 37^0C until they reached confluency. Cells were differentiated into a neuronal phenotype by the addition of 50 ng/ml of nerve growth factor to the culture medium.

HEK-293 cells stably transfected with cDNA for the human dopamine transporter (hDAT), were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum at 37^0C and 5% CO_2.

The cells were grown in monolayer cultures for 24-48 h until confluent. Before all assays, cells were thoroughly washed to remove residual drug after the *completed* incubation times.

[^{3}H]Dopamine uptake assay

The treated cells were suspended in Krebs bicarbonate buffer (20 mM $NaHCO_3$, 122 mM NaCl, 0.5 mM Na_2HPO_4, 4.8 mM KCl, 1.2 mM $MgSO_4$, 0.2 μg/ml ascorbic acid, 2 mg/ml glucose, 100 μM pargyline, pH 7.4) and allowed to equilibrate (approximately 75 μg protein was added to each assay tube). The aliquoted cells were then incubated with 20 nM [^{3}H]dopamine (68 Ci/mmol) for 10 min at 37^0C. Non-specific uptake was determined by adding 10 μM nomifensine to the incubation solution before adding radioactive dopamine. The uptake was terminated by rapid vacuum filtration over Whatman GF/C. The cells were washed on the filter rapidly three times with 3 ml of Krebs bicarbonate buffer. Accumulate radioactivity was determined by liquid scintillation counting.

Dopamine transporter phosphorylation assay

Cells were labelled with [^{32}P]orthophosphatic acid, then immunoprecipitated with anti-human dopamine transporter antibodies followed by sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. For assays, cells were plated into six-well plates so as to be nearly confluent on the days of experiments. Cells were rinsed and incubated for 7 h at 37^0C in phosphate-free, serum-free DMEM. The cells were then incubated for 7-12 h in medium containing 300 mCi/mL [^{32}P]orthophosphatic. Labelling medium was removed and cells were rinsed once with PBS buffer, and solubilized for 1 h at 4^0C in 1 ml of solubilization buffer (150 mm NaCl, 500 mm Tris-Cl, 1% Triton X-100, 0.1% SDS, 5 mm EDTA, 10 mm NaF, 10 mm sodium pyrophosphate, 0.1 mm PMSF, 0.5% sodium deoxycholate) with shaking. Solubilization was advanced by sonication, and the extracts were clarified by the addition of 50 mL of a 50% slurry of prewashed Protein G-Agarose in solubilization buffer for 3 h and centrifugation. The cell extracts were incubated with 4 mg goat-raised hDAT specific antibody for 2 h at 4 ^{0}C on a rocker, followed by addition of 50 mL Protein G-Agarose and incubated overnight at 4^0C on the rocking platform. The beads were centrifuged and washed five times with 400 ml of solubilized buffer, recovering them after each wash by centrifugation. The proteins eluted the beads for 20 min at room temperature with 2x Laemmli buffer containing 10% β-mercaptoethanol. The samples were electrophoresed in 10% SDS-PAGE followed by autoradiography using Bio-Imaging Analyser BAS-1500. Each band was quantified using Quantity One Software.

Radioligand binding assay

Binding assays were carried out on intact cells and membrane fractions using [^{3}H]WIN 35428 (85 Ci/mmol) as radioligand. Radioligand binding for total cell membrane was carried out as described previously (Whitehead et al., 2001). After treatment, culture medium was removed, washing twice with 5 mL PBS, followed by adding 4 mL lysis buffer (2 mM HEPES, 1 mM EDTA) for 10 min at 0°C. The lysed cells were scraped, centrifuged at 31,000xg for 20 min, and resuspended in 500 µL of Krebs bicarbonate buffer followed by sonication for 5 second and used immediately. The treated cells were resuspended in Krebs bicarbonate buffer containing 3 nM [^{3}H] WIN 35428 as final concentration, and a concentration range of 1-12 nM for saturation assays. Non-specific binding was determined by adding 10 µM nomifensine to the incubation solution before adding the radioactive labelled. Tubes

were incubated for 2 h at 4^0C, and the incubations were terminated by rapid vacuum filtration over Whatman GF/C. The filters were rinsed three times with 3 ml of ice-cold buffer. Accumulate radioactivity was determined by liquid scintillation counting. Estimated dissociation equilibrium constant (K_d) and a transporter density (B_{max}) were determined from non-linear plots of the binding data and Scatchard linear transformation plots using GraphPad Prism 3.0 Software.

Cell surface biotinylation and immunoblotting

Confluent cells after treatment, were washed three times with 1 ml of ice-cold calcium- and magnesium-supplemented PBS (Ca/Mg-PBS, 138 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.3). There were then incubated with Sulfo-NHS-biotin solution (1.5 mg/ml) in Ca/Mg-PBS for 1 h at 4^oC with agitation. Free sulfo-NHS-biotin was removed by washing with ice-cold 0.1 M glycine in 1 ml Ca/Mg-PBS twice. The reaction was further quenched by incubation with 100 mM glycine for 30 min after which the cells were washed with Ca/Mg-PBS three times. Biotinylated cells were lyzed in 0.5 ml of radioimmunoprecipitation assay buffer, (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate), supplemented with a protease inhibitor mixture tablet (Roche Diagnostics) for 1 h at room temperature with gentle shaking. The lyzed samples were then clarified at 20,000xg for 30 min at 4^oC. Supernates were incubated with immobilized neutravidin beads (3mg of protein/ml of beads) for 1 h at room temperature to separate biotinylated from nonbiotinylated protein. The beads were washed three times with 1 ml of radioimmunoprecipitation assay buffer and the biotinylated proteins eluted with 50 µl of 2x laemmli sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 10% SDS, 0.1 M dithiothreitol, and 0.2% bromphenol blue) for 30 min at room temperature. Aliquots of total cell lysates represent from equal amounts of protein and nonbiotinylated proteins were precipitated with TCA (5% final concentration) and the pH was neutralized with 5.0 M Tris-base before being resuspended in laemmli sample buffer. Biotinylated and nonbiotinylated proteins were resolved by SDS-PAGE (10% acrylamide). Western blots were performed. Blots were probed with a rat anti-dopamine transporter

monoclonal antibody diluted 1:1000. Immunoreactive bands were visualized by ECL on Hypersensitive ECL film and analyse with Quantity One Software.

[^{3}H]Dopamine release assay

Confluent monolayers of cells in 24 well plates were treated with pre-warmed stimulation buffer (10 mM HEPES, pH 7.2, 150 mM NaCl, 3 mM CaCl2, 2 mM KCl, 1 mM $MgSO_4$) The cells were allowed to equilibrate in this buffer for 20–30 min at 37^0C and 5% CO_2 before receiving fresh stimulation buffer containing a secretagogue (55 mM KCl, 1–5 μM ionomycin), after which they were returned to treated with fresh medium and equilibrated at 37^0C for 30 min and then loaded with 0.3-1 μCi/ml [^{3}H]dopamine for 3 h at 37^0C. The medium was removed and the cells were washed once with complete medium and twice with serum-free medium containing 1 mM ascorbic acid. Fresh medium was added, and the cultures were incubated with 100 μM MPP^+ for various time intervals. Basal levels of [^{3}H]dopamine release were measured in cultures incubated at 37^0C for the same time period as untreated cells. The sample (500 μl) from each well was collected from the medium, centrifuged to remove any cell debris (10 min at 2,000xg) and the radioactivity was measured in a liquid scintillation counter. To measure total radioactivity, the cells were washed with phosphate buffer saline and dissolved in 500 μl of 1 N NaOH. Accumulate radioactivity was determined by liquid scintillation counting.

Coimmunoprecipitation and Western blot analysis

After the cells were treated with the specified drug, the culture medium was removed and cells were lyzed in 500 μl of lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS and 50 mM DL-dithiothreitol), supplemented with a protease inhibitor mixture tablet. The lysates were subsequently centrifuged at 20,000xg for 20 min at 4^0C and the protein content was determined using Lowry's method (Lowry et al., 1951). Cell lysates (2 mg/ml) were incubated with anti-synaptophysin at 1:100 dilutions and incubated at 4^0C overnight. Precipitated synaptophysin was separated by adding 30 μl per sample protein A agarose beads, incubated for an additional hour, and then the beads were separated by centrifugation (2 min, 2,000xg). Beads were

washed 3 times with lysis buffer, centrifuged, and the pellet was boiled 5 min with 30 µl Laemmli sample buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% glycerol).

For Western blot analysis, each sample was loaded onto a 12% polyacrylamide gel, and transferred onto nitrocellulose membrane. Membranes were usually blocked for at least 60 min with 2% bovine serum albumin in a washing buffer (25 mM Tris, 150 mM NaCl, 0.05% Tween 20). Blots were probed with a phosphotyrosine monoclonal antibody diluted 1:1000. Immunoreactive bands were visualized by ECL on Hypersensitive ECL film and analyze with Quantity One Software.

Intracellular ROS formation assay

A fluorescent spectrophotometer was used to detect formation of intracellular peroxides using a nonfluorescent compound, $DCFH_2$-DA (DCF). Briefly, after exposure to the several drugs, the cells were harvested and washed in Krebs-Ringer solution, resuspended at 1 to 3×10^6 cells/ml. The cultured cells were resuspended in 200 µl phosphate buffer saline, pH 7.4. After loading with DCF for a final concentration 20 µM and incubation at 37°C for 30 min, cells were lysed with 100 µl DMSO and production of 2,7-dichlorofluorescein ($DCFH_2$-H), a fluorescent product of hydrolyzed $DCFH_2$-DA, was monitored over 1 h by spectrofluorometer at 490 nm excitation and 530 nm emission.

Assay of NADH: ubiquinone oxidoreductase (complex I) activity

Complex I activity was assayed in cell mitochondrial preparations according to Shults et al. (1995) with minor modifications as described below. The assay was performed in phosphate buffer (35 mM, pH 7.2) containing sodium cyanide (2.65 mM), magnesium chloride (5 mM), EDTA (1 mM), bovine serum albumin (1 mg/ml), and antimycin (2 µg/ml). Mitochondria from cultured cells were prepared by discontinuous Percoll gradient centrifugation (Barrientos et al., 1998), coenzyme Q-1 (ubiquinone-1, 0.05 mM final concentration) were added to the assay buffer such that the final assay volume was 0.48 ml. After preincubation of the reaction mixture at room temperature for 2 min, the reaction was initiated by the addition of 0.02 ml of a

5 mM solution of NADH. The decrease in absorbance at 340 nm was monitored over time.

Cell viability assay

The treated cells were plated in a 96-well plate and processed with the 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MTT) to measure the activity of mitochondrial dehydrogenase enzymes, which cleave the tetrazolium ring to produce formazan (Mosmann, 1983). MTT prepared in PBS at 5 mg/ml, was diluted 10 fold in the culture medium and incubated at 37^{o}C for 4 h. After incubation, the MTT medium was removed; 100 μl of 0.04 N HCl in isopropanol was added to each well for 15 min in a dark area. The amount of solubilized MTT formazan product was determined by spectrophotometer at 540 nm.

Statistical analysis

Indicated data are reported as percentages of control values. Statistical evaluation was performed using one-way analysis of various followed by student's paired *t*-test, and values of $p<0.01$ were considered statistically significant for all analyses.

CHAPTER 5
RESULTS

1. Effect of MPP^+ on dopamine transporter function

Results presented in Figure 5.1 indicate that exposure of hDAT-HEK to MPP^+ for 24 h caused a concentration-dependent decrease in [^{3}H]dopamine uptake. MPP^+ at concentrations as low as 1 μM significantly reduced [^{3}H]dopamine uptake (58±13% of control), and maximal inhibition was observed at 1000 μM concentration (25±5% of control, Table 5.1). As shown in Figure 5.2, after incubation with MPP^+ (100 μM) for 30 min, there was clear and statistically significant decreased in [^{3}H]dopamine uptake. The reduction in [^{3}H]dopamine uptake remained the same after 1h and 24h incubations, (38±8% of control and 30±13% of control, respectively; Table 5.2).

Table 5.1 Effects of MPP^+ treatment on [^{3}H]dopamine uptake

Treatment	% of Control ± SEM
Control	100
MPP^+ 0.1 μM	58±13*
MPP^+ 1 μM	43±7*
MPP^+ 10 μM	36±10*
MPP^+ 100 μM	29±8**
MPP^+ 1000 μM	25±5**

The hDAT-HEK cells were grown to confluence, and then treated with MPP^+ at the indicated concentrations (0.1μM to 1000 μM) for 24h at 37^0C prior to analysis for dopamine transport. Experiments used dopamine final concentration 20 nM, and results are expressed as the relative amount of dopamine transported compared with control at least four independent experiments.

*P<0.01 when compared to control

**P<0.001 when compared to control

Table 5.2 Time course of MPP^+ effects on [^{3}H]dopamine uptake

Treatment duration (hour)	% of Control ± SEM
0	100
0.5	58±9*
1	46±9*
3	39±8**
24	31±14**

hDAT-HEK cells were grown to confluence, and then treated with 100 μM MPP^+ at the indicated times (0.5 to 24h) at 37^0C prior to analysis for dopamine transport. Experiments used dopamine final concentration 20 nM, and results are expressed as the relative amount of dopamine transported compared with control at least six independent experiments.

*P<0.01 when compared to control

**P<0.001 when compared to control

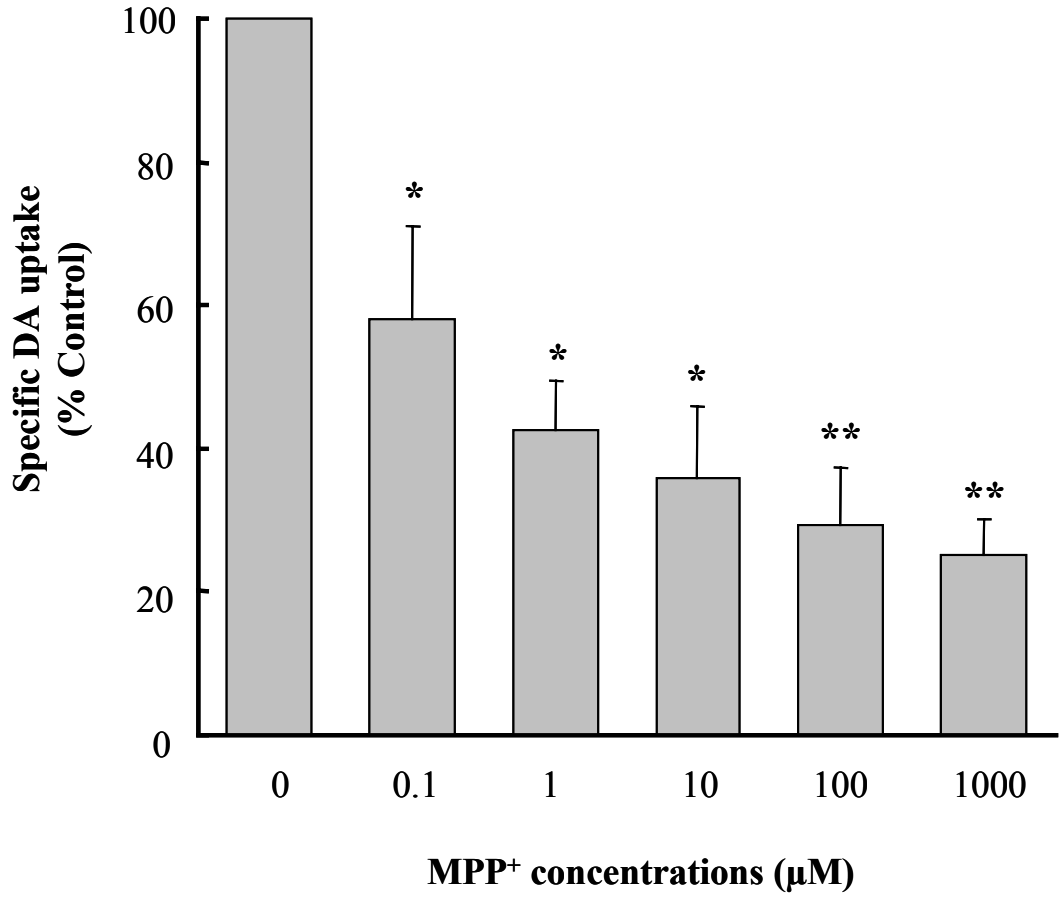


Figure 5.1 Concentration-dependent inhibition of [^{3}H]dopamine uptake by MPP^+ (Chagkutip et al., 2003).

$^*P<0.01$ when compared to control

$^{**}P<0.001$ when compared to control

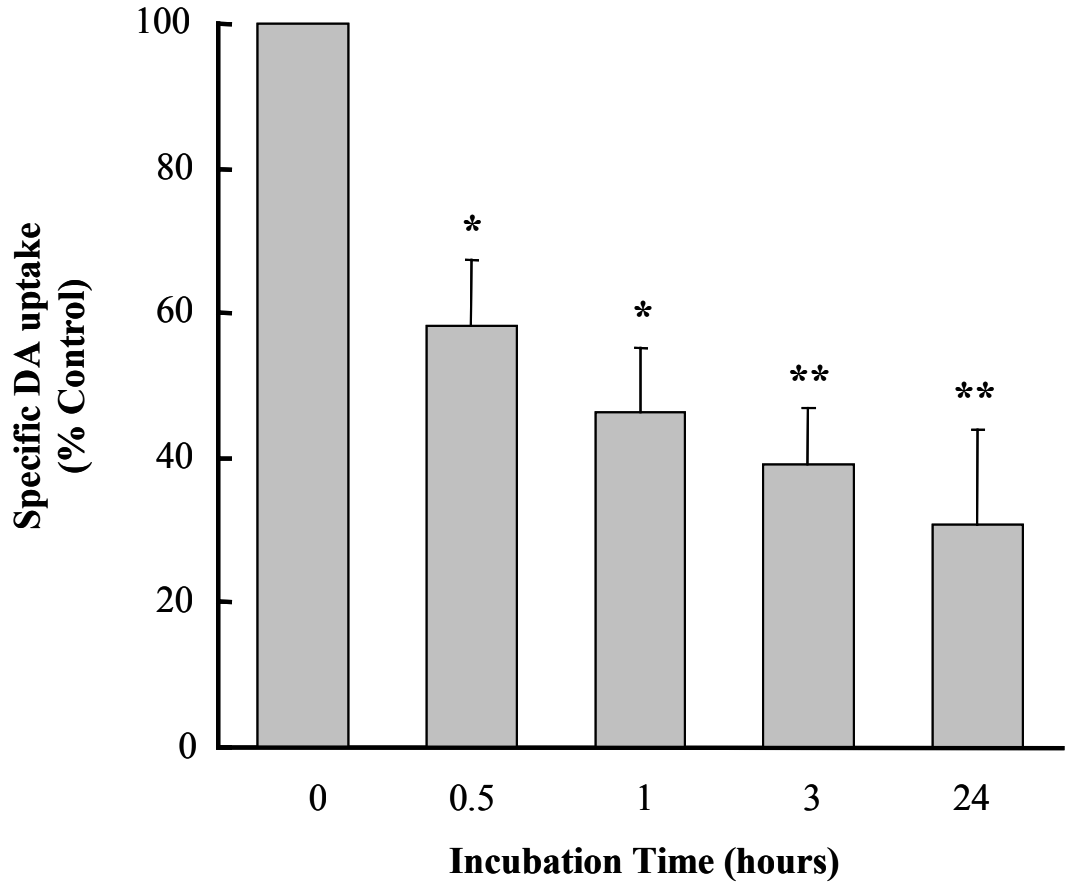


Figure 5.2 Time course of MPP^+ effects on [^{3}H]dopamine uptake (Chagkutip et al., 2003)..

$^*P<0.01$ when compared to control

$^{**}P<0.001$ when compared to control

2. Phosphorylation of the dopamine transporter produced by MPP^+ treatment

To investigate the possibility that the dopamine transporter is a phosphoprotein, hDAT-HEK cells were labelled with [^{32}P]orthophosphate, cell lysates were subjected to immunoprecipitation with anti-dopamine transporter antibodies, and sample were analysed by SDS-PAGE and autoradiography. Figure 5.3A displays immunoprecipitation of an 88-kDa phosphoprotein that migrates with the same relative mobility as [^{32}P]orthophosphate-labelled dopamine transporter prepared from hDAT-HEK cells. Treatment of hDAT-HEK cells with 100 μM MPP^+ during [^{32}P] orthophosphate labelling resulted in a dramatic increase in the level of DAT phosphorylation (Table 5.3, Figure 5.3). The time course studies showed increases levels of phosphorylated DAT by 10 min (126±5% relative to basal) of treatment, with maximum levels reached by 20-40 min (176±2%, 187±4%, and 176±7% relative to basal, respectively).

Treatment of hDAT-HEK cells with protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) for 30 min, during [^{32}P]orthophosphate labelling, resulted in an increase in the level of DAT phosphorylation (182±20% relative to basal). Similarly, the increased phosphorylation produced by 100 μM MPP^+ was also observed (Table 5.4, Figure 5.4).

Table 5.3 Time course of MPP^+effects on DAT phosphorylation

Treatment duration (hour)	% of Control ± SEM
0	100
10	126±5
20	176±2**
30	187±4**
40	176±7**
60	147±7

The hDAT-HEK cells were metabolically labelled with [^{32}P]orthophosphate for 7-12 h and treated with 100 μM MPP^+ for 10-60 min. Cells were washed, lysed, and then centrifuged to pellet the membranes, followed by DAT immunoprecipitation, electrophoresis and autoradiography. The autoradiogram was developed for 1-3 days. Autoradiograms were analysed by densitometry. Results are presented as mean ± SEM from four independent experiments.

** $P<0.001$ when compared to control

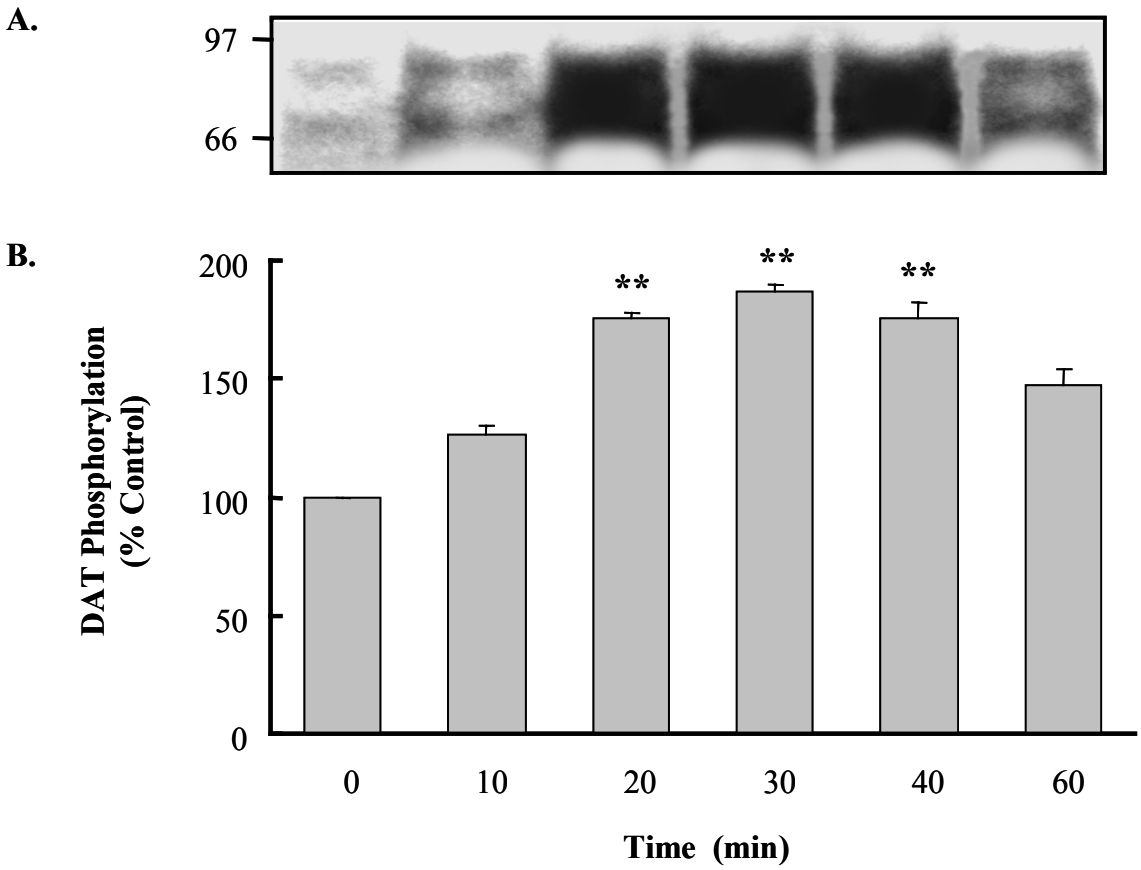


Figure 5.3 Time course of MPP^+ effects on DAT phosphorylation. (A) hDAT-HEK cells were metabolically labelled with [^{32}P]orthophosphate for 7-12 h and treated with 100 μM MPP^+ during the course of the incubation to produce the treatment times shown. (B) Quantification of autoradiograms by densitometry obtained from four independent experiments.

$^{**}P<0.001$ when compared to control

Table 5.4 Phosphorylation of DAT produced by MPP^+ and PMA treatment

Treatment	% of Control ± SEM
Control	100
MPP^+	$174 \pm 12^*$
PMA	$182 \pm 20^{**}$

hDAT-HEK cells were metabolically labelled with [^{32}P]orthophosphate for 7-12 h and treated with or without 10 μM PMA for an additional 30 min. Cells were washed, lysed, and then centrifuged to pellet the membranes, followed by DAT immunoprecipitation, electrophoresis and autoradiography. The autoradiogram was developed for 1-3 days. Autoradiograms were analysed by densitometry. Results are presented as mean ± SEM from three independent experiments.

$^*P<0.01$ when compared to control

$^{**}P<0.001$ when compared to control

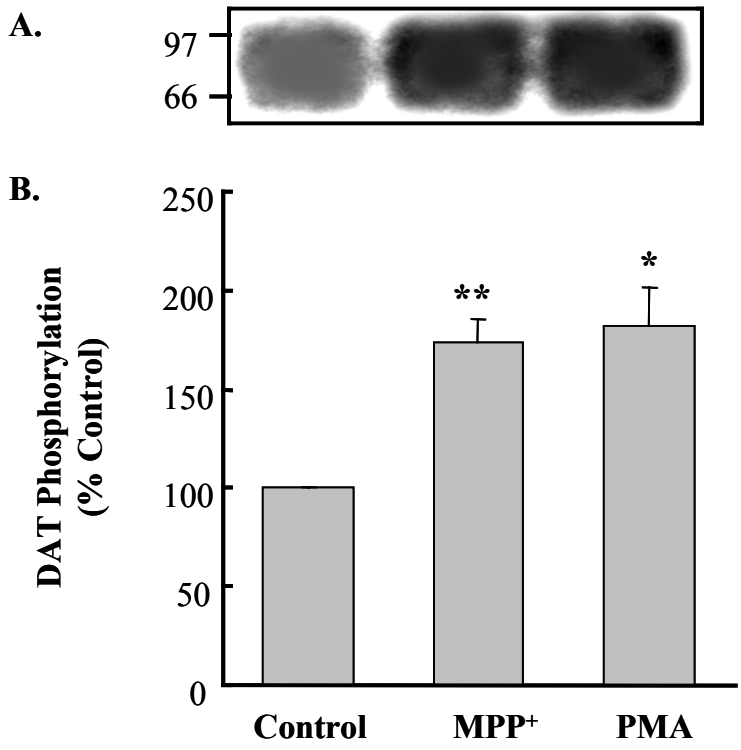


Figure 5.4 Phosphorylation of DAT produced by MPP^+ and PMA treatment. (A) hDAT-HEK cells were metabolically labelled with [^{32}P]orthophosphate for 7-12 h and treated with 100 μM MPP^+ (*lane2*) or 10 μM PMA (*lane 3*). Samples were then immunoprecipitated and subjected to electrophoresis and autoradiography. (B) Quantification of autoradiograms by densitometry obtained from three independent experiments.

$^{*}P<0.01$ when compared to control

$^{**}P<0.001$ when compared to control

3. Effect of MPP^+ on dopamine transporter cell surface expression

The effect of MPP^+ on [^{3}H]WIN 35428 binding was investigated in hDAT-HEK cells. As shown in Figure 5.5, after 3 h of incubation with increasing concentrations of MPP^+, there was a concentration-dependent reduction in [^{3}H]WIN 35428 binding sites in the intact cells with a maximal reduction at 100 μM (39±10% of control) and 1000 μM concentrations (33±7% of control). Apparently, there was no significant difference in the reduction of [^{3}H]WIN 35428 binding between 100 μM and 1000 μM MPP^+. Importantly, MPP^+ treatment reduced the density (B_{max}) of [^{3}H] WIN 35428 binding sites (5.7±1.1 and 2.7±1.4 fmol/mg in control and treated groups, respectively), whereas the K_d values in the membrane fraction increased (Figure 5.5).

Table 5.5 Effects of MPP^+ treatment on [^{3}H]WIN 35428 binding

Treatment	% of Control ± SEM
Control	100
MPP^+ 1 μM	63±1**
MPP^+ 10 μM	43±10**
MPP^+ 100 μM	39±7**
MPP^+ 1000 μM	32±7**

Concentration-dependent inhibition of [^{3}H]WIN 35428 binding by MPP^+ to intact hDAT-HEK cells. Cells were grown to confluence, and then treated with fresh medium containing either MPP^+ or the same volume of medium only. Cells were incubated with MPP^+ for 3h at 37^0C prior to an 30 min exposure to [^{3}H]WIN 35428. Cells were washed and supplied with fresh medium. Data are shown as percent of specific binding from three independent experiments.

** $P<0.001$ when compared to control

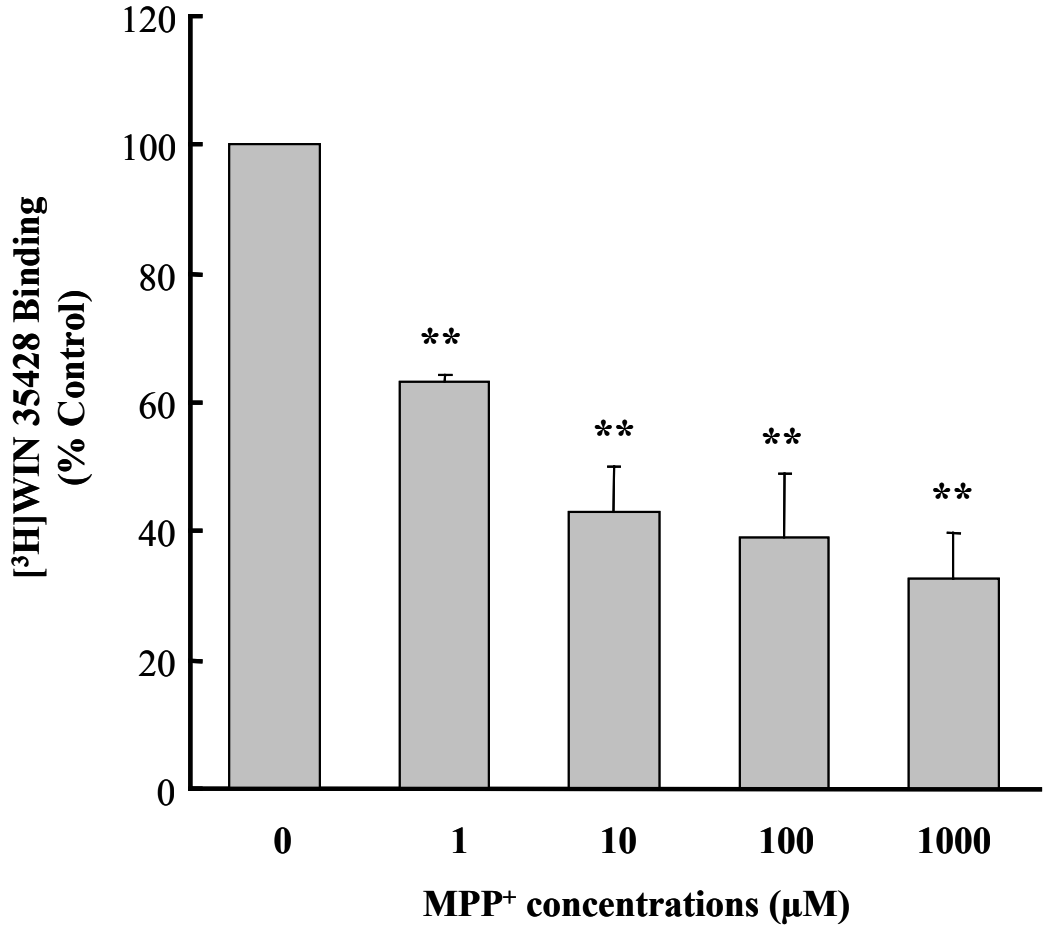


Figure 5.5 Effects of MPP^+ treatment on [^{3}H]WIN 35428 binding (Chagkutip et al., 2003).

$^{**}P<0.001$ when compared to control

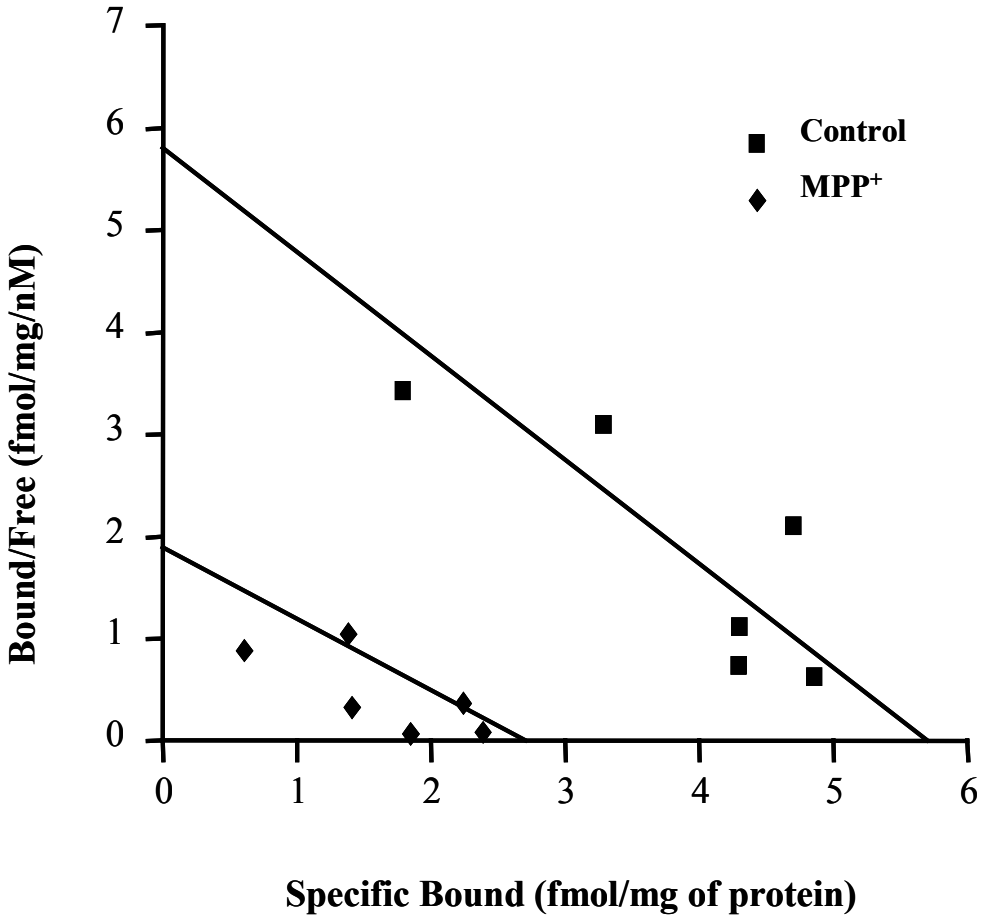


Figure 5.6 Saturation analysis of [^{3}H]WIN 35428 binding. Cells were grown to confluence, and then treated with fresh medium containing either MPP^+ or the same volume of medium only. Cells were washed after 3 h and supplied with fresh medium. Eight to six concentrations of [^{3}H]WIN 35428 were used in each experiment ranging from 1-10 nM, and the data were fit to a one-site model. The experiment shown is representative of four identical experiments, and the raw data was analysed as matched pairs by using GraphPad Prism 3.0 Software (Chagkutip et al., 2003).

Table 5.6 Saturation parameters of [^{3}H]WIN 35428 binding from hDAT-HEK cell membrane. The shown B_{max} and K_d of [^{3}H]WIN 35428 binding are presented as means±SEM.

	B_{max} (fmol/mg protein)	**K_d** (nM)
Control	5.7±1.1	1.0±0.6
MPP^+	2.7±1.4*	1.4±0.1*

*P<0.01 when compared to control

Saturation analysis of [^{3}H]WIN 35428 binding to hDAT-HEK cell membranes produced a monophasic Scatchard plot (Figure 5.6). Consistent with the reduction observed in intact hDAT-HEK cell, treatment with MPP^+ 100 μM for 3 h significantly decreased the B_{max} value from 5.7±1.1 to 2.7±1.4 fmol/mg protein ($p<0.01$) and slightly increased K_d value from 1.0±0.6 to 1.4±0.1 nM.

The next experiment was designed to investigate whether the negative effect of MPP^+ on dopamine uptake and [^{3}H]WIN 35428 binding correlates with DAT protein expression. An intensive study using biotinylation assays showed for the first time that MPP^+ significantly reduced the levels of biotinylated and nonbiotinylated DAT protein (53±7 and 77±1 % of control, respectively; Figure 5.7A and B). This parallels closely the reduction in [^{3}H]dopamine uptake (Figure 5.1) and the level of cell surface [^{3}H]WIN 35428 binding after MPP^+ treatment (Figure 5.5).

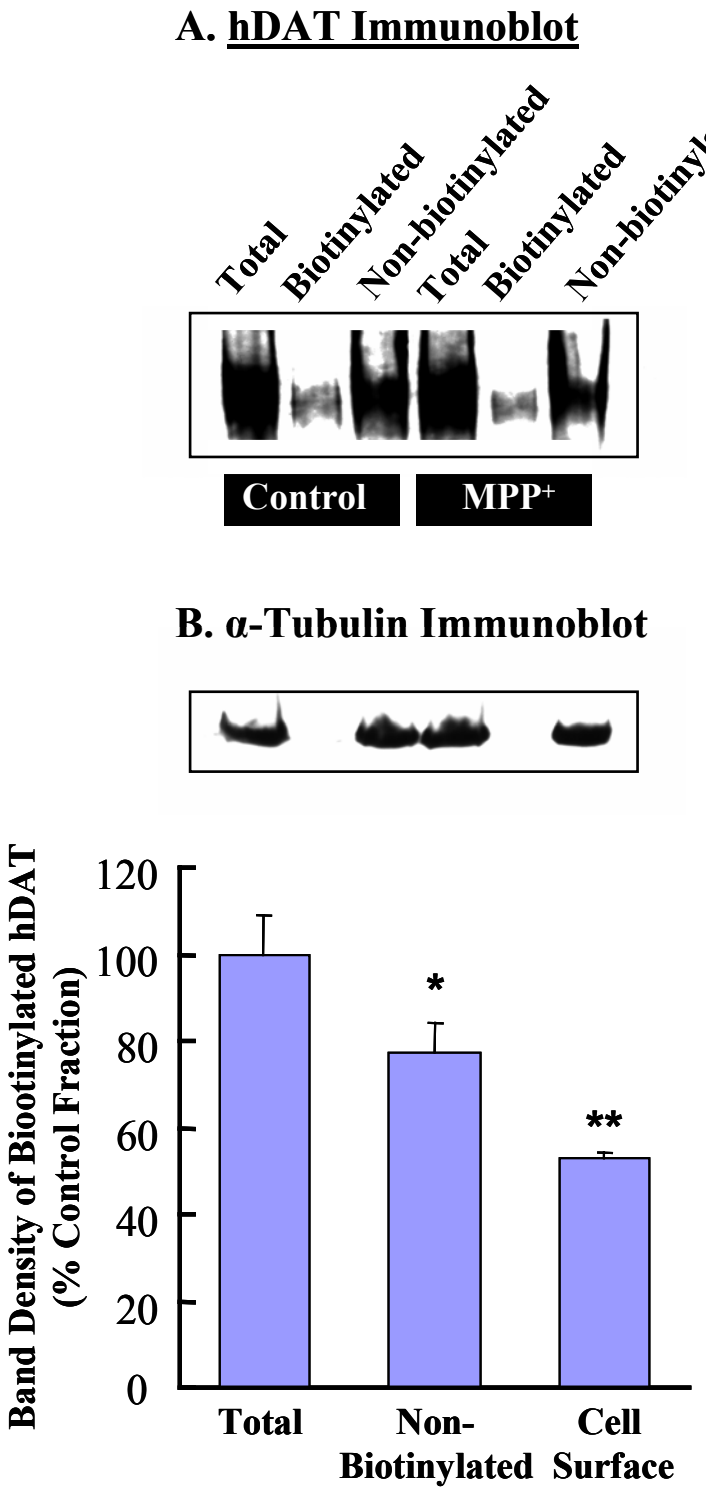

A. hDAT Immunoblot
Total
Biotinylated
Non-biotinylated
Total
Biotinylated
Non-biotinyl
Control
MPP+
B. α-Tubulin Immunoblot
Band Density of Biootinylated hDAT
(% Control Fraction)
120
100
80
60
40
20
0
*
**
Total
Non-
Biotinylated
Cell
Surface

Figure 5.7 MPP^+ decreases cell surface expression of DAT. (A) A representative immunoblot showing total cell extracts, biotinylated extracts, and nonbiotinylated extracted after treatment with MPP^+. (B) Immunoblot with anti alpha-tubulin antibody. After detection of hDAT, the blot was stripped and probed to identify the cytoskeletal membrane protein alpha-tubulin. Intensity of alpha-tubulin specific band was used to normalize hDAT specific immunoreactivity in control and MPP^+ treated cell. Quantification of immunoblots obtained from four independent experiments. Results are presented as mean±SEM (Chagkutip et al., 2003).

$^{*}P<0.01$ when compared to control

$^{**}P<0.001$ when compared to control

4. Effect of MPP^+ on dopamine release

The experiments shown in Figure 5.8 revealed the cumulative effects of MPP^+ during a 60 min exposure period. To investigate the time course of MPP^+ action, PC12 cells were pre-incubated in the stimulation buffer in the absence of MPP^+ and then stimulated in the presence of 100 μM MPP^+, the maximally effective concentration. DA release was unaffected by MPP^+ until about 10 min, after which time, increased release was observed. When PC12 cells were pre-treated with MPP^+, for 5-60 min prior to the addition of secretagogue, significantly increased DA release was apparent after 10 min of stimulation (Figure 5.8).

When PC12 cells were treated with 100 μM MPP^+, there was a significant increase (138±8% of control) in DA release at 10 min of incubation, as indicated in Figure 5.8. The level of DA release remained constant for up to 60 min of incubation with MPP^+.

Table 5.7 Time course of MPP^+ effects on dopamine release (1)

Treatment duration (min)	% of Control ± SEM
0	100
5	111±7
10	138±8**
20	134±10*
40	139±12*
60	129±10*

Cells were loaded with [^{3}H]dopamine for 3 h, washed and treated with 100 μ M MPP^+ for 0-60 min. [^{3}H]dopamine release was measured as described in Materials and Methods. The results shown are expressed as percentages of control values from six independent experiments. The results shown are expressed as percentages of control values.

*P<0.01 when compared to control

**P<0.001 when compared to control

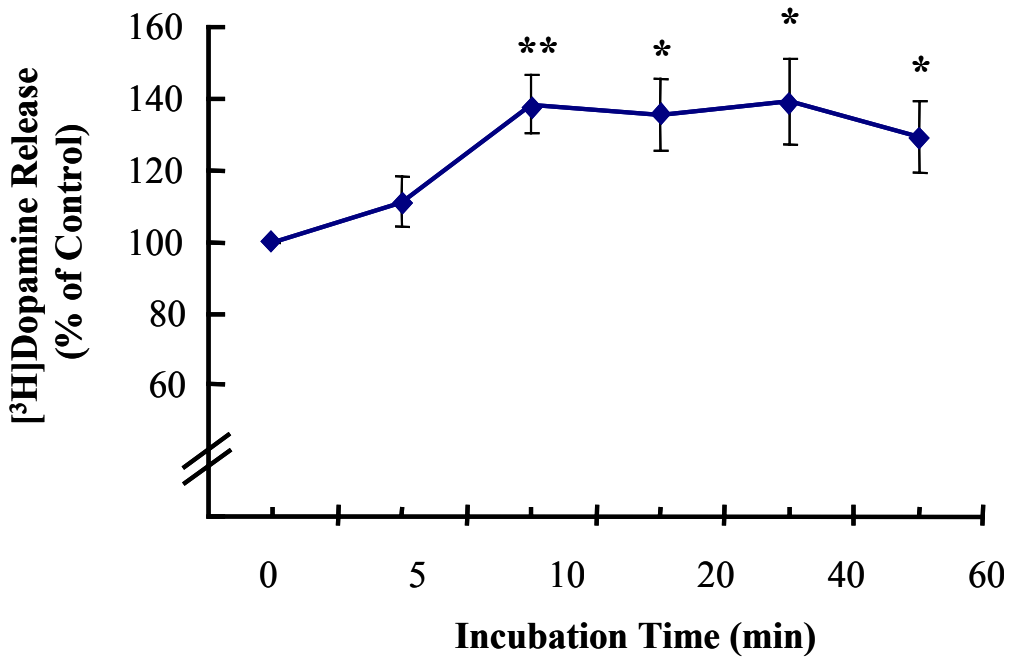


Figure 5.8 Time course of MPP^+ effects on dopamine release.

$^{*}P<0.01$ when compared to control

$^{**}P<0.001$ when compared to control

To assess whether MPP^+-enhanced DA release is dependent on Ca^{2+}, PC12 cells were pre-incubated in the presence or absence of the Ca^{2+} chelating agent EGTA (1mM) for 15 min. In one set of experiments without EGTA, MPP^+ at 100 μM enhanced the release of DA as compared with control (135±5% of control; Fig. 2), and as indicated previously (Figure 5.9). In the presence of EGTA, MPP^+-mediated increase in DA release was significantly reduced to 109±5% of control (Figure 5.9). These results suggest that Ca^{2+} is required for MPP^+-induced DA release.

Table 5.8 Time course of MPP^+-mediated dopamine release (2)

Treatment	% of Control ± SEM
Control	100
EGTA 1 mM	91±1
MPP^+ 100 μM	135±5**
MPP^+ 100 μM + EGTA 1 mM	109±5

Cells were loaded with [^{3}H]dopamine for 3 h, washed and treated with 100 μ M MPP^+ for 60 min, in the presence and absence of EGTA (1mM). [^{3}H]-Dopamine release was measured as described in Materials and Methods. The results shown are expressed as percentages of control values from six independent experiments. The results shown are expressed as percentages of control values.

** $P<0.001$ when compared to control

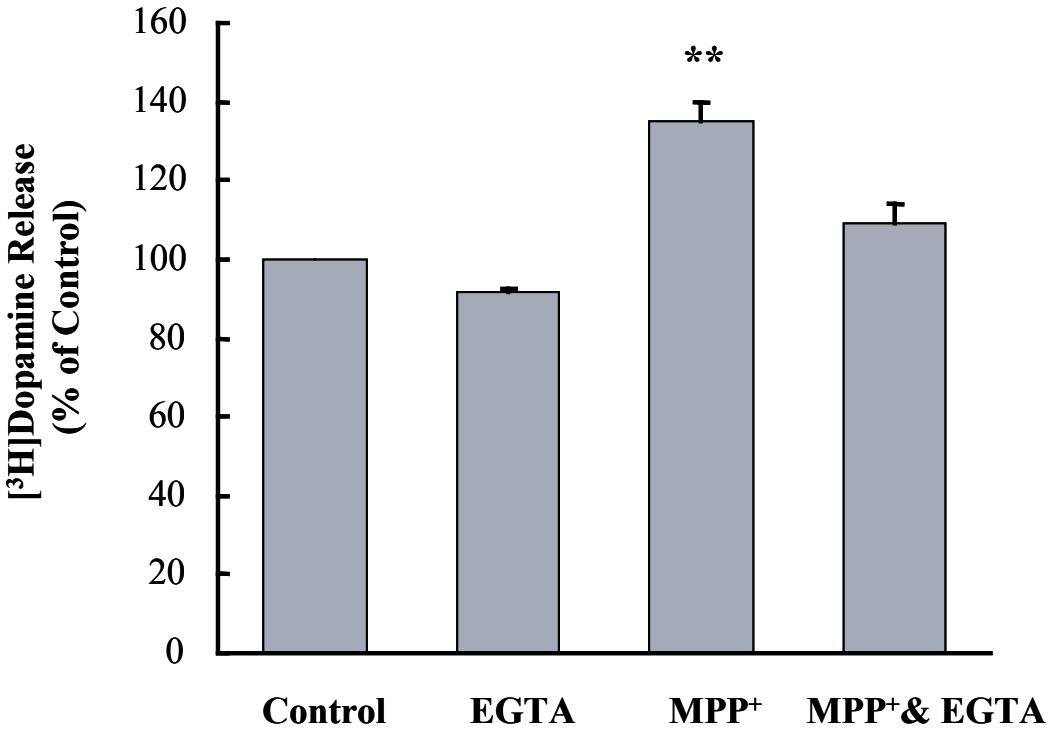


Figure 5.9 Potentiation of dopamine release by MPP^+ depends on extracellular Ca^{2+}. PC12 cells were treated with 100 μM MPP^+ for 60 min, in the presence and absence of EGTA (1mM). [^{3}H]-Dopamine release was measured as described in Materials and Methods. The results shown are expressed as percentages of control values from six independent experiments.

$^{**}P<0.001$ when compared to control

5. Time course of protein tyrosine phosphorylation by MPP^+

To determine whether MPP^+ treatment of PC12 cells would mediate modifications of proteins tyrosine phosphorylation, we investigated its effects by using a monoclonal phosphotyrosine antibody to discover any overt differences. After treating the cells with 100 μM MPP^+ there was an initial rapid increase in the phosphotyrosine immunoreactivity of several proteins varying in size from 28, 35, 38 and 57 kDa (Figure 5.10). The maximal increase in protein tyrosine phosphorylation occurred within 10 min of exposure of the cells to MPP^+ (117$\pm$4 % of control; Figure 5.10B). The increased phosphotyrosine immunoreactivity (up to 10 min) was followed by a decrease between 20-60 min of MPP^+ incubation to levels slightly higher than the control (0 min).

To examined whether MPP^+ stimulated the tyrosine phosphorylation of synaptophysin, a protein implicated in exocytosis, during 0-60 min exposure. By coimmunoprecipitation of synaptophysin using protein-A-coupled synaptophysin monoclonal followed by Western blot analysis with phosphotyrosine monoclonal antibody, synaptophysin was identified with an apparent molecular weight of 38 kDa. Among the proteins that underwent tyrosine phosphorylation in response to MPP^+ is synaptophysin (Figure 5.10). As can be seen, MPP^+ caused a maximally tyrosine-phosphorylated synaptophysin after 10 min (142$\pm$12% of control; Figure 5.11B), and which increased minimally over a period of 20-60 min, as shown in Figure 5.11A and B. The time-response profile parallels the synaptophysin phosphorylation and dopamine release (Figure 5.11 and 5.8), suggesting the association of synaptophysin phosphorylation with dopamine release mediated by MPP^+.

Table 5.9 Time course of MPP^+ effects on protein tyrosine phosphorylation

Treatment duration (hour)	% of Control ± SEM
0	100
5	103±2
10	117±4*
20	113±8**
40	106±9*
60	109±7*

PC12 cells were incubated with 100 μM MPP^+ for 0-60 min. Total protein lysates were resolved by 12% SDS-PAGE, and tyrosine phosphorylation immunoreactivity was determined as described in Materials and Methods using Western blot analyses. The results presented represent one of four experiments that gave similar results. Each phosphorylation band was quantified by densitometry, and control cells compared with cells that were treated with 100 μM MPP^+. The results shown are expressed as percentages of control values.

*$P<0.01$ when compared to control

**$P<0.001$ when compared to control

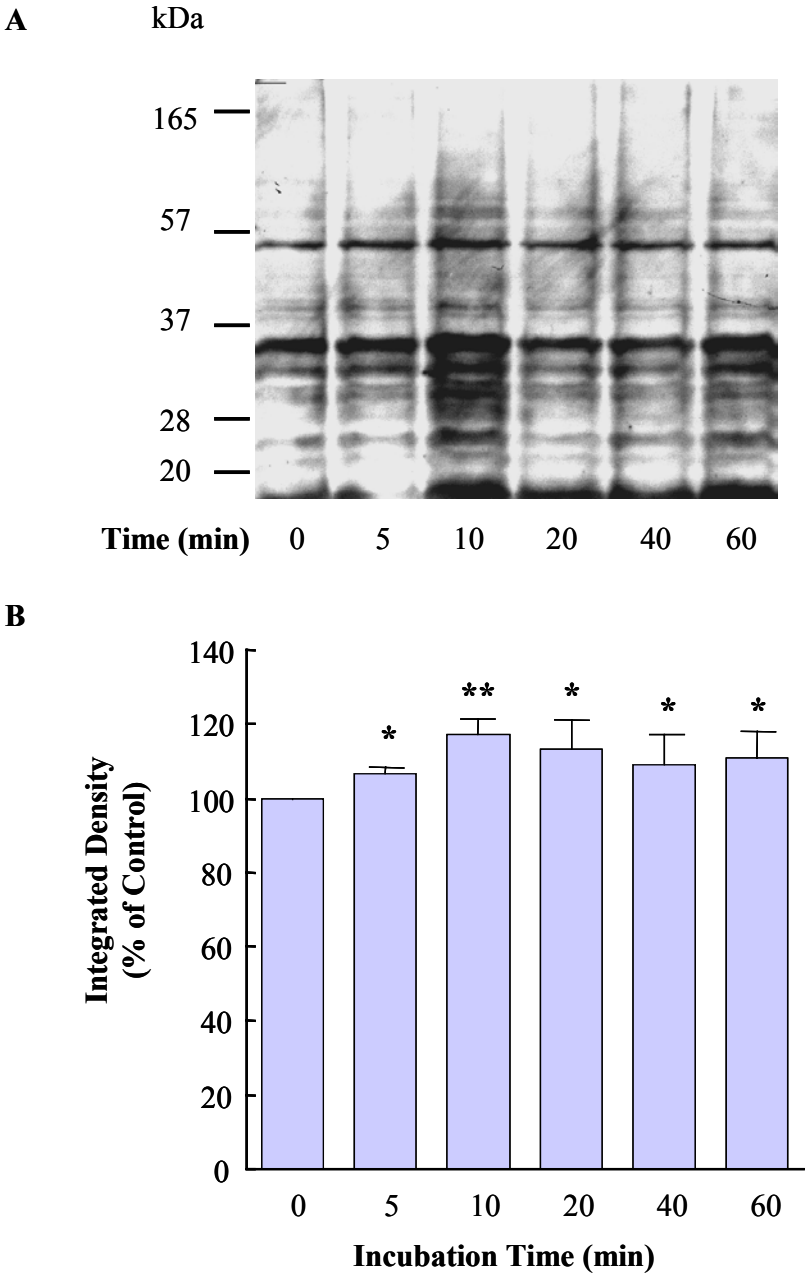

A
kDa
165
57
37
28
20
Time (min) 0 5 10 20 40 60
B
Integrated Density (% of Control)
140
120
100
80
60
40
20
0
*
**
*
*
*
0 5 10 20 40 60
Incubation Time (min)

Figure 5.10 Time course of MPP^+ effects on protein tyrosine phosphorylation. PC12 cells were incubated with 100 μM MPP^+ for 0-60 min. Total protein lysates were resolved, and tyrosine phosphorylation immunoreactivity was determined (A). Each phosphorylation band was quantified by densitometry. The results shown are expressed as percentages of control values. (B).

$^{*}P<0.01$ when compared to control

$^{**}P<0.001$ when compared to control

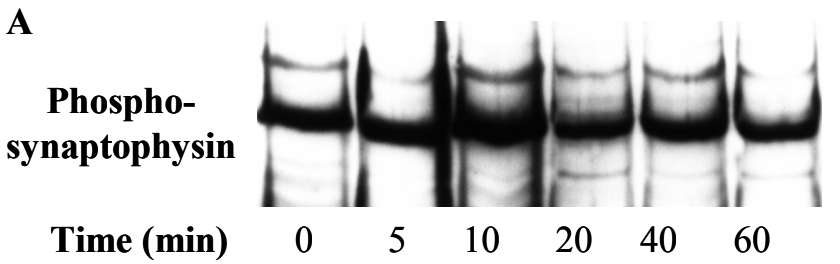
Table 5.10 Time course of MPP^+ effects on synaptophysin phosphorylation

Treatment duration (hour)	% of Control ± SEM
0	100
5	129±7
10	142±12*
20	137±10**
40	128±12*
60	122±10*

PC12 cells were treated with 100 μM MPP^+ for 0-60 min, and whole cell lysates were prepared and normalized to equal amounts of total protein. Synaptophysin was immunoprecipitated and phosphotyrosine was detected by Western blotting using a monoclonal anti-phosphotyrosine antibody. The data represent one of four experiments that gave similar results. Each phospho-synaptophysin band was quantified by densitometry, and control cells compared with cells that were treated with 100 μM MPP^+. The results shown are expressed as percentages of control values.

*$P<0.01$ when compared to control

**$P<0.001$ when compared to control


A
Phospho-
synaptophysin
Time (min) 0 5 10 20 40 60
IP: Anti-synaptophysin
WB: Anti-phosphotyrosine

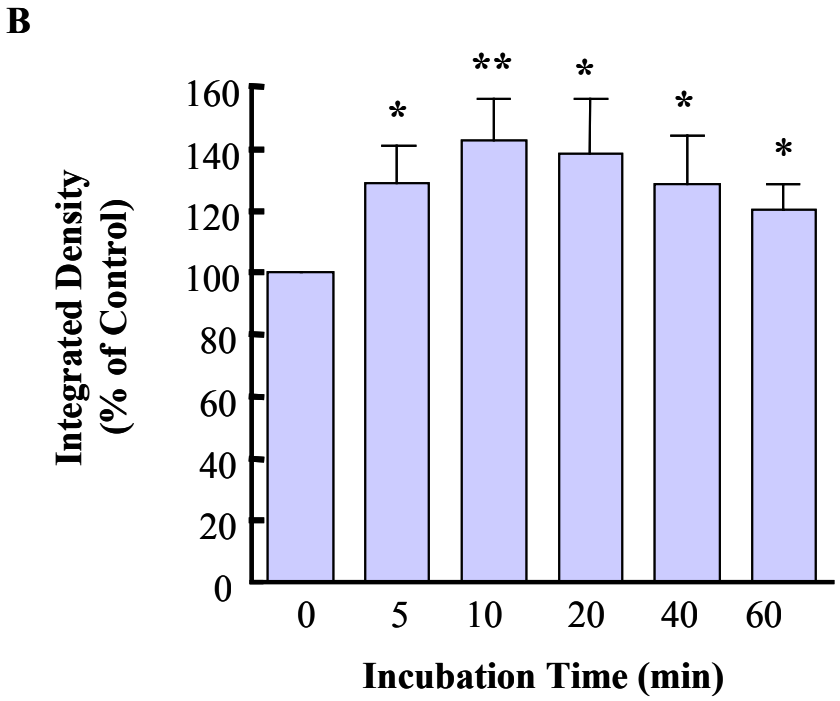

B
Integrated Density
(% of Control)
160
140
120
100
80
60
40
20
0
* ** * * *
0 5 10 20 40 60
Incubation Time (min)

Figure 5.11 Time course of MPP^+ effects on synaptophysin phosphorylation. PC12 cells were treated with 100 μM MPP^+ for 0-60 min. Synaptophysin was immunoprecipitated and phosphotyrosine was detected by Western blotting (A). Each phospho-synaptophysin band was quantified by densitometry. The results shown are expressed as percentages of control values (B).

$^{*}P<0.01$ when compared to control

$^{**}P<0.001$ when compared to control

6. Effect of MPP^+ on ROS generation

As shown in Figure 5.12, incubation of PC12 cells with MPP^+ for 60 min resulted in a significant increase in ROS generation that could be fully blocked by pretreating the cells for 15 min with the dopamine uptake inhibitor, mazindol. In addition, when cells were pretreated with 100 μM selegiline for 30 min prior to MPP^+ exposure, a reduction in MPP^+-induced ROS generation was observed from 179±18 to 144±20% of control, respectively (Table 5.11, Figure 5.12).

Table 5.11 Effect of MPP^+ treatment on ROS generation

Treatment	% of Control ± SEM
Control	100
MPP^+ 100 μM	179±18[a]
Selegiline 100 μM	71±10[b]
Mazindol 10 μM	128±17
MPP^+ 100 μM + Selegiline 100 μM	144±20[c]
MPP^+ 100 μM + Mazindol 10 μM	114±15[d]

PC12 cells were pre-treated with 100 μM selegiline or 10 μM mazindol for 30 min, and exposed to 100 μM MPP^+ for 1 h, and then ROS generation was measured. Production of 2,7-dichlorofluorescein (DCF-H), a fluorescent product of hydrolysed DCF-DA, was monitored over 1 h by spectrofluorometer (488/525 nm). Data represent the mean ± SEM. of seven independent experiments and are expressed as percentage of control. [a]$P<0.01$ when compared to control; [b]$P<0.05$ when compared to control; [c]$P<0.05$ when compared to MPP^+ group; [d]$P<0.05$ when compared to MPP^+ group.

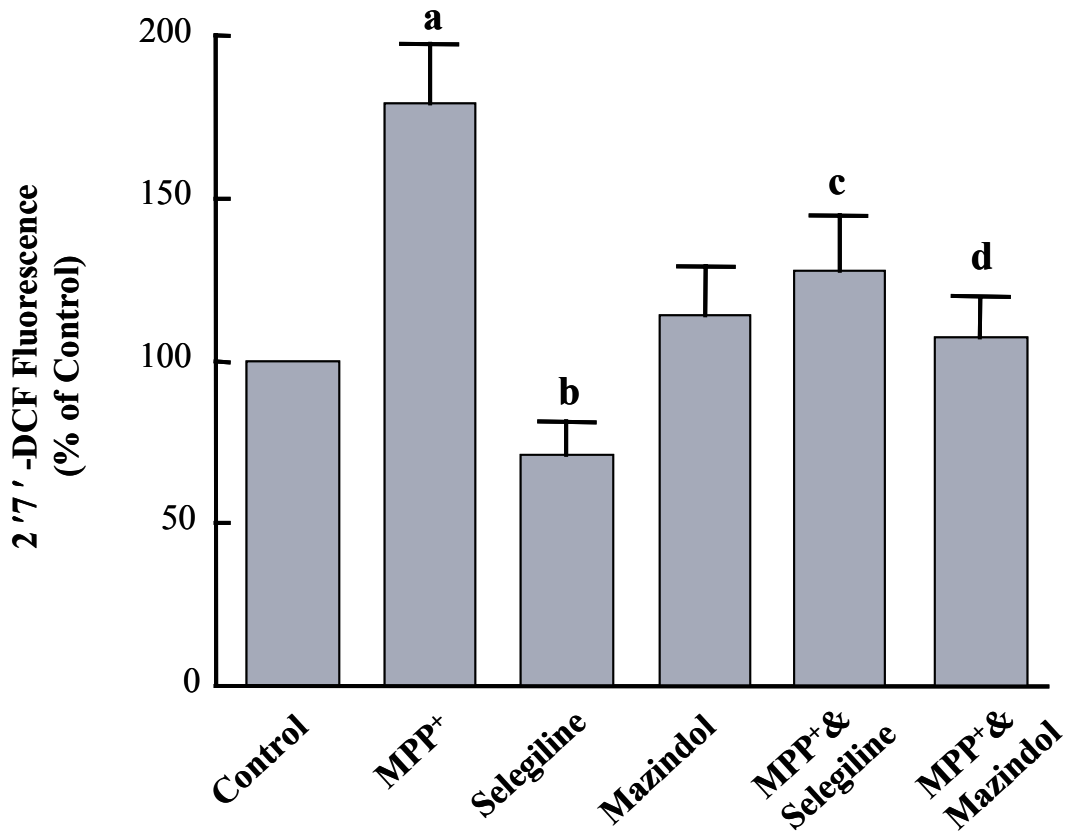


Figure 5.12 Effects of MPP^+ treatment on ROS generation. PC12 cells were pre-treated with 100 μM selegiline or 10 μM mazindol, and then exposed to 100 μM MPP^+, or left untreated. [a]$P<0.01$ when compared to control; [b]$P<0.05$ when compared to control; [c]$P<0.05$ when compared to MPP^+ group; [d]$P<0.05$ when compared to MPP^+ group.

7. Effect of MPP^+ treatment on mitochondrial complex-1 activity

As seen in Table 5.11, complex I activity in hDAT-HEK cells decreased to 56±12% and 42±18% of control after 3 h of treatment with 100 and 1000 μM MPP^+, respectively.

Table 5.12 Effects of MPP^+ treatment on mitochondrial complex-1 activity

Treatment	% of Control ± SEM
Control	100
MPP^+ 100 μM	56±12**
MPP^+ 1000 μM	42+18**

hDAT-HEK cells were incubated in MPP^+ with varying concentrations (100 μ M and 1000 μM) for 3 hr, and the activity of complex I was assayed as described. NADH oxidation was monitored spectrophotometrically at 340 nm and 37°C using a kinetic microplate reader. The enzyme activity is expressed as percentages of control values from five independent experiments.

** $P<0.001$ when compared to control

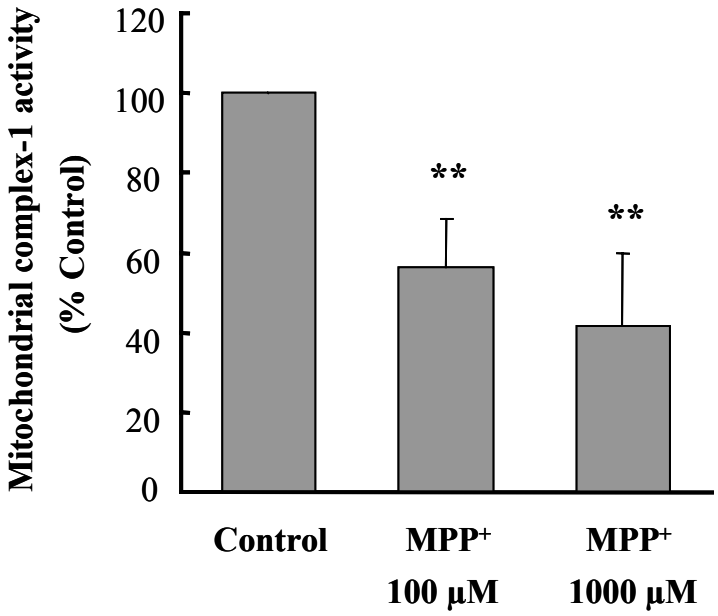


Figure 5.13 Effects of MPP^+ treatment on mitochondrial complex I activity.

$^{**}P<0.001$ when compared to control

8. Effect of MPP^+ treatment on cell viability

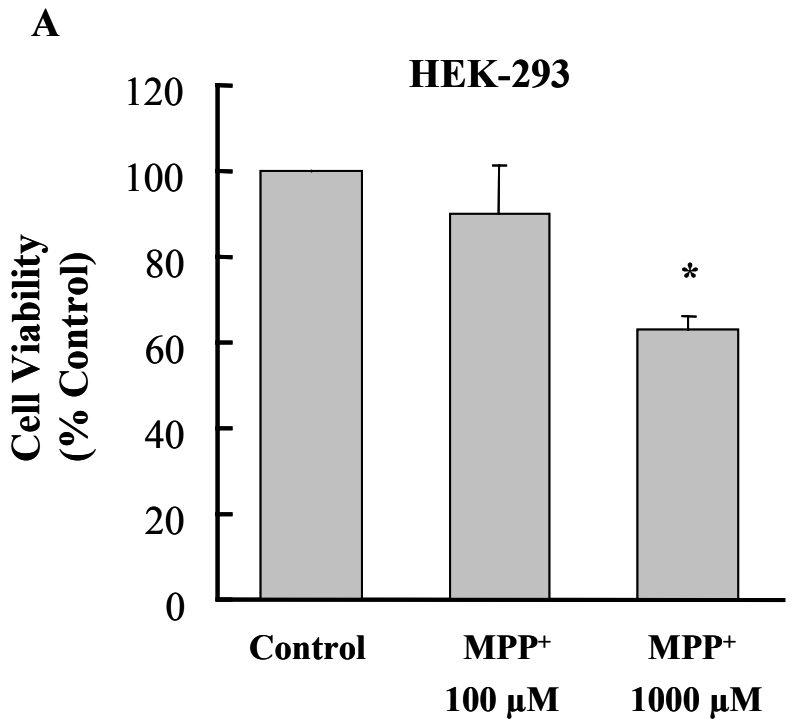
To define MPP^+-mediated cytotoxicity selectively via the dopamine transporter, The viability of HEK-293 and hDAT-HEK cells was assessed following incubation with different concentrations of MPP^+ using the MTT assay. As shown in Figure 5.14, treatment of hDAT-HEK cells for 24 hours at 37^0C with MPP^+ produced a significant reduction in cell viability (100 µM: 58±8% of control, 1000 µM: 49±4% of control). MPP^+ at 1000 µM produced mild toxic effects on HEK-293 cells with a notable decrease in cell viability, but no significant changes in cell viability at 100 µM.

Table 5.13 Effects of MPP^+ treatment on cell viability

	Treatment	**% of Control ± SEM**
HEK-293 cells	Control	100
	MPP^+ 100 μM	90±11
	MPP^+ 1000 μM	63±3*
hDAT-HEK cells	Control	100
	MPP^+ 100 μM	58±8*
	MPP^+ 1000 μM	49±4*

HEK-293 and hDAT-HEK cells were treated with 100 or 1000 μM MPP^+ for 24 h. After incubation, 0.2 mg/ml MTT were added to the medium for 4 hr at 37°C, then the medium was removed and the formazan product dissolved in DMSO. Absorbance was measured at 540 nm. Data represent the mean ± SEM. of eight independent experiments and are expressed as percentage of control.

*$P<0.001$ when compared to control


A
HEK-293
Cell Viability
(% Control)
120
100
80
60
40
20
0
*
Control
MPP+
100 µM
MPP+
1000 µM

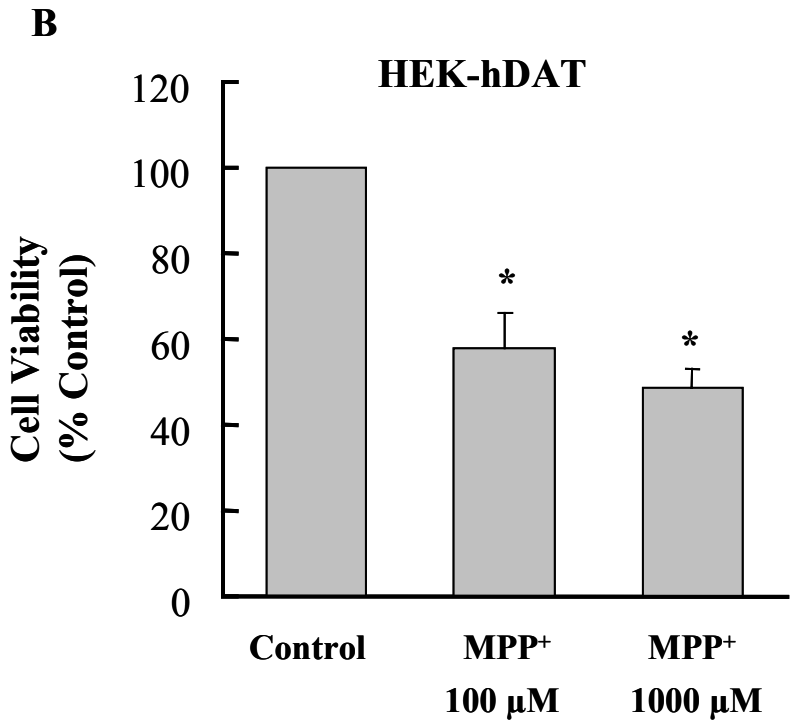

B
HEK-hDAT
Cell Viability
(% Control)
120
100
80
60
40
20
0
*
*
Control
MPP+
100 µM
MPP+
1000 µM

Figure 5.14 Effects of MPP^+ treatment on cell viability. (A) HEK-293, and (B) hDAT stably expressing HEK-293 cells were treated with MPP^+ (100 μM and 1000 μM) for 24 h and cell viability was assessed by the MTT assay.

$^*P<0.001$ when compared to control

CHAPTER 6
DISCUSSION

Effect of MPP^+ on dopamine transporter function

In the present study, the mechanism by which MPP^+ lowers the level of dopamine transporter activity, and asserts the role of DAT in MPP^+-mediated cytotoxicity was investigated. Several studies have demonstrated that MPP^+ elicits a marked decrease in hDAT transport activity, but the mechanisms involved largely remain hypothetical and equivocal. Consistent with earlier studies (Barc et al., 2001; Fonck and Baudry, 1996, 2003; Harrington et al., 1996), we have shown that the down-regulation of hDAT observed in response to MPP^+ (Figure 5.1 and 5.2) occurred in a concentration- and time-dependent fashion. MPP^+ at a concentration of 100 μM was used to investigate the time course of dopamine uptake because this concentration produced the highest reduction in dopamine uptake without affecting the cell culture conditions.

The micromolar concentrations of MPP^+ required for inhibiting dopamine transporter function in the current system are considerably lower than the submicromolar concentrations employed in most studies including the report of Fonck and Baudry, (2001) on the rat PC12 cells. It is possible that the current HEK-293 expression system is more sensitive to MPP^+ perhaps by a more easily redistribution of MPP^+ into the membrane or a less favourable membrane environment for expression of activity of translocated MPP^+.

In this context, it should be pointed out that translocation of dopamine by the transporter is a complex process consisting of multiple steps (Runick and Clark, 1993). Required ion gradients maintained by ion pumping membrane proteins may not be identical at different stages of the cycle of cell growth of the hDAT-HEK cell system used in the present experiments, even though the dopamine transporter protein is stably expressed in this transfected cell line. This may explain the observed variability in [^{3}H]dopamine uptake activity from experiment to experiment.

Functional regulation of DAT could have profound effects on the intensity and duration of dopaminergic synaptic transmission, mechanisms of neurodegeneration and neurotoxicity, and actions of psychostimulant drugs. These studies bring one step forward to understanding the mechanism of DAT regulation. Following experiments are needed to clearly establish discrete molecular basis of the regulation of DAT.

MPP^+-mediated phosphorylation of the dopamine transporter

The mechanism whereby MPP^+ may cause a reduction in DAT activity is phosphorylation. In recent observations it was proposed that dopamine transporter activity was regulated by phosphorylation of DAT (Vaughan et al., 1997; Huff et al., 1997; Zhang et al., 1997). A consequence of phosphorylation is the possible sequestration of the transporter proteins (Pristupa et al., 1998; Melikian and Buckley, 1999; Bauman et al., 2000) and lower activity. The present data however provide evidence that MPP^+ may reduce the activity of DAT by lowering level of DAT on both plasma membrane and in the cytoplasm.

The results also suggest that regulation of dopamine transporter phosphorylation could play a significant role in the modulation of transporter function that accompanies by MPP^+. These findings could have implications for the ability of rapid presynaptic regulatory events to modulate the transporter's role in terminating dopaminergic neurotransmission in vivo. Several lines of evidence support the identification of the phosphoprotein labelled in these studies as the dopamine transporter. The protein was immunoprecipitated using an antiserum demonstrated previously by immunoprecipitation to be specific for the dopamine transporter (Freed et al., 1995; Vaughan, 1995). The protein was extracted from hDAT-HEK cells. Immunoprecipitations were blocked by addition of the immunizing but not an irrelevant peptide. Phosphoproteins of similar apparent molecular weights have been found. This evidence documents the identity of the dopamine transporter as a phosphoprotein.

MPP^+ treatment increased dopamine transporter phosphorylation 0.5-1 fold and decreased dopamine transport activity by 50-60%. These findings suggest that the increase in dopamine transporter phosphorylation and decrease in dopamine uptake each resulted from MPP^+ stimulation. Phosphorylation of transporters may initiate

their removal from the cell surface or may cause a conformational change that interferes with transport. These results do not exclude the possibility that phosphorylation of a protein that interacts with the transporter could indirectly impact transporter function.

MPP^+-decreased dopamine transporter cell surface expression

[^{3}H]WIN 35428, which is a specific ligand for DAT was used to evaluate the effects of MPP^+ on the expression of DAT proteins. The [^{3}H]WIN 35428 binding assay in intact cells was conducted at low temperature to limit endocytosis of the ligand. A significant reduction in the binding of [^{3}H]WIN 35428 was observed after 3 hours incubation with MPP^+ at each of the concentration ranges used (1, 10, 100, and 1000 μM) and this consistent with a decline in DAT activity. Moreover, a notable decrease in B_{max} value for [^{3}H]WIN 35428 binding (Table 5.6) in the total membrane fractions isolated from lysed cells after treatment with 100 μM MPP^+ was observed (Figure 5.6), and this indicates that the MPP^+-induced decrease in DAT correlated with the reduction in the number of available [^{3}H]WIN 35428 binding sites at the cell surface (and also in the cytoplasm). The present results stand to the suggestion that phosphorylation creates transporter protein that is unable to bind [^{3}H]WIN 35428, causing a reduction in the B_{max} value.

The cell-impermeable biotinylated sulfo-NHS-biotin reagent and Western blot analysis were used to demonstrate that MPP^+ decreased both the biotinylated and nonbiotinylated DAT protein levels (Figure 5.7), and this is consistent with the data obtained from the Scatchard analysis (Figure 5.6). The data further indicate a smaller percentage change for the intracellular DAT, compared with that of the plasma membrane (72% of control and 53% of control, respectively). This probably reflects the greater absolute amounts of measurable intracellular DAT, which also includes cell surface membrane DAT. Taken together, these findings suggest that MPP^+-mediated down-regulation DAT activity correlates with an overall lowering of DAT protein expression.

MPP^+-effects on dopamine release are time-dependent

The results show that MPP^+ produces a rapid increase in DA release within a period of 5 min and this increase maximizes after about 10 min of incubation. The time course of synaptophysin phosphorylation by MPP^+ also parallels the potentiation of DA release (Figure 5.8 and 5.11). This apparent action of the kinase (within 10 min) concomitant with an increase in DA release suggests that MPP^+ may affect an early release of DA from the synaptic vesicles.

The regulation of neurotransmitter exocytosis is known to be influenced by intracellular levels of Ca^{2+} (Pristupa et al., 1998). Therefore the role of Ca^{2+} in the mechanism of MPP^+-mediated DA release was investigated. When the cells were treated with EGTA in order to remove Ca^{2+}, the enhancement of DA release by MPP^+ was abolished (Figure 5.9). Accordingly, the results suggest that MPP^+-induced DA release occurs through a Ca^{2+}-dependent exocytosis mechanism, which involves tyrosine phosphorylation. DA can also be released via a Ca^{2+}-independent mechanism (Chang and Ramirez, 1986; Melikian and Buckley, 1999). Using EGTA showed that MPP^+ caused DA to be released in the absence of Ca^{2+}, but at a lower level than with Ca^{2+} (Figure 5.9). MPP^+ is a lipophilic cation, which may be accumulated in nerve terminals through alterations in the plasma membrane potential (Bauman et al., 2000). Thus MPP^+ influx and subsequent depolarisation of the nerve terminal could account for these observations. This influx of MPP^+ may displace DA from vesicular storage causing an increase in cytosolic concentration of DA, which would account for the Ca^{2+}-independent MPP^+-evoked release of DA.

The effect of MPP^+ on dopamine transporter activity is a subject of considerable investigation. MPP^+ has been shown to inhibit uptake of dopamine (Kitayama et al., 1994; Copeland et al., 1996; Huff et al., 1997; Zhang et al., 1997). Phosphorylation of dopamine transporter by MPP^+ has been demonstrated in dopamine transporter transfected cultured cells. This phosphorylation was correlated with inhibition of dopamine uptake (Huff et al., 1997; Vaughan et al., 1997). Similarly, phosphorylation of the serotonin plasmalemma transporter, SERT, has been correlated with a reduction of serotonin uptake and reduction of surface SERT (Blakely et al., 1998). These studies, however, demonstrate that MPP^+ has an additional pronounced effect on the dopamine transporter, that of releasing dopamine.

The fact that the MPP^+-mediated release of dopamine was dependent of extracellular Ca^{2+}, since it was not completely blocked by the Ca^{2+} chelating agent EGTA. Taken together, it is reasonable to suggest that MPP^+ induces exocytosis and reverses transport of dopamine as well.

MPP^+-induced an increase in protein tyrosine phosphorylation

Several lines of study have suggested that protein tyrosine phosphorylation appears to be a critical intracellular signalling event that controls such cellular activities as cell growth, differentiation, motility and apoptosis (Ebadi and Sharma, 2003; Cassarino et al., 2000, Eberhardt and Schulz, 2003). Tyrosine phosphorylation is known to be regulated by the opposing actions of the protein tyrosine kinases and phosphatases (Monteiro and Stern, 1996). Alterations in the normal balance of phosphorylation/ dephosphorylation reactions play a role in a number of clinically important pathologies including PD (Ebadi et al., 2001, Ellis et al., 2001; Cohen, 2001). This study demonstrates that MPP^+ induces rapid tyrosine phosphorylation of several cellular proteins including synaptophysin, which is known to be essential for exocytosis.

One protein potentially involved in the enhancement of DA release by MPP^+ is synaptophysin, a known component of the exocytosis apparatus (Wiedenmann and Franke, 1985; Navone et al., 1986). Mammalian synaptophysin is highly conserved with 88% homology between human, rat and cow and several synaptophysin-like proteins have been identified in various species (Volknandt, 1995). Although the exact role of synaptophysin is not clear, there is evidence for its involvement in various aspects of neurotransmitter release and membrane recycling (Thoidis et al., 1998; Janz and Sudhof, 1998; Becher et al., 1999). Synaptophysin expression levels depend on PC12 clonal identity (Shoji-Kasai et al., 2001). Synaptophysin can be phosphorylated by calcium calmodulin kinase II (Rubinstein et al., 1993) and on tyrosine residues by the protooncogene product pp60c-src (Barnekow et al., 1990). In these studies, the time course of synaptophysin phosphorylation by MPP^+ (Figure 5.11) seems to parallel the potentiation of DA release by MPP^+ (Figure 5.8 and 5.11). Further studies are necessary to establish a causal link between these two events.

MPP^+-mediated mitochondrial complex-1 down-regulation and ROS generation

MPP^+ has been shown to impair cell energy metabolism over time (Ebadi and Shama, 2003, Monteiro and Stern, 1996; Di Stasi et al., 1999; Liu, 1997; Becher et al., 1999Shibaguchi et al., 2000). MPP^+ is believed to induce cellular toxicity through ROS generation and mitochondria functional impairment (Lotharius and O'Malley, 2000; Storch et al., 1999; Ramsay et al., 1986; Fonck and Baudry, 2001). Dopamine and MPP^+ accumulate in the cell via monoamine transporters, in particular the dopamine transporter. Therefore mazindol, a potent inhibitor of DA uptake, should reduce MPP^+ accumulation inside the cell and reduce the level of ROS. The results (Figure 5.12) show that mazindol blocks MPP^+-mediated ROS generation. Selegiline, a selective MAO-B inhibitor, has been shown to lower the metabolic oxidation of DA thereby reducing ROS generation from DA autooxidation (Lai et al., 1993; Lai and Yu, 1997). After pretreating the cells with selegiline the MPP^+-induced generation of ROS is significantly attenuated (Figure 5.12).

Several investigations have indicated that ROS may also be an important contributory factor in the modulation of tyrosine phosphorylation (Monteiro and Stern, 1996; Di Stasi et al., 1999; Lu et al., 2002; Li et al., 1998). In the present study MPP^+ induced a rapid increase in the tyrosine phosphorylation of several proteins (Figure 5.10). However ROS was not immediately induced (data not shown), but became significantly elevated after 60 min of MPP^+ exposure. These results suggest that MPP^+ as well as ROS may play a role in modulating protein tyrosine phosphorylation.

In summary, the data demonstrate that the events mediated by MPP^+ in PC12 cells include early protein tyrosine phosphorylation and elevated DA release via a Ca^{2+} dependent exocytosis mechanism with subsequent overproduction of ROS. Other reports (Lotharius and O'Malley, 2000, Ebadi et al., 2001, Ebadi and Shama, 2003) and these results indicate that ROS may act as an initiator of a downstream cellular toxicity cascade in cells. This present study provides evidence that the neurotoxin MPP^+ elicits unique intracellular events (Figure 6.1). Since, impaired ROS homeostasis has been implicated in PD, elucidating the mechanisms underlining

MPP^+-mediated intracellular events may prove to be important in our understanding of this disorder.

MPP^+-mediated cell degeneration

Reactive free oxygen radicals are as by-products of many metabolic processes (Maher and Davis, 1996). Several factors as inhibition of the mitochondrial respiration, generation of $^{\cdot}OH$ and reduced free radical defence mechanisms causing oxidative stress, have been postulated to contribute to the degeneration of dopaminergic neurons (Muralikrishman and Ebadi, 2001). Cytotoxic $^{\cdot}OH$ has been implicated in dopaminergic neurotoxicity caused by MPTP (Gerlach et al., 1994). The toxic action of MPTP is mediated by its major metabolite MPP^+ (Markey et al., 1984), which is known to be taken up into dopaminergic neurons, destroy nigrostriatal pathway, and produce a Parkinson's disease syndrome (Gerlach et al., 1994). Following uptake of MPP^+, radical generation occurs by both enzymatic and non-enzymatic auto-oxidation of DA (Chiueh et al., 1992). MPP^+ induces a massive release of DA in the striatum, which leads to increased $^{\cdot}OH$ radicals (Obata et al., 2001).

The results have shown that cultured hDAT-HEK cells exposed to MPP^+ exhibit a significant decrease in cell survival. This is consistent with recent reports (Di Stasi et al., 1999; Pang et al., 1988; Schweizer et al., 1995), which suggest that the dopamine transporter is required for MPP^+ mediated cytotoxicity. For instance, MPP^+ treated HEK-293 cells show minimal cytotoxicity compared to hDAT-HEK cells at corresponding concentration of MPP^+. HEK-293 cell death occurred at very high MPP^+ concentration (1000 μM), which most likely was caused by changes in pH or osmolarity of the culture medium.

Thus, MPP^+-induced cell death would be determined by a balance of events including but not necessarily limited to: 1) the capacity of a cell to sequester MPP^+, which in turn would depend upon the number of transporters, storage vesicles, vesicular proton gradient, affinity and rate of uptake, etc.; 2) the propensity of the redistributed neurotransmitter to form oxidative products; and 3) the rate of mitochondrial accumulation of MPP^+ and its ability to access its Complex I binding

site. MPP^+ toxicity *in vivo* is certain to be more complex and affected by many more variables than are present in the simple system used here.

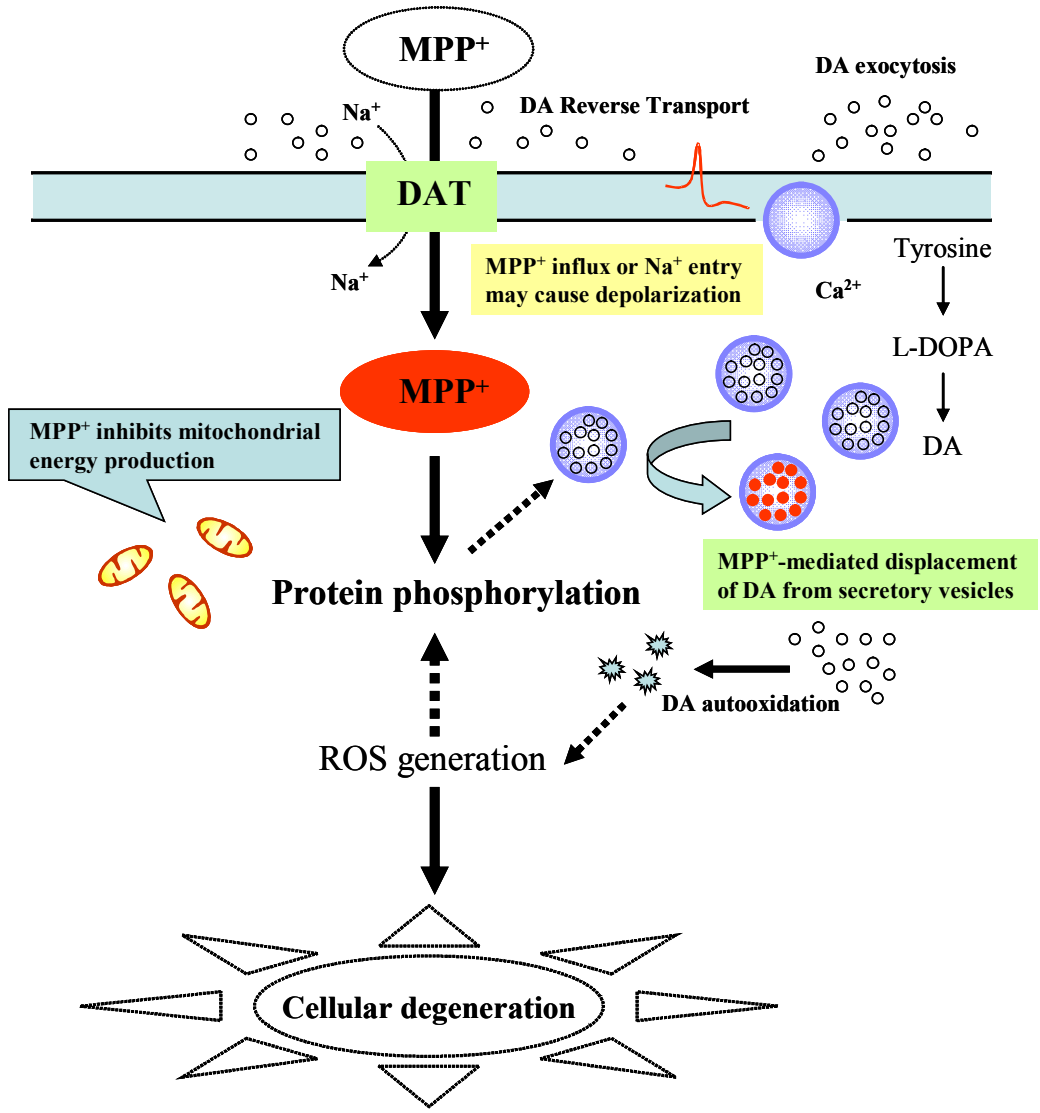


Figure 6.1 Schematic representations the events of how MPP^+ may mediate cellular toxicity. MPP^+ is selectively taken up into the cell via dopamine transporters (DAT) and mediates the displacement of DA from secretory vesicles. Excess cytosolic DA is oxidized to DA metabolites resulting in ROS generation. MPP^+ influx together with Na^{2+} entry may cause depolarisation at nerve terminal and induced DA exocytosis. Inside the cell, MPP^+ triggers protein tyrosine phosphorylation, and DA release from synaptic vesicles with the generation of ROS. These unique events may contribute to neuronal degeneration.

Mechanism for reduced dopamine transporter function

The neuropathological hallmark of Parkinson's disease is an irreversible loss of DA-containing neurons in the substantia nigra. Although the underlying cellular and molecular events in Parkinson's disease are still unknown, post-mortem studies suggest that both oxidative stress and impaired energy metabolism are involved (Bowling and Beal, 1995). Because the neurotoxin 1-methyl-4-phenyl-l, 2,3,6-tetrahydropyridine (MPTP) has been shown to produce Parkinsonian symptoms in primates and rodents (Przedborski and Jackson-Lewis, 1998), it has been extensively used as an animal model of Parkinson's disease. Thus, elucidation of its mode of action has been of paramount importance in understanding and potentially treating this disorder. Toward this end, studies have indicated that l-methyl-4-phenylpyridinium (MPP^+), the active metabolite of MPTP, can block electron transport by binding to the same site as the classic Complex I inhibitor, rotenone, leading to a loss of ATP production (Nicklas et al., 1985; Ramsay et al., 1986, 1991; Denton and Howard, 1987; Krueger, 1990). Rotenone or MPP^+ also produces superoxide anions in submitochondrial particles (Takeshige and Minakami, 1979; Turrens and Boveris, 1980; Hasegawa et al., 1990; Gerlach and Riederer, 1996), adding further support to the basic premise that MPP^+ acts primarily as a mitochondrial toxin.

However, MPP^+ does affect other processes. For example, MPP^+ rapidly induces DA release and subsequent formation of hydroxyl radicals both *in vivo* and *in vitro* (Markstein and Lahaye, 1984; Schmidt et al., 1984; Chang, G. D., and Ramirez, 1986; Rollema et al., 1986; Obata and Chiueh, 1992). Because the timing of this process is comparable with exocytosis (Clarke and Reuben, 1995), the deleterious effects of extracellular DA oxidation may actually precede mitochondrial dysfunction, which occurs more slowly (Chan et al., 1991; Denton and Howard, 1987). Moreover, the rapidity of this process suggests that MPP^+ release DA from intracellular pools *versus* toxin-induced degeneration of dopaminergic terminals.

A consequence of sequestration, however, might be the displacement of DA from vesicular stores. Once in the cytoplasm, DA would be readily auto-oxidized or deaminated (Maker et al., 1981) to produce hydrogen peroxide, superoxide, and reactive quinone species capable of covalently modifying cellular macromolecules (Graham, 1978). Thus, MPP^+-mediated displacement of DA from secretory vesicles

could not only lead to cytoplasmic DA oxidation and generation of free radicals but also to extracellular DA release and oxidation, both of which could contribute to dopaminergic neuronal degeneration.

The fact that MPP^+ can result in both reduction of substrate uptake and enhanced dopamine release is not necessarily conflicting. MPP^+ could have multiple actions affecting the dopamine transporter and it is entirely conceivable that inward and outward transport could be differentially regulated. The data that MPP^+ activation elicits reverse transport of the dopamine transporter could be reconciled with the data showing that MPP^+ mediates transporter trafficking in two ways. There could be two separate phosphorylation events mediated by MPP^+. An alternative scenario is that MPP^+ initially mediates reverse transport but the outward conformation of the transporter triggers internalization and trafficking of the transporter (Melikian and Buckley, 1999). The theory that reversal of transport is due to direct phosphorylation of dopamine transporter is depicted in Figure 6.2. Phosphorylation of the dopamine transporter could affect rate constants for orientation of the transporter in the membrane (Zhang et al., 1997), shifting the equilibrium of transporter conformation to favor outward as opposed to inward transport. MPP^+ could be changing the stoichiometry of Na^+ and Cl^- coupling so that reverse transport is favored, as opposed to inward transport. Phosphorylation of dopamine transporter or an accompanying protein could affect rate constants for orientation of the transporter in the membrane (Zhang et al., 1997) and affinity of the transporter for ions or intracellular dopamine. In support of this theory, the constitutive leak current associated with the dopamine transporter allows the export of cations and is modulated by MPP^+ (Zhu et al., 1997). On the other hand, alterations in activity of ion pumps, such as Na^+, K^+-ATPase activity, could be involved. Phosphorylation of Na^+, K^+-ATPase leads to a reduction in enzyme activity (Logvinenko et al., 1996), which could increase internal sodium and enhance outward transport through dopamine transporter. The actions of MPP^+ and amphetamine on the dopamine transporter are quite similar. Both can block uptake of dopamine and elicit a Ca^{2+}-independent release of dopamine.

The actions of MPP^+ on potentiating DA release appear to depend on extracellular Ca^{2+}, as were demonstrated in the experiments in the presence of EGTA. This MPP^+ may induce the mobilization of Ca^{2+} in order to affect the exocytosis of

DA. Indeed, calcium has previously been implicated in the exocytosis of neurotransmitters and most recently in the release of d-aspartate stored in secretory vesicles in PC12 cells (Nakatsuka et al., 2001). In summary, the results demonstrate that MPP^+ can lead to a Ca^{2+}-dependent release of dopamine and reverse transport through the plasmalemmal dopamine transporter.

Together, these observations suggest a complex rapid regulation of DAT by DA (Figure 6.2). It is not clear if all of these mechanisms are engaged by synaptic release of DA, or even if they are, whether they would oppose one another or occur sequentially. However, one scenario based on the latter idea would be that depolarization would elicit release of DA, as well as transiently lower DAT velocity by reducing DAT cell surface expression. Also, by being a substrate, DA would further reduce DAT cell surface expression by a PKC-dependent mechanism. Both of these mechanisms would allow the released DA to diffuse across the synaptic cleft and interact with its postsynaptic receptors, initiating signaling cascades. Subsequently, by interaction with presynaptic D_2 autoreceptors, DA would not only increase DAT surface expression and velocity of uptake, but also inhibit further stimulation-evoked release of DA, thereby eliciting mechanisms that would work in concert to limit prolonged stimulation of postsynaptic DA receptors. Since cocaine can transiently up-regulate DAT expression, as well as increase extracellular DA by DAT inhibition, the net result of DAT regulation by cocaine may be even more complex than that for DA. These questions are likely to be resolved by using a combination of approaches, such as electrophysiological recording of DAT associated currents and real-time imaging techniques in cultured DA neurons (Ingram et al., 2002).

While these results suggest that the substrate-induced trafficking of the transporter may shape dopamine transport capacity, many questions remain unanswered. Particularly, does DAT undergo functional modification in response to MPP^+ prior to its cell surface redistribution? Or alternatively does DAT, in response to MPP^+, simply leave the plasma membrane as an active carrier. New types of experiments monitoring both DAT activity and trafficking at a high time resolution are required to answer these questions.

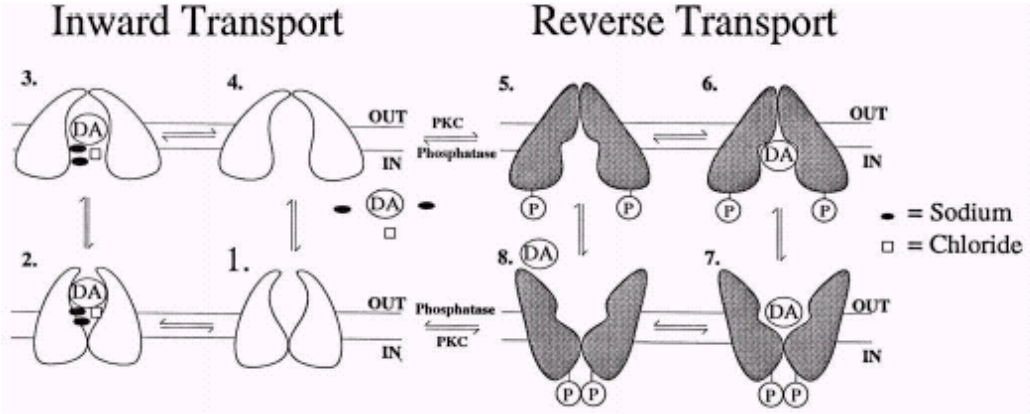


Figure 6.2 Schematic representing MPP^+-induced phosphorylation of dopamine transporter resulting in enhanced outward transport of dopamine. The left panel 1–4 shows the normal activity of the dopamine transporter under normal conditions, whereby extracellular dopamine, sodium ions and chloride ions are transported to the intracellular side of the membrane. In the right panel 5–8, a protein kinase C-mediated phosphorylation of the transporter or accompanying protein would change the conformation of the transporter such that its affinity for intracellular dopamine is significantly increased. Inward transport of either dopamine or amphetamine could increase the number of inward facing transporters, expose the relevant protein kinase C consensus sites and permit the necessary conformational change. The presence of dopamine transporter antagonists could stabilize the outward facing transporters and block reverse transport of dopamine.

Reactive oxygen species, the signaling system involved in dopamine transporter regulation

The monoamine and GABA transporters contain two conserved cysteine residues on the large extracellular loop. These cysteine residues are highly susceptible to oxidation and have been shown in DAT to be critical for transporter expression and activity (Wang et al., 1995). Uptake of [^{3}H]DA, [^{3}H]5-HT, and [^{3}H]GABA was inhibited by generators of ROS, likely via a mechanism that involves oxidation of these cysteine residues (Berman et al., 1996; Braughler, 1985; Debler et al., 1985; Fleckenstein et al., 1997; Haughey et al., 2000; Kokoshka et al., 1998; Pogun et al., 1994). Interestingly, norepinephrine transporter was not affected by ROS generators. While norepinephrine contains these same cysteine residues, it has been hypothesized that amino acid residues flanking the cysteines confer a protein-folding pattern prohibiting ROS interaction with the cysteine residues, rendering the NET resistant to down-regulation by ROS (Haughey et al., 1999). Administration of methamphetamine increased ROS and decreased uptake of [^{3}H]5-HT and [^{3}H]DA, but not [^{3}H]GABA (Fleckenstein et al., 1997; Haughey et al., 2000; Kokoshka et al., 1998; Metzger et al., 2000). DA and 5-HT are easily oxidized, and can lead to the production of ROS after their release by methamphetamine (Wrona et al., 1997). GABA, unlike 5-HT and DA, is not readily oxidized at physiological pH. Furthermore, it is not released by methamphetamine. Consequently, it is likely that ROS generated by methamphetamine administration was not close enough in proximity to GABA transporters to affect them. Indeed, ROS may play a role in MPP^+-induced inhibition of DAT.

Presynaptic receptor-mediated dopamine transporter regulation

Presynaptic "autoreceptors" are activated by the same neurotransmitter that is released from the neurons on which the receptors are localized. They can be localized on the soma and dendrites of the neuron, where they modulate neuronal firing, the so-called "activity modulating autoreceptors." They are also associated with the terminal regions or axon varicosities, where they modulate synthesis ("synthesis-modulating") and release ("release-modulating") of the neurotransmitter. In most cases, activation

of presynaptic autoreceptors results in inhibition of firing, synthesis, and/or release, thus mediating a short inhibitory feedback loop. Autoreceptors respond not only to their endogenous ligand, but also to exogenously administered receptor agonists and antagonists. Since autoreceptors and transporters are present on the same neurons, autoreceptors provide another potentially important mechanism by which neurotransmitters and drugs could regulate Na^+/Cl^--dependent neurotransmitter transporters.

In studies investigating the regulation of transporter activity by presynaptic autoreceptors, there appears to be a correlation between the experimental paradigm used and whether positive or negative results were obtained. Receptor modulation of transporter activity has been observed most frequently with high-speed voltametric measurements of exogenous neurotransmitter clearance. In contrast, results measuring radiolabeled neurotransmitter release/uptake in synaptosomes have been largely negative.

Like α_2-adrenergic receptors, DA D_2 receptors function both as presynaptic autoreceptors and as postsynaptic receptors (Figure 6.3). It is well established that activation of D_2 autoreceptors inhibits DA neuronal firing, DA synthesis, and DA release. More recently, several studies have provided in vivo evidence that D_2 receptors, presumably autoreceptors, also regulate DAT activity. The first evidence, albeit somewhat indirect, was based on the "zero net flux" quantitative microdialysis method to measure changes in DA uptake (Parsons et al., 1993). Repeated cocaine administration led to an up-regulation of DA uptake. However, when the D_2 receptor antagonist pimozide was co-administered with cocaine, DAT up-regulation did not occur. Subsequently, Cass and Gerhardt (1994) provided direct in vivo evidence that D_2 receptors regulate DAT activity. They used high-speed chronoamperometry to measure the clearance of locally applied DA in three DA neuronal projection areas – the striatum, nucleus accumbens, and prefrontal cortex – in urethane-anesthetized rats. They found that local application of the selective D_2 receptor antagonist raclopride, just prior to DA, increased both the amplitude and time course of the DA signals in all three areas, changes consistent with reduced DAT activity. Using a similar experimental approach, local application of the D_2 receptor antagonist haloperidol

was shown to decrease DA clearance rates by 62% in rat dorsal striatum (Rothblat and Schneider, 1997).

A study by Meiergerd and colleagues (1993) provided several more critical pieces of evidence supporting the idea that D_2 autoreceptors regulate DAT function. They first conducted in vitro kinetic experiments using rotating disk electrode voltametric and suspensions of minced rat striatum. With this approach, they were able to test the effect of a D_2 receptor agonist and found that the initial velocity of DA uptake was increased by 130% in the presence of the D_2 agonist quinpirole (100 nM). The effect of quinpirole was blocked by the D_2 receptor antagonist sulpiride (5 μM). Importantly, using isolated striatal tissue ruled out the possibility that the D_2 receptors that affected DAT activity did so via long-loop neuronal feedback. Thus, these results strongly suggested that it was D_2 autoreceptors, such as those present on the nigrostriatal neuronal terminals that regulated DAT.

It could be that D_2 receptor modulation of DAT activity is a rapid and modest effect providing transient, fine-tuning of uptake and requires a high-speed system such as voltametry for detection. Alternatively, it could be that critical signaling components necessary for this regulation are lost in the preparation of synaptosomes. Results with null-mutant mice lacking D_2 receptors have further strengthened the idea that it is the D_2 receptor subtype that interacts with the DAT (Dickinson et al., 1999).

Taken together, these studies suggested that in addition to modulating DAT activity, DAT trafficking, and DA release have a fourth function: providing fine-tuning of DAT activity. However, in the latter case, autoreceptor activation results in acceleration of DAT activity, in contradistinction to the usual inhibitory role played by autoreceptors. Thus, D_2 autoreceptor activation cannot only reduce DA release, but also can increase DA uptake (Figure 6.3). Overall, following release, DA will interact with postsynaptic receptors to trigger downstream effects and with presynaptic autoreceptors to produce a coordinated set of effects to limit further postsynaptic receptor stimulation.

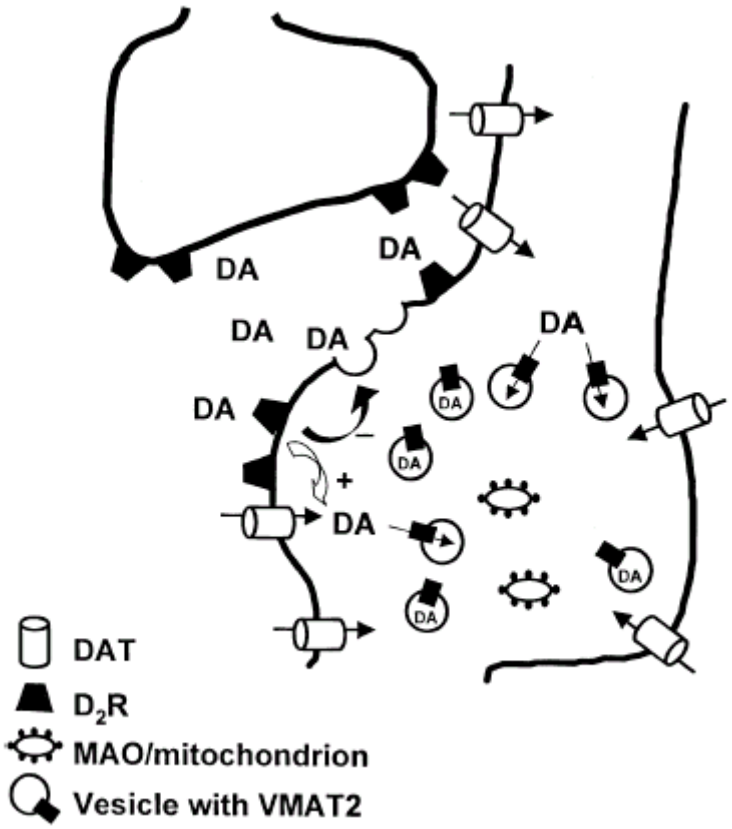


Figure 6.3 Activation of presynaptic DA D_2 autoreceptors on DA neurons transiently inhibits DA release and accelerates DAT activity. When DA is released, it interacts with both presynaptic and postsynaptic D_2 receptors (D_2Rs). It is not clear whether the D_2 autoreceptors that regulate release and transport are exactly the same receptors and which signaling mechanisms are involved. Nonetheless, this regulation will produce a coordinated set of effects, reduced release and accelerated uptake, to limit further postsynaptic receptor stimulation.

MPP^+ and acute changes in dopamine transporter function: implications for dopamine neurotoxicity

It is well established that high-dose administration of some psychostimulants and neurotoxins causes profound and persisting deficits in central dopamine neurons in rodents (Hotchkiss et al., 1980; Wagner and Ricaurte, 1980) non-human primates (Woolverton et al., 1989) and humans (Wilson et al., 1996), but with different expressions. For example, MPP^+ treatment causes long-term decreases in tyrosine hydroxylase activity, dopamine transporter function, and/or concentrations of associated neurotransmitters and metabolites. These deficits are thought to be neurotoxic responses that likely reflect destruction of corresponding monoamine axons and/or terminals.

Changes in dopamine disposition resulting from administration of MPTP analogs appear necessary for long-term toxicities. Still, it is unclear whether increases in extraneuronal dopamine levels or a redistribution of intraneuronal dopamine are responsible for these effects. Early data suggested that extraneuronal dopamine might be of importance; for instance, pretreatment with dopamine post-synaptic receptor antagonists prevents the long-term dopaminergic deficits (Sonsalla et al., 1986). In contrast to these findings, other evidence implicates changes in the disposition of intraneuronal dopamine as being important. For instance, LaVoie and Hastings (1999) demonstrated dissociation between extracellular dopamine concentrations; formation of dopamine oxidation products and long-term dopaminergic deficits is not exclusively the result of increases in extracellular dopamine concentrations. Based on these findings, it is suggested that MPP^+ might redistribute dopamine from the reducing environment within synaptic vesicles to extravesicular intracellular oxidizing environments, thus generating oxygen radicals and reactive metabolites within dopamine neurons that trigger selective dopamine terminal loss.

Although the question of whether intra- or extra-neuronal dopamine mediates persistent deficits in monoaminergic systems remains unresolved, it is also possible that MPP^+ mediates neurotoxicity by increasing the sensitivity of DAT to lysosomal degradation resulting in a shorter half-life (Figure 6.4). The data presented here forms the basis for further studies towards understanding the neurotoxic processes that occur

in PD. The decrease in DAT function could involve i) DAT phosphorylation; ii) enhanced internalisation and degradation of DAT, iii) reduction in DAT protein expression, or a combination of all three (Figure 6.4). However, these avenues remain to be explored further.

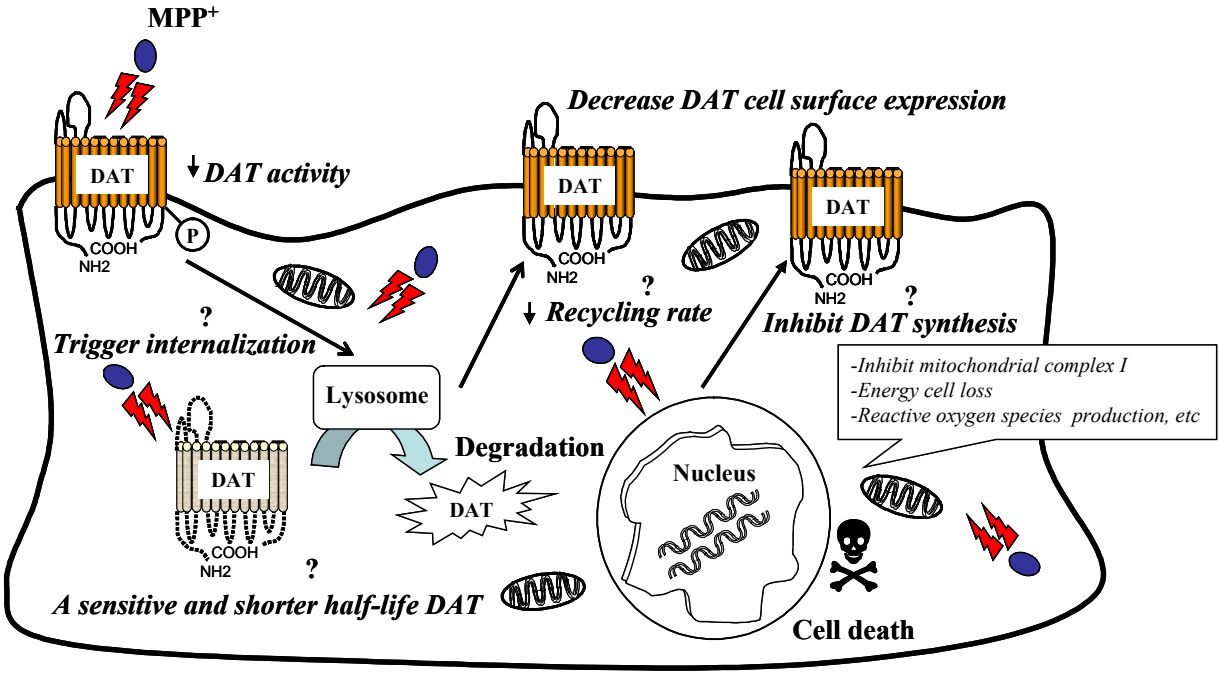


Figure 6.4 schematic representations the events of how MPP^+ may mediate cellular toxicity by involvement with DAT in the cells. MPP^+ is selectively taken up into the cell via DAT, triggering internalisation and degradation of DAT. A consequential reduction in DAT at the cell surface and in the cytoplasm lower DAT activity, related to PD while, MPP^+ promotes the development of cytotoxicity neurological sequalae (Chagkutip et al., 2003).

CHAPTER 7
CONCLUSION

The present experiments were designed to determine the dose response and time-dependence of MPP^+ effects on DAT activity and binding sites, the degree to which binding site changes paralleled dopamine uptake changes, and the mechanism involved. Given the alternative and possibly overlapping hypotheses as to the source and sequelae of MPP^+-induced free radicals, the present study used radioactive ligands together with pharmacological and molecular techniques to determine the role of DAT in MPP^+-mediated toxicity in cell culture model.

The [^{3}H]dopamine uptake experiments were performed to study the effect of MPP^+ on dopamine transporter function.

- Incubation with MPP^+ caused a concentration- and time-dependent decrease in [^{3}H]dopamine uptake.

The [^{32}P]orthophosphate labelling and immunoprecipitation experiments were performed to characterization of dopamine transporter phosphorylation.

- Treatment of the cells with 100 μM MPP^+ during [^{32}P]orthophosphate labelling resulted in a dramatic increase in the level of DAT phosphorylation by 10 min, with maximum levels reached by 20-60 min.

The [^{3}H]WIN 35428 binding and dopamine transporter cell surface biotinylation experiments were performed to examine the effect of MPP^+ on dopamine transporter cell surface expression.

- Incubation with increasing concentrations of MPP^+ (1-1000 μM) for 3 h, there was a concentration-dependent reduction in [^{3}H]WIN 35428 binding sites in the intact cells.
- Consistent with the reduction observed in the intact cell, treatment with MPP^+ 100 μM for 3 h significantly decreased the B_{max} value in the cell membrane fractions.
- MPP^+ significantly reduced the levels of biotinylated and nonbiotinylated DAT protein. This parallels closely the reduction in [^{3}H]dopamine uptake and the level of cell surface [^{3}H]WIN 35428 binding after MPP^+ treatment.

The [^{3}H]dopamine release experiments were performed to study the effect of MPP^+ on dopamine release.

- When the cells were pre-treated with MPP^+, for 5-60 min prior to the addition of secretagogue, significantly increased DA release was apparent after 10 min of stimulation. The level of DA release remained constant for up to 60 min.
- In the presence of EGTA, MPP^+-mediated increase in DA release was significantly reduced. These results suggest that Ca^{2+} is required for MPP^+-induced DA release.

The coimmunoprecipitation and Western blot experiments were performed to characterization of protein tyrosine phosphorylation.

- After treating the cells with 100 μM MPP^+ there was an initial rapid increase in the phosphotyrosine immunoreactivity of several proteins varying in size from 28, 35, 38 and 57 kDa. The increased phosphotyrosine immunoreactivity (up to 10 min) was followed by a decrease between 20-60 min of MPP^+ incubation to levels slightly higher than the control.

- Among the proteins that underwent tyrosine phosphorylation in response to MPP^+ is synaptophysin. MPP^+ caused a maximally tyrosine-phosphorylated synaptophysin after 10 min, and which increased minimally over a period of 20-60 min. This time-response profile parallels the synaptophysin phosphorylation and dopamine release.

Intracellular ROS formation and ubiquinone oxidoreductase (complex I) activity assays were performed to examine the effect of MPP^+ on mitochondrial complex-1 activity and ROS generation.

- MPP^+-induced mitochondrial complex-1 activity impairment and ROS formation that could be fully blocked by pretreating with the dopamine uptake inhibitor, mazindol and the MAO-B inhibitor, selegiline.

MTT experiments were performed to investigate the effect of MPP^+- on cell viability.

- MPP^+ produced a significant reduction in cell viability in hDAT-HEK cells but not HEK cells.

In summary, these observations demonstrate that MPP^+ alters DAT activity in a manner that parallels with a reduction in dopamine transporters cell surface expression. Furthermore, the results speculate that this MPP^+-induced decreased in dopamine uptake may be mediated by internalisation of DAT via a phosphorylation process. Other related events such as dopamine release, overproduction of ROS, impairment of Complex I activity, and cell death perhaps relates to an important means of physiologically regulating the cellular toxicity.

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