STUDIES OF CELL DEATH IN PARKINSON’S DISEASE USING ORGANOTYPIC CELL CULTURES

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B.Sc (Hons)

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A thesis submitted to the University of Adelaide in fulfillment of the requirements for the degree of Doctor of Philosophy
DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and that, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due references has been made in the text.

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Tuyet Tran

Date:
The following articles have been published or accepted for publication or presentation during the period of my PhD candidature, and sections of these articles are included in the present thesis.

**Publications under review:**

**Tuyet T.B Tran, Peter C. Blumbergs and James A. Temlett (2008)**

The effects of MPTP and rotenone on Dopaminergic neurons in Organotypic cell culture.

*Journal of Neurotoxicology*

**Abstracts:**

**Tuyet T.B Tran, Peter C. Blumbergs and James A. Temlett (2007)**

The Effects of MPTP and rotenone on Dopaminergic neuronal growth in Organotypic Cell Culture. Abstracts of the 8th International Conference AD/PD 2007, Salzburg, Austria. March 14 – 18, p327
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## ABBREVIATIONS

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<tr>
<td>AA</td>
<td>ascorbic acid</td>
</tr>
<tr>
<td>AA(A/D)C</td>
<td>aromatic l-amino acid decarboxylase</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis-inducing factor</td>
</tr>
<tr>
<td>α-syn</td>
<td>alpha synuclein</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
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<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AMPT</td>
<td>alpha-methyl-p-tyrosine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>antero posterior</td>
</tr>
<tr>
<td>APTP</td>
<td>1-amino-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>APP+</td>
<td>1-amino-4-phenyl-pyridinium</td>
</tr>
<tr>
<td>ARs</td>
<td>aldose reductase</td>
</tr>
<tr>
<td>ART</td>
<td>artemin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated protein X</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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BG - basal ganglia
BJAB - B lymphoma cell line
BMPs - bone morphogenic factors
BSA - bovine serum albumin
BSS - balanced salt solution
Ca$^{2+}$ - calcium
cAMP - 3'5'-cyclic adenosine monophosphate
CGC - cerebellar granule cells
CoQ(10) - coenzyme Q(10)
COMT - catechol-O-methyl transferase
CNS - central nervous system
Cu-SOD - copper superoxide dismutase
cGMP - Guanosine 3,5-cyclic monophosphate
DA - dopamine
DAergic - dopaminergic neurons
D1 - dopamine receptor, type 1
D2 - dopamine receptor, type 2
DAB - 3,3'-diaminobenzidine-Tetrahydrochloride
DAPI - 4,6-diamino-2-phenolindol dihydrochloride
DAT - dopamine transport
DBS - deep brain stimulation
DCF - 2',7'-dichlorofluorescin
DIV - days in vitro
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<td>DMEM</td>
<td>modified basal medium, Eagle</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DLBD</td>
<td>dementia with LB disease</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DOPAL</td>
<td>dihydroxyphenylacetaldehyde</td>
</tr>
<tr>
<td>DOPET</td>
<td>3, 4-dihydroxyphenylethanol</td>
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<tr>
<td>DPX</td>
<td>DePex mounting medium</td>
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<tr>
<td>DTC</td>
<td>dithiocarbamate</td>
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<td>DV</td>
<td>dorso ventral</td>
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<td>E</td>
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<td>EDTA</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>enkephalin</td>
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<tr>
<td>Epo</td>
<td>erythropoietin</td>
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<tr>
<td>ERKs</td>
<td>extracellular signal-regulated kinases</td>
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<td>ETC</td>
<td>electron transport chain</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FGF8</td>
<td>fibroblast growth factor-8</td>
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<tr>
<td>FD</td>
<td>fluorodopa</td>
</tr>
<tr>
<td>FJC</td>
<td>fluoro jade C</td>
</tr>
<tr>
<td>Foxa2</td>
<td>Forkhead box protein A2</td>
</tr>
<tr>
<td>FP</td>
<td>floor plate</td>
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FPD  - familial Parkinsons Disease
GABA  -gamma-aminobutyric acid
GADPH - glyceraldehyde-3-phosphate dehydrogenase
GBSS -Geys balanced salt solution
GbX2  - gastrulation brain homeobox 2
GDNF -glial-derived neurotrophic factor
GDF5 -growth/differentiation factor 5
GFAP -glial fibrillary acidic protein
GLU  -glutamate
GM-CSF -granulocyte macrophage colony-stimulating factor
GP  -globus pallidus
GPe  -external segment of the globus pallidus
GPi  -internal segment of the globus pallidus
GPx  -glutathione peroxidase
GSSG -oxidised glutathione
GSH  -glutathione
h/hr -hour
H&E  -haemoatoxylin and eosin
HBSS -Hank’s balanced salt solution
HB-EGF -heparin-binding epidermal growth factor
Hesc -human embryonic stem cells
HL-60 -human promyelocytic leukemia cell line
HP100 - $H_2O_2$-resistant cell line
HRP - horseradish peroxidase
HT1080 - human fibrosarcoma cell line
H₂O₂ - hydrogen peroxide
H₂SO₄ - sulphuric acid
5-HT - serotonin
6-OHDA - 6-Hydroxydopamine
Fe²⁺ - iron
IBMX - isobutylmethylxanthine
IMVS - Institute of Medical and Veterinary Science
IFN-γ - interferon gamma
IL-1β - interleukin-1β
IL-6 - interleukin-6
iNOs - inducible nitric oxide synthase
IPD - idiopathic Parkinson’s disease
JNK - Jun N-terminal kinase
LB - Lewy body
L-dopa - levodopa
LDH - lactate dehydrogenase
Lmx1 - Lim-domain transcription factors
LPS - lipopolysaccharide
MAPK - Mitogen-activated protein (MAP) kinases
mDNs - midbrain/mesencephalic dopaminergic neurons
M - molar
MAO-B - monoamine oxidase B

MHB - mid-hindbrain organizer

MHJ - midbrain–hindbrain junction

μm - micromolar

μl - microlitres

mg - milligrams

mins - minutes

MN9D - midbrain-derived dopaminergic neuronal cell line

Mn-SOD - magnesium superoxide dismutase

Mn–EBDC - manganese ethylene-bis-dithiocarbamate

MPDP - 1-methyl-4-phenyl-2,3-dihydropyridinium

MPPC - 1-methyl-4-phenylpyridine

MPPP - 1-methyl-phenyl-propion-oxy-piperidine

MPP+ - N-methyl-1-4-phenylpyridinium ion

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

Mw - molecular weight

NA - noradrenaline

NAC - N-acetylcysteine

NDI1 - single-subunit nicotinamide adenine dinucleotide (reduced) dehydrogenase of Saccharomyces cerevisiae

NMDA - N-methyl-D-aspartic acid

NADPH - Nicotinamide adenine dinucleotide phosphate

NADH - Nicotinamide adenine dinucleotide
NADH-DH  - NADH-dehydrogenase
3-NT  - 3-Nitrotyrosine
n  - number
nM  - nanomolar
NGF  - nerve growth factor
NM  - neuromelanin
NO\textsuperscript{*}  - nitric oxide
NTF  - neurotrophic factor
NRT  - neurturin
NSAIDs  - nonsteroidal anti-inflammatory drugs
NT-3  - neurotrophin-3
NT-4/5  - neurotrophin-4/5
NMDA  - N-methyl-D-aspartate
NF-κB  - nuclear factor κB
Nurr1  - Nuclear receptor related 1
O\textsubscript{2}  - oxygen
OH\textsuperscript{*}  - hydroxyl radical
\cdot O\textsubscript{2}\textsuperscript{-}  - superoxide anion radical (superoxide)
ONOO–  - peroxynitrite
OS  - oxidative stress
Otx2  - homeobox protein
PARP  - poly (ADP-ribose) polymerase
Pax-  - paired-like homeodomain proteins
PBS - phosphate buffered saline
PC12 cells - rat pheochromocytoma cell line
PD - Parkinson’s disease
P7 - postnatal day 7 (seven day old)
P75 - low affinity neurotrophin receptor
PBS - phosphate buffered saline
PET - positron emission tomography
Pitx3 - paired-like homeodomain transcription factor 3
PKC - Protein Kinase C
PPX - pramipexole
PSP - persephin
Ref - reference
ROI - reactive oxygen intermediates
ROP - ropinirole
ROS - reactive oxygen species
RRF - retrorubral field
SAPK - stress-activated protein kinase
SD - standard deviation
SEM - standard error of the mean
sFas - soluble Fas
Shh - Sonic hedgehog
SH-SY5Y - human dopaminergic cells
SK-N-SH - human neuroblastoma cell line
SN  - substantia nigra (A9)
SNpc  - substantia nigra pars compacta
SNpr  - substantia nigra pars reticulate
SOD  - superoxide dismutase
SP  - substance P
SPC  - Pierce peroxidase conjugated streptavidin tertiary
STN  - subthalamic nucleus
ST  - striatum
RPM  - revolutions per minute
TBARS  - thiobarbituric acid reactive substances
TBS  - Tris buffered saline
TH  - tyrosine hydroxylase
TH-ir  - tyrosine hydroxylase immunoreactive
TGF-β  - transforming growth factor beta
TMB  - 3,3′,5,5′-Tetramethylbenzidine
TNF  - tumor necrosis factor
TNFα-R1  - tumor necrosis factor alpha receptor 1
TRADD  - TNFRSF1A-associated via death domain
TUNEL  - terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
Trk  - tyrosine receptor kinase
UV  - ultraviolet
VM  - ventral mesencephalon
VMAT  - vesicular monoamine transporter
VMB - ventral midbrain
VTA - ventral tegmental area (A10)
Wnt1 - Wingless-type MMTV integration site family
WT - wild type
Zn-SOD - zinc superoxide dismutase
Z-DEVD-fmk - Caspase-3 Inhibitor
°C - degrees Celsius
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ABSTRACT

In this study we aimed to investigate the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone neurotoxins on dopaminergic (DAergic) neuronal survival using ventral mesencephalic (VM) organotypic cell culture derived from postnatal rat pups (P4-5) and immunocytochemistry for tyrosine hydroxylase (TH) as a marker of DAergic cells. In addition, we examined the neuroprotective effects of glial cell line-derived neurotrophic factor (GDNF) on TH-ir cells exposed to MPTP and rotenone as a possible treatment for PD.

The TH-ir cells in co-cultures with striatum (ST) as a target grew better than when VM was cultured alone and that TH-ir cells in co-cultures could be maintained without using conditioned and trophic media. We treated 7 day and 14 day co-cultures at different times with varying MPTP and rotenone concentrations and found 14 day old cultures were more vulnerable than 7 day old co-cultures to the effects of either neurotoxin with TH-ir cell numbers significantly lower in 14 day cultures compared to 7 day cultures. Both neurotoxins induced a dose-dependent TH-ir cell reduction in the co-cultures. In addition we compared the toxicity of MPTP and its active metabolite 1-methyl-4-phenylpyridinium (MPP+) as the neurotoxic effects of MPTP on DAergic cells depends on its conversion to MPP+ by astrocytes. We found no significant difference in TH-ir cell reduction in co-cultures treated with MPTP and MPP+. Rotenone was more toxic than MPTP with less TH-ir cell survival in the weeks post treatment. GDNF exposure produced increased cell size and significant increases in TH-ir cell branching in co-cultures in a dose-dependent manner. Post treatment of GDNF against MPTP and
rotenone provided significant neuroprotection as TH-ir cell survival was at the lower neurotoxin doses and not at the higher doses.
CHAPTER 1:
OVERVIEW OF PARKINSON’S DISEASE
1.0 INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder that is estimated to affect approximately 1% of the population older than 65 years of age (Lang and Lozano 1998a, b). Clinically the cardinal symptoms was first described by James Parkinson (1817) (Parkinson 2002) with most patients presenting bradykinesia, resting tremor, rigidity, and postural instability. A number of patients also suffer from autonomic, cognitive, and psychiatric disturbances (Aarsland et al. 2007). The major symptoms of PD result from the profound and selective loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNC), but there is widespread neuropathology with the SNC becoming involved later toward the middle stages of the disease (Braak et al. 2003). The pathological hallmarks of PD are round eosinophilic intracytoplasmic proteinaceous inclusions termed Lewy bodies (LBs) and dystrophic neurites (Lewy neurites) present in surviving neurons (Forno 1996). PD is primarily a sporadic disorder and its specific etiology is incompletely understood, but with the recent insights provided through studying the genetics, epidemiology, and neuropathology of PD, and the development of new experimental models have improved our understanding.

1.1 INCIDENCE AND PREVALENCE OF PD

PD is a common neurodegenerative disorder, characterized by neuronal cell loss in the substantia nigra (SN) and subsequently reduced secretion of dopamine (DA). PD is the second most common neurodegenerative disease after Alzheimer’s disease, affecting up to 1% of the elderly population over the age of 65. Epidemiological studies have estimated a cumulative prevalence of PD of greater than one per thousand with the
prevalence being limited to senior populations; this proportion increases nearly 10-fold. A family history of risk factors is a key factor for PD development. Indeed, the estimated genetic risk ratio for PD is approximately 1.7 (70% increased risk for PD if a sibling has PD) for all ages, and increases over 7-fold for those under 66 years of age (Langston 1998; Rocca et al. 2005; Levy 2007). The age-adjusted prevalence rate of PD revealed from a pilot study of at least a 42.5% increase in the disease compared to 1966 (Chan et al. 2001). The role for genes contributing to the risk of PD is also significant. Recently, monogenically PD as familial PD (FPD) has been identified and seven causative genes for FPD have been identified so far. Thus, the role of genetic factors and also increasing age play an important risk factor for the development of PD.

1.2 CLINICAL CHARACTERISTICS OF PD

Resting tremor at a frequency of 4-6 Hz occurs at rest but decreases with voluntary movement in about 70% of Parkinson’s patients. Rigidity refers to increased resistance (stiffness) initially occur only to trunk region but also extends to passive movement of limbs. Bradykinesia (slowness of movement), hypokinesia (reduction in amplitude, and akinesia (absence of normal initiation of movement) manifest as a variety symptoms, including paucity of normal facial expression, drooling, microphagia, and decreased stride length during walking. PD patients also develop a stooped posture and possibly lose postural reflexes. Freezing of gait, the inability to begin a voluntary movement such as walking is a common symptom of Parkinsonism. Impairment in different cognitive domains such as executive functions, language, memory, and visuospatial skills occurs frequently in PD even in the early stages of the disease (Caballol et al. 2007).
1.3 NEUROCHEMICAL AND NEUROPATHOLOGICAL FEATURES OF PD

1.3.1 Cell loss in PD

Idiopathic Parkinsonism (IPD) is characterized by progressive and profound loss of neuromelanin containing DAergic neurons in the substantia nigra pars compacta (SNpc) (Forno 1996). Moreover, slight gliosis and neuronal loss in the locus coeruleus, dorsal vagal nucleus with variable involvement of the nucleus basalis Meynert, and other subcortical nuclei have been reported (Chung et al. 2003). Degeneration of pigmented neuronal systems located in the brain stem, particularly in SNpc, is the most striking pathological feature of PD. This causes striatal DA deficiency and all the major motor PD symptoms.

The pattern of SNpc cell loss correlates with the level of expression of DA transporter (DAT) mRNA (Uhl et al. 1994) and is consistent with the level of DA depletion being most pronounced in the dorsolateral putamen (Jones 1999), the main site of projection for these neurons. At onset of symptoms, putam ental DA is depleted ~80%, and ~60% of SNpc DAergic neurons have already been lost. The mesolimbic DAergic neurons, cell bodies of which reside adjacent to the SNpc in the ventral tegmental area (VTA), are much less affected in PD (Horn 1979) and significantly less depletion of DA in the amygdala (Marsden 1987), the main site of projection for these neurons.

Neuropathological studies of PD-related neurodegeneration reveal DAergic neuronal loss to have a characteristic topology, which is distinct from the pattern seen in the aging process. In PD, cell loss is concentrated in ventrolateral and caudal portions of SNpc,
whereas during normal aging the dorsomedial aspect of SNpc is affected (Marsden 1987). The degree of terminal loss in the striatum (ST) appears to be more pronounced than that of SNpc DAergic neuronal loss, suggesting that ST DAergic nerve terminals are the primary target of the degenerative process. The neuropathology of PD is characterized solely by DAergic neuron loss which correlates with the progressive motor decline. However the neurodegeneration extends well beyond DAergic neurons (Di Monte 2003), with many “non-DAergic” PD features. Neurodegeneration and Lewy bodies (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 cingulated and entorhinal cortices), olfactory bulb, and autonomic nervous system. Degeneration of hippocampal structures and cholinergic cortical inputs contribute to high rate of dementia that accompanies PD, particularly in older patients. The clinical correlation to lesions in serotonergic and noradrenergic pathways is not clearly characterized as DAergic systems.

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. The symptoms worsen and prior to the introduction of levodopa, the mortality rate among PD patients were three times that of normal age-matched subjects. Although levodopa has dramatically improved the quality of life for PD patients, surveys revealed that these patients continue to display decreased longevity compared to the general population (Katzenschlager and Lees 2002; Marinus et al. 2003; Katsarou et al. 2004; Muller and Russ 2006). Furthermore, most PD
patients suffer considerable motor disability after 5-10 years of disease, even after beneficial medications.

1.3.2 Lewy Bodies in PD
Apart from the loss of melanized (Mason 1984) nigrostriatal DAergic (DAergic) neurons another major pathological hallmark of PD includes the presence of intraneuronal proteinacious cytoplasmic inclusions “Lewy Bodies” (LBs). This results in the classical neuropathological finding of SNpc depigmentation where the SNpc DAergic cell bodies project primarily to the putamen. However LBs are not specific for PD as they are also found in Alzheimer’s disease (AD), other α-synucleinopathies such as dementia with LB disease (DLBD), and as “an incidental” pathologic finding in people of advanced age than the prevalence of PD (Spillantini and Goedert 2000). The role of LB in neuronal cell death is controversial, as the reasons for their increased frequency in AD and the relationship of incidental LB to the occurrence of PD. LBs are spherical eosinophillic protein aggregates composed of numerous proteins, including intracytoplasmic α-synuclein, parkin, ubiquitin, and neurofilaments, which are found in all affected brain regions (Chung, 2003; Fahn, 2004; Marsden, 1987; De Girolami, 1999). LBs are more than 15μm in diameter with an organized structure containing a dense hyaline core surrounded by a clear halo. Electron microscopy reveals a dense granulovesicular core surrounded by radiating 8-10 nm fibrils (Marsden 1987).

1.4 ETIOLOGY OF PD
1.4.1 Environmental Factors
The specific etiology of PD is not known. It has been proposed to be multifactorial because of its sporadic nature. Epidemiological studies show that a number of factors may increase the risk of developing PD (Abbott et al. 2003; Baldereschi et al. 2003). These include the exposure to well water, pesticides, herbicide, industrial chemicals, farming and living in rural environment (Di Monte et al. 2002; Di Monte 2003). A number of exogenous toxins have been associated with the development of parkinsonism, including trace metals, cyanide, carbon monoxide, and carbon disulfide. In addition, the possible role of endogenous toxins such as tetrahydro-isoquinolines and beta-carbolines has also been implicated (McNaught et al. 1996; Storch et al. 2000; Maruyama and Naoi 2002). However, no specific toxin has been found in the human brain of PD patients, and also in many instances of Parkinsonism associated with toxins is not that of typical LB PD.

A study of monozygotic and dizygotic pairs of twins was conducted to assess the genetic versus environmental factors in the etiology of PD (Tanner et al. 1999). Results show that the contribution of environmental factors to both early and late onset forms can never be completely excluded; however it is more evident in late onset forms (onset beyond age 50). Human exposure to chemical compounds of synthetic origin, including pesticides, herbicides and insecticides has been the focus of several epidemiologic studies since the first description of Parkinson-like symptoms among individuals who had taken drugs contaminated with 1-methyl-4-phenyl-2,3,6-tetrahydropyridine (MPTP; (Langston et al. 1999)). MPTP is converted to 1-methylphenylpyridinium (MPP⁺) by MAO-B in glial cells (Nicklas et al. 1985; Schmidt and Ferger 2001), and subsequently selectively
concentrated in mitochondria of DAergic neurons where it interacts with elements of the mitochondrial respiratory chain (Langston et al. 1999), leading to ATP depletion, and eventually cell death of the DAergic neurons (Fisher A. 1986; Schmidt and Ferger 2001; Chung et al. 2003; Di Monte 2003). Paraquat is an herbicide structurally similar to MPTP; human exposure to paraquat has also been associated with an increased risk of PD (McCormack et al. 2002), and studies on animal models demonstrate that paraquat induces a selective loss of DAergic neurons (Liu et al. 2003). Conflicting results have been produced, in recent years, in association studies between exposure to pesticides, fungicides and herbicides and PD risk (Gorell et al. 1998; McCann et al. 1998; Baldi et al. 2003).

Human activities related to a possible pesticide exposure, including farming, living in rural areas and drinking water, are among risk factors associated with PD, whereas smoking and drinking coffee are protective factors (Hernan et al. 2002). A chronic occupational exposure to manganese is the cause of manganism, a condition characterized by tremor, rigidity and psychosis due to the accumulation of the metal in the basal ganglia (Mergler and Baldwin 1997). Although no changes in manganese brain concentration has been observed in PD, exposure to manganese has been linked to the risk of PD in some epidemiological studies (Gorell et al. 1997; Gorell et al. 1998). Copper exposure has been associated with PD, whereas iron exposure alone was not; however exposure to combinations of iron and lead, iron and manganese and iron and copper was associated with PD. Controversial or negative results have been obtained for exposure to zinc, mercury and aluminium (Gorell et al. 1997; Gorell et al. 1998; Powers
et al. 2003). Inflammation of the brain in early life as a consequence of head injuries, viral or bacterial infections or exposure to neurotoxicants, has been indicated as a possible contributor to the development of PD later in life (Liu et al. 2003); moreover it has been suggested that occupational exposure to viral (or other) respiratory infections might be one of the risk factors for the observed increased incidence of PD cases among teachers and healthcare workers (Tsui et al. 1999).

1.4.2 Genetic Factors
In a great majority of individuals with PD it is thought to occur sporadically, as the family history does not indicate affected individuals in their first degree relatives. However, a number of genetic forms of the disease have been recently discovered, and research into these hereditary forms has helped to understand the pathophysiology of this condition. To date, 10 loci (PARK1-10) have been mapped and found to be linked to familial forms of PD (Riess et al. 2000; Riess et al. 2002; Steece-Collier et al. 2002; Dawson and Dawson 2003; Fahn and Sulzer 2004; Pankratz and Foroud 2004) with genes identified in five (see Table 1.1). They are presented either as autosomal dominants or autosomal recessives.

The first gene discovered and mostly studied is the PARK-1 gene which encodes the alpha synuclein (α-syn) protein (Polymeropoulos et al. 1996; Polymeropoulos 1998) and is present in PD and dementia with LBs (DLB) but not in normal human brains. It is normally densely accumulated within presynaptic axon terminals and beta (β)-positive vesicles but gamma (γ)-negative are also present in the hippocampal dentate, hilar, and
CA2/3 regions (Galvin et al. 1999). Genetic variability in the α-syn gene has been shown to be a risk factor for the development of PD (Conway et al. 2000; Hsu et al. 2000; Saha et al. 2000; Lo Bianco et al. 2002; Lotharius and Brundin 2002). Immunohistochemical studies have indicated that LBs of the brainstem and also cortical types in the brains of PD patients with sporadic PD or DLB are strongly positive for α-syn (Hishikawa et al. 2001; Junn et al. 2003; Liani et al. 2004; Murakami et al. 2004) thus may be an important component of LBs. α-syn knock out mice revealed a striking resistance to MPTP induced degeneration of DA neurons and DA release. This resistance appeared to result from the inability of the toxin to inhibit complex I (Dauer et al. 2002). The accumulation of α-syn intracellularly at abnormally high quantities, in an aggregated form within the neurons and sometimes glial cells, have been shown to contribute to the pathology of PD and also to generate hydroxyl radicals (OH•) upon the addition of iron (Fe ii). The past 10 years has seen a shift in our etiological concepts of Parkinson's disease, moving from a nearly exclusively environmentally mediated disease towards a complex disorder with important genetic contributors. The identification of responsible mutations in certain genes, particularly alpha-synuclein, Parkin, PINK1, DJ-1 and LRRK2, has increased our understanding of the clinical and pathological changes underlying Parkinson's disease, with implications for patient diagnosis, management and future research.
### Table 1.1 Genes involved in Parkinsons Disease

<table>
<thead>
<tr>
<th>Gene</th>
<th>Inheritance</th>
<th>Locus</th>
<th>Clinical Features</th>
<th>Histopathology</th>
</tr>
</thead>
</table>
| α-synuclein | AD          | PARK1/4 (4q21) | Ala53Thr: early onset, rapid progression  
Ala30Pro: typical parkinsonian phenotype  
Glu46Lys: cognitive decline, hallucinations  
Duplication: typical parkinsonian phenotype, dementia, early death | SN degeneration, Lewy body pathology  
in substantia nigra, cortex and hippocampus          |
| Parkin   | AR          | PARK2 (6q25-27) | Early disease onset, resembles idiopathic PD phenotype, slow disease progression, good response to levodopa, early dyskinesias, diurnal fluctuation and sleep relief | SN degeneration in the absence of Lewy bodies.  
Lewy body pathology reported in compound heterozygous carriers |
| Unknown  | AD          | PARK3 (2p13)   | Typical parkinsonian phenotype  
Nigral Degeneration with Lewy bodies | N/A                                                 |
| UCH-L1   | AD          | PARK5 (4p14)   | Typical parkinsonian phenotype | N/A                                                 |
| PINK1    | AR          | PARK6 (1p35-36) | Early disease onset, levodopa responsive, slow progression, dyskinesias | N/A                                                 |
| DJ-1     | AR          | PARK7 (1p36)   | Early disease onset, levodopa responsive. Dystonia and psychiatric features reported | N/A                                                 |
| LRRK2    | AD          | PARK8 (12q12)  | Predominantly levodopa responsive parkinsonism for all mutations.  
Supranuclear gaze palsy, dystonia, dementia and motor neuron disease is described in some individuals | All had SN degeneration. Variable additional pathology: novel ubiquitin positive inclusions and MND (Tyr1699Cys), tau pathology (Arg1441Cys), Lewy bodies (Gly2019Ser, Arg1441Cys), no additional pathology (Arg1441Cys) |
| Unknown  | N/A         | PARK10 (1p32)  | Typical parkinsonian phenotype | N/A                                                 |
| Unknown  | N/A         | PARK11 (2q36-37)| Typical parkinsonian phenotype | N/A                                                 |

AD, autosomal dominant; AR, autosomal recessive; SN, substantia nigra; N/A, not available; MND, motor neuron disease (amyotrophic lateral sclerosis) (adapted from Gosal et al., 2006).
1.4.3 Ageing and PD development

Age is an important factor in PD expression (Figure 1.1) (Fahn and Sulzer 2004). After the age of 30-40, the incidence increases with the age reaching a maximum at the 8th decade with a decline in prevalence thereafter (Marsden 1987). The progression of the aged in the total population is also expected to rise from 12.2% in year 2000 to 15.5% in the year 2020. Aging in individuals is affected to a great extent by genetic factors, diet, social conditions, and the occurrence of age-related diseases. There is evidence that age-induced alterations in cells are an important component of aging of the organism (Cotran R.S 1999). A number of cell functions decline progressively with age, for example oxidative phosphorylation in the mitochondria, the synthesis of nucleic acids and structural enzymatic proteins are reduced. Oxidative damage to mitochondrial DNA increases with ageing and can induce point mutations thus, contributing to mitochondrial dysfunction and neuronal loss (Simon et al. 2004) [Simon, 2003]. Biochemical analysis revealed a shift in the extractability of parkin upon aging in humans but not in mice thus there is an effect of aging upon parkin in humans (Pawlyk et al. 2003).

1.5 MECHANISMS OF PARKINSONIAN CELL DEATH

1.5.1 Oxidative stress (OS)

OS and the consequent cell death in SNpc usually results from either an increased DA turnover, resulting in excess peroxide formation, a deficiency in glutathione (GSH), thus diminishing the brains capacity to clear H$_2$O$_2$ or an increase in reactive iron thereby, promoting OH$^\cdot$ formation (Olanow and Tatton 1999; Foley and Riederer 2000; Bharath et al. 2002; Koutsilieri et al. 2002; Ischiropoulos and Beckman 2003; Jenner 2003; Klein
and Ackerman 2003). Most of the evidence suggesting OS is linked to the pathogenesis of PD comes from postmortem studies (Jenner 1993; Yoritaka et al. 1996; Alam et al. 1997; Jenner 1998; Serra et al. 2001; Giasson et al. 2002; Hald and Lotharius 2005; Sofic et al. 2006) with increased iron levels are increased in the SNpc in PD, (Gu et al. 1998; Faucheux et al. 2003) and the reduced levels of GSH where excessive oxidative DAergic cell burden occurs which disrupts mitochondrial complex I function (Shults et al. 1997; Gu et al. 1998). Although ROS levels cannot be measured directly in living patients, their reaction by products and the damage in postmortem tissue is used as an indirect indicator (see Table 1.2).

![NOTE:]
This figure is included on page 13 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.1** Diagrammatic representation of the age predilections for typical, sporadic Parkinson’s disease and genetically determined Parkinsonism.

Illustrating the concept that genetically determined Parkinsonism tends to start at a younger age, but constitutes only a small proportion of all patients with typical, progressive Parkinsonism (Langston, 2002).
1.5.2 Iron

Iron is richest in the basal ganglia with the highest levels being in the SNC, pallidus and putamen (Dexter et al. 1989b; Foley and Riederer 2000). In IPD, the shift of iron Fe$^{2+}$/Fe$^{3+}$ ratio is almost 2:1 to 1:2 (Riederer et al. 1989; Sofic et al. 1991; Gerlach et al. 1994; Foley and Riederer 2000), this is consistent with the increased Fe$^{2+}$-catalysed conversion of H$_2$O$_2$ to the highly reactive OH$^\bullet$ known as the Fenton reaction: H$_2$O$_2$ + Fe$^{2+}$ → OH$^\bullet$ + OH$^\bullet$ + Fe$^{3+}$ (Foley and Riederer 2000). The reduction of Fe$^{3+}$ by the superoxide radical increases the reaction rate which drives the production of OH$^\bullet$. Iron levels are increased in IPD by about 35% specifically in the SNC (Gu et al. 1998; Bartzokis et al. 1999; Graham et al. 2000; Berg et al. 2001); similar increases have also been noted in other neurodegenerative diseases (Qian et al. 1997; Ke and Ming Qian 2003). Iron participates in the free-radical generating reaction only in the free ferrous form (Rouault and Cooperman 2006). Ferric iron (Fe$^{3+}$) in the SNC is normally bound either by ferritin (about 90%) or neuromelanin (NM) and, is associated with both LB and NM in IPD (Hirsch et al. 1991; Jellinger et al. 1992; Mann et al. 1994; Foley and Riederer 2000).

Infusion of iron into rat brains produces a model of PD characterized by a concentration-dependent loss of striatal DA, and the degeneration of SNC neurons with the behavioral changes (Fisher A. 1986; Berg et al. 1999; Santiago et al. 2000; Ponting 2001). How iron accumulates within the SNC in PD is not clear but an increased lactoferrin receptors have been detected on nigral neurons in PD patients which readily cross the BBB and are synthesized in the brain. This may explain the iron accumulation in SN and subsequent degeneration of DAergic neurons in PD (Berg et al. 2002).
Table 1.2 Summary of increased Oxidative stress (OS) in Idiopathic Parkinson’s disease (IPD)

<table>
<thead>
<tr>
<th>Evidence for an increase of OS in IPD</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; membrane peroxidation in SNc</td>
<td>Ala., 1997; Floor, 1998; Buhmann, 2004</td>
</tr>
<tr>
<td>&gt; ROS-induced protein modification</td>
<td></td>
</tr>
<tr>
<td>&gt; protein carbonyls in all IPD brain</td>
<td></td>
</tr>
<tr>
<td>&gt; thiobarbituric acid-reactive substance levels (measures the secondary products of lipid peroxidation)</td>
<td>Dexter, 1989</td>
</tr>
<tr>
<td>&lt; polyunsaturated fatty acid levels (peroxidant substrate)</td>
<td></td>
</tr>
<tr>
<td>&gt; 8-hydroxy-2’ deoxyguanosine levels in the SNc + other brain regions (indicates ROS-mediated DNA damage)</td>
<td>Zhang, 1999</td>
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</tbody>
</table>

Several studies have suggested that transcranial ultrasound (TCS) may able to demonstrate the presence of increased iron content in the SN of PD patients, even prior to the development of the disease symptoms (Berg et al. 2002). The echogenicity in PD patients substantially increases the echogenicity of the SNc (Berg et al. 2001). The elevated tissue echogenicity has been examined in postmortem studies, revealing a close correlation between echogenicity of the SN and tissue iron content. Iron accumulation in
the SN in rats confirmed that iron that is not protein-bound may lead to an increase of tissue echogenicity (Berg et al. 1999). Exposures to welding fumes have been shown to alter trace metal levels such as, manganese, iron, zinc and copper in the body. Serum levels of iron in the welders were 1.9 fold higher than those of controls and the level of erythrocytic superoxide dismutase activity was 2.4% less thus, occupational exposure to these toxic fumes disturbs the homeostasis of trace elements in the systemic circulation which induces OS (Li et al. 2004).

1.5.3 Neuromelanin (NM)

It’s been hypothesized that NM may act as an endogenous storage molecule for iron and possibly influence the production of free radicals. Iron-binding studies have demonstrated that both NM and synthetically-produced DA-melanin contained equivalent numbers of high and low-affinity binding sites for iron but, that the affinity of NM for iron was higher than that of synthetic melanin (Double et al. 2003). The iron-binding capacity of NM is 10-fold greater than that of the synthetic melanin. This is consistent with the hypothesis. The function or effect of NM on neuronal function is unclear; it is generally regarded as a waste product of DA auto-oxidation, and accumulates with age in nigral neurons (Zecca et al. 2004) but is dramatically decreased in PD patients (Zecca et al. 2002). NM has been described as a ‘double-edged sword’ with respect to its sequestering of redox-active metal ions such as iron [Zecca, 2002; Zecca, 2003] induced by free radical reactions (Enochs et al. 1994), and inhibiting lipid peroxidation as an oxidant (Korytowski et al. 1995). NM may also promote ROS-generating and act as a depot for cellular toxins like MPP⁺ (D’Amato et al. 1987b, c; D’Amato et al. 1987a; Zecca
et al. 2003). In IPD, cell death occurs mainly in the DA-containing SN. It is suggested that there is a selective vulnerability of neuromelanin-pigmented subpopulation of DA-containing mesencephalic neurons in PD [(Hirsch et al. 1998). High levels of Cu/Zn superoxide dismutase mRNA have been observed in the SN indicating high levels of oxygen free radicals are being produced in PD (Hirsch 1992). Catecholaminergic neurons surrounded by a low density of glutathione peroxidase cells were more susceptible to degeneration in PD than those well protected against OS, indicating the selective vulnerability of catecholaminergic neurons (Hirsch 1992).

1.5.4 Glutathione (GSH)

A defect in one or more of the naturally occurring antioxidants defences could lead to the neurodegeneration in PD (Jenner 1996) although no basic defects have been detected in levels of ascorbic acid, α-tocopherol, catalase, or glutathione peroxidase (Riederer et al. 1989; Dexter et al. 1994; Sian et al. 1994b; Sian et al. 1994a). The cellular radical detoxification system (the most important being superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity unfortunately declines with the ageing process. The activity of SOD is increased in discrete regions of the brain according to different neurodegenerative diseases, in IPD it is characteristically increased in SNC (Marttila et al. 1988; Saggu et al. 1989). Of the two isozymes, mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD, only levels of Mn-SOD are elevated in IPD (Yoshida et al. 1993; Yoshida et al. 1994), suggesting increased OS levels in the mitochondria although there have been report of high Cu/Zn-SOD levels occurring as well (Serra et al. 2001). This increase may be a neuroprotective response to the elevated levels of OS however; an increase in H₂O₂
production might be a problem in the absence of corresponding catalase and GPX activity. In genetically modified rodents, high SOD expression protected the rat nigral cells against MPTP and 6-OHDA (Asanuma and Cadet 1998; Asanuma et al. 1998). Similar results have been observed in the postnatal midbrain cultures from mice with increased amounts of WT SOD enzyme, promoting cell survival and providing protection against L-dopa-induced DAergic neurotoxicity whereas, increased amounts of mutated Cu/Zn-SOD enzyme had inverse effects (Mena et al. 1997).

Most attention has been directed to the finding of a selective decrease in the reduced form of GSH in the SNc in PD (Sofic et al. 1992; Sian et al. 1994b; Sian et al. 1994a). Reduced levels of GSH have not been detected in other brain areas in PD and have not been reported in any other neurodegenerative disorder. A reduction in GSH impairs H$_2$O$_2$ clearance thus, promoting OH$^\cdot$ formation, particularly in the presence of high levels of iron. The cause of GSH reduction in PD is still unknown and no defects have been detected in the enzymes associated with GSH synthesis. However, a significant increase in γ-glutamyltranspeptidase (γ-GTT), the enzyme responsible for the translocation of GSH precursor and the metabolism of the oxidised form of glutathione (GSSG) (Sian et al. 1994b; Sian et al. 1994a; Jenner 1996; Foley and Riederer 2000), could reflect the attempt of surviving cells to recruit and replenish diminished levels of GSH or act as a compensatory mechanism to remove the toxic GSSG. In the normal human brain, nigral GSH levels are low in comparison to with other brain regions (Perry et al. 1982; Riederer et al. 1989; Sofic et al. 1992; Pearce et al. 1997; Bharath et al. 2002). However, there is a further decline in IPD nigra of the total and reduced GSH levels (Dexter et al. 1994).
GSH levels are similar in incidental LBs but are not accompanied by changes in iron levels or mitochondrial function thus, a decline in GSH levels could possibly occur early in the course of the disorder. An artificially induced 40-60% loss of GSH by the administration of BSO (buthionine sulfoximine, a selective inhibitor of α-glutamylcysteine synthetase) led to a similar loss as in IPD however, no nigral striatal cell loss or changes in the mitochondrial SOD activity in rats were observed but the sensitivity to both 6-OHDA and MPP⁺ were enhanced (Pileblad et al. 1989; Wullner et al. 1996; Toffa et al. 1997). This suggests a reduction in GSH itself may not damage DAergic neurons but possibly rendering these vulnerable to other toxins.

1.5.5 Mitochondrial Dysfunction

There is a significant body of evidence which supports the hypothesis that the progressive reduction in mitochondrial respiration (a feature of the ageing brain) is involved in a number of neurodegenerative diseases including IPD (Schapira et al. 1989; Schapira et al. 1990; Di Monte 1991). A selective 38-49% decrease in complex 1 activity of the mitochondrial respiratory chain has been found in the SNc of PD patients (Schapira et al. 1990; Reichmann et al. 1993a; Reichmann et al. 1993b). A complex 1 defect has also been found in platelet and muscle tissues of PD patients (Schapira et al. 1990; DiMauro 1993; Reichmann et al. 1993a; Reichmann et al. 1993b) however, there have been no reports on detectable structural or mitochondrial DNA changes. The other transport chain complexes generally appear to be unaffected despite the report of a reduction in SNc complex IV activity (Itoh et al. 1997). The cause of the reduced complex I activity in IPD remains unknown. No MPTP-like toxin has been detected or any specific
abnormality in the subunits of complex I, DNA, nor nuclear gene which encodes complex I protein.

A mitochondrial complex I defect could contribute to cell degeneration in PD through decreased ATP synthesis and a bioenergetic defect. Complex I inhibition by MPTP or MPP⁺ have been shown to lead to a depletion of cellular ATP in mouse brain synaptosomes (Scotcher et al. 1990; Swerdlow et al. 1996). Studies in experimental animals indicate that a decrease in complex I activity of 40% or less does not compromise cellular ATP levels (Davey and Clark 1996). Complex I defect could also lead to cell damage through the generation of free radicals or the compensatory mechanism of increasing the respiration through complex II (Schulz et al. 1995a).

Factors that contribute to the progression of PD include brain defects in mitochondrial respiration and the generation of H₂O₂ by MAO. One study revealed that these two factors were linked (Cohen et al. 1997). Addition of glutathione disulfide (GSSG; oxidized form of glutathione) to the mitochondria resulted in a similar reversible inhibition of the electron transport (Cohen et al. 1997). A complex I defect may also contribute to the development of apoptosis (Ozawa et al. 1997; Tatton et al. 1998), as complex I defect is the major site of proton pumping; it is possible that this could lead to cell death via neuronal vulnerability and contributing to the pathogenesis of PD (Liu and Kane 1996; Susin et al. 1996).

1.5.6 Excitotoxicity
In the past decade, there has been wide acceptance of the theory of excitotoxicity as a viable process underlying a variety of acute and chronic degeneration disorders (Plaitakis and Shashidharan 2000). Activation of more than one ionotrophic glutamate receptor subtype can produce neuronal death. The N-methyl-D-aspartate (NMDA) receptor mediated toxicity is so far the most characterised pathway (Dickie et al. 1996; Plaitakis and Shashidharan 2000). It has been shown that excitatory amino acids given systemically to immature animals can cause neuronal degeneration in areas of the brain that lacks an intact BBB (Plaitakis and Shashidharan 2000). Unlike the classic neuromodulators –DA, norepinephrine and acetylcholine, which show marked regional distribution in the brain, amino acids is present at high concentration in all cells (Plaitakis and Shashidharan 2000).

Postsynaptic transduction of excitatory transmission is mediated by several classes of glutamate receptors. These include the NMDA, the AMPA (amino-3-[3-hydroxy-5-methylisooxazol-4-1]), proprionic (acid)/kainite, and the metabotrophic receptor. SNpc DAergic neurons are rich in glutamate receptor, receiving extensive glutamate innervation from the cortex and subthalamic nuclei (STN) with a pattern of burst firing in response to exogenous administration of glutamate (Johnson et al. 1992; Rothstein et al. 1994). DA lesions disinhibit the STN inducing an increase in the firing rate of its excitatory output neuron (DeLong 1990). Glutamate has a dual function in synaptic transmission being an excitatory neurotransmitter and mediating an inhibitory postsynaptic potential in DAergic neurons (Fiorillo and Williams 1998). A reduction in energy metabolism due to mitochondrial dysfunction is shown to result in a loss of ATP-
dependent Mg-blockade of NMDA receptors, allowing glutamate to mediate an influx of Ca\(^{2+}\) into the cell (Greenamyre et al. 1999). The role of excitotoxicity in PD are supported by reports of NMDA antagonists protecting DAergic cell loss against MPP\(^{+}\) infusion into the SNc of rats (Turski et al. 1991), and MPTP treatment in primates (Greenamyre et al. 1994). NMDA receptor mediated neuroprotection has also been shown to be dependent on nuclear factor-k\(\beta\) (NF- k\(\beta\)) and the release of brain derived neurotrophic factor (BDNF) (Lipsky et al. 2001).

1.5.7 Nitric Oxide (NO)

Excitotoxic damage is thought to be mediated at least in part via NO. Increased NO released from glial, microglia or macrophages could also be responsible for the elevation of local OS (Dawson et al. 1992). Although NO is an effective free radical scavenger (Rubanyi et al. 1991) it can also react with the superoxide radical to form the peroxynitrite anion (\(^{•}\)ONOO\(^{−}\)). The oxidative radical `ONOO' decomposes to form OH\(^{•}\) + NO\(_2\), which are potent initiators of lipid peroxidation (Beckman et al. 1990; Radi et al. 1991). NO also directly inhibits mitochondrial respiration at the level of complex IV and I, by liberating ferritin-bound iron thus, promoting lipid peroxidation (Bolanos et al. 1996) with mitochondrial respiratory chain being damaged by sustained exposure to NO and that GSH is an important defense. This has implications for PD where GSH levels are decreased.

NO-mediated toxicity has been implicated in nigral damage induced by MPTP. The neuronal NOS inhibitor 7-nitroindazole (7-NI), which blocks NO formation, protects
DAergic neurons from MPTP toxicity in both rats and baboons (Schulz et al. 1995b; Hantraye et al. 1996). Similarly, MPTP toxicity is diminished in NOS knock-out mice (Przedborski et al. 1996). A recent report noted that 7-NI inhibits MAO-B (Castagnoli et al. 1997), raising the possibility that it may act by blocking the conversion of MPTP to MPPC. Inducible NO synthetase activity (NI) has been shown to be greater in SNc of IPD patients, possibly secondary to reactive gliosis (Hunot et al. 1996). Damage due to NO can be estimated by mediating the formation of 3-Nitrotyrosine (3-NT), product of the peroxynitrite induced nitration of tyrosine residues on cellular probes [Ischiropoulos et al 1992]. Elevated levels of 3-NT have been reported in MPTP treated mice and monkeys (Schulz et al. 1995b) 3-NT levels in LB core have also been demonstrated to be increased in IPD, indicative of NO-induced damage (Jenner 1996; Good et al. 1998).

1.5.8 Inflammation

Inflammation plays a role in the pathogenesis of PD. Autopsy reports have shown a glial response found in both ST and nigra of PD patients and MPTP-mice which may likely exert deleterious effects on the remaining DAergic neurons (Przedborski et al. 2000; Przedborski et al. 2001; McGeer et al. 2003; Teismann and Schulz 2004; McGeer and McGeer 2008). This view has led many investigators to aggressively examine the potential role of neuroinflammation in the pathogenesis of PD. The SN DAergic neurons are vulnerable to a variety of insults due to their reduced antioxidant capacity with a high number of microglia cells, compared to other areas of the brain (Liu et al. 2003). When microglia becomes activated they release proinflammatory factors and such cytokines as interleukin-1 (IL-1), IL-2, IL-6 and tumor necrosis factor alpha (TNFα), which can be
cytotoxic (Blum et al. 2001). Autopsy reports from sporadic PD patients have shown at early stages of the disease the activated microglia were mainly associated with tyrosine hydroxylase positive (TH$^+$ve) neurites in the putamen and at the advanced stage with damaged neurons in the SN (Sawada et al. 2006). The number of activated microglia was also TNF$\alpha$-, and IL-6-positive. These increase in the SN during the progress of PD.

Not only is there an indication of cytokine elevation but a decrease in neurotrophins such as BDNF and nerve growth factor (NGF), in the nigrostriatal DA regions and ventricular and lumbar cerebral spinal fluid of PD patients (Nagatsu et al. 2000a). Furthermore, levels of TNF$\alpha$ receptor R1 (TNF$\alpha$-R1, p55), bcl-2, soluble Fas (sFas), and the activities of caspase-1 and -3 were also elevated in the nigrostriatal DA regions. Activated microglia and increased levels of inflammatory mediators have been detected in the ST of PD patients, in vivo (Waters et al. 1987; Nagatsu et al. 2000a; Wu et al. 2002; Hald and Lotharius 2005; Novikova et al. 2006; Sawada et al. 2006) and in vitro studies (Le et al. 2001; Gao et al. 2003b; Gao et al. 2003a; Wang et al. 2005; Shavali et al. 2006; Mount et al. 2007) which all point to an inflammatory component of DAergic cell loss. Acute toxin exposure in these models or decades after drug use in humans have shown that the presence of activated microglia still persists, suggesting a brief insult can induce ongoing inflammatory response (Shavali et al. 2006; Wilms et al. 2007). Moreover, not only do activated microglia phagocytose damaged cell debris but also neighbouring intact cells, further supporting their active participation in self-perpetuating neuronal damaging cycles (Kim and Joh 2006).
1.6 CELL DEATH IN PD

1.6.1 Apoptosis

There has been much debate about the mode of cell death in PD. Apoptosis has been suggested by some researchers (Agid and Blin 1987; Mochizuki et al. 1997; Tatton and Olanow 1999; Tatton et al. 2003) and others who disagree (Kosel et al. 1997; Jellinger 2000). A recent study by Tatton (Tatton 2000) shows that there is an increased occurrence of DNA fragmentation and chromatin condensation in the melanised cells of SN of PD patients compared to controls. There was also an increase in caspase-3 and Bax (a proapoptotic member of Bcl-2 protein family) immunoreactivity and nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GADPH) in PD nigral DAergic neurons. This demonstrates the occurrence of ongoing apoptotic processes and an involvement of mitochondrial processes.

1.6.2 Apoptotic markers in PD

Apart from caspase-3, Bax is another protein which plays a critical role in apoptosis is p53 which has been found to be up-regulated in the midbrain and ST of PD patients (de la Monte et al. 1998). This supports the role of intrinsic apoptotic pathways involving the mitochondria. Interestingly, tumor necrosis factor receptor 1 (TNFR1, a death receptor)-positive DAergic neurons have also been found in the SN of controls and PD patients (Boka et al. 1994). The level of tumor necrosis factor (TNF-α)-positive glial cells were markedly elevated (Hunot et al. 1999), and that of melanized neurons expressing death the receptor adaptor protein Fas-associated death domain (FADD) and caspase-8 were reduced in this area of PD patients (Hartmann et al. 2000; Hartmann et al. 2001). This
implicates the existence of an extrinsic mechanism known as the TNF receptor ligand system.

Caspase-8 is also activated early on in mice treated with MPTP, possibly as result of of a downstream consequence of cytochrome $c$ release from mitochondria as inhibitopn of caspase-8 inhibition resulted in necrosis rather than rescue of the DAergic cells (Hartmann et al. 2001). Thus intervention as this point may be too late in the cascade of events. Different toxins and models induce different outcomes, as comprehensively reviewed by Blum (2001), 6-hydroxydopamine (6-OHDA) induces strong reactive oxygen species (ROS) production, weakening cellular antioxidant defenses, lipid peroxidation, DNA strand breaking, cytoskeletal changes, and some evidence of mitochondrial impairment. Both in vivo and in vitro models, 6-OHDA induced both apoptosis and necrosis and an upregulation of p53 and caspase activations. Although overexpression of Bcl-2 and caspase inhibitors provide some protection from death it did not maintain functionality of the cultured DA cells, thus these models provide useful insights only into the aim of slowing disease progression.

MPP$^+$ exposure in cell culture and in animal models also showed evidence of apoptosis with p53 involvement, Bax upregulation, and caspase-3 activation (Blum et al. 2001). It is well received that the common final pathway in DAergic degeneration involves oxidative stress (OS) and/or mitochondrial impairment (Mattson 2000). In addition, some neurons that die due to energy loss thus can not complete apoptosis die by necrosis, resulting in gliosis and microgila activation inducing overproduction of nitric oxide (NO),
reinforcing the damages (Hirsch et al. 2000). Another complex I inhibitor is rotenone, a widely used model of apoptosis in cell culture models. Chronic rotenone administration leads to complex I inhibition in the brain, resulting in selective DAergic nigrostriatal degeneration and typical parkinsonian symptoms and cytoplasmic inclusions reminiscent of LBs (Bezard et al. 1997).

### 1.7 CURRENT TREATMENTS AND POTENTIAL THERAPIES FOR PD

#### 1.7.1 Symptomatic Treatment

There is no cure for PD, since currently available therapies can neither arrest or reverse the progression of the disease nor provide neuroprotection. However, the motor symptoms can be initially managed with several different drugs. The drugs used to treat PD either boost the levels of DA in the brain or mimic the effects of DA. The use of L-DOPA which is still considered the gold standard aims to relieve PD motor symptoms by replacing the deficient neurotransmitter, DA. However, the therapeutic efficacy of L-DOPA tends to fade with time and the development of motor and/or psychiatric side effects. An issue that is currently debated is the hypothesized toxicity of L-DOPA. Experimental data suggest that prolonged use of the drug may contribute to the degenerative process of PD. Various *in vivo* and *in vitro* studies have shown that L-DOPA can be toxic, as a result of its pro-oxidant properties (Ziv et al. 1997; Melamed et al. 1998). Whether L-DOPA is toxic per se or because it converts to DA whose metabolism is associated with formation of ROS, as well is unclear (Coyle and Puttfarcken 1993).
These considerations have led to the introduction of DA agonists in PD therapy. This class of drugs, that includes compounds such as pergolide, bromocriptine, lisuride or the more recent ropinirole and pramipexole, acts directly on the DA receptors mostly of the D₂ type mimicking the effect of DA. DA agonists reduce the occurrence of motor complications, in both experimental and clinical studies and are used as an adjunct to L-DOPA in patients with dyskinetic movements and fluctuations in the motor response to the drug (Olanow 1992a, b) by restoring the normal efficiency of the DAergic pathways.

Evidence suggests that DA agonists may have also neuroprotective properties. Such properties would be related to the ascertained efficacy of DA agonists in antagonizing OS, at various levels (Olanow et al. 1998). Apart from levodopa, drugs that are currently prescribed for the management of PD include DA receptor agonists, selegiline, amantadine, catechol-O-methyl transferase (COMT) inhibitors and anticholinergics (Figure 1.2, Table 1.3). Most current drugs are still in development and/or clinical phase and continual research is employed to produce the most effective treatments which will have all desired effects such as neuroprotection, less severe side effects, optimal delivery of drugs to site of problems, relieve/reduce motor symptoms and delayment of the neuronal degeneration.

1.7.2 Cell Transplantation

Transplantation of DA-producing neurons to replace those degenerated during the pathogenesis of PD is a promising approach to treatment. Thus far this is the only advancement that has shown the capacity to allow patients to achieve full restoration of their functional capacity. The grafts have shown minimal immunological rejection in
recipients and in the most successful trials have even allowed patients to withdraw from levodopa therapy (Lindvall and Hagell 2001). However, the majority of studies have derived allogenic ventral mesencephalic (VM) tissue from human foetuses (Subramanian 2001; Levy et al. 2004). This presents, apart from the ethical issues, the implication that sources of such tissues will be limited and cannot provide a consistent and reproducible response. Furthermore, substantial quantities are required to be adequately efficacious. Stem cells as alternative neuronal sources are receiving a great deal of focus as they can provide an ‘unlimited’ source of tissue that can be employed to generate mature DAergic neurons to innervate the affected ST (Sonntag et al. 2005). The most promising is the bone marrow stem cells (Lu et al. 2004) and ordinary epithelial skin cells (Chunmeng and Tianmin 2004) may possess the potential to be sources of inducible stem cells. Although studies have shown promising results in preclinical trials, generated neurons have survived poorly after transplantation in animal subjects (Lindvall and Hagell 2001; Levy et al. 2004)). One of the most difficult hurdles to overcome is the poor rate of graft cell survival as 90% of the transplanted cells fail to survive following intracerebral grafts (Sortwell et al. 2000; Emgard et al. 2003). Furthermore, few studies have been able to investigate the long term implications of transplantation; thus the safety of such treatment at this stage remains questionable.

1.7.3 Gene therapy

Transplanted neurons in PD patients are a potential target for the development of gene therapy procedures. A variety of different viral and non-viral methods for achieving gene delivery have been described (Latchman and Coffin 2001). Among the numerous
potential genes that have been evaluated for therapeutic efficacy for PD, those encoding tyrosine hydroxylase (TH), guanosine triphosphate cyclohydrolase I and aromatic l-amino acid decarboxylase all allow for an increase in the production of DA (Duan et al. 2005). The differentiation of these neurons is mediated by transforming growth factor beta (TGF-β) and bone morphogenic factors (BMPs) (Sonntag et al. 2005). Glial cell line-derived neurotrophic factors (GDNF) have thus far proven to be the most promising option available to restore DAergic activity in the SN (Pahnke et al. 2004). Adenoviral and lentiviral nigrastral implants to liposomes (Muramatsu et al. 2003; Azzouz et al. 2004; Muramatsu et al. 2005; Wong et al. 2006) have also been studied. However more recent studies have indicated conflicting results regarding its effectiveness and the numerous side effects (Nutt et al. 2003; Lang and Obeso 2004; Lang 2006).

Thus the development of a stem cell that has the capacity to differentiate into DAergic neurons as well as produce such factors is much needed (Sonntag et al. 2005). Genes encoding for the vesicular monoamine transporter-2 and glutamic acid decarboxylase have also demonstrated some benefit. Kang et al. (Kang et al. 2001) investigated potential genes that would be optimal for such therapy and found that the enzyme TH, which has been used in earlier studies, functions only when the essential cofactor, tetrahydrobiopterin is present. Thus, new developments into the delivery of genetically modified cells that can convert levodopa to DA and store it for gradual release is required. Alternatively, it has been proposed that nanomaterials like nanotubes may be employed therapeutically as ligand carriers or vectors for drug, DNA or gene delivery for PD (Singh et al. 2005). However, so far, this technology remains in the experimental
stages of development and far from clinical trials or commercial application (Pereira and Aziz 2006).

1.7.4 Surgical methods

Prior to the commercial availability of L-DOPA, treatment for PD emphasized surgical intervention (Kelly 1995). Such intervention such as thalamotomy focused on the reduction of tremor, but failed to address the more debilitating symptom, bradykinesia (Kelly 1995). However, pharmacological therapy for PD often becomes inadequate over long-term use and during significantly progressed stages of the disease. The patient's disability increases despite maximal drug management and many patients develop motor fluctuations and dyskinesias. Surgical interventions for PD have been shown to be beneficial for refractory symptoms. However their role is limited to being the ‘means of last resort’ due to the high risk of potential complications and limited long-term efficacy. Recent advances in this field have provided a greater range of surgical options. Thalamotomy, pallidotomy are destructive lesions thought to improve motor deficient. Deep brain stimulation (DBS) however offers subthalamic nucleus stimulation improves levodopa-induced ‘off’ period function, decreases ‘off’ time, and reduces dyskinesia (Zesiewicz and Hauser 2001). Surgery is considered for people with intolerable adverse side effects from medication, and those patients who have significant cognitive capacity (Olanow and Brin 2001; Olanow 2002; Betchen and Kaplitt 2003).

Possible adverse side effects of surgery include brain haemorrhage, infarction, seizures, and even death (Beric et al. 2001; Seijo et al. 2007). Furthermore, successful surgical
DBS procedure can still lead to side effects that include worsening dyskinesia, paraesthesias, speech and gait disturbances (Umemura et al. 2003). Developments in nanotechnology have indicated the potential for implantable carbon nanotubes and nanochips to be employed in this arena (Linazasoro 2008). These systems promise to allow greater safety and precision for the delivery of impulses in the SN, and therefore reduce side effects the aforementioned problems with regard to surgery and equipment malfunctions. Studies are still in preliminary phases of development for eliminating repeated surgeries; however animal studies have yielded exceedingly promising results.

**Figure 1.2.** Action sites of pharmacological therapies currently available for PD (Singh et al. 2007).
Table 1.3 Summary of current PD treatment

<table>
<thead>
<tr>
<th>Class</th>
<th>Drugs</th>
<th>Effect or comments</th>
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<td>Trihexyphenidyl, biperiden, piroheptine, profenamine, metixene</td>
<td></td>
</tr>
<tr>
<td>B. DA replacements</td>
<td>L-DOPA (levodopa), Amantadine, Carbidopa, Benserazide, Selegiline (deprenyl), Lazabemide, Tolcapone, Entacapone</td>
<td>DA precursor, DA releaser, NMDA receptor antagonist, Inhibits peripheral AADC (DCI)</td>
</tr>
<tr>
<td>C. DA receptor agonists</td>
<td>Ergot-derivatives, Bromocriptine, Pergolide, Cabergoline, Non-ergot-derivatives, Ropinirole, Pramipexole, Talipexole</td>
<td>D₂-R agonist, scavenger of ROS, D₂/D₁-R agonist, scavenger of ROS, reduction of lipid peroxidation, D₂/D₃-R agonist, scavenger of ROS, increases GSH with an induction of regulating enzymes, D₂/α₂-R agonist, induction of Bcl-2 (at low conc.), inhibits α-synuclein aggregation (at high conc.)</td>
</tr>
<tr>
<td>D. Others</td>
<td>L-threo-DOPS (droxidopa), GDNF, GPI-1046, Estrogens, KW6002, SIB1508Y, ABT418</td>
<td>Precursor of noradrenaline, Neurotrophic factor, induces Bcl-2 and Bcl-x, Immunophilin ligand, Neurite outgrowth and regenerative sprouting, Female sex steroid hormone, induces Bcl-2, Adenosine A₂A receptor antagonist, inhibits indirect pathway, Nicotine receptor agonists, Transplantation of DA-releasing cells, stem cells, etc., Gene therapy</td>
</tr>
</tbody>
</table>
1.8 BASAL GANGLIA

1.8.1 INTRODUCTION

The basal ganglia (BG) circuit plays a key role in the regulation of voluntary movements as well as in behavioural control and cognitive functions (Graybiel et al. 1994). In the BG circuit, cortical inputs reach separate subpopulations of striatal γ-aminobutyric acid (GABA)-containing medium sized spiny neurons. From the striatum (ST), cortical information are transmitted to SNpr through parallel routes named “direct” and “indirect” pathways (Chesselet and Delfs 1996) and to the others output structures of the BG (globus pallidus, subthalamic nucleus and thalamus) and finally link back to the frontal cortex (see Figure 1.3). This circuit plays a key role in the regulation of voluntary and purposive movements as well as in behavioural control and cognitive functions (Wise et al. 1996). The selective neuronal loss in one of the structures within the circuit results in clinical syndromes characterized by motor and cognitive dysfunctions such as Parkinson's disease and Huntington's disease (Albin et al. 1995). PD’s core pathological features are represented by the heterogenous loss of pigmented DAergic neurons in the SNpc and of their projecting fibers in the ST (Lang and Lozano 1998a, b), whereas in Huntington's disease the neurodegenerative disorder primary involves the degeneration of striatal spiny neurons with sparing of striatal large cholinergic interneurons (Ferrante et al. 1985; Price et al. 1998).

1.8.2 STRIATUM (ST)

The ST, which is the major component of the BG is situated in the forebrain and consists of the caudate nucleus (CN), putamen (Put) and ventral ST (VST). The major neuronal
population in the ST is represented by spiny projection neurons, accounting for almost 95% of total striatal cells (Kemp and Powell 1971) with γ-amino-butyric acid (GABA) as the main neurotransmitter (Kita and Kitai 1988). Within the projection neurons, GABA can be co-localized, alternatively, with enkephalin or substance P/dynorphin (Beckstead and Kersey 1985). The remaining 5% of striatal cells consists of aspiny interneurons containing, alternatively, acetylcholine, somatostatin, NADPH-diaphorase or GABA associated with parvalbumin or calretin (Kawaguchi et al. 1995). Recently, the presence of DAergic neurons intrinsic to the ST has also been suggested (Betarbet et al. 1997).

The main targets of striatal projections are the medial and lateral segments of the globus pallidus and the SNpr (Parent and Hazrati 1995a, b). Neurons containing enkephalin are believed to project to the lateral globus pallidus, while neurons containing substance P/Dynorphin project to the medial globus pallidus and SNpr. This striatal output is known as the direct and indirect pathway model of BG functional organization (figure 1.3a).

The ST is the main input structure of the BG circuit. The major neural input to the ST is excitatory. Glutamatergic projections from all cortical areas (McGeorge and Faull 1989) converge onto striatal neurons with other excitatory inputs to the ST arise from the thalamus (Berendse and Groenewegen 1990), and from limbic structures, particularly the amygdala (Kelley et al. 1982). Another input to the ST originates from DAergic neurons located in the SNpc and in the ventral tegmental area (VTA) (Nieuwenhuys et al. 1982). The ST also receives serotonergic afferent projections from the nucleus of the raphe (Halliday et al. 1990) and a sparse noradrenergic innervation from the locus coeruleus (Aston-Jones et al. 1986). The ST exhibits a variety of neurotransmitter receptors, which
also show a considerably higher density at the striatal level, compared to the other BG nuclei.

1.8.2.1 Dopamine receptors (D1R & D2R)

Striatal neurons express both D1 and D2 DA receptors, which mediate the modulatory effect of DA released from nigrostriatal terminal. D1 and D2 receptors are functionally segregated to different subsets of striatal neurons. D1 receptors are expressed by neurons projecting to the SNpr and medial globus pallidus, while D2 receptors are expressed by neurons projecting to the lateral globus pallidus (Gerfen and Keefe 1994). A small population of projection neurons expresses both D1 and D2 receptors ((Surmeier et al. 1996). The role played by DA at the striatal level has been extensively studied. Electrophysiological studies suggest that DAergic transmission modulate the striatal responses to incoming inputs, particularly those mediated by glutamate (Calabresi et al. 1993a; Cepeda and Levine 1998). Release of glutamate in the ST is modulated partly by nigrostriatal DAergic projections.

Chronic blockade of D2 dopamine receptors causes an increase in the levels of both basal extracellular and potassium-releasable glutamate in ST (Yamamoto and Cooperman 1994). In addition striatal DA depletion increases spontaneous glutamate release in ST (Calabresi et al. 1993b). Behavioral studies conducted in freely moving animals show that intra-striatal administration of DA attenuates neuronal excitation elicited by cortical activation in both rats (Kiyatkin and Rebec 1996) and monkeys (Rolls et al. 1984). Studies carried out in anaesthetized animals (Ohno et al. 1987; Johansen et al. 1991) or
using *in vitro* preparations (Calabresi et al. 1987; Nicola et al. 1996) have suggested that the inhibitory modulation might be mediated primarily by D1 receptors (Kiyatkin and Rebec 1999a, b).

**1.8.3 PALLIDAL COMPLEX**

In primates, the pallidal complex is comprised of two segments the medial globus pallidus and lateral globus pallidus. In rodents, medial and lateral segments correspond to the entopeduncular nucleus and globus pallidus, respectively. Both pallidal segments are populated by GABAergic neurons (Oertel and Mugnaini 1984; Oertel et al. 1984).

**1.8.3.1 Entopeduncular nucleus (medial globus pallidus)**

The entopeduncular nucleus is the smallest nucleus of the BG circuit. It plays a central role in the transmission of the BG output to the thalamus and to the motor cortex. Along with the SNpr which shares many histologic and functional properties with the medial globus pallidus, this area is the main output nucleus of the BG circuitry. The entopeduncular nucleus projects primarily to the motor thalamus. In particular, to the ventral anterior and ventral lateral thalamic nuclei which in turn, project diffusely to the motor cortex (Carter and Fibiger 1978; Nauta 1979; Parent and Hazrati 1995b).

Entopeduncular neurons receive a combination of inhibitory (GABAergic) and excitatory (glutamatergic) projections. The balance between the two opposite systems determines the functional activity of the nucleus. The main source of GABAergic fibers is the ST (Parent and Hazrati 1995b) with other GABAergic projections to the nucleus arising from
the adjacent globus pallidus (Parent and Hazrati 1995a). The excitatory innervation of the entopeduncular neurons is provided by the subthalamic nucleus (STN) (Parent and Hazrati 1995a), and the frontal cortex (Naito and Kita 1994a).

1.8.3.2 Globus pallidus (lateral globus pallidus)

Pallidal neurons use mainly GABA (co-localized with enkephalin) as neurotransmitter (Fonnum et al. 1978) and project to structures localized within the BG circuit. These are mainly the STN, SNpc, entopeduncular nucleus, pedunculopontine nucleus and reticular thalamic nucleus (Parent and Hazrati 1995a). There have been reports of the existence of pallidal, GABAergic projections to the SNpr (Smith et al. 1998) and, cholinergic and non-cholinergic projections to the cortex have also been described. The main sources of afferent projections to the globus pallidus are the ST-sending GABAergic fibers and the subthalamic nucleus-sending glutamatergic fibers (Parent and Hazrati 1995a). Fèger (Feger 1997) recently proposed the existence of another excitatory input to the globus pallidus, originating from the thalamus. In addition, the nucleus seems to receive a DAergic innervation from fibers originating from the nigrostriatal pathway (Lindvall and Bjorklund 1979). This finding has been recently confirmed by Cossette et al. (Cossette et al. 1999), who showed that, in the human brain, nigrostriatal axons provide collaterals that reach the pallidal complex.

Like the entopeduncular nucleus, the globus pallidus is particularly enriched with GABA receptors α1 and β2 subunits (Wisden and Seeburg 1992; Boyes and Bolam 2007). D1
and D2 dopamine receptors are also present in the globus pallidus (Richfield et al. 1987), which further supports the potential role of DA at this level.

1.8.4 SUBSTANTIA NIGRA (SN)

Two distinct structures can be recognized within the SN: a densely populated, pigmented area called substantia nigra pars compacta (SNpc) and an adjacent cell-sparse portion, located ventrally, called substantia nigra pars reticulate (SNpr).

1.8.4.1 Substantia nigra pars compacta (SNpc)

Neurons in the SNpc contain neuromelanin and use DA as neurotransmitter. The recipients of nigral projections are the striatum, subthalamic nucleus and globus pallidus. Another group of DAergic neurons, homogeneous to nigral neurons, is located more in the ventral tegmental area (VTA). These neurons project to the ventral ST, amygdala and cerebral cortex (Hauber 1998). Recently, electrophysiological and morphological studies have suggested the existence of a small percentage (5–8%) of nigrostriatal neurons containing GABA instead of DA (Rodriguez and Gonzalez-Hernandez 1999).

Afferent projections to the SNc originate from various structures. Both the ST (Ribak et al. 1980) and the globus pallidus (Smith et al. 1989) send GABAergic projections to nigral DAergic neurons. On the basis of electrophysiological evidence, the existence of GABAergic afferent projections from the SNpr has been suggested (Tepper et al. 1995). The SNc receives glutamatergic projections from the prefrontal cortex, subthalamic nucleus and pedunculopontine tegmental nucleus (which also sends cholinergic
projections) (Naito and Kita 1994a, b; Bezard et al. 1997). Nigral DAergic neurons also receive serotonergic projections from the medial and dorsal raphe nuclei (Hauber 1998).

Among the diverse neurotransmitters that affect nigral activity, it has been pointed out the importance of the glutamatergic input. Both NMDA and AMPA receptors are located on the soma and dendrites of DAergic neurons and regulate their electrical activity (Meltzer et al. 1997a). Iontophoretic administration of NMDA, AMPA and glutamate increase the firing rate of nigral DAergic neurons. These effects are prevented by previous administration of selective antagonists, such as the NMDA antagonists or the AMPA antagonist (Christoffersen and Meltzer 1995; Meltzer et al. 1997b). Recent evidence shows that nigral DAergic neurons express primarily GluR1, GluR2/3 and NR1 subunits (Albers et al. 1999). The presence of metabotropic receptors mGluR1 has been demonstrated within the cell body, axons and dendrites of SNpc neurons (Kosinski et al. 1998).

1.8.4.2 Substantia nigra pars reticulate (SNpr)

The SNpr is located ventral and adjacent to the SNpc, and is populated by GABAergic neurons (Oertel and Mugnaini 1984; Oertel et al. 1984). The SNpc sends its inhibitory projections to the ventral anterior and ventral lateral nuclei of the thalamus. Other targets of nigral projection include the superior colliculus and the pedunculopontine nucleus (Parent and Hazrati 1995b). The similarity with the entopeduncular nucleus extends to the afferent projections, thus the neurons of the SNpc receive a combination of inhibitory (GABAergic) and excitatory (glutamatergic) inputs from diverse structures. The main
source of inhibitory projections is the ST (Chevalier et al. 1985). Other sources of GABAergic afferents include the GP (Smith and Bolam 1989; Smith et al. 1989), nucleus accumbens (Deniau et al. 1994) and ventral pallidum (Groenewegen et al. 1993). Glutamatergic projections to the SNpc originate in the subthalamic nucleus (Kita and Kitai 1987), playing an important role in the regulation of nigral activity. Selective subthalamic lesion reduces the activity of mitochondrial enzymes complex I, II and IV in the SNpc. This reflects the reduced activity of nigral neurons resulting from the abolition of the subthalamic excitatory input (Blandini 2001; Blandini et al. 2001b; Blandini et al. 2001a). The pars reticulata shares many histologic and functional properties including the input/output connections with the entopeduncular nucleus. The two nuclei are considered the major output structures of the BG circuitry and are often referred to as the BG output nuclei.

1.8.5 SUBTHALAMIC NUCLEUS (STN)

The STN is the only glutamatergic nucleus of the BG circuit (Smith et al. 1998) that sends excitatory projections primarily to the BG output nuclei -SNpr and medial globus pallidus (entopeduncular nucleus), and to the lateral GP. Other targets include the ST, SNpc and motor cortex (Kita 2007). Subthalamic neurons receive an important inhibitory innervation from GABAergic neurons of the lateral GP. Other inhibitory projections arise from the ventral pallidum and ventral ST (Groenewegen and Berendse 1990). The STN also receives excitatory projections from the sensory-motor cortex (Fujimoto and Kita 1993), thalamic parafascicular nucleus, and pedunculopontine nucleus (Canteras et al. 1990).
DAergic innervation of the STN, originating from the SNpc has also been described in rats (Hassani et al. 1996) and recently has been demonstrated in the human STN, as well (Cossette et al. 1999; Cossette et al. 2005b; Cossette et al. 2005a). Both GABA and glutamate receptors are abundantly expressed in the STN (Wisden et al. 1992; Wisden and Seeburg 1992). As for the glutamate receptors, both the ionotropic and the metabotropic families have been described (Tallaksen-Greene and Albin 1996; Tallaksen-Greene et al. 1998). DA receptors are present at the subthalamic level, where they play an important role in the context of the BG functional organization. Both D1 and D2 receptors have been repeatedly described in the nucleus (Dawson et al. 1990; Bouthenet et al. 1991; Johnson et al. 1992). Recently, Flores et al. (Flores et al. 1999) reported the presence of mRNAs and binding sites for D1, D2 and D3 receptors.

1.8.6 THE BASAL GANGLIA CIRCUITRY

1.8.6.1 The direct and indirect pathway model

The functional architecture of BG has attracted numerous researchers in the last decade leading to the formulation of a model of BG functioning (Gerfen et al. 1987b; Gerfen et al. 1987a; Albin et al. 1989; Alexander and Crutcher 1990; Graybiel 1990; Gerfen 1992; Albin et al. 1995). According to the model, the ST is the main input nucleus of the circuit, transmitting the flow of information received from the cortex to the BG output nuclei, SNpr and medial globus pallidus, via a direct and an indirect pathway. The two pathways originate from different subsets of striatal neurons and, remain functionally segregated. In the direct pathway, striatal GABAergic neurons, containing dynorphin as a co-transmitter and expressing D1 DA receptors, project mono-synaptically to the SNpr
and medial globus pallidus. In the indirect pathway, the striatal output reaches the target
nuclei via a more complicated route, with different subset of GABAergic neurons
containing enkephalin and expressing D2 receptors, these projects to the lateral globus
pallidus, which sends GABAergic projections to the subthalamic nucleus. The STN, in
turn, sends its glutamatergic efferents to the output nuclei and to the lateral globus
pallidus. From the output nuclei, inhibitory, GABAergic projections reach the ventral
lateral and ventral anterior nuclei of the motor thalamus. Thalamic nuclei then send
 glutamatergic projections to the motor cortex, thus closing the loop (Figure 1.3).

The functional consequence of such organization is that the activation of the direct or the
indirect pathway leads to opposite changes in the net output of the BG circuitry. The
activation of the striatal GABAergic neurons giving rise to the direct pathway causes
inhibition of GABAergic neurons of the output nuclei, leading to the disinhibition of
thalamic nuclei, which are under the inhibitory control of the output nuclei projections.
Conversely, activation of the striatal neurons that project to the lateral globus pallidus, in
the indirect pathway, causes inhibition of the lateral globus pallidus and subsequently
disinhibits the STN. The activation of the subthalamic nucleus which is glutamatergic
increases the activity of the output nuclei. Consequently, their inhibitory control over the
motor thalamus results is enhanced (Alexander and DeLong 1985a, b).

Subthalamic excitatory projections modulate the neuronal activity in the SNpr and medial
globus pallidus. Electrical subthalamic stimulation increases metabolic rates for glucose
in these nuclei (Tzagournissakis et al. 1994) with unilateral ablation of the STN, causes
an ipsilateral reduction in the activity of mitochondrial enzymes complex I, complex II and complex IV in the recipients of subthalamic projections (Blandini et al. 1997; Blandini 2001; Blandini et al. 2001b). Thus, the final output of the basal ganglia processing is under the direct control of the STN.

1.8.6.2 BG changes in PD

The neurodegenerative process in PD causes a functional re-arrangement of the BG circuitry. The current model (Graybiel et al. 1990; Gerfen and Engber 1992; Albin et al. 1995) states that the DAergic denervation of the ST induces a cascade of events leading ultimately to the increased activity of the BG output nuclei. The enhanced activity of the output nuclei would be a result of the enhanced glutamatergic drive from the STN. This model also predicts that the enhanced activity from the output nuclei results in an increased inhibitory control over the motor thalamus and thus the reduction of the thalamic glutamatergic output to the motor cortex. These changes are believed to be the main reason for the parkinsonian symptoms (Figure 1.3b).

These functional changes occurring in PD has been mainly provided by animal studies in particular rodent and non-human primates. The most significant alterations in rodent models include increases in neuronal firing rate (Bergman et al. 1994), glucose metabolism (Mitchell et al. 1992), and mitochondrial enzyme activity (Porter et al. 1994; Vila et al. 1997) in the STN or its projection nuclei. In rats, lesion of nigrostriatal pathway causes down-regulation of glutamate receptors in projection nuclei of STN this is consistent with the current hypothesized hyperactivity of subthalamic glutamatergic
projections (Porter et al. 1994). Subthalamic ablation leads to increases in mitochondrial enzyme activity in the entopeduncular nucleus and SNpr of 6-OHDA lesioned rats (Blandini et al. 1997) and abolishes rotational response to apomorphine (Burbaud et al. 1995; Blandini et al. 1997). Also it normalizes the firing rate and discharge pattern of pars reticulate neurons in addition preventing the changes in gene expression in the ST, entopeduncular nucleus and GP (Delfs et al. 1995) of rat with unilateral nigrostriatal lesion.

Human studies also support the view that subthalamic hyperactivity plays a central role in PD pathophysiology. Not only reports of PD symptoms disappearance in a PD patient after the occurrence of a subthalamic hematoma (Sellal et al. 1992) but also a down-regulation of NMDA receptors in the medial GP as result of increased activity of subthalamic projections to the medial GP (Lange et al. 1997). Thus the introduction of electrophysiological techniques in the therapy of PD was rationally by blocking the hyperactivity of subthalamic neurons resulting in relievement of PD symptoms.

The STN is also a key player in the compensatory mechanisms that sustain the DAergic function in the pre-symptomatic phase of PD. The gradual loss of in the SNpc is initially accompanied by an increased DA at the ST level by efficiency of residual DAergic neurons (Zigmond et al. 1990b; Zigmond et al. 1990a). Monkeys rendered parkinsonian by MPTP have increased glutamatergic inputs to SNpc as temporary blockade of these inputs reveals the PD motor abnormalities (Bezard et al. 1997). This compensatory mechanism masks the PD symptoms in an early phase of PD but as it progresses and the
degenerative process reaches a threshold at which the functional changes leads to PD symptoms are triggered.
Figure 1.3 The current model of the basal ganglia.

The diagrams illustrate the functional organization of the basal ganglia in (a) normal conditions, (b) Parkinson’s disease. Thick and broken lines indicate pathways that are believed to be respectively hyperactive or hypoactive in the pathological conditions. The blue and red indicate inhibitory (Glutamate) and excitatory (GABA) projections, respectively. $D_1 =$ Direct pathway, $D_2 =$ Indirect pathway. Substance P (SP) + GABA (excitatory) = $D_1$ pathway, Enkephalin + GABA (excitatory) = $D_2$ pathway. Dopamine on $D_2$ receptor (inhibitory), dopamine on $D_1$ (excitatory) (Parent A et al., 2000). With the current model it is thought that dopamine inhibits neuronal activity in the indirect pathway and to excite neurons in the direct pathway. Whereas in the parkinsonian state, dopamine depletion leads to disinhibition of D2-receptor-bearing striatal neurons in the GPi/SNr and overinhibition of thalamo-cortical and brainstem motor centers resulting in parkinsonism.
1.9 THE NIGROSTRIATAL PATHWAY

1.9.1 INTRODUCTION

Nigrostriatal DAergic neurons are critically involved in the control of voluntary movements (DeLong 1990; Grillner and Mercuri 2002). As a result, their loss in PD leads to profoundly disabling motor symptoms (Agid 1991). The development of the mDA cell type during mammalian embryogenesis can be subdivided into three distinct processes: (1) the induction of a progenitor field within the neuroepithelium competent to generate mDA precursors at early stages of neural development (approx. from embryonic day (E) 8.5 to E10.5 in the mouse); (2) the specification of a mDA neuronal fate in these precursors at intermediate stages (approx. from mouse E10.5 to E12.5); and (3) the acquisition of the mature phenotype or terminal differentiation of mDA neurons at relatively late stages of neural development (i.e. from E12.5 onwards).

1.9.2 Ontogeny of Midbrain DAergic neurons

During the development of the rat, the cells of the SN and VTA are produced between embryonic day 11 and 15 (E11 and E15). This occurs in the ventricular zone where the greatest numbers of cells are undergoing division between E13 to E15 (Noisin and Thomas 1988; Voorn et al. 1988; Santana et al. 1992; Aubert et al. 1997; Park et al. 2000; Gates et al. 2006). It has been demonstrated that rat mDN are specified by the floor plate (FP-specialised group of cells at the ventral midline of the neural tube) which is mediated by contact-dependent mechanism (Hynes 1995). The FP, secretes the lipid-modified glycoprotein Sonic hedgehog (Shh), which is known for its pivotal role in the specification of the different ventral populations in the hindbrain and spinal cord by
controlling the expression of different transcriptional regulators (Placzek and Briscoe 2005) in addition mediates the DAergic induction.

Following induction of DAergic neurons, migration occurs. Initially the neurons migrate ventrally in a radial pattern (Marchand and Poirier 1983) along glia paths (Shults et al. 1990) which extends to the ventral surface of the mesencephalon and in a rostral direction. During the migration process the cells differentiate and become immunoreactive for TH from E12.5 (Specht et al. 1981b). The D1 (excitatory receptors) are initially present in striatal patches then gradually the homogenous distribution characteristic of the adult ST emerges. D2 (inhibitory) receptor expression within the ST is a homogeneous population which finally forms clusters.

1.9.3 In vivo development of the nigro-striatal pathway

TH-ir terminals are first detected in the caudal putamen from E14.5 (Specht et al. 1981a, b). Thereafter, the innervation of the rostral striatum shows a ventrolateral to dorsomedial gradient and the innervation of caudal regions is from a ventromedial position (Voorn et al. 1988). As they reach the ventral mesencephalon the dopaminergic neurons then project axons to the ST. At E14, DAergic neuron bundles move towards the ST and by E17 the bundles are dense and are present in the dorsolateral ST.

The DAergic neurons become more closely packed together within each ventral mesencephalon during stages E15-16 along each side of the midline. These two groups in the more caudal regions merge along the midline to form the origin of the VTA, at E17.
the dendrites can be clearly seen and from E19 onwards the SNpc, SNpr and VTA can be
distinguished throughout the whole VM. During the first postnatal week, the VM
develops the typical adult organization with DAergic neurons of the SN projecting dendrites
both within the pars compacta and reticulata (Voorn et al. 1988). There is a functional
and anatomical diversity among mDNs (Carlsson 1959; Dahlstroem and Fuxe 1964a, b).
mDN located within the SNpc (SN or A9) form the nigrostriatal pathway to the
dorsolateral ST and are essential for the control of voluntary movement; mDNs within the
ventral tegmental area (VTA or A10) form the mesolimbocortical system and controls
brain mechanisms of novelty, reward, and addiction; and mDNs within the retrorubral
field (RRF or A8) are involved in both local midbrain circuitry and the SN pathway.

1.9.4 *In vivo* development of patch/matrix innervation

The adult ST is functionally and structurally separated into “patch” and “matrix” regions.
These differences arise from the development of DAergic innervation of the ST, as
innervation to patch regions of the ST occurs before formation of the matrix (Voorn et al.
1988). By E19, the DAergic axons produce ramifications in “patch” regions throughout
the ST. Following birth a second group of neurons project axons to the matrix regions of
the ST, by third week postnatal week the characteristic adult pattern of innervation has
formed. Studies by Gerfen (Gerfen et al. 1987a) confirm these observations by 6-OHDA
injection into nigro-striatal pathway into newborn rats destroyed DAergic innervation of
patch regions whereas the matrix region which develops later, remained intact.
The subdivision of the ST into two major compartment—the striosomes (or patches) and the extrastriosomal matrix, was first proposed by Graybiel and Ragsdale in 1978 (Graybiel and Ragsdale 1978). These investigators noted small AChE-poor striosomes that represented approximately 10–20% of the total striatal volume and formed complex three-dimensional labyrinths embedded in an AChE-rich matrix (Graybiel and Ragsdale 1978; Penney and Young 1986; Johnston et al. 1990). The notion of striosome/matrix compartmentalization of the ST was further supported by studies of the distribution of a wide variety of transmitter-related substances and by the organization of striatal afferent and efferent connections (Pickel et al. 1981a; Pickel et al. 1981b; Ferrante et al. 1986; Gerfen et al. 1987b; Gerfen et al. 1987a; Graybiel et al. 1987; Holt et al. 1997; Prensa et al. 1999).

1.9.5 *In Vitro* modeling of nigrostriatal pathway

*In vitro* studies have been utilized to study the development of the nigrostriatal pathway. Both dissociated neuronal cultures of DAergic nigro-striatal neurons and their target region the striatum (Prochiantz et al. 1979; di Porzio et al. 1980; Hemmendinger et al. 1981; Prochiantz et al. 1981; Kotake et al. 1982; Denis-Donini et al. 1983, 1984) and organotypic co-cultures (Whetsell et al. 1981; Jaeger et al. 1989; Ostergaard et al. 1990) have been used to investigate the development of nigrostriatal neurons.

DAergic neurons in both organotypic (Ostergaard et al. 1990) and dissociated neuronal cultures (Prochiantz et al. 1979) can survive in the absence of their striatal target region. The TH-ir neurons of the VTA and SN derived from rats aged between E21 and postnatal
day 7 retain their basic morphological characteristics in organotypic cultures (Ostergaard et al. 1990) however, the maturation and development of DAergic nigro-striatal neurons measured by studies of DA synthesis and uptake, is enhanced by the presence of striatal cells (Prochiantz et al. 1979; di Porzio et al. 1980; Shalaby et al. 1983) (or striatal membranes (Prochiantz et al. 1981). Also, the DAergic neurons do not develop axons in the absence of target cells but in their presence DAergic neurons form axons characteristic of those formed in vivo (Hemmendinger et al. 1981).

The growth of these DAergic neurons is also shown to be target specific by innervation of the ST. Total neurite outgrowth of the neurons is significantly reduced when DA neurons are cultured with striatal cells but not with the predominant mesencephalic non-DA cell population or with cerebellar cells at any concentration tested (Denis-Donini et al. 1983). Direct neuro-neuronal interactions between DA neurons and striatal cells regulate the development or maturation of the afferent DA cells is in agreement on the basis of biochemical data (di Porzio et al. 1980). Further support that co-cultures containing the VTA/SN-complex and the PFC or the striatum, dopaminergic mesencephalic neurons able to innervate the target areas in the PFC and the STR but no TH+ve fibers and cells were found in the prefrontal as well as in the striatal tissue cultured alone and also in co-cultures which had not developed a bridge (Franke et al. 2003).

Co-cultures of dissociated striatal and mesencephalic cells have been used to demonstrate the influence of target cells on the development of DA arbors (Prochiantz et al. 1979; Manier et al. 1997). However, tissue organization and spatial cues related to this
organization are lost in dissociated cultures, and mechanisms relying on these aspects, such as selectivity of the innervation, can hardly be studied in such cultures. Organotypic slice cultures might represent a convenient compromise between in vivo models and the use of dissociated cocultures (Bahr 1995; Gahwiler et al. 1997).

1.10 FACTORS INFLUENCING THE DEVELOPMENT OF MESENCEPHALIC DOPAMINE NEURONS (mDNs)

Many factors have been identified that may be involved in the formation of the nigrostital pathway by promoting survival, differentiation and maturation of nigrostriatal neurons in vitro. The development of mDNs can be divided into 4 stages (Figure 1.4). First, progenitors at the ventral mesencephalic surface that have self-renewing properties and give rise to multiple cell types (‘stem-like’) arise at embryonic day 7.5 (E7.5). In a second step, these are specified to a DAergic neuronal precursor cell fate, and several molecular markers are associated with this population (see below). In a third stage, the DAergic precursors exit the cell cycle and begin to display early mDN markers. Finally, the early mDNs functionally mature, express mature mDN markers establishing appropriate connectivity.

1.10.1 Early development

Mesencephalic organization is initiated by the positioning of key signaling centers such as the floor plate, present at the ventral midline, and the midbrain–hindbrain junction (MHJ, also known as the isthmic organizer) at the caudal extreme of the mesencephalon. In addition, early patterning events also position the dividing stem-like precursors that are
compotent to become mDNs in the context of instructive cues from signaling centers. Genetic loss-of-function studies have identified a network of transcription regulators and other signaling factors that underlie patterning of the midbrain and hindbrain precursors (Joyner et al. 2000). Expression of two homeodomain transcription factors, Otx2 in the midbrain and Gbx2 in the hindbrain, initially form a boundary at the MHJ at E7.5 in the mouse and becomes well-defined by E9.5. Multiple signaling and regulatory molecules function as effectors downstream of Otx2 and Gbx2 at the MHJ, including fibroblast growth factor-8 (FGF8), Engrailed (En)-1 and -2 homeobox transcription factors, the secreted factor Wnt1, the Lim-domain transcription factors Lmx1a and Lmx1b, Foxa2, and the paired-like homeodomain proteins Pax-2, -5, and -8 (Liu and Joyner 2001).

1.10.2 Specification of mitotic mesencephalic precursors to the mDN fate

In the early embryonic midbrain (E7.5–E9.5 in the mouse), the floor plate organizer at the ventral surface of the neural tube (Hynes et al. 1995) and the midbrain/hindbrain junction at the caudal extreme of the midbrain (MHJ or isthmus) (Alvarado-Mallart 1993) instruct the fate of adjacent neural progenitors. A number of secreted proteins encode the action of these signaling centers such as Sonic hedgehog (SHH), secreted by the floor plate organizer, is necessary to instruct the ventral cell fate of progenitors in vivo and in vitro and fibroblast growth factor-8 (FGF8) a key MHJ-derived signal that is required for specification of the mesencephalic progenitors (Ye et al. 1998). Wnt1 is an additional secreted factor that promotes mDN development (Schulte et al. 2005). Transgenic markers demonstrate Wnt1 expression (lateral to the floor plate) at the region of DAergic
neuronal progenitors at the time point when postmitotic mDNs are born (murine E9.5–E11) (Zervas et al. 2004)

1.10.3 Postmitotic development of mDNs

As midbrain precursor cells exit the cell cycle (E10.5–E11.5 in mice), they migrate away from the ventricular surface. Early phenotypic markers of mDNs, such as TH and other enzymes in the dopamine biosynthesis pathway are induced at this time (E11.5 in the mouse; (Lauder and Bloom 1974; Wallen et al. 1999). Subsequent maturation of mDNs (E12–15) is characterized by the expression of common synaptic markers and the DAT. DAT expression is relatively specific to the mDN population, unlike TH, which is also expressed in other catecholaminergic cell types. Several transcription factors have been implicated in the postmitotic development of mDNs (Figure 1.5). Within the postmitotic mesencephalic cell population, expression of these factors is restricted to the early mDNs but, none of these transcription factors (TF) appear sufficient individually to instruct the mDN phenotype, suggesting a network model. One important TF is Nurr1 an orphan nuclear receptor/transcription factor which is induced in postmitotic mDN precursors at approximately E10.5, just preceding TH expression (Wallen and Perlmann 2003). Nurr1 is necessary for mDN specification as Nurr1-deficient animals fail to express early mDN phenotypic markers including TH and cRet, a component of the Glial cell derived neurotrophic factor (GDNF) receptor.
Figure 1.4 Stages of MDN development.
Initially mature stem cells are patterned to a ventral cell fate and away from a dorsal fate (in grey). Subsequently ventral dividing mesencephalic precursors are specified towards a MDN fate away from other fates, such as serotonergic neurons (in grey). A network of transcription regulatory factors promotes (blue) or inhibits (green) the process. An array of secreted factors is implicated at each step of the developmental program (red) (adapted from (Abeliovich and Hammond 2007)).
**Figure 1.5 Signaling cascades in the MDN development.**

Soluble factors (green) and transcription factors (blue). (A) At the dividing mesencephalic progenitor stage, SHH induces Gli1 activation and Foxa2 induction (which can induce SHH expression i.e. autocrine loop). (B) SHH also induce transcription cascade involving activation of Lmx1a, Msx1, and Ngn2, and inhibition of Nkx6.1. Otx2 induces Ngn2 expression of Nkx2.2. Nurr1, Lmx1b, and En1/2 function in parallel to induce aspects of the postmitotic mDN fate. Nurr1 and Pitx3 act cooperatively to induce late markers of the mDN phenotype (adapted from (Abeliovich and Hammond 2007)).
1.10.4 Development of postmitotic mDNs

Several secreted factors have been implicated in postmitotic mDN development including Wnt family members such as Wnt5a (Schulte et al. 2005) and FGF family members such as FGF-20 (Ohmachi et al. 2003). It is unclear whether secreted factors that act at an earlier stage in the development of mitotic precursors, such as SHH or FGF8, serve an additional function in postmitotic mDN development. Overexpression of Nurr1 in neuronal cell lines (Wagner et al. 1999), hippocampal neural progenitor cultures (Sakurada et al. 1999), midbrain precursors (Kim et al. 2003), and embryonic stem cells (Sonntag et al. 2004; Kim et al. 2006) appears to promote the generation of mDNs, but this may reflect a broad proneural activity of Nurr1 (Kim et al. 2003). Furthermore, only a subset of mDN markers appears to be induced by Nurr1 alone. Pitx3 overexpression in undifferentiated ES cultures (Chung et al. 2005) or in neuron progenitor cells (Sakurada et al. 1999) leads to either no changes or the induction of a limited set of nigral mDN markers suggesting Pitx3 drives the expression of markers specific to the SN but not the VTA, as VTA neurons are relatively spared in Pitx3 mutant mice (Chung et al. 2005). These transcription factors in mDN maturation may function within a network as Nurr1 alone is sufficient to induce early mDN markers such as TH, and late events in mDN maturation can only be induced by the combination of Nurr1 and Pitx3 (Martinat et al. 2006).

1.10.4 Functional maturation and axonal pathfinding

Functional maturation of mDNs is distinguished by axonal pathfinding, synaptogenesis, and depolarization-induced vesicular DA release. Several studies have suggested that
interaction with ST target tissue promotes mDN maturation events (Perrone-Capano and Di Porzio 2000). Newly born mDNs extend axonal and dendritic processes that must be correctly targeted to their destinations. mDN axons project rostrally at E11 in the mouse (Nakamura et al. 2000b) by E14.5, many of these axons have reached their targets. mDN axonal pathfinding is likely governed by some of the same guidance cues that regulate CNS projections (Tessier-Lavigne 2002). Expression of Neuropilin-1, a co-receptor for the semaphorin family ligands, appears to be induced by Nurr1 (Hermanson et al. 2006), but its role for semaphorin signaling in the guidance of mDN axons has not been clearly defined. In addition, the homeodomain proteins En-1 and En-2, which are implicated in mDN maturation, can induce the expression of the guidance molecule receptors ephrin-A2 and ephrin-A5 (Logan et al. 1996; Kimura et al. 2004).

Slits and Netrins may also be involved in target recognition by nigrostriatal mDNs as they express the Slit receptors, Robo1 and Robo2, and the Netrin receptor DCC (Lin et al. 2005). The Slit family of guidance cues has also been implicated in the guidance of mDN projections, as mice deficient in Slit2 or both Slit1 and Slit2 show DAergic projections that are displaced ventrally in the diencephalon (Bagri et al. 2002). Furthermore, in the double mutants some axons aberrantly cross the ventral midline. mDNs are repelled by Slit2-expressing cells in culture, whereas they are attracted by Netrin-1-expressing cells, suggesting that Slit2 and Netrin-1 act in concert to guide these axons to the lateral ganglionic eminence (LGE), the precursor to the ST (Lin et al. 2005).
Once the nigrostriatal mDNs reach the ST their targeting may be regulated by ephrin signaling, as these mDNs express the EphB1 receptor, while ephrin-B2 ligand is expressed in the ST. Cultures of SN mDNs, but not VTA mDNs, show reduced axonal outgrowth on monolayers of ephrin-B2表达ing cells (Yue et al. 1999). Such signaling could ensure accurate (topographical) innervation of the ST by the nigrostriatal mDNs. Interestingly application of ephrin-B2 to midbrain neuronal cultures results in an upregulation of Nurr1, suggesting that correct target recognition is required for the maintenance of DAergic identity and better survival (Calo et al. 2005).

Upon target innervation, mDNs axons likely compete to establish synapses and survive. In support of this model, a wave of apoptotic mDN cell death is observed perinatally in rodents (Burke 2003). Growth factors, notably GDNF and BDNF, have long been implicated in this process, as this increases the survival (Beck et al. 1995) and arborization of mDNs (Costantini and Isacson 2000a, b). In vivo these data have been corroborated by the observation that transplantation of GDNF-expressing cells into mice lesioned with the mDN toxin 6-hydroxydopamine (6-OHDA) results in increased survival of mDNs and sprouting of DAergic axons into the graft site (Akerud et al. 1999). Notably Nurr1 mutant mice lack expression of the GDNF receptor cRet (Wallen and Perlmann 2003), suggesting that defective GDNF signaling may partially account for the migration and target innervation defects seen in such mice.

Finally, there is surprising functional and anatomical diversity among mDNs (Carlsson 1959; Dahlstroem and Fuxe 1964b). mDN located within the SNpc (SN or A9) form the
nigrostriatal pathway to the dorsolateral ST and are essential for the control of voluntary movement; mDNs within the ventral tegmental area (VTA or A10) form the mesolimbocortical system and controls brain mechanisms of novelty, reward, and addiction; and mDNs within the retrorubral field (RRF or A8) are involved in both local midbrain circuitry and the SN pathway. The guidance cues that underlie this diversity remain to be identified. This may also relate to the apparent cell-intrinsic differences among types of mDNs, as described above (Maxwell et al. 2005).

1.11 NEUROTROPHINS AND THE DEVELOPMENT OF mDNs

The experimental evidence on which the neurotrophic factor concept was built came mainly from studies of the first-discovered and purified neurotrophic factor (NTF), nerve growth factor (NGF), and its capacity to increase survival, neurite growth, and neurotransmitter synthesis of paravertebral sympathetic neurons (Levi-Montalcini 1987). NGF is synthesized by the target cells of these neurons, is released, binds to high affinity binding receptors of the nerve terminals, is subsequently internalized, and is retrogradely transported together with its receptors to the neuronal cell soma where it exerts its action (Lewin 1996; Lewin and Barde 1996; Campenot and MacInnis 2004). NGF has been shown to act in vitro and in vivo (Levi-Montalcini 1987; Snider 1994; Klein 1994), and has become more clear during the last few years that cell death from Caenorhabditis elegans to mammals is a tightly regulated process that requires both specific death-promoting external signals and the coordination and synergies of several intracellular signaling networks (Frade and Barde 1998; Krieglstein et al. 2000; Raoul et al. 2000; Barker et al. 2001). This is demonstrated by glial-cell-line-derived neurotrophic factor
(GDNF) promoting the survival of impaired mDN more efficiently when it is co-applied with anti-apoptotic agents (Eberhardt et al. 2000; Perrelet et al. 2002).

Five established growth factors may be considered to act as target-derived neurotrophic factors for nigrostriatal DAergic neurons: GDNF, neurturin (NRT), brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NT-4), and fibroblast-growth factor-2 (FGF-2).

1.11.1 Glial-cell-line-derived neurotrophic factor (GDNF)

GDNF was identified and purified from a glioma cell line, B49, by its ability to promote survival of mesencephalic DAergic neurons (Unsicker et al. 1991; Lin et al. 1993; Airaksinen and Saarma 2002). GDNF is expressed throughout the CNS, with the highest levels occurring in the P0 and P10 rat, in the human ST (Springer et al. 1994; Choi-Lundberg and Bohn 1995), and at lower levels, in the SN (Schaar et al. 1993; Choi-Lundberg and Bohn 1995) suggesting that GDNF may act both as a local and a target-derived NTF for nigrostriatal DAergic neurons. The developmental pattern of expression correlates with striatal target innervation and with ontogenetic cell death (Burke 2004). The selectivity of GDNF for nigrostriatal DAergic neurons is still debatable as other neuron populations have been shown to be responsive to GDNF (Henderson et al. 1994; Arenas et al. 1995; Buj-Bello et al. 1995; Trupp et al. 1995; Farkas et al. 1997; Krieglstein et al. 1998a), the relevance of and excitement for GDNF as the prime candidate for a NTF-based Parkinson’s therapy are however still unaltered (Kordower 2003).
In addition to promotion of survival, GDNF has also been shown to induce neurite growth, sprouting (Akerud et al. 1999), synaptic efficacy (Bourque and Trudeau 2000), and TH expression (Xiao et al. 2002). GDNF has also been shown to enhance long-lasting changes on DAergic afferents, including increased DA turnover in vivo (Hudson et al. 1995). In animal models of PD, the effect of GDNF has a major impact on the metabolic strengthening and increased DA synthesis of the remaining neurons (Gash et al. 1996). However, there are also reports of aberrant effects of GDNF in certain experimental situations. GDNF has been shown to induce aberrant sprouting and downregulation of TH in 6-OHDA-lesioned nigrostriatal DAergic neurons (Georgievska et al. 2002a).

1.11.2 GDNF family- ligands

GDNF is part of a family of growth factors which includes neurturin, artemin, and persephin (Baloh et al. 2000; Saarma 2000) which share the transmembrane tyrosine kinase receptor with its intracellular tyrosine kinase, c-Ret. Their specificity, however, is through their individual ligand-binding receptor, which is a GPI-anchored receptor interacting with c-Ret upon ligand binding (Baloh et al. 2000; Sariola and Meng 2003). They all have survival-promoting activity of GDNF on MDN under many in vitro conditions (Lin et al. 1993) such as preventing apoptosis in vitro (Burke 1998) and in vivo (Oo et al. 2003). Administration of GDNF into the ST during the biphasic period of ontogenetic cell death suppresses apoptosis of nigral DAergic neurons. Neutralizing
endogenous GDNF in the ST diminishes cell death, although only during the first period of cell death (Kholodilov et al. 2004; Baloh, 2000 #1300).

1.11.2.1 Neurturin

The other GDNF family ligands NRT, artemin, and persephin, are also expressed in the nigrostriatal system. NRT is of interest as it is also expressed in the ST (Widenfalk et al. 1997; Horger et al. 1998; Akerud et al. 1999). NRT mRNA has been found in the mouse VMB and ST during development with peaks in the MB floor at E11.5–E13.5 as DAergic progenitors differentiate. As DAergic neurons extend their axonal projections to targeted areas, NRT expression decreases in the SN and gradually increases in the ST with a peak at P10. NRT expression levels are relatively low compared with those of GDNF and do not precisely match the time course of ontogenetic cell death (Cho et al. 2004a; Cho et al. 2004b) suggesting NRT’s role in regulating ontogenetic cell death in DAergic neurons, either as a target-derived or as a local paracrine NTF. Its ability to promote the survival of DAergic neurons in vitro is comparable to GDNF (Horger et al. 1998; Akerud et al. 1999) but does not induce sprouting and hypertrophy of developing DAergic neurons like GDNF. In vivo, NRT protects 6-OHDA-lesioned nigrostriatal neurons (Horger et al. 1998; Akerud et al. 1999), again does not induce TH staining and sprouting (Akerud et al. 1999). There are also regionally distinct effects of NRT in vivo beeing reported with infusion into the lateral ventricle in rats’ leads to higher DA levels in the mediodorsal ST, relative to the ventrolateral ST, whereas such a distinct regional distribution could not be identified upon GDNF infusion (Hoane et al. 1999).
1.11.2.2 Neublastin/artemin and persephin

In addition to GDNF and NRT, neublastin/artemin (ART) and persephin (PSP) also promote the survival of DAergic neurons in vitro and in vivo (Balah et al. 1998; Milbrandt et al. 1998; Rosenblad et al. 2000b). PSP is expressed in relatively high levels in the MB floor and in the ST (Jaszai et al. 1998; Akerud et al. 2002). DAergic neurons also express the preferred ligand-binding receptor for PSP, GFR-4 (Enokido et al. 1998; Akerud et al. 2002). However, there are too few data available to discuss their physiological relevance in the context of promoting DAergic neuron survival.

1.11.3 Neurotrophins

Neurotrophins represent a family of growth factors that are closely related to NGF, namely brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 (Lindsay et al. 1993; Seroogy and Gall 1993; Unsicker 1994; Lykissas et al. 2007; Skaper 2008). BDNF and NT-4 signal via the tyrosine kinase receptor trkB, and NT-3 does so via trkC (Huang and Reichardt 2003; Segal 2003). Neurotrophins and NGF have been shown to promote survival of MDN (Chaturvedi et al. 2006; Hirata et al. 2006; Jiang et al. 2006). Nigral DAergic neurons express the high affinity receptors trkB and trkC (Merlio et al. 1992; Numan and Seroogy 1999), suggesting that DAergic neurons direct response to the corresponding trk ligands. BDNF mRNA has been detected in the ST, and BDNF and NT-3 occur in the VMB (Hofer et al. 1990; Friedman et al. 1991; Gall et al. 1991; Seroogy et al. 1994). In contrast to other neurotrophins, the expression of NT-4 is relatively low in the CNS (Berkemeier et al. 1991). BDNF and NT-3 have been shown to be retrogradely transported by DAergic neurons when injected into the ST (DiStefano et
al. 1992; Mufson et al. 1994), whether BDNF acts as a target-derived factor for nigrostriatal DAergic neurons is unknown; none of the mice carrying null mutations of the neurotrophins nor their receptors provide supportive evidence (Yan et al. 1993; Yan and Miller 1993; Klein 1994).

Chronic BDNF infusion results in an increased number of spontaneously active DAergic neurons, showing an increased firing rate and an increased number of action potentials within bursts (Shen et al. 1994) which may be the basis for the increased locomotor behavior and striatal DA turnover as observed during supranigral BDNF infusions. Similarly, the reduction of endogenous BDNF by the intranigral and intrastriatal application of anti-sense BDNF in rats modulates nigrostriatal neurotransmission (Lau et al. 1998), and homozygous BDNF mutations produce severe catecholamine depletion within the nigrostriatal DAergic system, whereas heterozygous mice exhibit a slight but significant elevation of striatal DA content (Dluzen et al. 1999). Rite et al. (Rite et al. 2003) have shown that the expression of BDNF in the SN is dependent on target integrity but is independent of neuronal activation. Suggesting BDNF is probably not a target-derived NTF for nigrostriatal DAergic neurons, but that it possibly acts in the SN in an autocrine or paracrine fashion to modulate nigrostriatal function anterogradely.

1.11.4 Local factors

Several other growth factors have been shown to promote the survival of DAergic neurons. These include fibroblast-growth factor-2 (FGF-2) (Otto and Unsicker 1990; Timmer et al. 2007; Peng et al. 2008), transforming growth factor beta (TGF-β)
(Krieglstein et al. 1995b; Farkas et al. 2003; Roussa et al. 2004; Roussa and Krieglstein 2004; Roussa et al. 2006), growth/differentiation factor 5 (GDF5) (Krieglstein et al. 1995c), GDF15 (Strelau et al. 2000), bone morphogenetic proteins (BMPs) (Jordan et al. 1997), TGF-α (Alexi and Hefti 1993), heparin-binding epidermal growth factor (HB-EGF) (Farkas and Krieglstein 2002), and sonic hedgehog (Shh) (Miao et al. 1997). FGF-2, BMPs, and HB-EGF survival-promoting capacity may be indirect and is possibly mediated via astroglial cells and represent a relevant endogenous source for dopaminotrophic growth factors (Nakayama et al. 2003). Their complete spectrum of survival-promoting molecules has not been analyzed (Schaar et al. 1994; Engele and Franke 1996; Engele et al. 1996; Engele and Schilling 1996).

1.11.4.1 TGF-β

This is a multifunctional growth factor (Bottner et al. 2000; Unsicker 2000; Unsicker and Strelau 2000). TGF-β2 and TGF-β3 are expressed in the developing and adult SN and in the ST (Flanders et al. 1991; Unsicker et al. 1991). In vivo, TGF-β’s role as endogenous promoter of DAergic neuron survival has been shown (Farkas et al. 2003; Roussa et al. 2006) and is supported with GDNF (Krieglstein et al. 1998b), Shh and FGF-8 (Roussa and Krieglstein 2004). Similar to BDNF, TGF-β acts directly on DAergic neurons, as demonstrated by TGF-β dependent translocation to the nucleus of TH’ve neurons, but may act additionally on other mesencephalic cells (Hyman et al. 1994; Roussa et al. 2004).

1.11.5 Low molecular weight compounds
The delivery of proteins into the brain is a problem (Fricker and Miller 2004; Pan and Kastin 2004) thus, there is an ongoing search to find small compounds that either mimic neurotrophic factors, enhance neurotrophic effects, or induce neurotrophic factors in the substantia nigra. Several low molecular weight compounds have been indentified that increases the production of BDNF in nigral DAergic neurons: salicylic acid, cGMP analogs, okadaic acid, IBMX, dipyridamole and glutamate (Chun et al. 2000) AMPA receptors have been found to serve as potential targets as their stimulation increases neuronal activation and activity-dependent signalling (Zafra et al. 1990). A potent and selective AMPA receptor potentiator, LY404187 protects against unilateral infusion of 6-OHDA into the SN or ST of rats (ONeill et al. 2004). The protective effect may be attributable to the increased expression of BDNF in DAergic neurons in vivo (O'Neill et al. 2004), as these compounds have previously demonstrated their capacity to increase BDNF expression in primary neuron culture (Legutko et al. 2001).

An alternative approach to enhance effects of endogenous BDNF is to block dephosphorylation of the activated tyrosine kinase receptor trkB. Inhibition of protein tyrosine phosphatases has been shown to protect axotomized nigral DAergic neurons in vivo (Lu et al. 2002). Application of an ineffective dose of BDNF synergistically increases the protective capacity of such nihbitors, suggests that tyrosine phosphatase inhibition prevents neuronal death by enhancing neurotrophic signalling.

1.11.6 Immunophilin ligands
The immunophilin ligands has been introduced as potential neurotrophic molecules (Guo et al. 2001a; Guo et al. 2001b) acting via their protein receptors and are known immunosuppressants. The first immunophilin ligand GPI-1046 came of interest due to its neurotrophic activities (Steiner et al. 1997). GPI-1046 bound to FK506-binding protein-12 induces neurite outgrowth in vitro similarly to NGF, and regenerative sprouting from spared nigrostriatal neurons following MPTP or 6-OHDA lesioning in rats. Systemically, GPI-1046 in MPTP treated rhesus monkeys failed to produce neuroprotective effects (Emborg et al. 2001). As the immunosuppressant effect of immunophilin ligands makes them unsuitable for neurological application, nonimmunosuppressant immunophilin ligands have been introduced. Novel compounds include V-10,367 (Costantini et al. 1998); and V-13,661 (Costantini et al. 2001) (Constantini et al. 2001). Both ligands prevent loss of striatal DA innervation in the MPTP mouse model following oral administration. However, only orally applied V-10,367, which binds to FKBP12 protects against a slow and progressive intrastriatal 6-OHDA lesion.

1.12 CHANGES IN CYTOKINE LEVELS, DECLINE OF ESSENTIAL NTFs AND LINKS WITH PD

1.12.1 Human evidence

Degeneration of the DAergic neurons of the SNpc and the resulting loss of nerve terminals accompanied by DA deficiency in the ST are responsible for most of the movement disturbances observed in PD. One hypothesis of the cause of degeneration of the nigrostriatal DA neurons is that PD is caused by programmed cell death (apoptosis) due to increased levels of cytokines and/or decreased ones of neurotrophins. There is a
marked increase in levels of cytokines (Nagatsu et al. 2000a; Nagatsu and Sawada 2005; Sawada et al. 2006), such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, IL-2, IL-4, IL-6, transforming growth factor (TGF)-alpha, TGF-beta1, and TGF-beta2, and decreased levels of neurotrophins (Siegel and Chauhan 2000; Chauhan et al. 2001), such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), in the nigrostriatal DA regions and ventricular and lumbar cerebrospinal fluid of PD patients. Furthermore, the levels of TNF-alpha receptor R1 (TNF-R1, p55), bcl-2, soluble Fas (sFas), and the activities of caspase-1 and caspase-3 were also elevated in the nigrostriatal DA regions in PD (Dragunow et al. 1997; Tatton et al. 2003; Nagatsu and Sawada 2007).

1.12.2 Evidence from experimental PD models

In experimental animal models of PD, IL-1 beta level is increased and NGF decreased in the ST of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonian mice, and TNF-alpha level increased in the SN and ST of the 6-hydroxydopamine (6-OHDA)-injected side of hemiparkinsonian rats (Grunblatt et al. 2000a, b). L-DOPA alone or together with 6-OHDA does not increase the level of TNF-alpha in the brain in vivo (Mogi et al. 1999). Increased levels of proinflammatory cytokines, cytokine receptors and caspase activities, and reduced levels of neurotrophins in the nigrostriatal region in PD patients, and in MPTP and 6-OHDA-produced parkinsonian animals suggest increased immune reactivity and programmed cell death (apoptosis) of neuronal and/or glial cells (Nagatsu et al. 2000a, b). These data indicate the presence of such proapoptotic environment in the SN in PD that may induce increased vulnerability of neuronal or glial cells towards a variety of neurotoxic factors. The probable causative linkage among the increased levels of proinflammatory cytokines and the decreased
levels of neurotrophins, candidate parkinsonism-producing neurotoxins such as isoquinoline neurotoxins (Nagatsu 1997), and the genetic susceptibility to toxic factors, remains for further investigation in the molecular mechanism of PD. The increased cytokine levels, decreased neurotrophin ones, and the immune response in the nigrostriatal region in PD indicate new neuroprotective therapy such as nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, immunosuppressive or immunophilin-binding drugs such as FK-506, and drugs increasing neurotrophins (Gold and Nutt 2002; McGeer and McGeer 2002; Tansey et al. 2008).
CHAPTER 2:

IN VIVO PARKINSONIAN MODELS: MPTP & ROTENONE
2.0 INTRODUCTION

As discussed in chapter 1 the etiology of PD is not completely understood, but it is likely to involve both genetic and environmental factors (Sherer et al. 2002a; Allam et al. 2005). Epidemiological studies suggest that exposure to environmental agents containing neurotoxins such as pesticides, may increase PD risk (Gorell et al. 1998; Menegon et al. 1998). Mitochondrial dysfunction has also been linked to PD. Specifically; there are systemic reductions in the activity of complex I of the mitochondrial electron transfer chain (ETC) in PD brain, muscle, and platelets (Mizuno et al. 1989; Parker et al. 1989; Schapira et al. 1989; Cardellach et al. 1993; Haas et al. 1995). Additional evidence for mitochondrial impairment in PD comes from the finding that MPP⁺ (1-methyl-4-phenyl-2,3-dihydropyridinium), the active metabolite of the parkinsonism toxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), acts as a complex I inhibitor (Nicklas et al. 1985). There are three major toxin models of PD: 6-hydroxydopamine (6-OHDA), MPTP, and rotenone (see Table 2.1), in which there is DAergic neuronal cell death associated with Parkinsonism. Since two of these well known selective DAergic cell neurotoxins are employed in the experiments discussed in this thesis, they are reviewed in more depth in this chapter.

2.1 MPTP

In the early 1980s the DAergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) a meperidine analogue 1-methyl-phenyl-propion-oxypiperidine (MPPP) was discovered by accident during synthetic heroin production (Davis et al. 1979; Langston et al. 1983). It is of interest to note the biochemical, pathological and clinical features
induced in these young addicts corresponded to the hallmarks of PD. A follow up of this study presented evidence that MPTP induced severe and unremitting Parkinsonism caused by an active nerve cell degeneration with characteristic features of neuroinflammation (Langston et al. 1999)

### Table 2.1 Characteristics of animal models of Parkinson’s disease

<table>
<thead>
<tr>
<th>Model</th>
<th>Gradual cell loss of DA neurons in adulthood</th>
<th>Easily detectable motor deficits</th>
<th>Development of Lewy bodies</th>
<th>Short timecourse</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OHDA</td>
<td>No</td>
<td>Yes (quantifiable rotation deficit)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Yes, but variable Individual susceptibility</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Acute MPP⁺ Drosophila α-synuclein overexpression</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Mouse α-synuclein overexpression</td>
<td>Yes, but not in SN</td>
<td>Yes</td>
<td>Nuclear as well as cytoplasmic inclusions</td>
<td>No (1 year)</td>
</tr>
</tbody>
</table>

* Chronic MPTP administration induces slow Parkinsonian syndrome and might produce Lewy bodies (Adapted from (Beal 2001)).

### 2.2 BIOCHEMISTRY OF MPTP

MPTP itself is not toxic in the brain but requires a two–step biotransformation process to form the metabolite MPP⁺ which is the ultimate toxin (See Figure 2.1). The lipophilic MPTP rapidly enters the blood brain barrier after systemic administration where MPTP is converted via MAO-B to form the 1-methyl-4-phenyl-1,2-dihydroxypyridinium ion (MPDP⁺) (Markey et al. 1984; Chiba et al. 1985). MAO-B is mainly located in
astrocytes (Takada et al. 1990; Di Monte et al. 1991) but not in DAergic neurons (Westlund et al. 1985). MPDP$^+$ is spontaneously oxidized to MPP$^+$. Theoretically both MAO-A and MAO-B isoforms are able to convert MPTP but MAO-B is the likely key enzyme as inhibition of MPTP metabolism by MAO-B inhibitors, but not MAO-A inhibitors, was effective in preventing MPTP neurotoxicity (Heikkila et al. 1984a; Nagatsu and Sawada 2006). MPP$^+$ is released by glial cells into the extracellular space by an active mechanism using the extracellular monoamine transporter (Russ et al. 1996). Subsequently, MPP$^+$ can be specifically taken up into DAergic nerve terminals via plasma membrane DA transporter (DAT) (Chiba et al. 1985; Javitch et al. 1985). It has been demonstrated that mice with a deficiency of the DAT are resistant against MPTP toxicity (Gainetdinov et al. 1997; Takahashi et al. 1997) thus overexpression of DAT may result in enhanced MPTP neurotoxicity. MPP$^+$ is then taken up by then intraneuronal vesicular monoamine transporters (VMATs) and sequestrated into synaptosomal vesicles or accumulated in mitochondria via energy-driven uptake (Singer 1987). Intravesicular storage of MPP$^+$ via VMAT suppressed its toxicity in vitro (Liu 1992). This was confirmed in vivo using VMAT-2 deficient mice which demonstrated an increased MPTP toxicity (Takahashi et al. 1997; Gainetdinov et al. 1998).
Figure 2.1 Schematic Representation of MPTP Metabolism.

After systemic administration, MPTP crosses the blood-brain barrier. Once in the brain, MPTP is converted to MPDP$^+$ by MAO-B within nondopaminergic cells, such as glial cells and serotonergic neurons (not shown), and then to MPP$^+$ by an unknown mechanism (?). Thereafter, MPP$^+$ is released, again by an unknown mechanism (?), into the extracellular space. MPP$^+$ is concentrated into dopaminergic neurons via the dopamine transporter (DAT) (Dauer and Przedborski 2003).
2.3 ANIMAL MODELS OF PD USING MPTP

2.3.1 Factors influencing the neurotoxic action of MPTP

Models of MPTP have been widely used and investigated (Heikkila and Sonsalla 1987; Gerlach et al. 1991; Heikkila and Sonsalla 1992; Tipton and Singer 1993). Neurological effects following systemic application of MPTP have been found in a variety of animals, including monkeys, mice, dogs, cats, sheep and goldfish (Heikkila and Sonsalla 1987; Zigmond and Stricker 1989; Gerlach et al. 1991; Heikkila and Sonsalla 1992; Tipton and Singer 1993). There are marked differences with regard to sensitivity to the neurotoxic action of MPTP (Table 2.2). Even large doses of MPTP elicit only slight neurotoxic effects in rats and guineapigs. To produce a DA loss similar to that observed and elsewhere in the monkeys in even the most sensitive strain of mice, the C57/Black mouse, a 50-fold dose of MPTP is required. The reasons for the differential sensitivities are still not completely understood, however the pharmacokinetics of MPTP, and the distribution and excretion rate of its main metabolite MPP⁺, may be responsible. Other factors that may influence these species differences include neuromelanin, distribution and localisation of MAO-subtypes in the brain, DA metabolism and the anti-oxidant content of the nigrostriatal system.

2.3.2 Behavioural changes

Monkeys treated with MPTP develop motor disturbances comparable to those in man (Stern 1990; Gerlach et al. 1991). The most prominent of these are akinesia and rigidity; resting tremor is only seen in isolated instances. The precise appearance of these neurologic abnormalities varies depending on the age, species and dosage. MPTP-
syndromes have been described in rhesus monkeys (Burns et al. 1983; Smith et al. 1993), squirrel monkeys (Irwin et al. 1990), macaques (Crossman et al. 1989), baboons (Hantraye et al. 1993) and marmosets (Ueki et al. 1989; Russ et al. 1991). The quantitation of the neurologic abnormalities in monkeys is achieved using the modified PD scales.

Table 2.2 Relative toxicity of MPTP in different animals

<table>
<thead>
<tr>
<th>Animal Family</th>
<th>Cumulative dose (mg/kg)</th>
<th>Dopamine concentration (% of normal values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rodents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprague Dawley</td>
<td>151</td>
<td>77 (Caudate nucleus)</td>
</tr>
<tr>
<td>- Guineapigs</td>
<td>105</td>
<td>50 (Striatum)</td>
</tr>
<tr>
<td>- Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57/Black</td>
<td>90</td>
<td>24 (Caudate nucleus)</td>
</tr>
<tr>
<td>CF/1</td>
<td>80</td>
<td>60 (Striatum)</td>
</tr>
<tr>
<td>Swiss-Webster</td>
<td>410</td>
<td>35 (Striatum)</td>
</tr>
<tr>
<td>Non-Human primates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Common marmoset</td>
<td>6.9-9.2</td>
<td>15 (Caudate nucleus)</td>
</tr>
<tr>
<td>- Rhesus monkey</td>
<td>1.5</td>
<td>3 (Striatum)</td>
</tr>
<tr>
<td></td>
<td>2.1-6.5</td>
<td>0.4 (Caudate nucleus)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 (Putamen)</td>
</tr>
<tr>
<td>- Squirrel monkey</td>
<td>2</td>
<td>30 (Caudate nucleus)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 (Putamen)</td>
</tr>
</tbody>
</table>

See (Gerlach and Riederer 1996)
MPTP-treated mice show only transient behavioral changes as an initial short-term toxic effect (Sundstrom et al. 1990; Zuddas et al. 1992b). They exhibit hypersalivation, piloerection, seizures and hypokinesia (Weihmuller et al. 1989; Zuddas et al. 1992b) and recover within 24–48h, although decreased locomotor activity (-66%) has been described. Moderate DA depletion does not produce overt motor symptoms, but performance in spatial working memory tasks was impaired when more difficult tasks were applied (Tanila et al. 1998). Long-term effects as a result of dopamine denervation including postural abnormalities, bradykinesia and tremor were observed by some groups (Teismann et al. 2001). The mode of MPTP administration and the strain of animals used may lead to apparently different observations (Schmidt and Ferger 2001).

2.3.3 Histopathological changes

A selective destruction of SN neurons has been described in MPTP-treated non-human primates (Burns et al. 1983; Ueki et al. 1989; Mavridis et al. 1991; Bernocchi et al. 1993; Hantraye et al. 1993). However, detailed examination shows that other regions of the brain are also affected (Mitchell et al. 1985; German et al. 1988; Gibb et al. 1989). This mainly occurs in older animals. A loss of neurons is also found in the locus ceruleus (Mitchell et al. 1985; Forno et al. 1993). In younger animals, losses of TH-ir neurons occur in the VTA and hypothalamus (German et al. 1988); quantitative evaluation revealed that MPTP-treated macaques observed cell loss between 46-93% in the SNpc leading to a stable Parkinsonian syndrome with more than 99% loss of DA in the ST compared to the 28-57% in VTA. Additionally, a decreased density of DA receptors was found after MPTP treatment (Mitsumoto et al. 1998). MPP⁺ acutely displaces DA from
synaptosomal vesicles and gives rise to DA-induced hydroxyl radical formation (Chiueh et al. 1992a; Chiueh et al. 1992b). Using the reverse microdialysis a 60-fold increase of DA release after striatal application of MPP⁺, and significantly increased hydroxyl radical formation was observed (Obata et al. 1992; Ferger et al. 1998b; Ferger et al. 1998a; Obata et al. 2001).

The presence of LBs has not been established in MPTP-treated non-human primates. In old monkeys eosinophil inclusion-bodies have been found in the same areas of where LBs are found in humans (Forno et al. 1993). In MPTP-treated C57/Black mice an average 40% loss of TH-ir neurons in the SN and 17% loss in the VTA are observed (Date et al. 1990b; Kupsch et al. 1992). This degree of damage led to a substantial depletion (~85%) of DA in the ST. This pattern of neuronal loss was further confirmed by Nissl-staining methods (Seniuk et al. 1990) and showed that the locus ceruleus and the hypothalamus are also affected.

2.3.4 Neurochemical changes

MPTP produces a series of neurochemical changes in primates and rodents. Decreased concentration of DA and its metabolites, diminished TH activity and fewer DA-uptake sites in the ST and GP indicate damage to the nigrostriatal DAergic system. MPTP treatment of monkeys’ results in striatal DA levels being reduced, followed by the reduction of DOPAC and HVA levels, whereas the noradrenergic and serotonergic system were less affected (Ueki et al. 1989; Pifl et al. 1991; Russ et al. 1991). In contrast, noradrenaline, serotonin and neuropeptides are less affected by MPTP.
Concentrations of enkephalins, cholecystokinin, substance P and neurotensin are unchanged in the BG of MPTP-treated marmosets (Taylor et al. 1991).

In marmosets (Russ et al. 1991) and squirrel monkeys (Moratalla et al. 1992) the putamen is more severely affected than the caudate nucleus, but in the macaque (Alexander et al. 1991) the opposite was found. This can be explained by the supersensitivity of the post-synaptic DA D2-receptor (Alexander et al. 1991). The essential features of the neurochemical effects observed in primates with MPTP-treatment, such as depletion of DA in the nigrostriatal and mesolimbic systems and depletion of adrenaline are also demonstrated in various strains of mice (Heikkila et al. 1989; Weihmuller et al. 1989; Date et al. 1990a; Seniuk et al. 1990; Sundstrom et al. 1990; Gerlach et al. 1993).

2.3.5 Invertebrates

Studies have shown that MPTP toxicity is lower in animals such as frogs and leeches. The frog *Rana pipiens*, showed a decrease in DA content and motor dysfunction with MPTP and MPP⁺ (Barbeau et al. 1985a; Barbeau et al. 1985b). However, MPP⁺ was more toxic and effective at lower doses than MPTP in frogs. The medicinal leech (*Hirudo medicinalis*), also showed decreased DA content and motor dysfunction with MPTP, but *Macrobdella decora* showed no MPTP sensitivity (Langston and Irwin 1986; Kopin and Schoenberg 1988). The planaria (*Dugesia japonica*) is the most primitive species to have a centralized nervous system (Kitamura et al. 1998) and is considered an ancestor of the mammalian brains (Sarnat and Netsky 1985). This flatworm contains neurotransmitters such as DA, NA and 5-HT. Treatment with L-dopa or reserpine
induces an increase or decrease DA content respectively, and DA agonists or antagonist influence the planarias behaviour (Sarnat and Netsky 1985). Not only does it have high capacity for regeneration and reorganization (Agata and Watanabe 1999; Kato et al. 1999; Kobayashi et al. 1999), MPTP induced degeneration is also completely rescued by anti-parkinsonian drugs such as talipexole and pramipexole (Kitamura et al. 1998).

2.3.6 Genetic mouse models and MPTP
Several gene polymorphisms have been identified which contribute to the development of PD in humans (See Table 2.3). This includes the different mutations in the α-synuclein gene (Polymeropoulos et al. 1997; Kruger et al. 1998; Zarranz et al. 2004). Mice with a targeted deletion of the α-synuclein gene are viable and develop normally (Abeliovich et al. 2000). Although they show normal brain architecture and DAergic neurons, they display a reduction in ST DA content and attenuated DA-dependent locomotor response following amphetamine treatment. In addition, the reserve pool of neurotransmitter vesicles in the hippocampus was reduced (Cabin et al. 2002).

2.4 MECHANISMS OF MPTP NEUROTOXICITY

2.4.1 Mitochondrial impairment
Within mitochondria, MPP⁺ acts by inhibiting the electron transport system of the mitochondrial complex I (NADPH-ubiquinone oxidoreductase I; see Figure 2.2) (Nicklas et al. 1985; Ramsay et al. 1991b). This leads to impairment in ATP production, to an elevation of the intracellular calcium concentration and to the generation of free radicals,
resulting in cellular energy failure (Nicklas et al. 1987) and in the formation of superoxide anions (Dawson 2000).

**Table 2.3 MPTP-induced effects upon the DAergic system in different mouse strains**

<table>
<thead>
<tr>
<th>Mouse lines</th>
<th>Neurodegeneration in the SNpc</th>
<th>ST denervation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>α-synuclein KO mice</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>α-synuclein (A30P) transgenic mice</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>α-synuclein (A53T) transgenic mice</td>
<td>like in normal mice</td>
<td>like in normal mice</td>
</tr>
<tr>
<td>α-synuclein overexpressing mice</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>DJ-1 KO mice</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
</tbody>
</table>

> as compared to normal mice, KO knockout mice, see (von Bohlen Und Halbach 2005; von Bohlen und Halbach et al. 2005).

There is a correlation between the time course of ATP changes with MPP⁺ brain levels (Chan et al. 1991, 1992). The selective toxicity of MPP⁺ to DAergic neurons derives, at least in part, from its high affinity for the dopamine transporter (Javitch and Snyder 1984; Javitch et al. 1984). In line with this, mice lacking this transporter are protected from MPTP toxicity (Bezard et al. 1999). MPP⁺ is also sequestered into synaptic vesicles by the vesicular monoamine transporter (VMAT), and sequestration into vesicles decreases
MPP⁺ toxicity by preventing its interaction with mitochondria (Reinhard et al. 1987; Liu et al. 1992). Thus, mice with a 50% depletion of VMAT2 show increased vulnerability to MPTP (Takahashi et al. 1997).

2.4.2 Energy Failure

MPP⁺ inhibits complex I and impairs ATP formation which in turn disables or reduces energy-dependent processes such as maintenance of calcium homeostasis, cellular membrane potential, and ion- and transmitter transport (Di Monte, 1991; Royland & Langston, 1998). The energy failure due to MPTP-induced ATP depletion is aggravated by secondary steps due to energy consuming repair processes. In particular enzymes such as poly (ADP-ribose) polymerase (PARP) which require ATP for DNA repair are critically involved in MPTP toxicity. This is supported by PARP inhibitors or PARP KO mice showing protection against MPTP toxicity (Cosi et al. 1996). Additionally MPP⁺ is thought to also inhibit the α-ketoglutarate dehydrogenase of the tricarboxylic acid cycle (complex II of the respiratory chain) (Mizuno et al. 1987b). This mechanism acts synergistically enhancing the MPTP-induced disruption of cellular energy metabolism.

2.4.3 Calcium homeostasis

As a consequence of severe energy impairment there is decreased activity of dependent calcium-ATPase which leads to intraneuronal calcium-overload. Elevated intracellular levels of calcium have been shown to activate degradative enzymes such as proteases and phosphatases. These results in cell membrane and cytoskeleton degradation, disruption of cell function, loss of cell membrane potential and ultimately neuronal death. Binding
of excess calcium and calcium channel blockers have shown protection against MPTP-induced nigral degeneration (German et al. 1992; Kupsch et al. 1995; Kupsch et al. 1996).

2.4.4 Glutamate release
Excessive MPP$^+$ concentrations may promote excitotoxicity since an enhanced glutamate release has been shown (Carboni et al. 1990b; Carboni et al. 1990a). Glutamate has been shown to be neurotoxic despite having a physiological role as the most abundant excitatory amino acid. Excessive glutamate release activates NMDA receptors leading to a massive influx of calcium thereby inducing the formation of reactive oxygen species (ROS) as well as a reducing intracellular glutathione synthesis (Murphy et al. 1989; Beal et al. 1993).

2.4.5 Reactive oxygen (ROS) and nitrogen species (NOS)
ROS are continuously formed in the body as byproducts from numerous biochemical reactions. Compared to other brain regions SNpc is exposed to higher levels of oxidative stress (OS) caused by the catabolism of DA via MAO-B mediated deamination, DA autoxidation and high levels of iron all result in a high degree of ROS formation (Coyle and Puttfarcken 1993). In addition, low levels of glutathione peroxidise diminish the ability of the SNpc to cope with OS (Sian et al. 1994b; Sian et al. 1994a). MPTP
Figure 2.2 Schematic representation of MPP⁺ intracellular pathways.

Inside dopaminergic neurons, MPP⁺ can follow one of three routes: (1) concentration into mitochondria through an active process (toxic); (2) interaction with cytosolic enzymes (toxic); (3) sequestration into synaptic vesicles via the vesicular monoamine transporters (VMAT; protective). Within the mitochondria, MPP⁺ blocks complex I (X), which interrupts the transfer of electrons from complex I to ubiquinone (Q). This perturbation enhances the production of reactive oxygen species (not shown) and decreases the synthesis of ATP (Dauer and Przedborski 2003).
administration increases DA turnover (Teismann et al. 2001) resembling the enhanced DA turnover in PD. In early stages of PD the remaining DAergic neurons try to compensate for the reduced number of DA neurons by producing more DA (Fahn and Cohen 1992).

Transgenic mice which overexpressed the superoxide detoxifying enzyme superoxide dismutase (SOD) showed significant protection against MPTP toxicity (Przedborski et al. 1992). Nitric oxide (NO) also seems to be directly and indirectly involved in MPTP neurotoxicity (Castagnoli et al. 1997; Di Monte et al. 1997). The reaction of NO with superoxide results in the formation of peroxynitrite (Beckman et al. 1990), which is neurotoxic by oxidation and nitration of biomolecules (Beckman 1996; Beckman and Koppenol 1996; Beckman et al. 1996). It has been shown that peroxynitrite inhibits complex I, II and III of the mitochondrial respiratory chain and irreversibly enhances energy depletion (Radi et al. 1991). NO synthase inhibitors and mice with a deficiency in NO synthesizing enzymes showed protection against MPTP toxicity (Schulz et al. 1995c; Schulz et al. 1995b; Hantraye et al. 1996; Przedborski et al. 1996; Liberatore et al. 1999). This has been confirmed by studies with 3-nitrotyrosine, which is a marker for nitration reactions and has been found to be elevated after MPTP administration (Pennathur et al. 1999).

2.4.6 Cytokines and inflammatory processes

ROS and NO production are enhanced by microglia infiltration and activation as well inflammatory reactions in the MPTP mouse model (Czlonkowska et al. 1996; Kurkowska-Jastrzebska et al. 1999a, b). Reactive microglia release cytotoxic species like
hydroxyl radicals, NO, glutamate and cytokines such as interleukin (IL)-1β, IL-6 and
tumor necrosis factor-α (TNF-α) (Grunblatt et al. 2000a, b). MPTP-treated mice showed
elevated levels of proinflammatory cytokines (Mogi et al. 1998; Kaku et al. 1999) which
are similar to the post mortem analysis of PD brains (Mogi et al. 1994b; Mogi et al.
1994a). Furthermore, humans who ingested MPTP up to 14 years later still showed
evidence of chronic inflammation (Langston et al. 1999). Anti-inflammatory drugs such
as meloxicam and acetlysalicylic acid showed a pronounced neuroprotection against
MPTP toxicity (Ferger et al. 1999; Teismann and Ferger 2001).

Further support for the implication of microglia in the loss of DAergic neurons in PD is
the increased number of major histocompatibility complex class II-positive microglial
cells in the SNpc of patients who developed PD (McGeer et al. 1988) or suffered from
parkinsonism due to accidental administration of MPTP (Langston et al. 1999). The
same observation was also made in the SNpc of MPTP-treated mice (Liberatore et al.
1999) and monkeys (Hurley et al. 2003; McGeer et al. 2003; Barcia et al. 2004),
suggesting that microglial cells might be involved in a putative antigen-specific immune
response. A marked increase in cytokine levels such as the pro-inflammatory cytokines
TNF-α, and IL-1β has been observed in the SNpc of PD patients compared to control
subjects (Hunot et al. 1999). Anti-inflammatory drugs like pioglitazone and minocycline
(Du et al. 2001; Breidert et al. 2002; Wu et al. 2002), as well as gene deletions targeting
pro-inflammatory molecules such as iNOS and cyclooxygenase type-2 (Liberatore et al.
1999; Dehmer et al. 2000; Teismann et al. 2003b; Teismann et al. 2003a; Hunot et al.
2004) have been shown to protect the nigral DA neurones in animal models of PD.


2.4.7 MPTP and apoptosis

In PD, estimations of apoptotic cells by terminal deoxynucleotidyl transferase-mediated 2'-deoxy-uridine-5'-triphosphate nick end labelling (TUNEL)-positive DAergic neurons vary between 2 and 12% with great interindividual variability (Mochizuki et al. 1997; Tompkins et al. 1997; Kingsbury et al. 1998; Tatton 2000). Additionally, apoptotic cell profiles have been demonstrated ultrastructurally (Anglade et al. 1997). In other small series, no neuronal TUNEL reactivity was observed (Kosel et al. 1997; Banati et al. 1998; Wullner et al. 1999; Jellinger 2000). Sometimes, solely apoptotic glial profiles were noted in SN (Kosel et al. 1997). Considering the slow evolution of the disease, it is to be expected that just a small percentage of cells will show signs of active cell death at any given time-point (Blum et al. 2001). The contradictory results regarding apoptosis may be due to differences in patient age, disease duration, postmortem time, and experimental protocol.

TUNEL staining cannot be considered entirely specific for apoptotic cell death as DNA strand breaks might also occur randomly in necrotic cell death (Andersen 2001) and may reflect deficient DNA repair (Jellinger 2000). Thus, TUNEL staining should be complemented by analysis of morphological markers of apoptosis (Burke 1998). Currently, it is unknown how long apoptotic profiles may persist in vivo and this is an area of ongoing research (Banati et al. 1998; Hirsch et al. 1999). Less well-known is the contribution of autophagic cell death to Parkinson's disease, characterized by prominent early cytoplasmic changes and vacuolization.
In vivo, apoptotic nuclei were detected in mice undergoing a 5-day treatment with MPTP, peaking on day 1 after the final injection (Tatton and Kish 1997), whereas this could not be demonstrated after a 1-day regimen of MPTP exposure (Jackson-Lewis et al. 1995). This may be related to the degree of ATP depletion, as apoptosis might shift to necrosis under conditions of extreme lack of energy substrates (Leist et al. 1997). Thus, only with prolonged cellular damage more in accordance with the human situation may apoptotic cell death be observed.

Another approach to showing apoptotic cell death relies on the demonstration of caspase cleavage (Figure 2.3). Caspases are a family of cysteine proteases with substrate specificity for aspartic acid. Currently 14 members are known. Ced-3 is the structural homolog in Caenorhabditis elegans, testifying to their marked conservation through phylogeny. Each caspase is comprised of two large and two small subunits. Activation requires a caspase-dependent cleavage of a precursor protein into a procaspase, and a large and a small subunit, the latter two then forming the heterotetramer. According to their differential substrate specificities, they may be divided into three major groups, which also provide insight into their biological roles in inflammation and apoptosis (Thornberry et al. 1997; Thornberry and Lazebnik 1998; Nicholson 1999).

Caspase-3 activation has been demonstrated in vivo after MPTP treatment (Eberhardt et al. 2000; Hartmann et al. 2000) (Viswanath et al. 2001). Activated caspase-3 has also been demonstrated in SNpc of PD (Hartmann et al. 2000; Tatton 2000), while this has been refuted by others (Jellinger 2000). Interestingly, caspase-mediated cleavage of both
wild-type and mutant parkin has been demonstrated (Kahns et al. 2002). The functional significance of this finding in relation to pathogenesis is presently unknown. Using electron microscopy, either apoptotic (Sheehan et al. 1997) or early necrotic and late apoptotic changes (Mukherjee et al. 1997) were observed after MPP⁺ in cell culture. Unfortunately, it is not known from the few pathological reports on patients with MPTP-related Parkinsonism whether apoptotic markers were present, although an ongoing neuronal degeneration was assumed (Langston et al. 1999).

2.5 DA AGONISTS AND MPTP

From a therapeutic perspective both humans and monkeys who received MPTP respond very well to anti-PD treatments such as L-DOPA/carbidopa. However, as in PD patients (Kostic et al. 1991), long-term treatment with L-DOPA leads to hyperkinetic motor complications called dyskinesia, which can be as disabling as the parkinsonian symptoms themselves. For instance, among the seven indexed MPTP human cases, five developed dyskinesia within the first year of treatment with L-DOPA/carbidopa (Langston and Ballard 1984). As of yet, the occurrence of L-DOPA-induced motor complications remains a major impediment to the proper management of PD patients. The MPTP monkey model has emerged as an invaluable tool to investigate the molecular basis of these drug-induced abnormal movements and to test therapeutic strategies to control these (Blanchet et al. 2004). This is elegantly illustrated in the study by Bézard (Bezard et al. 2003) in which the administration of a D3-dopamine partial agonist markedly improved L-DOPA-induced dyskinesia in MPTP monkeys, without exacerbating the parkinsonian symptoms.
Figure 2.3 Pathways implicated in MPTP-mediated toxicity.

Two major pathways leading to caspase activation have been characterized (Kaufmann and Hengartner, 2001). One involves the activation of death receptors on the cell surface, e.g. Fas/CD95 or TNFα. Both share a common motif, the death domain, necessary for the binding of cytoplasmic adaptor molecules, e.g. FADD or TRADD. Through involvement of a common death effector domain, the prodomain of procaspase-8 is bound, bringing about the autocatalytic activation of the enzyme. This, in turn, leads to the activation of downstream effector caspases, directly or via a mitochondrial pathway as an amplification step. The mitochondrial pathway involves the release of cytochrome c from mitochondria, forming a ternary complex with procaspase-9 and with the scaffolding protein Apaf-1 and procaspase-9. Alongside with cytochrome c, other proapoptotic effectors, such as Smac/Diablo or apoptosis-inducing factor (AIF), are released from mitochondria into the cytosol. In the ternary complex, the so-called apoptosome, caspase-9 activation can take place. Again, in further steps effector caspases, such as caspase-3, are activated and are responsible for the cleavage of at least 200 protein substrates involved in the degenerative process (Eberhardt and Schulz 2003).
The diagram illustrates the complex network of interactions involved in neuronal death and oxidative stress. Key components include:

- **Death receptors**: Initiators of the intrinsic and extrinsic apoptotic pathways.
- **MPP+ (Methylamine)**: Induces oxidative stress and apoptosis.
- **PARP activation**: Promotes cell death via DNA damage.
- **NO production**: Linked to oxidative stress.
- **NOS neurons**: Nitric oxide synthase action.
- **Oxidative phosphorylation**: Central to ATP production.
- **Bid and Bax/Bim**: Regulators of mitochondrial permeability transition.
- **Caspases**: Enzymes that cleave structural proteins.
- **p53 and JNK pathways**: Key regulators of cell death.
- **NFκB**: Modulates immune and inflammatory responses.

The diagram highlights the role of various proteins and pathways in neuronal death, including death substrates (CAD, PARP, etc.), cytochrome c, AIF, and effector caspases (caspase-3). The interplay between these components underscores the complexity of neuronal death mechanisms.
2.6 ROTENONE

2.6.1 INTRODUCTION

Several epidemiological studies suggest that pesticides and other environmental toxins may be involved in the pathogenesis of PD (Beal 2001). Rotenone, an agricultural toxin used as an insecticide and fish poison, produces Parkinsonism in rats when administered intravenously (Betarbet et al. 2000). Rotenone is a mitochondrial complex I inhibitor, but differs from MPTP in that rotenone acts uniformly throughout the brain. This model is unique as the rats develop progressive degeneration of nigrostriatal DAergic neurons and inclusions that stain with antibodies against ubiquitin and α-synuclein. These inclusions contain a dense core surrounded by fibrils, similar to LBs, supporting the link between dysfunction in complex I with alterations in α-synuclein. Furthermore, the rats have clinical signs suggestive of PD including bradykinesia, postural instability, unsteady gait, and tremor that respond to the DA agonist apomorphine. Although this model satisfies most of the criteria for an excellent model of PD, its utility may be limited since there is a high mortality rate, and less than 50% of surviving rats treated with rotenone develop consistent lesions.

2.6.2 Biochemistry of rotenone

Rotenone is widely used around the world as an insecticide and pesticide. Rotenone readily breaks down by exposure to sunlight with most of the toxicity of the compound lost in 2–3 days. It is also rapidly broken down in soil and in water with the half-life in both of these environments being between 1 and 3 days (Caboni et al. 2004). Because of its short half-life and because it does not readily leach from soil, it is not considered to be
a groundwater pollutant. Consequently, the likelihood of PD being caused by environmental exposure to rotenone is low; however epidemiological data show that the risk of PD increases with exposure to pesticides (Butterfield et al. 1993; Gorell et al. 1998). The most common way that rotenone exposure to humans would take place is through ingestion. However, absorption in the stomach and intestines is slow and incomplete, and the liver breaks down the compound effectively, thus enormous quantities would need to be ingested. Consistent with this view is the fact that chronic ingestion of rotenone by rats for 24 months at doses 30 times greater than that used to model PD by systemic infusion (Betarbet et al. 2000) failed to cause any behavioral or neuropathological features of the disease (Marking 1988). In a single reported case of fatal rotenone poisoning after acute ingestion (De Wilde et al. 1986) rotenone was not found in the brain although present in blood, liver and kidney.

2.7 ROTENONE-TREATED ANIMAL PD MODELS

2.7.1 Rodents

Rotenone has been used extensively as a prototypic mitochondrial poison in cell cultures, but less frequently in living animals. In animals, rotenone has been administered by different routes. As stated above, oral delivery of rotenone appears to cause little neurotoxicity in animals (Gorell et al. 1998). Systemic administration, on the other hand, often causes toxicity and lethality, the degree of which is related to the dose used. Stereotaxic injection of rotenone into the median forebrain bundle depletes striatal DA and serotonin (Gao et al. 2002). Rats treated for a week with rotenone by intravenous infusion show bilateral lesions of the striatum and the globus pallidus, characterized by
neuronal loss and gliosis (Heikkila et al. 1985). In that study, the nigrostriatal DAergic pathway remained unaffected. Similarly, subcutaneous injection of rotenone once or multiple times, although causing fatality, failed to affect striatal DAergic content in mice (Thiffault et al. 2000). Conversely, Greenamyre and collaborators (Betarbet et al. 2000; Sherer et al. 2003b) found that intravenous and subcutaneous infusion of rotenone for about 3 weeks to rats does produce nigrostriatal DAergic neurodegeneration. By quantitative analysis, SN DAergic neuron numbers are reduced by about 30% in rotenone-infused rats compared with vehicle controls (Hoglinger et al. 2003b). This study also shows that the numbers of mesolimbic DAergic neurons, the cell bodies of which reside adjacent to the SN in the ventral tegmental area (VTA), are unchanged by rotenone administration. In the ST, the average loss of DAergic fibers was estimated to be 55% after rotenone infusion in rats, similar in PD, was greater than the loss of SN DAergic neurons. Despite the use of the exact same regimen of rotenone, the severity of the striatal DAergic damage in rats within a given experiment appears highly variable, ranging from none to near complete (Betarbet et al. 2000; Hoglinger et al. 2003b; Sherer et al. 2003a; Lapointe et al. 2004; Zhu et al. 2004). After the infusion of rotenone, the loss of tyrosine hydroxylase-positive (TH’ve) fibers in the ST is either focal, showing a zone of maximal loss at the center, or diffuse; whether the latter represents a more severe lesion of the striatal DAergic fiber network than the former remains to be demonstrated. Of note, the focal loss in the center of the ST seen in some of the lesioned rats is a pattern that differs from that of PD in which the dorsolateral quadrant of the ST is typically the most affected.
2.7.2 Invertebrates

Rotenone has also been shown to produce behavioural impairments related to the DAergic system in invertebrates. In *Drosophila*, application of rotenone (25-500 μM) produces a reduction in locomotion and selective loss of TH (Coulom and Birman 2004; Meulener et al. 2005). *Drosophila* lacking DJ-1 function (Meulener et al. 2005), despite being viable, fertile and with a normal lifespan, show striking selective sensitivity to environmental toxins, such as paraquat and rotenone, that are linked epidemiologically to sporadic PD in humans (Hertzman C. 1990; Semchuk et al. 1992; Liou et al. 1997; Gorell et al. 1998; Fall et al. 1999; Uversky 2004). In flatworms, rotenone evokes neuronal degeneration which is prevented by antiparkinsonian drugs (Kitamura et al. 2003). The pond snail (*Lymnaea stagnalis*) is widely used in invertebrate neurobiology (Vehovszky et al. 2007). Both acute and chronic rotenone treatment inhibited spontaneous locomotion and feeding, both behaviours being dependent on muscle movement. Biochemical assays revealed a significantly lower DA content in addition to TH loss in the cell bodies and axons (Beal 2001; Vehovszky et al. 2007). Snails have advantages over *Drosophila* that their neurons in the CNS are large and individually identifiable from preparation to preparation (Syed and Winlow 1991; Skingsley et al. 1993; Sakharov and Tsyganov 2000; Tsyganov and Sakharov 2000; Zhu et al. 2004; Haque et al. 2006). DAergic neurons have also been identified in the buccal ganglia of snails (Kyriakides et al. 1989; Kyriakides and McCrohan 1989; Elekes et al. 1991; Rosen et al. 1991; Quinlan et al. 1997; Kabotyanski et al. 2000). This suggests that vertebrates and invertebrates share some common mechanisms in rotenone-triggered changes leading to syndromes of Parkinsonism.
2.8 ROTENONE AND PD PATHOLOGY

In contrast to the 6-OHDA and MPTP models, rotenone-infused rats show proteinaceous inclusions in some of the remaining SN DAergic neurons. Like LBs in PD, these inclusions are immunoreactive for both ubiquitin and α-synuclein (Betarbet et al. 2000), and by electron microscopy they appear composed of a dense core with fibrillar peripheral elements. Like PD in which neurodegeneration extends beyond the DAergic system (Agid and Blin 1987), rotenone infusion is associated with 35% reduction in serotonin transporter density in the striatum, 26% reduction of noradrenergic neurons in the locus coeruleus, and 29% reduction in cholinergic neurons in the pedunculopontine nucleus (Hoglinger et al. 2003a).

Although the initial descriptive studies did not report any striatal lesion (Betarbet et al. 2000), the number of DA-regulated phosphoprotein-32 projecting neurons, cholinergic interneurons and nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase-positive neurons in the ST were all significantly reduced by the infusion of rotenone in rats (Hoglinger et al. 2003a; Lapointe et al. 2004). Unexpectedly, even at doses of rotenone that did not damage the nigrostriatal DAergic pathway in rats, despite the significant loss of intrinsic striatal neurons. Remarkably, Zhu and collaborators (Zhu et al. 2004) found that the rotenone-induced intrinsic striatal neuronal loss occurs especially in those rats exhibiting the central striatal loss of tyrosine hydroxylase immunoreactivity (TH-ir) mentioned above. These results indicate that rotenone exerts a much more widespread neurotoxicity than initially thought and, contrary to the initial contention that, it does not consistently spare striatal postsynaptic DAergic neurons.
2.9 ROTENONE AND L-DOPA

Behaviorally, rotenone-infused rats exhibit reduced mobility, flexed posture, and in some cases rigidity (Sherer et al. 2003a) and even catalepsy (Alam and Schmidt 2002). Four weeks after the infusion of rotenone, rats show more than 70% reduction in spontaneous motor activity (Hoglinger et al. 2003b). These motor abnormalities appear to be reversed by L-DOPA administration (Alam and Schmidt 2004). However, some rotenone-infused rats without a nigrostriatal DAergic lesion have been reported to exhibit a similar set of motor abnormalities (Sherer et al. 2003a). In addition, indices of DAergic damage across different doses of rotenone did not correlate with motor behavior in individual rats (Fleming et al. 2004). Therefore, whereas the rotenone-related motor abnormalities are dramatic, it is still questionable that they result from a loss of nigrostriatal DAergic neurons and thus the use of these behavioral alterations as an experimental correlate of PD symptoms must be done with caution.

2.10 MECHANISMS OF ROTENONE TOXICITY

2.10.1 The Complex 1 Inhibitor

Like MPTP, rotenone is highly lipophilic and thus readily gains access to all organs including the brain. After a single intravenous injection, rotenone reaches maximal concentration in the CNS within 15 min and decays to about half of this level in less than 2 hours (Talpade et al. 2000). Its brain distribution is heterogeneous, paralleling regional differences in oxidative metabolism. It also freely crosses all cellular membranes and can accumulate in subcellular organelles such as the mitochondria where it impairs oxidative phosphorylation by inhibiting reduced NADH-ubiquinone reductase activity through its
binding to the PSST subunit of the multipolypeptide enzyme complex I of the electron transport chain (Schuler and Casida 2001a). Aside from its action on mitochondrial respiration, rotenone also inhibits the formation of microtubules from tubulin (Brinkley et al. 1974; Marshall and Himes 1978). This effect may be quite relevant to the mechanism of DAergic neurodegeneration because excess of tubulin monomers may be toxic to cells (Burke et al. 1989; Weinstein and Solomon 1990). Interestingly, a protein implicated in some familial forms of PD, parkin, appears to bind to tubulin, thereby enhancing the ubiquitination and degradation of misfolded tubulins, an effect that is lacking with the PD-linked parkin mutants (Ren et al. 2003).

2.10.2 Rotenone and OS

Brains of PD patients show evidence of OS, including decreased levels of reduced glutathione and oxidative modifications to DNA, lipids, and proteins (Dexter et al. 1989a; Alam et al. 1997; Pearce et al. 1997; Floor and Wetzel 1998), and oxidative damage is hypothesized to contribute to the neurodegenerative process in PD (Jenner 1998). The source of this oxidative damage is unknown. ROS are generated during dopamine metabolism and by mitochondrial respiration. Within complex I, upstream of the rotenone-binding site, is a site of electron leakage that produces ROS (Hensley et al. 1998; Kushnareva et al. 2002) and impaired complex I activity enhances ROS formation (Cassarino et al. 1997; Barrientos and Moraes 1999; Kushnareva et al. 2002).

As in vivo rotenone infusion resulted in uniform complex I inhibition across brain regions (Betarbet et al. 2000), ROS production by complex I dysfunction cannot fully explain the
selectivity of oxidative damage resulting from rotenone exposure. Nevertheless, rotenone infusion caused relatively selective oxidative damage to certain DA-rich brain regions known to degenerate in PD. It has been hypothesized that there may be an interaction between ROS synthesized during rotenone-induced mitochondrial inhibition and ROS produced during DA metabolism. DAergic neurons are believed to exist normally in a state of compensated OS because they contain DA. Normal metabolism of DA by monoamine oxidase produces H$_2$O$_2$ (Maker et al. 1981), which is sufficient to increase levels of oxidized glutathione {Maker, 1981 #533; Spina and Cohen 1988), and which suggests ongoing oxidative stress. In addition, in the presence of iron (abundant in the SN), H$_2$O$_2$ is converted to the reactive and highly damaging hydroxyl radical. Furthermore, DA may be oxidized nonenzymatically to form reactive DA semiquinones (Hastings et al. 1996). For these reasons, the greatest oxidative damage after rotenone exposure may occur in DAergic neurons. Thus, the extent of oxidative damage in DAergic neurons may be much greater than that observed for the entire homogenate because protein from DAergic neurons comprises only a small percentage of the total midbrain homogenate. Oxidative damage after rotenone exposure may explain, in part, the aggregation of α-synuclein caused by rotenone (Betarbet et al. 2000). In the PD brain, α-synuclein becomes oxidatively modified and insoluble, leading to aggregation (Giasson et al. 2000).

2.10.3 Rotenone and Inflammation

The rotenone-induced degeneration of DAergic neurons may not be solely attributable to an impairment of neuronal mitochondrial complex I activity but may also involve the
activation of microglia (Gao et al. 2003a; Sherer et al. 2003b). Microglial cells, which are the resident macrophages in the brain, respond to many insults by rapid proliferation, hypertrophy, and expression of a number of cytokines (Floyd et al. 1999; Raivich et al. 1999). SN has the highest density of microglia in the brain ((Kim et al. 2000). Importantly, there is an increase in reactive microglia in the ST and SN of patients with idiopathic PD (McGeer et al. 1988; Beal 2003). In vitro, rotenone-induced microglial activation before apparent neurodegeneration (Gao et al. 2002). Activated microglia upregulate cell surface markers such as the macrophage antigen complex I and produce a variety of proinflammatory cytokines. One of the consequences of microglia activation is the production of ROS.

The reaction of $O_2^\bullet$ with $NO^\bullet$ generates peroxynitrite $ONOO^\bullet$, which is strongly implicated in PD pathogenesis (Beal 2003). Prior studies have shown that the LB-positive SN neurons in PD stain with antibodies to 3-nitrotyrosine, which is thought to be a specific marker for peroxynitrite-mediated damage (Good et al. 1998). Furthermore, LBs are known to react with antibodies to nitrated $\alpha$-synuclein, the major protein component of LBs and LNs (Duda et al. 2000; Giasson et al. 2000).

2.10.4 Rotenone and Apoptosis

Rotenone is assumed to exert its cytotoxicity through the induction of apoptosis (Duan and Mattson 1999; Duan et al. 1999; Isenberg and Klaunig 2000; Armstrong et al. 2001), a form of programmed cell death that normally occurs during the development of the nervous system. The term apoptosis is applied to a group of characteristic structural and
molecular events that separate this type of cell death from necrosis. In contrast to necrosis, which involves a group of cells simultaneously, apoptosis may occur in a single cell surrounded by a group of viable cells. There is a distinct and precisely localized control over the fate of specific cells in a mixed cell population that undergo apoptosis. The biochemical cascades that lead to apoptotic cell death seem to involve the activation of one or more members of a family of cysteine proteases, called caspases, and the release of factors, such as cytochrome c from mitochondria, that ultimately induce nuclear DNA condensation and fragmentation (Kiechle and Zhang 2002). There are two major pathways through which apoptosis are induced; one involves death receptors and is exemplified by Fas-mediated caspase-8 activation, and the other is the stress-mediated or mitochondria-mediated caspase-9 activation pathway. Both pathways converge on caspase-3 activation, resulting in nuclear degradation and cellular morphological change (Ueda et al. 2002). Most evidence has stemmed from in vitro models (see Chapter 3).

2.11 OTHER PARKINSONIAN-INDUCING NEUROTOXINS

2.11.1 6-hydroxydopamine (6-OHDA)

6-Hydroxydopamine (6-OHDA), a hydroxylated derivative of DA, was introduced as the first animal model of PD (Porter et al. 1963; Ungerstedt 1968; Blum et al. 2001). In contrast to MPTP and rotenone, 6-OHDA does not cross the rat BBB, therefore it is administered by stereotaxic injection along the pathway of the DAergic fibers into the SNc, the median forebrain bundle, or the ST. The stereotaxic injection allows a unilateral lesion avoiding generalized symptoms and providing a contralateral control (Perese et al. 1989; Cenci et al. 2002). The extent of the lesion can be quantified in vivo by measuring
the asymmetric turning behaviour of the rats induced by systemic administration of DA receptor agonists, L-DOPA or DA-releasing drugs (Ungerstedt 1968; Hefti et al. 1980). However, the turning behaviour due to unilateral lesions may also cause contralateral modifications at striatal level or in the STN (Salin et al. 1996; Perier et al. 2000). In rodents, cats and non-human primates, 6-OHDA-induced toxicity is relatively selective for monoaminergic neurons, as a result of its uptake by DA and noradrenaline transporters (Luthman et al. 1989). Once inside the neurons 6-OHDA produces toxic effects by inhibiting mitochondrial complex I and causing OS (Sachs and Jonsson 1975a, b; Glinka et al. 1997).

The 6-OHDA model has provided considerable amount of information. DA denervation is known to affect corticostriatal glutamatergic neurotransmission (Calabresi et al. 1993a; Schwarting and Huston 1996a, b; Dunah et al. 2000). In the parkinsonian rat, L-DOPA therapy is able to reverse the alterations observed in glutamatergic neurotransmission (Calabresi et al. 2000; Picconi et al. 2004). However, similar to humans after prolonged L-DOPA treatment dyskinesias appear. The 6-OHDA model does not reproduce all the pathological and clinical features of human Parkinsonism. It induces DAergic neuron death with preservation of non-DAergic neurons, whereas the formation of cytoplasmatic inclusions (Lewy bodies) does not occur. 6-OHDA does not affect other brain areas involved in PD, such as the anterior olfactory structures, lower brain stem areas or the locus coeruleus (Betarbet et al. 2002b; Del Tredici et al. 2002). Reports of Parkinsonian-like tremor are rare in studies of 6-OHDA-lesioned rodents however occasional akinesia, rigidity and tremor have been described (Lindner et al. 1999; Cenci et al. 2002). Finally, the regimen of the 6-OHDA model with intrastriatal injections may be more useful for
neuroprotective studies, whereas the regimen with 6-OHDA injections into the SN–VTA complex appears to be a more useful approach for testing new pharmacological or cell replacement therapies (Hirsch et al. 2003). In general, this model exclusively induces acute effects, which differs significantly from the slowly progressive pathology of human PD (Betarbet et al. 2002b).

2.11.2 Maneb

The mechanism of maneb toxicity is not well known (Thiruchelvam et al. 2002). Maneb contains a major active fungicidal component, manganese ethylene-bis-dithiocarbamate (Mn–EBDC), and belongs to the dithiocarbamate (DTC) fungicide family. Chronic exposures of humans to maneb have been linked to the development of Parkinsonism (Ferraz et al. 1988; Meco et al. 1994). Furthermore, in experimental models, maneb appears to decrease locomotor activity (Morato et al. 1989), potentiate MPTP effects on locomotor activity and catalepsy (Takahashi et al. 1989), and modulates the toxicity of paraquat (see above).

Commercial maneb contains not only Mn–EBDC but also many other minor reagents without clearly defined functions. Other constituents of maneb, rather than the fungicidal Mn–EBDC, may be responsible for maneb-mediated neurotoxicity, as is the case of MPTP-contaminated synthetic heroin. Alternatively, the neurotoxic effect can be attenuated by the combined action of several constituents of maneb. Furthermore, Mn–EBDC, being relatively stable in vitro, could potentially degenerate to manganese and EBDC in vivo, both of these compounds being potentially neurotoxic. Indeed, manganese is known to be relatively non-toxic to the adult organism except for the brain, where it
causes PD-like symptoms when inhaled, even at moderate amounts over longer periods of
time (Carpenter 2001; Gerber et al. 2002).

Occupational exposure to manganese occur mainly in mining, alloy production,
processing, ferro-manganese operations, welding, and work with agrochemicals. Among
the neurologic effects is an irreversible parkinsonian-like syndrome, manganism (Levy
and Nassetta 2003; Takeda 2003). However, although the neurological signs of
manganism have received close attention because they resemble several clinical disorders
collectively described as extrapyramidal motor system dysfunction and in particular,
idiopathic PD (IPD) and dystonia, there are well-established distinct dissimilarities
between IPD and manganism. Therefore, whether manganese plays an etiologic role in
idiopathic PD remains to be determined (Aschner 2000).

On the other hand, the EBDC component (and not the manganese moiety of maneb) has
been suggested to contribute to toxicity. This conclusion follows from the finding that
both MANCOZEB (Mn–Zinc–EBDC) and ZINEB (Zinc–EBDC) produce neurotoxicity
in cell cultures (Soleo et al. 1996). Indeed, EBDC per se enhances MPTP-induced
neurotoxicity (McGrew et al. 2000). The direct involvement of manganese ethylene-bis-
dithiocarbamate (Mn–EBDC) in selective DAergic neurodegeneration was recently
demonstrated in a rat model (adult male Sprague-Dawley rats), in which Mn–EBDC was
directly delivered to the lateral ventricles (Zhang et al. 2003). Use of this model has
shown that Mn–EBDC is able to induce extensive striatal dopamine efflux comparable
with that induced by MPP⁺. Furthermore, Mn–EBDC preferentially inhibits
mitochondrial complex III. As mitochondrial dysfunction is pivotal in the pathogenesis of PD, these results support the proposal that exposure to pesticides such as maneb, or other naturally occurring compounds that inhibit mitochondrial function, may contribute to PD development (Zhang et al. 2003).

2.11.3 Paraquat

Acute poisoning by high levels of paraquat is known to cause lung, liver, kidney, and brain injury. This herbicide is poorly absorbed when inhaled but causes severe illness when ingested orally, usually causing death within 2 days of ingestion. At lower doses, death may be delayed for several weeks. Paraquat has been shown to accumulate in lung tissue, where free radicals are formed, lipid peroxidation is induced, and NADPH is depleted, producing diffuse alveolitis, followed by extensive pulmonary fibrosis (Bismuth et al. 1990). Free radical production and the activation of cholinergic and glutamatergic transmission are related events palying a crucial role in paraquat-induced neurotoxicity (Corasaniti et al. 1998). Furthermore, paraquat is known to be a potent redox cycler, being readily converted to a free radical, which, in reaction with molecular oxygen, generates superoxide anions and subsequently other redox products (Jones and Vale 2000; Yumino et al. 2002).

Paraquat induces NADPH-dependent lipid peroxidation (Hara et al. 1991a, b, c, d; Jones and Vale 2000; Yumino et al. 2002). Subcutaneous injections in rats resulted in a glutamate efflux initiating excitotoxicity of nitric-oxide-synthase-containing neurons and eliciting the depolarization of N-methyl-d-aspartate (NMDA) receptor channels and Ca$^{2+}$
penetration into cells by activation of non-NMDA receptor channels (Shimizu et al. 2003b). The influx of Ca^{2+} into cells stimulates nitric oxide synthase release and diffusion into DAergic terminals inducing mitochondrial dysfunction and DA overflow (Shimizu et al. 2003b). Importantly, paraquat dose-dependently reduces the number of DAergic neurons in cultured rat organotypic midbrain slices (Shimizu et al. 2003a). Since this damage is prevented by GBR-12909, a selective DAT inhibitor, DAT has been assumed to be the initial step in paraquat-induced DAergic neurotoxicity. The death of DAergic neurons is prevented by inhibitors of NMDA, nitric oxide synthase, cycloheximide, and the caspase cascade (Shimizu et al. 2003a). Neurodegeneration is also inhibited by l-deprenyl and dopamine D2/3 agonists. These results strongly support that constant exposure to low levels of paraquat leads to the vulnerability of DAergic neurons in the nigrostriatal system via excitotoxic pathways (Shimizu et al. 2003a).

Another line of evidence emphasizes the pathological importance of the direct interaction between paraquat and α-synuclein. Paraquat markedly accelerates the in vitro rate of α-synuclein fibril formation (Uversky et al. 2001; Uversky et al. 2002), with the accelerating effects being dose-dependent (Manning-Bog et al. 2002). Furthermore, when mice are exposed to the herbicide, brain levels of α-synuclein are significantly increased. This up-regulation follows a consistent pattern, with higher α-synuclein levels at 2 days after each of three weekly paraquat injections and with protein levels returning to control values by day 7 post-treatment (Manning-Bog et al. 2002). This is accompanied by the formation of intraneuronal aggregates with histological properties of amyloid fibrils (thioflavine S staining), to which α-synuclein co-localizes (Manning-Bog
et al. 2002). Thus, paraquat exposure triggers α-synuclein fibrillation in the mouse brain. This suggests that the up-regulation of α-synuclein as a consequence of toxicant insult and the direct interaction between the protein and environmental agents are potential mechanisms leading to α-synuclein pathology in neurodegenerative disorders.

2.12 SUMMARY

It is clear that certain environmental neurotoxins can promote and accelerate the development of PD, whereas other environmental agents may be neuroprotective; for example, caffeine consumption and cigarette smoking may decrease the risk of PD {Manning-Bog, 2002 #631; Ross, 2000 #659; Ross, 2000 #660; Ragonese, 2003 #662; Ross, 2001 #661; Tan, 2003 #663; Schwarzschild, 2003 #665; Ascherio, 2001 #664; Gale, 2003 #1537; Paganini-Hill, 2001 #1538}. PD-promoting environmental toxins are metals (Tanner et al. 1989; Hirsch et al. 1991; Good et al. 1992; Yasui et al. 1992; Hertzman et al. 1994; Hellenbrand et al. 1996b; Hellenbrand et al. 1996a; Altschuler 1999; Gorell et al. 1999), solvents (Uitti et al. 1994; Pezzoli et al. 1996; Seidler et al. 1996; Davis and Adair 1999; Hageman et al. 1999), carbon monoxide (Klawans et al. 1982), and MPTP (Langston et al. 1983). Epidemiological studies have shown an the association between increased PD risk and such environmental factors as rural residence (Morano et al. 1994; Liou et al. 1997; Marder et al. 1998), farming (Semchuk et al. 1992; Liou et al. 1997; Gorell et al. 1998; Fall et al. 1999), the drinking of water from wells (Morano et al. 1994; Marder et al. 1998), and exposure to agricultural chemicals, pesticides, and/or herbicides (Semchuk et al. 1992; Liou et al. 1997; Gorell et al. 1998; Fall et al. 1999; Vanacore et al. 2002). The ability of different environmental factors to
increase PD risk and produce PD characteristics in some experimentally has resulted in the development of valuable models for the analysis of mechanisms of neurodegeneration targeting the nigrostriatal system (Di Monte 2003).

Figure. 2.4. A–C Chemical structure of rotenone (A), paraquat and MPP+ (B), and maneb (C) (Uversky 2004).
CHAPTER 3:

*IN VITRO MODELS OF PD*
3.0 INTRODUCTION

Neural tissue grown and maintained in vitro provides an alternate way of studying in situ properties of neurons and glial cells under simple and controlled conditions. A given neural tissue culture preparation retains its physiological and biochemical properties representing those exhibited by the intact brain making tissue culture a useful model for studying the nervous system. There are four broad classes in which in vitro cultivation and maintenance of living biological units from multicellular organisms techniques fall into:

1) Organotypic (explant) cultures are explanted from specific neuroanatomical loci to substrates as small tissue fragments.
2) Dissociated cell cultures involve the seeding of enzymatically or mechanically dispersed single cells on various substrates.
3) Reaggregate cultures require reassociation of dissociated single cells into small aggregates.
4) Cultures of purified cell populations or monotypic cultures are prepared by the isolation of particular cell types by gradient centrifugation or other separation technique.

3.1 DISSOCIATED CULTURES

This form of cell culture involves the isolation of the brain region containing the cells of interest mainly from embryonic tissue, and the dissociation of the tissue into a single cell suspension. These cells are then plated onto a surface that they will adhere to such as laminin or poly-D-lysine coated coverslips in multiwell dishes. Alternatively, neurons
can be cultured within the culture medium without attachment to a surface, thus allowing aggregation to take place (Prochiantz et al. 1979; di Porzio et al. 1980; Prochiantz et al. 1981; Denis-Donini et al. 1983).

Many neuronal populations have been studied using this form of cell culture including the DAergic neurons of the VM (Prochiantz et al. 1979; Daguet et al. 1980; Prochiantz et al. 1981; Berger et al. 1982; Dal Toso et al. 1988; Soderback et al. 1989; Niijima et al. 1990; O'Malley et al. 1991; Masuko et al. 1992; Fawcett et al. 1995). These dissociated cultures of DAergic cells develop in vivo morphological characteristics such as the release and synthesis of DA (Prochiantz et al. 1979; Daguet et al. 1980; Prochiantz et al. 1981; Dal Toso et al. 1988), respond to a variety of neurotrophic factors (Ferrari et al. 1989; Knusel et al. 1990b; Knusel et al. 1990a; Ferrari et al. 1991; Kushima et al. 1992; Kushima and Hatanaka 1992; Lin et al. 1993; Nikkhah et al. 1993; Hyman et al. 1994), in addition express many of the electrophysiological characteristics that have been reported for midbrain DAergic neurons in vivo and in slice cultures (Ort et al. 1988; Masuko et al. 1992; Cardozo 1993).

Disadvantages of this technique come from the mechanical destruction of all components of the tissue matrix and alterations of cell/cell interactions during the culture process. Cell plating density has been shown to influence the survival of DAergic neurons in several studies such as increasing cell density enhances the specific high affinity DA uptake per cell as well as cell survival in dissociated cultures of E13 mouse mesencephalic cells (Dal Toso et al. 1988). Others such as O’ Malley et al., (O'Malley et
al. 1991) have demonstrated that high plating conditions of embryonic rat mesencephalic cells selectively enhanced the survival of the DAergic subpopulation; Fawcett et al., (Fawcett et al. 1995) found that 87% of DAergic neurons in a dissociated monolayer culture system died which was far greater than cell death seen in a 3-dimensional culture system where cell/cell contacts were increased.

3.2 REAGGREGATE CULTURES

Reaggregate cultures involve the dissociation of neural tissues into single cells and subsequently the dispersion of cells resembling themselves under controlled conditions. The dissociated cell suspensions are maintained in rotating flasks. These single cell suspensions can be cultured alone or in combination with single cells from other areas of the embryonic brain. Over a period of 12-24 hours these cells form spherical reaggregates of typical 300 μm in diameter (Heller et al. 1988). It was observed that dissociated DAergic neurons from the rostral mesencephalic tegmentum of embryonic day 14 mouse brain, co-aggregated with dissociated cells from the ST, developed an increasing ability to synthesise, accumulate and store DA with time (Kotake et al. 1982; Shalaby et al. 1983). This temporal development resembled the in vivo development of DAergic neurons. Furthermore, an increased survival of mouse embryonic DAergic neurons when co-cultured with striatal target neurons (Hoffmann et al. 1983) and a selective association of DA axons with their ST target cells have been shown and also the formation of synaptic contacts between embryonic mouse DAergic and striatal neurons within reaggregate cultures (Won et al. 1989).
A major advantage is that these cells develop into 3-dimensions. This technique has also proven useful in allowing studying of transition from unicellular to a multicellular system and be maintained long term (Choi et al. 1993). There is electron microscopic evidence of synapse and myelin formation in these aggregate cultures (Seeds 1975). In reaggregate cultures, a disadvantage is that cells cannot be visualized under direct observation, and the morphological examination requires time-consuming and tedious histological methods.

### 3.3 ORGANOTYPIC EXPLANT CULTURES

The goal for an explant culture system is to culture small fragments of tissue that will maintain the cytoarchitecture, simulate differentiation and maturation patterns, and preserve the functions of the original tissue. Explants maybe maintained in special Maximow chambers (Bornstein 1958) or in the culture dishes. The development of DAergic neurons within explant cultures of postnatal DAergic neurons co-cultured with ST tissue have been described (Whetsell et al. 1981; Mytilineou et al. 1983). These nigro-striatal explant cultures survived for up to 15 weeks were capable of DA synthesis, uptake and release.

The explant culture system has the advantage that cells may be maintained for months in vitro, unlike dissociated and reaggregate cultures; the cytoarchitecture of the culture is maintained and preserved within the explants. Most of the important structural and functional attributes of neurons and glial cells have been found to be maintained in culture. These include the formation of synapses, the appearance of normal myelin, rise
in enzyme activity associated with neurotransmitter metabolism, the increase in myelin-specific enzymes, and the development of the bioelectric activity characteristic of interneuronal communication (Burton and Bunge 1975; Crain and Peterson 1975; Crain et al. 1975). The explant technique also enables the study of the formation of connections between two co-culture explants. However, despite these advantages of explant cultures due to the thickness of the tissue sections (1 mm), cells within the explant are almost as inaccessible as they are in situ. Furthermore, also as a consequence of the thickness of the culture, examination by light microscopy has poor results.

3.4 ORGANOPTYC SLICE CULTURES

This technique will be employed throughout this thesis and was developed by Gahwiler (Gahwiler 1981, 1988). Organotypic tissue culture techniques allow neuronal tissue to be maintained for weeks in vitro, and have thus expanded the utility of in vitro preparations for neurological study. When prepared from thick (200–400 μm) sections of neuronal tissue, organotypic cultures permit the maintenance of the three-dimensional tissue architecture observed in vivo. These cultures maintain fundamental neuronal circuitries, neuronal and glial interactions and have the ability to form new neuronal synapses with other appropriate brain regions when cultured together.

Two organotypic culture techniques are predominately utilized. The first is the roller-tube method (Gahwiler 1988; Ostergaard et al. 1990), in which tissue is continuously rotated within a bath of medium. The second is the static membrane model developed by Stoppini and colleagues (Stoppini et al. 1991); in this technique, tissue rests on a porous
membrane exposed to culture medium below while the upper surface is exposed to air so that tissue oxygenation can occur. Regardless of the culture method used, the organotypic preparation allows the study of an \textit{in vivo}-like anatomical system with the ease of \textit{in vitro} sample manipulations, such as administration of controlled levels of growth factors (Hoglinger et al. 1998; Schatz et al. 1999; Meyer et al. 2001; Jaumotte and Zigmond 2005) or neurotoxicants (Kotake et al. 2003; Shimizu et al. 2003a; Kress and Reynolds 2005; Testa et al. 2005).

The organotypic slice culture technique is an adaptation of the explant culture technique that permits the long-term preservation of tissue structure within cultures that are highly accessible to observation and experimental manipulation. This approach involves culturing slices of neuronal tissue attached to glass coverslips, and are rotated in tissue culture tubes. The main advantage over dissociated neuronal cultures is that the organotypic cultures retain a high degree of their original morphology and characteristics (including electrophysiological characteristics and the formation of functional synaptic networks) and have been described using source tissue from various brain regions. These include the VM (Jaeger et al. 1989; Ostergaard et al. 1990; Sorensen et al. 1994; Holmes et al. 1995; Jones et al. 1995; Steensen et al. 1995; Dickie et al. 1996) hippocampus (Zimmer and Gahwiler 1984; del Rio et al. 1991), cerebellum (Calvet and Calvet 1988a, b; Mouginot and Gahwiler 1995), visual cortex (Caeser et al. 1989), retina (Feigenspan et al. 1993), and ST (Ostergaard 1993; Ostergaard et al. 1995). Organotypic co-cultures of slices from two (and sometimes three) brain regions may also be prepared. Other regions of the brain have also been employed such as co-cultures of locus coeruleus with
hippocampus (Knopfel et al. 1989), cortex with thalamus (Bolz et al. 1992), hippocampus and medial septum (Gahwiler and Brown 1985), ST with neocortex (Ostergaard 1993), visual cortex and lateral geniculate nucleus or superior colliculus (Bolz et al. 1992; Novak and Bolz 1993) and VM with ST (Jaeger et al. 1989; Ostergaard et al. 1990) have been described. The co-culture technique is used in experiments described in Chapter 5 to study the development of the nigrostriatal pathway in organotypic cultures.

The roller-tube technique for slice cultures were introduced by Hogue (1947) and modified by Costero (Costero and Pomerat 1951). Since then Gahwiler (Gahwiler 1981) has developed this technique culturing postnatal rat tissue from several brain regions including the cerebellum, hippocampus and hypothalamus (Gahwiler 1984a, b). This technique also allows the culturing of two different regions of the brain together such as the ST and neocortex (Knopfel et al. 1989; Ostergaard et al. 1991; Bolz et al. 1992) and more importantly the ventral mesencephalon with the striatum (Whetsell et al. 1981; Jaeger et al. 1989; Ostergaard et al. 1990).

The organotypic slice culture system has been used to study the development and survival of DAergic neurons of the VM employing different donor ages (E21- P12) and different species, including human (Ostergaard and Rasmussen 1991) have been shown to survive for up to nine months. The morphology of DAergic neurons within organotypic slice cultures resemble that of the postnatal rat in vivo: they exhibit several shapes including bipolar, pyramidal and multipolar (Ostergaard et al. 1990). Electrophysiological characteristics of DAergic neurons in organotypic cultures of VM have been shown to
resemble those seen in other in vitro preparations and in vivo (Steensen et al. 1995). However, one fundamental difference between organotypic slice cultures and other in vitro preparations is the presence of spontaneous burst firing activity in organotypic cultures (Steensen et al. 1995), which resembles a firing pattern characteristically only seen in the intact animal. It has been suggested that burst firing results from afferent inputs (Yung et al. 1991) thus these afferents are likely to be present in organotypic cultures and so are a more realistic representation of the intact animal.

Cultures of purified cell populations have been recognized as important development in the field of neural tissue culture. Reports of cultures enriched for neurons (Mains and Patterson 1973a, b; Wood 1976), astrocytes (McCarthy and de Vellis 1980; Kim et al. 1983a), oligodendrocytes (McCarthy and de Vellis 1980; Lisak et al. 1981; Kim et al. 1983b), and Schwann cells (Brockes et al. 1979; Brockes and Raff 1979). These culture systems have contributed to a better understanding of morphological, functional, and metabolic properties of individual cell types and neuron-glia interactions.

All of these culture systems are considered to be primary cultures as they started from material obtained directly from the brain tissue of origin without any subculturing. The inability of normal neurons to divide continually not only makes the maintenance of neural cultures challenging but those cultures that do survive will grow, mature, differentiate, and eventually die within the course of 3 to 6 months and no longer than a year. Primary cultures can not divide indefinitely and die thus clonal cell lines are defined as “immortal”. Their source usually being neoplastic tissue either explanted from
in situ neural tumors and subcultured or taken cell lines derived from cloning of chemically, virally or transformed cells.

3.5 In Vitro SYSTEMS AND THEIR RELEVANCE TO PD

At present, all forms of pharmacological treatment are mainly aimed at increasing DAergic neurotransmission which only control the symptoms. The underlying cause of neuronal loss remains unknown. It has been speculated that the etiology of PD is concerned with the four pathomechanisms:

1) Direct effect of neurotoxins MPTP, 6-OHDA and rotenone on SN DAergic neurons, resulting in neuronal loss in SN (Davis et al. 1979; Burns et al. 1983; Calne and Langston 1983; Langston et al. 1983; Bywood and Johnson 2003; Testa et al. 2005)

2) Toxicity of excitatory amino acids acting on DAergic neurons in SN (Spencer et al. 1987b; Spencer et al. 1987a; Choi 1988).

3) The involvement of free radical-induced OS leading to degeneration of SN neurons (Ambani et al. 1975; Dexter et al. 1986).

4) Deficiency in target-derived neurotrophic factor that is responsible for the maintenance and survival of SN DAergic neurons (Prochiantz et al. 1979; Hyman et al. 1991).

3.5.1 MPTP & IN VITRO STUDIES
As previously mentioned in chapter 2, MPP+ the toxic metabolite of MPTP has been used numerously in animal models and other *in vitro* systems. *In vitro* models of PD have been developed and employed for decades in an attempt to better understand the mechanisms underlying the causes of DAergic cell death and the PD pathology. These include primary cell lines derived from rat or mouse mesencephalon either from fetal or postnatal rodents (Hartikka et al. 1992; Chen et al. 1995; Koutsilieri et al. 1996; Marini and Nowak 2000; Pong et al. 2000; Shinpo et al. 2000; Bilsland et al. 2002; Callier et al. 2002; Gille et al. 2002; Han et al. 2003; Hoglinger et al. 2003a; Andres et al. 2005; Chu et al. 2005; Du et al. 2005; Mercer et al. 2005; Li et al. 2006; Bains et al. 2007; Grammatopoulos et al. 2007; Radad et al. 2007a; Radad et al. 2007b), human neuroblastoma cell line, SH-SY5Y (Fang et al. 1995; Fall and Bennett 1999; Conn et al. 2002; Gomez-Santos et al. 2002; Ba et al. 2004; Mathiasen et al. 2004), PC12 cells, a cell line established from rat adrenal pheochromocytoma cell (Greene and Tischler 1976), possess intracellular substrates for DA synthesis, metabolism and transport (Rebois et al. 1980; Hatanaka 1981; Tuler et al. 1989). The cells contain the enzymes required for synthesis and decomposition of DA such as TH and MAO. The membrane receptors and synthesized transmitters in PC12 cells are similar to DAergic neurons located in midbrain. Therefore, PC12 cell line has been used as a cellular model in PD studies (Denton and Howard 1987; Marongiu et al. 1988; Basma et al. 1990; Basma et al. 1992; Itano et al. 1994; Cappelletti et al. 1995; Desole et al. 1996; McNaught et al. 1996; Wei et al. 1996; Desole et al. 1997b; Desole et al. 1997a; Hom et al. 1997; Ara et al. 1998; Kohda et al. 1998; Soldner et al. 1999; Lee et al. 2000; Seyfried et al. 2000; Fonck and Baudry 2001; Guo et al. 2001a; Bai et al. 2002; Gelinas and Martinoli 2002; Quigney et
al. 2003; Shimoke et al. 2003; Noda et al. 2004; Virmani et al. 2004; Xu et al. 2005; Chalimoniuk et al. 2006; Chiasson et al. 2006; Gesi et al. 2006; Meuer et al. 2006; Weinreb et al. 2006; Iuvone et al. 2007; Kang et al. 2007), DAergic cell line MN9D has features such as the transport activity for DA transporter (DAT) and VMAT2, and secretory vesicle properties makes this in vitro model advantageous for studies in vesicular monoamine transporters (VMAT2) neuroprotection and molecular mechanisms involved in the protection and its regulation (Chen et al. 2005), and slice cultures of SN (Mytilineou et al. 1983; Mytilineou and Cohen 1984; Schmidt et al. 1997; Liu et al. 2001; Madsen et al. 2003).

3.5.1.2 DAergic Neurons-Target Specific Toxicity

Exposure to MPP+ not only significantly reduces the number of TH+ve cells and neurites (Sanchez-Ramos et al. 1986; Sanchez-Ramos et al. 1988b; Michel et al. 1989; Michel et al. 1990; Dickie and Greenfield 1997; Goto et al. 1997; Madsen et al. 2003) but also a significant decrement in TH, aromatic L-amino acid decarboxylase (AAAD) activities, reduced DA and DOPAC content and, DA uptake. In addition, TH mRNA was decreased, whereas AAAD mRNA was no different from control cultures (Michel et al. 1990; Dalia et al. 1993, 1996). In vivo, high doses of MPTP induces a significant reduction in both striatal DAconcentrations (95%), and midbrain DAergic cells; 69% loss of nucleus A8 cells, 75% loss of nucleus A9 cells, and in nucleus A10 subnuclei there was 42% loss of VTA cells, 55% loss of interfascicular nucleus cells, and no loss of cells in the central linear nucleus. This provides evidence for differential susceptibility to MPTP toxicity among different mouse strains and provides us the precise locations of
midbrain DAergic cells that are vulnerable to MPTP (Zuddas et al. 1992a; Del Zompo et al. 1993; German et al. 1996). The strong belief by which-MPTP selectively destroys only certain midbrain DAergic neurons is further supported by in vitro studies (Sanchez-Ramos et al. 1986; Sanchez-Ramos et al. 1988a; Sanchez-Ramos et al. 1988b; Michel et al. 1990; Saporito et al. 1992; Zuddas et al. 1992a; Del Zompo et al. 1993; German et al. 1996).

3.5.1.3 Complex 1 Inhibitor

Despite intensive investigation, the molecular mechanism of MPTP has not been definitively demonstrated. MPP⁺, the active metabolite of MPTP, has been shown to inhibit complex I of the mitochondrial electron transport chain, which was thought to be the mechanism by which MPTP induced parkinsonism (Nicklas et al. 1985; Cheeseman and Clark 1987; Pai and Ravindranath 1991; Sayre et al. 1991; Basma et al. 1992; Rollema et al. 1994; Sriman et al. 1995; Sriman et al. 1997; Stephans et al. 2002; Richardson et al. 2007). A study by Soldner (Soldner et al. 1999) reported otherwise. It was found that treatment of undifferentiated PC12 cells with MPP⁺ primarily inhibited proliferation of PC12 cells and secondarily led to cell death after the depletion of all energy substrates by glycolysis. This cell death showed no morphological characteristics of apoptosis and was not blocked by treatment with caspase inhibitors. It would seem that the inhibition of cell growth was not dependent on an inhibition of complex I activity since MPP⁺ also inhibited cell proliferation in SH-SY5Y cells lacking mitochondrial DNA and complex I activity (Soldner et al. 1999).
However, the weak inhibitory ability of MPP⁺ at complex I (Ramsay et al. 1991b; Ramsay et al. 1991a; Richardson et al. 2005) has led some to question the role of complex I inhibition in MPTP toxicity (Lotharius and O'Malley 2000; Nakamura et al. 2000a). It has been demonstrated that the single-subunit nicotinamide adenine dinucleotide (reduced) dehydrogenase of *Saccharomyces cerevisiae* (NDI1) can serve as a replacement for complex I in mammalian cells and restore electron transfer in cell devoid of mitochondrial DNA (Seo et al. 1998; Seo et al. 2000; Bai et al. 2001) and that NDI1 expression in neuroblastoma cells abolishes the toxicity of rotenone, a complex I inhibitor that has been demonstrated to reproduce features of PD in rats (Betarbet et al. 2000; Sherer et al. 2003b; Sherer et al. 2003a). Recently, it has been shown that NDI1 can be expressed *in vivo* using viral-mediated techniques and that unilateral expression of NDI1 partially protected against reduced TH-ir resulting from an acute high-dose regimen of MPTP (4 x 15 mg/kg over 1 day; (Seo et al. 2006; Richardson et al. 2007). These findings suggested that NDI1 could provide some protection from MPTP neurotoxicity. Others have hypothesized alternate mechanisms of action for MPTP including release of vesicular stored DA and subsequent oxidative damage, release of stored iron deposits, increased cytoplasmic calcium and intraneuronal calcium release, and redox cycling of MPP⁺ (Trevor et al. 1987b; Trevor et al. 1987a; Obata et al. 1992; Chen et al. 1995; Lotharius and O'Malley 2000; Obata et al. 2001; Obata 2002; Obata et al. 2002; Kooncumchoo et al. 2006; Obata 2006). Thus, the molecular mechanism of MPTP remains to be established.

### 3.5.1.4 Oxidative Stress (OS)
There is growing evidence indicating that OS and mitochondrial dysfunction play a role in the pathogenesis and progression of PD. Toxic build up of superoxide and NOS are major OS indicators such as lipid peroxidation and protein oxidation, are increased in the cell membrane and/or mitochondria in the ventral MB and the ST may trigger pathogenesis in PD (Fahn and Cohen 1992; Przedborski and Jackson-Lewis 1998; Reiter 1998; Heales et al. 1999; Munoz et al. 2006; Zeng et al. 2006).

Exposure of mouse brain to MPTP resulted in significant increases of ROS and malondialdehyde (MDA, the product of lipid peroxidation) and decreases in GSH content (Okawara et al. 2007). Pretreatment of mouse brain slices with GSH or GSH isopropyl ester attenuated MPTP toxicity as assessed by the tissue activity of the mitochondrial enzyme, NADH-dehydrogenase (NADH-DH), and by leakage of the cytosolic enzyme, lactate dehydrogenase (LDH), from the slice into the medium. This supports that MPTP exposure generates ROS resulting in OS (Sriram et al. 1997). In addition transgenic mice with the overexpression of copper/zinc containing SOD were shown to be resistant to MPTP-induced DAergic toxicity (Przedborski et al. 1992).

While it is generally acknowledged that exposure to MPTP can possibly lead to the generation of oxygen free radicals, it is debatable whether this is a primary mechanism of neurotoxicity of MPTP or a secondary event caused by the inhibition of mitochondrial respiration and resultant energy depletion (Tipton et al. 1993; Tipton and Singer 1993). The latter hypothesis is supported by the evidence that inhibition of NADH-DH by rotenone (Turrens and Boveris 1980) or MPP⁺ (Hasegawa et al. 1990) results in the
generation of superoxide. The observation that the irreversible inhibition of complex I by MPP\(^+\) in a non-neuronal system such as bovine heart mitochondria is totally prevented by the addition of GSH, ascorbate or catalase in the incubation medium, suggests that free radical generation may be a primary event (Cleeter et al. 1992).

However, the role of OS in MPTP-induced neurotoxicity is seemingly controversial. The generation of oxygen free radicals by MPTP has been demonstrated in non-neuronal systems such as cerebellar granule cells (CGC) (Oyama et al. 1993). The addition of MPP\(^+\) induced an increased in the percentage of ROS-positive cells (Oyama et al. 1993). Interestingly the neuronal non-calcium antagonistic 1,4-dihydropyridine (DHP) derivatives cerebrocrast, glutapyrone and tauropyrone was shown to decrease the generation of ROS and the loss of mitochondrial membrane potential which provided protection against MPP\(^+\)-induced deterioration of mitochondrial bioenergetics (Klimaviciusa et al. 2007). The formation of free radicals through redox cycling of MPP\(^+\) has been demonstrated in purified hepatic microsomal NADPH cytochrome P-450 reductase (Sinha et al. 1986; Frank et al. 1987; Lambert and Bondy 1989).

### 3.5.1.5 Inflammation: The Contribution of Microglia and Cytokines

The evidence so far suggests that chronic inflammation, mitochondrial dysfunction, and OS play significant and perhaps synergistic roles in PD, where the primary pathology is significant loss of the DAergic neurons in the SN. Mesencephalic neuron-glia cultures treated with MPTP and an inflammogen lipopolysaccharide (LPS) synergistically, induced a progressive and selective degeneration of DAergic neurons. The synergistic
neurotoxicity was observed when both agents were applied either simultaneously or in tandem. The synergistic neurotoxicity was more prominent when lower doses of both agents were applied for a longer period of time. In addition, MPTP and LPS synergistically stimulated the NADPH oxidase-mediated release of superoxide free radical in which pharmacological inhibition and genetic inactivation of NADPH oxidase prevented superoxide production and the synergistic neurotoxicity. Furthermore, inhibition of NOS significantly provided neuroprotection suggesting the involvement of nitric oxide in the synergistic neurotoxicity (Gao et al. 2003b; Gao et al. 2003a).

There is increasing evidence that suggests an involvement of glia in MPTP neurotoxicity, the nature of this involvement remains unclear. In particular microglia, but not astroglia is significantly involved in enhancing the progression of MPTP-induced DAergic neurodegeneration through the release of NADPH oxidase-derived ROS, microglia contribute to the progressive neuronal damage. Among the factors measured, the production of extracellular superoxide was the most prominent. NADPH oxidase inhibitor, apocynin were shown to attenuate MPTP-induced DAergic neurodegeneration but only in the presence of glia. More importantly, DAergic neurons from mice lacking NADPH oxidase, a key enzyme for superoxide production, were significantly more resistant to MPTP neurotoxicity than those from wild-type controls. This suggests that NADPH oxidase may also be a promising target for therapeutic interventions in PD (Gao et al. 2003b; Gao et al. 2003a).
Microglial cells also produce cytokines which are important in the inflammation process. Studies of neuronal/glial mesencephalic cell cultures treated with MPP⁺ not only induced the proliferation of microglial cells, the inflammatory cytokine granulocyte macrophage colony-stimulating factor (GM-CSF), which could be produced by glial cells contributing to microgliosis (Henze et al. 2005). On the other hand, some cytokines have been shown to be protective such as transforming growth factor-beta 1 (TGF-beta) (Krieglstein and Unsicker 1994; Akaneya et al. 1995a, b; Krieglstein et al. 1995b; Krieglstein et al. 1995c; Henze et al. 2005) in DAergic neurons against MPP⁺ toxicity. IL-6 is a major immunomodulatory cytokine with neuroprotective activity. The absence of IL-6 resulted in increased vulnerability of DAergic neurons to MPTP, and a compromised reactive microgliosis with significantly greater amounts of the pro-inflammatory cytokines IL-1α, IL-1β and TNF-α, IL-10 and IL-12 (Bolin et al. 2005). Taken together, these data strongly suggest that microglial cells can participate in the death of DA neurons via the production of pro-inflammatory cytokines.

3.5.1.6 Cell Death

Apoptosis has been suggested as a mechanism of cell death in MPTP-induced parkinsonism, since MPP⁺ induces apoptosis in cultured cerebellar granular neurons (Dipasquale et al. 1991), in PC12 cells (Hartley et al. 1994) and in rat ventral mesencephalic-striatal co-culture (Mochizuki et al. 1994). The syntheses of 1-amino-4-phenyl-1,2,3,6-tetrahydropyridine (APTP) and 1-amino-4-phenyl-pyridinium salt (APP⁺), the 1-amino analogues of the DAergic neurotoxins, MPTP and MPP⁺, respectively, have also demonstrated that APTP and APP⁺ are both cytotoxic to PC12
cells inducing apoptotic cell death (Noda et al. 2004). The major contributors to DAergic cell death mainly involve ROS cell death pathways such as caspase-3 activation post MPP⁺ treatment (Du et al. 1997; Leist et al. 1998; Shimoke et al. 2003). Another regular marker for apoptosis is TUNEL. Evidence for TUNEL-positive DAergic cells after application of MPP⁺ in cell culture has been demonstrated repeatedly (Dodel et al. 1998) while denied by other researchers (Mochizuki et al. 1997).

Apoptosis is the mode of cell death induced in SH-SY5Y cells by MPTP, and this was confirmed with nick end labeling and bisbenzimide staining (Sheehan et al. 1997). The human neuroblastoma cell line SH-SY5Y possesses many of the qualities of human neurons and, as such, has served as a model. Analysis by electronic microscopy revealed ultrastructural changes consistent with the morphological characteristics of apoptosis (Sheehan et al. 1997). These changes included plasma membrane blebbing, altered cytosolic density, nuclear condensation and fragmentation, pronounced vacuole formation, ribosomal dispersion, and the disappearance of the Golgi complex, microtubules, and smooth endoplasmic reticulum. The in vitro induction of apoptosis by a parkinsonian neurotoxin might be reflective of the mechanisms of in vivo nigral degeneration occurring during PD (Ba et al. 2003).

Caspase-3 activation following DAergic cell death there are also evidence of time-dependent increases in ROS generation, cytochrome c release, and also caspase-9 activation (Kaul et al. 2003). The caspase-3 inhibitor Z-DEVD-fmk effectively blocked MPP⁺-induced PKC delta cleavage and kinase activity, suggesting that the proteolytic
activation is caspase-3 mediated. Similar results were seen in MPP⁺-treated rat midbrain slices. Z-DEVD-fmk and the PKC delta specific inhibitor rottlerin almost completely blocked MPP⁺-induced DNA fragmentation. The SOD mimetic, MnTBAP also effectively attenuated MPP⁺-induced caspase-3 activation, PKC delta cleavage, and DNA fragmentation. Intracellular delivery of catalytically active recombinant PKC delta significantly increased caspase-3 activity, further indicating that PKC delta regulates caspase-3 activity. Finally, over-expression of a kinase inactive PKC delta K376R mutant prevents MPP⁺-induced caspase activation and DNA fragmentation, confirming the pro-apoptotic function of PKC delta in DAergic cell death (Kaul et al. 2003).

Another pathway involved in DAergic cell death is the stress-activated protein kinase (SAPK) cascade. Recent in vitro and in vivo evidence has directly implicated this kinase in the death of DAergic nigral neurons in the MPTP model of PD. MPP⁺ specifically induces apoptotic changes in nuclear morphology in TH⁺ve (DAergic) cells and a nuclear phospho-c-Jun-ir (a key transcription factor substrate of SAPK). This indicates a role for the SAPK/JNK pathway including its c-Jun transcriptional target in the apoptotic death of DAergic nigral neurons in the MPTP model of PD (Chun et al. 2001; Gearan et al. 2001; Xia et al. 2001). Inhibition of the proapoptotic c-Jun N-terminal kinase (JNK) signaling cascade blocked JNK, c-Jun, and caspase activation, the death of DAergic neurons, and the loss of catecholamines in the ST in mice (Xia et al. 2001).

3.5.1.7 DA Agonists & Antioxidants

Anti-parkinsonian agents, pramipexole (PPX) and ropinirole (ROP) have been reported to possess neuroprotective properties, both in vitro and in vivo. Incubation of primary
mesencephalic cultures with PPX and ROP or the conditioned medium from PPX- or ROP-treated primary cultures induced a marked increase in the number of DAergic neurons in the cultures (Du et al. 2005). Similar effects can be observed after incubating with the conditioned medium derived from PPX- and ROP-treated SN astroglia. Meanwhile, PPX and ROP can protect the primary cells from the MPP⁺ insult. Furthermore, the neurotrophic effects of PPX and ROP on mesencephalic DAergic neurons could be significantly blocked by D3 receptor antagonist, but not by D2 receptor antagonist (Du et al. 2005).

Most antioxidants and DA agonists provide neuroprotection by blocking DA depletion in the ST such as minocycline, a semisynthetic tetracycline (Du et al. 2001) and/or reduce/inhibit ROS production. OS has been implicated in the selective degeneration of DAergic neurons in PD in particular as a mechanism of MPTP induced neuronal death. However not all antioxidants and DA agonists can provide such protection. Apoptosis induced by MPP⁺ in PC12 cells was unable to be rescued by the antioxidant drugs N-acetylcysteine (NAC) and ascorbic acid (AA) or DA agonist deprenyl (Desole et al. 1996; Desole et al. 1997a) again confirming that apoptosis is an important mechanism of cell death in MPTP. There are still potential therapeutic agents such as the bioenergetic coenzyme Q (10) (CoQ (10)) which has beneficial effects on TH and complexes I and II of the respiratory chain in striatal slice cultures induced by MPP⁺ (Gille et al. 2004).

The neuroprotective effects of anti-parkinsonian agents are suggested to be associated with marked reductions in inducible NO synthase (iNOS) and caspase 1 expression (Du
et al. 2001) since Peroxynitrite anion, an active metabolite of NO has been shown to cause significant cytotoxic effects against DAergic neurons (Sawada et al. 1996). But MPP+-induced neurotoxicity by NO is inhibited only in the presence of glia when treated with minocycline (Du et al. 2001) pointing out the involvement of glial/astrocytic cells presence contributing to cell death (McNaught and Jenner 1999).

MPP+ and other mitochondrial inhibitors (e.g., rotenone) induce apoptosis in vitro is believed to be via stimulation of autocrine excitotoxicity (Leist et al. 1998). Cell death, increase in intracellular Ca\(^{2+}\) concentration, release of cytochrome c, and all biochemical and morphological signs of apoptosis were prevented by blockade of the N-methyl-D-aspartate receptor (NMDA) with noncompetitive, glycine-site or glutamate-site inhibitors. In addition, MPP+-induced apoptosis was reduced by high Mg\(^{2+}\) concentrations in the medium or by exocytosis with clostridial neurotoxins (Leist et al. 1998). Many anti-excitotoxic agents have been shown to be neuroprotective by inhibiting blocking NMDA receptors and/or release of excessive glutamate such as memantine (Volbracht et al. 2006), IV cAMP phosphodiesterase inhibitor, Ro20-1724 (Dickie and Greenfield 1997), NMDA/glutamate antagonist MK-801 (McNaught and Jenner 1999; Shimizu et al. 2003b).

Antioxidant enzymes such as SOD and glutathione peroxidase (GSHPx) have also been shown to be affected and the target area for therapeutic intervention. Following MPTP treatment, a decreased GSHPx activity was observed in both SN and ST, while erythropoietin (Epo) restored nigral GSHPx activity decreased by MPTP, SOD enzyme
activity was not significantly changed by MPTP and Epo treatment. Furthermore, Epo stimulated astroglial GSHPx production in astroglial cell culture (Genc et al. 2002). SOD treatment has been shown to be neuroprotective against MPP⁺ exposure (Gonzalez-Polo et al. 2004), pretreatment of cultures with EUK-134 (a SOD and catalase mimetic) completely protected DAergic neurons against MPP⁺-induced neurotoxicity and prevented MPP⁺-induced nitration of tyrosine residues in TH (Pong et al. 2000). Tetrahydrobiopterin, an essential cofactor for TH, may also act as an antioxidant in DAergic neurons and protect against the toxic consequences of glutathione depletion. The increasing intracellular tetrahydrobiopterin levels may protect against OS by complex-I inhibition (Madsen et al. 2003). Dextromethorphan (DM), a widely used antitussive agent, attenuated endotoxin-induced DAergic neurodegeneration in vitro. The neuroprotective effect of DM in mesencephalic neuron-glia cultures significantly reduced the MPTP-induced production of both extracellular superoxide free radicals and intracellular ROS. More importantly, due to its proven safety record of long-term clinical use in humans, DM may be a promising agent for the treatment of degenerative neurological disorders such as PD (Zhang et al. 2004).

Studies have also focused on the potential of growth factors as neuroprotective and/or neuroregenerative therapeutic agents for PD. Treatment with nerve growth factor (NGF) promote acute cell proliferation when exposed with MPP⁺ via the sustained extracellular signal-regulated kinases (ERKs) and the p38 MAPK pathway in PC12 cells (Shimoke and Chiba 2001; Shimoke and Kudo 2002). Another growth factor that has been focused lately is glial cell line-derived neurotrophic factor (GDNF). Exposure to MPP⁺ was
found to be also toxic for hESC-derived DAergic neurons (Human embryonic stem cells can proliferate indefinitely yet also differentiate in vitro, allowing normal human neurons to be generated in unlimited numbers). Treatment with (GDNF) protected TH+ve neurons against MPP+-induced apoptotic cell death and loss of neuronal processes as well as against the formation of intracellular ROS (Zeng et al. 2006). Moreover, we found that the levels of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) in the conditioned medium of mesencephalic cultures treated with PPX and ROP were significantly increased. Blocking GDNF and BDNF with the neutralizing antibodies, the neurotrophic effects of PPX and ROP were greatly diminished. These results suggest that D3 dopamine receptor-preferring agonists, PPX and ROP, exert neurotrophic effects on cultured DA neurons by modulating the production of endogenous GDNF and BDNF, which may participate in their neuroprotection (Du et al. 2005).

3.5.2 ROTENONE & IN VITRO STUDIES

Similar to MPP+, rotenone uptake into brain synaptosomes is similar among primates and rodents, and hence is used in neuronal cultures. Cultures of dissociated mesencephalic neurons from fetal rats and DA-neuron-derived cell lines such as human SH-SY5Y and rat PC12 (see Table 3) is suitable for studying mechanisms of DAergic neuronal degeneration and for screening new pharmacological agents. Rotenone toxicity is believed to be a result from OS generated during DA metabolism and by mitochondrial respiration. In addition, progressive depletion of glutathione, oxidative damage to proteins and DNA, release of cytochrome c from mitochondria to cytosol, activation of
caspase 3, mitochondrial depolarization, and eventually apoptosis (Greenamyre et al. 2001).

### Table 3 Summary of Experimental PD In Vitro models

<table>
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<tr>
<th><strong>In Vitro model</strong></th>
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<tr>
<td>Primary Embryonic/Postnatal mouse mesencephalon</td>
<td>(Danias et al. 1989; Lotharius and O’Malley 2001; Gao et al. 2002; Ahmadi et al. 2003; Gao et al. 2003b; Gao et al. 2003a; Gille et al. 2004; Diaz-Corrales et al. 2005; Grammatopoulos et al. 2005; Radad et al. 2006)</td>
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<tr>
<td>Mouse MN9D cell line</td>
<td>(Seo et al. 2002; Kweon et al. 2004; Cao et al. 2007)</td>
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<tr>
<td>SH-SY-5Y cells</td>
<td>(Seaton et al. 1997; Lamensdorf et al. 2000b; Lamensdorf et al. 2000a; Imamura et al. 2006; Klintworth et al. 2007)</td>
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<tr>
<td>Synaptosomes</td>
<td>(Bougria et al. 1995; Fonck and Baudry 2003)</td>
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### 3.5.2.1 DAergic Toxicity

Rotenone treatment induces a dose- and time-dependent destruction of SNpc neuron processes, morphologic changes, some neuronal loss, and decreased TH protein levels. Chronic complex I inhibition also caused oxidative damage to proteins, measured by protein carbonyl levels. This oxidative damage was blocked by the antioxidant alphatocopherol (vitamin E). At the same time, alpha-tocopherol also blocked rotenone-induced reductions in TH protein and TH immunohistochemical changes (Testa et al. 2005). The number of TH+ve neurons was shown to be reduced by 50 +/- 6%
[Grammatopoulos TN], along with a reduction in DA, dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) levels in PC12 cells, DOPA formation with an accompanying decrease in ATP and increase in lactate in rat striatal slices (Hirata and Nagatsu 2005).

Decreases in ATP levels, changes in catechol levels, and increased DA oxidation is the general outcome of rotenone treated neurons. Whether endogenous DA makes PC12 cells more susceptible to rotenone is still unknown. Cells treated with the TH inhibitor alpha-methyl-p-tyrosine (AMPT) to reduce DA levels prior to rotenone exposure revealed no changes in rotenone-induced toxicity with or without AMPT treatment. However, a potentiation of toxicity was observed following coexposure of PC12 cells to rotenone and methamphetamine. PC12 cells were depleted of DA prior to methamphetamine and rotenone cotreatment to determine whether this effect was due to DA, resulted in a large attenuation in toxicity. These findings suggest that DA plays a role in rotenone-induced toxicity and possibly the vulnerability of DA neurons in PD (Dukes et al. 2005).

Cell death and reduced DA release has been observed in PC12 cells induced by rotenone (Yang et al. 2004b), this response was abolished by removal of extracellular Ca$^{2+}$. Mitochondrial inhibitors and uncouplers evoke catecholamine secretion from PC12 cells which is wholly dependent on Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels (Taylor et al. 2000). Rotenone also induces a rapid accumulation of DOPAL and DOPET in the medium of cultured PC12 cells but is decreased by MPP$^+$. 3,4-Dihydroxyphenylacetaldehyde (DOPAL) is a toxic metabolite formed by the oxidative
deamination of DA. This aldehyde is mainly oxidized to 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase (ALDH), but is also partly reduced to 3, 4-dihydroxyphenylethanol (DOPET) by aldehyde or aldose reductase (ARs) combined inhibition of ALDH and ARs potentiated rotenone-induced toxicity (Lamensdorf et al. 2000a).

The neurotoxic vulnerabilities between DAergic subgroups have also been studied. Rotenone induced severe dendrite loss among SN DAergic neurons, whereas hypothalamic A11 DAergic neurons were spared. Adjacent sections that were immunolabeled for calbindin or stained with cresyl violet also revealed a striking dendritic degeneration of SN neurons in rotenone-exposed slices, whereas noncatecholamine neurons, such as those in the perifornical nucleus were more resistant (Bywood and Johnson 2003).

3.5.2.2 Oxidative Stress (OS)

One possible result of complex I inhibition is increased formation of ROS, creating oxidative damage within the cell. Oxidative damage, rather than a bioenergetic defect, is also seen in the in vivo rotenone model (Sherer et al. 2003b; Sherer et al. 2003a; Bashkatova et al. 2004). PC12 cells treated with rotenone causes an apoptotic cell death and elevated intracellular ROS and lactic acid accumulation (Lotharius and O'Malley 2001; Dargusch and Schubert 2002; Liu et al. 2005; Radad et al. 2006), the formation of ROS and the depletion of GSH in differentiated PC12 cells (Sousa and Castilho 2005; Kim et al. 2007).
Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial ROS production. Recently, it has been shown that fraxetin (coumarin) and myricetin (flavonoid) have significant neuroprotective effects against apoptosis induced by rotenone, increases the total glutathione levels in vitro, and inhibit lipid peroxidation., such as Mn and CuZn superoxide dismutase (MnSOD, CuZnSOD), catalase, glutathione reductase (GR), and glutathione peroxidase (GPx) on rotenone neurotoxicity in neuroblastoma cells. N-acetylcysteine (NAC), a potent antioxidant, was employed as a comparative agent. SH-SY5Y-rotenone treated cells significantly increased the expression and activity of MnSOD, GPx and catalase (endogenous antioxidant defense system). Cells preincubated with fraxetin resulted in a decrease in the protein levels and activity of both MnSOD and catalase, in comparison with the rotenone treatment. Activity and expression of GPx were increased by rotenone and pre-treatment with fraxetin did not modify those levels significantly. This suggests that rotenone-induced neurotoxicity is partially mediated by free radical formation and OS, and that it can be partially protected by fraxetin providing the main protection system of the cells against oxidative injury (Molina-Jimenez et al. 2005).

The effect of the complex I-inhibitor rotenone on glutathione redox status and the generation of reactive oxygen intermediates (ROI) in rat pheochromocytoma PC12 cells induce a time-dependent loss of GSH, whereas treatment with lower concentrations of rotenone increases cellular GSH. Both MPP⁺ and rotenone increase cellular levels of oxidised glutathione (GSSG) and the higher concentrations of both compounds lead to an
elevated ratio of oxidised glutathione (GSSG) vs total glutathione (GSH + GSSG) indicating a shift in cellular redox balance. However MPP⁺ or rotenone does not induce the generation of ROI or significant elevation of intracellular levels of thiobarbituric acid reactive substances (TBARS) for up to 48 h (Seyfried et al. 2000).

3.5.2.3 α-Synuclein

In vitro, rotenone-induced α-synuclein aggregation (Lee et al. 2002) is correlated with elevations in insoluble protein carbonyls (Lee et al. 2002; Sherer et al. 2002b). In addition, in vivo rotenone infusion results in selective upregulation of α-synuclein in the substantia nigra and the increased occurrence of higher-molecular-weight, aggregated forms of α-synuclein (Betarbet et al. 2000; Betarbet et al. 2002a; Hoglinger et al. 2003a). An elevation in insoluble protein carbonyls in the midbrains of rotenone-treated animals suggests that rotenone treatment may cause oxidative modifications to α-synuclein that make it more likely to aggregate (Giasson et al. 2000; Krishnan et al. 2003). On the other hand, elevated α-synuclein expression can itself cause oxidative damage and render cells more vulnerable to oxidative insults (Hsu et al. 2000; Kanda et al. 2000; Ko et al. 2000).

Aggregation of gamma-tubulin protein, which is a component of the centrosome matrix and recently identified in LBs of PD, was observed in primary cultures of mesencephalic cells treated with rotenone. Rotenone-treated neurons and astrocytes showed enlarged and multiple centrosomes. These centrosomes also displayed multiple aggregates of α-synuclein protein. Neurons with disorganized centrosomes exhibited neurite retraction
and microtubule destabilization, and astrocytes showed disturbances of mitotic spindles (Diaz-Corrales et al. 2005).

3.5.2.4 Complex I Inhibitor

Complex I inhibition has several potential functional consequences, including decreased ATP production, altered calcium handling, and oxidative damage. Complex I is the first of four multisubunit protein complexes in the mitochondrial electron transport chain responsible for converting energy from cellular metabolism into the proton gradient used by complex V to generate ATP. Electron transport chain dysfunction could lead to loss of the proton gradient, impaired ATP production, and a bioenergetic defect. However, in dissociated culture systems, rotenone does not kill cells via ATP depletion instead rotenone increases OS (Zhang et al. 2001; Sherer et al. 2003b).

Complex I inhibition provokes the following events: 1) activation of specific kinase pathways; 2) release of mitochondrial proapoptotic factors, apoptosis inducing factor, and endonuclease G. AS601245, a kinase inhibitor, exhibited significant protection against these apoptotic events. The traditional caspase pathway does not seem to be involved because caspase 3 activation was not observed. This suggests that overproduction of ROS caused by complex I inhibition is responsible for triggering the kinase activation (Ishiguro et al. 2001; Lee et al. 2005; Bal-Price et al. 2006; Hirata et al. 2006; Ito et al. 2006; Radad et al. 2006; Hirata and Kiuchi 2007; Marella et al. 2007; Tan et al. 2007; Yim et al. 2007).
Rotenone causes the loss of mitochondrial membrane potential, released cytochrome c into the cytosol, and reduced cytochrome c content in mitochondria addition of bHB blocked this toxic effect and also attenuated the rotenone-induced activation of caspase-9 and caspase-3 (Imamura et al. 2006). Numerous studies also suggest that dysfunction of mitochondrial proton-translocating NADH-ubiquinone oxidoreductase (complex I) is associated with neurodegenerative disorders, such as PD and Huntington's disease. It was previously shown that the single-subunit NADH dehydrogenase of *Saccharomyces cerevisiae* (Ndi1P) can work as a replacement for complex I in mammalian cells such as DAergic cell lines rat PC12 and mouse MN9D. The cells expressing the Ndi1 protein were resistant to rotenone (Seo et al. 2002).

### 3.5.2.5 Inflammation

Increasing evidence suggests an important role for environmental toxins such as pesticides in the pathogenesis of PD. Rotenone-induced DAergic neurodegeneration has been associated with both its inhibition of neuronal mitochondrial complex I and the enhancement of activated microglia. Previous studies with NADPH oxidase inhibitors, diphenylene iodonium and apocynin suggest that NADPH oxidase-derived superoxide might be a major factor in mediating the microglia-enhanced rotenone neurotoxicity. However, because of the relatively low specificity of these inhibitors, the exact source of superoxide induced by rotenone remains to be determined. Primary mesencephalic cultures from NADPH oxidase--null (gp91phox-/−) or wild-type (gp91phox+/+) mice, demonstrate the critical role for microglial NADPH oxidase in mediating microglia-enhanced rotenone neurotoxicity. In neuron--glia cultures, DAergic neurons from
gp91phox−/− mice were more resistant to rotenone neurotoxicity than those from gp91phox+/+ mice. However, in neuron-enriched cultures, the neurotoxicity of rotenone was not different between the two types of mice. These results show that the greatly enhanced neurotoxicity of rotenone was attributed to the release of NADPH oxidase-derived superoxide from activated microglia (Gao et al. 2003a).

Observations of rotenone and the inflammogen lipopolysaccharide (LPS) synergistically induced DAergic neurodegeneration. The synergistic neurotoxicity of rotenone and LPS is observed when the two agents were applied either simultaneously or in tandem. Mechanistically, microglial NADPH oxidase-mediated generation of ROS appeared to be a key contributor to the synergistic DAergic neurotoxicity. This conclusion is based on the facts that inhibition of NADPH oxidase or scavenging of free radicals induced significant neuroprotection. Finally, rotenone and LPS failed to induce the synergistic neurotoxicity as well as the production of superoxide in cultures from NADPH oxidase-deficient animals. This is the first demonstration that low concentrations of pesticide and an inflammogen work in synergy to induce a selective degeneration of DAergic neurons (Gao et al. 2003b).

Neuron-enriched and neuron/glia cultures from the rat mesencephalon, has been used to study the role of microglia in rotenone-induced neurodegeneration. Significant and selective DAergic neurodegeneration was observed in neuron/glia cultures 2d after treatment with rotenone. The greatly enhanced neurodegenerative ability of rotenone was attributed to the presence of glia, especially microglia, because the addition of microglia
to neuron-enriched cultures markedly increased their susceptibility to rotenone. Mechanistically, rotenone stimulated the generation of superoxide from microglia that was attenuated by inhibitors of NADPH oxidase. Furthermore, inhibition of NADPH oxidase or scavenging of superoxide significantly reduced the rotenone-induced neurotoxicity (Gao et al. 2002).

3.5.2.6 Cell death via Apoptosis

Two cell models, human cultured cells HL-60 and BJAB, have shown that the exposure of cells to rotenone induces the generation of H$_2$O$_2$, which leads to significant changes in the mitochondrial membrane potential, and which is accompanied by the fragmentation of internucleosomal DNA and the formation of DNA-ladders (Tada-Oikawa et al. 2003). Furthermore, caspase-3 activity increases in rotenone-treated HL-60 cells in a time-dependent manner. These apoptotic events are delayed in HP100 cells, an H$_2$O$_2$-resistant clone of HL-60, confirming the involvement of H$_2$O$_2$ in apoptosis. In the same study, the expression of anti-apoptotic protein, Bcl-2, in BJAB cells, was shown dramatically to inhibit rotenone-induced changes in the mitochondrial membrane potential and the formation of DNA ladders, confirming the involvement of mitochondrial dysfunction in apoptosis (Tada-Oikawa et al. 2003).

Nanomolar concentrations of rotenone are also able to induce caspase-3-mediated apoptosis in primary DA neurons in VM cultures from E15 rats (Ahmadi et al. 2003; Hirata et al. 2006). After 11h of exposure to 30 nM rotenone, the number of DA neurons identified by TH immunostaining decreases rapidly, and only 20% of the neurons
survive. On the other hand, more than 70% of total neurons survive rotenone treatment, implying that DAergic neurons (TH⁺-neurons) are more sensitive to rotenone (Ahmadi et al. 2003). Rotenone significantly increases the number of apoptotic TH⁺-neurons (Hartley et al. 1994; Seaton et al. 1997, 1998; Radad et al. 2006; Hirata and Kiuchi 2007; Tan et al. 2007), which is correlated with an increase in immunoreactivity for active caspase-3 in DAergic neurons (Seaton et al. 1997; Lee et al. 2005; Wang et al. 2005; Hirata et al. 2006; Ito et al. 2006). Furthermore, the caspase-3 inhibitor, DEVD, rescues a significant number of TH⁺-neurons from rotenone-induced death. Thus, at low (nanomolar) concentrations, rotenone is able to induce caspase-3-mediated apoptosis in primary DA neuron cultures, with TH⁺-neurons being more sensitive to rotenone-induced toxicity than the total VM cell population (Ahmadi et al. 2003). This immediately raises a question concerning the mechanisms involved in the selectivity of dopaminergic neuronal death by rotenone.

Nitric oxide (NO) can also trigger either necrotic or apoptotic cell death. A 24-h incubation of PC12 cells with NO donors or specific inhibitors of mitochondrial respiration rotenone, in the absence of glucose, caused ATP depletion and resulted in 80-100% necrosis. Glucose addition almost completely prevented the decrease in ATP level and the increase in necrosis induced by the NO donors or mitochondrial inhibitors. This suggests that the NO-induced necrosis in the absence of glucose was due to the inhibition of mitochondrial respiration and subsequent ATP depletion. However, in the presence of glucose, NO donors and mitochondrial inhibitors induced apoptosis of PC12 cells as determined by nuclear morphology. The presence of apoptotic cells is prevented
completely by benzyloxycarbonyl-Val-Ala-fluoromethyl ketone (a nonspecific caspase inhibitor), this shows apoptosis was mediated by caspase activation. Indeed, both NO donors and mitochondrial inhibitors in PC12 cells caused the activation of caspase-3- and caspase-3-processing-like proteases. Caspase-1 activity was not activated. Cyclosporin A (an inhibitor of the mitochondrial permeability transition pore) decreased the activity of caspase-3- and caspase-3-processing-like proteases after treatment with NO donors, but was not effective in the case of the mitochondrial inhibitors. The activation of caspases was accompanied by the release of cytochrome c from mitochondria into the cytosol, which was partially prevented by cyclosporin A in the case of NO donors. These results show that NO donors may trigger apoptosis in PC12 cells partially mediated by opening the mitochondrial permeability transition pores, release of cytochrome c, and subsequent caspase activation (Bal-Price and Brown 2000).

Rotenone exposure caused apoptosis in SH-SY5Y cells but not in PC12 cells. This selective ability of paraquat and rotenone to induce apoptosis in different cell lines correlates with their ability to activate c-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinases. Furthermore, JNK and p38 are required for rotenone-induced apoptosis in SH-SY5Y cells (Newhouse et al. 2004) as well as primary neurons (Klintworth et al. 2007). Along with nuclear damage, the changes in the mitochondrial membrane permeability, leading to the cytochrome c release and caspase-3 activation, the formation of reactive oxygen species and the depletion of GSH in differentiated PC12 cells was induced by rotenone (Sousa and Castilho 2005; Kim et al. 2007).
3.5.2.7 DA Agonists and Antioxidants

Markers of DAergic neurons are more altered than those of \(\gamma\)-aminobutyric acid (GABA) neurons in PD-toxin models, suggesting greater susceptibility of DAergic neurons. Thus DA agonists are employed to replace the DA lost. Intracellular DA depletion with reserpine significantly attenuated rotenone-induced ROS accumulation and apoptotic cell death but not with deprenyl (Lotharius and O'Malley 2001; Dargusch and Schubert 2002; Liu et al. 2005; Radad et al. 2006). Another DA agonist which has been shown to possess anti-apoptotic actions is pramipexole, which also reduces caspase-3 activation, decreases the release of cytochrome c and prevents the fall in the mitochondrial membrane potential induced by rotenone (Schapira 2002a, b; Gu et al. 2004). The oxidative damage and DAergic neuronal loss caused by rotenone were shown to be blocked by alpha-tocopherol (Seyfried et al. 2000; Sherer et al. 2003b).

3.6 ORGANOTYPIC CELL CULTURE: IN VITRO MODEL FOR PD

The ability to conduct morphological assessments of ventral mesencephalic (VM) and striatum co-cultures provides support for the use of the organotypic co-culture model of the basal ganglia, given that DA neurons present in the VM will project axons to an adjacent cultured striatal tissue slice (Snyder-Keller et al. 2001; Gates et al. 2004) and form functional synapses (Tseng et al. 2007). Further analyses have shown that even the complex patch/matrix organization of the striatal tissue is maintained when donor tissue of VM and ST is age-appropriate (Snyder-Keller et al. 2001). These studies suggest that it is important to use VM tissue in which the dopamine (DA) neurons have differentiated,
yet have not begun to project DA axons/terminals to the striatum; in this way, the likely
degeneration incurred with axotomy during tissue dissection can be limited.

PD research has utilized VM organotypic cultures, containing the DA neurons affected in
PD, in the study of tissue explants for transplantation purposes, as well as to assess
potential DA neurotoxicants. Researchers have used the VM cultured alone (Dickie et al.
1996; Hoglinger et al. 1998; Meyer et al. 2001; Shimizu et al. 2003a), the VM in co-
culture with ST (the target of VM DA neuron terminals) (Schatz et al. 1999; Katsuki et
al. 2001; Snyder-Keller et al. 2001; Kotake et al. 2003; Gates et al. 2004), and the VM,
striatum, and cortex in triple cultures to model the BG DAergic system (Plenz and Kitai
1996; Snyder-Keller et al. 2001). However, to date, studies have not adequately
described the DA development, in terms of neurochemical and protein analyses, of the
VM and striatum in co-culture (Lyng et al. 2007). Here, we have used organotypic co-
cultures of P5-6 VM and P5-6 rat ST in the roller tube system with slight modifications
(Stoppini et al. 1991; Snyder-Keller et al. 2001) to model the developing nigrostriatal DA
system.

3.6.1 MPTP application in organotypic cell cultures
Using midbrain slice cultures a few studies have demonstrated MPP\(^+\) to be a complex I
inhibitor (Madsen et al. 2003; Xu et al. 2003; Gille et al. 2004), specifically toxic to
DAergic neurons by reduction in DA levels, its metabolites and tyrosine hydroxylase-
positive cells in a dose-dependent manner (Reinhardt 1993; Madsen et al. 2003; Shimizu
et al. 2003b; Shimizu et al. 2003a; Kress and Reynolds 2005). MPP\(^++\)'s toxic action was
attenuated by NMDA inhibitor MK801 (Kress and Reynolds 2005). DA agonists such as L-Dopa, antioxidant and bioenergetic coenzyme Q(10) (CoQ(10)) had preserving potential on the activities of TH, complexes I and II of the respiratory chain along with the survival rate of DAergic neurons (Gille et al. 2004). Cell death process is believed to occur via apoptosis evident by the loss of cell bodies and the fragmentation of processes along with nucleus shrinkage (Kress and Reynolds 2005). This cell death was prevented by the inhibitors of NMDA, nitric oxide synthase (NOS), cycloheximide and caspase cascade and also rescued by L-deprenyl and dopamine D2/3 agonists (Shimizu et al. 2003b; Shimizu et al. 2003a). The influence of target tissue was also examined on co-cultures of DAergic neurons forming dense innervation to the striatal tissue. DAergic neurons in midbrain--striatum co-cultures were more resistant to the cytotoxic actions of NMDA and a nitric oxide donor NOC-18, than the same neuronal population in single midbrain cultures. On the other hand, the toxicity of MPP+ was more prominent in midbrain-ST co-cultures than that in single midbrain cultures (Katsuki et al. 1999). The neurotrophic factor GDNF was also examined in this in vitro model showing that pre-treatment and post-treatment with GDNF is important to obtain maximal protection against MPP+ toxicity (Jakobsen et al. 2005).

### 3.6.2 Rotenone and Organotypic Cell Culture

To a lesser extent, the use of rotenone in both animal and cell cultures is fewer than MPP+ and has mainly been studied in primary cell cultures deriving from fetal mesencephalon. Only a few studies have employed rotenone in this culture system. Similar to MPP+, it is observed to be a complex I inhibitor and specific DAergic toxin
leading to a dose- and time-dependent destruction of SNpc neuron processes, morphologic changes, neuronal loss, and decreased TH protein levels (Xu et al. 2003; Kress and Reynolds 2005; Testa et al. 2005). Chronic complex I inhibition also caused oxidative damage to proteins, measured by protein carbonyl levels. This oxidative damage was blocked by the antioxidant alpha-tocopherol (vitamin E). At the same time, alpha-tocopherol also blocked rotenone-induced reductions in TH protein and TH immunohistochemical changes (Testa et al. 2005). Addition of L-Dopa, DA agonists and antioxidants such as CoenzymeQ were able to counteract rotenone’s toxicity (Gille et al. 2004).
4.0 AIMS OF STUDY

1) To grow and maintain the development of dopaminergic neurons *in vitro* using the organotypic slice culture technique.

2) To establish a period of optimal dopaminergic neuronal growth for application of neurotoxins.

2) To assess the effects of neurotoxins MPTP and rotenone on the survival of dopaminergic neurons.

3) To examine the neuroprotective effects of neutrophic factor GDNF on dopaminergic neurons with or without the toxic stress from the neurotoxins.
CHAPTER 5:
GENERAL MATERIAL AND METHODS
5.1 ANIMAL CARE

5.1.1 Ethics
The experimental studies presented in this thesis were performed within the guidelines established by the National Health and Medical Research Council (NH&MRC) and were approved by the ethics committees of the Institute of Medical and Veterinary Science (IMVS; 64A/04, 152A/07) and the University of Adelaide (M-64-2004) to minimize animal suffering and to reduce the number of animals used.

5.1.2 General
Sprague-Dawley rats from postnatal day of 4 to 5 (P 4-5) were kept in a heated pad insulated container until required.

5.2 ORGANOTYPIC SLICE CULTURES
All the culture procedures were carried out under sterile conditions, within a laminar flow hood. Similarly, all equipment was sterilized in an oven for four hours at 180°C and solutions were either sterile filtered or obtained pre-sterilized or autoclaved.

5.2.1 Dissection of the ventral mesencephalon and striatum
Organotypic slices were prepared from brains of pups 4-5 d of either sex according to the procedure described by Stoppini (Gahwiler 1981; Stoppini et al. 1991) and Gahwiler (Gahwiler, 1988 #27) with slight modifications. Briefly, pups were quickly decapitated; small dissecting scissors were used to make a cut along the midline of the skull before the skull was opened to expose the dorsal surface of the brain. A spatula was used to cut
through cranial nerves and ease the brain, dorsal surface downwards onto a sterile chilled Petri dish. Following the rapid removal of the brain, placed on its dorsal side with the ventral side up on a sterile glass cold Petri dish, under a dissecting microscope.

In order to dissect the striatum (ST), two cuts in the coronal plane were made; one through the forebrain anterior to the level of the optic chiasm, the second through the optic chiasm itself. The block was placed on its anterior cut surface and another two horizontal cuts separated the striatum from the cerebral cortex and basal forebrain, whilst two vertical cuts removed the septum and any remaining cortex from the striatum. Any meninges attached to the tissue were removed with tweezers. The tissue block was transferred to a McIlwain tissue sectioner where serial coronal sections of 300μm thickness were cut. After transferring the ST to a petri dish containing cooled ringers balanced salt solution (BSS; see appendices), the sections were gently teased apart. Up to 10 slices were obtained from each tissue block.

A similar procedure was used to dissect the ventral mesencephalon (VM). A coronal cut was made through the posterior thalamus at the level of the mamillary bodies. A second cut was made through the brain at the level of the pons in order to isolate the midbrain. A horizontal cut removed the dorsal midbrain and cortical tissue from the ventral midbrain. The tissue was then turned onto the dorsal cut surface and any attached meninges were removed with fine tweezers before making a vertical midline cut to separate the two VM. Then 200μm thick slices were cut. Up to ten cultures could be produced from each tissue block. Finally, the sections were transferred to a solution of cooled ringers and the
sections were teased apart under the dissecting microscope. Petri dishes with ringer’s solution containing either SN or ST were then transferred into another sterile Petri dish containing chilled Gey’s balanced salt solution (GBSS; JRH biosciences) and 0.1 % D-glucose and 0.1% potassium chloride. The Petri dishes containing either ST or SN placed in GBSS were placed at 4°C for 90min in a fridge before mounting.

5.2.2 Mounting the slices

The slices were mounted on glass coverslips (11cm x 22cm). The coverslips were cleaned for fifteen minutes in a 1% solution of sodium thiosulphate and potassium hexacyano ferrate, then boiled in distilled water for two hours, immersed in ethanol for two days and sterilised.

A plasma clot was used to mount the tissue onto the cover slips. This involved embedding the tissue in a plasma clot which was formed by mixing a solution of chicken plasma with thrombin. A stock solution of chicken plasma was prepared from lyophilised chicken plasma to which 5ml of sterile purified water was added. The solution was centrifuged at 2500 revolutions per minute (RPM) for thirty minutes and the supernatant was sterile filtered before being frozen in 1ml aliquots. The thrombin solution was prepared from 0.1g of bovine thrombin by addition of 5ml of sterile purified water. The solution was centrifuged for thirty minutes at 2500 RPM and the supernatant was sterile filtered before being frozen in 200μl aliquots.
On the day of preparation of cultures, 150μl of the prepared thrombin solution was diluted with 5ml of sterile GBSS. Both the chicken plasma (Sigma P-3266-5mL) and the thrombin (Sigma T-4648-10KU) solutions were kept on ice. A 20μl drop of chicken plasma solution was placed on one end of the pre-cleaned coverslip. A fine spatula was then used to gently lift a section of ventral mesencephalon followed by the ST, from the GBSS and into the drop. A 20μl drop of thrombin was then placed on the cover slip and a spatula was used to mix the two solutions around the sections. Finally, the tissues were carefully positioned approximately 1mm apart within the plasma clot which had formed.

5.2.3 Slice culture by the roller-tube technique

Each coverslip with the mounted cultures was placed in screw cap, diagonal bottomed culture tube (NUNC TC tube; NC-156758, SI 110 X 6 mm) and 0.75ml of culture medium was added to each tube. The culture medium contained 25% horse serum which had been inactivated by heating at 56°C for thirty minutes, 50% Eagle minimum essential medium and 25% Hanks BSS ([10 mM HEPES] IMVS; Infectious disease lab media unit). The medium was also supplemented with d-glucose to a final concentration of 6.5mg/ml and L-glutamine to a final concentration of 0.15 mg/ml. New culture medium was made each week. Penicillin and gentamycin were also added to the medium.

The culture tubes were placed in a roller drum which was tilted at an angle of 5° from the horizontal axis to ensure that the cultures were immersed by the medium for about half of the rotating cycle. The culture tubes were rotated at a rate of approximately ten revolutions per hour. The roller drum was positioned within an incubator which kept the
cultures at a temperature of between 35.5 and 36.5°C. The culture medium was changed twice a week by pouring away the old medium and adding 0.75ml fresh medium to each tube. In order to control the number of non-neuronal cells, anti-mitotic drugs were added to the medium for 24 hours on the third or fourth day in culture. The anti-mitotic substances used were uridine and cytosine-β-D-arabino-furanoside, both at concentrations of 10⁻³ M. Cultures were maintained for variable lengths of time, most cases for 7 weeks.

Co-cultures included whole intact hemispheres of ventral mesencephalon containing SN were plated 1mm apart from the ST on glass coverslips (LOMB Scientific) with 25 μl of chicken plasma and 20 μl thrombin. Placed in a humidified chamber for 15 min to allow the chicken plasma and thrombin to clot then into a flat-bottom culture tubes with screw caps containing 1 ml medium [50% DMEM (IMVS; Infectious disease lab media unit), 25% Hanks balanced salt solution with 10 nM HEPES (IMVS; Infectious disease lab media unit), 25% heat inactivated horse serum (JRH biosciences; 50120-100 M), supplemented with glucose (1 ml 50% D-glucose to 100 ml of medium) and glutamine (500 μl of 1 mM L-glutamine to 100 ml of medium), and 0.5% gentamycin and penicillin (IMVS; Infectious disease lab media unit). The medium was changed twice a week.

After 4 to 6 days in vitro (DIV), co-cultures were treated with antimitotic agents 1 ml of cytosine-β-D-arabinofuranoside (Sigma C-6645) and 1 ml of uridine (Sigma U-3750) to 100 ml of medium for 24 h to retard glial and fibroblast growth, then discarded and fresh media was added. Cultures were maintained for variable lengths of time, most cases for 7 weeks, in a cell culture incubator at 35-36.5 °C in a roller drum at 5 revolutions per hour.
5.3 FIXATION OF CULTURES

Cultures were fixed at 1 week intervals with 4% paraformaldehyde (pH 7.4) overnight or minimum 4 hours at room temperature. Then endogenous peroxidases were inactivated with treatment with 0.5 % H₂O₂ (8.3 mL) plus 100% of methanol (500 mL) for 30 min. Sections were the rinsed twice (5 min) in fresh PBS (pH 7.4) 0.3 % Triton X-100 (Sigma T9284-500 mL).

5.3.1 Immunoperoxidase antigen retrieval

For antigen retrieval, the cultures on coverslips or 7μM sections of paraffin embedded tissue were placed in coplin glass staining jars or slide rack (respectively) (Proscitech; L056) containing citrate solution (see appendices) with lid screwed slightly, the coplin jars are then placed within a kartel slide dish filled with distilled water (approximately 250 ml) and lid placed on, then microwave as follows. Place side dishes in Panasonic microwave oven (Model NE-1037) press 0 the start button. Microwave the coverslips until they start to boil. Once boiled remove the boiled dishes and place in a second microwave oven (NEC). If there is 2 dishes select timer fro 10 min and power level at 2, for 3 dishes select timer for 10 min and power level at 3. Once microwaving is complete remove dishes from microwave take lids off and allow 30 min to cool (approximately below 40°C). Cultures are then washed twice (5 min) fresh 0.3% Triton X-100 PBS.

5.3.2 Immunohistochemistry

Antisera: The following mono- and polyclonal antibodies were used: rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody (1:2000; Chemicon AB151), rabbit monoclonal
anti-glial fibrillary acidic protein (GFAP) antibody (DAKO, Z0334; 1:20 000), mouse polyclonal anti- Neu-N (1:4000; Chemicon MAB 377). Antigen retrieval was mainly citrate unless primary antibodies were Caspase -3 in which EDTA solution was used instead. Secondary antibodies were Vector Biotinylated anti-mouse IgG (BA-2000) or anti-rabbit IgG (BA-1000) at 1/250. Followed by the 3° step where the Pierce peroxidase conjugated streptavidin tertiary (21127) at 1/1000 (is also referred to as SPC).

**Paraffin sections:** Following immunoperoxidase antigen retrieval procedure the cultures were blocked with normal donor horse serum [1 ml to 30 ml 0.3% Triton X-100 PBS (CSL Biosciences 09532301)] for 30 min at room temperature then incubated with primary antibodies overnight at RT. Rinsed twice in Triton X-100 PBS and incubated with 30 min secondary antibodies (1/250) at room temperature, rinsed twice then incubated with tertiary antibodies SPC (1/1000) at room temperature for 1 hour. Cultures were rinsed twice before antigen-antibody complexes were revealed using 3,3’ – diaminobenzidine-Tetrahydrochloride (DAB; Sigma, D8001) as chromogen for 7 min until a dark brown colour can be seen. The cultures were then brought to tap water for 10 min to wash off any excess DAB, and were further counterstained with Lillie Mayers alum haematoxylin (see appendices for procedure). 5 μm sections of paraffin sections from P5-7 rat pups were de-waxed in heater for 10 min then placed in 2 changes of xylene (2 min) and alcohol (2 min) before treatment with H₂O₂ and methanol as described with the cultures.
Controls and cultures were dehydrated with two changes of alcohol and cleared with two lots of histolene and mounted onto microscope glass slides (coverslipped for controls) with mounting glue (D.P.X).

**Cultures:** Procedure is the same as paraffin sections however no need for antigen retrieval procedure and incubation of primary antibodies is overnight at 4 °C.

**5.3.3 Co-localisation of TH and active Caspase-3**

Cultures were treated as above however the secondary antibodies were conjugated with a fluorochrome. TH (Chemicon; AB152) polyclonal.

**5.4 LACTATE DEHYDROGENASE (LDH) ELISA**

LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes and released from necrotic cells with damaged membranes. Briefly, LDH catalyzes the conversion of lactate to pyruvate upon reduction of NAD⁺ to NADH/H⁺, the added TMB (yellow) is formed, the amount of yellow correlates with LDH activity. Culture medium samples were collected and snapped frozen (~ 80° C) and thawed in 60° C water bath when required. The bovine serum albumin (BSA) standard was used as an internal control (1 mg/1 mL) in tris buffered saline (TBS) and also as a blocking solution. Samples (20 μL) were pre-mixed with TBS (80 μL) with a total volume of 100 μL per well in a 96-well plate (Maxicorp) in triplicates per treatment group, overnight at 4° C. Following day plates were aspirated (tipped out) and blocked for 1 hr at RT with 3% BSA on orbital shaker at low. Washing in TBS x 2 then incubated with the 1° Ab LDH...
(AB2101-1; AbCam) 1/1000 min 1 hr in humidifier. Another TBS wash (2 x) and incubation with 2° Ab HRP-conjugated anti-goat (Jomar Diagnostics; 611-701-127) in the humidifier for an 1 hr. Final TBS x3 wash then revealed with TMB (Sigma-Aldrigde; T-8665 100 ml) 100 μL per well) forming a yellow by-product and reaction terminated with addition of H₂SO₄ (50 μL). Colour intensity was assessed using a plate reader (Ascent Multiwell Plate reader) at 450 nm (see Figure 5.4).

5.5 QUANTIFICATION AND STATISTICAL ANALYSES OF CELL COUNTS
Cultures were examined and photographed by light microscopy at 400 x with camera attached to microscope (Olympus) and a monitor. Cell counts were estimated by using unbiased, blind fashion, uniform and systematic random samples of sections throughout the cultures. A minimum 3 cultures per group and 4 or more images per cultures were taken and TH-ir cells with clear nucleus and neuritic or axonal projections observed were counted. Areas of where the tissues were still thick were not counted as single cell labeling was not defined.

For experimental Chapters 6, 7 and 8 an assessment of the survival and growth of the TH-ir neurons within the organotypic slice culture was required. Therefore, using a light microscope the number of TH-ir cells surviving in each culture was determined by counting the cells under high power (400 x) magnification. This process was facilitated by the fact that the cultures were approximately only one cell layer thick.
Data are shown as mean ± standard error of measurement (SEM). Repeated measures analysis of variance (ANOVA) followed by Bonferroni t-tests were used to analyse the growth of TH-ir cells. 1-way ANOVA was used to compare cell counts between groups and weeks in vitro and a 2-way ANOVA was used when comparing treated groups of various concentrations and following weeks. A $P$ value of $< 0.05$ was considered significant. Prism (Graphpad TM Software, SanDiego, CA) statistics computer program was used for all analyses.
Figure 5.1. Schematic illustration of the preparation protocol of slice cultures from 4 to 5-day-old rats.

Tissue blocks were dissected at the position A and B to obtain the ventral tegmental area/substantia nigra-complex (A), the striatum (B). Blocks were trimmed along the dashed lines and coronal sections of the regions of interest (250-300 μm; asterisks) were selected and mounted on a glass coverslip by means of a plasma clot. The coverslip was then placed in a flat-bottom culture tube containing culture medium. Continuous rotation of the culture was achieved by placing the culture tube in a roller within an incubator (36°C). STR, striatum; VTA/SN-complex, ventral tegmental area/substantia nigra-complex. (Adapted from Heike Franke (Franke et al. 2003)).
Figure 5.2  Schematic diagram of the culturing process.

Each coverslip contains a piece of VM and ST tissue placed under a chicken plasma cloth and thrombin; it is then inserted into a flat-bottom screw cap tube with 1 mL medium the finally placed into a roller drum in a 36 °C incubator rotating at 5 rev/min.
Figure 5.3  Schematic representation of the biotin-avidin peroxidase procedure for the identification of TH-ir neurons.

Each arrow between the steps represents 3 x 5 minute washes with Triton X-100 with PBS.
CULTURE FIXED
4% paraformaldehyde (30mins)
↓
ENDOGENOUS PEROXIDASE ACTIVITY BLOCKED
0.2% H2O2 in methanol (30mins)
↓
CELL MEMBRANES PERMEABILISED
1% Triton X-100 in PBS (3x 5mins)
↓
NON-SPECIFIC BINDING BLOCKED
10% normal horse serum in Triton X-100 in PBS (30mins)

TYROSINE HYDROXYLASE LABELLED
Rabbit anti-TH, 1:2000 dilution with 10% NHS Triton X-100 in PBS (O/N cold room)
↓
SECONDARY ANTIBODY
Vector Biotinylated anti-rabbit IgG (BA-1000) at 1/250 dilution with 10% NHS Triton X-100 in PBS (30mins at room temp.)
↓
PEROXIDASE LABEL ATTACHED
Pierce peroxidase conjugated streptavidin tertiary (# 21127) at 1/1000 (referred to as SPC) with 10% NHS Triton X-100 in PBS (1hr at room temp.)
↓
ANTIGEN-ANTIBODY COMPLEXES REACTION VISUALISED
0.05% DAB with 0.01% in PBS (<10mins)
↓
COUNTERSTAIN
Lillie Mayers haematoxylin (1min)
↓
DEHYDRATE TO HISTOLENE
2x100% ethanol then 2x100% histolene (30mins)
Figure 5.4 LDH ELISA.

(A) Samples loaded into wells with buffer (TBS) and incubated overnight (4°C).  
(B) Samples become attached to the well surface excess sample is aspirated.  
(C) Incubation with BSA to block unoccupied sites on well surface with neutral protein on orbital shaker for 1 hr at RT (low).  
(D) Add 1° Ab and incubate in humidifier (1 hr).  
(E) Unattached Abs is removed followed by incubation of 2° Ab (min 1 hr) in humidifier.  
(F) Add substrate and incubate-peroxidase enzyme breaks down substrate and turns yellow, reaction terminated by H₂SO₄ (blue).
Marker Protein → TBS x2 wash
Primary antibody
Secondary Antibody
CHAPTER 6:
DOPAMINERGIC NEURONS GROWN IN ORGANOTYPIC SLICE CULTURES: TH-ir CELLS ARE DEPENDENT ON TARGET REGION-STRIATUM
6.0 INTRODUCTION

There are two main populations of DA neurons that innervate the forebrain, the substantia nigra (SN; A9) cell group, and the ventral tegmental area (VTA; A10) cell group (Ungerstedt 1971; Beckstead et al. 1979). The SN confines its outgrowth to the dorsal striatum (ST; or caudate-putamen) (Huang 1990), whereas the VTA also innervates several other structures, including the nucleus accumbens (Chronister et al. 1980) and cerebral cortex (Kalsbeek et al. 1988). The determinants of these innervation patterns are not known, however, evidence for specific factors that influence the survival and outgrowth of dopaminergic (DAergic) neurons has been accumulating since the 1970s. Prochiantz and colleagues showed that DAergic neurons from the ventral mesencephalon (VM) grew toward and formed terminals with neurons from the ST, and they further demonstrated that this resulted at least in part from a diffusible factor released from striatal glia (Barbin, 1985 #1642; Prochiantz, 1981 #120; Prochiantz, 1982 #1643; Denis-Donini, 1983 #75). Bohn and co-workers then showed that glial cell lines released one or more substances that promoted the survival and outgrowth of DAergic neurons (Engele et al. 1991).

6.1 AIMS

Using postnatal SD rats, we aim to grow and maintain a DAergic system and study the trophic effects of ST target, which could be used as a model for PD in vitro.

6.2 METHOD

The slice cultures were prepared according to the method described in Chapter 5.
Co-cultures of ventral mesencephalon (VM) and striatum (ST) were prepared from 4-5 day old (P4-5) Sprague-Dawley rats. Cultures of VM with and without ST were prepared. The cultures were incubated by the roller tube technique according to Gahwiler (1981) for 49 days. The live cultures were inspected twice a week. Cultures producing yellow colour medium indicated healthy live cells whereas a pink to orange buffer is indicative of poor growth. Once every week a minimum of 3 tubes were removed, fixed in paraformaldehyde and stained for tyrosine hydroxylase immunoreactivity (TH-ir), a marker of DAergic neurons and counter-stained with haematoxylin to show the cell cytology. The cultures were then analyzed in a blind fashion. The numbers of TH-ir cells in a minimum of 3 fields under 400x magnification were counted using specific criteria to include only cells with neurites and a nucleus. These were photographed and independently checked by a second researcher.

Analysis of the number of TH-ir cells was performed by using One-way ANOVA and Two-way ANOVA, followed by Bonferroni's Multiple Comparison Test. A p value of <0.05 was considered significant.

6.3 RESULTS

6.3.1 TH-ir neurons of the ventral mesencephalon in culture

The primary TH antibody was tested for its specificity as a DAergic marker. In all our cultures only whole VM slices were used instead of halves to minimize the variability as both sides of the VM do not have the same amount of DAergic neurons (Figure 6.1A & B), thus if VM halves were cultured this would result in some co-cultures with good
growth and others with poor growth of DAergic neurons. Culture controls showed no staining in the negative control (Figure 6.1C) and brown DAB staining with no background staining in positive control (Figure 6.1D).

The characteristic organization of the VM disappeared with progressive cultures and neither the substantia nigra pars compacta (SNpc), nor the VTA, could be detected separately. The cultures consisted of overlaying layers of neurons upon non-neuronal cells. A monolayer was formed after 14-28 days incubation with both non-TH-ir cells and TH-ir neurons extended fibres from the centre of the tissue towards the surrounding plasma clot (Figure 6.2A). Some of the TH-ir cells were able to direct their axons, to innervate nearby striatal tissue, forming an axon bridge that between VM-ST (Figure 6.2B). Different morphological types of TH-ir cells were present (Figure 6.2C), projecting their axons and dendrites in all directions within the tissue forming neuronal networks (Figure 6.2D).

### 6.3.2 TH-ir cell growth in the Co-cultures

Between 7-14 days, the co-cultures were thick with many layers of non-neuronal and neuronal cells emerging from the tissue surfaces. Individual TH-ir cells could be clearly seen as they grew from the edge of the tissue outwards into the plasma clot. After 21 days, the co-cultures were reduced to a 1-2 cells thick layer and by the 28th day single cells formed distinct cell bodies, axons and dendrites. These TH-ir cells formed networks with neighbouring cells.
6.3.3 The influence of trophic ST target on VM TH-ir cells

Single cultures of VM (SVM) grew poorly compared to the co-cultures. These cultures did not flatten to a monolayer and neuronal network formation was sparse (Figure 6.3A) compared to co-cultures (Figure 6.3B). Generally TH-ir cells in SVM were smaller and had fewer processes (Figure 6.3C) compared to co-cultures (Figure 6.3D). SVM cultures grown for 42 days had significantly lower TH-ir cells (Figure 6.4) when compared to co-cultures (Figure 6.5 & Figure 6.6).

6.3.4 Glial cells contribute to the regulation of DAergic outgrowth

Cultures were also stained for the astrocytic marker glial fibrillary acidic protein (GFAP). GFAP-ir cells in SVM cultures were large with numerous projections (Figure 6.7B) compared to the co-cultures (Figure 6.7C). GFAP-ir cell numbers were initially lower in the first weeks in co-cultures (Figure 6.8) and increased to similar numbers compared to the SVM cultures (Figure 6.9). In SVM cultures GFAP-ir cell numbers were initially higher than co-cultures and remained stable in the following weeks (Figure 6.9). The GFAP-ir cell numbers were not significantly higher than the TH-ir cell numbers in both co-cultures and SVM cultures (Figure 6.10 & Figure 6.11).
**Figure 6.1 Dopaminergic (TH-ir) controls**

A) TH-positive control [VM rat pup 4-5d, paraffin section 5-7 μm] (x200).  
(B) Whole VM hemisphere of figure (A) at x40. Note: left side has less TH-ir neurons than the right side (C) TH-negative control from co-cultures (x400).  
(D) TH-positive control from co-cultures (x400). Bar = 10 μm (C & D), bar = 20 μm (A), bar = 100 μm (B).
Figure 6.2 Dopaminergic (TH-ir) neuronal growth in VM & ST co-cultures.

(A) 7 -14 days, TH-ir cells emerge from tissue (x100). (B) Bridge between VM & ST targets (x40). (C) Subtypes of DAergic neurons (x400). (D) 4 weeks, formation of axons and dendrites and networks with neighbouring cells (x 200). Bar = 10 μm (C), bar = 20 μm (A), bar = 50 μm (B), bar = 100 μm (D).
Figure 6.3 Dopaminergic (TH-ir) neuronal growth in VM and ST co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x for each week (wk). The Means are calculated from the raw data and data expressed as mean ± SEM followed by One-way ANOVA which was calculated using STDEV, ** p<0.01 [1 wk compared to 3-6 wk; 2 wk compared to 3-6 wk], *** p<0.001 [1 and 2 wk compared to 7 wk].
Figure 6.4 TH-ir cell growth in co-cultures vs single cultures (SVM).

(A) Poor TH-ir cell growth in SVM slice culture at 3 weeks (x 40). (B) TH-ir cells from SVM cultures at 2 weeks (x400). (C) TH-ir cells in co-cultures SVM at 2 weeks (x 400).

Bar = 10 μm (B & C), bar = 100 μm (A).
Figure 6.5 Dopaminergic (TH-ir) neuronal growth in single VM cultures (SVM). The number of cells per field (0.2290mm²) viewed at 400x for each week (wk). The Means are calculated from the raw data and data expressed as mean ± SEM followed by One-way ANOVA which was calculated using STDEV followed by Bonferroni’s Multiple Comparison Test. * p<0.05 [1 wk compared 2 wk], *** p<0.001 [2 wk compared to 3-6 wk].
Figure 6.6 Dopaminergic (TH-ir) neuronal growth in co-cultures vs SVM.

The number of cells per field (0.2290mm²) viewed at 400x for each week (wk). The Means are calculated from the raw data and data expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV followed by Bonferroni post-tests, *** p<0.001.
Figure 6.7 GFAP growths in co-cultures vs single cultures.

(A) GFAP-positive control (VM rat pup 4-5d, paraffin section 5-7μm; x 200).  (B) GFAP-ir cells from co-cultures at 7 DIV (x 400).  (C) GFAP-ir cells in SVM at 7 DIV (x 400).  Bar = 10 μm (B & C), bar = 20 μm (A).
Figure 6.8 Glial fibrillary acidic protein (GFAP-ir) growth in VM & ST co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x for each week (wk). The means are calculated from the raw data and data expressed as mean ± SEM followed by One-way ANOVA which was calculated using STDEV followed by Bonferroni’s Multiple Comparison Test, * p<0.05, ** p<0.01, *** p<0.001.
Figure 6.9 Glial fibrillary acidic protein (GFAP-ir) growth in single VM (SVM) cultures.

The number of cells per field (0.2290mm²) viewed at 400x for each week (wk). The means are calculated from the raw data and data expressed as mean ± SEM followed by One-way ANOVA which was calculated using STDEV followed by Bonferroni's Multiple Comparison Test, * p<0.05, ** p<0.01.
Figure 6.10 Dopaminergic (TH-ir) & Glial fibrillary acidic protein (GFAP-ir) growth in VM & ST co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x for each week (wk). The Means are calculated from the raw data and data expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV and Bonferroni’s Multiple Comparison Test, *** p<0.001.
Figure 6.11 Glial fibrillary acidic protein (GFAP-ir) growth in VM & ST co-cultures and SVM.

The number of cells per field (0.2290mm²) viewed at 400x for each week (wk) from co-cultures & SVM. The Means are calculated from the raw data and data expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV and Bonferroni's Multiple Comparison Test, *** p<0.001.
6.4 DISCUSSION

6.4.1 General findings

In this study we were able to maintain the growth of DAergic cells without any special conditioned medium using the organtotypic culture technique. In particular, we demonstrated that growth and maturation of VM DAergic neurons was in some way regulated and dependent on its specific trophic target- namely the ST. The DAergic neurons were able to innervate the ST and that glial cells played a supportive role in this process.

Ostergaard (Ostergaard et al. 1990) described 5 distinct groups of TH-ir cell types which we were able to identify in our slice cultures. The multipolar neurons were the most commonly observed throughout the period of culture which mainly is comprised of three primary dendrites and a single unbranched axon from the cell body with a small number had spines on the dendrites. Pyramidal shaped neurons have a triangular cell body shape with both smooth axons and dendrites that are spineless and have three dendrites emerging from the cell body. The bipolar neurons commonly have two dendrites and like the pyramidal neurons, their axons and dendrites are spineless whereas the small, multiform neurons have either round, pyramidal or bipolar cell body shape with two or three dendrites.

6.4.2 Advantage and disadvantages of the culture system-organotypic slice culture system
This DAergic in vitro system has been employed widely over the last 5 decades and shown to be reliable and reproducible. Slice cultures are usually derived from early postnatal rodents. Early postnatal periods (p0 to p7) are ideally suited for culturing because cytoarchitecture is already established in most brain networks, the brain is larger and easier to dissect, and the nerve cells survive explantation readily. Tissue that is derived from embryonic animals survives in slice cultures (Steensen et al. 1995; Ostergaard et al. 1996; Becq et al. 1999; Berglof et al. 2008) but the organotypic organization often becomes distorted since in early development, the majority of nerve cells are still in their migratory phase from the periaqueductal area, where they originate, to the SNpc. Few attempts have been made to culture adult tissue (Sorensen et al. 1994; Wilhelmi et al. 2002). The organotypic slice culture system provides a highly accessible system for studying the development of neuronal tissues. The TH-ir cells in organotypic cultures of VM showed morphological characteristics similar to those described in intact animals (Ostergaard et al. 1990; Tepper et al. 1994), consistent with their in vivo morphology.

Variability was the main disadvantage of this culture technique. The numbers of TH-ir cells and their neuritic outgrowth varied widely between the cultures. This variability impeded quantitative analysis. However, we overcame this problem by only selecting fields containing single DA cell growth, with a clearly identified cell body and processes with a nucleus. Areas which had dense growth were avoided because of overlapping DAergic neurons originating from the VM. One explanation for the increased variability of slice cultures is that the numbers of cells present in each slice at the start of the culture...
period is unknown compared to dissociated cultures where the plating density can be calculated. Another factor is that each VM slice contains DAergic neurons from a different VM level (rostral to caudal nigral) and since the whole rostral-caudal extent of SN and ST were dissected in all experiments and then combined randomly to form co-cultures; it is possible that the specific topographical projection of DAergic neurons to the ST resulted in good growth in certain cultures and poor in others. To reduce the variability only whole VM slices were used and large numbers of cultures were prepared for each experimental condition to reduce the standard error of deviation (SD) number of TH-ir cells in each culture. Cultures which detached from the coverslips (overall < 10% of plated VM-ST cultures) and cultures that did not grow were discarded. To obtain unbiased counting, slides were coded and cells were scored blindly.

6.4.3 The influence of target region-Striatum on TH-ir neurons

As discussed in chapter 1 and 3 during development, DA neurons follow a specific temporal pattern by which their axons project from the VM and innervate the ST, a process that has been well documented both in vivo (Voorn et al. 1988; Gates et al. 2004) and in vitro in VM/ST co-cultures (Voorn et al. 1988; Schatz et al. 1999; Snyder-Keller et al. 2001; Gates et al. 2004). The number of DAergic neurons grown in SVM cultures in the absence of the ST target started low and remained low for the whole culture period compared to co-cultures with the ST. Although there were no significant differences in the morphology of the TH-ir neurons between SVM and co-cultures it was apparent that SVM cultures yielded poor TH-ir cell growth compared to co-cultures. These results are supported by previous studies in which the ST exerted a trophic influence on the
development of the DAergic neurons in dissociated cell cultures (Prochiantz et al. 1979; Prochiantz et al. 1981). Further studies also demonstrated that ST extracts exerted neurotrophic effects on mesencephalic DAergic cells (Dal Toso et al. 1988). In addition, the development of dendritic and axonal patterns of TH-ir aggregates was also influenced by the presence of ST cells (Hemmendinger et al. 1981).

6.4.4 The growth of TH-ir neurons with increasing culture duration

Significant differences between SVM and VM-ST cultures were observed regarding the numbers of TH-ir cells. There is an increase in TH-ir cell number from week two to three in the co-cultures in agreement with Ostergaard (Ostergaard et al. 1990) who found that following six days in co-culture no TH-ir neurons had innervated the ST but by day twenty five innervation had occurred in the majority of co-cultures. Following the third and fourth week, growth of TH-ir cells were at its maximum in VM-ST cultures. In contrast in the SVM cultures, the numbers of TH-ir cells remained low as they had no target tissue to innervate. Once the TH-ir cells from the VM had innervated the ST in the co-cultures, the number of TH-ir cells did not significantly increase in the remaining weeks of culturing. One possibility is that the target region could regulate this innervation by producing a limited amount of neurotrophic factor (Barde 1989) as BDNF and GDNF are produced by the ST and enhance survival of TH-ir neurons in culture (Knusel et al. 1990b; Hyman et al. 1991; Oo et al. 2003; Jaumotte and Zigmond 2005) controlling innervation by TH-ir neurons. Thus, axons which are deprived of any neurotrophic factor would die.
6.4.5 Possible influences on TH-ir cell development

The prenatal development of DAergic neurons of the SN is characterized by their birth, specification and migration to their final positions. Their postnatal development is characterized by the establishment of contact and interactions between the SN and other neural nuclei, particularly the ST target, by extension of axons, terminal differentiation, and synapse formation. In the postnatal process there is natural cell death, which is apoptotic and biphasic in time course, initially peaking on postnatal day (PND) 2, and a second on PND14 (Mahalik et al. 1994; Jackson-Lewis et al. 2000; Burke 2003; Oo et al. 2003) and by PND20 apoptotic events have largely subsided. This natural cell death event is regulated in vivo by interaction with ST target. This target dependence is present largely within the first two postnatal weeks (Burke 2003). Since our co-cultures derived from P4-5 day old rat pups the DAergic cells would have gone through the initial apoptotic phase however due to the dissection of the VM and ST regions resulting in severed afferent/efferent connections this may have augmented the natural cell death process. After the tissue slices were plated next to each other the regenerative process would have taken a while to establish and to allow the TH-ir cells to migrate and project axons innervating the ST. This may explain the low levels of TH-ir cells in the first two weeks of culture and then increased in the following third week. It is well accepted that the ST regulates DAergic cell death as suggested by in vitro (Prochiantz et al. 1979; Hemmendinger et al. 1981; Hoffmann et al. 1983; Tomozawa and Appel 1986) and in vivo studies (Coyle and Schwarcz 1976; Burke et al. 1992; Macaya et al. 1994).

6.4.6 Role of GFAP-ir cells in TH-ir neuronal development
Glial cells play an important role in CNS development (Hatten 1993). In our co-cultures the numbers of GFAP-ir cells were always less than TH-ir cell numbers. However as the TH-ir cell numbers increased so did the number of GFAP-ir cells. In contrast in the SVM cultures GFAP-ir cell numbers were significantly higher than the TH-ir cell numbers in the first week of incubation and remained similar numbers as the TH-ir cells throughout the culturing period. Studies have shown that DAergic neurons in culture may be guided by astroglial cells (Johansson and Stromberg 2002; Johansson et al. 2005). The long-term surviving outgrowth of DAergic nerve fibers were shown to be guided by astrocytes DAergic. Little is known about how cell types other than astrocytes may influence nerve fiber formation from DA neurons. It is known that conditioned medium from oligodendrocyte-type-2 astrocyte progenitors and microglia may enhance survival of cultured DAergic neurons (Nagata et al. 1993b; Nagata et al. 1993a; Takeshima et al. 1994a, b; Takeshima et al. 1994c; Zietlow et al. 1999; Sortwell et al. 2000), although the presence of both oligodendrocytes and microglia is known to exert inhibitory effects (Ling et al. 1998b; Wang et al. 2002; Park et al. 2007).

The GFAP-ir cells in SVM may provide support since no ST target tissue is available to innervate and in turn regulate development as astroglia are thought to be involved in the guidance of neurons and axonal growth cones (Jacobs and Goodman 1989; Hidalgo et al. 1995; Hidalgo and Booth 2000). It is likely that the interaction between glia and neurons are mutual, where astroglia and neurons are active team players and thus regulate mutual survival (Gates and Dunnett 2001; Chotard and Salecker 2004). It has been shown that astroglia-associated TH-positive neurite outgrowth development only innervates fetal ST,
the innervation pattern overlapping astrocytic-dense areas (Johansson and Stromberg 2003). Although it is not known how the astrocytes might attract the TH-positive axons, in situ, studies have shown that extracellular matrix molecules, which are produced by astrocytes, are concentrated in striatal patches when DA innervation occurs (O'Brien et al. 1992; Charvet et al. 1998a, b). In addition, enhanced migration of astrocytes increases formation of TH-positive nerve fibers (Johansson and Stromberg 2002). However, it cannot be determined from our study as to what factors influence the astrocytes to terminate their migration.

In vitro, DAergic neurones from the mesencephalon plated on glial monolayers either from the striatal or the mesencephalic region on mesencephalic glial cells, the DAergic neurones develop highly branched and varicose neurites, whereas on striatal glia they only exhibit one long, thin and rather linear neurite (Denis-Donini et al. 1984). This demonstrates that glial cells from two different brain regions have distinct properties. Studies have also shown that neurotrophic factors are produced by glial cells which protect DAergic neurons against oxidative stress (Hou et al. 1997). Furthermore, an astrocyte conditioned medium promotes the survival of DAergic cells (Engele et al. 1991; O'Malley et al. 1994) and protects these cells against L-DOPA toxicity (Mena et al. 1996).
CHAPTER 7:
THE EFFECTS OF NEUROTOXINS: MPTP & ROTENONE
ON TH-ir CELLS GROWTH IN ORGANOTYPIC SLICE
CULTURES
7.0 INTRODUCTION

As discussed in chapter 2 the neurotoxin MPTP has been studied extensively in both in vivo and in vitro PD models (Uversky 2004; Bove et al. 2005; Smeyne et al. 2005; Smeyne and Jackson-Lewis 2005). Most in vitro studies have employed MPP⁺ compared to rotenone with its application mainly in specific types of primary cell lines or derived from embryonic mice (Fonck and Baudry 2001; Gao et al. 2003b; Gao et al. 2003a; Hirata and Nagatsu 2005; Zeng et al. 2006). Rotenone has been shown to be more toxic to DAergic neurons (Sherer et al. 2003b; Sherer et al. 2003a) compared to MPTP. Both toxins have been reported to act through mitochondrial dysfunction causing reactive oxygen species (ROS) and subsequent cell death (Storch et al. 2000; Fonck and Baudry 2001; Richardson et al. 2007). Astrocytes have been recognized as the key player in converting MPTP to its active metabolite MPP⁺ (Brooks et al. 1989; Di Monte et al. 1991; Di Monte et al. 1992) and also contribute to ROS production (Block and Hong 2007; Block et al. 2007). Rotenone on the other hand is highly lipophilic and does not require astrocytes for its conversion into an active metabolite to be toxic.

7.1 AIMS

This study aims to characterize the effects of both MPTP and rotenone on TH-ir cell growth and survival in organotypic slice cultures derived from 4-5d postnatal rats.

7.2 METHOD

The slice co-cultures were prepared according to the method described in Chapter 5.
### 7.2.1.1 MPTP Effects on 7 Day old VM & ST co-cultures

Co-cultures of ventral mesencephalon and striatum were prepared from P4-5 Sprague Dawley rats, cytostatics were added on day 4 post co-culture and media changed twice a week. Co-cultures were then divided into groups which were incubated in different toxin doses (see Table 7.1). 24 hours post treatment with MPTP the media was changed with fresh medium, and thereafter changed twice a week. The following 1, 2, and 3 weeks post-treatment, a minimum of 3 tubes per group were immunocytochemically stained for tyrosine hydroxylase (TH-ir). Thereafter, the number of TH-ir cells were counted in a blinded fashion and analysed as previously described (chapter 5).

#### Table 7.1

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<tr>
<th>GROUPS</th>
<th>TREATMENTS</th>
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<td>1</td>
<td>Naïve controls (normal media)</td>
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7.2.1.2 Rotenone Effects on 7 Day old VM & ST co-cultures

Co-cultures were divided into the following groups which were incubated in different doses (see Table 7.2). 24 hours post treatment with rotenone the media was changed with fresh medium, and thereafter changed twice a week. The following 1, 2, and 3 weeks post-treatment, a minimum of 3 tubes per group were immunocytochemically stained for tyrosine hydroxylase (TH-ir). Thereafter, the number of TH-ir cells were counted in a blinded fashion and analysed as previously described (chapter 5).

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<th>GROUPS</th>
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<td>1</td>
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<td>Vehicle controls (DMSO + media)</td>
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7.2.2.1 MPTP Effects on 14 Day old VM & ST co-cultures

Co-cultures of ventral mesencephalon and striatum were prepared from P4-5 Sprague Dawley rats, cytostatics were added on day 4 post co-culture and media changed twice a week. Co-cultures were then divided into groups which were incubated in different doses (see Table 7.3). 24 hours post treatment with MPTP the media was changed with fresh medium, and thereafter changed twice a week. The following 1, 2, and 3 weeks post-treatment, a minimum of 3 tubes per group were immunocytochemically stained for tyrosine hydroxylase (TH-ir). Thereafter, the number of TH-ir cells were counted in a blinded fashion and analysed as before.

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<td>Vehicle controls (PBS + media)</td>
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7.2.2.2 Rotenone Effects on 14 Day old VM & ST co-cultures

Co-cultures were divided into the following groups which were incubated in different doses (see Table 7.4). 24 hours post treatment with rotenone the media was changed with fresh medium, and thereafter changed twice a week. The following 1, 2, and 3 weeks post-treatment, a minimum of 3 tubes per group were immunocytochemically stained for tyrosine hydroxylase (TH-ir). Thereafter, the number of TH-ir cells were counted in a blinded fashion and analysed as before.

Table 7.4

14 day old co-cultures treated with rotenone

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7.2.2.3 Effects of MPP⁺ on 14 Day old VM & ST co-cultures

Co-cultures were divided into 2 groups which were incubated in different doses and neurotoxin (see Table 7.5). 24 hours post treatment with MPTP or MPP⁺ the media was changed with fresh medium, and thereafter changed twice a week. The following first week of post-treatment, a minimum of 3 tubes per group were immunocytochemically stained for tyrosine hydroxylase (TH-ir). Thereafter, the number of TH-ir cells were counted in a blinded fashion and analysed as before.

<table>
<thead>
<tr>
<th>MPTP</th>
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<td>Control</td>
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7.2.4 Cytotoxicity of Neurotoxins

Lactate dehydrogenase (LDH) is released from dead or degenerating cells with permeabilized membranes into the culture medium, the medium was analysed at 7 days post toxin treatment by a fully automatic spectrophotometer (Ascent Multiwell plate reader). The medium was collected for LDH determination and frozen immediately with
storage at −20°C until analysed. Two independent series of LDH measurements were performed.

7.2.5 Neuronal Degeneration: Fluoro-Jade C (FJC)

Neuronal cell death was assessed using the neuron-specific cell death marker FJC using a standard protocol (Schmued et al. 2005), with modification. (1) cultures were transferred to 0.06% postassium permanganate solution for 10 min, then rinsed in DH2O for 2 min; (2) incubation with 0.0001% solution of FJC (Chemicon, Tememcula, CA) for 20 min; (3) FJC was immediately before use bt diluting a stock solution of 0.01% FJC by 100-fold in 0.1% acetic acid; (4) followed by washes x3 time for 1 min in DH2O, dried in a 45°C oven, cleared in histolene and coverslipped using DPX; (5) cultures were examined using epifluorescence microscope with an excitation wavelength of 488 nm.

7.2.6 Apoptotic Cell Death: Caspase-3

Cultures were treated as previously described in chapter 5, incubated overnight with TH and active caspase-3 at 4°C, following day incubation with appropriate biotinylated secondary antibody for 30 min, followed by streptavidin-conjugated AlexaFluor 488, 546 or 660.
Figure 7.1 Schematical diagram of the experiments.

A) 7 day old co-cultures were treated with MPTP or rotenone for 24 hours then fixed at 1, 2 & 3 weeks post treatment.  B) 14 day old co-cultures were treated with MPTP or rotenone.  * = Normal media change x2/wk.
7.3 RESULTS

7.3.1 Neurotoxin treatment of VM and ST co-cultures

All experiments included both a naïve control and vehicle control and were compared at the same time as toxin treated co-cultures. All naïve and vehicle controls had TH-ir cell numbers that were similar to each other and correlated to their respective weeks in culture. There was no significant difference between controls and vehicles thus, suggesting neither had any effect on TH-ir cell growth during the treatments. Both MPTP and rotenone induced dose-dependent cell death and 14 day old co-cultures were more vulnerable than 7 day co-cultures.

7.3.2 Dose-dependent death of TH-ir cells-MPTP

MPTP treatment of 7 day co-cultures induced a reduction in TH-ir cell numbers and neuritic outgrowth. The greatest reductions were seen at the highest dose (100 μM) (Figure 7.2). All MPTP concentrations induced a significant reduction in numbers of TH-ir cells compared to naïve and vehicle controls. One week post treatment at lower doses (1 μM) induced a 13 % significant decrease in TH-ir cell number compared to naïve controls with further significant decreases at highest dose (100 μM) of 35 % compared to naïve controls. At two weeks post treatment (Figure 7.3.2) the dose-response was similar to one week post treatment. TH-ir cell numbers were lower than one week post at 1 μM (11 %) but the reduction continued to increase at the highest dose (100 μM) 47 % compared to naïve controls. The reduction in TH-ir cells continued steeply during third week of post treatment (Figure 7.3.3) as 1 μM caused a significant reduction of 32 % and 61 % at 100 μM compared to naïve controls.
Figure 7.2. MPTP treated co-cultures at 7 days.

(A), control; (B), 5 μM; (C), 10 μM; (D), 50 μM; (E), 100 μM, all 1 week post. *

Degenerating cells. Bar = 10 μm.
Figure 7.3.1 Dopaminergic (TH-ir) neuronal growth following 1 week post MPTP treatment of 7 day co-cultures.

The number of cells per field (0.2290mm$^2$) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.3.2 Dopaminergic (TH-ir) neuronal growth following 2 week post MPTP treatment of 7 day co-cultures.

The number of cells per field (0.2290 mm$^2$) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV, * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.3.3 Dopaminergic (TH-ir) neuronal growth following 3 week post MPTP treatment of 7 day co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.4. MPTP treated co-cultures at 14 days.

(A), control; (B), 5 μM; (C), 10 μM; (D), 50 μM; (E), 100 μM, all 3 week post. Bar = 10 μm.
Figure 7.5.1 Dopaminergic (TH-ir) neuronal growth following 1 week post MPTP treatment of 14 day co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.5.2 Dopaminergic (TH-ir) neuronal growth following 2 week post MPTP treatment of 14 day co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV, * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.5.3 Dopaminergic (TH-ir) neuronal growth following 3 week post MPTP treatment of 14 day co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
The number of cells per field (0.2290mm$^2$) viewed at 400x following different doses and treatments. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.6. 7 day & 14 day old co-cultures MPTP treated. The number of cells per field (0.2290mm²) viewed at 400x following different doses and at all weeks of recovery. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV, *** p<0.001 7 days compared to their respective 14 day groups. (A), 1 week post; (B), 2 week post; (C), 3 week post.
7.3.3 MPTP toxicity varies with the time of co-culture exposure

14 day old co-cultures treated at lower doses were more affected than 7 day old treated co-cultures. TH-ir cells showed less intense immunostaining as well as loss of neural processes (Figure 7.4). MPTP treated 14 day old co-cultures produced a more consistent dose-response cell count than 7 day old co-cultures. One week post treatment at 5 μM induced a significant reduction in TH-ir cell numbers (38%) whereas 7 day old co-cultures treated at the same dose reduced TH-ir cells by 22 % (Figure 7.5.1). At the 100 μM a significant decrease of 72 % was observed in 14 day old co-cultures. Reductions in TH-ir cell number during second week of post treatment at low doses (5 μM) 46 % (7 day cultures at same dose and week; 12.5 %) continued to increase but at higher doses (100 μM) were slowed down to 64 % compared to naïve controls (Figure 7.5.2). By the third week reduction in TH-ir cell numbers at low doses (5 μM) were also slowing down to 41% (7 day old cultures treated at same dose and week; 32 %). However the higher doses of 100 μM continued to cause significant TH-ir cell reductions (79 %) compared to naïve controls (Figure 7.5.3). To compare whether MPTP or its by product MPP⁺ was more toxic we treated 14 day old co-cultures with varying concentrations and analysed TH-ir cells at one week post treatment. There was no significant difference in toxicity between the two toxins but a dose-response was observed for both (Figure 7.5.4).

We compared dose effect at each point for 7 and 14 day old co-cultures (Figure 7.6). 7 day co-cultures was observed to be more resistant to MPTP compared to 14 day co-cultures. The number of TH-ir cells was significantly reduced at all concentrations compared to naïve controls but recovered much faster in all three weeks of post treatment
in 7 day old co-cultures. In contrast in 14 day co-cultures even at the lower doses MPTP induced significant reductions in TH-ir cell numbers and continued to decrease at all weeks post treatment compared to naïve controls.

**7.3.4 Dose-dependent death of TH-ir cells-Rotenone**

Rotenone co-cultures treated at 7 days old induced reductions in TH-ir cell numbers and a few projections compared to naïve controls (Figure 7.7). The intensity of TH-immunostaining was clearly less intense compared to naïve controls. A dose-response was observed with rotenone treated co-cultures. 7 day old co-cultures treated with rotenone at the lowest dose (1 nM) induced a 3 % reduction in TH-ir cell numbers in the first week of post treatment compared to naïve controls (Figure 7.8.1). Significant TH-ir cell reduction was observed at the higher doses 100 nM (67 %) compared to naïve controls. TH-ir cell reductions continued to increase significantly at 1 nM (38 %) and at 100 nM (89 %) during the second week compared to naïve control (Figure 7.8.2). Third week of recovery TH-ir cell numbers were reduced to 31 % (1 nM) and 51 % (100 nM) compared naïve controls (Figure 7.8.3).

14 day old co-cultures treated with rotenone induced swelling of TH-ir cell bodies and reduction in TH-ir neurites (Figure 7.9). The dose response was similar to MPTP treated co-cultures. First week of recovery resulted in a significant reduction in TH-ir cell numbers by 31 % at 5 nM whereas at 100 nM induced a greater reduction by 93 % compared to naïve controls (Figure 7.10.1). This percentage is much larger than 7 day old co-cultures where 5 nM resulted in 3.5 % and 100 n M was 67 % in the first week.
Second week of recovery TH-ir cell number reduction continued to increase to 53 % at 5 nM and 92 % at 100 nM compared to naïve controls (Figure 7.10.2). Again these numbers were much higher compared to 7 day old co-cultures (5 nM = 43 % & 100 nM = 67 %). Finally third week of recovery TH-ir cell number decreased to to 26 % at 5 nM and 86 % at 100 nM compared naïve controls (Figure 7.10.3). Compared to 7 day old co-cultures treated at 5 nM which resulted in 44 % TH-ir cell number reduction this percentage were much higher than 14 day old co-cultures.

7.3.5 Rotenone toxicity varies with the time of co-culture exposure

Similar to MPTP treatment, rotenone was observed to be more toxic to 14 day co-cultures than 7 day co-cultures (Figure 7.11). There was less variability between the treatment groups and a more consistent dose-response effect in the weeks post treatment. Lower doses of rotenone treated in 7 day co-cultures resulted in low TH-ir cell reductions but progressively increased in the weeks of recovery whereas the higher doses varied between 67 % in the first week, 89 % in the second and 51 % in the last week. 14 day old co-cultures on the other hand when treated with low doses of rotenone a 31 % reduction in TH-ir cell numbers in the first week was observed which continued to increase to 53 % in second week and reduce to 26 % in the final week. At the highest concentration of 100 nM 14 day treated co-cultures induced a substantial reduction in TH-ir cell numbers by 93 % in the first week and were stable in the second week (92 %) and declined slightly to 86 % in the third week. Thus, 14 day co-cultures were observed to be more prone to rotenone at all doses whereas 7 day old co-cultures were more vulnerable at the higher doses as indicated in weeks of post treatment.
Figure 7.7. Rotenone treated co-cultures at 7 days.

(A), control; (B), 5 nM; (C), 10 nM; (D), 50 nM; (E), 100 nM, all 1 week post treatment.

* Degenerating neuron. Bar = 10 μm.
Figure 7.8.1 Dopaminergic (TH-ir) neuronal growth following 1 week post rotenone treatment of 7 day co-cultures.

The number of cells per field (0.2290mm$^2$) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.8.2 Dopaminergic (TH-ir) neuronal growth following 2 week post rotenone treatment of 7 day co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x following different doses and 2 weeks of recovery. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV, * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.8.3 Dopaminergic (TH-ir) neuronal growth following 3 week post rotenone treatment of 7 day co-cultures.

The number of cells per field (0.2290 mm$^2$) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.9. Rotenone treated co-cultures at 14 days.

(A) control; (B), 5 nM; (C), 10 nM; (D), 50 nM; (E), 100 nM, all 1 week post. Bar = 10 μm
Figure 7.10.1 Dopaminergic (TH-ir) neuronal growth following 1 week post rotenone treatment of 14 day co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.10.2 Dopaminergic (TH-ir) neuronal growth following 2 week post rotenone treatment of 14 day co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.10.3 Dopaminergic (TH-ir) neuronal growth following 3 week post rotenone treatment of 14 day co-cultures.

The number of cells per field (0.2290mm²) viewed at 400x following different doses. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV, * p<0.05, ** p<0.01, *** p<0.001 compared to controls.
Figure 7.11. 7 day & 14 day old co-cultures rotenone treated.

The number of cells per field (0.2290mm$^2$) viewed at 400x following different doses and at all weeks of recovery. The Means are calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV. ** p<0.01, *** p<0.001 7 days compared to their respective 14 day groups. (A), 1 week post; (B), 2 week post; (C), 3 week post.
7.3.6 TH-IR CELL DEATH BY MPTP AND ROTENONE.

7.3.6.1 Cytotoxicity of MPTP and rotenone by LDH assay.

To evaluate the degree of cell death, we used an LDH cytotoxicity assay, apoptotic marker caspase-3 and neuronal degenerating marker fluoro Jade-C (FJ-C) which are different methods. The LDH cytotoxicity assay detects LDH released from the cytosol of dead cells and indicates destruction of the cell membrane (Korzeniewski and Callewaert, [1983]; Decker and Lohmann-Matthes, [1988]). Exposure of co-cultures to 100 nM of rotenone increased LDH release from the cytosol by 58% compared to controls. This was greater than at the lower toxic doses 31% (5 nM) and 42% (10 nM). MPTP on the other hand resulted in 52% release of LDH compared to controls at 100 μM. This was lower than with rotenone but greater when compared to relative lower doses (34% at 5 μM, 42% at 10 μM) (Figure 7.12).

7.3.6.2 Fluoro Jade C (FJ-C)

The Fluoro Jade C antibody was used to detect degenerating neurons following MPTP and rotenone treatment. Both high and low doses of MPTP and rotenone induced degeneration of neurons despite their intact processes and cell bodies compared to control (Figure 7.13A). The loss of cell body integrity and processes however, were clearly marked by FJ-C. The toxicity of rotenone and MPTP can be shown by the loss of cell body and processes (Figure 7.13 B, C, D, E).
Figure 7.12. Lactate Dehydrogenase Cytotoxicity Assay (LDH) of MPTP & Rotenone treated co-cultures.

The lactate dehydrogenase (LDH) cytotoxicity assay was used to detect LDH in the culture medium after its release from the cytosol of dead cells. 14 day cultures were treated with either rotenone or MPTP at 5, 10 and 100 (nM/μM) for 24 hrs then allowed to recover for 7 days in normal culture medium. On 7th day medium was collected and snap frozen in – 80 °C and thawed when analysis was carried out. Data is represented as the mean (absorbance) calculated from raw data and expressed as mean ± SEM followed by Two-way ANOVA which was calculated using STDEV, # p<0.01 BSA vs. control; *** p<0.001 BSA vs. all doses both MPTP & rotenone; control vs all doses both MPTP & rotenone; *** p<0.001 5 nM vs 100 nM; * p<0.05 5 μM vs. 100 μM. n = 5 per group.
7.3.6.3 MPTP and rotenone induced Caspase-3 activation

Treatment of co-cultures with MPTP and rotenone at high doses (100 μM; 100 nM, respectively) induced caspase-3 activation at one week of recovery. However only a small number were TH+ve compared to controls (Figure 7.14, 7.15, 7.16). Caspase-3 was more prominent at the lower doses of MPTP and rotenone (5 μM; 5 nM, respectively) compared to controls (Figure 7.17, 7.18).
Figure 7.13. MPTP & rotenone treated co-cultures 1 week post treatment-
Degenerating neurons-FJC.

(A) No degenerating neurons were seen in control co-cultures; 3 weeks. (B) MPTP: 100 μM; (C) 10 μM; (D) rotenone: 100 nM; (E) 10 nM. Bar = 10 μm.
Figure 7.14. Co-localisation of TH-ir and caspase-3 in co-cultures.

Control 3 weeks, no neurotoxin treatment (A) TH-ir (Red); (B) caspase-3 (green); (C) merge; (x 400). Bar = 10 μm.
Figure 7.15. Co-localisation of TH-ir and caspase-3 in co-cultures following MPTP treatment at one week recovery period.

MPTP treatment at 100 μM (A) TH-ir (Red); (B) caspase-3 (green); (C) merge; (x 200).
Bar = 10 μm.
Figure 7.16. Co-localisation of TH-ir and caspase-3 in co-cultures following rotenone treatment at one week recovery period.

Rotenone treatment at 100 nM (A) TH-ir (Red); (B) caspase-3 (green); (C) merge; (x 200). Bar = 10 μm.
Figure 7.17. Co-localisation of TH-ir and caspase-3 in co-cultures following MPTP at one week recovery period.

MPTP treatment at 10 μM (A) TH-ir (Red); (B) caspase-3 (green); (C) merge; (x 200). Bar = 10 μm.
Figure 7.18. Co-localisation of TH-ir and caspase-3 in co-cultures following rotenone treatment at one week recovery period.

Rotenone treatment at 10 nM (A) TH-ir (Red); (B) caspase-3 (green); (C) merge; (x 200). Bar = 10 μm.
7.4 DISCUSSION

7.4.1 Dose and time dependent toxicity of neurotoxins

A dose and time dependent response was observed when the co-cultures were treated with either neurotoxin. 7 day old co-cultures treated with MPTP were not affected as much at the lower doses as compared with the higher doses, post treatment, in a dose dependent manner. However 14 day old co-cultures demonstrated more vulnerability at both low and high doses, as shown by the significant reductions in TH-ir cells at all weeks post treatment. Our results confirm multiple other reports indicating a dose-dependent toxicity by either MPTP (Chang and Ramirez 1986; Notter et al. 1988; Christie-Pope et al. 1989; Michel and Agid 1992; Koutsilieri et al. 1993; Schmidt et al. 1997; Lannuzel et al. 2003; Madsen et al. 2003) and rotenone (Gao et al. 2002; Ahmadi et al. 2003; Xu et al. 2003; Gille et al. 2004; Kress and Reynolds 2005; Testa et al. 2005; Radad et al. 2006; Klintworth et al. 2007; Ren and Feng 2007) in different animal models and organotypic cell cultures.

As shown in the 7 and 14 day co-cultures, toxin application at a critical time (or age of cells) has different effects on the TH-ir cells in the following recovery weeks. This was an unexpected finding. Previous studies applied toxins at different doses for a specific time and analysed cell response within a week. This study has demonstrated that acute exposure to the complex I inhibitors MPTP or rotenone that cell death and/or neurodegeneration may occur within hours, days or weeks post toxin application. Therefore, DAergic survival after neurotoxin treatment is dependent on the time and dose at which the toxin is applied and when the cells are analysed. Studies have suggested that
rotenone causes cell death of TH-ir cells at a very high concentration of rotenone (2μM vs. 100nM rotenone) or where cells have been treated for longer periods of time (48 vs. 24 h) (Hartley et al. 1994; Bal-Price and Brown 2000; Klintworth et al. 2007). Cultures treated with rotenone at a low dose of 20 nM for 24 hrs resulted in a 50 % reduction of TH-ir cells (Radad et al. 2006).

*In vivo* studies have shown that MPTP induces neuronal destruction in the SNpc and the VTA with the active phase of degeneration beginning 12 h post-injection and lasting up to 4 days. During this period, there was a greater decrease in TH-ir neurons than in Nissl-stained neurons suggesting that MPTP can cause a functional synthetic loss in TH, without necessarily destroying the neuron (Jackson-Lewis et al. 1995). *In vitro*, MPP⁺ treatment (30 μM MPP⁺ for 3, 6 or 24 hrs) can induce cell death, with increased caspase-3 in western blot analysis and the DEVDase activity assay suggesting apoptotic death (Chu et al. 2005). Whereas MPP⁺ has been shown to elicit a minimal amount of caspase-3 activation (Hartmann et al. 2000) which was detectable only at the 24-h time point by the DEVDase activity assays (2-fold increase vs. control). This may explain that even at the lower doses of MPTP and rotenone, TH-ir cell reduction continued in the weeks post treatment (Hartley et al. 1994). Apart from the effects observed in these cells there are also functional changes. Following exposure of cells to 1 μM MPP⁺ 3H-DA uptake was decreased to 38% compared to controls within the first two hours of incubation and to 8% after 48 hours (Koutsilieri et al. 1993). Loss of TH-ir cells became evident at 0.1 μM MPP⁺ (80% of control) leading to maximal toxicity at 10 μM (20% of control).
7.4.2 Toxicity of neurotoxins on Dopaminergic co-cultures varies with the age of co-cultures

Although there was a significant dose dependent TH-ir cell loss following MPTP and rotenone treatment in both 7 and 14 day co-cultures, the effects were most potent in 14 day co-cultures especially at the higher doses. It was unexpected that the TH-ir cells were still affected after a single dose exposure and three weeks of recovery. The vulnerability of 14 day compared to 7 day co-cultures to both toxins was confirmed by cell counts and changes in the intensity of TH-ir and cytologic alterations. A possible reason for this is that neurotoxins may have a greater effect on the cells that have already migrated away from the thick tissue. By the second week in vitro there are more single TH-ir cells to be exposed to the neurotoxins. This method while retaining organotypic connections allows examination of a thinned out region of tissue where individual DA cells can be morphologically examined and counted free of influence of overlying DAergic cells in the thicker regions of tissue. Short exposure to both neurotoxins at low and high doses were more deleterious in 14 day than 7 day co-cultures as indicated by minimal TH-ir cell recovery at all time periods of post treatment. 7 day co-cultures were able to maintain a greater number of TH-ir cells during the weeks of post treatment especially after the lower doses. The mesencephalic DAergic cells are more vulnerable to MPTP or rotenone mitochondrial insult and in addition rotenone is a more potent toxin compared to MPTP or MPP+ in this DAergic cell model.

The vulnerability of 14 day to 7 day co-cultures is at variance with previous studies. Jakobsen et al., (Jakobsen et al. 2005) observed that one week old cultures derived from
embryonic day 12 mice were more vulnerable to MPP⁺ than three week old cultures. Others have demonstrated that immature or newly plated neurons were more sensitive to MPP⁺ and as lengths of time in culture increased the neurotoxin effects were diminished (Dania et al. 1989; Golde et al. 2002; Jakobsen et al. 2005). These studies however employed embryonic dissociated cultures of the mesencephalon and compared one week cultures with 4 week cultures. Thus, different experimental paradigms were used and cannot be compared directly with this current study. In our study 14 day co-cultures were more vulnerable and showed significant reduction in TH-ir cell numbers compared to 7 day old cultures. Thus, it would seem that the age of the DAergic neurons in organotypic cultures is an important factor in toxin-induced degeneration. *In vivo* studies have supported our finding where a low systemic dose of rotenone had no effect on young rats, but led to a 20–30% reduction of TH-ir neurons in the SN of older rats (Phinney et al. 2006).

The establishment of axonal projections and innervation of ST by TH-ir cells of VM plays an important role in the vulnerability to these neurotoxins. Some studies have implicated an excitotoxic component to the injury induced by DAergic neurotoxins in animal models because drugs such as the N-methyl-d-aspartate (NMDA) receptor antagonist MK801 can protect against the loss of DAergic neurons caused by MPTP (Turski et al. 1991). In addition, DAergic toxicity after chronic application of MPP⁺ or rotenone does not occur unless an excitotoxic stimulus such as NMDA is co-applied (Kress and Reynolds 2005). NMDA receptor blockade with MK801 lead to a rescue of 60-70% of mature neurons. The expression of NMDA receptors increases with
maturation in culture (Phinney et al. 2006). This could explain why 7 day co-cultures were less vulnerable than 14 day co-cultures as TH-ir cell innervation of ST would be greater in 14 day co-cultures. Interestingly Maeda et al., (Maeda et al. 1998) observed that VM and ST slices co-cultured at a wide distance from each other neither resulted in failure of outgrowth of DAergic fibres to the ST and no cytotoxic response to NMDA. VM slices co-cultured at a close distance to the ST were affected even at the lowest concentration of neurotoxins. Thiruchelvam (Thiruchelvam et al. 2003) and Li (Li et al. 2005) also report application of neurotoxins to be age-dependent as catecholamine synthesis and uptake in immature cells renders these vulnerable to oxidative stress. In addition, DAergic neurons innervate and develop synaptic connections in the ST over the course of 2–3 weeks in vitro (Plenz and Kitai 1998) and as glutamatergic connections increase so do their vulnerability to excitotoxic stress.

### 7.4.3 Glial cells play an important role in DAergic degeneration

The role of nigral glial cells is important in PD and in this model of DA cell growth. As previously discussed in chapter 2, MPTP is only toxic when it is converted to its metabolite MPP⁺ and transported into the cell via the DAT (Chiba et al. 1985; Gainetdinov et al. 1997; Miller et al. 1999) and concentrated in the mitochondria to inhibit complex I (Ramsay et al. 1991b; Ramsay et al. 1991a; Russ et al. 1996). MPTP is taken up by glial cells where inside monoamine oxidases convert it to the toxic metabolite. Administration of MPTP to mice lacking MAO-B shows that SNpc neurons do not die (Shih and Chen 1999) further demonstrating that MAO conversion of MPTP to MPP⁺ is necessary to cause cell death. Several articles suggest that glial cells can contribute
directly to the toxic effects of MPTP, primarily through the action of free radical mediators such as inducible nitrous oxide synthase (iNOS) (McGeer et al. 1988; Hirsch et al. 1998; McNaught and Jenner 1999). MPTP administration leads to a rapid gliosis (Schneider and Denaro 1988), followed by an increase in the production of iNOS (Zietlow et al. 1999). iNOS has been shown to interact with free radicals to produce peroxynitrite, which can interact with tyrosine residues in cellular proteins and inhibit neuronal signal transduction (Ferrante et al. 1999; Kuhn et al. 1999; Kuhn and Geddes 1999). In a model of iNOS action that extends the role of glia, Hirsch and Hunot (Hirsch and Hunot 2000) suggest that MPTP acts directly to induce cytokines which in turn activate iNOS. The iNOS is then released from the glial cells to act upon DAergic neurons, inducing damage within these cells. Although differences in iNOS levels have not been examined in different strains of mice, it is possible that this pathway could underlie some of the differences in toxicity that occur after administration of MPTP or MPP⁺ (Smeyne et al. 2001).

The organotypic cell culture system differs from primary cell lines and dissociated cultures in that it does not just contain a single pure population of a specific cell type but maintains the different cell types and original morphology and characteristics of the tissue of interest. Neurons in slice culture are still associated with glial cells and more likely to express their in vivo morphology and characteristics than dissociated neuronal cultures although reaggregated (Hemmendinger et al. 1981) and plated (Berger et al. 1982) cultures have produced DAergic neurons retaining their ability to interact and form morphologically characteristic pattern of fibres. MPTP was applied in our studies to slice
cultures containing glia. MAO-A and MAO-B are the major enzymes that catalyze the oxidative deamination of monoamine neurotransmitters such as DA, noradrenaline, and serotonin in the central and peripheral nervous systems. MAO B is mainly localized in glial cells which convert MPTP to MPP⁺ (Bove et al. 2005; Nagatsu and Sawada 2006). MAO-B inhibitors such as L-deprenyl (selegiline) and rasagiline have been shown to be effective in the treatment of PD (Nicotra and Parvez 2000; Nagatsu and Sawada 2006), preventing both PD behavioural manifestations and cell loss in the SN of monkey (Heikkila et al. 1984a; Langston and Ballard 1984) and reduction in DA levels in rodents (Heikkila et al. 1984b). Our results have shown that MPTP induced TH-ir cell loss in a dose-dependent manner and maintained significant cell loss in weeks of post treatment. It would be of interest to compare the actions between MPP⁺ and MPTP as other studies have only used slice cultures with embryonic rat midbrain and MPP⁺ and/or excitotoxic toxins (Ito et al. 1999; Katsuki et al. 1999).

Rotenone is highly lipophilic and freely crosses brain cellular membranes to accumulate in subcellular organelles including the mitochondria. In mitochondria, rotenone impairs oxidative phosphorylation by inhibiting reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone reductase activity through its binding to the subunit of the multipolypeptide enzyme complex I of the electron transport chain (Syed and Winlow 1991; Schuler and Casida 2001a, b). The mechanism of cell death may also occur from withdrawal of trophic support from glia and microglia. Little neurotoxicity was detected in the mesencephalic neuron-enriched cultures after treatment for 8 day with up to 20 nm rotenone compared to significant and selective DAergic neurodegeneration in co-cultures.
with glia two days after treatment with 20 nm rotenone or 8 days after treatment with 1 nm rotenone (Gao et al. 2002). This is a result of glial presence especially microglia as it markedly increases neuronal susceptibility to rotenone. Rotenone stimulated the release of superoxide from microglia that was attenuated by inhibitors of NADPH oxidase. Furthermore, inhibition of NADPH oxidase or scavenging of superoxide significantly reduces the rotenone-induced neurotoxicity (Gao et al. 2002).

In vivo studies further support the notion that glial cell activation contributes to toxicity as rotenone administration in young rats has no effect but produces a 20-30% reduction in TH-ir cells in the SN of older rats (Pasinetti et al. 1999). GFAP-ir is an astrocytic marker which has been observed to be less intense in young rats when compared to aged rats (Pasinetti et al. 1999). It is recognised that there is an increase in the basal level of glial cell activation with aging (Linnemann and Skarsfelt 1994; Rozovsky et al. 1998; Gao et al. 2003b) and studies have also shown involvement of microglia and increased inflammatory markers following rotenone toxicity (Hogue 1953; Gao et al. 2002; Gao et al. 2003a). Several identified growth factors for DA neurons are present in diminished quantity or absent in the adult and aging organism (Wilcox and Derynck 1988; Schaar et al. 1993; Seroogy and Gall 1993; Stromberg et al. 1993; Choi-Lundberg and Bohn 1995; Kaseloo et al. 1996; Nosrat et al. 1996; Kornblum et al. 1997; Widenfalk et al. 1997) these include BDNF, NGF, NT-3, CNTF and GDNF which could contribute to the diminished neurite-promoting activity and increased sensitivity to neurotoxins.

7.4.3 Rotenone vs MPTP neurotoxicity
Results from our current study show that rotenone is more toxic than MPTP. There is a large unit difference (nM to μM) between the toxins and the 14 day old co-cultures treated with rotenone at the higher doses (50 and 100 nM) induced a marked TH-ir cell reduction at all three weeks of recovery compared to MPTP. In addition, the neurotoxic effects of MPTP were variable. The observation that complex I was uniformly inhibited throughout the brain (and the rest of the body) but that only the SN neurons degenerated (Betarbet et al. 2000; Hoglinger et al. 2003b; Sherer et al. 2003a) indicates that the DAergic neurons of the SN have a higher sensitivity to rotenone, presumably because of the interplay between rotenone-induced defects in complex I and the oxidation of DA. Rotenone is a high affinity inhibitor of complex I, and interacts specifically with the ND1 and PSST subunits of complex I (Nicolaou et al. 2000; Schuler and Casida 2001a, b) compared to MPP⁺ which has a low affinity for complex I and typically produces 104- to 106-fold less complex I inhibition than rotenone (Mizuno et al. 1987b; Mizuno et al. 1987a; Hasegawa et al. 1990; Ramsay et al. 1991b; Ramsay et al. 1991a; Hasegawa et al. 1995).

7.4.4 MPTP and rotenone activate caspase-3

Both MPP⁺ and rotenone have been shown to activate caspase-3 in DAergic cells (Ahmadi et al. 2003; Kaul et al. 2003; Yang et al. 2004a; Ramachandiran et al. 2007). Co-cultures treated with high doses of MPTP and rotenone (100 μM; 100 nM) did not induce high numbers of caspase-3 co-localised with TH-ir following one week of recovery. However, at the low doses of both neurotoxins (5 and 10 μM; nM) there were a greater number of apoptotic cells compared to controls. Other studies have shown
otherwise (Abbott et al. 2003; Chee et al. 2005). An explanation for this is that different apoptotic pathways may be activated. As there are more than 10 caspases that have been identified, caspase-3 is believed to be the final executor of apoptotic DNA damage and activated caspase-3 has been detected (Cohen 1997; Naoi et al. 2000). Therefore, severity of insult and duration of MPP⁺ treatment may be responsible for the inconsistent results. In addition, the inhibition of caspase in primary cultures of DAergic neurons treated with MPP⁺ can trigger a switch from apoptosis to necrosis (Hartmann et al. 2001). MPP⁺ and rotenone may have the potential to induce caspase-dependent and -independent cell death, and the final outcome may be determined by several extrinsic factors placed on cells in defined situations (Han et al. 2003). Despite this, caspase-3 inhibitors such as DEVD has been successful in preventing apoptosis occurring in DAergic neurons treated with either rotenone or MPP⁺ (Dodel et al. 1998; Viswanath et al. 2001; Bilsland et al. 2002; Ahmadi et al. 2003).
CHAPTER 8:
THE NEUROPROTECTIVE EFFECT OF GLIAL CELL-LINE DERIVED NEUROTROPHIC FACTOR (GDNF) ON TH-ir CELLS IN CO-CULTURES
8.0 INTRODUCTION

The first trophic factor for DAergic neurons was termed glial cell line-derived neurotrophic factor (GDNF) (Lin et al. 1993). Subsequently, it was shown as a survival factor in dissociated cultures of DA neurons, and promoted the survival and outgrowth of DAergic neurons (Engele et al. 1991). GDNF protected DAergic neurons against the toxic effects of 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Lapchak et al. 1997c; Lapchak et al. 1997b; Lapchak et al. 1997a; Bohn et al. 2000). These observations have raised the possibility that GDNF can serve as a therapeutic agent in PD, a condition associated with the premature loss of DAergic neurons (Olanow and Tatton 1999; Airaksinen and Saarma 2002) as well as in other neurological diseases, including amyotrophic lateral sclerosis (Manabe et al. 2002; Manabe et al. 2003), and aging (Ai et al. 2003).

Mesencephalic DAergic neuronal development has been suggested to be dependent on GDNF (Krieglstein et al. 1995a; Krieglstein 2004) thus we employed organotypic cultures which develop nigrostriatal connections to examine the role of GDNF in protecting and/restoring DAergic neurons from toxic stress. Current DAergic therapies for PD specifically target the symptoms of PD and are not known to alter disease progression. In addition, the effectiveness of these treatments wanes with time, and patients develop significant side effects including dyskinesias that can become disabling. For these reasons, the development of neuroprotective or neuroregenerative therapies for PD would represent a tremendous advance in our ability to treat the disease. Collectively, in vitro and in vivo data, in both the rodent and nonhuman primate, provide
strong support for a role for GDNF in treating PD, showing considerable promise for continued investigation as a drug candidate for the treatment of PD. Not much work has been performed using GDNF and neurotoxins-MPTP and rotenone in organotypical cell culture derived from postnatal rats, thus we aim to further characterise this system.

8.1 AIMS
Assess:
i) Dose-response of GDNF on TH-ir cells.

ii) Neuroregenerative properties of GDNF post toxin exposure on TH-ir cells.

iii) Neuroprotective effects GDNF pre-toxin exposure on TH-ir cells.

8.2 METHOD
The slice co-cultures were prepared according to the method described in Chapter 4.

8.2.1 Dose-response of GDNF on TH-ir cells
Co-cultures of ventral mesencephalon (VM) and striatum (ST) were prepared from P4-5 Sprague-Dawley rats. Co-cultures received cytostatics between 4-5 days in culture for 24 hrs then fresh media was added and changed twice a week. The co-cultures were then divided into four groups (see Table 8.1).

24 hrs after cytostatic exposure co-cultures received fresh media change, co-cultures that were treated with GDNF (Promega; human glial cell-line derived neurotrophic factor;
G2781-5 mg) were added in the media according to their assigned concentrations at every media change.

**Table 8.1**

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TREATMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (normal medium)</td>
</tr>
<tr>
<td>2</td>
<td>10ng/mL GDNF + medium</td>
</tr>
<tr>
<td>3</td>
<td>20ng/mL GDNF + medium</td>
</tr>
<tr>
<td>4</td>
<td>100ng/mL GDNF + medium</td>
</tr>
</tbody>
</table>

At one, two, and three weeks during the treatment a minimum of three tubes were stained for tyrosine hydroxylase-immunoreactivity (TH-ir), a marker of DAergic neurons, and counter stained with haematoxylin. The cultures were then analyzed in a blind fashion. The number of TH-ir cells was counted in a minimum of 3 fields under 400x magnification with a light microscope. Analysis of number of TH-ir cells was performed by a 2 way-ANOVA followed by Bonferroni's Multiple Comparison Test. P value of <0.05 was considered significant.
8.2.2 TH-ir cells response to GDNF following neurotoxin treatment

Co-cultures were prepared as described in Chapter 4, and grown for two weeks before receiving their treatments (see Table 8.2). Neurotoxins were incubated with the co-cultures for 24 hrs after which the media was changed with fresh media; GDNF was added to the media twice a week for a week in the designated co-cultures. One week post recovery period, a minimum of 3 tubes per group were immunocytochemically stained for tyrosine hydroxylase (TH-ir). The number of TH-ir cells was counted in a blind fashion and analysed.

8.2.3 TH-ir cell response to pre-treatment with GDNF followed by neurotoxin insult.

Co-cultures were prepared as described in Chapter 4, and grown for two weeks with GDNF supplement at 20ng/mL at each media change before receiving their neurotoxin treatments (see Table 8.3). Co-cultures were incubated with neurotoxins for 24 hrs and then the media was exchanged with fresh media. Media changes were carried out twice a week for a week. One week post recovery period a minimum of 3 tubes per group were immunocytochemically stained for tyrosine hydroxylase (TH-ir). The number of TH-ir cells was counted in a blind fashion and analysed. See Figure 8.4.1A, 8.4.1B & 8.4.1C for schematical diagram of experiments.
Table 8.2
Post treatment of Co-cultures with GDNF following MPTP/rotenone exposure

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>TREATMENT</th>
<th>GDNF (20ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control/normal medium</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Control/supplement</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>100 μM MPTP</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>100 μM MPTP</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>50 μM MPTP</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>50 μM MPTP</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>100 nM rotenone</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>100 nM rotenone</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>50 nM rotenone</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>50 nM rotenone</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>10 μM MPTP</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>10 μM MPTP</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>5 μM MPTP</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>5 μM MPTP</td>
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</tr>
<tr>
<td>15</td>
<td>10 nM rotenone</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>10 nM rotenone</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>5 nM rotenone</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>5 nM rotenone</td>
<td>+</td>
</tr>
</tbody>
</table>
### Table 8.3
Pre-treatment of Co-cultures with GDNF prior to MPTP/rotenone exposure

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>TREATMENT</th>
<th>PRE-TREATMENT GDNF (20ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (normal medium)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Control (medium with supplement)</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>10 μM MPTP</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>10 μM MPTP</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>5 μM MPTP</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>5 μM MPTP</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>10 nM rotenone</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>10 nM rotenone</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>5 nM rotenone</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>5 nM rotenone</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 8.1 Schematic diagram of the experiments.

A) 5 day old co-cultures were treated with GDNF at 10, 20 or 100 ng/mL for 3 weeks at every media change, TH-ir cell analysis at 1 week intervals.  
B) 14 day old co-cultures were treated with MPTP or rotenone, 24 hrs post toxin co-cultures were treated with GDNF (20 ng/mL) for 1 week at media changes followed by TH-ir cell analysis.  
C) Co-cultures were pre-treated with GDNF (20 ng/mL) straight after cytostatic treatment up until toxin exposure, 1 week of recovery in normal media followed by TH-ir cell analysis.  

* GDNF supplementation x2/wk;  
# normal media change x2/wk.
8.2 Analysis of cell death
The medium from toxin treated at 7 day recovery and GDNF post treatment (same time at toxin recovery day) will be collected for LDH determination as previously described in chapter 7. FJC staining will also be carried out to assess whether GDNF post treatment of co-cultures provided neuroprotection.

8.3 RESULTS

8.3.1 GDNF at varying doses did not induce a significant increase in TH-ir cells
Despite the increasing concentration of GDNF in the media, the number of TH-ir cells did not significantly increase throughout the weeks compared to normal controls except at the highest dose (100 ng/mL) during the first week. There was, however a trend (statistically not significant) for the number of TH-ir cells to increase with increasing GDNF dose (Figure 8.2A; B; C & E, 8.3).

8.3.2 GDNF treatment promotes increase in cell size and branching.
The main difference between cells treated with GDNF and those that were not is that the size and number of branches of TH-ir cells increased markedly compared to normal controls (Figure 8.2A; E, 8.4). The difference in intensity of TH-ir staining between the normal controls and GDNF-treated cells was distinctive as they were much darker (Figure 8.2A; B).

8.3.3 Post GDNF treatment following MPTP and rotenone exposure.
GDNF treatment at 20ng/mL does not provide any significant protection to the TH-ir cells at the higher doses of MPTP (50 & 100 μM). However at the lower doses of 5 and 10 (μM) MPTP there is a significant increase of TH-ir cells when treated with GDNF (Figure 8.5 & 8.6). An obvious change was in the morphology of the cells, where the cell body and processes were intact compared to toxin treated cells. Similar results were seen with rotenone (Figure 8.7 & 8.8). Further support of GDNF protection against MPTP and rotenone was observed by LDH assays (Figure 8.9 & 8.10) showing protection is significant only at the lower doses of toxins but not at the higher doses. FJC staining shown GDNF post treatment was able to provide partial protection of TH-ir cells indicated by the decreased number of degenerating neurons (Figure 8.11).

### 8.3.4 Pre-treatment with GDNF followed by MPTP and rotenone exposure.

Since treatment with GDNF of co-cultures exposed to higher doses of MPTP and rotenone resulted in no protection, we decided to pre-treat co-cultures with GDNF in conjunction with MPTP and rotenone at the lower doses. Unlike post-treatment with GDNF, TH-ir cells were not significantly protected against MPTP and rotenone during pre-treatment with GDNF (Figure 8.12, 8.13 & 8.14). However, a trend is observed with co-cultures pre-treated with GDNF. These had higher numbers of TH-ir cells after MPTP and rotenone exposure compared to co-cultures that did not receive any GDNF treatment post-toxin exposure. In addition, the morphology of TH-ir cells improved with increased neuritic branching and cell body size.
Figure 8.2. TH-ir cells in co-cultures treated with GDNF.

7 day co-cultures treated with GDNF (A) control, DAB; (B) 5 ng/mL; 10 ng/mL; (C) 10 ng/mL; (D) 100 ng/mL for 1 week followed by 1 week of recovery, DAB + nickel solution for dark grey colour. (E) 100 ng/mL treated TH-ir cell. Note: enlarged cell body and long processes plus multiple branching, in addition boutons/nodules on them. Image analysis at 400 x; bar = 10 μm.
Figure 8.3. TH-ir analysis of 7 day co-cultures treated at varying doses of GDNF.

Cultures were treated for two weeks and allowed to recover in fresh normal media; following consecutive weeks, co-cultures were randomly fixed and processed for TH-ir cell analysis. Data is presented as Mean ± SEM. Two-way ANOVA followed by Bonferroni’s post tests was performed on Mean ± SD. * p < 0.05 (Control vs 5 ng at 3 weeks); *** p <0.001 (Control vs 100 ng at 1 week); * p < 0.05 (5 ng vs 100 ng at 1 & 3 weeks), n = 5 for each group.
Figure 8.4. 7 day co-cultures treated at varying doses of GDNF.

Cultures were treated for two weeks then allowed to recover in fresh normal media; following consecutive weeks co-cultures were randomly fixed and processed for TH-ir process analysis. Data is presented as Mean ± SEM. Two-way ANOVA followed by Bonferroni’s post tests was performed on Mean ± SD. *** p < 0.001 (Control vs 5, 10 & 100 ng/mL at 1, 2 & 3 weeks); α p < 0.01 (5 ng vs 10 ng at 3 weeks); # # # p <0.001 (5 ng vs 100 ng/mL at 1, 2 & 3 weeks); § p <0.001 (10 ng vs 100 ng/mL at 1, 2 & 3 weeks), n = 50 for each group.
Figure 8.5. The neuroprotective effects of GDNF on TH-ir cells following MPTP exposure.

14 day co-cultures treated with MPTP followed by GDNF treatment (A) control; (B) 5 μM; (C) 5 μM + GDNF (20 ng/mL); (D) 10 μM; (E) 10 μM + GDNF. Image analysis at 400 x; bar = 10 μm.
Figure 8.6. The effects of GDNF post treatment following MPTP exposure.

14 day co-cultures treated with varying doses of MPTP for 24 hrs, followed by 7 days of media changes with GDNF supplementation (20 ng/mL). Cultures were fixed and processed for TH-ir cell analysis. Data are presented as Mean ± SEM, one-way ANOVA was performed on Mean ± SD followed by Bonferroni’s Multiple comparison test. *** p <0.001 (Naïve control vs 5, 10, 50 & 100 µM); # # # p <0.001 (GDNF vs 5, 10, 50 & 100 µM); * p < 0.05 (5 µM vs 5 µM GDNF); * p <0.05 (10 µM vs 10 µM + GDNF).
Figure 8.7. The neuroprotective effects of GDNF on TH-ir cells following rotenone exposure.

14 day co-cultures treated with rotenone followed by GDNF treatment (A) control; (B) 5 nM; (C) 5 nM + GDNF (20 ng/mL); (D) 10 nM; (E) 10 nM + GDNF (20 ng/mL). Image analysis at 400 x; bar = 10 μm.
**Figure 8.8. The effects of GDNF post treatment following rotenone exposure.**

14 day co-cultures treated with varying doses of rotenone for 24 hrs, followed by 7 days of media changes with GDNF supplementation (20 ng/mL). Cultures were fixed and processed for TH-ir cell analysis. Data are presented as Mean ± SEM, Two-way ANOVA was performed on Mean ± SD followed by Bonferroni’s Multiple comparison test. *** p <0.001 (Naïve control vs 5, 10, 50 & 100 nM); # # # p < 0.001 (GDNF vs 5, 10, 50 & 100 nM); * p < 0.05 (5 nM vs 5nM +GDNF); * p <0.05 (10 nM vs 10 nM +GDNF).
Figure 8.9. LDH ELISA of GDNF post treatment following MPTP exposure.

14 day co-cultures treated with varying doses of MPTP for 24 hrs, followed by 7 days of media changes with GDNF supplementation (20 ng/mL). Culture media were collected on final day of recovery and snapped frozen in -80 °C. Bovine serum albumin (BSA) used an internal control and as blocking. Data are presented as Mean absorbance levels ± SEM, one-way ANOVA was performed on Mean absorbance levels ± SD followed by Bonferroni’s Multiple comparison test. *** p <0.01 (Control vs 5, 10, & 100 μM); *** p < 0.001 (GDNF Control vs 5, 10 & 100 μM); * p <0.05 (10 μM vs 10 μM + GDNF); ** p <0.01 (5 μM vs 5 μM + GDNF).
Figure 8.10. LDH ELISA of GDNF post treatment following rotenone exposure.

14 day co-cultures treated with varying doses of rotenone for 24 hrs, followed by 7 days of media changes with GDNF supplementation (20 ng/mL). Culture media were collected on final day of recovery and snapped frozen in -80 °C. Bovine serum albumin (BSA) is used an internal control and as blocking. Data are presented as Mean absorbance levels ± SEM, one-way ANOVA was performed on Mean absorbance levels ± SD followed by Bonferroni’s Multiple comparison test. *** p <0.001 (Control vs 5, 10 & 100 nM); *** p < 0.001 (GDNF control vs 5, 10 & 100 nM); * p <0.05 (5 nM vs 5 nM + GDNF); * p <0.05 (10 nM vs 10 nM + GDNF).
Figure 8.11. FJC-staining of GDNF Post-treatment on co-cultures following MPTP and rotenone exposure.

(A) control; (B) 14 day co-cultures treated with MPTP 5 μM + GDNF (20 ng/mL); (C) 5 μM; (D) rotenone 5 nM + GDNF (20 ng/mL); (E) 5 nM. Image analysis at 400 x; bar = 10 μm.
Figure 8.12. The neuroprotective effects of GDNF Pre-treatment on TH-ir cells following MPTP and rotenone exposure.

(A) control; (B) 14 day co-cultures treated with MPTP 5 nM; (C) 5 nM + GDNF (20 ng/mL); (D) rotenone 5 nM; (E) 5 nM + GDNF (20 ng/mL). Image analysis at 400 x; bar = 10 μm.
Figure 8.13. The effects of GDNF Pre-treatment following MPTP exposure.

GDNF (20 ng/mL) treatment followed cytostatics up to 14 day; co-cultures were then treated with low doses of MPTP for 24 hrs, followed by 7 days of media changes with normal media. Data are presented as Mean ± SEM, one-way ANOVA was performed on Mean ± SD followed by Bonferroni’s Multiple comparison test. *** p < 0.001 naïve control compared to all treatments excluding GDNF control; # # # p < 0.001 GDNF control compared to all treatments excluding naïve control.
Figure 8.14. The effects of GDNF Pre-treatment following rotenone exposure.

GDNF (20 ng/mL) treatment followed cytostatics up to 14 day; co-cultures were then treated with low doses of rotenone for 24 hrs, followed by 7 days of media changes with normal media. Data are presented as Mean ± SEM, one-way ANOVA was performed on Mean ± SD followed by Bonferroni’s Multiple comparison test. *** p < 0.001 naïve control compared to all treatments excluding GDNF control; # # # p < 0.001 GDNF control compared to all treatments excluding naïve control.
8.4 DISCUSSION

8.4.1 The neurotrophic effects of GDNF on DAergic neurons survival and development.

GDNF supports the development of embryonic DAergic neurons (Lin et al. 1993) and is particularly important for postnatal survival of mesencephalic DAergic neurons (Granholm et al. 2000). Despite being named glial-derived growth factor, it is mainly present in the striatal medium spiny neurons that receive DAergic input from the SN (Oo et al. 2005). GDNF levels in various parts of the brain are reported to be no different in parkinsonian and control patient brains (Mogi et al. 2001). However, GDNF mRNA expression is increased in the putamen of PD patients compared with controls (Backman et al. 2006), and so there is no evidence that loss of GDNF is responsible for development of PD.

Although GDNF’s neurotrophic effect is on a smaller scale than expected, it did demonstrate a positive outcome on postnatal DAergic neuron development. The DAergic neurons were larger with increased cell branching and lengthening of processes and greater staining intensity which may be the result of an increase in tyrosine hydroxylase and/or VMAT2 expression induced by GDNF (Emborg et al. 2008). *In vivo* studies have shown similar results where GDNF injected into the ST promotes cell survival by inhibiting cell death and induced regeneration by fiber outgrowth (Hou et al. 1996; Zeng et al. 2006; Kowsky et al. 2007; Elsworth et al. 2008). It may be that a constant supply of GDNF over a longer period of time is needed to produce a much greater effect as these cultures were only exposed to GDNF for a short period of time. Previous studies have
used embryonic cultures (Meyer et al. 2000; Jakobsen et al. 2005; Zeng et al. 2006), genetically modified cells expressing GDNF (Bjorklund et al. 2000; Park et al. 2001; Kordower 2003) or implantation of a pump in vivo (Palfi et al. 2002) for continuous supply of GDNF.

### 8.4.2 Neuroprotection and regeneration of DAergic neurons by GDNF.

A variety of experiments in rodent models have shown that GDNF injected directly into the SN or ST protects DAergic neurons from neurotoxins (Kearns and Gash 1995; Tomac et al. 1995; Kirik et al. 2000a; Kirik et al. 2000b; Kirik et al. 2004). Elevating GDNF in the ST by gene therapy is also protective (Kordower et al. 2000; Georgievksa et al. 2002b; Eslamboli 2005; Eslamboli et al. 2005). Unlike the case with BDNF, there is also evidence for restoration of function of injured neurons by GDNF after toxic insults (Tomac et al. 1995; Kirik et al. 2000a; Kirik et al. 2001)—although restorative actions are generally not as dramatic as the neuroprotective actions.

A series of influential studies from Gash, Gerhardt, and coworkers (Gash et al. 1996; Zhang et al. 1997; Grondin et al. 2002; Grondin et al. 2003) have demonstrated GDNF-induced improvement in bradykinesia, rigidity, and postural instability in monkeys with stable MPTP-induced hemiparkinsonism. GDNF was effective when given by bolus or by constant infusion, via three routes of administration: intranigral, intrastriatal, and intracerebroventricular (ICV). These studies, showing clear improvement in monkeys with established hemiparkinsonism, stimulated the clinical trials that followed. In addition to neuroprotective and neurorestorative actions, GDNF also has direct effects on
DAergic neurons, modulating excitability via changes in A-type potassium channels (Yang et al. 2001). This may be a mechanism by which GDNF acutely increases DA release (Hebert et al. 1996).

8.4.3 GDNF neuroprotection against neurotoxins in slice cultures.

Studies so far have demonstrated protection by growth factors against MPP+ in embryonic systems such as stem cells (Zeng et al. 2006); midbrain from mice (Son et al. 1999; Jakobsen et al. 2005) or primary DAergic neurons (Chu et al. 2005; Anantharam et al. 2007a; Anantharam et al. 2007b) and only a few experiments have been carried out on postnatal slice cultures exposed to MPP+ (Schmidt et al. 1997) and rotenone (Yang et al. 2008). A majority of current findings are based on in vivo studies using MPTP, MPP+ or 6-OHDA as the neurotoxin. Our current study employs MPTP and rotenone in slice cultures derived from postnatal rats in conjunction with GDNF. We were able to show neuroprotection from MPTP or rotenone but only at the low doses of neurotoxins. This may be due to the fact that higher doses (50-100 μM, nM) induce a 75-90% decrease in DAergic neurons and thus, not a lot of surviving cells are present for treatment with GDNF. Although TH-ir cells were rescued by GDNF treatment it did not prevent these cells from degenerating. It may be that the GDNF concentration was too low to provide neuroprotection against the ongoing degenerating process.
CHAPTER 9:

GENERAL DISCUSSION
9.1 INTRODUCTION
Organotypic slice cultures are widely used in CNS research to investigate neuronal functioning and cell-cell interactions in situ. The present study employed organotypic slice cultures to investigate the effects of two neurotoxins on brain DAergic neurons together with the neuroprotective role of GDNF against these toxins. Chapter 6 described the characterization of DAergic neurons using this culture system. The effects of two toxins, MPTP and rotenone, on DAergic neurons, were described in chapter 7 and the neuroprotective effects of GDNF were outlined in chapter 8.

9.2 ORGANOTYPIC SLICE CULTURE OF VM AND ST
9.2.1 The advantages of using organotypic slice cultures.
The advantages of the organotypic slice culture model over an in vivo preparation include its relative simplicity, and ease of access. These factors readily allow studies into the DAergic innervation of the ST. Furthermore, unlike a typical in vitro system, the physiological environment of the developing ST neurons is preserved. The slice culture thus represents a system that is more anatomically and physiologically relevant than cultures of cell lines (Cattaneo and McKay 1990; Evrard et al. 1990) or dissociated primary cells (Panula et al. 1979; Bockaert et al. 1986). The maintenance of cell-cell interactions in slice cultures allows the development and cell biology of VM neurons and their connections with the ST to be analyzed as if in situ. These properties, and the experimentally accessible condition of cells in the slice model, make the organotypic culture of VM and ST a valuable compromise paradigm between the in vivo whole animal and in vitro dissociated or cell culture systems. Additionally, it is possible with
this method to co-culture different brain regions and form neuronal connections between them (Gahwiler 1988; Ostergaard et al. 1990; Yamamoto et al. 1992). Novel findings can thus be obtained when the co-culture system is used in neurotoxicologic studies (Maeda et al. 1998; Katsuki et al. 2001). Organotypic slice co-cultures of VM and ST are also suitable for electrophysiological studies of the nigrostriatal pathway as the morphological and electrophysiological characteristics of SN neurons in such preparations are similar to those in other non organotypic *in vitro* and *in vivo* studies (Steensen et al. 1995; Rohrbacher et al. 2000). Importantly, slice cultures have demonstrated that DAergic axons do actually form functional synapses with neurons of the ST *in vivo* (Ostergaard et al. 1990; Ostergaard et al. 1991; Ostergaard 1993; Holmes et al. 1995; Ostergaard et al. 1995; Ostergaard et al. 1996; Franke et al. 2003).

### 9.2.2 Disadvantages of using organotypic slice cultures

Unfortunately, organotypic slice cultures do not *exactly* replicate the *in vivo* environment of the cells. This is due to unavoidable severance of existing cell-cell connections during removal of the tissue. As the *in vitro* environment is not exactly the same as the *in vivo* environment it is unlikely that all the factors which regulate the innervation of ST by DAergic neurons, *in vivo*, are present in culture. Despite these drawbacks, the co-culture system is still closer to representing *in vivo* conditions, in terms of preservation of the basic structural organization of the tissue, than single-cell culture systems usually employed for studies of DAergic neurodegeneration.

### 9.2.2 Summary of experimental findings
In this study we were able to maintain the growth of DAergic cells without any conditioned medium using the organotypic culture technique. Furthermore, the morphology of TH-ir cells observed in the current study resembled that of those seen in the postnatal rat in vivo (Mytilineou et al. 1983; Østergaard et al. 1990; Tepper et al. 1994). Our study focused initially on the important role of the ST in the DAergic development of the VM. This is because it has been shown to have a trophic influence on the development of DAergic neurons of the SN in vitro (Prochiantz et al. 1979). It was demonstrated that the growth and maturation of VM DAergic neurons was regulated by, and dependent on, its specific trophic target (the ST). Growth of TH-ir cells in co-culture remains regular throughout the 7 weeks of culture and such cells formed dense networks of neuritic projections. Similar observations were reported earlier by Østergaard et al. (1991). Single VM (SVM) cultures showed poor TH-ir cell growth with such cells being smaller with fewer processes during an analogous 6 week period. Poor growth of SVM DAergic neurons was likely to be due to the absence of the trophic target (ie the ST) and the lack of contact-dependent mechanisms specific for DAergic fibers (Østergaard et al. 1990). Striatal membranes or extracts have been demonstrated to have a neurotrophic effect on mesencephalic DA cells (Prochiantz et al. 1981; Dal Toso et al. 1988). Furthermore, the development of axonal and dendritic connections have also been shown to be influenced by the presence of ST cells (Hemmendinger et al. 1981).

Our studies also revealed the role that GFAP-ir astrocytes may play in the co-cultures and the SVM cultures. Growth of GFAP-ir astrocytes in the SVM was better in the first two weeks of culture compared to that in the co-cultures. There was, however, no
significance difference in GFAP-ir cell numbers between the co-cultures and the SVM cultures from the third week onwards. GFAP-ir cell numbers, however, never exceeded the number of TH-ir cells in co-cultures but did so in SVM. These observations suggest that astrocytes may play a supportive role in the development of DAergic neurons particularly, in the absence of their trophic ST-target in the SVM.

Astrocytes represent the major non-neuronal cells in the brain, and these cells have been implicated in the segregation, maintenance, and support of neurons. There is emerging evidence that astrocytes are an important source of neuroactive substances such as growth factors, eicosanoids, and neurosteroids, which may subsequently influence neuronal development, survival, and neurosecretion (Kabbadj et al. 1993; Ojeda et al. 2000; Araque et al. 2001; Azcoitia et al. 2001). Many studies have demonstrated the therapeutic potential of astrocyte transplantation in neurodegenerative diseases (Smith et al. 1987; Lundberg et al. 1996; Ridet et al. 2003; Ericson et al. 2005). It is also believed that astrocytes may represent ideal vehicles for delivery of neurotrophic factors such as GDNF into the CNS to provide protection in PD animal models (Ericson et al. 2005; Yang et al. 2008).

Astrocytes have several unique properties that make them an ideal resource for cell or gene therapy.

1) Astrocytes can provide growth factors or other molecules to support the growth and functional maintainence of neurons (Ridet et al. 1997; Araque et al. 2001).

2) Astrocytes represent excellent carriers to help deliver therapeutic molecules into the brain.
3) Astrocytes have the ability to survive for prolonged periods when transplanted into their native environment (Andersson et al. 1993).

9.3 THE EFFECTS OF MPTP AND ROTENONE ON VM AND ST SLICE CULTURES

9.3.1 MPTP and rotenone
The discovery of MPTP (Langston et al. 1983) paved the way for understanding the molecular mechanisms of DAergic cell death in various animal and cell culture models (Speciale 2002). MPTP is itself non-toxic, but conversion by MAO-B in astrocytes to its active metabolite, MPP⁺, renders a much more toxic species which is selectively taken up by DAergic neurons expressing DAT (Chiba et al. 1985; Javitch et al. 1985; Gainetdinov et al. 1997). The mechanism of toxicity of MPP⁺ has been proposed to be mediated through inhibition of mitochondrial complex I (Nicklas et al. 1985). The rotenone model of PD (Betarbet et al. 2000) has reinforced the idea that complex I inhibition may actually be a key factor involved in the death of DAergic neurons and the subsequent development of Parkinsonism.

9.3.2 Summary of experimental findings

A dose- and time-dependent response was observed when the co-cultures were treated with either MPTP or rotenone. 7 day old co-cultures treated with MPTP showed less of a loss in numbers of their TH-ir cells, at lower doses, as compared to the higher doses during the post-treatment period. 14 day old co-cultures, however, demonstrated a
greater vulnerability at both low and high doses, as shown by the significant reductions in TH-ir cells at all analysis times during the recovery period. Interestingly, the higher doses of rotenone (50 & 100 nM) were cytotoxic to most cells, whether they were DAergic or not, whereas with the lower doses TH-ir cells were most affected. This could be due to its highly lipophilic nature, thus, such that it freely crosses all cellular membranes and accumulates in subcellular organelles such as the mitochondria (Talpade et al. 2000; Schuler and Casida 2001a, b; Caboni et al. 2004). In contrast, MPTP, once converted to MPP⁺, is specifically taken up by DAT in DAergic neurons, as mentioned previously.

An MPTP concentration of 100 μM was required to dramatically reduce the number of TH-ir neurons in the culture. Like rotenone, MPTP not only decreased the number of TH-ir cells but also of all other cells in culture, indicating that high concentrations of MPTP produced a general toxic effect. A similar result has been reported for MPP⁺ and although most in vitro studies have used MPP⁺ in rat dissociated embryonic mesencephalon and primary cell lines (Cohen and Mytilineou 1985; Mytilineou and Cohen 1985; Mytilineou et al. 1985; Friedman and Mytilineou 1987; Mytilineou and Cohen 1987; Schinelli et al. 1988; Danias et al. 1989; Otto and Unsicker 1993). Low doses of 0.1 – 10 μM MPP⁺ produced a concentration-dependent decrease in the number of surviving TH-ir neurons. Concentrations equal to, or higher than, 30 μM, however, were toxic to all cell types (Sanchez-Ramos et al. 1986). Our data did not show any significant difference in TH-ir cell loss between MPTP and MPP⁺ in the culture either at low (5 & 10 μM) and high doses (50 & 100 μM).
The numbers of GFAP-ir astrocytes in culture plays an important role in the toxicity of MPTP. MPTP is not toxic itself, as mentioned previously, its conversion to MPP⁺ has been shown to be the main reason for DAergic cell death (Chiba et al. 1985; Javitch et al. 1985; Gainetdinov et al. 1997). MPTP conversion to MPP⁺ by MAO-B mainly occurs in astrocytes (Markey et al. 1984; Takada et al. 1990; Di Monte et al. 1991) and not in DAergic neurons (Westlund et al. 1985). MPP⁺ is then specifically taken up into DAergic nerve terminals via plasma membrane-bound DA transporter (DAT) (Chiba et al. 1985; Javitch et al. 1985). Genetically modified mice with a deficiency of DAT have been shown to be resistant to MPTP toxicity (Gainetdinov et al. 1997; Takahashi et al. 1997). Thus, the amount of TH-ir cell death is likely to be dependent on the number of astrocytes available to convert MPTP to its toxic by-product and on the presence of DAT.

Death of TH-ir and other cells at the higher treatment doses may be due to a number of reasons for example, MPTP, once converted to MPP⁺, could enter DAergic neurons and subsequently cause displacement of DA from secretory vesicles (Chang and Ramirez 1986. As a result, cytoplasmic oxidation occurs, due to the generation of oxygen free radicals upon extracellular DA release (Sulzer, 2000 #1759; Speciale et al. 1998; Sulzer et al. 2000; Inazu et al. 2001). Oxidation of DA produces free radicals and reactive quinones (Tse et al. 1976; Graham 1978; Graham et al. 1978), which damage cell components and therefore exert a toxic effect on neurons (Berman and Hastings 1999; Olanow and Tatton 1999). It is generally accepted that MPP⁺ is one of the most potent DA releasing agents (Markstein and Lahaye 1984; Pileblad et al. 1984; Chang and
The common variability in TH-ir cell neurodegeneration was more obvious in the 7 day old compared with the 14 day old co-cultures, since these cells are less likely to have established a monolayer neuronal network. The toxic effects of both toxins may depend on the numbers/density of the cells present. The VM slice contained different subsets of DAergic neurons and other cells. Thus, the variation in TH-ir cell number after treatment could be caused by the different susceptibility of the requisite subsets of DAergic neurons present. For example, MPTP treatment in mice caused the loss of midbrain DAergic cells; this was quantified as a 69% loss of nucleus A8 cells, a 75% loss of nucleus A9 cells and A10 subnuclei, and a 42% loss of ventral tegmental area cells (German et al. 1996). A single dose of MPTP induced a toxic effect that remained in the post treatment period. This was demonstrated by using FJ-C staining, which is a marker for neuronal degeneration.

Rotenone was more toxic than MPTP, as shown by the difference in dose concentrations required to elicit toxicity. 100 nM rotenone induced a greater TH-ir cell loss than 100 μM MPTP. Even though the inhibition sites of both neurotoxins are thought to be the same (Ramsay et al. 1991b; Ramsay et al. 1991a) rotenone is highly competitive whereas MPP⁺ is a very weak complex I inhibitor (Tipton et al. 1993; Tipton and Singer 1993; Schuler and Casida 2001a, b). Similar to MPTP, Rotenone has also been shown to
induce DA release from DAergic neurons (Zoccarato et al. 2005; Sai et al. 2008). Not only does rotenone cause a decrease in TH and VMAT2 protein levels, it also increases DAT protein level and MAO activity in a dose dependent manner (Sai et al. 2008). This could contribute to increased ROS production and subsequent neuronal degeneration (Radad et al. 2006; Sai et al. 2008). Both neurotoxins were able to induce caspase-3 activation particularly at the lower doses indicating cell death occurred by apoptosis. FJC demonstrated that TH-ir cell exposed to MPTP or rotenone at low doses is able to induce neuronal degeneration which continued in the following weeks of recovery.

9.4 THE NEUROTROPHIC ROLE OF GDNF ON TH-ir NEURONS IN SLICE CULTURES

9.4.1 GDNF and DAergic neurons

Since the discovery that GDNF is a trophic factor for embryonic midbrain DAergic neurons in culture (Lin et al. 1993) this compound has been widely studied as a survival factor. This is the case for DAergic neurons in vivo after grafting (Stromberg et al. 1993; Rosenblad et al. 1996) and in vitro (Meyer et al. 1999; Meyer et al. 2001). GDNF has also been studied as a neuroprotectant and/or factor which can enhance regeneration of injured neurons or augment phenotypic expression of surviving DAergic neurons in models of PD, both in vitro (Krieglstein et al. 1995b; Krieglstein et al. 1995c; Hou et al. 1996; Eggert et al. 1999; Nicole et al. 2001) and in vivo (Choi-Lundberg et al. 1997; Rosenblad et al. 2000b; Rosenblad et al. 2000a).

9.4.2 Summary of experimental findings
Co-cultures treated with increasing GDNF concentrations showed increases in both TH-ir cell size and number of dendritic branches. However, the numbers of DAergic neurons were not significantly influenced by GDNF in a dose-dependent manner, as compared to controls. The neurotrophic influences of GDNF on TH-ir cell size and branching have been reported in previous in vitro studies (Lin et al. 1993; Lin et al. 1994; Costantini and Isacson 2000b; Jakobsen et al. 2005; Hirata and Kiuchi 2007) but not previously in studies using postnatal SN tissue. Levels of the dopamine metabolites, DOPAC and HVA, in the culture medium, as well as the amount of high-affinity $[^3]$H-DA uptake were also significantly increased by GDNF (Hou et al. 1996; Jakobsen et al. 2005). In vivo studies have shown GDNF to increase the survival, growth and function of DAergic neurons in particular in intrastriatal fetal nigral DAergic grafts (Lin et al. 1993; Stromberg et al. 1993; Johansson et al. 1995; Rosenblad et al. 1996; Sinclair et al. 1996; Apostolides et al. 1998).

Studies of GDNF neuroprotection mainly stem from PD toxin-induced animal models. MPTP-treated non-human primates receiving intrastriatal and intranigral injections of GDNF was observed to have more TH- and VMAT2-positive fibers in the areas of delivery, as well as reductions in their clinical rating scores (Tomac et al. 1995; Gash et al. 1996; Bjorklund et al. 2000; Kordower et al. 2000; Palfi et al. 2002; Oiwa et al. 2006; Emborg et al. 2008). Furthermore, administration of GDNF not only improved MPTP-induced disability and reversed DAergic cell loss in the SN but also diminished L-DOPA-induced dyskinesia (Iravani et al. 2001). Gene transfer of GDNF to the ST of MPTP
monkeys also enhanced the survival and outgrowth of co-implanted fetal DAergic neurons (Elsworth et al. 2008).

In the current study, GDNF treatment after toxin exposure did not provide any significant neuroprotection against the higher doses of MPTP or rotenone but, at the lower doses there was significant protection against both toxins, as indicated by the greater number of TH-ir cells in GDNF-treated co-cultures compared to non-GDNF treated co-cultures. These results are supported by various in vitro studies of DAergic neurons exposed to both MPP+ and GDNF together. GDNF shown to has been shown to protect TH+ve neurons against both MPP+-induced apoptosis and loss of neuronal processes, as well as against formation of intracellular reactive oxygen species (ROS) (Krieglstein et al. 1995c; Hou et al. 1996; Jakobsen et al. 2005; Zeng et al. 2006).

Similar to the current study, it was also observed that GDNF did not alter the total number of TH+ve neurons, but increased (at high GDNF concentrations) DA cell soma size and – most dramatically – the production of DA and its metabolites DOPAC and HVA (Jakobsen et al. 2005). The restorative effect of GDNF on DA levels indeed, was much stronger than its effect on preservation of TH+ve cell numbers. Additionally, in vitro studies using MPP+ in primary mesencephalic cell cultures reported that GDNF could not prevent the acute loss of TH+ve cells, but could prevent delayed cell death and/or enhance recovery of injured neurons (Hou et al. 1996). Furthermore, pretreatment with GDNF significantly protected these cultures against MPP+ toxicity (Chao and Lee 1999); (Jakobsen, 2005 #40). This suggests that GDNF pre-treatment stimulates
expression of protective factors which prepares the cellular defence mechanisms against possible toxic insults (eg. oxidative stress, apoptosis). This was not the case in the current study, as pre-treatment with GDNF did not provide any significant neuroprotection against MPTP and rotenone. However, there was a marked morphological improvement of TH-ir cells treated with GDNF compared to co-cultures that were not treated with GDNF. The low GDNF dose treatment (20 ng/mL) may be too low to provide such a significant neuroprotection prior to MPTP and rotenone toxic insult.

Fewer studies have compared the effect of GDNF against rotenone compared to those against MPTP/MPP⁺. TH+ve cell-loss as induced by rotenone exposure was significantly attenuated when mesencephalon cultures were co-incubated with GDNF (Yang et al. 2008). Blockade of GDNF with specific antibodies dramatically abolished the GDNF-induced neuroprotective effect (Yang et al. 2008). Interestingly, rotenone and MPP⁺ have been shown to down-regulate the GDNF receptor, Ret, but unlike MPP⁺, rotenone did not affect the expression of other cellular components, including TH and actin, in cultured cells (Hirata and Kiuchi 2007). This finding is important since there is known to be a progressive and late-onset loss of DAergic neurons in mice where Ret has been conditionally deleted (Kramer et al. 2007). These findings establish Ret as a critical regulator of long-term maintenance for the nigrostriatal DAergic system.

The effects of other NTFs, including nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), have been studied in cultures exposed to rotenone (Siegel
and Chauhan 2000; Hirata et al. 2006; Jiang et al. 2006). These NTFs and GDNF, as well as their receptors have been localized by immunocytochemistry to DAergic neurons in the SN (Nishio et al. 1998; Walker et al. 1998; Chauhan et al. 2001), suggesting both autocrine and paracrine functions for these neurotrophins. All three NTFs have been shown to significantly reduce rotenone toxicity to TH+ve neurons in midbrain neuronal cultures (Jiang et al. 2006). The protective effect of NGF was completely abolished by inhibiting both the microtubule-associated protein kinase (MEK) and by treating with the microtubule-stabilizing drug taxol. In a MEK-dependent manner, NGF, BDNF and GDNF also significantly attenuated rotenone-induced microtubule depolymerization and ensuing accumulation of vesicles in the soma and elevation in protein carbonyls (Jiang et al. 2006). NGF in particular, however, did not attenuate the rotenone-induced increase in lactate levels indicating that NGF may not be involved in the inhibition of mitochondrial respiration by rotenone (Hirata et al. 2006). However NGF was able to completely prevent rotenone-induced DNA fragmentation and subsequent cell death. In the current study GDNF treatment was able to reduce cytotoxicity levels induced by both rotenone and MPTP measured by LDH assay. The intracellular signaling of NGF-induced cell survival is largely dependent on Trk, a high affinity NGF receptor (Schramm et al. 2005; Arevalo et al. 2006). Rotenone exposure of PC12 cells did not affect Trk expression, but treatment with NGF caused down-regulation of Trk (Hirata et al. 2006), and internalization of the activated receptor. Furthermore, a specific Trk inhibitor, K252a (Ohmichi et al. 1992), completely attenuated the protective effect of NGF on rotenone-induced DNA fragmentation (Koizumi et al. 1988). These results suggest that NGF attenuates rotenone-induced apoptosis via the Trk signaling pathway. NGF, EGF and
FGF-b have also been shown to attenuate rotenone-mediated apoptosis, however IL-6 and GDNF had little effect on rotenone-induced DNA fragmentation (Hirata et al. 2006).

GDNF may protect DAergic neurons through its activation of antioxidant enzyme systems since both acute and chronic treatments with this factor caused an elevation glutathione peroxidase (GPx) activity (Chao and Lee 1999). Acute GDNF treatment also increased superoxide dismutase (SOD) and catalase (CAT) activities in rat striatum (Chao and Lee 1999). In addition, chronic GDNF treatment decreased the DA level by increasing DA turnover (Chao and Lee 1999). These results suggest that GDNF treatment can activate antioxidant defences, resulting in the efficient removal of ROS. Another antioxidant enzyme, manganese superoxide dismutase (MnSOD), a primary antioxidant defence enzyme against superoxide radicals produced within mitochondria, was overexpressed in transgenic mice (Klivenyi et al. 1998) and this overexpression significantly attenuated MPTP toxicity (Przedborski et al. 1992). There have also been conflicting reports in DAergic neuron cultures with overexpression of human SOD (hSOD1) activity showing no significant resistance to MPP⁺ treatment when compared to normal cultures (Sanchez-Ramos et al. 1997). This has been further supported by a more sensitive measure of DA neuron integrity and function ([³H]-DA uptake) which failed to demonstrate resistance of hSOD1 cultures to the MPP⁺ toxin (Sanchez-Ramos et al. 1997).

Apart from increases various antioxidant enzyme activities in midbrain DA neurons (Chao and Lee 1999) GDNF also protects mesencephalic neurons against apoptosis by
up-regulation of bcl-2 and bcl-x through PI3 kinase activation (Sawada et al. 2000). Further, MAPK/ERK pathway activation is necessary for GDNF to protect against NMDA-induced neuronal death through reduction of Ca^{2+} influx (Nicole et al. 2001).

So far most GDNF studies have been undertaken in non-human primates (Gerhardt et al. 1999; Kordower et al. 2000; Iravani et al. 2001; Palfi et al. 2002; Elsworth et al. 2008) and rodents (Cheng et al. 1998; Date et al. 1998; Date et al. 2001; McLeod et al. 2006; Schober et al. 2007; Chen et al. 2008; Zhang et al. 2008) in conjunction with MPTP/MPP+. In vitro studies mainly involved primary midbrain DAergic cells derived from embryonic rats or mice (Krieglstein et al. 1995b; Krieglstein et al. 1995a; Hou et al. 1996; Jordan et al. 1997; Ling et al. 1998a; Ma et al. 2000; Jakobsen et al. 2005) or human embryonic stem cells (Zeng et al. 2006) whereas the present study has been carried out on postnatal rat slice co-cultures. Studies of the effects of GDNF on rotenone exposure are limited (Hirata and Kiuchi 2007; Cho et al. 2008; Yang et al. 2008).

**9.5 Summary**

In this study we were able to maintain the growth of DAergic neurons derived from postnatal rat pups using the organotypic slice cultures. The importance of the trophic target-ST in the development and survival of DAergic neurons in culture was demonstrated by poor growth in its absence. Both neurotoxins MPTP and rotenone, induced DAergic cell degeneration in a dose-dependent manner and TH-ir cell vulnerability which increased with culture time. Exposure of TH-ir cells to GDNF in co-cultures increased cell size and induced significant cell branching and lengthening despite
no difference in cell numbers compared to controls. TH-ir cells in co-cultures treated with MPTP and rotenone was protected by GDNF but only at the lower doses of the neurotoxins.

9.6 Conclusion

Thus, this study has shown the importance of using organotypic slice cultures of VM and ST, demonstrating TH-ir cell growth and development is regulated by its trophic target-ST. By establishing this culture system we were able to study cell death of TH-ir cells, in addition, not only are TH-ir cells vulnerable to MPTP and rotenone treatment, but they can be protected by GDNF. These results represent important findings in the continued investigation of the underlying basis against PD.
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APPENDICES

Table 1. Co-cultures of ST & VM (TH-ir cells)

<table>
<thead>
<tr>
<th>Weeks</th>
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<th>5 (n=11)</th>
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Table 2. Single VM cultures (TH-ir cells)

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Table 3. Co-cultures of ST & VM (GFAP-ir cells)

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Table 4. Single VM cultures (GFAP-ir cells)

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Table 5. 7 day co-cultures MPTP treated (TH-ir cells)

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<th>1 uM (n=9)</th>
<th>5 uM (n=9)</th>
<th>10 uM (n=9)</th>
<th>50 uM (n=9)</th>
<th>100 uM (n=9)</th>
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<td>0.712125</td>
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<td>0.237375</td>
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<td></td>
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<td></td>
<td></td>
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<td>0.888355</td>
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<td><strong>3 weeks post</strong></td>
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Table 6. 14 day co-cultures MPTP treated (TH-ir cells)

<table>
<thead>
<tr>
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<th>Control (n=9)</th>
<th>Vehicle (n=9)</th>
<th>5 uM (n=9)</th>
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<th>50 uM (n=9)</th>
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<tbody>
<tr>
<td><strong>1 week post</strong></td>
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<td>11.51111</td>
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<tr>
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<td></td>
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<td>0.264214</td>
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<td>0.254477</td>
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<tr>
<td><strong>2 weeks post</strong></td>
<td>AVG</td>
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<td>12.13333</td>
<td>6.177778</td>
<td>5.511111</td>
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</tr>
<tr>
<td></td>
<td>STDEV</td>
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<tr>
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<tr>
<td><strong>3 weeks post</strong></td>
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<td>10.57778</td>
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Table 7. 7 day co-cultures Rotenone treated (TH-ir cells)

<table>
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<th>Control (n=12)</th>
<th>Vehicle (n=10)</th>
<th>1 nM (n=9)</th>
<th>5 nM (n=9)</th>
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<th>50 nM (n=9)</th>
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AVG = Average, STDEV = Standard Deviation, SEM = Standard Error of the Mean.
Table 8. 14 day co-cultures Rotenone treated (TH-ir cells)

<table>
<thead>
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<th></th>
<th>Control (n=9)</th>
<th>Vehicle (n=9)</th>
<th>5 nM (n=9)</th>
<th>10 nM (n=9)</th>
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Table 10. GDNF Dose-Dependent increase in TH-ir Cell branching

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<td>1 week</td>
<td>Control n=50</td>
<td>2.714286</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>5 ng n=50</td>
<td>4.367348939</td>
<td>0.698029296</td>
</tr>
<tr>
<td></td>
<td>10 ng n=50</td>
<td>4.510204</td>
<td>0.649437</td>
</tr>
<tr>
<td></td>
<td>100 ng n=50</td>
<td>5.979592</td>
<td>0.749716</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2 week</th>
<th>AVG</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control n=50</td>
<td>3.020408</td>
<td>0.594762</td>
</tr>
<tr>
<td></td>
<td>5 ng n=50</td>
<td>3.897959184</td>
<td>0.467479855</td>
</tr>
<tr>
<td></td>
<td>10 ng n=50</td>
<td>4.16326531</td>
<td>0.65659915</td>
</tr>
<tr>
<td></td>
<td>100 ng n=50</td>
<td>5.632653061</td>
<td>0.487077918</td>
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<table>
<thead>
<tr>
<th>3 week</th>
<th>AVG</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control n=50</td>
<td>3.061224</td>
<td>0.555584</td>
</tr>
<tr>
<td></td>
<td>5 ng n=50</td>
<td>3.673469</td>
<td>0.473804</td>
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<tr>
<td></td>
<td>10 ng n=50</td>
<td>4.040816</td>
<td>0.454569</td>
</tr>
<tr>
<td></td>
<td>100 ng n=50</td>
<td>5.102041</td>
<td>0.684498</td>
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### Table 11. GDNF Post-treatment of TH-ir cells in 14 day old co-cultures exposed to MPTP & Rotenone

<table>
<thead>
<tr>
<th>MPTP</th>
<th>Naive Control (n=9)</th>
<th>GDNF Control (n=9)</th>
<th>5 uM</th>
<th>5 uM +</th>
<th>10 uM</th>
<th>10 uM +</th>
<th>50 uM</th>
<th>50 uM +</th>
<th>100 uM</th>
<th>100 uM +</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVG</td>
<td>10.4</td>
<td>10.2963</td>
<td>6.444444</td>
<td>7.666667</td>
<td>5.6</td>
<td>7.266667</td>
<td>3.133333</td>
<td>3.866667</td>
<td>1.966667</td>
<td>2.433333</td>
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<tr>
<td>STDEV</td>
<td>1.069966</td>
<td>1.067521</td>
<td>0.800641</td>
<td>0.620174</td>
<td>0.894427</td>
<td>0.691492</td>
<td>0.681445</td>
<td>0.730297</td>
<td>0.718395</td>
<td>0.85836</td>
</tr>
<tr>
<td>SEM</td>
<td>0.338353</td>
<td>0.355843</td>
<td>0.26688</td>
<td>0.206725</td>
<td>0.282843</td>
<td>0.218669</td>
<td>0.215492</td>
<td>0.23094</td>
<td>0.227177</td>
<td>0.271437</td>
</tr>
<tr>
<td>Rotenone</td>
<td>5 nM (n=9)</td>
<td>GDNF (n=9)</td>
<td>6.253259</td>
<td>7.888889</td>
<td>5.066667</td>
<td>6.133333</td>
<td>4</td>
<td>4.266667</td>
<td>0.6</td>
<td>0.933333</td>
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<tr>
<td></td>
<td></td>
<td>GDNF (n=9)</td>
<td>0.764229</td>
<td>0.57735</td>
<td>0.691492</td>
<td>0.730297</td>
<td>0.830455</td>
<td>0.868345</td>
<td>0.498273</td>
<td>0.639684</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GDNF (n=9)</td>
<td>0.254743</td>
<td>0.19245</td>
<td>0.218669</td>
<td>0.23094</td>
<td>0.262613</td>
<td>0.274595</td>
<td>0.157568</td>
<td>0.202286</td>
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</tbody>
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Table 12. GDNF Pre-treatment of TH-ir cells for 14 days in co-cultures prior to exposure to MPTP & Rotenone

<table>
<thead>
<tr>
<th></th>
<th>MPTP Naive Control (n=9)</th>
<th>GDNF Control (n=9)</th>
<th>5 uM (n=5)</th>
<th>5 uM + GDNF (n=5)</th>
<th>10 uM (n=5)</th>
<th>10 uM + GDNF (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AVG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.6</td>
<td>8.933333</td>
<td>6</td>
<td>7.133333</td>
<td>4.866666</td>
<td>6.1333333</td>
</tr>
<tr>
<td><strong>STDEV</strong></td>
<td>0.968468</td>
<td>0.784915</td>
<td>0.755929</td>
<td>0.639940</td>
<td>0.74322335</td>
<td>0.6399407</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>0.306257</td>
<td>0.248212</td>
<td>0.338062</td>
<td>0.286190</td>
<td>0.33237959</td>
<td>0.28619008</td>
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<table>
<thead>
<tr>
<th></th>
<th>Rotenone 5 n M (n=5)</th>
<th>5 nM +GDNF (n=5)</th>
<th>10 nM (n=5)</th>
<th>10 nM +GDNF (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AVG</strong></td>
<td>5.533333</td>
<td>7.26667</td>
<td>4.8</td>
<td>6.6666667</td>
</tr>
<tr>
<td><strong>STDEV</strong></td>
<td>0.915475</td>
<td>0.883715</td>
<td>0.7745967</td>
<td>0.89973541</td>
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<tr>
<td><strong>SEM</strong></td>
<td>0.409413</td>
<td>0.395209</td>
<td>0.34641016</td>
<td>0.40237391</td>
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</tbody>
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