Effects of inhibitors of apoptotic pathways in Parkinson's disease models

by

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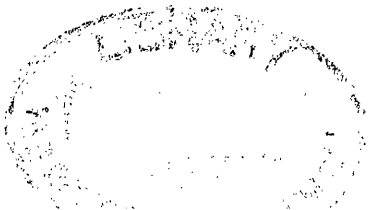

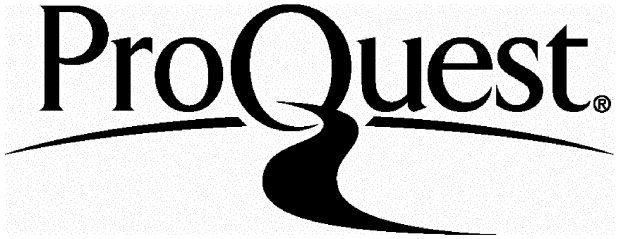

To Elaine and Alexander

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Abstract

Dopaminergic cell death in models of Parkinson's disease has been extensively studied, but the mechanisms underlying the toxicity remain unclear. Some reports have indicated that the cell death is apoptotic and involve the caspase family of cysteine proteases; there is some controversy in the literature regarding this. The current study was undertaken to evaluate the mechanism of cell death and apoptotic pathways activated in the 1-methyl-4-phenlypyridinium (MPP^+) model of dopaminergic cell death.

A culture model for primary foetal rat mesencephalon was established and the presence of dopaminergic neurones validated. The role of the caspases was evaluated, using both commercially available and novel selective inhibitors of the caspase pathway. Immunocytochemical staining was used to visualise active caspase, and to evaluate apoptotic nuclear morphology. A similar approach was used to examine the role of the stress activated mitogen activated protein kinases c-jun-N-terminal kinase and p38. Novel selective inhibitors were used to block these pathways; and immunocytochemistry used to evaluate survival effects on dopaminergic neurones and on expression of phosphorylated c-jun. Finally, a model of *in vivo* dopaminergic cell death was established using the toxin 6-hydroxydopamine; the effects of toxin treatment on expression of phosphorylated c-jun in the nigra was evaluated.

The results show that MPP^+ induced apoptosis is mediated through caspases, especially caspase 3, and that inhibition of the JNK pathway but not the p38

pathway spares cells by inhibiting caspase 3 activation. In the *in vivo* studies it was shown that 6-OHDA causes nigral cell death and phosphorylation of c-jun.

Publications

Papers

Bilsland J, Roy S, Xanthoudakis S, Nicholson DW, Han Y, X, Grimm E, Hefti F, Harper SJ (2002) Caspase inhibitors attenuate 1-methyl-4-phenylpyridinium toxicity in primary cultures of mesencephalic dopaminergic neurons. Journal of Neuroscience 22: 2637-2649.

Review articles

Bilsland J and Harper S (2002) Caspases and neuroprotection. Current Opinion in Investigational Drugs 3: 1745-1752

Abstracts

Bilsland J, Roy S, Nicholson D, Hefti F, Harper, S (1999) Effects of caspase inhibitors on MPP^+ toxicity in primary cultures of mesencephalic dopaminergic neurones in vitro. Presented at British Neuroscience Association 15^{th} Annual Meeting, Harrogate, England, May, 1999.

Harper S, Bilsland J, Roy S Nicholson DW, Hefti F (1999) Caspase inhibitors prevent MPP^+ toxicity on cultured mesencephalic dopaminergic neurones. Presented at 29th Annual Meeting of the Society for Neuroscience, Miami, October 1999

Bilsland J, Harper S, Roy S, Nicholson D, Hefti F (1999) Caspase inhibitors prevent MPP^+ toxicity on cultured mesencephalic dopaminergic neurones. Presented at FENS Winterschool, Kitzbuhel, Austria, December 1999

Bilsland J, LoGrasso P, Hefti F, Harper S (2000) Inhibition of caspases and c-Jun N-terminal kinase, but not p38, is neuroprotective in primary cultures of rat mesencephalic dopaminergic neurones treated with 1-methyl-4-phenylpyridinium. Presented at Federation of European Neuroscience Societies, Brighton, UK, June 2000.

Bilsland J, LoGrasso P, Hefti F, Harper S (2000) Inhibition of caspases and c-Jun N-terminal kinase, but not p38, is neuroprotective in primary cultures of rat mesencephalic dopaminergic neurones treated with 1-methyl-4-phenylpyridinium. Presented at British Society for Cell Biology Apoptosis Meeting, Edinburgh, September 2000.

Bilsland J, LoGrasso P, Hefti F, Harper S (2000) Inhibition of caspases and c-Jun N-terminal kinase, but not p38, is neuroprotective in primary cultures of rat mesencephalic dopaminergic neurones treated with 1-methyl-4-phenylpyridinium. Presented at 30th Annual Meeting of the Society of Neuroscience, New Orleans USA, November 2000

Harper SJ, Bilsland J, Roy S, Xanthoudakis S, Nicholson D, Han Y, Grimm E, Hefti F (2001) Caspase inhibition prevents toxicity of MPP^+ in primary cultures of dopaminergic neurones. Presented at The Developing and Regeneration Brain, London, September 2001

Bilsland J, Harper S. (2002) MPP^+ induces expression of phosphorylated c-jun and apoptotic morphology in primary mesencephalic dopaminergic neurones; regulation by caspase, JNK and p38 inhibitors and growth factors. FENS 2002, Paris

Table of contents

Text

Title page	1
Dedication	2
Acknowledgements	3
Abstract	4
Publications	6
Contents	8
List of abbreviations	15
Chapter 1: General introduction	19
1.1. Parkinson's Disease	20
1.2. MPTP/MPP^+ as a model of Parkinson's disease	21
1.3. Apoptosis	27
1.4. Caspases	29
1.4.1. The caspase family – structure and regulation	29
1.4.2. Caspase pathways and apoptosis	33
1.4.3. Inhibitors of caspases	37
1.5. Stress activated MAP kinases	41
1.5.1. The MAP kinase family	41
1.5.2. JNK and p38 signalling pathways	44
1.5.3. Inhibitors of JNK and p38	47
1.6. Aims and outline of project	50
Chapter 2: Materials and Methods	52
2.1. Materials	53
2.2. Cell culture	54
2.2.1. Mesencephalic dopaminergic neurone preparation	54
2.2.2. Coating of tissue culture plastic with poly-D-lysine	55

2.2.3. Preparation of Sato serum substitute 56
2.2.4. Treatment of cultures with MPP^+ 56
2.2.5. Compound preparation 57
2.3. Analysis techniques 58
2.3.1. Fixation of cells 58
2.3.2. Immunostaining for TH – peroxidase 58
2.3.3. Quantification of TH-immunoreactive neuronal survival and morphology 60
2.3.4. Analysis of ^{3}[H]-DA uptake by dopaminergic neurones. 61
2.3.5. Analysis of apoptotic profiles 62
2.3.6. Double immunocytochemistry for TH and apoptotic markers 62
2.4. Evaluation of cell death pathways in 6-OHDA lesioned rat brain *in vivo* 63
2.4.1. 6-OHDA lesions and perfusions 63
2.4.2. Quantification of TH-immunoreactive neurones *in vivo* models 65
2.4.3. Immunostaining for phosphorylated c-jun 67
2.5. Statistical analyses 68

Chapter 3: Validation of culture model 70
3.1 Introduction 71
3.2. Results 73
3.2.1. Culture characterisation 73
3.2.2. MPP^+ toxicity for dopaminergic neurones in primary mesencephalic culture 81
3.2.3. Growth factors attenuate MPP^+ dopaminergic toxicity 85
3.5. Discussion 89

Chapter 4: MPP^+ induces caspase mediated apoptosis in primary dopaminergic neurones 93
4.1. Introduction 94
4.1.1. Caspases in neurodegeneration 94

4.1.2. Caspase mediated apoptosis in Parkinson's disease and models 98
4.1.3. Chapter aims 99
4.2. Results 100
4.2.1. Evidence for apoptosis and caspase activation following MPP^+ treatment of dopaminergic neurones. 100
4.2.2. Neuroprotection by caspase inhibition 104
4.2.2.1. Effects of zVAD-fmk 104
4.2.2.2. Peptide caspase inhibitors 112
4.2.2.3. Novel caspase inhibitors 115
4.2.3. Effects of caspase inhibition on expression of apoptotic features. 123
4.3. Discussion 127
4.3.1. Results summary 127
4.3.2. Apoptosis/caspase activation 128

Chapter 5: MPP^+ toxicity is partially mediated by JNK but not p38 induced caspase activation. 141
5.1. Introduction 142
5.1.1. Neuronal apoptotic regulation by JNK and p38 142
5.1.2. Neuroprotection by inhibitors of JNK and p38 147
5.1.3. JNK and p38 in Parkinson's Disease and Parkinsonian models 151
5.1.4. Aims of this chapter 152
5.2. Results 154
5.2.1. Neuroprotection by JNK but not p38 inhibition 154
5.2.2. MPP^+ increases expression of phosphorylated c-jun 163
5.2.3. JNK activation is upstream of caspase 3 activation 172
5.3. Discussion 175
5.3.1. Results summary 175
5.3.2. General discussion 176

Chapter 6. Unilateral 6-OHDA administration to adult rats increases c-jun phosphorylation in dopaminergic neurones 183

6.1. Introduction 184
6.1.1. *In vivo* models of Parkinson's Disease 184
6.1.2. The 6-OHDA model of Parkinson's Disease 185
6.1.3. Aims of this chapter 188
6.2. Results 189
6.3. Discussion 198

Chapter 7: General discussion 202
7.1. General discussion 203
7.2. Future directions 216
7.3. Final conclusion 218

Reference list 219
Publications 271

Figures

Figure 1.1 The caspase family. 32
Figure 1.2 Primary pathways of caspase activation. 34
Figure 1.3 Simplified diagram of MAP kinase signalling pathways. 45
Figure 2.1 Localisation and dissection of ventral mesencephalon from 14 day gestation rat brain. 55
Figure 3.1 TH-immunoreactive neurones comprise a small proportion of the total cells in primary cultures of embryonic rat ventral mesencephalon. 74
Figure 3.2 The number of TH-immunoreactive neurones in primary mesencephalic cultures declines with time in culture. 76
Figure 3.3 Comparison of mesencephalic cultures maintained in 10% foetal bovine serum (FBS) with those maintained in Sato serum substitute. 78
Figure 3.4 Primary cultures of mesencephalic neurones take up ^{3}[H]-DA through the dopamine transporter. 80

Figure 3.5	MPP^+ reduces number of TH-immunoreactive neurones.	82
Figure 3.6	MPP^+ reduces uptake of ^{3}[H]-DA in primary mesencephalic neurones.	84
Figure 3.7	BDNF and GDNF pretreatment attenuates loss . of TH-immunoreactive neurones induced by MPP^+.	86
Figure 3.8	GDNF and BDNF pretreatment attenuates MPP^+ mediated decrease in $^{[3H]}$DA uptake.	88
Figure 4.1	MPP^+ causes nuclear chromatin condensation, a characteristic feature of apoptosis, in dopaminergic neurones.	101
Figure 4.2	MPP^+ increases expression of activated caspase 3 in TH-immunoreactive cells in primary cultures of mesencephalic neurones.	103
Figure 4.3	Effects of the broad-spectrum caspase inhibitor zVAD-fmk on MPP^+ treated TH-immunoreactive neurones.	106
Figure 4.4	zVAD-fmk attenuates MPP^+ mediated loss of TH-immunoreactive neurones in primary mesencephalic cultures.	107
Figure 4.5	zVAD-fmk prevents MPP^+ mediated loss of somatic area in TH-immunoreactive cells, and partially restores longest neurite length	108
Figure 4.6	MPP^+ mediated decrease in ^{3}[H]DA uptake by primary mesencephalic cultures is only partially attenuated by zVAD-fmk co-administration.	110
Figure 4.7	Pretreatment of mesencephalic cultures with zVAD-fmk does not prevent MPP^+ mediated loss of ^{3}[H]DA uptake.	111
Figure 4.8	Peptide inhibitors of caspases 2, 3 and 9, but not of caspase 1, prevent MPP^+ mediated loss of TH-immunoreactive neurones.	113
Figure 4.9	Novel inhibitors of caspases prevent MPP^+ toxicity for primary cultures of mesencephalic dopaminergic neurones.	118
Figure 4.10	Effectiveness of novel caspase inhibitors in preventing MPP^+ toxicity for dopaminergic neurones correlates well with their reported IC_{50} values for caspase 3 inhibition in human NT2 cells.	119
Figure 4.11	Novel caspase inhibitors have little effect on the number of TH-immunoreactive neurones when added for 48 hours in the absence of MPP^+.	120

Figure 4.12	M-920 and M-791 are less effective in restoring ^{3}[H]DA uptake in MPP^{+} treated dopaminergic neurones.	122
Figure 4.13	Caspase inhibition prevents MPP^{+} mediated increase in cells with apoptotic nuclei, but has little effect on the expression of active caspase 3.	125
Figure 5.1	Neuroprotective effects of the JNK pathway inhibitor CEP-1347 on TH-immunoreactive neurones in primary mesencephalic cultures.	154
Figure 5.2	IC_{50} values for JNK and p38 inhibitors on three JNK isoforms, JNK3α1, JNK2α1, JNK2 α2, and p38 in both whole cell and cell free assays using purified enzymes.	156
Figure 5.3	The direct JNK/p38 inhibitor Compound 1 promotes survival of mesencephalic TH-immunoreactive neurones.	157
Figure 5.4	Effects of a range of inhibitors of JNK/p38 on survival of TH-immunoreactive neurones exposed to MPP^{+}.	158
Figure 5.5	Selective inhibitors of p38 have little neuroprotective effect on MPP^{+} treated TH-immunoreactive neurones.	159
Figure 5.6	The dual JNK/p38 inhibitor Compound 1 increases uptake of ^{3}[H]DA in primary mesencephalic cultures exposed to MPP^{+} 0.1μM.	161
Figure 5.7	The selective p38 inhibitor compound 2 does not restore ^{3}[H]-DA uptake in primary mesencephalic cultures exposed to MPP^{+} 0.1μM for 48 hours with no recovery period.	162
Figure 5.8	MPP^{+} induces expression of phosphorylated c-jun in TH-immunoreactive cells in primary cultures of mesencephalic neurones.	165
Figure 5.9	Time course of induction of phosphorylated c-jun (serine 63) immunoreactivity and nuclear chromatin condensation in control and MPP^{+} treated TH-immunoreactive neurones and total cell population.	166
Figure 5.10	Photomicrographs of mesencephalic neurones treated with MPP^{+} in the presence and absence of inhibitors of JNK, p38 or caspases for 24 hours.	169
Figure 5.11	Quantification of P-jun immunoreactivity and apoptotic nuclear morphology in TH-immunoreactive neurones exposed to MPP^{+} 10μM for 24 or 48 hours in the presence or absence of JNK inhibitor, p38 inhibitor, caspase inhibitors or growth factors.	170
Figure 5.12	Quantification of P-jun immunoreactivity and	171

	apoptotic nuclear morphology in mesencephalic neurones exposed to MPP^+ 10μM for 24 or 48 hours in the presence or absence of JNK inhibitor, p38 inhibitor, caspase inhibitors or growth factors.	
Figure 5.13	Effects of JNK and p38 inhibitors on expression of active caspase-3 in TH-immunoreactive neurones exposed to MPP^+ 10μM for 24 or 48 hours.	173
Figure 6.1	Effects of unilateral 6-OHDA infusion into the medial forebrain bundle on the number of nigral TH-immunoreactive neurones.	190
Figure 6.2	Photomontage of induction of expression of phosphorylated c-jun (serine 63) in the ipsilateral substantia nigra and ventral tegmental area 7 days following unilateral 6-OHDA injection.	192
Figure 6.3	Quantification of timecourse of c-jun induction in substantia nigra and ventral tegmental area in ipsilateral and contralateral hemispheres following unilateral 6-OHDA injection	193
Figure 6.4	Phosphorylated c-jun (serine 63) is expressed in TH-immunoreactive cells in the ipsilateral substantia nigra and VTA 7 days following unilateral 6-OHDA injection into the medial forebrain bundle	195
Figure 6.5	Quantification of surviving and phosphorylated c-jun expressing TH-immunoreactive neurones in the substantia nigra and VTA of 6-OHDA lesioned rat brain 7 days following unilateral 6-OHDA administration.	196

List of Abbreviations

6-OHDA	6-hydroxydopamine
AAV	adeno-associated virus
ABC	avidin-biotin complex
AIF	apoptosis inducing factor
AP-1	activator protein 1
APAF	apoptosis activating factor
ASK-1	apoptosis signal-regulating kinase-1
ATF	activating transcription factor
Bax	bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
BDNF	brain derived neurotrophic factor
BH	Bcl homology
Bid	BH3 interacting death agonist
Bim	Bcl-2 interacting mediator of cell death
Boc-Asp-fmk (BAF)	benzyloxycarbonyl-aspartyl (OMe) fluoromethylketone
BSA	bovine serum albumin
CARD	caspase recruitment domain
Caspase	cysteine aspartase
C. elegans	Caenorhabditis elegans
Ced	cell death abnormal
CHOP	C/EBP homologous protein
CNS	central nervous system
CREB	cyclic AMP response element binding protein
Cox	cyclooxygenase

DA	dopamine
DAB	diaminobenzidine
dATP	deoxyadenosine triphosphate
DED	death effector domain
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DPM	disintegrations per minute
DRG	dorsal root ganglion
ELK	eph-like kinase
ERK	extracellular signal-related kinase
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
H_2O_2	hydrogen peroxide
HPLC	high pressure liquid chromatography
IAP	inhibitor of apoptosis
ICE	interleukin-1β converting enzyme
JIP	JNK inhibitory protein
JNK	c-jun amino-terminal kinase
MAO	monoamine oxidase
MAPK	mitogen activated protein kinase
MAPKAP	MAP kinase activated protein kinase

MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MCAO	middle cerebral artery occlusion
MEF	myocyte enhancer factor
MFB	median forebrain bundle
MLK	mixed lineage kinase
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADH	nicotinamide adenine dinucleotide
NAIP	neuronal apoptosis inhibitory protein
NFAT	nuclear factor of activated T-cells
NGF	nerve growth factor
NGS	normal goat serum
NHS	normal horse serum
PBS	phosphate buffered saline
PD	Parkinson's disease
P-jun	phosphorylated c-jun
RNA	ribonucleic acid
SAP Kinase	stress activated protein kinase (JNK)
SMAC/DIABLO	second mitochondrial factor activating caspases/direct inhibitor of apoptosis binding protein with low pi
SNPC	substantia nigra pars compacta
TH	tyrosine hydroxylase
TNF	tumor necrosis factor
TRITC	tetramethylrhodamine isothiocyanate
TX-100	t-Octylphenoxypolyethoxyethanol

VTA	ventral tegmental area
XIAP	X-chromosome linked inhibitor of apoptosis
zDEVD-FMK	benzyloxycarbonyl-Asp-Glu-Val-Asp (OMe) fluoromethylketone
zIETD-fmk	benzyloxycarbonyl-Ile-Glu-Thr-Asp (OMe) fluoromethylketone
zLEHD-fmk	benzyloxycarbonyl-Leu-Glu-His-Asp (OMe) fluoromethylketone
zVAD-FMK	benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone
zVAD-DCB	benzyloxycarbonyl-Val-Ala-Asp dichlorobenzoyloxymethylketone
zVDVAD-fmk	benzyloxycarbonyl-Val-Asp-Val-Ala-Asp (OMe) fluoromethylketone
zYVAD-fmk	benzyloxycarbonyl-Tyr-Val-Ala-Asp (OMe) fluoromethylketone

Chapter 1:

General introduction

1.1. Parkinson's disease

Parkinson's disease is a movement disorder primarily characterised by rigidity, tremor, and bradykinesia (reviewed in Marsden 1990). The pathological hallmark of Parkinson's disease is a loss of catecholaminergic neurones in various areas of the brain (reviewed in Agid et al., 1990). While the loss of these catecholaminergic neurones is not restricted to the dopaminergic neurones of the substantia nigra pars compacta, this has been the most discussed area of cell loss, as it is implicated in the generation of the movement disorders which typify the condition. The dopaminergic neurones in the substantia nigra pars compacta project axons through the median forebrain bundle to the striatum, where they innervate widely. Disruption of striatal innervation in animal models mimics the movement disorders seen in idiopathic Parkinsonism. A wide array of potential causative factors for Parkinson's disease has been suggested, which include environmental toxins such as pesticides and a genetic susceptibility to the condition (reviewed in Olanow and Tatton, 1999). It seems plausible that the mechanism triggering the disease may be any of these, or a combination of factors. The loss of cells in the substantia nigra is gradual, taking place over the course of years; by the time patients present clinically, however, it is likely that in the order of 70% of the cells in this region will have been lost.

This gradual cell loss, together with the low numbers of remaining cells at post mortem, have meant that the aetiology of cell death in Parkinson's disease has remained elusive. A great deal of research has accordingly been directed towards

understanding the mechanisms underlying the actual neuronal loss. There are a number of animal models which mimic the condition, and the mechanism of cell death in these models is the subject of intense research. Among the most commonly used models of Parkinson's disease are the dopaminergic toxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA). The primary aim of this research project was to examine the *in vitro* toxicity of the active metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP^+) for primary dopaminergic neurones to attempt to elucidate intracellular signalling pathways activated by the toxin. As well as the MPP^+ work *in vitro*, an *in vivo* model of Parkinson's disease was set up using 6-OHDA as a dopaminergic toxin. The data generated in the *in vitro* models give an insight into the complexities of the signalling pathways activated by the neurotoxin MPP^+.

1.2. MPTP/MPP^+ as a model of Parkinson's disease

The toxic effects of MPTP were first identified when it was synthesised as a by-product in a 'synthetic heroin' mixture, and intravenously injected by a number of drug users in California. A number of these patients developed symptoms closely resembling those of idiopathic Parkinson's disease, including immobility, resting tremor, and Parkinsonian postural abnormalities; these symptoms initially responded to levodopa therapy (Langston et al., 1983, Langston and Ballard, 1984, Ballard et al., 1985). An autopsy of one patient with MPTP induced Parkinson's disease showed a selective loss of the dopamine neurones in the substantia nigra, with no effect on other cell types within the brain. This finding

was confirmed by studies in a number of species of primates, where MPTP treatment caused a Parkinson's disease like condition. Histological evaluation of the brains of these MPTP treated primates showed highly selective loss of nigral dopaminergic neurones and their striatal terminals, with little or no damage to other areas of the brain (Langston et al., 1984a, Burns et al., 1983). Rodent studies showed that in mouse, MPTP treatment led to damage to the substantia nigra similar to that observed in primates, with a particularly marked decrease in dopaminergic nerve terminals (Heikkila et al., 1984, Hallman et al., 1985). In rat there was no evidence of dopaminergic cell loss (Boyce et al., 1984), though there were locomotor deficits and neurochemical changes through increased dopamine release (Sahgal et al., 1984, Schmidt et al., 1984, Sirinathsinghji et al., 1986). In rat embryonic mesencephalon *in vitro*, however, treatment with MPTP resulted in toxicity to dopaminergic neurones and decreased dopamine uptake, effects which were inhibited by treatment of the cultures with pargyline, an inhibitor of monoamine oxidase (Mytilineou and Cohen, 1984).

Following MPTP treatment in primates, a pyridinium metabolite, 1-methyl-4-phenylpyridinium (MPP^+) was detected in the brain (Markey et al., 1984, Langston et al., 1984b). When MPTP was administered with MAO inhibitors, the formation of MPP^+ was prevented in both primates (Langston et al., 1984c) and mice (Markey et al., 1984); MAO inhibition also caused a significant decrease in the neurotoxicity produced by MPTP treatment (Heikilla et al., 1984). The effect was greater with inhibitors of MAO B than with inhibitors of MAO A. This led to the conclusion that the toxin responsible for the degeneration of the nigral

neurones following MPTP treatment was the metabolite MPP^+ rather than MPTP itself. A number of studies confirmed that MPP^+ was indeed a potent neurotoxin for dopaminergic neurones. *In vivo*, intraventricular injection of MPP^+ into both mice and rats produced a marked toxicity for dopaminergic neurones in the substantia nigra (Bradbury et al., 1986). *In vitro*, MPP^+ treatment of organotypic explants and dissociated cultures of rat midbrain caused selective toxicity for dopaminergic neurones (Mytilineou et al., 1985, Sanchez Ramos et al., 1986); in the dissociated cultures, there was no toxic effect when MPTP was added.

Thus the neurotoxicity following MPTP treatment is mediated by the metabolite MPP^+. The oxidation of MPTP by MAO B into MPP^+ does not occur in the dopaminergic neurones themselves, which have little MAO B, but in astrocytes (Ransom et al., 1987). The selective oxidation of MPTP by the MAO B isoform also explains the lack of effect in rat, which has a decreased level of MAO B compared to MAO A in comparison to human or primate (Kinemuchi et al., 1985). The MPP^+ produced is then released from the astrocytes and taken up into dopaminergic neurones through the dopamine transporter; it is this specific uptake which confers the selectivity of toxicity for dopaminergic neurones. Blockade of the dopamine transporter with mazindol or nomifensine prevents degeneration of dopaminergic neurones following treatment with either MPTP or MPP^+ *in vivo* (Ricaurte *et al.*, 1985, Sundstrom et al., 1986). Additionally, transfection of either the rat or human dopamine transporter into mammalian cell lines of both neuronal and non-neuronal origin confers toxicity to low concentrations of MPP^+, an effect which is dependent on the expression level of the dopamine transporters. This

toxicity can be inhibited with mazindol (Pifl et al., 1993), indicating that it is indeed the uptake of MPP^+ through the dopamine transporter which induces the selective toxicity for dopaminergic neurones.

This selective toxicity for dopaminergic neurones has been studied extensively using dopaminergic neurones *in vitro*. In primary cultures of dissociated rat mesencephalon, MPP^+ treatment for 48 hours at 10μM results in a selective decrease in tyrosine hydroxylase (TH) immunoreactive cells, TH enzymatic activity, TH mRNA, ^{3}[H]-dopamine (^{3}[H]-DA) uptake and levels of catecholaminergic immunofluorescence within the cultures (Sanchez Ramos et al., 1988, Michel et al., 1989, Michel et al., 1990, Beck et al., 1991). The toxicity of MPP^+ for dopaminergic neurones is irreversible (Michel et al., 1990). There is no toxicity for non-dopaminergic cells present within the cultures, as measured by uptake of ^{3}[H]-GABA, and by counting the number of non-TH immunoreactive cells within the cultures at MPP^+ concentrations up to 10μM. In addition, treatment of septal neurones in culture with 10μM MPP^+ for 48 hours caused neither a decrease in numbers of cholinergic neurones, choline acetyltransferase activity, nor high affinity choline uptake. Thus, at concentrations up to 10μM, MPP^+ is a selective toxin for dopaminergic neurones. At concentrations above 10μM, there are reported to be toxic effects on the other cell types within mesencephalic cultures (Sanchez Ramos et al., 1988, Michel et al., 1990). At high concentrations, MPP^+ is also toxic to cerebellar granule cells. This toxicity may be mediated by uptake of MPP^+ through the glutamate transporter; this uptake proceeds at a slower rate than uptake through the dopamine transporter,

but the intracellular MPP^+ concentration reached is comparable to that in dopaminergic neurones (Du et al., 1997).

Following uptake, MPP^+ accumulates intracellularly, where it is taken up into mitochondria via an energy dependent system for cations. Within the mitochondria, MPP^+ causes a specific, reversible inhibition of NADH ubiquinone oxyreductase (Complex I), the first step of the mitochondrial electron transport chain (Degli, 1998, Schapira, 1998). Complex I deficits are also a feature of idiopathic Parkinson's disease (Swerdlow et al., 1996). Intrastriatal administration of rotenone, a potent inhibitor of complex I, caused similar neurotoxicity to MPP^+ treatment (Langston and Irwin, 1986), implicating Complex I inhibition as an important factor in induction of MPP^+ toxicity. Complex I inhibition results in decreased production of ATP together with increased production of free radicals from the mitochondrial respiratory chain. The decrease in ATP production results in reduced cellular energy stores, leaving the neurones hypoenergetic and unable to perform functions necessary for survival (Gerlach *et al.*, 1991). The increased production of free radicals also damages the neurones through oxidative stress; free radical scavengers including catalase and vitamins E and C partially inhibit degeneration of dopamine neurones *in vitro* following MPP^+ treatment (Akaneya et al., 1995).

A wide range of factors have been shown to have neuroprotective effect against MPP^+/MPTP toxicity *in vitro* and *in vivo*. A partial list includes the growth factors brain derived neurotrophic factor (BDNF) (Beck et al., 1992), epidermal

growth factor (Hadjiconstantinou et al., 1991, Yoshinaga et al., 1998, 2000), fibroblast growth factors 1 and 2 (Otto and Unsicker, 1990), and glial cell line derived neurotrophic factor (GDNF) family members (Hou et al., 1996, Horger et al., 1998, Fan et al., 1998); of these, GDNF is the most widely characterised in animal models, and has been tested in clinical trials for Parkinson's disease. Other than growth factors, there are reports that immunophilin ligands such as FK506 and GPI-1046 also protect dopaminergic neurones from MPP^+/MPTP toxicity (Ross et al., 2001;Guo et al., 2001), though there is debate in the literature over the efficacy of GPI-1046 (Harper et al., 1999, Bocquet et al., 2001). A number of transgenic animals are also resistant to MPP^+ toxicity; some of these will be discussed later in the thesis.

Much research has been carried out for many years into the regulation of MPP^+ mediated cell death. Despite this, the machinery which couples MPP^+ uptake and Complex I inhibition to the death of the cell was largely unknown at the time of commencement of this research project. Data had emerged indicating that the cell death induced by MPTP/MPP^+ was apoptotic, a form of programmed cell death; however, there was, and is, some controversy in the literature regarding this. Apoptosis is a fundamental physiological programme, but aberrant activation can lead to neurodegeneration. Control of apoptosis is complex, and the next sections will provide an overview of the regulation of the process.

1.3. Apoptosis

The term apoptosis describes a form of programmed cell death which is characterised by cell shrinkage, membrane blebbing, chromatin condensation and breakdown of the cell into discrete membrane bound compartments followed by phagocytosis (Kerr et al., 1972). Apoptosis is crucial in development during the periods of naturally occurring cell death. In the adult, apoptosis is crucial for normal tissue turnover and homeostasis, and for the most part is a crucial physiological process (reviewed in Jacobson et al., 1997). Inappropriate regulation of apoptosis has, however, been implicated in the pathogenesis of a wide range of disorders. Where there is a lack of appropriate apoptosis, diseases of cellular ~~aggregation~~ proliferation such as cancer can occur. Where apoptosis is activated inappropriately, diseases of cell loss can occur. Inappropriate apoptosis has been implicated as a possible mechanism underlying the cell loss in a range of neurodegenerative conditions; these include Parkinson's disease, Alzheimer's disease, Huntingdon's disease, amyotrophic lateral sclerosis, and peripheral neuropathies.

Much of the early information on the mechanisms of apoptosis came from studies in the free living nematode worm Caenorhabditis elegans (*C. elegans*). A defined population of cells within *C. elegans* die by apoptosis during development. The cell death in *C. elegans* is regulated by the products of three genes *ced-3*, *ced-4* and *ced-9*, as demonstrated by genetic studies. These gene products have either pro- or anti-apoptotic effects.

CED-9 is anti-apoptotic; disruption of this pathway results in inappropriate death of cells which do not normally die during *C. elegans* development (Hengartner et al., 1992). The mammalian homologs of CED-9 are members of the Bcl-2 family of proto-oncogenes (Hengartner and Horvitz, 1994a, b). The Bcl-2 family is large and it contains three main groups of proteins. There are a group of anti-apoptotic family members, which includes Bcl-2 and bcl-X_L, and a group of pro-apoptotic members such as Bax and Bad. These proteins are localised on, or can insert into, the mitochondrial membrane, and pro- and anti- apoptotic activity is regulated by a dynamic balance between them. The third group contains a family of proteins which have one related domain to the rest of the family, known as a Bcl homology 3 (BH3) domain. These are primarily localised in the cytoplasm, but can be cleaved by a number of factors; this exposes a hydrophobic terminal which targets the protein to the mitochondrial membrane. Once inserted into the mitochondrial membrane, these proteins act as accessory factors to pro-apoptotic family members; they can be sequestered by anti-apoptotic family members (reviewed in Bratton and Cohen, 2001).

CED-4 is pro-apoptotic. Loss of function mutation of CED-4 results in lack of appropriate apoptosis (Yuan and Horvitz, 1992). The mammalian homolog of CED-4 was identified as apoptosis activating factor-1 (APAF-1) (Zou et al., 1997). This factor acts downstream of mitochondrial pro-apoptotic factors released during cellular stress; this will be discussed in more detail in the next section of this chapter. CED-9 and APAF-1 couple into apoptosis through

interactions with the last component of the signalling cascade, CED-3 and related proteases.

CED-3 is pro-apoptotic, and mutation again results in a failure of naturally occurring cell death in C. elegans (Horvitz et al., 1983, Avery and Horvitz, 1987, Yuan and Horvitz, 1990). The mammalian homologs of CED-3 are a family of enzymes which play a critical role in the execution of apoptosis, the caspases. The caspases have been demonstrated to play a pivotal role in apoptosis during development, during normal tissue homeostasis, and possibly also in the pathogenesis of many neurodegenerative conditions. The structure, function and regulation of the mammalian caspase family is complex, and will be discussed in detail in the next section of this chapter.

1.4. Caspases

1.4.1. The caspase family – structure and regulation

The prototypic caspase is interleukin-1β converting enzyme (ICE), which was cloned in 1992 and shown to cleave the inactive 31 kDa precursor of interleukin-1β at Asp116-Ala117 to generate the active form of the cytokine (Cerretti et al., 1992, Thornberry et al., 1992). In 1993, Yuan and coworkers (Yuan et al., 1993) demonstrated that the C. *elegans* cell death gene *ced-3* encoded a protein with 29% sequence homology to ICE. In this paper, it was also shown that both ICE and CED-3 proteins shared 27% sequence homology with another protein, mouse Nedd-2, which is developmentally expressed in neurones at the time of naturally

occurring cell death. It was proposed that the ICE/CED-3 family of proteases might be of importance in mediating apoptosis. In 1994, a third ICE like protease was cloned and shown to have significant homology to CED-3, ICE and Nedd-2 (Fernandes Alnemri et al., 1994). The discovery that this enzyme was responsible for the cleavage of cellular substrates such as poly (ADP-ribose) polymerase (Nicholson et al., 1995;Tewari et al., 1995) proved the involvement of this family of proteases in the execution of apoptotic cell death.

14 members of this family of proteases have been characterised to date, though not all appear to be conserved across species. All share a number of features; they are cysteine proteases containing the active site QACXG, which cleave cellular substrates specifically after aspartate residues (reviewed in (Salvesen and Dixit, 1997, Stennicke and Salvesen, 1998). This activity gave rise to the term caspase (cysteine aspartase), with ICE being caspase 1, Nedd-2/ICH-1 being designated caspase 2, CPP-32/Yama/Apopain being caspase 3 and other caspases being assigned names according to when they were cloned (Alnemri et al., 1995). The only other mammalian protease to share the caspase specificity for an aspartate residue in the P1 position is the T cell derived protease Granzyme B (Darmon et al., 1994, Gorman et al., 1998). All caspases are synthesised in a proenzyme form. The proenzyme is termed the prodomain and consists of an N-terminal polypeptide which is involved in self-processing of certain caspases to the active form, and two domains of approximately 10 and 20 kDa (reviewed in Stennicke and Salvesen, 1998, and Stennicke and Salveson, 2000). The proenzyme is cleaved at two sites by an enzyme such as granzyme B (Darmon et al., 1995) or

another caspase (Muzio et al., 1997), detaching the prodomain and separating the p10 and p20 domains. The p10 and p20 domains form a heterodimer, which then joins another heterodimer to form the active tetramer (Walker et al., 1994, Wilson et al., 1994, Fernandes Alnemri et al., 1994, Munday et al., 1995, Fernandes Alnemri et al., 1995). The activated enzyme then cleaves pro-cytokines, other caspases, or the cellular substrates that bring about the apoptotic response depending on the substrate specificity of the particular caspase.

Caspase family members can broadly be divided into three groups based on their activity, structure and substrate preferences (reviewed in (Stennicke and Salvesen, 1998, Nicholson and Thornberry, 1997); the substrate preferences will be discussed in detail in the section on caspase inhibitors below (4.1.3). Group 1 caspases include caspase 1, 4, 5, 11 and 13, and are involved more in the mediation of cytokine activation than the apoptotic response. The other two groups of caspases are more involved in the apoptotic cascade, being the activators and executioners of apoptosis. Group 2 caspases can be broadly termed the activators of apoptosis, and includes caspases 6, 8, 9, 10 and 12. These are caspases that function upstream of, and are capable of activating, the executioner caspases, and are homologous to Granzyme B and CED-3. The third group of caspases is the executioners of apoptosis, which cleave protein substrates involved in cellular homeostasis. This group contains caspases 2, 3, and 7. As well as this division of caspases based on function, they can also be divided based on their structural similarities or substrate preference; this is illustrated in Figure 1. For the purposes of this thesis, the functional designation will be used.

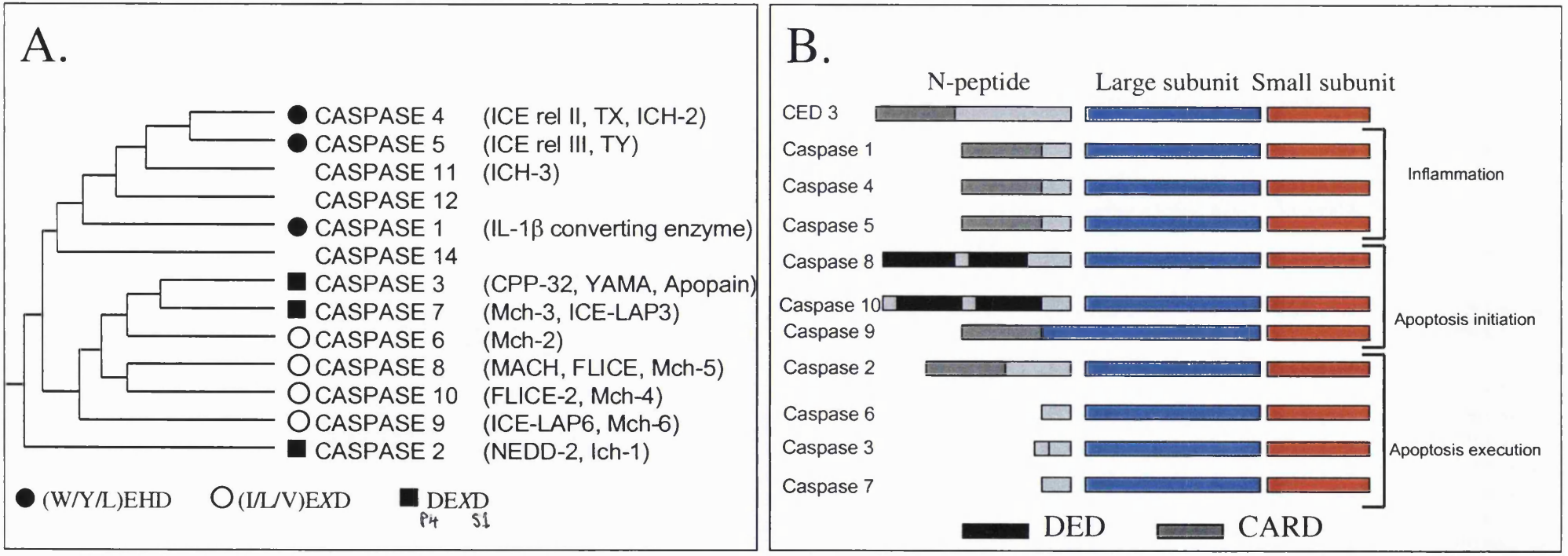


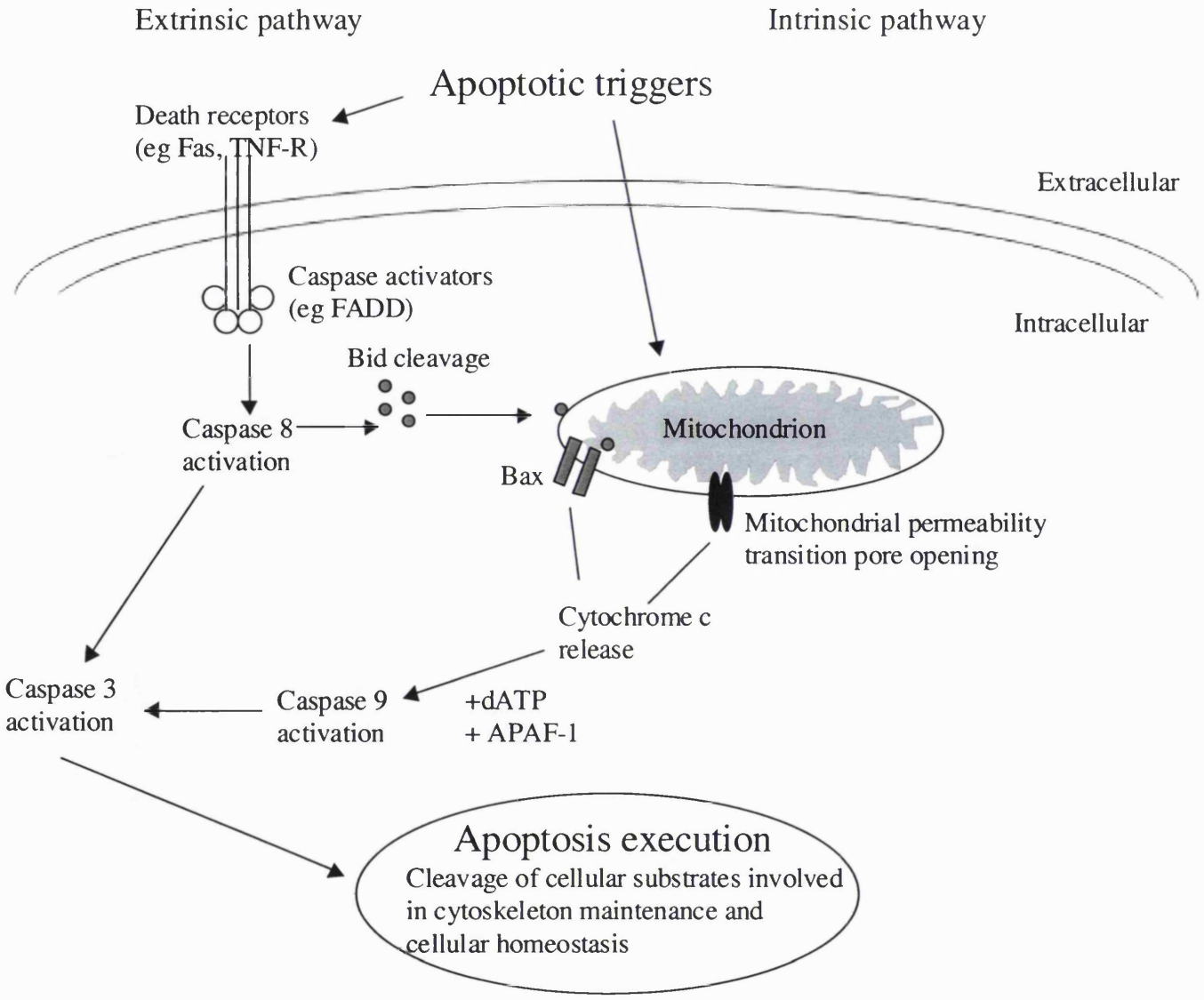
Figure 1.1. The caspase family. The phylogenetic relationship of caspase family members is shown in (A), together with their preferred substrate sequence (shown below; X can be any amino acid). By phylogenetic relationship, the caspases divide into 3 groups, with caspases 1, 4, 5, 11, 12, and 14 in one group, caspases 3, 6, 7, 8, 9, 10 in another, and caspase 2 in a separate third group. When organised by preferred substrate, the caspases fall into groups which are more aligned with their function. Thus, caspases 1, 4, and 5 form one group and these enzymes are thought to be more involved in the inflammatory response than in apoptotic execution. Caspases 6, 8 , 9, and 10 form a second group; caspases 8 and 9 are involved more in the initiation of apoptosis, cleaving and activating other downstream caspases. Caspases 2, 3 and 7 are the executioners of apoptosis, cleaving a wide range of cellular proteins involved in cytoskeleton maintenance and cellular homeostasis to bring about apoptosis Much of the structural variety of caspases is in the N-terminal prodomain (B). This contains, in the cases of caspases 10 and 8, tandem death effector domains (DED) which recruit the enzyme to death receptor adaptor molecules. CED-3, and caspases 1, 4, 5, 9, and 2 have caspase recruitment domains (CARD) which selectively target the enzyme to other caspases.

1.4.2. Caspase pathways and apoptosis

There are a number of well defined intracellular caspase cascades which culminate in caspase mediated apoptosis. In simplified form, two of the most extensively studied pathways are illustrated in Figure 1.2. The two pathways can broadly be described as a cell intrinsic pathway and a cell extrinsic pathway. The cell intrinsic pathway involves release of pro-apoptotic factors from the inner leaflet of the mitochondrial membrane into the cytoplasm, which then activate a caspase cascade. The cell extrinsic pathway involves ligation of cell surface death receptors such as tumour necrosis factor receptor (TNF-R) or CD95/Fas/Apo-1. This recruits a number of adaptor proteins to the intracellular receptor domain, resulting in a cascade of caspase activation and apoptosis. Though this defines a rough division between the two pathways, there is cross talk between the pathways at a number of levels, and a number of feedback loops exist which can link one to the other.

The cell intrinsic pathway has been the subject of intensive study in recent years. Mitochondria have been shown to have a crucial role in apoptotic execution in many models of cellular toxicity. Mitochondria couple into the apoptotic response through the release of pro-apoptotic factors into the cytoplasm following cellular stress. These apoptotic factors include cytochrome c (Liu et al., 1996, Kluck et al., 1997, Yang et al., 1997), second mitochondrial factor activating caspases/direct inhibitor of apoptosis (IAP) binding protein with low pi (Smac/DIABLO) (Verhagen et al., 2000, Du et al, 2000), and apoptosis inducing

Figure 1.2. Primary pathways of caspase activation in simplified form. Two major pathways are involved in activation of the apoptotic caspase cascade. The first is triggered by signals extrinsic to the cell through ligation of death receptors such as fas or TNF-α receptor. Ligation of these receptors recruits a number of adaptor molecules to the intracellular domain of the receptor, which culminate in activation of the initiator caspase 8 . This caspase in turn cleaves executioner caspases such as caspase 3, which cleave a wide range of intracellular substrates to effect the apoptotic response. The second major pathway which activates the caspase cascade is an intrinsic cellular response to a range of stressors, and involves release of a number of pro-apoptotic factors from the mitochondrion ; there is cross-talk between the two pathways at the level of caspase 8. Mitochondria are involved in apoptotic regulation through the release of pro-apoptotic factors into the cytoplasm. The best characterised of these factors is cytochrome c , which can be released either following a decrease in mitochondrial membrane potential and opening of the mitochondrial permeability transition pore,or through pores formed by pro-apoptotic Bcl-2 family members, such as Bax . Upon release into the cytoplasm, cytochrome c forms a complex designated the apoptosome with dATP and APAF-1 (apoptosis activating factor-1). This complex cleaves and activates procaspase 9, which in turn activates caspase 3 and thus brings about the apoptotic response . Both of the caspase cascade pathways converge upon activation of executioner caspases, of which the best characterised is caspase 3.


Extrinsic pathway
Intrinsic pathway
Apoptotic triggers
Death receptors
(eg Fas, TNF-R)
Extracellular
Caspase activators
(eg FADD)
Intracellular
Bid cleavage
Caspase 8
activation
Mitochondrion
Bax
Mitochondrial permeability
transition pore opening
Cytochrome c
release
Caspase 3
activation
Caspase 9
activation
+dATP
+ APAF-1
Apoptosis execution
Cleavage of cellular substrates involved
in cytoskeleton maintenance and
cellular homeostasis

factor (AIF). The first two of these factors interact with the caspase pathway; most of the research carried out to date has involved apoptotic regulation by cytochrome c.

Cytochrome c is released through the mitochondrion through one of two routes. Firstly, cellular stress can cause a catastrophic loss of mitochondrial membrane potential; this results in opening of a multi-protein large diameter channel in the membrane called the mitochondrial permeability transition pore (sometimes designated 'megachannel') through which cytochrome c and other pro-apoptotic factors can pass under certain conditions (Ichas and Mazat, 1998, Petronilli et al., 2001). The other route of cytochrome c release is through pores formed by pro-apoptotic members of the Bcl-2 family of proto-oncogenes such as Bax (Antonsson et al., 1997, Schendel et al., 1997). This pore formation is facilitated by mitochondrial insertion of BH-3 domain only family members (reviewed in Bratton and Cohen, 2001). Once released into the cytoplasm, cytochrome c forms a complex, known as the apoptosome, with dATP and apoptosis activating factor-1 (APAF-1), a mammalian homolog of the *C. elegans* death gene CED-4 (Liu et al., 1996, Chinnaiyan et al., 1996, Li et al., 1997, Zou et al., 1997). This complex then cleaves and activates the activator caspase 9; active caspase 9 then activates caspase 3 leading to cell death (Bossy-Wetzel et al., 1998, Pan et al., 1998, Cecconi et al., 1998, Yoshida et al., 1998, Kuida et al., 1998, Hakem et al., 1998, Cai et al., 1998, Cain et al., 2002). Blockade of cytochrome c activity or release spares compromised neurones from executing the apoptotic programme (Neame

et al., 1998). Many reviews of this system have been published (Jacobson, 1997, Reed, 1997, Green, 1998, Susin et al., 1998, Adrain and Martin, 2001, Bratton and Cohen, 2002). Smac/DIABLO is also released through the opening of the mitochondrial permeability transition pore, and also interacts with the caspase 9 pathway through inhibiting activity of an endogenous caspase 9 inhibitor, X-linked inhibitor of apoptosis (XIAP) (Srinivasula et al., 2000).

The cell extrinsic route to apoptosis is activated by death receptor ligation. This results in recruitment of a number of adaptor molecules to the receptor, which recruit and cleave caspase 8 (Chinnaiyan et al., 1995, Muzio et al., 1996, Srinivasula et al., 1996, Vincenz and Dixit, 1997). Activated caspase 8 can then activate caspase 3 like proteases directly (Los et al., 1995, Enari et al., 1995, Schlegel et al., 1996, Enari et al., 1996, Muzio et al., 1997), or feed into the mitochondrial pathway to cell death through cleavage of the BH3 domain only Bcl-2 family member Bid – this causes translocation from the cytoplasm to the mitochondrial membrane, and facilitation of Bax pore formation. Caspase 8 has also been implicated in the cleavage of caspases other than caspase 3, such as caspase 1.

Thus there are a number of routes through which caspases can be activated, and a number of well defined but interacting intracellular caspase cascades. Caspases have been implicated in the cell death following a number of models of neuronal toxicity; this will be discussed in more detail in the introduction to Chapter 4.

1.4. 3. Inhibitors of caspases.

The differing substrate specificities of the caspase family members has allowed the development of a number of peptide inhibitors (Zhou et al., 1997, Talanian et al., 1997, Garcia-Calvo et al., 1998, Rano et al., 1997, Thornberry et al., 1997, Margolin et al., 1997, reviewed in Grutter, 2000). Caspases have a near absolute requirement for an aspartic acid residue in the S1 subsite. See Figure 1.1 Substitutions in the p4 positions confer a degree of specificity, with caspases 1, 4, 5 and 13 preferring a hydrophobic residue, caspases 2, 3 and 7 requiring aspartic acid, and caspases 6, 8, 9, and 10 requiring a branched aliphatic residue (Figure 1.1). Peptide inhibitors contain a tetrapeptide which binds to the catalytic cysteine of the active caspase tetramer and prevent substrate cleavage. The inhibitors can be separated into reversible or irreversible inhibitors based on the modifications to the peptide. Irreversible inhibitors are α-substituted ketones with the structure peptide-CO-CH2-X with X being either a halide ion (eg fluoromethylketone (FMK), chloromethylketone (CMK)) or similar. Reversible inhibitors are aldehyde, ketone or nitrile modified peptides. The most commonly used caspase inhibitor is benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (z-VAD-FMK); this is a poly-caspase inhibitor, though it does not inhibit all members of the family, a notable exception being caspase 2 (Garcia-Calvo et al, 1998). Inhibitors based on the preferred cleavage sequence for specific caspases include Asp-Glu-Val-Asp (DEVD) for caspase 3, Tyr-Val-Ala-Asp (YVAD) or Trp-Glu-His-Asp (WEHD) for caspase 1, Leu-Glu-His-Asp (LEHD) for caspase 9, and Ile-Glu-Thr-Asp (IETD) for caspase 8. It is essential to note that while these inhibitors are based

upon the cleavage sequence for specific caspases, they have little or no selectivity for individual caspases. The LEHD sequence, for example, also inhibits other activator caspases such as 8 and 6, and possibly also some group I caspases. At the high concentrations typically used in reported studies, there is likely to be very little selectivity with these inhibitors, ~~certainly across related groups of caspases~~ across caspase families; there may be selectivity ~~between caspase groupings.~~ within caspase families

A number of companies have been actively developing small molecule caspase inhibitors. The current situation with regard to development of novel caspase inhibitors has recently been the subject of two excellent reviews (Denner, 1999, Ashwell, 2001); the review by Ashwell contains the structures of many of the compounds mentioned here. Merck Frosst, Warner Lambert, Vertex Pharmaceuticals and Idun Pharmaceuticals have developed series of caspase inhibitors, a number of which are claimed to have efficacy in *in vivo* models of neurodegeneration (Ashwell, 2001); Glaxo Smithkline have developed compounds with efficacy in *in vitro* models of neurodegeneration; they have also developed a novel class of non-peptide istatin sulphonamide derivative selective caspase 3/7 inhibitors which do not bind in the S1 aspartic acid binding pocket (Lee et al., 2000). These compounds have greater selectivity than the classical peptide inhibitors and thus may be more useful in dissecting the caspase pathways; as yet, however, they have not been tested in models of neurodegeneration.

With all classical peptide inhibitors and most of the novel inhibitors poor brain permeability has limited their usefulness *in vivo*. In most studies, the compounds require administration intracerebroventricularly – development of blood-brain barrier permeable compounds is thus important if caspase inhibitors are to be used *in vivo* or developed for clinical use. A range of blood brain barrier permeable compounds has been synthesised by Idun Pharmaceuticals (Deckwerth et al., 2001) and shown to be neuroprotective against cerebral ischaemia *in vivo*.

A further complication in interpreting these studies is the extent to which the caspases must be inhibited to prevent all activity. In both *in vivo* and *in vitro* models, a sustained inhibition of more than 95% of caspase activity is required to fully prevent cleavage of apoptotic substrates; in *in vivo* models of sepsis, where inhibitors do not need to cross the blood brain barrier, continuous IV infusion of caspase inhibitor is required to reach these levels (D.W. Nicholson, personal communication). Thus, it is difficult to interpret studies in which one ICV administration of caspase inhibitor is made, as it is unclear both for how long and to what extent caspases are inhibited, and also for how long and to what extent caspases require to be inhibited for a full functional recovery. This may particularly be a problem with peptide caspase inhibitors given the lack of selectivity associated with these compounds.

A number of endogenous cellular or virally encoded direct caspase inhibitors also exist. Cellular caspase inhibitors include the inhibitor of apoptosis family (IAP) of proteins; six members of this family have been identified to date, including

neuronal apoptosis inhibitory protein (NAIP) and X-chromosome linked inhibitor of apoptosis (XIAP). All members of this family inhibit processing of pro-caspase 9 and thus prevent activation of executioner caspases, as well as directly inhibiting caspase 3. Two virally encoded caspase inhibitors are cytokine response modifier A (crm-A) from cowpox virus, which inhibits all caspases apart from 3, 6 and 7, and p35 from baculovirus which inhibits caspases 1, 2, 3 and 4 (reviewed in Nicholson and Thornberry, 1997). Genetic manipulation of these factors has been used in *in vivo* models of neurodegeneration to examine the effects of caspase inhibition. Another means of blocking the activation of the caspase cascade is to target the release of pro-apoptotic factors from the mitochondrion. Release of cytochrome c through pores formed by Bax (see Figure 1.2) can be blocked by overexpression of anti-apoptotic Bcl-2 family members, and a number of compounds such as cyclosporin A and bongkrekic acid block opening of the mitochondrial permeability transition pore – this provides another means of examining the caspase cascade.

As well as targeting the caspases themselves, another possible means to prevent apoptosis is to target other factors upstream of caspases. A number of other factors are activated in response to cellular stress, and some of these cause apoptosis by activating the caspase cascade either through the intrinsic or extrinsic pathways. Among these factors are the group of stress activated protein kinases which have been shown to be involved in initiating the apoptotic programme in many models of neuronal injury.

1.5. Stress activated MAP kinases

1.5.1. The MAP kinase family

The family of mitogen activated kinases (MAP kinases) comprises the extracellular signal ~~-related~~ regulated kinase (ERK), p38 kinase and c-jun-N-terminal kinase (JNK), also known as stress activated kinase (SAPK). The MAP kinases are involved in a wide range of cellular functions; the ERK and JNK/p38 subgroups are reported generally to have opposing effects on apoptosis (Xia et al., 1995), though this is to some extent an oversimplification and is cell type and stimulus dependent. ERK is activated in response to growth factor binding and has been reported in a number of neuronal models to promote survival and neurite outgrowth. JNK and p38, conversely, are activated by a range of cellular stresses such as death receptor ligation and intracellular stress, and they are involved in activating a number of elements of the cell death cascade and inflammatory responses.

JNK and p38 both have a number of isoforms, with differing expression patterns. JNK exists in 10 isoforms, encoded by three genes, *jnk1, jnk2* and *jnk3* with alternative splicing giving rise to four JNK1 isoforms, 4 JNK 2 isoforms and 2 JNK 3 isoforms (Gupta et al., 1996). The different isoforms are called α or β depending on size, with the α subunits having a size of approximately 55kDa and the β subunits having a size of approximately 46kDa in JNK1 and JNK2; JNK3 has an extended N-terminus, and the subunits are thus larger, the α subunit being

approximately 57kDA and the β subunit being 49kDa. No functional differences between these splice variants have been reported. In *jnk1* and *jnk2* transcripts, there is another level of alternative splicing that has not as yet been reported for the *jnk3* transcript, giving rise to the four isoforms for each of JNK1 and JNK2; this alternative splicing involves exons within the kinase subdomains and results in slight modifications to the interaction with and modification of substrates *in vitro*. These splice variants are designated, for example, JNK1α1, JNK1α2, JNK1β1 and JNK1β2.

The expression patterns of the JNK isoforms differ. JNK1 and JNK2 are expressed at high levels throughout the body. In contrast, JNK3 is primarily expressed in the brain, with low levels of expression in the heart, the kidney and the testes. In the brain, *jnk* mRNAs form a specific pattern (Carletti et al., 1995), with JNK1 being restricted to the endopiriform nucleus and the medial habenula; this may not, however, give a true picture of JNK1 protein expression, as JNK1 protein has been found in cortical homogenates (Mielke et al., 1999), and has been reported to be downregulated in cortex of kainic acid treated animals (Ferrer et al., 1997). *Jnk2* and *jnk3* mRNAs have a more widespread distribution in the brain. Carboni and coworkers (Carboni et al., 1997, 1998) show expression of *jnk1* mRNA with a more widespread distribution; in this study, *jnk1* is expressed in the hippocampus, the cerebellar granule layer, the medial habenulae, the red nucleus, the anterodorsal thalamic nucleus, the pontine nucleus, the facial nucleus, the motor and mesencephalic nuclei of the trigeminal nerve, the hypoglossal and vestibular nuclei and the nucleus ambiguus. *Jnk2* mRNA expression follows a

similar pattern to that of *jnk1*, and both have high levels of expression elsewhere in the body, particularly the immune system. JNK3 expression is more restricted to the brain, and is observed at high levels in the endopiriform cortex, the medial habenula and at lower levels in the hippocampus (Kuan et al., 1999). Expression of jnk3 is developmentally regulated, with higher levels being observed in the postnatal period; this may indicate a role in developmental programmed cell death.

Transgenic animals lacking JNK isoforms have been developed, and offer insight into the role of the various JNK isoforms. Animals lacking any one of the three JNK isoforms are developmentally normal, and have no gross differences from wildtype animals. Double deletions of either JNK1 or JNK2 with JNK3 again results in animals which are apparently normal; double deletion of JNK1 and JNK2 however, results in embryonic lethality at around gestation day 11-12, with severe brain abnormalities caused by both reduction of developmental cell death in some brain areas such as the hindbrain, and increased apoptotic cell death in others. Thus JNK1 and JNK2 together appear to play a role in developmental cell death regulation (Kuan et al., 1999); JNK3 appears to play a role in mediating cellular responses to stress, as JNK3 null mice are resistant to kainic acid induced seizures (Yang et al., 1997) – given the primarily neuronal distribution of JNK3, this may make it an attractive target for neuroprotective strategies.

p38 exists in four homologs, p38α, p38β, p38γ, and p38δ, with additional alternative splicing. The major p38 isoform in the brain is p38β2 (Enslen et al.,

1998), though high levels of p38α are also expressed. In the brain, p38 is expressed at high levels in the cortex and in the hippocampus (Mielke et al., 1999). There is no expression of p38γ or p38δ in brain.

1.5.2. JNK and p38 signalling pathways

The signalling pathways of JNK and p38 are complex, and contain 'crosstalk' with other pathways at various points. They involve kinase cascades which culminate in both cases in dual phosphorylation of JNK or p38, and subsequent phosphorylation of nuclear and non-nuclear substrates. There are several levels of activation of the JNK and p38 pathways (shown in simplified form in Figure 1.3); the kinases which act directly upstream of JNK and p38 are the MAP kinase kinases (MKKs), which in turn are phosphorylated and activated by the MAP kinase kinase kinases(MKKKs), a range of factors which includes enzymes such as mixed lineage kinase (MLK), MEKK1 and Ask-1. These factors are activated by a range of cellular stresses including death receptor ligation by TNF or fas ligand, UV irradiation, treatment with toxic compounds, osmotic shock, cytokines, growth factor signalling or oxidative stress (reviewed in Minden and Karin, 1997, Cobb, 1999, Kyriakis and Avruch, 2001).

Activation of JNK is mediated by phosphorylation at two sites, a tyrosine residue and a threonine residue. The MKKs which phosphorylate JNK are MKK4 and MKK7, also collectively known as the JNK kinases (JNKKs). Either MKK4 or MKK7 is required for JNK activation; and for maximal JNK1 (Lawlor et al.,

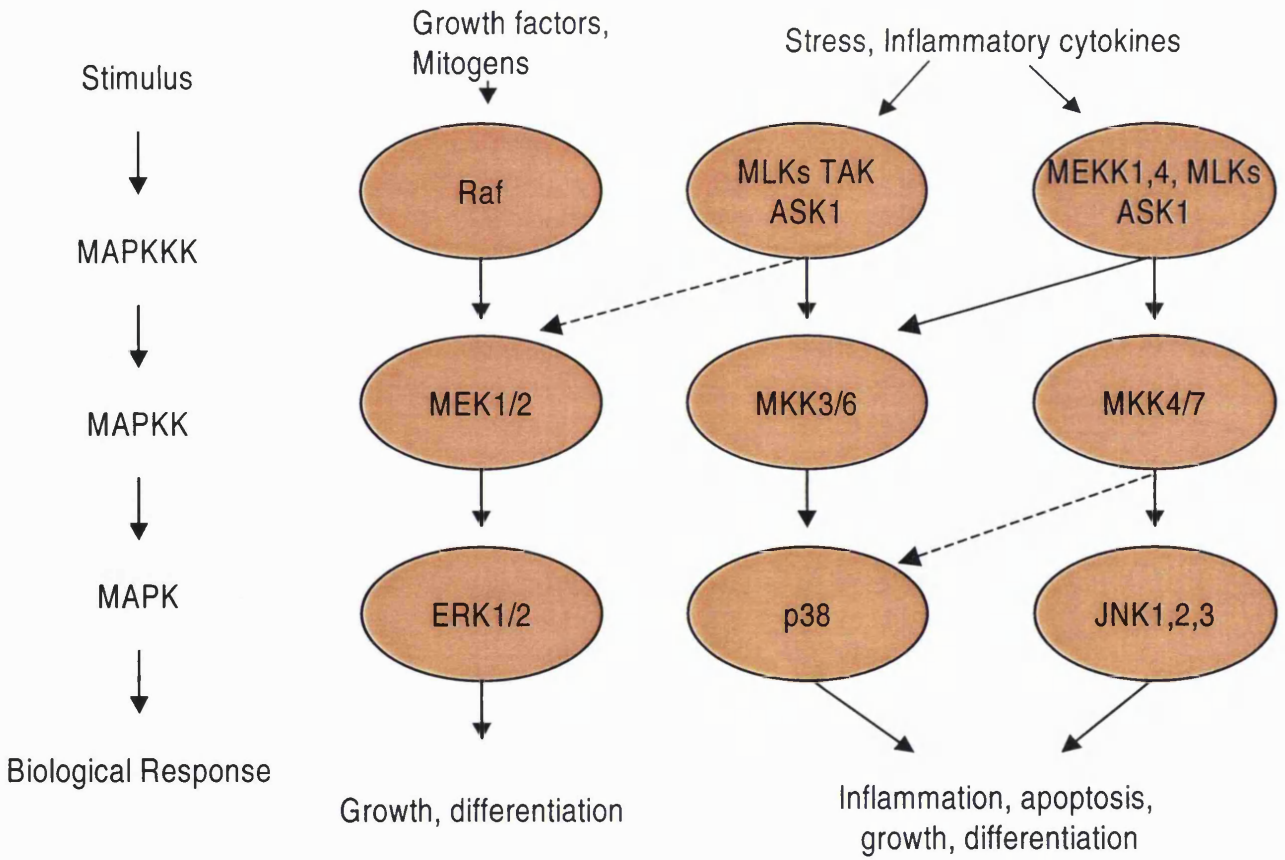


Figure 1.3. Simplified diagram of MAP kinase signalling pathways. MAP kinases (MAPK) are activated by phosphorylation by upstream MAP kinase kinases (MAPKK) which are themselves activated upon phosphorylation by MAP kinase kinase kinase (MAPKKK). MAPKKK are activated by a range of factors. The ERK pathway is activated by growth factor binding; JNK and p38 pathways can also be activated by growth factor binding, but are also activated by UV irradiation, oxidative stress, osmotic shock, and cytokines such as interleukin-1β. p38 is also activated by lipopolysaccharide.

1998) or JNK3 (Lisnock et al., 2000) activation *in vitro*, both of these kinases are required. This latter study also demonstrated that MKK7 phosphorylation of the JNK3 threonine residue was required prior to MKK4 mediated phosphorylation of the tyrosine residue.

The primary nuclear targets of JNK include the transcription factor c-jun, which is phosphorylated at two serine residues in the amino terminus, at positions 63 and 73. Other transcription factors activated by JNK include ATF2 and ELK1; NFAT4 is phosphorylated and inactivated by JNK. Activation of transcription factors by JNK leads to transcription of a number of factors, which includes the death receptor ligand, fas ligand. Non-nuclear targets of JNK include Bcl-2 and glucocorticoid receptors, both of which are inactivated by phosphorylation by JNK. Tau proteins and neurofilament are also phosphorylated by JNK. Activation of the JNK pathway also has a modulatory effect on pro-apoptotic Bcl-2 family members; JNK activation induces BH-3 domain only Bcl-2 family members in neurones (Harris and Johnson, 2001) which lead to Bax dependent cell death; auto-oxidised dopamine has also been reported to activate the JNK pathway, leading to decreased Bcl-2 and increased Bax expression (Kang et al., 1998). JNK signalling substrates are reviewed in (Mielke and Herdegen, 2000, Harper and Lograsso, 2001). Thus there are a number of possible ways in which the JNK pathway can activate the caspase pathways described above.

Activation of p38 occurs when it is dually phosphorylated on tyrosine and threonine residues. There is overlap of the nuclear targets of JNK and p38; p38

also phosphorylates ATF2 and ELK1. Other nuclear targets of p38 include CREB, MEF2C and CHOP; all of these are activated upon p38 phosphorylation. Non-nuclear targets include Tau proteins, as in the case of JNK, and the family of MAP kinase activated protein kinases (MAPKAP) which in turn interact with other factors such as heat shock protein 27 (reviewed in New and Han, 1998, Harper and LoGrasso, 2001, Mielke and Herdegen, 2000).

Thus there is some overlap in the signalling pathways leading to activation of JNK and p38, and a degree of overlap in the targets of the active JNK and p38 kinases. It is hypothesised that these enzymes might act synergistically in some cases; both JNK and p38 can increase activity of the AP-1 promoter through induction of *c-jun* and *c-fos* by phosphorylation of ATF2 and ELK1. JNK, however, selectively phosphorylates c-jun, and phosphorylated c-jun is implicated in a number of models of neurodegeneration. It is interesting, then, to attempt to unravel to what extent the JNK and p38 kinases are involved in mediating neuronal toxicity; the development of inhibitors with selectivity between the two enzymes has allowed this.

1.5.3. Inhibitors of JNK and p38

Until comparatively recently there were no small molecule inhibitor of JNK or the JNK pathway. A number of studies utilised overexpression of dominant negative JNK or c-jun mutants to examine the contribution of the JNK pathway to neuronal apoptosis (discussed in more detail in Chapter 5). Other approaches used transgenic animals lacking JNK isoforms or endogenous inhibitors of JNK such

as JNK inhibitory protein-1 (JIP-1), a scaffold protein which holds JNK in the cytoplasm preventing c-jun phosphorylation. Recently, however, several companies are developing compounds which inhibit JNK or the JNK pathway.

The best characterised of these JNK inhibitory compounds is the Cephalon compound CEP-1347. This compound, also called KT-7515, is a bis-thioethylmethyl analog of the indolcarbazole K252a (Kaneko et al., 1997), which exerts a trophic effect on neurones at lower concentrations but is a potent non-selective protein kinase inhibitor at higher concentrations. CEP-1347 is itself reported to inhibit upstream of JNK, at the level of MLK-3 (Maroney et al., 2001, Xu et al., 2001); the compound also inhibits with less potency at a number of other kinases, however. CEP-1347 has been tested widely in models of neurodegeneration and has been shown to exert neuronal survival promoting effects in many *in vivo* and *in vitro* models; the compound also has neurite outgrowth promoting activity. These data in neuronal models will be discussed in more detail in Chapter 5. CEP-1347 has recently entered Phase II clinical trials in Parkinson's disease. Cephalon have developed similar compounds, of which the most widely available is CEP-11004 (Murakata et al., 2002).

A number of other companies have been actively developing inhibitors of JNK; Signal Pharmaceuticals have developed a range of JNK inhibitory compounds (Bennett et al., 2001), which have been tested in models of neurodegeneration; as yet the effects of these compounds in neuronal toxicity models have been presented only in abstract form (Raymon et al., 2000). A number of other

companies have pursued JNK inhibitors, including GlaxoSmithkline, Applied Research Systems, and Hoffmann-La-Roche (reviewed in Harper and LoGrasso, 2001). Thus far, few of these compounds have been tested in neuronal models.

With regard to p38 inhibitors, a number of these have been reported on in the literature. Unfortunately two of the most widely used p38 inhibitory compounds, SB-203580 and SB-202190, are also potent inhibitors of JNK. This has made many of the reported studies using these compounds impossible to interpret. Recently, a number of more selective compounds have been reported; SB-239063 is reported to have 2000-fold selectivity for p38 over other kinases, including JNK, and to protect neurones in a number of models of toxicity (see Chapter 5). Merck have developed a range of highly selective p38 inhibitors (Liverton et al., 1999), and effects of some of these compounds will be described within the work presented here. Counterscreens for p38 inhibitory compounds also revealed a number of related structures with activity at JNK; a number of these compounds will also be described in this thesis.

1.6. Aims and outline of project

The primary aim of this project was to investigate apoptotic pathways in Parkinson's disease toxin models *in vitro* by using novel and commercially available inhibitors of components of the intracellular apoptotic cascades. At the time of commencement of the project, in 1997, there was some debate in the literature regarding the contribution of apoptosis to MPP^+ toxicity. Thus, early experiments were designed to investigate whether the cell death was apoptotic and caspase mediated.

To investigate this a primary culture model of embryonic rat mesencephalic dopaminergic neurones was established and validated; the toxicity of MPP^+ was confirmed and a number of compounds from the literature tested to validate the model. The apoptotic nature of the cell death induced by MPP^+ was confirmed by assessment of nuclear morphology in the cultures.

To investigate apoptotic processes in dopaminergic neurones exposed to MPP^+, selected commercially available inhibitors of various caspases were tested for neuroprotective effect. Several of these compounds were neuroprotective; the peptide inhibitors are of little selectivity and potency, however, so a number of selective caspase 3-like protease inhibitors from the Merck-Frosst caspase programme were tested. The most potent of these caused near complete neuroprotection. These latter inhibitors gave an insight into the important contribution of caspase 3 in effecting the cell death; none of the inhibitors tested

were sufficient to restore functional responses, however, as measured by uptake of ^{3}[H]-DA.

Given the importance of the caspase cascade in mediating the toxicity of MPP^+, experiments were undertaken to evaluate the possible contributions of the JNK and p38 pathways in mediating the toxicity. Initially the Cephalon compound CEP-1347 was tested; this partially protected neurones from MPP^+ toxicity. Novel inhibitors identified as having activity at JNK and p38 were also tested for neuroprotective effect. A direct dual JNK/p38 inhibitor caused partial protection to a similar extent as CEP-1347, and decreased activation of caspase 3. Inhibition of p38 alone was ineffective; this clearly demonstrated the greater importance of the JNK pathway than the p38 pathway in modulating MPP^+ toxicity. The partial neuroprotection afforded by JNK inhibition was investigated; total JNK activity inhibition did not result in a total blockade of caspase activation or of apoptotic profiles indicating that other, possibly compensatory, mechanisms may also be activated.

Finally, given the efficacy of JNK inhibition in protecting cells from MPP^+ toxicity *in vitro* and reported protective effects of CEP-1347 against MPTP toxicity *in vitro*, experiments were undertaken to evaluate the role of JNK in another *in vivo* Parkinson's disease model, 6-hydroxydopamine toxicity. A model of 6-OHDA toxicity for nigral dopaminergic neurones was established and validated, and it was demonstrated that 6-OHDA treatment indeed activated JNK and caused c-jun phosphorylation prior to the majority of nigral cell death.

Chapter 2:

Materials and Methods

2.1. Materials

14 day gestation Sprague-Dawley rats were obtained from Harlan (UK) Ltd. Male Sprague Dawley rats for *in vivo* experiments were purchased from Bantin and Kingman (Hull, UK). Hank's balanced salt solution, Dulbecco's modified Eagle's medium, 2.5% trypsin solution, 70μm cell strainers, 48 well culture clusters and 8 well chamber slides were purchased from Invitrogen Corp. (Paisley, UK). Antibiotic/antimycotic solution, foetal bovine serum, poly-D-lysine hydrobomide, phosphate buffered saline tablets, Trypan Blue solution, boric acid, bovine serum albumin fraction V, progesterone, putrescine, L-thyroxine, sodium selenite, tri-iodo-thyronine, 1-methyl-4-phenylpyridinium, Triton X-100, hydrogen peroxide 30% solution, ascorbic acid, EDTA, mazindol, 6-hydroxydopamine with ascorbic acid, Cy-3 conjugated horse anti-mouse IgG, Cy-3 conjugated goat anti-rabbit IgG, FITC conjugated goat anti-rabbit IgG, Extravidin FITC and Extravidin Cy-3 were purchased from Sigma-Aldrich Co. (Poole, UK). Bottle top filters were purchased from Becton-Dickinson Labware, (UK). Vectastain Elite ABC kits, normal goat serum, normal horse serum, biotin conjugated horse anti-mouse IgG and Vector SG insoluble peroxidase substrate were purchased from Vector Laboratories (Peterburgh, UK). Rabbit anti-tyrosine hydroxylase antiserum was purchased from Institut Jacques Boy SA (Reims, France). Mouse monoclonal anti-tyrosine hydroxylase was purchased from Chemicon Inc (Harrow, UK). All peptide caspase inhibitors were purchased from Calbiochem/Novabiochem Inc (Nottingham, UK). Rabbit polyclonal anti-active caspase 3 and rabbit polyclonal anti phosphorylated c-jun (serine 63) were

purchased from Cell Signalling Technologies Inc (Hitchin, UK). Hoechst 33342 was purchased from Molecular Probes Inc (Oregon, USA). Haematoxylin, Optimax buffer, and DAB immunostaining kits were purchased from Biogenex (UK) Ltd.

2.2 Cell culture

2.2.1. Mesencephalic dopaminergic neurone preparation.

All tissue culture preparation and treatments were carried out under aseptic conditions in a laminar flow cabinet. Primary cultures of mesencephalic dopaminergic neurones were prepared from 14-day gestation Sprague-Dawley rat embryos. Pregnant female rats were killed by stunning and decapitation, and embryos removed. The embryos were decapitated, and the heads transferred to 10ml sterile Hank's balanced salt solution. Using fine forceps, the skull was peeled from each head to expose the brain; brains were then transferred to fresh sterile HBSS. The cortices were detached from the midbrain, and the ventral mesencephalon dissected from each brain (see diagram below, Figure 2.1). Meninges were stripped from the tissue, which was transferred into 1.8ml HBSS. After all ventral mesencephali dissected, 0.2ml 2.5% trypsin stock was added to the tissue, to give a final concentration of 0.25% trypsin. Tissue was incubated in trypsin for 15 minutes at 37°C/5% CO_2; trypsin was then stopped by the addition of 5ml Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotic/antimycotic solution and 10% foetal bovine serum (FBS). Tissue was transferred to a centrifuge tube, and spun at 1000rpm for 10 minutes. The

supernatant was aspirated, and the cell pellet resuspended in 1ml DMEM/10%FBS. The pellet was triturated to give a single cell suspension; this suspension was then passed through a 70μm cell strainer. Cell suspension was diluted 1:10 in Trypan Blue, and viable (i.e. Trypan Blue excluding) cells counted in an improved Neubauer haemocytometer. Cell suspension was diluted to the required density with DMEM/10% FBS, and plated onto poly-D-lysine coated tissue culture plates (as described below). Cultures were incubated at 37°C/5%CO_2 for two hours, then the medium was aspirated and replaced with DMEM supplemented with 10% serum or serum substitute as described below.

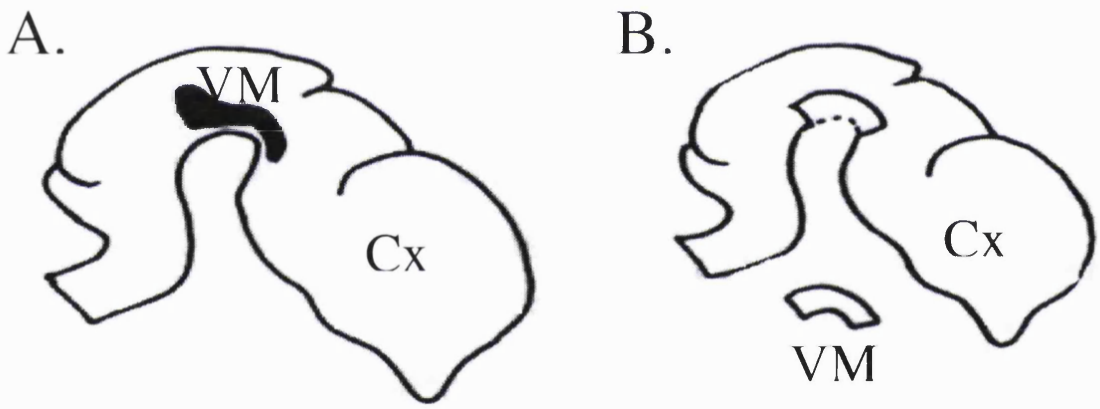


Figure 2.1. Localisation and dissection of ventral mesencephalon from 14 day gestation rat brain. In A, ventral mesencephalon is shaded region. This area is microdissected from the brain (B). VM, ventral mesencephalon; Cx, Cortex. Diagram adapted from PhD thesis of Eric Meyer, University of Cambridge.

2.2.2. Coating of tissue culture plastic with poly-D-lysine.

Tissue culture plates and 8-well chamber slides were coated with poly-D-lysine hydrobromide with a molecular weight greater than 300,000. 3.09g boric acid

was dissolved in sterile dH_2O, and the pH adjusted to 8.4. The solution was then passed through a bottle top filter with 0.22μm pore size, and 30mg poly-D-lysine added to the sterilised solution. Once prepared, poly-D-lysine was stored in aliquots at –20°C. For coating of plates and chamber slides, poly-D-lysine was added to each well for at least 30 minutes at room temperature. After 30 minutes, poly-D-lysine was aspirated, and wells washed with two changes of sterile dH_2O. Plates or slides were allowed to air dry in a laminar flow cabinet prior to plating of cells.

2.2.3. Preparation of Sato serum substitute

For a majority of experiments, cells were maintained in Sato serum substitute, a modification of the supplement defined by Bottenstein and Sato (1979). Final concentration of Sato in well 4.3mg/ml BSA, 0.77μg/ml progesterone, 20μg/ml putrescine, 0.49μg/ml L-thyroxine, 0.048μg/ml selenium and 0.42μg/ml tri-iodo-thyronine. A stock solution of this supplement was prepared and stored at –20°C until required for use. For preparation of medium, 2.75ml of stock solution was added to 100ml DMEM supplemented with antibiotic antimycotic solution.

2.2.4. Treatment of cultures with MPP^+.

For survival assays, cultures were maintained at 37°C/5% CO_2 for 5 days following plating. Medium was then aspirated, and compounds added 15 minutes prior to addition of MPP^+. For MPP^+ toxicity experiments, and for MPP^+ signalling analysis experiments in 8-well chamber slides, medium was aspirated and replaced with 250μl DMEM/Sato. 15 minutes following change of medium,

25µl MPP^+ was added at 11X final concentration directly to the medium in the well. For the majority of toxicity experiments, 10µM MPP^+ was used; to prepare this, a stock of 110µM MPP^+ was prepared, and 25µl added to relevant wells. Control wells were treated with 25µl DMEM/Sato alone. Cultures were typically incubated for timepoints up to 48 hours for signalling experiments, and for 48 hours for survival experiments, then fixed by the addition of 4% paraformaldehyde as described below.

In ^{3}[H]-DA uptake assays, MPP^+ was again added directly to the medium present in the wells at 11X stock concentration. Compounds were added either 15 minutes, six hours or 24 hours prior to MPP^+ addition, and in a number of experiments MPP^+ was added for only 24 hours, with a further 24 hour recovery period prior to assay. Compounds were tested for neuroprotective effects against a number of MPP^+ concentrations, ranging from 0.1µM to 10µM.

2.2.5 Compound preparation

Typically, compounds were prepared in DMEM supplemented with Sato and antibiotic/antimycotic solution at the required concentrations, and added to dopaminergic neurones 15 minutes prior to MPP^+ addition. For some experiments, compounds were preadministered for a longer time; where this is the case, the method is indicated in the individual chapter. When stock compound concentrations were prepared in DMSO, stocks were prepared such that the final DMSO concentration added to cells did not exceed 1%.

2.3. Analysis techniques

2.3.1. Fixation of cells

For immunocytochemistry experiments, cultures were fixed using 4% paraformaldehyde. One volume of 4% paraformaldehyde was added directly to the medium in wells, and incubated for 10 minutes at room temperature. This was then pipetted off using a 1ml Gilson pipette to minimise disruption of the cultures, and a further volume of 4% paraformaldehyde was added for 15 minutes at room temperature. This was aspirated, and the cells washed 3 times with PBS/0.3% t-Octylphenoxypolyethoxyethanol (TX-100).

2.3.2. Immunostaining for tyrosine hydroxylase - peroxidase

Immunostaining for tyrosine hydroxylase for cell survival assays was carried out using a rabbit polyclonal antiserum raised against TH. These assays typically were carried out on mesencephalic neurones prepared on 8-well chamber slides. Volumes for 8-well chamber slides are 250µl per well, except where otherwise stated; wash steps used 400µl per well. Cultures were fixed using 4% paraformaldehyde as described above; cells were then washed three times using phosphate buffered saline (PBS) supplemented with 0.3% TX-100. 400µl per well 5% normal goat serum was prepared in PBS/0.3% TX100, and added to the cultures for one hour at room temperature to block non-specific binding sites. This was then aspirated without washing, and primary antiserum added at a dilution of 1:5000 in 5% NGS/PBS/0.3% TX100. Cultures were refrigerated overnight in primary antiserum at 4°C.

The next day, antibody was aspirated and the cultures washed three times with PBS/0.3% TX100. Biotin conjugated goat anti-rabbit IgG (2.5μg/ml) prepared in 5% NGS/PBS/0.3% TX100 was added to the cultures as secondary antibody for one hour at room temperature. After incubation in secondary antibody cultures were washed three times in PBS/0.3% TX100, and peroxidase conjugated avidin-biotin complex (ABC reagent) prepared in PBS/0.3% TX100 was added for one hour at room temperature. Both the secondary antibody and ABC reagent were prepared from the Vectastain Elite ABC kit according to the manufacturer's instructions. For secondary antibody one drop (approximately 50μl) of antibody was added per 10ml buffer immediately before use; for ABC reagent two drops of avidin solution and two drops of biotin solution were added per 5ml buffer and incubated for at least 30 minutes prior to use. After incubation in ABC reagent, cultures were washed three times in PBS/0.3% TX100, and the staining visualised with Vector SG insoluble peroxidase substrate according to manufacturer's instructions. To prepare substrate, three drops of chromogen and three drops of H_2O_2 were added per 5ml PBS/0.3% TX100 immediately before use. Staining of dopaminergic neurones was typically apparent within 1- 3 minutes of substrate addition, and the reaction was monitored using an inverted microscope throughout. Once staining was apparent, the substrate was aspirated and the reaction stopped by the addition of 500μl dH_2O per well for 10 minutes. This was then aspirated and the cultures washed three times with PBS/0.3% TX100. The buffer was then aspirated and the gasket removed from each chamber slide; slides were mounted with coverslips using Shandon Immunomount aqueous mountant.

2.3.3. Quantification of TH-immunoreactive neuronal survival and morphology.

Prior to quantification, all slides were blinded by another investigator and only unblinded once all quantification was complete. To determine TH-immunoreactive cell survival, cells were observed under transmitted light on a Zeiss Axiovert inverted microscope using a 10X objective. Counts were made of all the TH-immunoreactive cells present in each well. The culture conditions described here typically produce a yield of around 0.5% – 1% TH-immunoreactive cells, or around 1500 cells in a control well. Data were meaned, and expressed as a percentage of the number of cells in untreated control cultures.

MCID image analysis (Brock University, Ontario, Canada) was used to evaluate the somatic area of TH-immunoreactive neurones. Area quantification was made from dopaminergic neurones in one experiment, from untreated control cultures, cultures treated with MPP^+ 10μM for 48 hours, and cultures treated with MPP^+ 10μM in the presence of zVAD-fmk 100μM or 300μM. One hundred cells were measured from random fields of view throughout each of four wells for each treatment group. To quantify area in μm^2, the image analysis system was first calibrated in μm using a graticule. Area of immunostained soma were then established using the Autoscan tool. For each neurone, a control density was set outside the area of the stained soma; the stained area of the soma was then established. Neurites were excluded from each measurement. Mean areas for

soma within each area were then established, and the results presented as the mean area across four wells.

For neurite length measurements, MCID image analysis was used to quantify the length of the longest neurite for each of 100 TH-immunoreactive neurones in four wells per treatment group. The image analysis system was calibrated as described above. Neurite length measurements were taken from either control cultures, cultures exposed to MPP^+ 10μM for 48 hours, or cultures exposed to MPP^+ 10μM in the presence of zVAD-fmk 300μM. To determine neurite length, a sample tool was used to draw manually along the length of the longest visible neurite. Results were expressed as mean neurite length for each group.

2.3.4. Analysis of ^{3}[H]-DA uptake by dopaminergic neurones.

To analyse ^{3}[H]-DA uptake, ^{3}[H]-DA uptake assay mix was prepared. DMEM was supplemented with 5.6mM D-glucose, 0.2mg/ml ascorbic acid, 1.3mM EDTA, and 0.5μCi/ml ^{3}[H]-DA. Negative control samples contained the dopamine transport blocker mazindol at 10μM. Medium was removed from samples, and wells washed once with DMEM. ^{3}[H]-DA uptake assay mix was added for 30 minutes at 37°C/5% CO_2. This was then removed, and the cultures washed twice with assay mix in the absence of ^{3}[H]-DA. Cells were lysed by the addition of 95% ethanol for 30 minutes at 37°C/5% CO_2, and samples pipetted into scintillation vials containing 4ml aqueous scintillant. To analyse uptake, disintegrations per minute were analysed on a Beckman LS 6000TA scintillation

counter. Mean scintillation count data were expressed as disintegrations per minute or normalised to percentage of control response.

2.3.5. Analysis of apoptotic profiles

To analyse apoptotic profiles, the nuclear dye Hoechst 33342 (2'-[4-Ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzamidole) was prepared in PBS at a concentration of 2μg/ml. Following immunostaining with fluorescent antibodies, the dye was added to cells for 30 minutes; cells were then washed and nuclei visualised under UV fluorescence on a Zeiss Axiovert 25 or Leitz DMRB microscope. To allow analysis of nuclear morphology in dopaminergic neurones, cultures were co-labelled for TH using a monoclonal antibody followed by a fluorophore as described below. Apoptotic nuclei showed chromatin condensation.

2.3.6. Double immunocytochemistry for TH and apoptotic markers

For double immunostaining experiments with polyclonal apoptotic marker antibodies, a monoclonal antibody raised against TH was used. Cultures were treated with MPP^+ and compounds as required, then fixed with 4% paraformaldehyde as previously described. Cells were washed, and non-specific binding sites were blocked with 5% normal horse serum in PBS/0.3% TX100 for one hour at room temperature. Blocking serum was then aspirated, and primary antibodies added together in 5% NHS/PBS/0.3% TX100 and refrigerated overnight at 4°C. The next day, slides were washed with PBS/0.3 TX100, and secondary antibodies added. Secondary antibodies were prepared in 5%

NHS/PBS/0.3% TX100. The optimal choice of secondary antibodies was determined during initial titration experiments with the non-TH antibody in single immunolabelling experiments. Typically, a biotinylated secondary antibody was applied together with an antibody conjugated to a fluorophore, generally Cy-3; TRITC was used in a number of experiments, but gave a fainter staining intensity. Cells were incubated in secondary antibodies for one hour at room temperature, then washed and incubated with the second fluorophore conjugated to avidin – typically extravidin fluorescein was used. Nuclei were counterstained using Hoechst 33342 (see above), and the slides mounted using Shandon Immunomount aqueous mountant. Double labelling experiments were carried out using primary antibodies raised against the active form of caspase 3 and serine 63 phosphorylated c-jun.

2.4. Evaluation of cell death pathways in 6-OHDA lesioned *in vivo* rat brain

For all *in vivo* experiments, stereotaxic surgery was carried out by Miss D. Pearce and Dr. S. Harper and rats perfused by Dr. M. Rigby. Sections were processed and embedded in paraffin wax by Mrs. A. Jennings. All cutting, immunostaining and quantification was carried out by myself.

2.4.1. 6-OHDA lesions and perfusions

Medial forebrain bundle lesion experiments were carried out in accordance with the Home Office Animals (Scientific Procedures) act 1986. Animals were housed in a pathogen free environment and maintained on a 12h:12h light dark cycle with free access to food and water. The numbers of animals used for these studies was

the minimum required to demonstrate reliable effects. Surgery was carried out under aseptic conditions. Following surgery the animals were placed in a heated incubator until fully awake. All animals were checked 4 times daily in the first 48h and then daily thereafter, by trained animal care staff. Body weights were recorded in order to monitor animals' welfare.

In these studies male Sprague Dawley rats in the weight range of 250-300g were used. Animals were anaesthetised with isoflurane until deep anaesthesia was obtained as determined by loss of paw withdrawal and blink reflexes. Animals were shaved over the top of the head and the skin was swabbed with antiseptic solution. Animals were placed in a David Kopf stereotaxic frame. A cut was made in the skin and bregma was exposed by scraping the surface of the skull. Once the location of bregma was determined a small hole was drilled in the skull at a position –2.2 in the anterior-posterior direction and –2.0mm in the medial-lateral direction. Once the depth of the dura was determined the canula was lowered to a position –7.9mm below dura. 8 μg of 6-hydroxydopamine/ascorbate solution (2.5mg/ml reconstituted with sterile PBS). Cannula was lowered into position, left for 1 minute, then 6OHDA was infused at 1μl per minute (3.2 min) and cannula was left in place for a further three minutes to prevent diffusion back up the tract left by the cannula.

After the required amount of recovery time for each experiment (48 hours to 14 days), animals were deeply anaesthetised with Euthatal (1ml/kg) until there was no paw withdrawal reflex. Animals were perfused with a solution of 4%

formaldehyde in saline, and brains were processed for paraffin embedding. 6μm sections were cut throughout the substantia nigra, and two placed on each slide. Typically, every 10th slide was immunostained throughout the substantia nigra.

2.4.2. Quantification of TH-immunoreactive neurones in *in vivo* models

To quantify the cell loss following unilateral 6-OHDA lesion, sections were immunostained using a polyclonal primary antiserum raised against TH. Sections were dewaxed and rehydrated through graded ethanol. Sections were dewaxed in two changes of two minutes each in xylene, then rehydrated through two changes of 100% ethanol, one change of 95% ethanol and one change of 70% ethanol, each at two minutes per treatment. Sections were placed in running dH_2O for two minutes, then washed 3x2 minutes in PBS. Endogenous peroxidase was blocked by incubating the sections in PBS/0.3% H_2O_2 for 30 minutes at room temperature. Slides were washed 3 times with PBS, and immunostaining carried out.

For immunostaining, the Biogenex Optimax automated slide staining system was used. A one day immunostaining protocol was used. All reagents were prepared in proprietary Optimax buffer, and all steps were carried out at room temperature. Prior to immunostaining, a pap pen was used to draw across the top and bottom of each slide above and below the sections. Non-specific binding was blocked by 30 minute incubation in 5% normal goat serum; blocking buffer was then removed without washing and rabbit polyclonal anti-TH was added at 1:1000 in 5% normal goat serum. Sections were incubated in primary antibody for two hours, then were washed twice and secondary antibody added. The secondary antibody used

was goat anti-rabbit IgG, biotin conjugate, prepared from the Vectastain Elite ABC kit by adding one drop of antibody to 10ml 5% normal goat serum (approximately 2.5μg/ml final antibody concentration). Sections were incubated in secondary antibody for 30 minutes; following this treatment, slides were washed and incubated in peroxidase conjugated avidin-biotin complex, prepared from the Vectastain Elite ABC kit as the manufacturer's instructions one hour prior to addition. Slides were incubated in ABC reagent for 30 minutes, then were washed and peroxidase visualised using diaminobenzidine (DAB) prepared from the Biogenex DAB kit. Slides were incubated in DAB for 10 minutes, then were washed once with dH_2O, and three times with PBS. Nuclei were counterstained using haematoxylin, which was fixed using acid alcohol (0.5% HCl in 70% ethanol) prepared immediately prior to use. Following counterstaining, sections were dehydrated and cleared following the reverse of the dehydrating and dewaxing protocol previously described, and slides mounted with coverslips using DPX.

For quantification of TH-immunoreactive cells in the substantia nigra and the VTA, slides from all animals within a study were pooled, randomised and blinded by another investigator. Sections were observed on a Leitz DMRB microscope. Counts were made of the TH-immunoreactive neurones in the nigra and VTA of both the ipsilateral and contralateral hemispheres. Once all sections were quantified, slides were unblinded. Data were presented in the form of ipsilateral counts versus contralateral counts to allow observation of uniformity of lesion throughout the nigra.

2.4.3. Immunostaining for phosphorylated c-jun

Expression of phosphorylated c-jun was visualised using a rabbit polyclonal antibody raised against serine 63 phosphorylated c-jun. For staining of phosphorylated c-jun, cultures were dewaxed and rehydrated as described above. Antigen retrieval was carried out; sections were immersed in 10mM citrate buffer (pH 6) and heated on full power in a microwave for two bursts of five minutes, with the buffer being topped up in between. The slides were then allowed to cool in the citrate buffer for 5 minutes and then washed with PBS. In the first experiments, comparisons were carried out of sections stained with and without antigen retrieval to allow comparison. Immunostaining was carried out as described above, using a primary antibody dilution of 1:100. All secondary antibody, ABC reagent and DAB development steps were as described above.

To colocalise phosphorylated c-jun expression with TH immunoreactivity, double immunolabelling was carried out. Phosphorylated c-jun and TH are expressed in different cellular compartments, with TH being expressed in the cytoplasm and phosphorylated c-jun being expressed in the nucleus; it was thus possible to carry out double peroxidase labelling using DAB and Vector SG. For this technique, cultures were first immunostained using the phosphorylated c-jun primary antibody at 1:100 dilution; this was detected using a biotinylated secondary antibody, followed by peroxidase conjugated avidin-biotin complex and the staining visualised using Vector SG. Vector SG staining produced intense black staining of phosphorylated c-jun immunoreactive nuclei. Slides were washed, and peroxidase quenched by 1 hour incubation in 0.3% H_2O_2 prepared in PBS. Slides

were then blocked using 5% normal goat serum, and immunostained for TH using the rabbit polyclonal TH antibody. This primary antibody was added at a higher dilution, 1:5000 compared to 1:1000 previously used; the staining was thus fainter, and allowed clearer visualisation of double immunolabelled cells. Following primary antibody, biotinylated secondary antibody was added, followed by peroxidase conjugated avidin-biotin complex and DAB. The TH immunoreactivity was thus visualised as light brown cytoplasmic staining, with the phosphorylated c-jun staining appearing as dark black nuclei. Sections were haematoxylin counterstained, dehydrated and mounted using DPX as described above.

To quantify P-jun immunoreactive cells, and double labelled TH/P-jun labelled cells, slides were blinded by another investigator. Labelled cells were counted in both ipsi- and contralateral nigra and VTA; only cells with a visible nucleus were counted. In double labelling experiments, the total number of TH cells and the number of TH/P-jun double labelled cells were counted in both the nigra and VTA in the ipsilateral and contralateral hemispheres. After all quantification was complete, slides were unblinded and the data analysed.

2.5 Statistical analysis

All statistical analyses carried out were using one way analysis of variance followed by Dunnett's test comparing all groups to either control cultures or to cultures treated with MPP^+ 10μM alone in the case of experiments examining

neuroprotection. For control MPP^+ experiments, all groups were compared to untreated control results. Significance was considered reached at $p<0.05$.

Chapter 3:

Validation of culture model.

3.1. Introduction

The aim of this chapter is to describe a series of experiments undertaken to validate the culture system used for the majority of *in vitro* work discussed later in the thesis. In Chapter 1, the use of MPP^+ treated primary dopaminergic neurones as a model for Parkinson's Disease was discussed. This model was chosen as a model system to evaluate neuroprotective effect of anti-apoptotic strategies.

Primary dopaminergic neurones can be prepared from the ventral mesencephalon of rat embryos at around 14 days gestation. Dissociated cultures of dopaminergic neurones derived from foetal tissue are not stable over time in culture; there is a progressive attrition of the cells within the cultures over time (Fawcett et al., 1995). This is the case both in cultures derived from rodents and in tissue from human embryos in transplantation studies, and preventing this attrition has been an area of intensive research for many years.

The presence of dopaminergic neurones within mesencephalic cultures may be demonstrated by a number of ways. Immunocytochemically, immunoreactivity for tyrosine hydroxylase (TH), the first step in the dopamine synthesis pathway, is a marker for dopaminergic neurones. TH is also a marker for noradrenergic neurones; in cultures derived from the ventral mesencephalon, however, there are likely to be no noradrenergic neurones, as the nearest population is in the developing locus coeruleus. Another method by which the presence of dopaminergic neurones can be demonstrated is by assaying for ability of cells to take up ^{3}[H]-DA. Dopaminergic neurones take up ^{3}[H]-DA through a selective

dopamine transporter, primarily located on the neurites. Diminished uptake of ^{3}[H]-DA is a commonly used functional marker for loss of or damage to dopaminergic neurones. Further methods of assaying for the presence of dopaminergic neurones are to evaluate release of dopamine under depolarising conditions, or to evaluate the levels of dopamine metabolites in the culture medium. In this study the former two methods are used, counts of the numbers of TH-immunoreactive neurones within the cultures and assay of ^{3}[H]-DA uptake.

MPP^{+} is reported to decrease both the number of TH-immunoreactive neurones within mesencephalic cultures and the ability of the remaining cells to take up ^{3}[H]-DA, as discussed in Chapter 1. The loss of ^{3}[H]-DA uptake is both due to loss of neurones and to damage to the neurites of the remaining TH-immunoreactive neurones. This MPP^{+} induced loss of dopaminergic neurones and ^{3}[H]-DA uptake is reported to be inhibited by many factors, some of which were outlined in Chapter 1. A wide range of growth factors are reported to prevent MPP^{+} toxicity for dopaminergic neurones, and two of the best characterised are BDNF and GDNF; both of these factors are reported to prevent the loss of TH-immunoreactive neurones and to prevent the loss of ^{3}[H]-DA uptake (Beck et al., 1992, Hou et al., 1996).

This chapter describes the preliminary experiments carried out to validate the culture system used. The presence of TH-immunoreactive neurones within the cultures was established along with their ability to take up ^{3}[H]-DA; that this ^{3}[H]-DA uptake was mediated by the dopamine transporter was confirmed using the selective dopamine transporter blocker mazindol. Experiments were carried

out to establish that MPP^+ was indeed toxic for the dopaminergic population, and to confirm that growth factors reported in the literature to protect dopaminergic neurones were also active under the culture conditions used here.

3.2. Results

3.2.1. Culture characterisation

The culture conditions utilised for these studies were a modification of the protocol described by Hefti and co-workers (1993). The conditions described do not provide a pure population of dopaminergic neurones; the population of dopaminergic neurones in culture is typically around 0.5 – 1% of the total cells plated. The cultures are also reported to contain both GABAergic and glutamatergic neurones, but not noradrenergic neurones from the developing locus coeruleus (Hefti et al., 1993).

In Figure 3.1.A., ventral mesencephalic cultures fixed at 24 hours after plating and immunostained for TH are shown under phase contrast microscopy, so no differentiation of cell types is possible. Figure 3.1.B. shows the same field of view under transmitted light; the subpopulation of dopaminergic neurones is visible.

Figure 3.2. shows characterisation of stability over time of the number of TH-immunoreactive neurones in mesencephalic cultures plated and grown for 7 days in DMEM/Sato. Cultures were fixed every 24 hours for 7 days following plating, with a medium change prior to the fifth day following plating. Once all samples

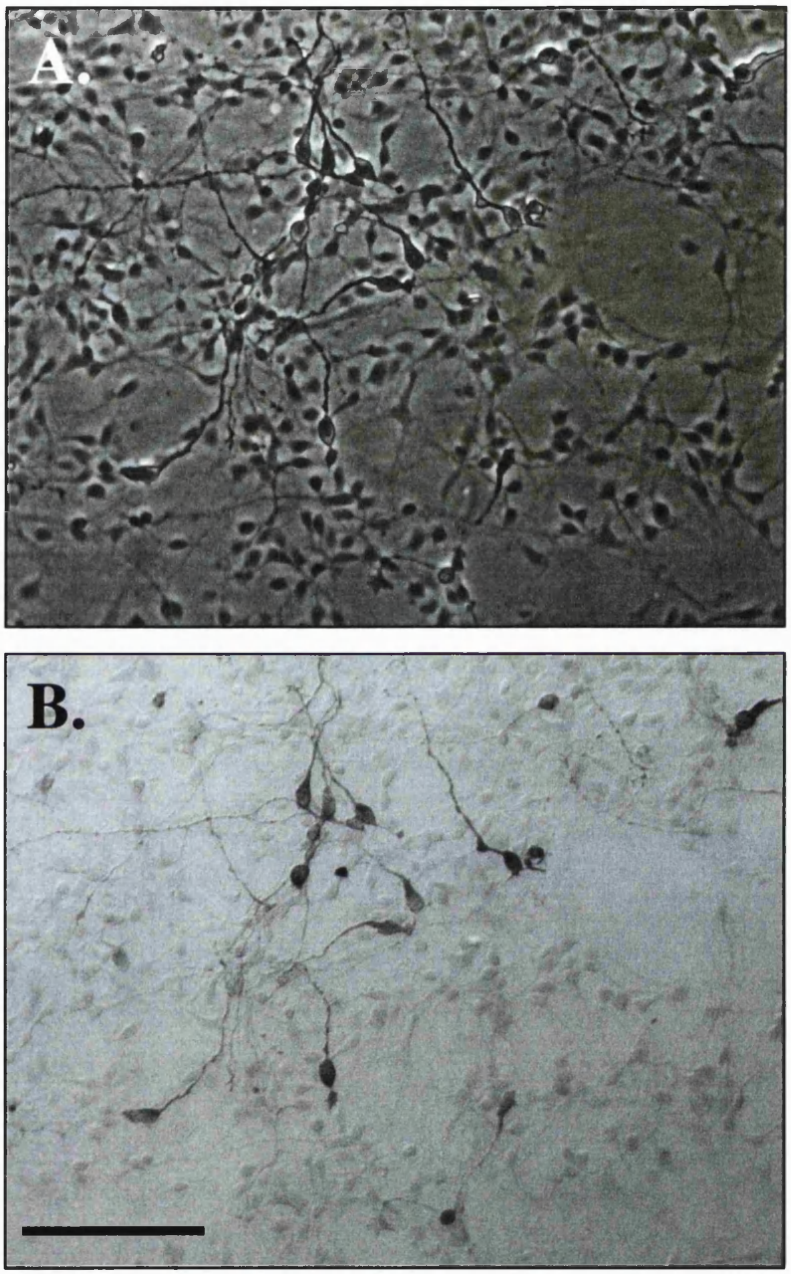


Figure 3.1. TH-immunoreactive neurones comprise a small proportion of the total cells in primary cultures of embryonic rat ventral mesencephalon. Ventral mesencephalic cultures were fixed and immunostained for TH expression 24 hours after plating. In (A), a field of view is shown under phase contrast illumination; the same field of view is shown in (B) under brightfield illumination. It is apparent that the majority of cells within the culture are non-TH-immunoreactive. Scalebar = 100μm.

were fixed, all samples were immunostained for TH expression. The subpopulation of dopaminergic neurones was not stable over time (Figure 3.2.A). There is a loss of the TH-immunoreactive neurones over the culture period, most of which occurs in the first 4 -5 days post plating. From 5 days *in vitro* to 7 days *in vitro*, the number of TH-immunoreactive neurones present within the cultures is relatively stable, though the numbers are reduced relative to the first days after plating. Photomicrographs of TH-immunoreactive neurones fixed and stained at 24 hours (3.2.B), 120 hours (3.2.C) and 168 hours (3.2.D) are also shown. Although the cell counts demonstrate an ongoing attrition of the TH-immunoreactive cells within the cultures, those neurones remaining appear viable and have extensive neuritic outgrowth. Given this loss of dopaminergic neurones over time, all experiments were carried out with an untreated control group fixed at the same time to allow effects of toxins and compounds to be evaluated. Additionally, experiments were performed in the 5 –7 day timepoint range, when the number of TH-immunoreactive cells under control conditions is relatively stable.

The serum supplement Sato allows consistency of growth conditions for mesencephalic cultures. This contrasts with cultures maintained in serum, as the composition of serum differs across batches. Serum may also contain differing levels of growth factors and cytokines, and thus may influence experimental outcome. One major difference between cultures maintained in Sato compared to those maintained in serum is the presence of astrocytes; Sato does not support

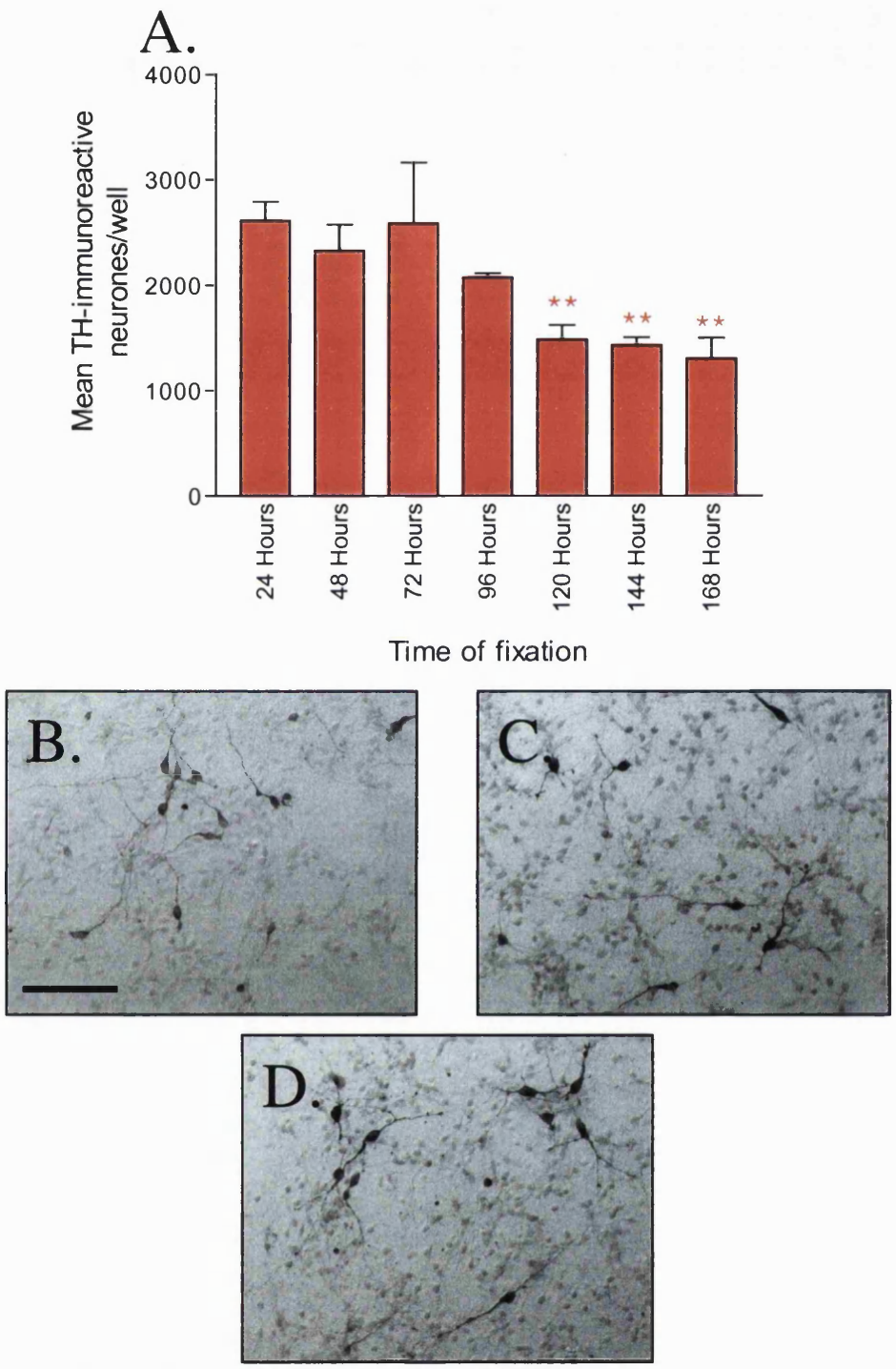


*Figure 3.2. The number of TH-immunoreactive neurones in primary mesencephalic cultures declines with time in culture. Ventral mesencephalic cells were dissociated and plated; cultures were fixed every 24 hours up to 7 days post plating, and immunostained for expression of TH. The mean number of cells per well at each timepoint is shown in (A); each data point shown is the mean ± s.e.m. of four independent wells per timepoint. There is a loss of TH immunoreactive cells after the first four days in culture but at timepoints of 5 days and above, the number of TH-immunoreactive cells is relatively constant (**p<0.01 by one-way ANOVA followed by Dunnett's test comparing all groups to 24 hour timepoint). Photomicrographs of cells at 24 hours (B), 120 hours (C) and 168 hours (D) are also shown; at these later timepoints there are fewer cells but those remaining retain extensive neuritic arbors. Scalebar = 100μm.*

growth or proliferation of astrocytes. A comparison of mesencephalic cultures maintained in Sato with those maintained in serum is shown in Figure 3.3.

Figure 3.3.A. shows primary mesencephalic neurones maintained in DMEM/10% FBS for 7 days. The cultures were fixed and immunostained for TH; a number of TH-immunoreactive neurones are visible. The TH-immunoreactive neurones appear similar in number to those maintained in Sato (Figure 3.3.B.), but also appear to be slightly larger, with more extensive neuritic outgrowth.

Although the number of TH-immunoreactive neurones in cultures maintained in serum is similar, striking differences were observed in the number of astrocytes present within the cultures. Cultures were maintained for 7 days in DMEM supplemented with either 10% FBS or Sato. Cultures maintained in 10% FBS contain a large number of astrocytes, visualised by GFAP immunoreactivity (Figure 3.3.C.); this contrasts with sister cultures maintained in Sato, where few astrocytes are visible (Figure 3.3.D.).

Thus, the culture of mesencephalic dopaminergic neurones in Sato serum substitute results in an equivalent number of neurones to cultures maintained in serum, and these neurones are free of glia. This astrocyte free model was chosen for further experimentation, as a number of reports indicate that MPTP/MPP^+ toxicity may also have a component mediated through release of toxic factors from glial cells. The essentially purified neuronal cultures described here allow for the study of the intracellular pathways activated by MPP^+ within neurones to be studied free from any astrocyte mediated component. The neurones obtained

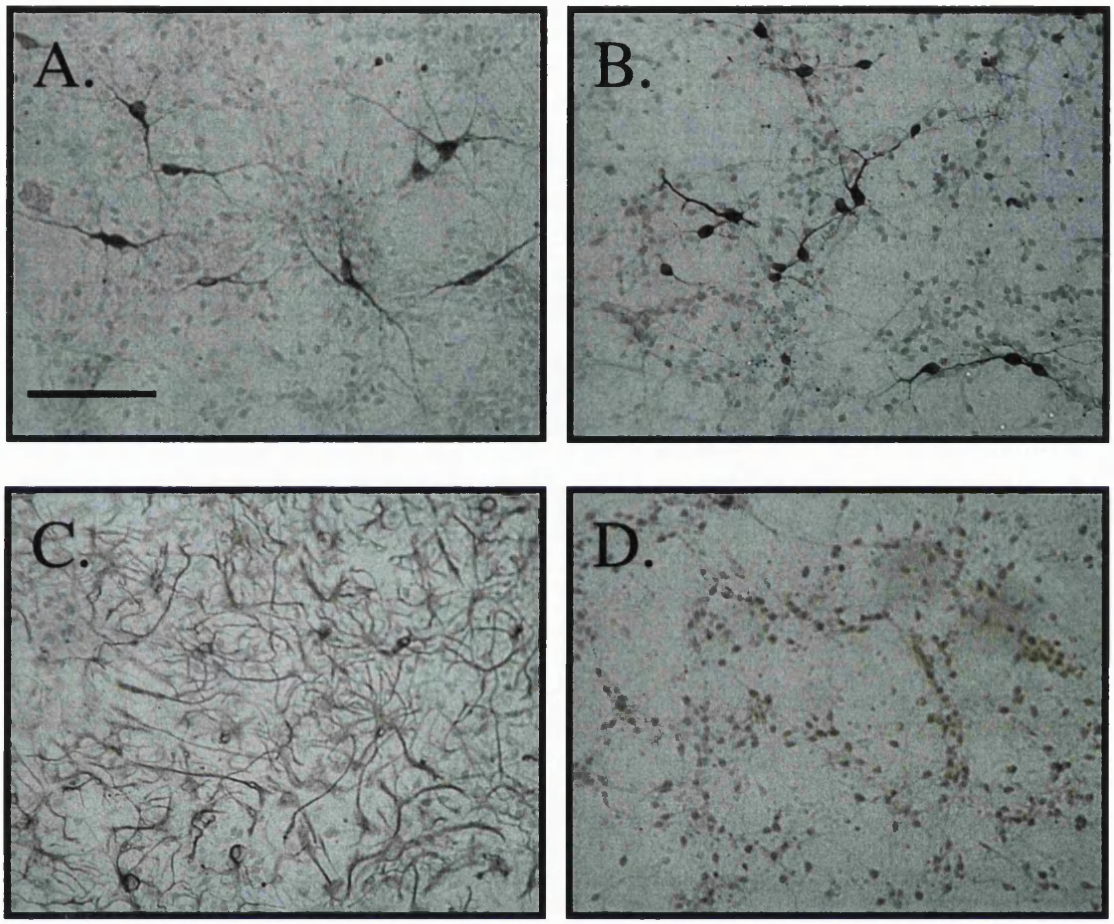


Figure 3.3. Comparison of mesencephalic cultures maintained in 10% foetal bovine serum (FBS) with those maintained in Sato serum substitute. Cultures maintained in 10% FBS (A) have equivalent numbers of TH-immunoreactive neurones to those maintained in Sato (B), but have larger somata and more extensive neuritic arbors. Cultures maintained in 10% FBS also have a large number of astrocytes (C), whereas those maintained in Sato are astrocyte free (D), as established by immunostaining for glial fibrillary acidic protein (GFAP). Cultures were maintained for 7 days in vitro prior to immunostaining for TH and GFAP. Scalebar = 100μm.

under these conditions do, however, appear smaller and have less extensive neurite outgrowth; the next series of experiments examined the functionality of these neurones by evaluating their ability to take up ^{3}[H]-DA.

To evaluate the functionality of the dopaminergic neurones by assaying ^{3}[H]-DA uptake, cultures were prepared and grown for 5 days. The medium was then removed and the cultures assayed for ^{3}[H]-DA uptake as described in Chapter 2. Briefly, cultures were washed and exposed to ^{3}[H]-DA at a concentration of 0.5μCi/ml for 30 minutes; the cultures were then washed with culture medium and cells lysed with 95% ethanol. Lysates were mixed with aqueous scintillant and the disintegrations per minute assayed. Background counts were subtracted prior to analysis. The dopamine transporter blocker mazindol was used as a control to demonstrate that the uptake was indeed into dopaminergic neurones and through the dopamine transporter; mazindol was added to the cultures at the same time as ^{3}[H]-DA. The results of these experiments are shown in Figure 3.4. The cultures take up ^{3}[H]-DA, and this is potently and significantly inhibited by mazindol at all the concentrations tested. These data indicate the presence of functional dopaminergic neurones within the cultures. Preliminary data using HPLC analysis of medium from mesencephalic cultures exposed to depolarising K^{+} concentrations also demonstrated increased release of dopamine but no noradrenaline release (not shown).

Thus cultures prepared and maintained using this methodology contain viable TH-immunoreactive neurones within an enriched neuronal population. Experiments were then carried out to evaluate the toxicity of MPP^{+} in these cultures.

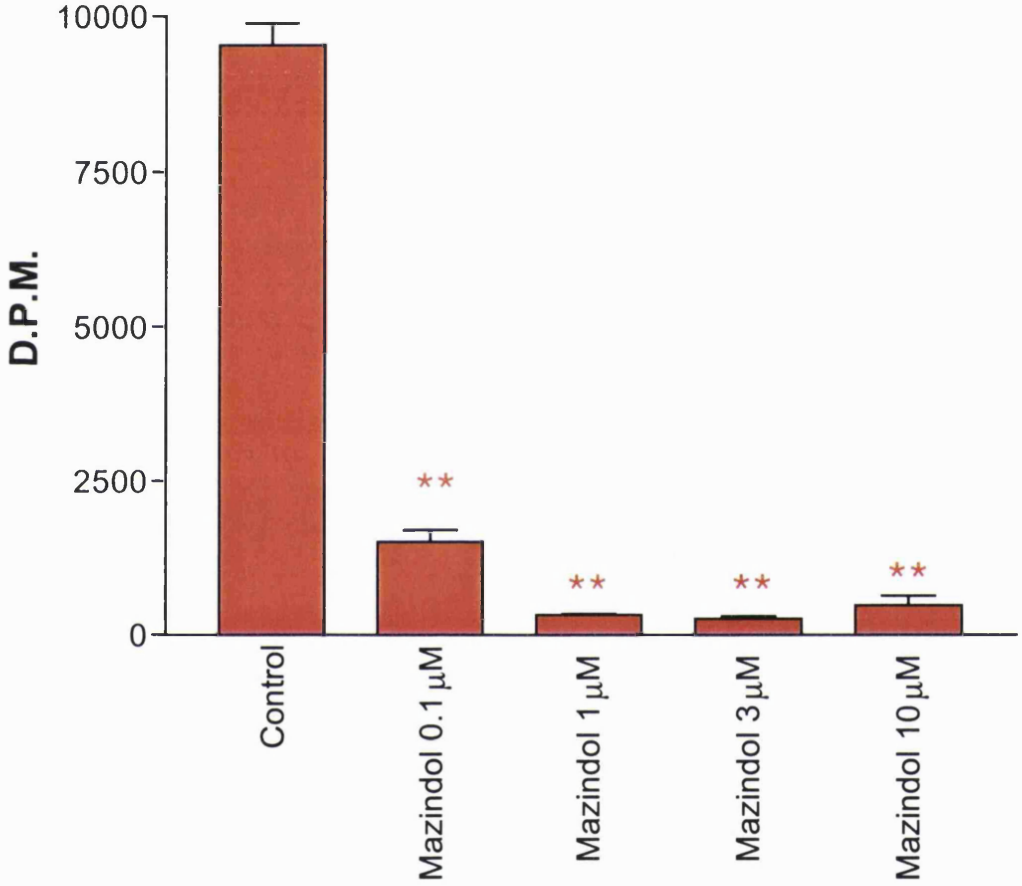


*Figure 3.4. Primary cultures of mesencephalic neurones take up ^{3}[H]-DA through the dopamine transporter. Cultures were prepared as described and maintained for 7 days in DMEM/Sato. Cultures were treated with 0.5µCi ml^{-1} ^{3}[H]-DA for 30 minutes in the presence or absence of the dopamine transporter blocker mazindol, then lysed using 95% ethanol. Data shown are the mean ± s.e.m. of four independently treated wells from one experiment (**p<0.01 by one-way ANOVA followed by Dunnett's test comparing all groups to control). Background counts were subtracted from all samples. Mazindol 10µM was included as a control condition in all further ^{3}[H]-DA experiments.*

3.2.2. MPP^+ toxicity for dopaminergic neurones in primary mesencephalic cultures.

The experiments described above demonstrate that mesencephalic cultures prepared and maintained under these conditions contain TH-immunoreactive neurones, and that these neurones are viable as demonstrated by ^{3}[H]-DA uptake. The next series of experiments were undertaken to validate the toxicity of MPP^+ for these cultures. MPP^+ was tested for ability to reduce both cell number and ^{3}[H]-DA uptake.

The effects of MPP^+ at various concentrations on the number of TH-immunoreactive neurones is shown in Figure 3.5. Cultures were prepared as described in Chapter 2, above, and maintained in 8-well chamber slides for 5 days *in vitro*. The culture area of the chamber slides was $0.81cm^2$. MPP^+ was prepared at 11X stock concentrations, and added directly to the medium within wells to give final concentrations in well ranging from 0.001μM to 100μM; culture medium alone was added to control wells. Cultures were incubated in the presence of MPP^+ for 48 hours, then the cells were fixed and immunostained for TH immunoreactivity. Remaining TH-immunoreactive cells within each well were quantified by counting all TH-immunoreactive cells within the well. The criteria for including cells in the count were that the cells should be both TH-immunoreactive, and possess at least one neurite, however rudimentary – these criteria were kept constant throughout all experiments. The data show a significant decrease in TH-immunoreactive cells with MPP^+ concentrations of 100nM and above (Figure 3.5.A), which accords well with previous reports. At

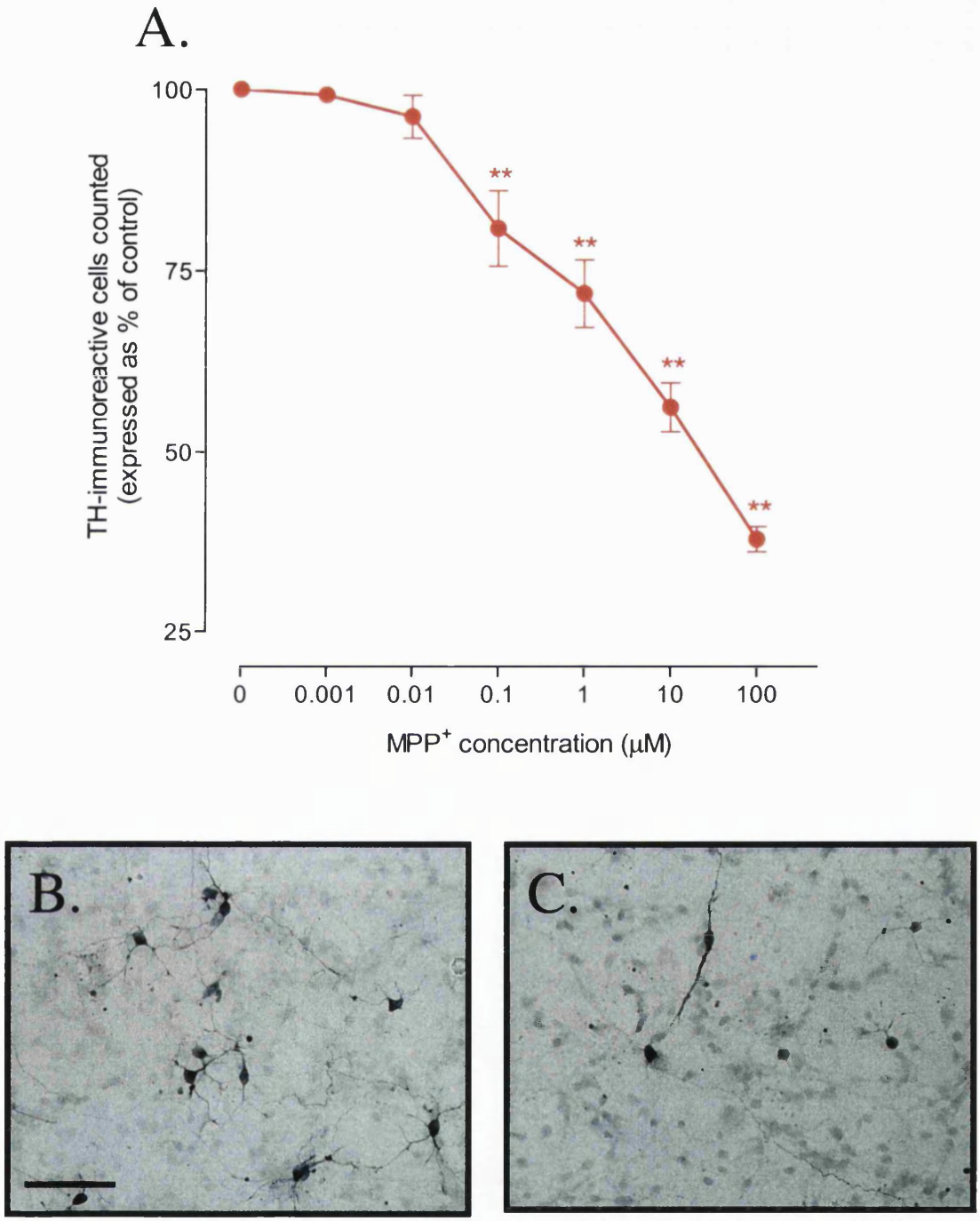


*Figure 3.5. MPP^+ reduces number of TH-immunoreactive neurones. Cultures were maintained for 5 days in vitro, then treated with MPP^+ at the concentrations shown for 48 hours. Significant (**p<0.01 by one way ANOVA followed by Dunnett's test) decreases in TH-immunoreactive cell number are observed with MPP^+ concentrations of 0.1μM and above (A). The photomicrographs show control (B) and MPP^+ 10μM treated cells (C) Results shown are the mean ± standard error margin (s.e.m.) of three independent experiments, each consisting of four independent wells per treatment group, and are normalised to % of untreated control. Scalebar = 100μm.*

an MPP^+ concentration of 10μM, the number of surviving TH-immunoreactive neurones decreased by approximately 50%; many of those neurones remaining also had shrunken cell bodies and a loss of neurites, quantified in Chapter 4, below. Representative photomicrographs of control and MPP^+ 10μM treated TH-immunoreactive neurones are shown in Figure 3.5.B and 3.5.C respectively.

When the ability of MPP^+ to decrease uptake of ^{3}[H]-DA in primary mesencephalic cultures was assessed, there was again a significant decrease (Figure 3.6). Cultures were prepared and maintained as described above for 5 days, then MPP^+ was added at 11X stock concentrations to give a final concentration in well ranging from 0.01μM to 100μM. Neurones were maintained in the presence of MPP^+ for 48 hours, then were assayed for ability to take up ^{3}[H]-DA. The data again show significant toxicity of MPP^+ at concentrations of 0.1μM and above. In these experiments, however, the relative toxicity of MPP^+ was more pronounced. MPP^+ 10μM reduced ^{3}[H]-DA uptake to less than 10% of untreated control levels, and to an equivalent extent as mazindol 10μM. This is in accordance with previous reports; MPP^+ is more potent in decreasing uptake of ^{3}[H]-DA than in decreasing TH-immunoreactive cell number, as it causes damage to the neurites of remaining neurones and thus decreases dopamine transporter expression (Mytilineou et al., 1985, Sanchez-Ramos et al., 1988, Michel et al., 1990).

Together these experiments show that the treatment of primary mesencephalic neurones with MPP^+ results in significant decreases both in the number of TH-

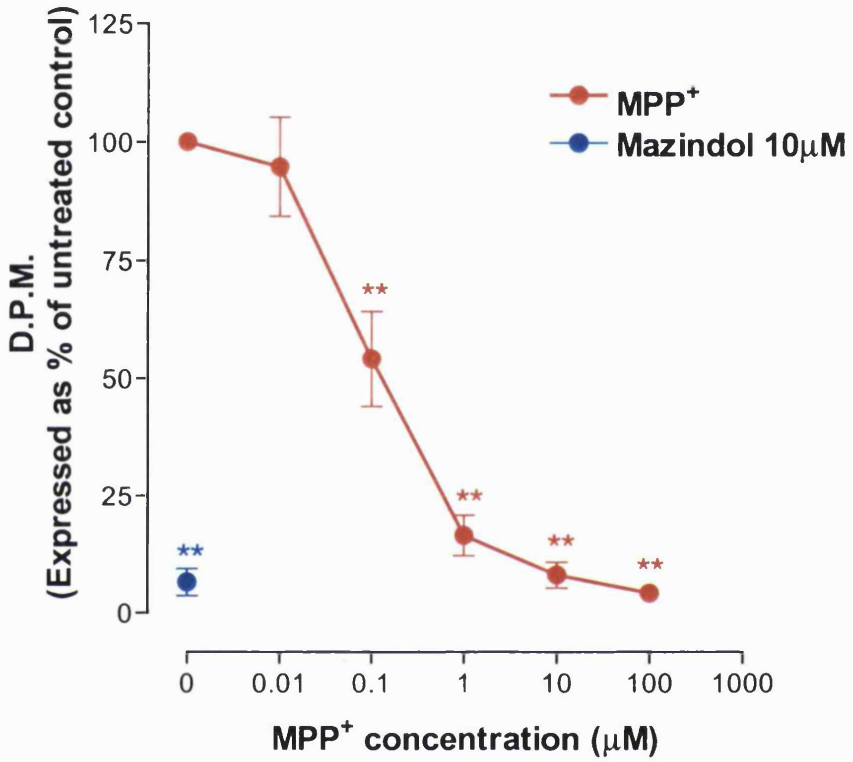


*Figure 3.6. MPP+ reduces uptake of ^{3}[H]-DA in primary mesencephalic neurones. Cultures were maintained for 5 days in vitro, then MPP+ was added at the concentrations shown. ^{3}[H]-DA uptake was assayed after a further 48 hours. MPP+ treatment decreased the ^{3}[H]-DA uptake in these cultures to an equivalent extent as the dopamine transporter blocker mazindol. Data shown are the mean ± s.e.m. of three independent experiments, each consisting of four independent wells (**p<0.01 by one-way ANOVA followed by Dunnett's test comparing all groups to control).*

immunoreactive neurones and in the uptake of ^{3}[H]-DA by those neurones remaining. Experiments were then undertaken to confirm that factors demonstrated in the literature to exert neuroprotective effect also do so in this model. The factors chosen were the growth factors BDNF and GDNF, both of which have been demonstrated to protect dopaminergic neurones from MPTP/MPP^{+} toxicity in both *in vivo* and *in vitro* models.

3.2.3. Growth factors attenuate MPP^{+} dopaminergic toxicity.

In order to determine whether growth factors reported in the literature to protect dopaminergic neurones from MPTP/MPP^{+} toxicity had similar effect in this culture system, mesencephalic cultures were prepared and cultured for 5 days. BDNF or GDNF were then added to the cultures at a range of concentrations up to 50ng/ml for 6 hours prior to MPP^{+} addition. This pre-administration has previously been reported to be necessary for BDNF to exert a neuroprotective effect against MPP^{+} toxicity *in vitro* (Beck et al., 1992). MPP^{+} was then added directly to the cultures at 11X stock concentration to give a final concentration in well of 10μM. Cultures were maintained for a further 48 hours prior to fixation and immunostaining for TH-immunoreactive neurones. Quantification of neuroprotection by growth factors was carried out by counting the number of TH-immunoreactive cells present within the cultures.

These data are shown in Figure 3.7. Both BDNF and GDNF increased the number of dopaminergic neurones present in MPP^{+} treated cultures. The response in the case of both factors was a partial protection of the dopaminergic neurones

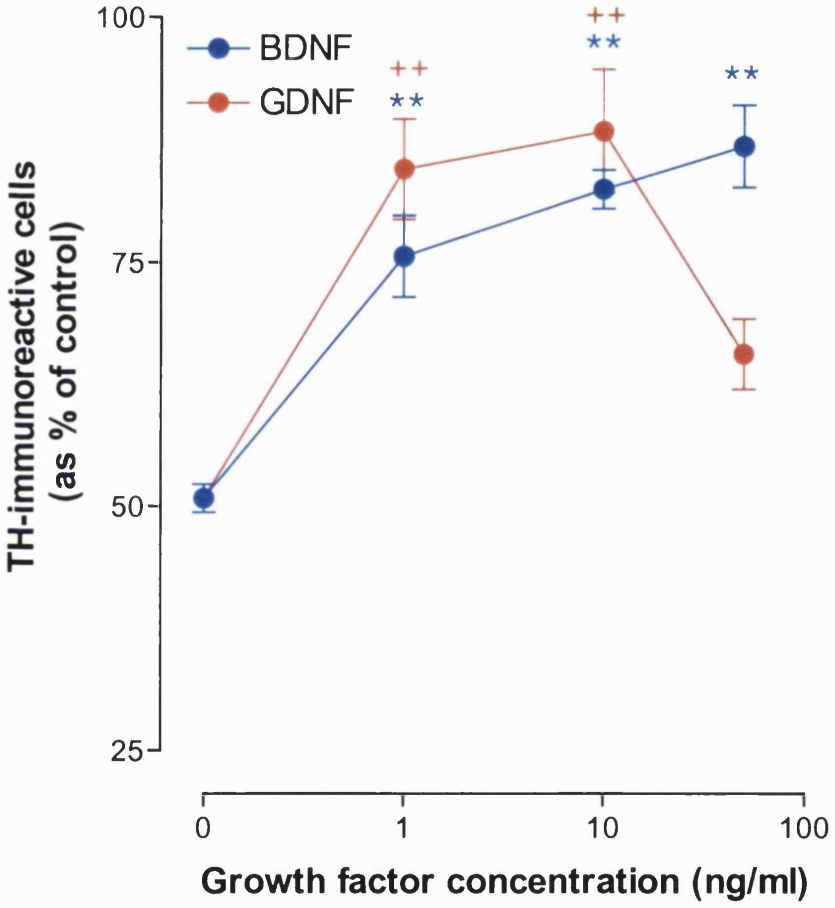


*Figure 3.7. BDNF and GDNF pretreatment attenuates loss of TH-immunoreactive neurones induced by MPP^+. Growth factors were pre-administered to mesencephalic neurones at 5 days in vitro for 6 hours, then MPP^+ was added to a final concentration in well of 10μM. Cultures were incubated for a further 48 hours, then were fixed and immunostained and TH-immunoreactive cells counted. Data points shown are the mean ± s.e.m. of four independent wells from one sample experiment (**,, ++ $p<0.01$ by one-way ANOVA followed by Dunnett's test comparing all groups to MPP^+ alone).*

at concentrations up to 10ng/ml. In the case of BDNF, the response was stable up to 50ng/ml; in the case of GDNF, however, the response declined after 10ng/ml.

The effects of BDNF and GDNF on ^{3}[H]-DA uptake by MPP^{+} treated primary mesencephalic cultures were also evaluated (Figure 3.8). Cultures were prepared and maintained for 5 days. BDNF and GDNF were then added to the cultures 6 hours prior to addition of MPP^{+}. MPP^{+} was added at concentrations of 0.1μM in this experiment. Cultures were either maintained in the presence of MPP^{+} for a further 48 hours, or washed free of MPP^{+} after 24 hours and maintained in growth factors for a further 24 hours. The data show a marginal increase in ^{3}[H]-DA uptake with both growth factors in the absence of a recovery period, and a more robust increase when the cultures were allowed to recover in the presence of growth factors for a further 24 hours. Thus both BDNF and GDNF protect mesencephalic dopaminergic neurones cultured under the conditions described above from MPP^{+} toxicity.

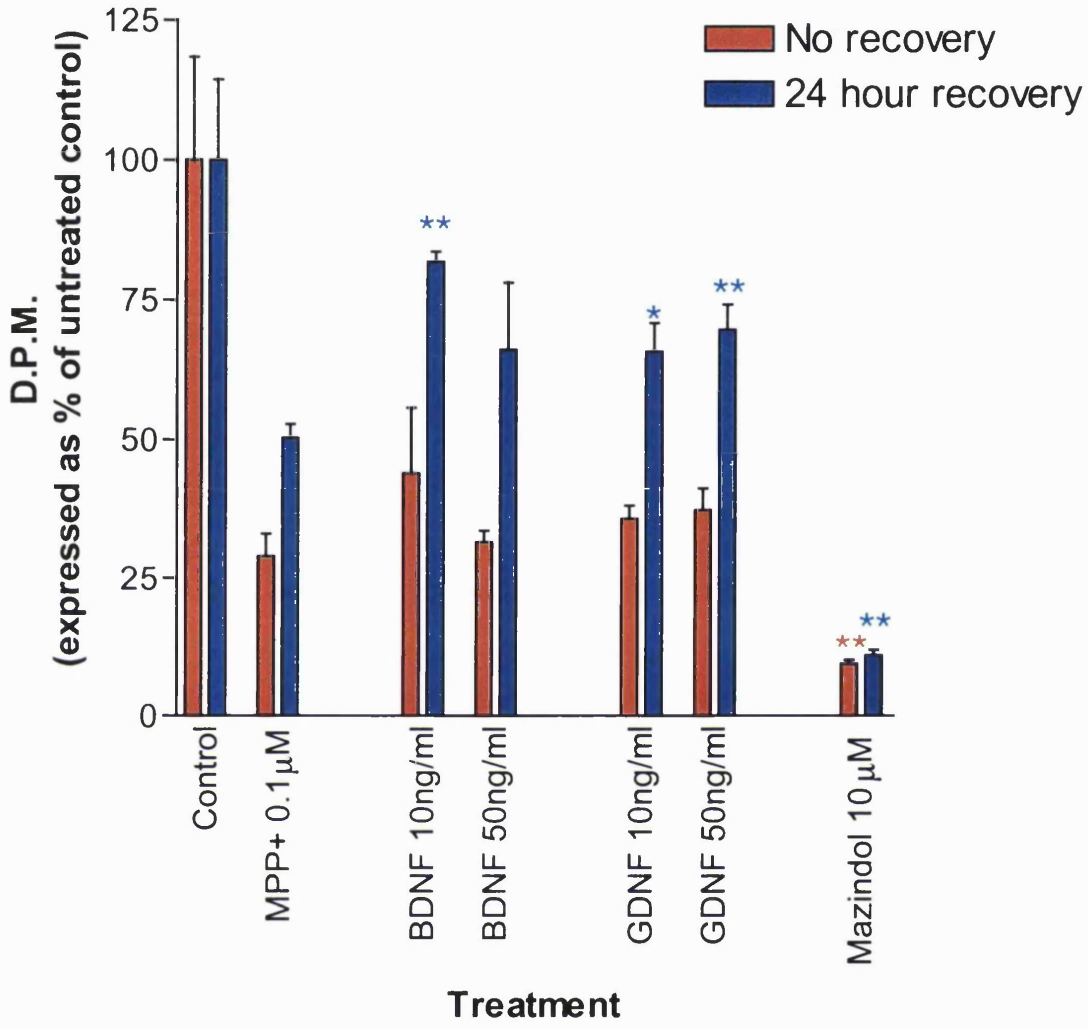


Figure 3.8. GDNF and BDNF pretreatment attenuates MPP^+ mediated decrease in $^{[3H]}DA$ uptake. Cultures were maintained for 5 days in vitro. Growth factors were pre-administered for 6 hours, then MPP^+ was added at a concentration of 0.1µM. Cultures were either incubated for a further 48 hours (no recovery) or for 24 hours, followed by washing the MPP^+ from the cultures and continuing growth factor treatment for a further 24 hours (24 hour recovery). The data show marginal increases in $^{[3H]}DA$ uptake with no recovery, and more pronounced increases ($p<0.05$, **$p<0.01$ compared to MPP^+ treated cultures) with 24 hour recovery. Data shown are from one sample experiment of two performed with each growth factor, and are the mean ± s.e.m. of four independent wells per condition.*

3.3. Discussion

There are a number of primary culture systems which have been used to evaluate survival of mesencephalic dopaminergic neurones, including explant cultures and dissociated cultures. In this study, dissociated cultures were used, and were grown in serum free conditions. 10% foetal bovine serum is a commonly used medium supplement; the use of serum, however, has a number of potential drawbacks. The exact composition of serum is unknown; different batches of serum may contain different levels of growth factors, cytokines, and antioxidants, and so adds a level of variability to the culture conditions. Serum also allows for proliferation and survival of glial cells within the cultures. In this study it was decided to culture as far as possible in glial cell free conditions. This was for a number of reasons. Firstly, the purpose of the study was evaluate the intracellular pathways activated by MPP^+ in dopaminergic neurones; MPP^+ may also be taken up by astrocytes and indirectly influence the pathways activated in dopaminergic neurones. Secondly, the presence of astrocytes within the cultures may also directly influence the survival of dopaminergic neurones in the culture system. There are a range of available defined serum substitutes available. A commonly used supplement is B27, a modification of B18 supplement (Brewer and Cotman, 1989). Growth of both cortical and hippocampal neurones in this supplement allows for high levels of neuronal survival (data not shown), and B27 is commonly used in the culture of central nervous system neurones. This supplement was not used in this study for several reasons. Firstly, the precise composition of B27 is not publicly available. Secondly, the formulation of B18,

and so presumably B27, has high levels of antioxidants; given that the formation of oxygen radicals following mitochondrial Complex I inhibition is widely reported, this may influence the survival of neurones following MPP^+ toxicity in itself. Thirdly, cortical or hippocampal cultures maintained in B27 also contain many astrocytes (not shown). There are a range of other serum supplements available, including N1 and N2 supplements, the formulation of which is public; it was decided, however, to use the serum substitute Sato, described by Bottenstein and Sato (1979), which allowed maintenance of mesencephalic cultures, survival of TH-immunoreactive neurones within the cultures, but did not support growth of astrocytes.

Mesencephalic cultures maintained under these conditions had a population of TH-immunoreactive neurones, which declined over time in culture, but was relatively stable from 5 days in culture to 7 days in culture; this was the period used in all other experiments. The cells were viable, as indicated by ^{3}[H]-DA uptake, and challenge with MPP^+ decreased both the number of TH-immunoreactive neurones and the ability of the remaining neurones to take up ^{3}[H]-DA. This reduction in the ability of ventral mesencephalic cultures to take up ^{3}[H]-DA is of equivalent extent to previous reports (Michel et al., 1990). The loss of TH-immunoreactive neurones was less than has been described by some investigators. At the 10μM concentration, the reduction in TH-immunoreactive neurones was only around 50%; in this series of experiments, this 50% reduction in cell number was a very robust finding, observed in more than 40 independently performed experiments. In some previous reports, the reduction has been

reported to be greater. The reported extent of dopaminergic cell death induced by MPP^+ in primary mesencephalic cultures *in vitro*, however, does vary. In one recent report, for example, the TH-immunoreactive cell loss in MPP^+ 3μM treated mesencephalic dopaminergic neurones after 24 hours was around 20% less than untreated control (Hartmann et al., 2001); in another, 5μM MPP^+ treatment for 24 hours reduced the number of TH-immunoreactive neurones to 60% less than untreated control (Viswanath et al., 2001). The extent of dopaminergic cell death, then, does vary, and this is likely dependent on the culture conditions. It is possible that the absence of glia under the culture conditions used in this study prevents a component of the MPP^+ mediated cell death. Alternatively, the fixation protocol used, where cultures were not washed prior to fixation and the fixative applied directly to the medium within the wells may allow for retention of some weakly adherent compromised cells which may otherwise have detached from the substrate. Given the robust nature of the 50% loss of dopaminergic neurones in cells cultured under the conditions described above, it was considered adequate for analysis of neuroprotection.

The neuroprotective effects of BDNF and GDNF are well documented in the literature in both *in vivo* and *in vitro* MPTP/MPP^+ models; both factors are reported to attenuate both the loss of TH immunoreactive neurones and the decrease in ^{3}[H]-DA uptake induced by MPP^+. These neuroprotective effects were confirmed; both of these factors increased the numbers of TH-immunoreactive neurones in MPP^+ treated mesencephalic cultures, and both factors increased uptake of ^{3}[H]-DA. The partial protection obtained is similar to

that previously reported for both BDNF (Beck et al., 1992) or GDNF (Hou et al., 1996), though both these studies utilised a different growth factor treatment protocol, where growth factors were added from the time of plating. These data demonstrate that the cultures respond to well characterised neuroprotective factors as would be predicted, and helps validate the model for the study of neuroprotective effects of inhibitors of components of the apoptotic cascade.

Thus, the data in this chapter demonstrate that under these defined conditions, cultures of 14 day rat ventral mesencephalon contain a population of viable dopaminergic neurones which are susceptible to MPP^+ toxicity and which are protected by the growth factors BDNF and GDNF. These data validate the culture system for further study of potential neuroprotective factors.

Chapter 4:

MPP^+ induces caspase-mediated apoptosis in primary dopaminergic neurones.

4.1. Introduction

4.1.1. Caspases in neurodegeneration

Caspase mediated apoptosis has been implicated in the cell death in a number of neurodegenerative conditions. These include Parkinson's Disease (discussed below), Alzheimer's Disease (Anderson et al., 1996, Li et al., 1997), Huntingdon's Disease (Butterworth et al., 1998), cerebral ischaemia (MacManus et al., 1993, Choi 1996), peripheral neuropathies, axonal transection and amyotrophic lateral sclerosis (Martin et al., 2000). Caspase inhibition has proved neuroprotective in a wide range of *in vivo* and *in vitro* models of these conditions, discussed below.

In vitro, caspases have been demonstrated to be active in many models of neurodegeneration. A widely used model of neuronal degeneration is trophic factor withdrawal from PC12 cells or peripheral neurones. Caspase inhibition protects PC12 neurones and sympathetic neurones from nerve growth factor withdrawal. Inhibition of caspases also protects primary cultured cortical or hippocampal neurones from oxygen and glucose deprivation (Nath et al., 1998) and a number of toxins such as β-amyloid peptide (Loo et al., 1993, Jordan et al., 1997); a recent report, though, has indicated that caspase activation is stimulus dependent in primary cortical neurones (Moore et al., 2002), and that with certain insults another family of apoptotic proteases, the calpains, are activated. Caspases have also been implicated in the apoptotic death of cerebellar granule neurones in

response to potassium deprivation (Ni et al., 1997, Simons et al., 1999) or excitotoxicity (Nath et al., 1998).

Thus, there is evidence for a neuroprotective role of caspase inhibition in a range of *in vitro* models of neurodegeneration. Caspase inhibition is also neuroprotective in *in vivo* models of neurodegeneration; of these, cerebral ischaemia is perhaps the most widely studied.

In cerebral ischaemia there is a central core of necrotic cell death. This is surrounded by a zone, the ischaemic penumbra, where the cell death is delayed and may be apoptotic. In 1996, the first report of neuroprotection by caspase inhibition in cerebral ischaemia was published; in this study, the poly-caspase inhibitor z-VAD-DCB decreased infarct volume following permanent middle cerebral artery occlusion (MCAO) in rat (Loddick et al., 1996). z-VAD-FMK has since been reported to be more potent than other peptide caspase inhibitors (Hara et al., 1997a), and it and another poly-caspase inhibitor, boc-Asp-FMK, have also been shown to be effective in a number of models of ischaemia (Cheng et al., 1998, Wiessner et al., 2000).

Evidence for a role of caspase 1 came from studies in transgenic mice. Both mice lacking the caspase 1 enzyme (Schielke et al., 1998, Liu et al., 1999) and mice expressing a dominant negative caspase 1 (Friedlander et al. 1997, Hara et al., 1997b) showed protection from ischaemic damage in permanent MCAO and transient MCAO with 24 hour reperfusion respectively. Inhibition of caspase 1-like proteases using zYVAD-CMK in permanent MCAO prevents both apoptosis

and inflammatory mechanisms indicating that there may be more than one neuroprotective mechanism (Rabuffetti et al., 2000). A novel peptidomimetic caspase 1 inhibitor developed by Warner Lambert also attenuates lesion size in transient MCAO in mice (c.f. Ashwell, 2001). It seems likely that the neuroprotective effect of caspase 1 inhibition may be mediated by both prevention of apoptosis and prevention of interleukin-1β production, as interleukin-1β has been shown to exacerbate ischaemic damage (Touzani et al., 2002).

A number of studies have also shown neuroprotection by caspase 3-like protease inhibition. The peptide inhibitor zDEVD-FMK has been shown to prevent delayed cell death in the hippocampus following transient global ischaemia in rat (Cheng et al., 1998) and MCAO in mouse (Hara et al., 1997); the effect was greatest when the inhibitor was administered prior to ischaemia, but was still apparent when zDEVD-FMK treatment was commenced following ischaemia (Fink et al., 1998). In addition to peptide inhibitors, novel small molecule caspase 3 inhibitors have been tested in ischaemia models. The Idun Pharmaceutical peptidomimetic caspase inhibitors IDN5370 and IDN7866 protected cells by around 20% after 28 days following permanent MCAO when administered intracerebroventricularly prior to MCAO; IDN7866 also protected from both permanent and transient MCAO by around 25% after 24 hours when administered intravenously prior to MCAO (Deckwerth et al., 2001). Peptidomimetic caspase 3 inhibitors developed by Merck Frosst attenuate damage following 45 minute regional cerebral ischaemia and three hour reperfusion; attenuation of damage following MCAO was observed with novel non-peptide caspase 3 inhibitors

(reviewed in Braun et al., 1999). A recent report from Merck-Frosst demonstrated that caspase 3 inhibition with a highly selective novel caspase 3 inhibitory compound, M826, blocked caspase 3 activation and delayed cell death in a neonatal hypoxic-ischemic brain injury model, but did not prevent activation of calpain and caspase 2 processing in the early post lesion period (Han et al., 2002).

As well as caspase inhibitory compounds, studies using overexpression of IAPs show neuroprotective effect. Overexpression of NAIP (Xu et al., 1997) or XIAP (Xu et al., 1999), delivered by adenovirus, attenuated neuronal loss following ischaemia; in the latter study behavioural deficits were also attenuated. Caspase inhibition may be synergistic with other potential therapies in ischaemia, notably the NMDA receptor antagonist MK801 (Ma et al., 1998).

Thus, the studies using caspase inhibitors indicate a potential therapeutic role for small molecule caspase inhibitors in cerebral ischaemia. There is, however, some controversy over caspase inhibition as a valid therapeutic strategy in ischaemia. In one study caspase inhibition spared CA1 neurones of the hippocampus but did not restore deficits in hippocampal LTP, indicating a possible lack of functional protection (Gillardon et al., 1999, for review see Loetscher et al., 2001).

In addition to models of cerebral ischaemia, inhibition of caspases has also been reported to be neuroprotective in axonal transection models such as optic nerve section (Chaudhary et al., 1999), and in models of Huntingdon's Disease and Alzheimer's Disease. The next section discusses the evidence for apoptosis and

caspase activation in Parkinson's Disease and MPTP/MPP^+ induced Parkinsonism.

4.1.2. Caspase mediated apoptosis in Parkinson's Disease and models

Caspase mediated apoptosis has been implicated both in idiopathic Parkinson's Disease and in a number of models of the condition. In idiopathic Parkinson's Disease, apoptosis has been reported by a number of groups in the substantia nigra (Mochizuki et al., 1996, Anglade et al., 1997, Tompkins et al., 1997, Hirsch et al., 1999); this is under debate, however, as other groups have found no evidence of apoptotic cell death (Banati et al., 1998, Wullner et al., 1999). Active caspase expression has also been observed in human PD brain; studies by Hartmann and coworkers (2000, 2001) have demonstrated expression of caspases 3 and 8 respectively. The evidence for a role of apoptosis in Parkinson's Disease is reviewed in (Schulz and Gerhardt, 2001, Andersen, 2001). Studies of apoptotic markers in human brain samples from Parkinsonian patients are hampered by a number of factors; the Parkinsonian dopaminergic cell death occurs over a period of many years, and thus there are likely to be few cells dying, by apoptosis or otherwise, in a given sample of tissue. Apoptosis is a dynamic process, an integral part of which is the expression of 'eat me' signals - such as phosphatidylserine - on the cell surface to attract scavenging cells. As apoptotic cellular debris is rapidly scavenged, apoptotic cells may be difficult to observe histologically. In addition, the post mortem time of tissue fixation may be important; longer delays lead to acidification of the tissue, and this may make DNA strand breaks more difficult to observe.

In the MPTP/MPP^+ model of Parkinson's Disease there is also evidence for apoptotic cell death. Apoptotic cells were detected in the nigrae of chronically MPTP treated mice (Tatton and Kish, 1997) by using TdT labelling and Acridine Orange staining to detect DNA strand breaks and chromatin clumping respectively; this appears to be stimulus dependent, however, and other investigators using a more acute dosing paradigm have found no evidence of apoptosis (Jackson-Lewis et al., 1995). In early *in vitro* studies, apoptosis was detected in MPP^+ treated neurones and mesencephalon/striatum co-cultures (Dipasquale et al., 1991, Mochizuki et al., 1994). This chapter describes a series of experiments to evaluate the contribution of apoptosis and caspase activation to the cell death induced by MPP^+ in primary cultures of mesencephalic dopaminergic neurones.

4.1.3. Chapter aims

The aim of this series of experiments was to examine apoptotic features and caspase activation in MPP^+ treated mesencephalic cells, using selective inhibitors to examine the contribution of different caspases to the cell death. Both commercially available peptide inhibitors of various caspases and a range of novel selective caspase 3 inhibitors from the Merck-Frosst caspase programme (Hotchkiss et al., 2000) were used. Additionally, functional recovery was evaluated using the ^{3}[H]-DA uptake assay, and a double immunocytochemistry approach was employed to examine caspase activation. The results give an insight into the caspase pathways activated by MPP^+ within dopaminergic neurones, and into the importance of caspase 3 in executing the cell death.

4.2. Results

4.2.1. Evidence for apoptosis and caspase activation following MPP^+ treatment of dopaminergic neurones.

These first experiments were designed to determine whether the cell death induced by MPP^+ was apoptotic, established by chromatin condensation. Nuclear morphology was assessed in dopaminergic neurones following MPP^+ exposure to determine whether the cell death induced was apoptotic by staining with the nuclear dye Hoechst 33322. Photomicrographs of mesencephalic cultures stained for TH and counterstained with Hoechst 33342 to visualise nuclei are shown in Figure 4.1. TH-immunoreactive neurones are stained green, and Hoechst stained nuclei fluoresce blue. Double exposures were also taken to confirm localisation of TH-immunoreactive cell nuclei. TH-immunoreactive neurones are shown in Figures 4.1.A., 4.1.D. and 4.1.G., Hoechst 33342 stained nuclei are shown in Figures 4.1.B., 4.1.E. and 4.1.H., and double exposed images to show colocalization are shown in Figures 4.1.C., 4.1.F. and 4.1.I. Figures 4.1.A., 4.1.B. and 4.1.C. show control cultures. TH-immunoreactive neurones have large cell bodies and extensive neurites; the nuclear morphology of these neurones show no chromatin condensation, illustrated by the yellow arrows. Figures 4.1.D., 4.1.E. and 4.1.F. are of a field of view from cultures exposed to MPP^+ 10μM for 48 hours. Within the field, a number of degenerating TH-immunoreactive neurones can be observed (white arrows). The nuclei of these neurones show chromatin condensation when stained with Hoechst 33342, a characteristic feature of apoptosis. Also within the well are a number of TH-immunoreactive neurones

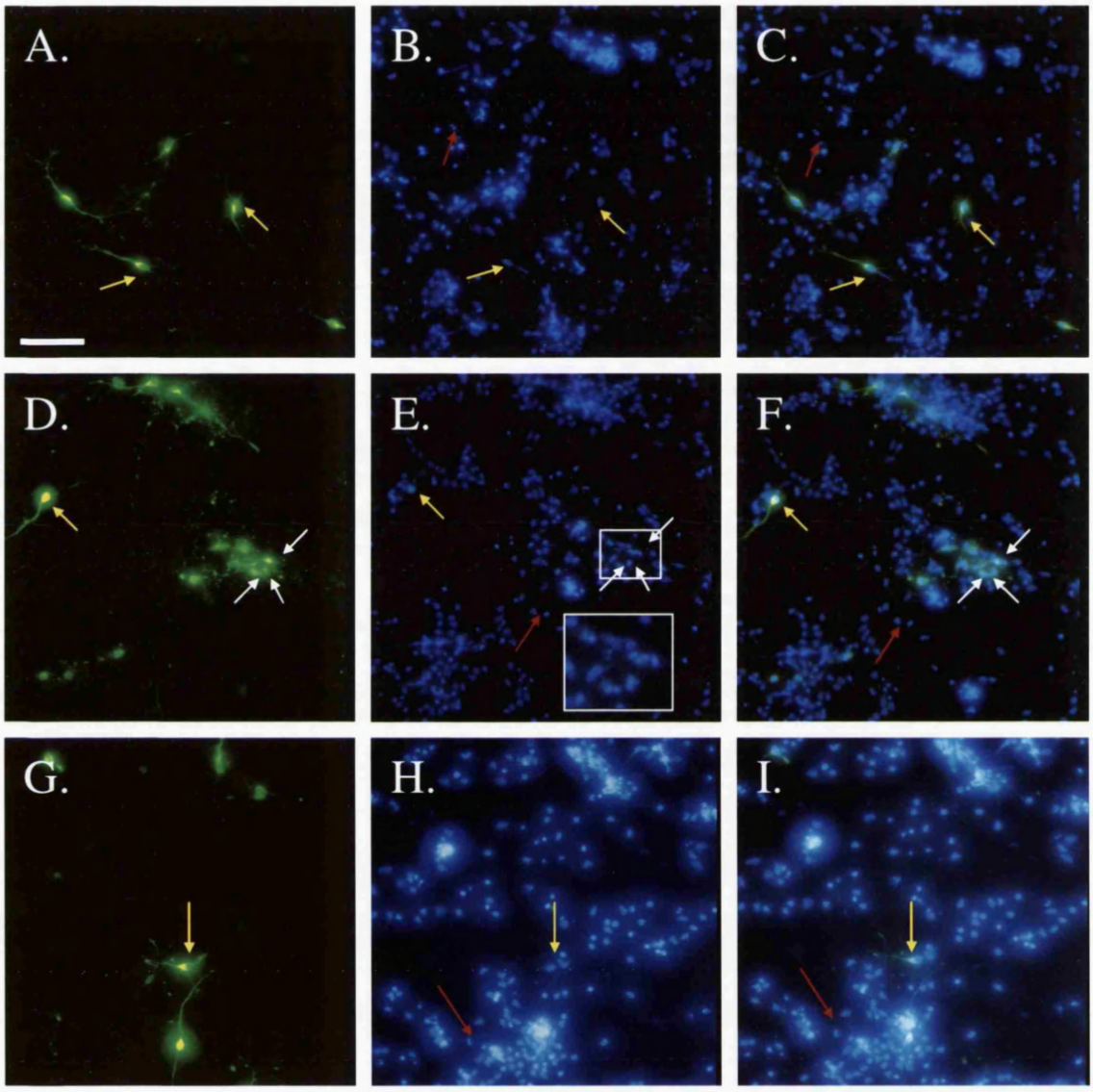


Figure 4.1. MPP^+ causes nuclear chromatin condensation, a characteristic feature of apoptosis, in dopaminergic neurones. A, D, and G show TH staining, B, E, and H show nuclear morphology visualised with Hoechst 33342, C, F, and I show combined images. Top row shows control cultures, middle row cultures exposed to MPP^+ 10 μM for 48 hours and the bottom row cultures exposed to MPP^+ 10 μM in combination with caspase inhibitor zVAD-fmk. Yellow arrows show sample non-apoptotic TH-immunoreactive neurones, white arrows show sample apoptotic TH-immunoreactive neurones and red arrows show sample apoptotic non-TH-immunoreactive neurones. Inset in E is a magnification of the highlighted area. Scalebar = 100μm.

which do not appear to have degenerated; the nuclei of these neurones do not show chromatin condensation (yellow arrow). Figures 4.1.G., 4.1.H., and 4.1.I. show cultures exposed to MPP^+ 10μM for 48 hours in the presence of zVAD-fmk 300μM. The TH-immunoreactive neurones within the culture do not appear to have degenerated, and their nuclei do not show chromatin condensation (yellow arrow). Also within each well, there is a population of cells which exhibit chromatin condensation, but are not TH-immunoreactive, highlighted by the red arrows. Such nuclei are observed in control, MPP^+ treated, and MPP^+ and zVAD-fmk treated cultures. These profiles may reflect a population of non-dopaminergic cells in the culture which are undergoing cell death, perhaps as a result of changing the medium on the cultures.

In order to visualise activated caspase 3 in dopaminergic neurones following MPP^+ treatment, double immunolabelling studies were carried out using primary antibodies to activated caspase 3 and to TH. Cultures were grown for 5 days, then returned to culture medium alone (Figures 4.2.A. – 4.2.C.), or treated with MPP^+ 10μM for 24 hours (Figures 4.2.D. – 4.2.F.) or 48 hours (Figures 4.2.7. – 4.2.I.). Figures 4.2.A., 4.2.D. and 4.2.G. show TH-immunoreactivity. Figures 4.2.B., 4.2.E. and 4.2.H. show activated caspase 3 immunoreactivity in the same field of view, and Figures 4.2.C., 4.2.F. and 4.2.I. show colocalisation of caspase 3 with TH-immunoreactivity.

In control cultures, a number of TH-immunoreactive neurones can be observed (Figure 4.2.A.), along with a population of cells expressing activated caspase 3

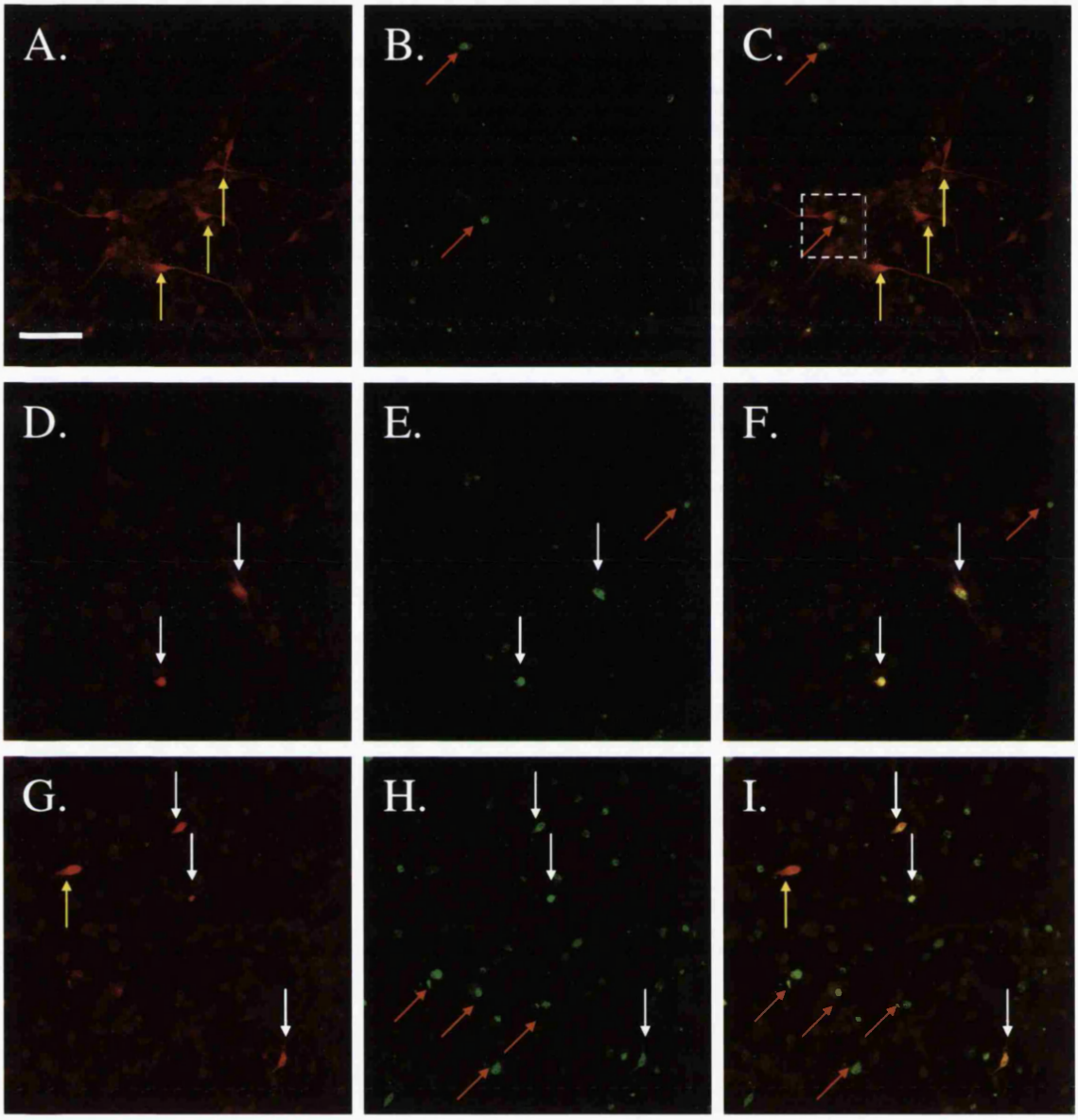


Figure 4.2. MPP⁺ increases expression of activated caspase 3 in TH-immunoreactive cells in primary cultures of mesencephalic neurones. A, B and C show untreated control cultures at 48 hours; D, E and F, and G, H and I show cells exposed to MPP⁺ 10μM for 24 and 48 hours respectively. TH-immunoreactivity is shown in red in A, D and G; active caspase 3 immunoreactivity is shown in green in B, E and H. Merged images showing colocalisation are shown in C, F and I. Increased numbers of TH/active caspase 3 positive cells (white arrows) are observed with MPP⁺ treatment, along with a population of non-TH immunoreactive active caspase-3 expressing cells (red arrows); a few non-TH immunoreactive active caspase 3 expressing cells are also observed in control cultures. Yellow arrows show TH-immunoreactive neurones negative for active caspase 3; note that the TH-immunoreactivity and caspase-3 immunoreactivity in the dashed box in panel C do not colocalise to the same cell. Scalebar = 100μm.

(Figure 4.2.B.). There is, however, little co-expression of activated caspase 3 with TH in these cultures (Figure 4.2.C.), indicating that caspase 3 is not active in dopaminergic neurones. In cultures treated with MPP^+ for 24 or 48 hours, however, a population of dopaminergic neurones which co-express TH and caspase 3 is apparent (Figures 4.2.F., 4.2.I.). In all treatment groups, a population of non-dopaminergic neurones are apparent which express activated caspase 3, indicating that there is a population of cells within the cultures undergoing apoptosis; this is in accordance with the presence of apoptotic profiles in a population of non-dopaminergic neurones observed in Figure 4.1. Thus, MPP^+ treatment of primary cultures of dopaminergic neurones for 24 or 48 hours causes activation of caspase 3 in these neurones. Quantification of the numbers of apoptotic and caspase 3 expressing TH and non-TH immunoreactive cells within these cultures in the presence and absence of caspase inhibitors was carried out; these data are presented below, in Figure 4.13.

4.2.2. Neuroprotection by caspase inhibition.

4.2.2.1. Effects of zVAD-fmk

The broad-spectrum caspase inhibitor zVAD-fmk was tested for neuroprotective effects on TH-immunoreactive neurones when coadministered with MPP^+ 10μM for 48 hours. Cultures treated with MPP^+ alone had fewer TH-immunoreactive cells; those cells remaining had shrunken cell bodies and loss of neurites. Treatment with zVAD-fmk increased the number of TH-immunoreactive neurones, together with increasing somatic size and partially restoring neurite

length. Photomicrographs illustrating these effects are shown in Figure 4.3.

Quantification of the neuroprotective effect of zVAD-fmk showed a concentration dependent increase in the number of TH-immunoreactive cells, with significant increases observed at zVAD-fmk concentrations of 30μM and above (Figure 4.4). MPP^+ alone decreased the survival of TH-immunoreactive neurones to around 50% of control; the maximal zVAD-fmk response, at 300μM, restored the number of cells counted to greater than 90% of untreated control. Thus, caspase inhibition using a broad-spectrum inhibitor significantly protects dopaminergic neurones from MPP^+ toxicity under these culture conditions. Increased numbers of TH-immunoreactive neurones in zVAD-fmk + MPP^+ 10μM treated cultures were also observed at timepoints up to 5 days following administration (not shown).

MPP^+ treatment results in morphological damage to the remaining TH-immunoreactive neurones after 48 hour treatment, with the remaining cells having shrunken cell bodies and neuritic damage. Image analysis was used to determine whether caspase inhibition was capable of attenuating these morphological deficits. The results show that the decrease in somatic size of MPP^+ treated TH-immunoreactive neurones is completely prevented by zVAD-fmk co-administration at 100μM or 300μM; at 300μM, the somatic size is also increased relative to neurones from the control group. MPP^+ also damaged the processes of dopaminergic neurones. In order to evaluate the extent of this damage, and any attenuation by caspase inhibition, the length of the longest neurite was evaluated in TH-immunoreactive cells using image analysis. The data show that MPP^+

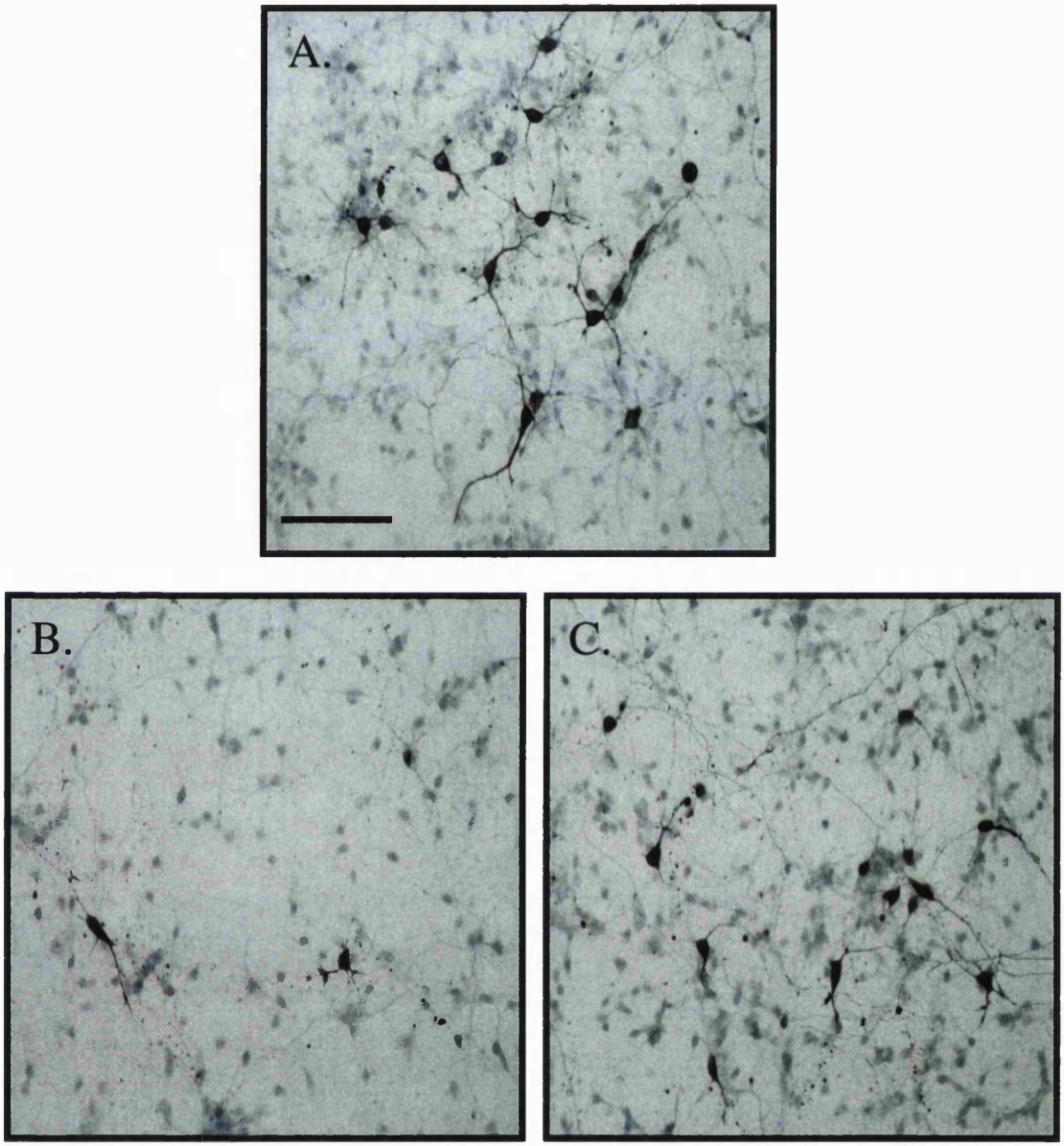


Figure 4.3. Effects of the broad-spectrum caspase inhibitor zVAD-fmk on MPP^+ treated TH-immunoreactive neurones. Cultures were maintained for 5 days in vitro, then treated for 48 hours with MPP^+ 10μM (B) or MPP^+ 10μM and zVAD-fmk 300μM (C). Control cells are shown in (A). zVAD-fmk increases the number of TH-immunoreactive neurones, and appears to attenuate the MPP^+ mediated somatic shrinkage and neurite loss. Scalebar = 100μm.

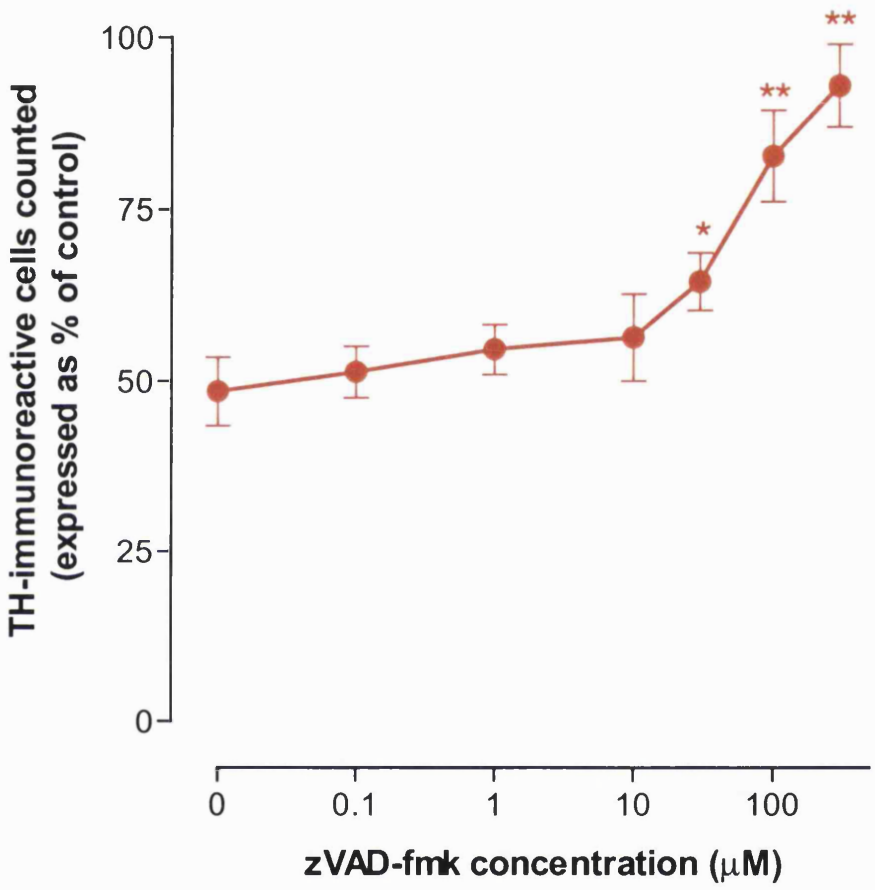


*Figure 4.4. zVAD-fmk attenuates MPP^+ mediated loss of TH-immunoreactive neurones in primary mesencephalic cultures. MPP^+ 10μM treatment decreased the number of TH-immunoreactive neurones to around 50% of untreated control; zVAD-fmk treatment attenuated this loss in a concentration dependent fashion. The maximal protection was observed with zVAD fmk 300μM. This concentration restored the number of TH-immunoreactive neurones to greater than 90% of control. Data shown are from 3 independent experiments, each consisting of four independent wells per treatment group. Statistically significant sparing was observed at zVAD-fmk concentrations of 30μM and above (*p<0.05, **p<0.01 by one way analysis of variance followed by Dunnett's test).*

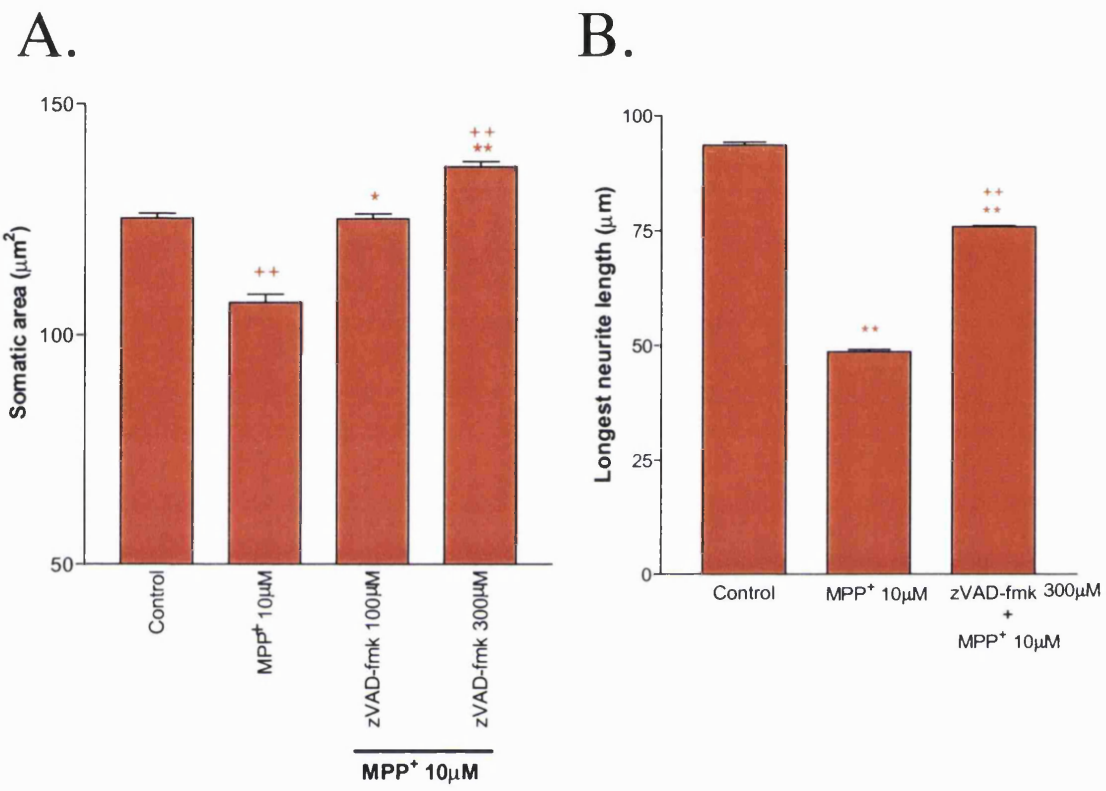


Figure 4.5. zVAD-fmk prevents MPP^+ mediated loss of somatic area in TH-immunoreactive cells, and partially restores longest neurite length, as quantified using image analysis. (A) shows the effects of zVAD-fmk on somatic size of the TH-immunoreactive neurones. 48 hour MPP^+ 10μM treatment significantly (++ $p<0.01$) decreased somatic size of remaining TH-immunoreactive neurones from around 125μm^2 to around 100μm^2. zVAD-fmk concentrations of 100μM and 300μM significantly ($p<0.05$, **$p<0.01$) prevented this somatic area loss. 300 μM zVAD-fmk significantly increased somatic are over control. (B) shows the effect of zVAD-fmk 300μM on 48 hour MPP^+ 10μM mediated loss of neurite length. Image analysis was used to quantify longest neurite length in TH-immunoreactive neurones. MPP^+ significantly (**$p<0.01$) reduced the length of TH-immunoreactive neurites, by around 50%; this neurite loss was partially attenuated by MPP^+ 300μM (**$p<0.01$), but this was significantly less than control. For both graphs, each data point shows the mean result for four independent wells from one experiment; in each well, measurements were taken from 100 randomly selected TH-immunoreactive neurones.*

significantly decreases longest neurite length in dopaminergic neurones; this deficit, however, was only partially attenuated by co-administration with zVAD-fmk 300μM. These data are shown in Figure 4.5.

Given that zVAD-fmk is capable of only a partial protection of dopaminergic processes even at high concentrations, it is perhaps unsurprising that the attenuation of MPP^+ mediated decreases in ^{3}[H]-DA uptake are modest (Figure 4.6.). When MPP^+ was coadministered at either 1μM or 10μM with zVAD-fmk at concentrations up to 300μM, significant increases in ^{3}[H]-DA uptake were observed only with the maximal zVAD-fmk concentration. The increase observed in ^{3}[H]-DA uptake was small, reaching only around 10% of the uptake in control cultures. In order to determine whether the lack of effect was due to insufficient penetration of the inhibitor into neurites, zVAD-fmk was pre-administered for 6 hours prior to MPP^+ addition; under these conditions, no increase in the protective response was observed with the inhibitor (Figure 4.7).

Thus, the data obtained with the broad-spectrum inhibitor zVAD-fmk show that caspase inhibition significantly spares TH-immunoreactive cell bodies in MPP^+ treated primary mesencephalic cultures, and increases somatic size of the spared neurones. There was a partial restoration of the length of the longest neurite in TH-immunoreactive neurones, but little increase in ^{3}[H]-DA uptake, perhaps due to a failure to prevent degeneration of the dopamine transporter sites on terminals. Experiments were then undertaken to identify which caspases might be involved in mediating MPP^+ induced apoptosis.

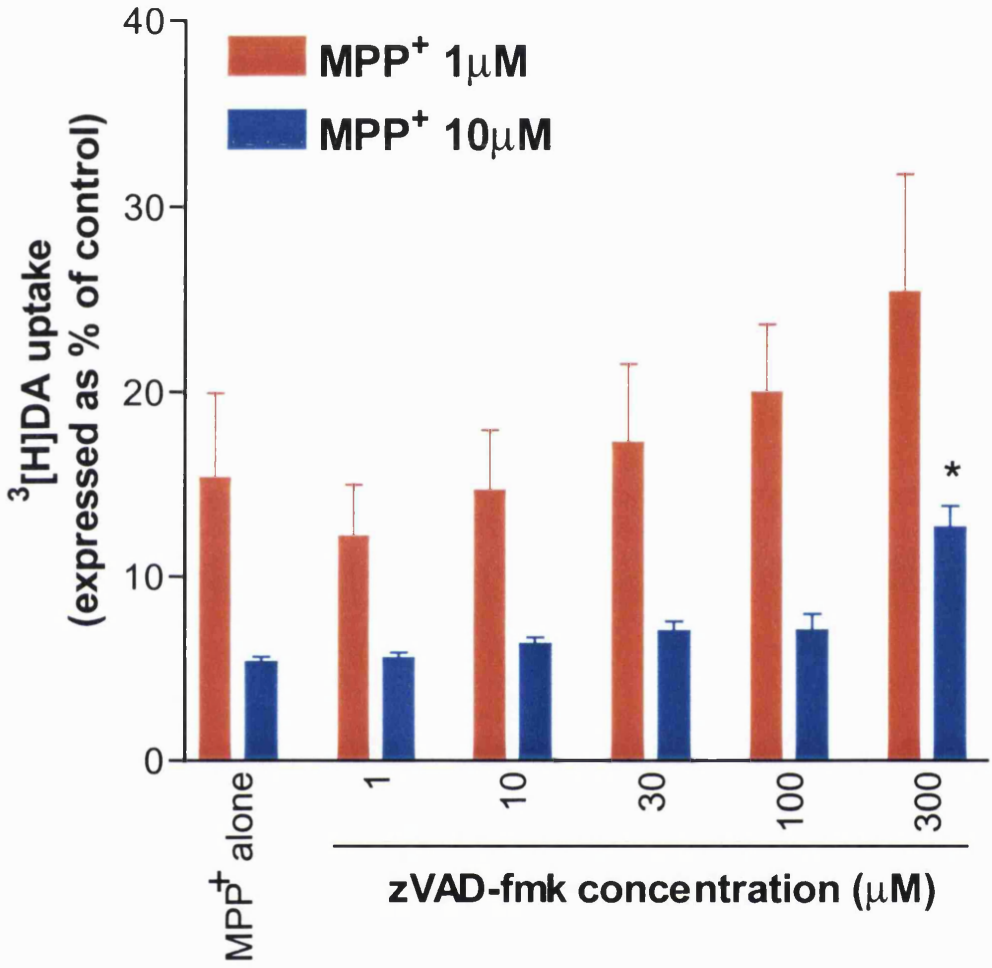


*Figure 4.6. MPP+ mediated decrease in ^{3}[H]DA uptake by primary mesencephalic cultures is only partially attenuated by zVAD-fmk co-administration. zVAD-fmk was added in combination with MPP+ at either 1μM or 10μM for 48 hours, then ^{3}[H]DA uptake was assayed. Data are expressed as % of the response of untreated control cultures. There was little sparing of ^{3}[H]DA uptake with zVAD-fmk; a significant increase (*p<0.05 by one way ANOVA followed by Dunnett's test comparing all groups to MPP+ alone) was observed with zVAD-fmk 300μM when added with MPP+ 10μM, but the effect was small by comparison with the survival effects. There was a non-significant trend towards increased ^{3}[H]DA uptake when zVAD-fmk was added with MPP+ 1μM. Data shown are the mean ± s.e.m. of three independent experiments, each consisting of four independent wells.*

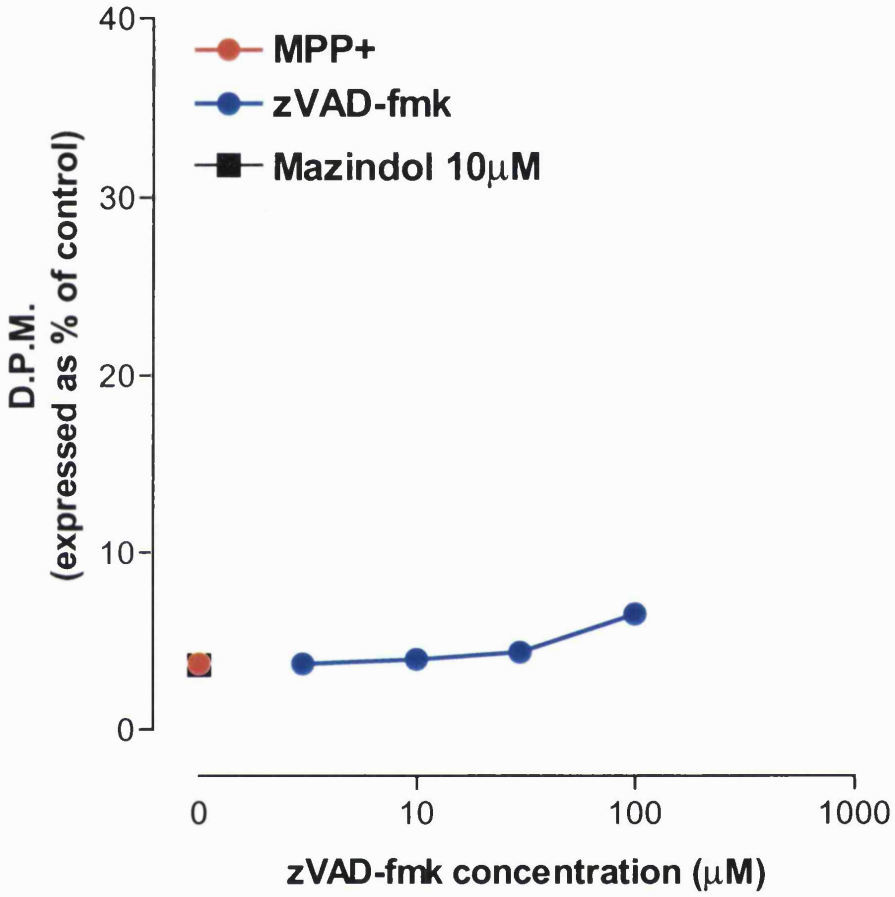


Figure 4.7. Pretreatment of mesencephalic cultures with zVAD-fmk does not prevent MPP⁺ 10μM mediated loss of ^{3}[H]DA uptake. zVAD-fmk was added 6 hours prior to MPP⁺ addition. There was a slight but non-significant (by one-way ANOVA followed by Dunnett's test comparing all groups to MPP⁺ alone) increase in ^{3}[H]DA uptake with zVAD-fmk 300μM. Data shown are from one sample experiment consisting of four independent wells per treatment group.

4.2.2.2. Peptide caspase inhibitors

Given the neuroprotective effect of zVAD-fmk for mesencephalic dopaminergic neurones exposed to MPP^+, it was of interest to establish which members of the caspase family were involved in mediating the MPP^+ toxicity. Peptide inhibitors based on the cleavage sequences of caspases 1, 2, 3 and 9 were tested for neuroprotective effects. The inhibitors tested were zYVAD-cmk for caspase 1, zVDVAD-fmk for caspase 2, zDEVD-fmk for caspase 3, and zLEHD-fmk for caspase 9. The results obtained with these inhibitors are shown in Figure 4.8. Concentration dependent increases were observed with three of the compounds, zDEVD-fmk (caspase 3), zLEHD-fmk (caspase 9), and zVDVAD-fmk (caspase 2) (Figures 4.8.A – 4.8.C), but no significant increases were observed with the caspase 1 inhibitor zYVAD-cmk (Figure 4.8.D.). Significant increases in TH-immunoreactive cell number were observed with zLEHD-fmk and zVDVAD-fmk concentrations of 100μM and above. The caspase 3 inhibitor zDEVD-fmk caused significant increases in dopaminergic neuronal survival only at 300μM, while no significant increases were observed with zYVAD-cmk at any concentration tested. When the compounds were added for 48 hours in the absence of MPP^+, no deleterious effects were observed with any of the compounds at any concentration (data not shown).

While zVDVAD-fmk is an inhibitor based on the preferred cleavage site for caspase 2, it is unlikely to be absolutely specific for caspase 2. The presence of an Asp residue in the P4 position of the inhibitor is a requirement for peptide

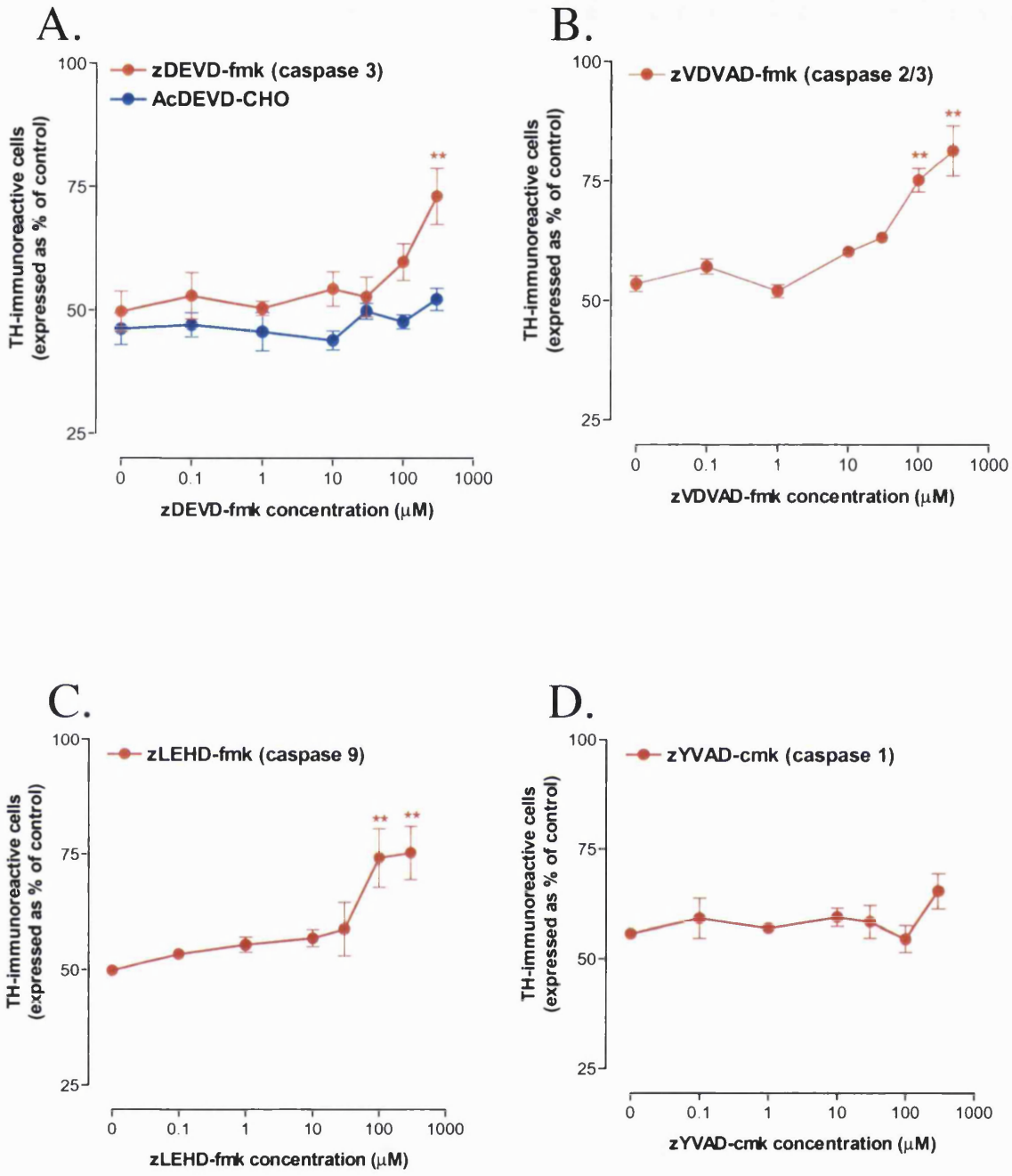


*Figure 4.8. Peptide inhibitors of caspases 2, 3 and 9, but not of caspase 1, prevent MPP⁺ mediated loss of TH-immunoreactive neurones. Cultures were maintained for 5 days, then treated with MPP⁺ 10μM in the presence of inhibitors at the concentrations shown for a further 48 hours. Cultures were fixed and immunostained for tyrosine hydroxylase, and surviving cells counted. Data shown are the mean ± s.e.m. of three independent experiments, each consisting of four independent wells per treatment group. Significant increases in survival were observed with zDEVD-fmk and, zLEHD-fmk and zVDVAD-fmk, but not with zYVAD-cmk (**p<0.01 by one-way ANOVA followed by Dunnett's test comparing all groups to MPP⁺ alone).*

inhibitors of caspases 3 and 7, and the VDVAD sequence has also been shown to inhibit these enzymes (Thornberry et al., 1997). Thus, the neuroprotection observed with this inhibitor may be due in part to an inhibition of caspase 3, though caspase 2 does have a preference for a pentapeptide structure. Neither zLEHD-fmk or zYVAD-cmk are likely to significantly inhibit executioner caspases; neither of these sequences has the required Asp in the P4 position. In cell free models, the YVAD sequence is around 10,000-fold more selective for caspase 1 than for caspase 2, 3, or 7, and around 1000-fold more selective for caspase 1 than for caspase 9 (Garcia-Calvo et al., 1998). The LEHD sequence does resemble the cleavage sites of caspases 4 and 5 and 8; there is likely to be some inhibition of these caspases.

Treatment of cultures with zDEVD-fmk resulted in only a partial neuroprotection which reached significance at only high concentrations; this may be due to the membrane permeability of the inhibitor. Charged tetrapeptide inhibitors such as DEVD typically have less membrane permeability than aromatic sequences such as YVAD; this may explain the complete lack of effect observed when MPP^+ was coadministered with AcDEVD-CHO; though the peptide sequence is identical, no neuroprotective effects are observed with the aldehyde compound, and this may reflect poorer cell permeability than the fluoromethyl ketone. These inhibitors typically have an intrinsic enzyme inhibitory activity in cell free systems in the low nM range, but require to be applied in the μM range in cell based systems. In such cell based systems, the intracellular concentrations of the compounds are unknown. Additionally, the peptide structure is unstable in the cellular

environment once the protecting group is cleaved off; the VAD peptide is stable for only 30 minutes once the fluoromethylketone is removed, and this is another reason why high concentrations of inhibitor are required (Donald W. Nicholson, personal communication)

Thus, while treatment of cultures with the caspase 3 inhibitor zDEVD-fmk causes only a partial neuroprotection from MPP^+ toxicity, this may reflect poor cell permeability or stability of the inhibitor rather than a limited role for the enzyme in mediating the toxicity. Development of inhibitors with greater potency in cell based assays has allowed closer analysis of this.

4.2.2.3. Novel caspase inhibitors

Compounds from a range of novel caspase inhibitors were tested for neuroprotective effects for TH-immunoreactive neurones in primary cultures of mesencephalic neurones exposed to MPP^+. Figure 4.9. shows the effects of two of these inhibitors, M-920, a non-specific inhibitor of caspases, and M-791, a selective caspase 3 inhibitor. M-725, an inactive diastereomer of M-920 was also tested. These inhibitors are described in a model of sepsis by Hotchkiss et al. (2000). The results of these experiments are shown in Figure 4.9.A. Both of the active caspase inhibitors caused significant increases in the number of surviving TH-immunoreactive neurones. Significant neuroprotection was observed with M-920 at concentrations of 10μM and above; at concentrations of 10μM and above, the survival was similar to that observed in untreated control cultures. Treatment of dopaminergic neurones with M-791 caused significant neuroprotection at

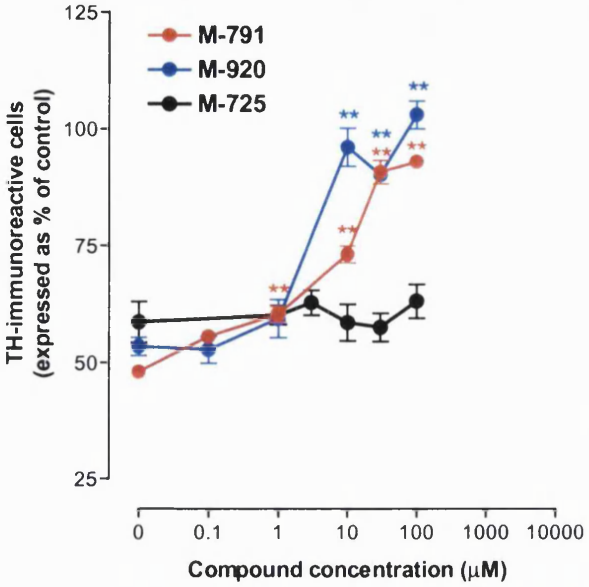
concentrations of 1μM and above; the maximal response observed with this inhibitor increased the number of surviving TH-immunoreactive neurones to greater than 90% of untreated control. The significant neuroprotective effects observed with M-791 at 1μM indicate that the neuroprotection is likely to be mediated by inhibition of caspase 3 like proteases. The survival response with this caspase 3 inhibitor is considerably higher than that observed with zDEVD-fmk, the peptidergic caspase 3 inhibitor, which might indicate limited cell permeability of the peptide caspase inhibitor. The magnitude of the survival effect of M-791 is equivalent to the effects observed with both zVAD-fmk (see Figure 4.4) and M-920, the broad spectrum caspase inhibitors. This suggests that inhibition of caspase 3 or a caspase 3 like protease is sufficient to prevent almost all the toxicity of MPP^+ in this culture system. When cultures were treated with the inactive compound M-725, no neuroprotective effects were observed at any of the concentrations tested. Photomicrographs of TH-immunoreactive neurones co-exposed to MPP^+ 10μM and M-791 at the maximal concentration of 100μM show that there are more cells in the M-791 treated cultures (Figure 4.9.E) compared to cultures exposed to MPP^+ alone (Figure 4.9.D.). Untreated control cultures are shown in Figure 4.9.C.

With regard to the specificity of the inhibitors, M-920 is reported to have an IC_{50} value of 0.002μM for caspase 3 in *in vivo* sepsis models, and sub-micromolar IC_{50} values for caspases 1, 4, 7, and 8. The IC_{50} values for caspase 5 and 6 are 2 and 1.5μM respectively. M-791 has an IC_{50} value of 0.008μM for caspase 3, and 0.23μM for caspase 7 in the sepsis model; the IC_{50} for caspase 8 is 4μM, and for

other caspases is in the mid-micromolar range (Hotchkiss et al., 2000). IC_{50} values on a range of caspases and in two whole cell *in vitro* models for these three compounds are shown in Figure 4.9.B; these data are provided courtesy of the Merck-Frosst caspase programme and are published data (Hotchkiss et al., 2000).

Further evidence for the importance of caspase 3 in mediating the cell death induced by MPP^+ comes from testing of further members of this series of compounds, data which are presented in Figure 4.10. This graph contains data generated with 6 compounds, with varying potencies in cell free and cell based systems for inhibiting caspase 3, together with the data obtained with M-920, M-791 and M-725. The neuroprotection offered by each of these inhibitors broadly follows the rank order of potency for the inhibition of caspase 3 by each inhibitor. Thus the least potent of the inhibitors is ~~L-818,078,~~ M-078 which has a reported IC_{50} in an NT-2 toxicity assay of 12μM; the most potent is ~~L-826,920,~~ M-920 with a reported IC_{50} of 0.12μM. The two most potent of the caspase 3 inhibitors, ~~L-826,643~~ M-643 and ~~L-826,791,~~ M-791 have IC_{50} values of 0.3μM and 0.49μM respectively. IC_{50} values for these compounds are provided courtesy of Sophie Roy, Merck-Frosst caspase inhibitor programme. Six of the inhibitors were tested for effects in dopaminergic neurones when applied to mesencephalic cultures for 48 hours in the absence of MPP^+; these data are shown in Figure 4.11. No deleterious effects were observed on the number of TH-immunoreactive cells after 48 hour treatment with these inhibitors at any of the concentrations tested.

A.



B.

Caspase inhibitor IC_{50} values			
Caspase	Cell free assays (μM)		
	M-920	M-791	M-725
Caspase 1	0.01	11	15
Caspase 2	>50	>450	
Caspase 3	0.002	0.008	45
Caspase 7	0.02	0.23	>100
Caspase 8	0.04	4	68
Caspase 9	>50	>450	
Cell type	Whole cell apoptosis IC_{50}		
hNT2	0.12 μM	0.49 μM	
1°mCGN	0.2 μM	1.0 μM	

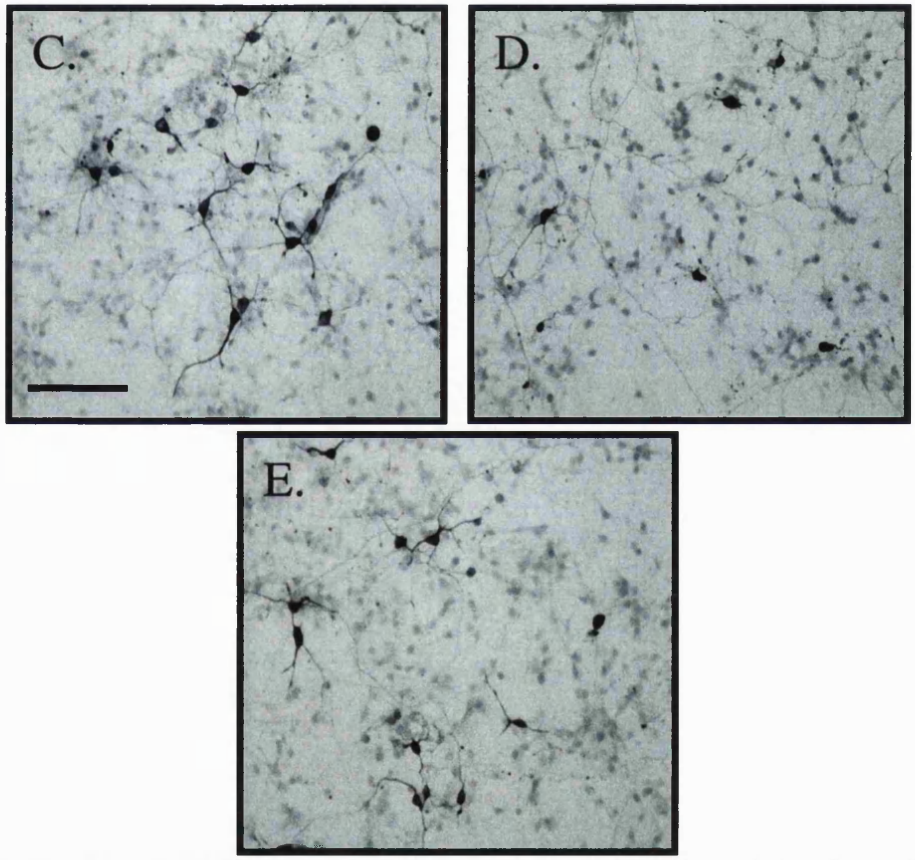


*Figure 4.9. Novel inhibitors of caspases prevent MPP⁺ toxicity for primary cultures of mesencephalic dopaminergic neurones; caspase 3 inhibition is as effective as a broad-spectrum inhibitor (**p<0.01 by one-way ANOVA followed by Dunnett's test comparing all groups to MPP⁺ alone). M-791 and M-920 both caused near complete sparing of TH-immunoreactive neurones; the control compound M-725 was ineffective (A). IC50 values of the compounds at selected caspases are shown in (B), together with IC50 values in whole cell models of neuronal cell death (provided courtesy of Dr Sophie Roy, Merck-Frosst Canada caspase program). Photomicrographs of control (C), MPP⁺ 10μM treated (D) and MPP⁺ 10μM + M-791 100μM treated (E) cultures are also shown (scalebar=100μm).*

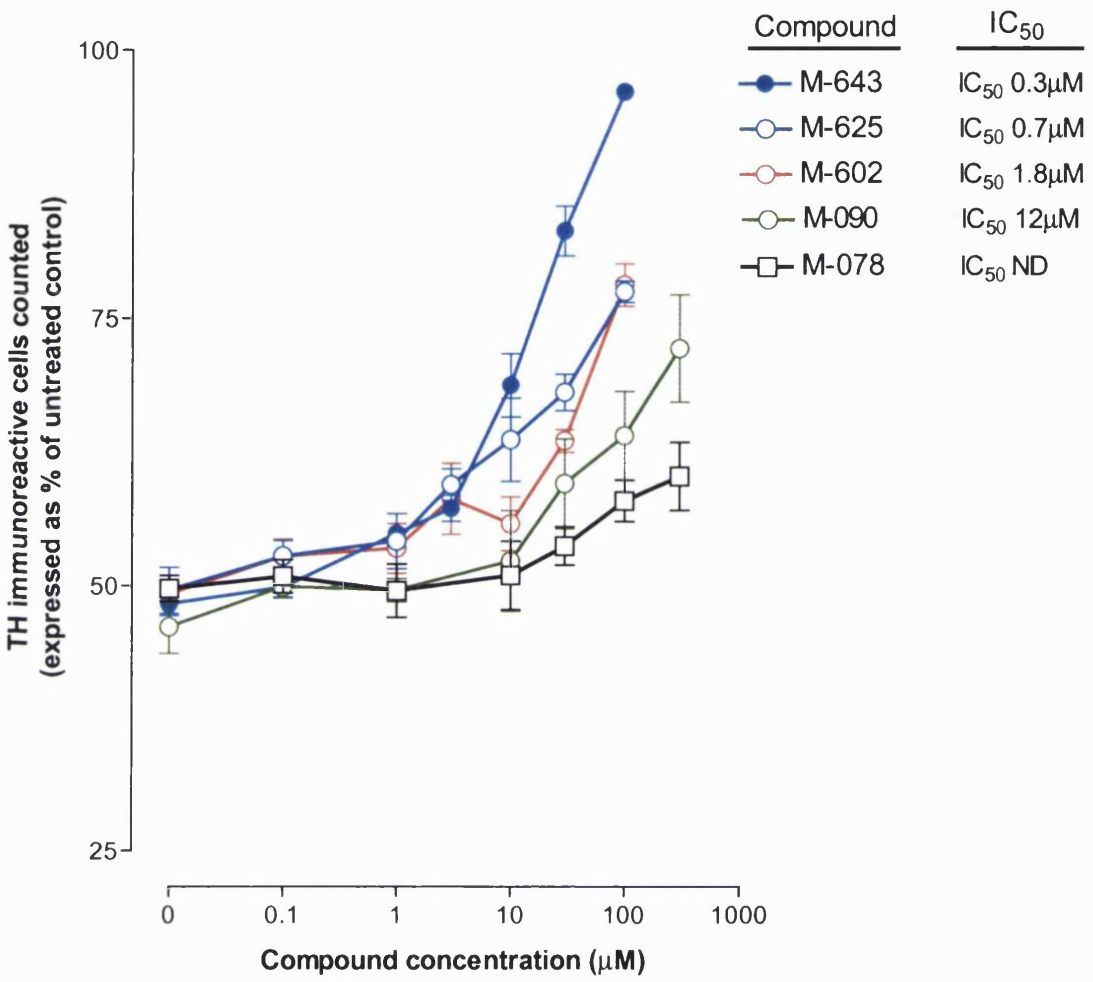


Figure 4.10. Effectiveness of novel caspase inhibitors in preventing MPP^+ toxicity for dopaminergic neurones correlates well with their reported IC_{50} values for caspase 3 inhibition in human NT2 cells. All of the inhibitors tested caused significant sparing of TH-immunoreactive neurones from MPP^+ 10μM toxicity. Compounds were added in the presence of MPP^+ for 48 hours. The potency of the inhibitors in sparing dopaminergic neurones correlated well with their caspase 3 IC_{50} values in NT2 cells (provided by Merck Frosst caspase programme). The most potent inhibitors restored the number of TH-immunoreactive neurones to control levels. All data points shown are from four independent wells per treatment group.

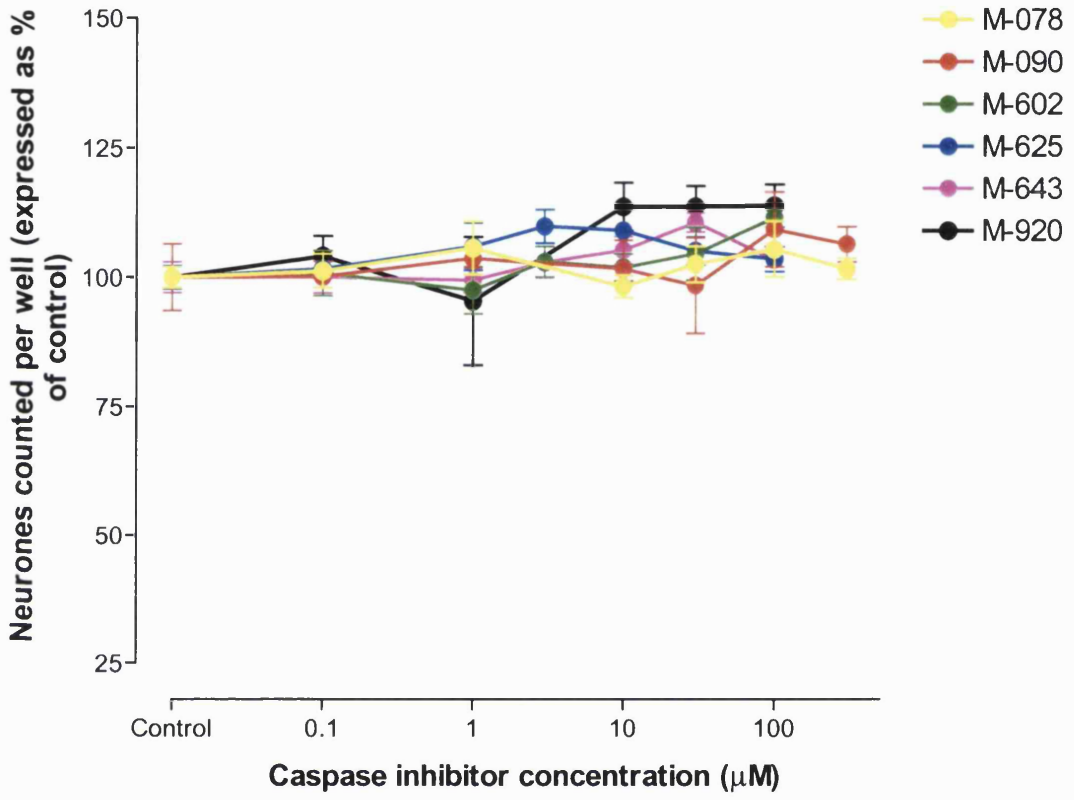


Figure 4.11. Novel caspase inhibitors have little effect on the number of TH-immunoreactive neurones when added for 48 hours in the absence of MPP^+. Compounds were added for 48 hours to mesencephalic cultures in the absence of MPP^+; cultures were then fixed and immunostained and the number of TH-immunoreactive neurones quantified. Slight increases in the number of TH-immunoreactive neurones were observed in cultures treated with M-920; this is possibly due to the prevention of apoptotic cell death in the cultures as a result of medium changing.

Thus, inhibition of caspases can restore the number of MPP^+ exposed TH-immunoreactive neurones to near control levels, and selective inhibitors of caspase 3 are equivalent in potency to broad spectrum inhibitors. This indicates that caspase 3 inhibition alone is sufficient to preserve the number of MPP^+ exposed TH-immunoreactive neurones. Experiments were undertaken to determine whether the novel caspase inhibitors were more potent than zVAD-fmk in preventing MPP^+ mediated loss of ^{3}[H]-DA uptake. The results of these experiments are shown in Figure 4.12. M-920 caused increased uptake of ^{3}[H]-DA at concentrations of 10μM and above; the compound was thus more potent at increasing ^{3}[H]-DA uptake than zVAD-fmk. The magnitude of the response, however, was broadly similar to the maximal response observed with zVAD-fmk, with ^{3}[H]-DA uptake being increased only to about 15% of control levels from 5% with MPP^+ alone. These data confirm that caspase inhibition is not sufficient to prevent the majority of MPP^+ mediated loss of ^{3}[H]-DA uptake. The results obtained with M-791, on the other hand, show little increase over cells treated with MPP^+ alone; it may be that inhibition of a caspase other than caspase 3 is required for the modest increase in ^{3}[H]-DA uptake observed with the broad spectrum inhibitors. What is clear is that caspase inhibition is less effective in restoring the ability of dopaminergic neurones to take up ^{3}[H]-DA than in preventing loss of the TH-immunoreactive cell bodies; this may be due to a failure to prevent degeneration of terminals and thus dopamine transporter sites, or else to an inability of these caspase inhibitors to retain functional dopaminergic neurones.

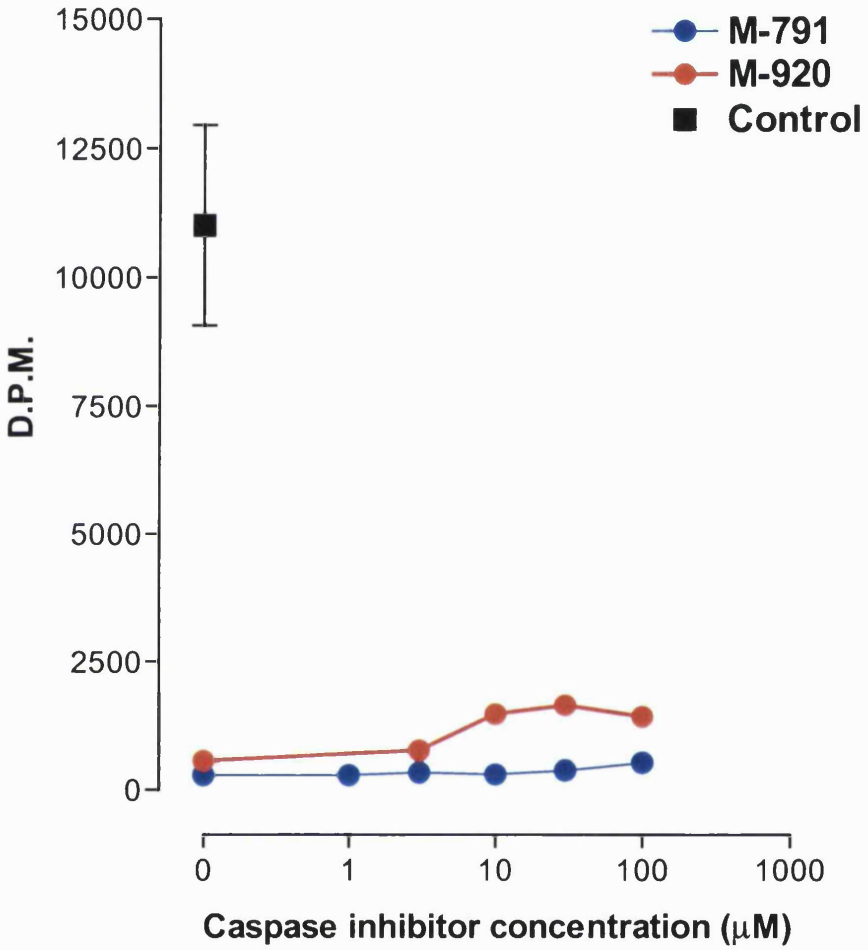


Figure 4.12. M-920 and M-791 are less effective in restoring ^{3}[H]DA uptake in MPP^{+} treated dopaminergic neurones. M-920 and M-791 were added in combination with MPP^{+} 10μM at the concentrations shown for 48 hours. Sample data from one experiment (of two performed) are shown for each compound. M-920 is the more potent of the two compounds, causing around a three-fold increase in ^{3}[H]DA uptake. For both compounds, however, the response was small by comparison to untreated control levels.

4.2.3. Effects of caspase inhibition on expression of apoptotic features.

In order to quantify MPP^+ induced caspase activation and chromatin condensation, and evaluate the effects of caspase inhibition on these parameters, triple fluorescent labelling was carried out. Cultures were treated with MPP^+ for 48 hours in the presence or absence of zVAD-fmk 300μM or the caspase 3 inhibitor M-791, then fixed and double immunostained for active caspase 3 and TH. Nuclei were counterstained using Hoechst 33342, and quantification was carried out. Ten fields of view containing at least three TH-immunoreactive cells were quantified in each of three independent wells. The total number of nuclei was established, and the number of these which showed apoptotic features established. The number of TH-immunoreactive neurones, and the number of active caspase-3 neurones was also counted. Each field of view was quantified for the number of neurones co-expressing TH / active caspase 3 and TH / condensed chromatin. In 4.13.A., the number of apoptotic cells and the number of active caspase 3-immunoreactive cells in each treatment group is shown, expressed as a percentage of the total number of cells within the cultures. In control cultures there is a population of around 20% of cells which express apoptotic morphology, likely as a result of stress through changing the medium or a natural attrition of cells within the culture. There is a slight increase in the number of apoptotic cells in the MPP^+ treated group, which is reduced by the caspase inhibitors. There is also a small population of active caspase 3-immunoreactive cells within control cultures, less than 10%. This is increased by MPP^+ treatment, but this increase is not reversed by the caspase inhibitors. Figure

4.13.B. shows the expression of apoptotic nuclei and active caspase 3 in TH-immunoreactive neurones. Around 10% of TH-immunoreactive neurones have apoptotic nuclei in control cultures; this is markedly increased by MPP^+ treatment, which increases the number of apoptotic nuclei in remaining TH-immunoreactive neurones to around 60%. Both of the caspase inhibitors tested completely reverse the increase in apoptotic nuclei induced by MPP^+. When co-expression of TH and activated caspase 3 was examined, there was again a marked increase in the number of co-expressing cells, from around 10% in control cultures to around 50% in MPP^+ 10μM treated cultures. When MPP^+ was coadministered with the caspase inhibitors, however, there was little decrease in the expression of activated caspase 3 in TH-immunoreactive neurones. This lack of decrease with the caspase inhibitors is likely due to the mode of action of the inhibitors, which bind to the cleavage site of the active caspase and prevent cleavage of cellular substrates rather than preventing formation of the active caspase from the inactive zymogen. Thus, in the inhibitor and MPP^+ treated dopaminergic neurones, the caspase appears to be activated as in cultures treated with MPP^+ alone, but inhibition prevents it from executing the apoptotic response; this leads to the decreased evidence of chromatin condensation and the increased neuronal number.

Not all MPP^+ treated dopaminergic cells visualised expressed chromatin condensation or active caspase 3; this may reflect a population which has not yet effected the apoptotic response following MPP^+ treatment. At 48 hours treatment, only around 50% of dopaminergic cells remain in the cultures compared to

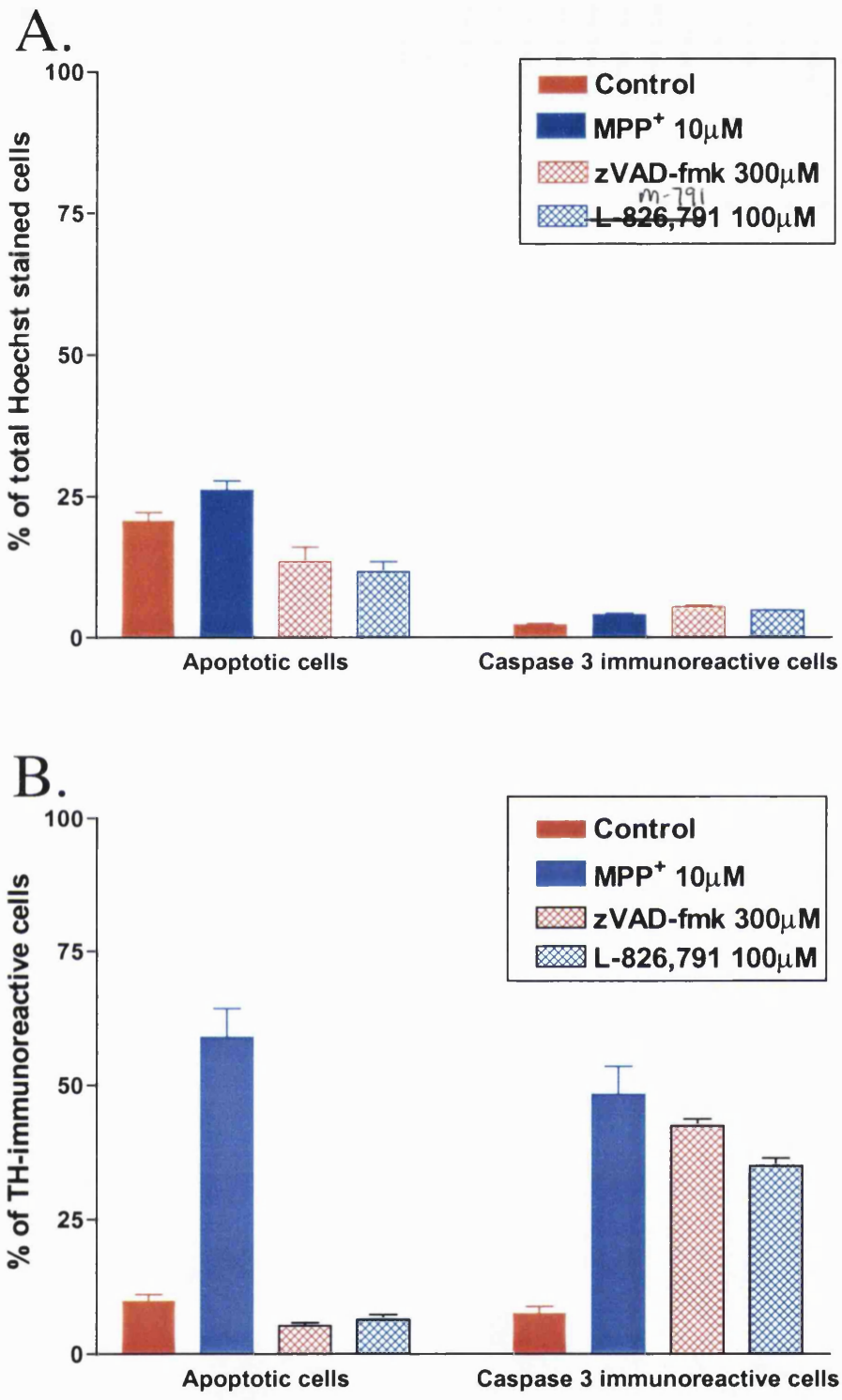


Figure 4.13. Caspase inhibition prevents MPP+ mediated increase in cells with apoptotic nuclei, but has little effect on the expression of active caspase 3. (A) shows the effect in the total population of cells, with a slight decrease in apoptotic nuclei. (B) demonstrates that caspase inhibition prevents the expression of apoptotic nuclei but not of active caspase 3 in TH-immunoreactive neurones.

untreated controls. Dopaminergic cells expressing active caspase 3 were also present within the cultures at earlier timepoints (shown in Figure 4.2.). It is likely that these cells which activate caspase 3 earlier in the timecourse undergo apoptosis and detach from the substratum resulting in this decrease in numbers, and that the number of dopaminergic neurones counted with the active enzyme at 48 hours underestimates the number which express this over the total treatment period.

4.3. Discussion

4.3.1. Results summary

To summarise, the data in this chapter show that MPP^+ treatment of primary mesencephalic dopaminergic neurones results in morphological features characteristic of apoptosis and in activation of caspase 3. Inhibition of caspases with the broad spectrum inhibitors zVAD-fmk or M-920 resulted in a sparing of MPP^+ 10μM treated TH-immunoreactive cells; survival increased to greater than 90% with zVAD-fmk treatment, and to control levels with the novel inhibitor M-920. Using peptide inhibitors of specific caspases, significant but partial neuroprotective effects were observed with inhibitors of caspases 2, 3, and 9, but not with an inhibitor of caspase 1. The effects observed with the caspase 3 inhibitor were observed with a fluoromethyl ketone inhibitor structure; the same peptide with an aldehyde group was ineffective, indicating that membrane permeability or stability of this inhibitor may be an issue. The most compelling evidence for a crucial role of caspase 3 in mediating MPP^+ mediated cell death is the data obtained with a range of novel selective inhibitors of caspase 3; the most potent of these inhibitors, M-791, restored the number of surviving TH-immunoreactive neurones to near control levels. The neuroprotective effect of this inhibitor was equivalent to that observed with either of the broad-spectrum inhibitors; this provides strong evidence for a pivotal role of this enzyme in mediating the MPP^+ toxicity.

zVAD-fmk coadministration with MPP^+ resulted in attenuation of MPP^+ mediated somatic shrinkage in TH-immunoreactive neurones, and a partial restoration of the length of the longest neurite in the cells, both evaluated by image analysis. Little increase was observed in ^{3}[H]-DA uptake by dopaminergic neurones when treated either with zVAD-fmk or with either of the novel caspase inhibitors M-920 or M-791.

Treatment of mesencephalic neurones with MPP^+ resulted in a small increase in the number of apoptotic cells counted in the population as a whole, and a large increase in the number of apoptotic cells in the TH-immunoreactive population. Similarly, MPP^+ treatment caused small increases in the number of active caspase 3 expressing cells in the general population, but a large increase in the TH-immunoreactive population. Coadministration with either zVAD-fmk or the selective caspase 3 inhibitor M-791 abolished the MPP^+ induced increase in apoptotic dopaminergic cells, but was ineffective at reducing the number of dopaminergic cells expressing the activated caspase.

4.5.2. Apoptosis/caspase activation

Since 1998, when this work commenced, a number of publications have appeared which indicate a role of caspases in mediating the toxicity of MPP^+/MPTP both *in vitro* and *in vivo*. Unusually, perhaps, the data obtained in *in vivo* studies have appeared more robust. In *in vitro* systems, there has been controversy in the literature, with a number of publications finding a protective role for caspase inhibition against MPP^+ toxicity, and others finding no evidence for such a role.

In this discussion the evidence for and against caspase activation in both *in vitro* and *in vivo* models will be reviewed in the light of the data presented here.

Early evidence for activation of caspases and for a neuroprotective role of caspase inhibition in *in vitro* systems was presented by Dodel and coworkers (1998). In this study, the authors reported protection of primary mesencephalic dopaminergic neurones by caspase inhibition using zVAD-fmk, and found no significant neuroprotection using a caspase 1 inhibitor. The authors hypothesised that a caspase 3 like protease was responsible for the execution of the cell death, though no direct evidence was presented to support this hypothesis. The same group (Du et al., 1997) had previously demonstrated that in cerebellar granule neurones exposed to MPP^+ at high concentrations, caspase inhibition by broad-spectrum or caspase 3 selective peptide inhibitors was neuroprotective, and that MPP^+ treatment led to a redistribution of cytochrome c from the mitochondria to the cytoplasm. In contrast, a study by Lotharius and coworkers (1999) failed to find evidence for a neuroprotective role of caspase inhibition in MPP^+ treated dopaminergic neurones using the poly-caspase inhibitor boc-Asp-fmk, though neuroprotection from 6-OHDA toxicity was observed. Differences were observed in the expression of markers of apoptosis in these studies; in both cerebellar granule cells (Du et al., 1997) and primary dopaminergic neurones (Dodel et al., 1998), apoptotic morphology was detected in neuronal nuclei, whereas Lotharius and coworkers found no evidence for phosphatidylserine externalisation, another marker of apoptosis. Studies in the MN9D dopaminergic cell line following MPP^+ exposure have also failed to find evidence for either apoptosis or caspase

activation (Choi et al., 1999, 2001, Kim et al., 2001). The intensity of the MPP^+ intoxication appears to be crucial in determining whether the cell death proceeds through an apoptotic or necrotic pathway (Chalmers-Redman et al., 1999), with lower concentrations causing apoptosis and higher concentrations causing necrosis. A recent study by Eberhardt and co-workers (2000) demonstrated that inhibition of caspases using zVAD-fmk spared MPP^+ treated dopaminergic cell bodies but not processes *in vitro* and *in vivo*; a study by Hartmann and coworkers (2001), however, demonstrated that zVAD-fmk, while neuroprotective against MPTP toxicity *in vivo*, potentiated dopaminergic neuronal death by necrosis in MPP^+ treated primary mesencephalic cultures unless cultures were maintained in elevated glucose. This study also demonstrated that caspase 8 like-protease inhibition using the tetrapeptide IETD was neuroprotective *in vivo*, but again potentiated toxicity *in vitro* unless cells were maintained in elevated glucose. The changes in the survival response of MPP^+ exposed dopaminergic neurones with different glucose concentrations in the Hartmann study is interesting; an interpretation of these data is that the culture conditions used are critical in determining whether caspase inhibition is neuroprotective against MPP^+ toxicity *in vitro*. Adjustment of one component of the culture medium, glucose, in this study is sufficient to cause a switch from potentiation of cell death to neuroprotection with both zVAD-fmk and zIETD-fmk, the caspase 8 inhibitor. Glucose has previously been shown to attenuate the cell death induced by MPP^+ (Chalmers-Redman et al., 1999); these authors hypothesised that this might be due to reverse proton pumping at ATP synthase by glycolytic ATP resulting in a

stabilisation of mitochondrial membrane potential and prevention of mitochondrial permeability transition pore opening. Thus, changes in culture conditions are sufficient to change the response of MPP^+ treated dopaminergic neurones to caspase inhibitors, making direct comparisons between these studies difficult.

The results in MPTP treated animals *in vivo* have been more robust; reports from a number of groups have demonstrated both activation of caspases and neuroprotection by caspase inhibition. It may, therefore, be fair to suggest that the culture conditions in those reports showing no effects of caspase inhibition on dopaminergic neurones *in vitro* reflect the *in vivo* situation less well. Despite this, the debate between apoptotic/necrotic cell death is still a vexed question, in this as in a number of other models; this will be addressed in more depth in the general discussion.

Under the culture conditions used in this study, it is clear that caspase inhibition is neuroprotective against MPP^+ toxicity. The finding that zVAD-fmk but not zYVAD-cmk increases TH-immunoreactive cell number confirms the findings of Dodel et al (1998); the limited restoration of ^{3}[H]-DA uptake and preservation of neurites with zVAD-fmk has also recently been confirmed in both MPP^+ and 6-OHDA treated dopaminergic neurones. A number of *in vivo* reports have now been published, utilising different approaches to target the caspase pathway. Few studies have been published using caspase inhibitory compounds *in vivo*, due to problems of administration and selectivity. As previously mentioned, the broad spectrum caspase inhibitor zVAD-fmk and the caspase 8-like protease inhibitor

zIETD-fmk are neuroprotective against MPTP toxicity for mice *in vivo* (Hartmann et al., 2000); zVAD-fmk has also been shown to protect mesencephalic dopaminergic neurones from 6-OHDA toxicity in rats *in vivo* (Von Coelln et al., 2001). Other reports have used transgenic animals or viral vector mediated gene delivery to examine the role of the caspase pathway in MPTP/MPP^+ toxicity.

A number of studies have examined the role of caspase 1 in mediating MPTP mediated cell death *in vivo*. Overexpression of dominant negative caspase 1 has been shown to protect nigral dopaminergic neurones from MPTP toxicity in one study (Klevenyi et al., 1999), but there is some variability in the literature with regard to a role this enzyme. This caspase is a member of the group I family which is typically thought to be more involved in the inflammatory response than in regulation of apoptosis; the lack of neuroprotective effect of inhibition in *in vitro* models would appear to confirm this. Two studies, including the current study, have demonstrated that caspase 1 inhibition with a peptide inhibitor is ineffective in protecting against MPP^+ toxicity *in vitro* (Dodel et al., 1998), though increased caspase 1 activity has been observed in the dopaminergic cell line SN4741 following MPP^+ exposure (Chun et al., 2001). Recombinant adeno-associated viral mediated expression of a dominant negative caspase 1 C285G construct had no effect on survival of dopaminergic neurones in another *in vivo* MPTP study in mouse (Mochizuki et al., 2001). MPTP treatment has also been reported to increase brain caspase 3 but not caspase 1 activity *in vivo* (Usha et al., 2000), though the caspase 1 data were not shown; precisely the opposite data

have, however, also been reported recently, where MPTP increases expression of both caspase 1 and iNOS (Du et al., 2001), and increases production of mature interleukin-1β (Wu et al., 2002). These studies also suggest the interesting possibility that the MPTP/MPP^+ mediated caspase 1 activation may take place in glial cells rather than in the neurones; if this is the case, it may be one reason why there was no indication of a neuroprotective role of enzyme inhibition in *in vitro* neuronal models. As in the *in vitro* models, it seems most likely that there are different potential pathways which may be activated by MPTP *in vivo*, and the severity of the initial insult determines which is activated.

Other studies have targeted elements upstream of caspase activation. The cell intrinsic pathway to caspase activation appears to be involved, based on the results of these studies. A number of studies have examined the role of the Bcl-2 family in MPTP/MPP^+ mediated cell death. Nigral dopaminergic neurones of mice overexpressing Bcl-2 are resistant to MPTP toxicity *in vivo* (Yang et al., 1998), and have reduced levels of the active form of caspase 2. Another study demonstrated decreased MPP^+ toxicity for cortical neurones *in vitro* derived from Bcl-2 overexpressing mice (Offen et al., 1998); in this study, rather high levels of MPP^+ were used to kill the cortical neurones, and it is unclear why this neuronal population was chosen rather than a population more responsive to MPP^+. In a similar vein, ablation of Bax protects nigral neurones from MPTP toxicity *in vivo* (Vila et al., 2001), indicating that the balance between pro- and anti- apoptotic Bcl-2 family members is of importance in mediating MPTP toxicity. MPTP has been shown to increase expression of Bax *in vivo* (Hassouna et al., 1996, Chen et

al., 2001). In the MN9D cell line, however, MPP^+ did not increase Bax expression, though overexpression of Bcl-2 was neuroprotective (Choi et al., 1999); a similar lack of effect on Bax expression with MPP^+ was observed in primary dopaminergic cultures by Hartmann and co-workers (2001b), though cytochrome c release and apoptosis were detected.

As well as the Bcl-2 family, mitochondria can release pro-apoptotic factors through the mitochondrial permeability transition pore in stressed cells. A number of studies have demonstrated that MPP^+ and other Complex I inhibitors can open the mitochondrial PTP *in vivo* and *in vitro* (Cassarino et al., 1998, Seaton et al., 1998, Cassarino et al., 1999, Chalmers-Redman et al., 1999, reviewed in Tatton and Olanow, 1999, Schapira, 1998). MPP^+ induced apoptosis has been reported to be inhibited by the mitochondrial PTP blocker cyclosporin A in PC12 cells (Seaton et al., 1998) and by bongkreckic acid in SH-SY5Y cells (Abramova et al., 2001). Cyclosporin A has also been reported to potentiate SH-SY5Y cell death, however (Fall and Bennett Jr., 1998), and it was without neuroprotective effect in the model described here, most likely due to toxicity at the higher concentrations (not shown).

Both pro-apoptotic members of the Bcl-2 family and opening of the mitochondrial permeability transition pore can lead to release of pro-apoptotic factors from the inner leaflet of the mitochondrion to the cytoplasm, as discussed in Chapter 1, above. Several approaches have examined this pathway. Cytochrome c release has been observed in response to MPP^+ treatment *in vitro* in cerebellar granule neurones (Du et al., 1997, Cassarino et al., 1999) and in the substantia nigrae of

MPTP treated mice *in vivo* (Viswanath et al., 2001). This released cytochrome c appears to activate the caspase cascade through caspase 9 activation. Viswanath and co-workers demonstrate active caspase 9, along with active caspases 3 and 8 and cleavage of Bid following MPTP treatment and cytochrome c release *in vivo.* The data presented in this chapter demonstrate that caspase 9 inhibition protects dopaminergic neurones *in vitro* from MPP^+ toxicity; Viswanath and co-workers also demonstrate that in PC12 cells caspase 9 inhibition prevents activation of caspase 3 and 8 and Bid cleavage, but not cytochrome c release. The presence of active caspases 3, 8 and 9 was also demonstrated in primary dopaminergic neurones and in Parkinsonian brain. Increased caspase 9 activity has also been reported in MPP^+ exposed SH-SY5Y cells (Gomez et al., 2001). Thus, caspase 9 is activated in MPP^+/MPTP toxicity, and appears to play a crucial role in mediating the cell death through activation of other caspases. That this toxicity is mediated through the formation of the apoptosome is demonstrated in a report by Mochizuki and co-workers (2001), who demonstrated that adeno-associated viral delivery of dominant negative Apaf-1 both prevented MPTP nigral toxicity in mice and retained functionality of the remaining neurones in rotation studies. Similarly adenoviral delivery of X-linked inhibitor of apoptosis protein, an endogenous caspase inhibitor which binds caspase 9, also reduces cell loss in the substantia nigrae of MPTP treated mice (Eberhardt et al., 2000). XIAP also has inhibitory activity at caspase 3; since caspase 3 has also been implicated as a crucial factor in the execution of cell death induced by MPTP/MPP^+ this may also be of importance.

The release of cytochrome c from the mitochondrion and subsequent activation of caspase 9 are clearly important in MPP^+ toxicity, but caspase 9 has little pro-apoptotic activity. Caspase 9 cleaves apoptotic executioners such as caspase 3. The data presented in the current study indicate that caspase 3 is activated in MPP^+ toxicity; moreover, the data presented with the novel caspase 3 inhibitors indicate that activity of this caspase is a critical step in MPP^+ toxicity, as inhibition causes near complete neuroprotection. A number of studies have implicated caspase 3 as an important component of the apoptotic response to MPP^+. *In vitro*, caspase 3 has been shown to be activated in MPP^+ exposed SH-SY5Y cells (King et al., 2001, Abramova et al., 2001), PC12 cells (Shimoke and Chiba, 2001, Viswanath et al., 2001) and primary dopaminergic neurones, as described here. Increased activation of caspase 3 is also reported in the substantia nigrae of MPTP treated mice (Turmel et al., 2001, Xia et al., 2001, Viswanath et al., 2001), and has, in fact, been reported in the brains of PD patients (Hartmann et al., 2001). Inhibition of caspase 3 has been shown to be neuroprotective against both MPP^+ (Du et al., 1997, Dodel et al., 1998) and 6-OHDA toxicity *in vitro* (Dodel et al., 1999). The data presented here with the novel caspase 3 inhibitors demonstrate the most robust *in vitro* effect of caspase 3 inhibition reported to date; this is likely due to the limitations of the commercially available inhibitors.

In addition to the pathway described above, where cytochrome c release induces activation of caspase 9 then 3, caspases 2 and 8 have been implicated in MPTP/MPP^+ toxicity. Bcl-2 overexpressing mice, discussed above, are resistant

to MPTP toxicity (Yang et al., 1998). These mice also show reduced caspase activation in response to MPTP; the caspase evaluated was caspase 2, which is an executioner caspase by substrate preference. Though in this study zVDVAD-fmk was tested, and this peptide is based on the preferred cleavage site of caspase 2, in reality there is likely to be little or no specificity for caspase 2 over caspase 3 or 7 with this compound (discussed above). Thus, the Bcl-2 study is the only evidence for a role of caspase 2 in the toxicity; given that activity is inhibited by Bcl-2 overexpression it is likely to be involved in the cell intrinsic pathway to apoptosis. Caspase 2 could be activated through at least three pathways. Firstly, a direct activation by cytochrome c release from the mitochondrion. This is possible, but has never yet been reported to my knowledge. Secondly, direct activation by caspase 9 – again, this has not been demonstrated. What has been demonstrated is the third route, which involves processing of caspase 2 by caspase 3, which is in turn activated by caspase 9 (Paroni et al., 2001). Alternatively, an as yet unspecified pathway may activate caspase 2, as caspase 3 independent caspase 2 activation has been described in trophic factor deprived sympathetic neurones and PC12 cells (Stefanis et al., 1998) and β-amyloid 1-42 treated neurones (Troy et al., 2000); the pro-domain of caspase 2 itself possesses intrinsic enzymatic activity capable of autoactivating the caspase. Interestingly, a number of recent publications have demonstrated that caspase 2 itself can lead to release of cytochrome c from mitochondria, both through interaction with BH3 domain only Bcl-2 family members such as Bid and through a direct effect on Bax. Thus this is a possible feedback loop, where caspase 2 is activated either through cleavage

by caspase 3 or another mechanism and then feeds back by enhancing cytochrome c release from mitochondria to potentiate the caspase cascade.

Caspase 2 is not the only caspase which can modulate mitochondrial cytochrome c release in this fashion; similar findings are reported for caspase 8. Caspase 8 has been shown to be activated in the MPTP/MPP^+ model of dopaminergic toxicity *in vivo* and *in vitro* (Hartmann et al, 2000, Viswanath et al., 2001), and pathway inhibition is reported to be neuroprotective in both models, at least under certain culture conditions. Viswanath and co-workers (2001) suggest that caspase 8 activation is downstream of caspase 9 and 3 activation in MPTP/MPP^+ toxicity *in vivo* and *in vitro*, as demonstrated by activity assays. While this may indeed be the case, it is important to note that the inhibitor used in their *in vitro* study, zLEHD-fmk, is likely also to inhibit the activity of caspase 8 (Garcia-Calvo et al., 1998). More convincing data may have been to demonstrate a reduction in immunostaining for active caspase 8 with zLEHD-fmk treatment. Caspase 8 is also activated by the 'extrinsic pathway' discussed in the introduction, and there is no evidence that this is not also activated in MPTP/MPP^+ toxicity (discussed further in Chapter 5, below). Caspase 8, once activated, again functions in a cascade which culminates on activation of the executioner caspases such as caspase 3. Caspase 8 can directly activate caspase 3. It is also well established that caspase 8 can interact with the mitochondrial apoptotic pathway; activated caspase 8 can cleave BH3 domain only Bid, causing mitochondrial translocation, facilitation of Bax-like pro-apoptotic pore formation, and potentiating release of

cytochrome c (discussed in Chapter 1, above); this may well form another feedback loop to potentiate the caspase cascade.

Though the majority of the published studies now appear to indicate that the loss of cells induced by MPTP/MPP^+ treatment is mediated at least in part by the caspase family, there is rather less evidence for a functional recovery. In the series of experiments described here, caspase inhibition caused at best a marginal recovery in ability of cells to take up ^{3}[H]-DA, and only a partial restoration of neurites. Similar findings were reported in MPP^+ treated dopaminergic neurones (Eberhardt et al., 2000) and in the 6-OHDA model of toxicity for SH-SY5Y cells and for dopaminergic neurones (von Coelln et al., 2001). In *in vivo* experiments with caspase inhibitors, sparing of a proportion of striatal innervation has been observed (Eberhardt et al., 2000); in this same study, however, it was reported that while adenoviral gene transfer of XIAP into nigral dopaminergic neurones spared them from MPTP toxicity, it was not sufficient in the absence of additional trophic support to restore the loss of striatal dopamine and metabolites. Other approaches to targeting the caspase pathway *in vivo* have resulted in functional sparing of the nigrostriatal pathway. These include p35 overexpression (Viswanath et al., 2001), Bcl-2 overexpression (Yang et al., 1998), Bax ablation (Vila et al., 2001) and dominant negative caspase 1 expression (Klevenyi et al., 1999). In all of these reports, however, the recovery of striatal dopamine or dopamine metabolite concentration, or striatal dopaminergic innervation density, was less robust than the sparing of the nigral dopaminergic neurones themselves. Thus, the functional recovery induced by inhibition of caspases or the caspase

pathway in MPTP/MPP^+ is rather questionable. This would lead to the conclusion that there may be additional, non-caspase mediated pathways which underlie the loss of striatal innervation. MPTP/MPP^+ has been shown to cause dramatic early depletion of catecholamine neurotransmitters (Sirinathsinghji et al., 1986), and this may be a mechanism underlying this effect; it is, however, also true to say that it is unclear in the studies to date how effective the approaches have been in blocking all caspase activity. It is possible that more complete caspase inhibition may increase the functional recovery observed in this model; unfortunately, there are currently no such approaches available.

Together, these data and the reported studies indicate that the activation of caspases is a crucial step in the execution phase of neuronal loss induced by MPTP/MPP^+. In both *in vivo* and *in vitro* models, interference with the caspase cascade protects neurones from MPTP/MPP^+ mediated apoptosis, though not all studies have reported robust functional recovery. These data on the recovery of function with apoptosis inhibitors will be discussed further in the final discussion. Though there is clearly activation of the apoptotic caspase cascade with MPTP/MPP^+ treatment, the factors which couple the toxin to activation of the caspases have yet to be clarified.

The next section of the thesis discusses the stress activated MAP kinase family as potential upstream factors activating caspases. The development of robust direct inhibitors of the JNK and p38 kinases allowed the contributions of these family members to be dissected and their relative contributions to MPP^+ toxicity evaluated, together with their effects on caspase activation.

Chapter 5:

MPP^+ toxicity is partially mediated by JNK but not p38 induced caspase activation.

5.1. Introduction

5.1.1. Neuronal apoptotic regulation by JNK and p38

Both JNK and p38 are reported to have a wide range of effects in non-neuronal cells *in vitro* and *in vivo*; these include regulation of inflammation, effects on proliferation and apoptotic effects, and are activated by a diverse range of stimuli. In neurones and neuronal cell lines, both p38 and JNK are reported to be activated by a number of stimuli. In neurones, activation of either or both enzymes has been linked to the apoptotic response to cellular stress, though JNK activation appears to link more strongly to apoptosis in most neuronal models.

A number of models of neuronal stress are reported to lead to JNK phosphorylation and to have effects on the expression and/or phosphorylation state of c-jun. In cell lines, early evidence for a role of c-jun in PC12 cell apoptosis in response to NGF withdrawal was presented by Xia and colleagues (1995), who reported increased activity of JNK and p38 in this model. Interestingly, the expression of JNK isoforms and activation in response to stress differs across cell lines. In a study comparing responses in rat PC12 cells, murine Neuro2A cells and human SHSY5Y cells to UV irradiation, H_2O_2 and TNF-α, it was found that the stressors induced not only different degrees of cell death in each cell type, but also different patterns of JNK isoform expression and activation (Mielke et al., 2000). These data indicate that the correlation between JNK activation and apoptosis is likely to be complicated and involve differential activation of JNK isoforms by different stressors.

Data from cell culture models also provide evidence for a role of JNK and c-jun activation in apoptosis. In primary sympathetic neurones deprived of NGF there is activation of JNK, and increased expression of c-jun mRNA (Estus et al, 1994) and protein, together with increased phosphorylation of c-jun (Ham et al., 1995, Harding et al., 2001). In contrast, no activation of p38 was observed in NGF deprived sympathetic neurones (Eilers et al., 1998). In cerebellar granule cells, which are dependent on activity for survival, deprivation of potassium and serum induces apoptosis, together with elevation of c-jun mRNA (Watson et al., 1998). In this study no evidence was found for p38 involvement in this model of cell death, though other groups have observed both JNK and p38 activation in potassium-deprived cerebellar granule cells (Harada and Sugimoto, 1999), and p38 activation in glutamate treated granule cells (Kawasaki et al., 1997). Other studies of cell death in these neurones show apoptosis induction is independent of JNK/p38; these include treatment with β-amyloid, with glutamate, and withdrawal of serum (Gunn-Moore and Tavare, 1998). Together these data indicate that the regulation of cell death in these cultures in response to various stimuli is likely to be both stimulus dependent, and also to be linked to the culture conditions in which the cells are grown. This accords well with the findings (discussed above) of Mielke and coworkers (2000) in cell lines. JNK regulation is complicated, and the response observed to a given stressor in one cell type may vary from that obtained either with a different stressor in the same cell type, or to the same stressor in another cell type. In other primary neuronal models, JNK3 and p38 activation is observed in primary cortical neurones in response to sodium

arsenite, with no activation of either JNK1 or JNK2 (Namgung et al., 2000). In cochlear explant cultures, neomycin exposure induces phosphorylation of both JNK and c-jun (Pirvola et al., 2000). Recently, increased expression of phosphorylated c-jun has been demonstrated in primary cultures of mesencephalic dopaminergic neurones treated with MPP^+ (Gearan et al., 2001), though the results in these neurones and various mesencephalon derived cell lines have varied (Choi et al., 1999).

In vivo, JNK activation and upregulation and/or activation of c-jun have been observed in a number of models of cellular stress. Early evidence that the JNK pathway was implicated in apoptosis in the nervous system came from JNK3 deficient transgenic mice; these mice were resistant to kainic acid induced seizure activity, and had decreased cell death in the hippocampus following kainic acid treatment (Yang et al., 1997). A number of other studies have since shown activation of JNK and upregulation and phosphorylation of c-jun in the hippocampus following seizure activity. Mielke and coworkers (1999) showed activation of JNK1 following kainate induced seizures, together with increased expression, phosphorylation and promoter binding of c-jun. These effects were apparent within three hours of kainate administration, and levels were elevated for at least 12 hours following kainate treatment. In this study, expression levels and activity of ERK and p38 fell following kainate treatment. The seizures and neuronal cell death induced by kainic acid was shown to be mediated by the phosphorylation of c-jun by Behrens and coworkers (1999), who examined kainic acid induced seizures and cell death in wildtype mice and mice expressing a

mutant c-jun with the serines at position 63 and 73 mutated to alanines. In the transgenic animals, both the number and intensity of seizures was reduced, along with the extent of cell death. Electrically induced status epilepticus also induces upregulation of the expression of c-jun protein (Dragunow et al., 1993). Further evidence for a role for JNK and c-jun in mediating seizure induced cell death comes from a study by Shauwecker (2000), who examined JNK and c-jun expression together with cell death following kainic acid induced seizures in two mouse strains, C57BL/6, which are resistant to excitotoxic cell death, and FVB/N, which are susceptible to excitotoxic cell death. In this study, there were no strain related differences in seizure activity, but there were pronounced differences in the extent of neuronal damage. The FVB/N mice had significantly more cell death than the C57B/6 animals, and also had significantly greater induction of JNK1 immunoreactivity. Both strains had increased expression of both c-jun and phospho-c-jun following seizures, though only the FVB/N strain had a prolonged upregulation of c-jun expression. This may indicate that the expression and phosphorylation of c-jun may not lead inexorably to cell death, but may lead to different effects in different strains of animal; indeed the author of this study suggested that expression of phosphorylated c-jun in this study correlated to areas protected from kainic acid induced cell death. Rather different results were obtained in adult rats which underwent repetitive electroconvulsive seizures (ECS); in this model, there was region specific activation of JNK1, but little hippocampal c-jun phosphorylation (Brecht et al., 1999). In this model there was also little hippocampal cell death, confirming that cell death is not dependent on

JNK activation *per se*, but requires phosphorylation of c-jun, and also indicating again that induction of c-jun phosphorylation is cell type and stimulus dependent. In this study it was also shown that repeated stimulation resulted in desensitization of c-jun phosphorylation, indicating perhaps a role for c-jun in plastic changes within the brain. A further study also demonstrated the importance of the JNK3 isoform in kainic acid induced seizures; JNK3 -/- mice, which have a less severe phenotype than JNK1 and JNK2 null animals, are resistant to kainic acid induced seizure activity, and have reduced hippocampal cell death (Yang et al., 1997).

Thus, JNK and c-jun are involved in the response of hippocampal neurones to seizures, and play a role in mediating the cell death; p38 induction is also described in the hippocampus of kainic acid treated mice (Che et al., 2001) and rats (Jeon et al., 2000). In the study by Che and co-workers there was a delayed induction of p38 following seizure; the p38 was not induced in neurones but rather in reactive astrocytes, and the authors hypothesis that the p38 induction may be implicated in the delayed component of the neuronal cell death and in reactive gliosis. This delayed induction was not observed in the study by Jeon and co-workers, who observed JNK, p38 and ERK activation by 30 minutes following kainate administration. Stress activated MAP kinases are also implicated in a number of other models of cell death *in vivo*. In ischaemia models, activation of JNK has been reported. Early evidence for this came from Dragonow and coworkers (1994), who showed elevation of c-jun in rat brain following carotid artery occlusion and hypoxia; upregulation of c-jun has also

been observed in gerbil brain following transient global ischaemia (Kiessling et al., 1993). Increased expression of phosphorylated JNK and c-jun has also been observed following transient middle cerebral artery occlusion (MCAO) in the rat (Herdegen et al., 1998). In this model there is a core of rapidly developing necrotic cell death, with an area of more slowly developing apoptotic cell death. Increased phosphorylated c-jun expression was detected within the necrotic infarct areas by three hours, and persisted for up to 72 hours; many of these cells are also exhibit apoptotic markers indicating that they are destined to die, though it is unclear whether the death of these cells is directly linked to the c-jun phosphorylation. This study also showed increased phosphorylation of JNK1; increased expression and phosphorylation of JNK following MCAO was also observed by Hayashi and colleagues (2000), and JNK phosphorylation has also been observed 24 hours following global forebrain ischaemia (Gillardon et al., 1999). Upregulation of p38 immunoreactivity has also been reported following global cerebral ischaemia; the increased p38 expression was again not in neurones, however, but in microglia in proximity to the degenerating hippocampal neurones (Walton et al., 1998).

5.1.2. Neuroprotection by inhibitors of JNK and p38

Thus JNK and p38 have been implicated in the apoptotic response to a wide range of stimuli; the development of selective inhibitors has allowed the neuroprotective effects of stress activated MAP kinase inhibition to be evaluated.

A number of naturally occurring inhibitors of JNK exist, including JNK inhibitory proteins (JIPs) and heat shock protein 72 (Park et al., 2001). Early evidence that inhibition of the JNK pathway may attenuate neuronal cell death came from a study by Ham and coworkers (1995), who showed that a dominant negative mutant c-jun protected sympathetic neurones *in vitro* from NGF withdrawal induced cell death. Similar results were observed by Xia and coworkers (1995), who demonstrated that interference with the JNK and p38 pathways in NGF deprived PC12 cells using dominant negative or constituently active enzymes blocked or potentiated apoptosis respectively. These data from sympathetic neurones have been confirmed using a number of pathway inhibitors, including small molecules and the JNK binding domain of JIP-1 (Eilers et al., 2001, Harding et al., 2001).

The most widely tested small molecule inhibitor of JNK is the Cephalon compound CEP-1347 (also known as KT-7515), a bis-thioethylmethyl analog of the indolcarbazole K252a (Kaneko et al., 1997). K252a, while a potent inhibitor of tyrosine kinase receptors at higher concentrations, exerts a neurotrophic effect in a range of cellular types at submicromolar concentrations *in vitro.* Among these effects are increased survival and neurite outgrowth of chick and rat dorsal root ganglion neurones (Kaneko et al., 1997, Borasio et al., 1998, Bilsland et al., 2000, Young et al., 2000, Bilsland and Harper, in press), and increased choline acetyltransferase (ChAT) activity in primary septal cholinergic neurones. The neurotrophic activity of CEP-1347 was identified based on this ability to increase ChAT activity, a neurotrophic effect which was confirmed *in vivo* in the fimbria-

fornix lesion model (Kaneko et al., 1997, Harper et al., 2000) and increased survival of spinal motoneurones (Glicksmann et al., 1998). This survival promoting effect was shown to correlate with the ability of the compound to inhibit JNK activation, thus indicating that the neurotrophic activity may be mediated through inhibition of the JNK pathway. The JNK inhibitory activity of CEP-1347 has since been shown to be mediated by inhibition upstream of JNK at the level of mixed lineage kinase (MLK-3) (Maroney et al., 2001).

CEP-1347 has been tested in a wide range of apoptotic models both *in vitro* and *in vivo*. In addition to the effect on cholinergic neurones *in vitro* and *in vivo* previously discussed, it is also neuroprotective for developing motoneurones of the developing spinal nucleus bulbocavernosus following testosterone withdrawal *in vivo*, and chick embryonic motorneurones (Glicksmann et al., 1998). CEP-1347 has also been shown to increase survival of rat sympathetic neurones deprived of NGF *in vitro*. It also has been shown to increase survival of gentamycin treated cochlear and vestibular hair cells *in vivo* (Ylikoski et al., 2001) and to restore hearing and protect auditory neurones following ototoxic stimuli and noise (Pirvola et al., 2000). In primary cortical neurones treated with sodium arsenite, CEP-1347 prevented apoptosis (Namgung et al., 2000); in this model, the p38 inhibitory compound SB203580 was also neuroprotective, though at a concentration which may also inhibit JNK. Perhaps the most interesting data on the activity of CEP-1347 has come from its reported activity in models of Parkinson's Disease; CEP-1347 has been reported to attenuate MPTP induced

toxicity in mouse (Saporito et al., 1999). These data will be discussed in detail later in this chapter.

Since the development of CEP-1347, a number of small molecule inhibitors which target JNK directly have been reported; these include the Signal Pharmaceuticals compounds SP600125 and SPC-0009766, which are reported to have around 20-fold selectivity for JNK over a range of other enzymes, including p38 (Bennett et al., 2001). The former of these compounds was recently reported to inhibit c-jun phosphorylation and consequently to decrease a range of inflammatory responses *in vitro*. Both of these compounds have also been reported in abstract form to have neurotrophic effect, and to protect dopaminergic neurones *in vitro* from toxicity of 6-OHDA and MPP^+ (Raymon et al., 2000).

With regard to inhibitors of p38, there have been many publications using the imidazole compounds SB203580 and SB202190, or a structurally similar compound PD169316; these compounds are often described as p38 inhibitors (and are marketed as such), but have poor selectivity for p38 over JNK. The IC_{50} values for SB203580 on JNK3 and JNK2α1, for example, are around 2μM (Eilers et al., 2001) and 290nM (Liverton et al., 1999) respectively, while the p38 IC_{50} is reported to be in the mid-nM range (Liverton et al., 1999, Lisnock et al., 2000). PD169316 spares PC12 cells from NGF withdrawal (Kummer et al., 1997), and SB203580 and SB202190 promote survival of chick sensory and motor neurones (Horstmann et al, 1998). More selective inhibitors of p38 have been developed, including a range of tetrasubstituted imidazole compounds developed by Merck

Research Laboratories, some of which will be described later in this chapter (Liverton et al, 1999).

5.1.3. JNK and p38 in Parkinson's Disease and Parkinsonian models

Perhaps the most compelling evidence for a role of stress activated MAP kinases in Parkinson's disease has come from the data obtained with CEP-1347 in animal models; CEP-1347 protects MPTP treated mice from loss of nigral neurones and from loss of striatal innervation (Saporito et al., 1999, 2000). There has been a report demonstrating immunocytochemical localisation of active, phosphorylated ERK, JNK and p38 in both Parkinson's disease tissue and that obtained from patients suffering dementia with Lewy bodies (Ferrer et al., 2001). The report described few positive phosphorylated JNK expressing cells in the nigra and slightly more p38 expressing cell. No quantification of these data was presented, however, and the photomicrographs presented were all taken at a high magnification so relative numbers of cells could not be determined. The low numbers of positive cells reported may reflect the gradual nature of Parkinsonian nigral cell death, as discussed in Chapter 4, above, and it is also possible that sustained activation of the kinases is not required for deleterious cellular effects; if so, detecting transient activation of a kinase within a gradually degenerating region might be a difficult task; this will be discussed later in the thesis.

5.1.4. Aims of this chapter.

The aim of this chapter is to examine the relative contribution of the JNK and p38 stress activated MAP kinase pathways in MPP^+ toxicity, and to evaluate

whether these pathways lead to caspase activation. Novel direct inhibitors of JNK and p38 will be employed to inhibit the pathway, and immunocytochemical techniques used to evaluate pathways activated by MPP^+ treatment.

5.2. Results

5.2.1. Neuroprotection by JNK but not p38 inhibition

CEP-1347 was identified as an inhibitor of the JNK pathway, and is reported to inhibit upstream of JNK at the level of mixed lineage kinase (MLK). The compound was reported to increase neurite outgrowth from chick and rat DRG explants, and to promote survival of dissociated chick and rat DRG neurones *in vitro* (Borasio et al., 1997). The reported neuroprotective and neurite outgrowth promoting effects of the compound in chick DRG explant and dissociated cultures were repeated, and the compound was also shown to maintain DRG neurones in an NGF responsive state in explants over 48 hours (Bilsland and Harper, in press). When this compound was tested for neuroprotective effect against 10μM MPP^+ 48 hour toxicity, there was a significant partial neuroprotection (Figure 5.1). MPP^+ alone decreased the number of dopaminergic neurones to around 50% of untreated control; the maximal CEP-1347 response, at 500nM, restored the number of TH-immunoreactive neurones to around 75% of control.

CEP-1347 is a JNK pathway inhibitor, which inhibits upstream of JNK, and also has inhibitory effect on a number of other kinases (Maroney et al., 2001). It was of interest to determine whether direct inhibition of JNK could protect dopaminergic neurones from MPP^+ toxicity.

A range of novel direct inhibitors of JNK and of p38 were identified from the proprietary Merck compound collection in kinase counterscreens. The IC_{50} values of these compounds are shown in Figure 5.2.A. The compounds have a

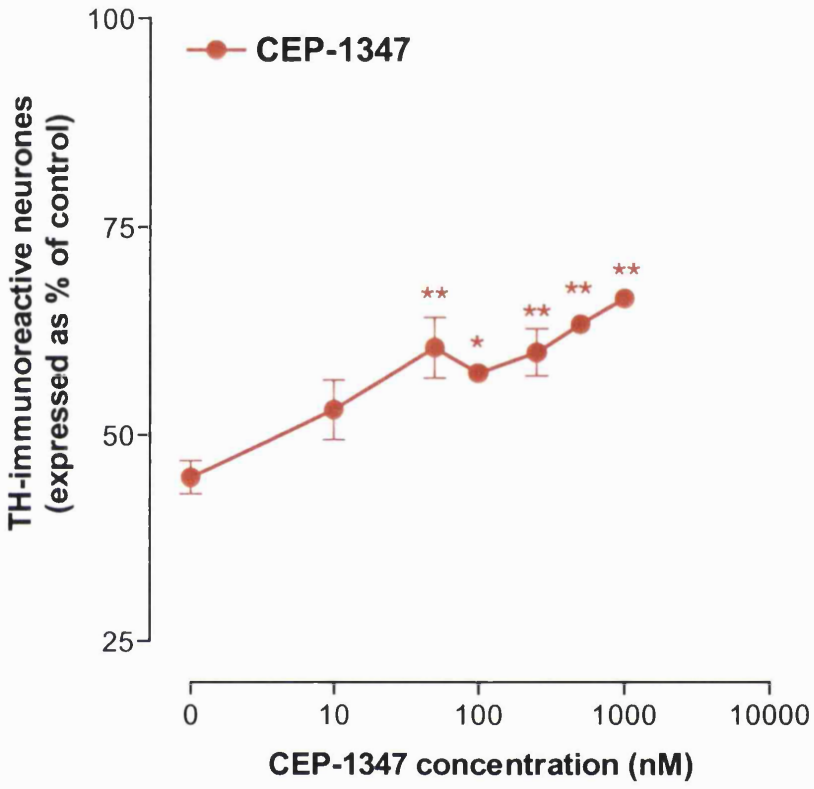


*Figure 5.1. Neuroprotective effects of the JNK pathway inhibitor CEP-1347 on TH-immunoreactive neurones in primary mesencephalic cultures. Significant neuroprotective effects were observed with CEP-1347 concentrations of 50nM and above (*p<0.05, **p<0.01 by one way ANOVA followed by Dunnett's test comparing all groups to MPP^+ alone). The maximal protection was observed with CEP-1347 1μM; this neuroprotection was partial. Results shown are the mean ± s.e.m. of 3 independent experiments each comprising four independent wells.*

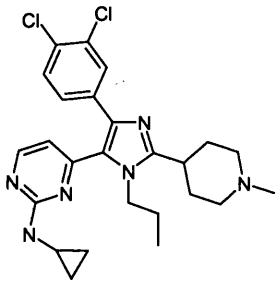
range of potencies in inhibiting JNK and p38. There are no selective JNK inhibitors; all the compounds which inhibit JNK also potently inhibit p38. There are, however, several highly selective p38 inhibitors, and this allows dissection of the contributions of JNK and p38 in the MPP^+ toxicity pathway. Due to a company policy, the compound identifiers are not available for public release; these compounds, therefore are designated Compound 1 through Compound 10. The two compounds selected for further experimentation were Compound 1, a dual JNK/p38 inhibitor, and Compound 2, a selective inhibitor of p38. As the compound identifiers cannot be disclosed, the structures of these compounds are shown in Figure 5.2.B. The crystal structure of compound 1 bound to the JNK 3 enzyme has recently been submitted for publication (Scapin et al., manuscript submitted); the compound binds competitively at the ATP site of the JNK and p38 enzymes.

When the direct inhibitors of JNK/p38 were tested for neuroprotective effects for TH-immunoreactive neurones in MPP^+ exposed mesencephalic cultures, significant neuroprotection was observed with all compounds. Compound 1 restored survival of dopaminergic neurones to a similar extent as CEP-1347, at equivalent concentrations; survival was increased from around 50% with MPP^+ alone to over 70% in cultures exposed to MPP^+ in the presence of Compound 1 500nM. This was the maximal response; the survival decreased slightly by 1μM Compound 1, and a pronounced toxicity was apparent by 10μM which was not specific for the dopaminergic neurones (Figure 5.3). A further 6 combined p38/JNK inhibitors were tested for neuroprotective effect in this model (Figure

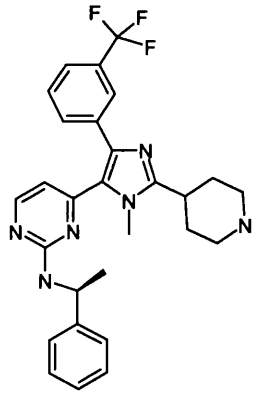
A.

Compound	JNK3α1	JNK2α1	JNK2α2	p38	
				Cell free	Whole cell
Compound 1	4nM	5nM	ND	0.15nM	
Compound 2	500nM	ND	ND	0.2nM	2nM
Compound 3	99nM	43nM	220nM	45nM	144nM
Compound 4	192nM	26nM	52nM	1nM	32nM
Compound 5	124nM	ND	286nM	6nM	ND
Compound 6	323nM	ND	204nM	29nM	440nM
Compound 7	128nM	95nM	469nM	23nM	3μM
Compound 8	46nM	ND	15nM	6nM	330nM
Compound 9	>30μM	>10μM	ND		1nM
Compound 10	700nM	600nM	1μM	0.23nM	0.78nM

B.



JNK/p38 inhibitor
(Compound 1)



p38 inhibitor
(Compound 2)

Figure 5.2. IC_{50} values for JNK and p38 inhibitors on three JNK isoforms, JNK3α1, JNK2α1, JNK2 α2, and p38 in both whole cell and cell free assays using purified enzymes. Compound 1 is a potent inhibitor of both JNK and p38 MAP kinases. All of the other JNK inhibitors also have activity at p38. The p38 inhibitors, however, are more selective for p38 than for JNK. Compound 2, which was utilised for further experimentation is around 1000-fold more potent in inhibiting p38 that JNK. These values are provided courtesy of Dr. Philip LoGrasso, Merck Research Laboratories, and are unpublished data.

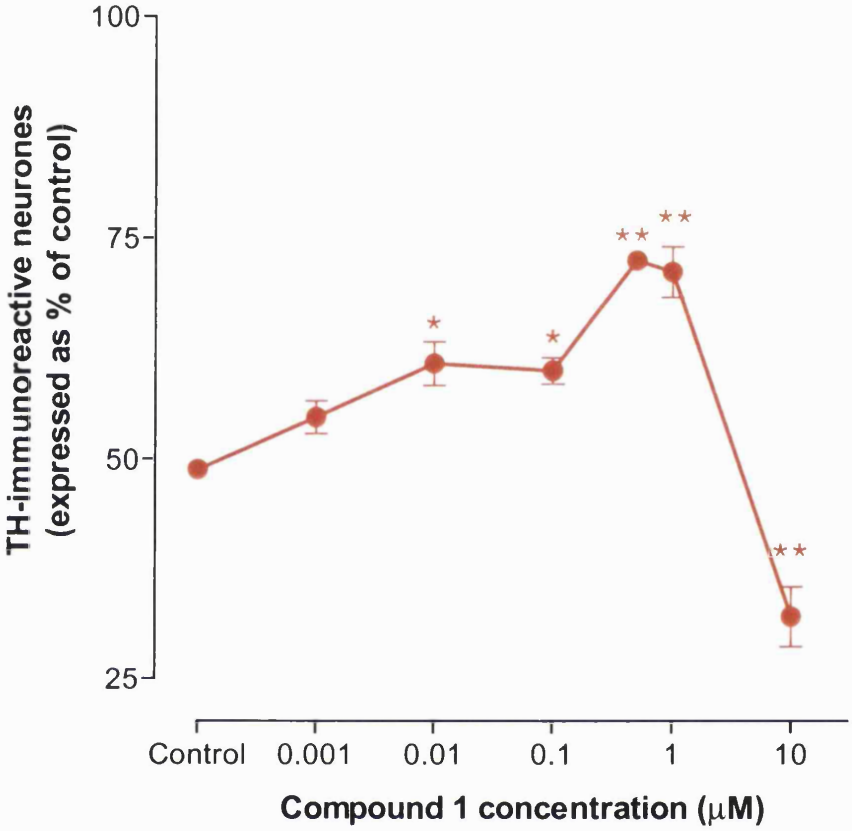
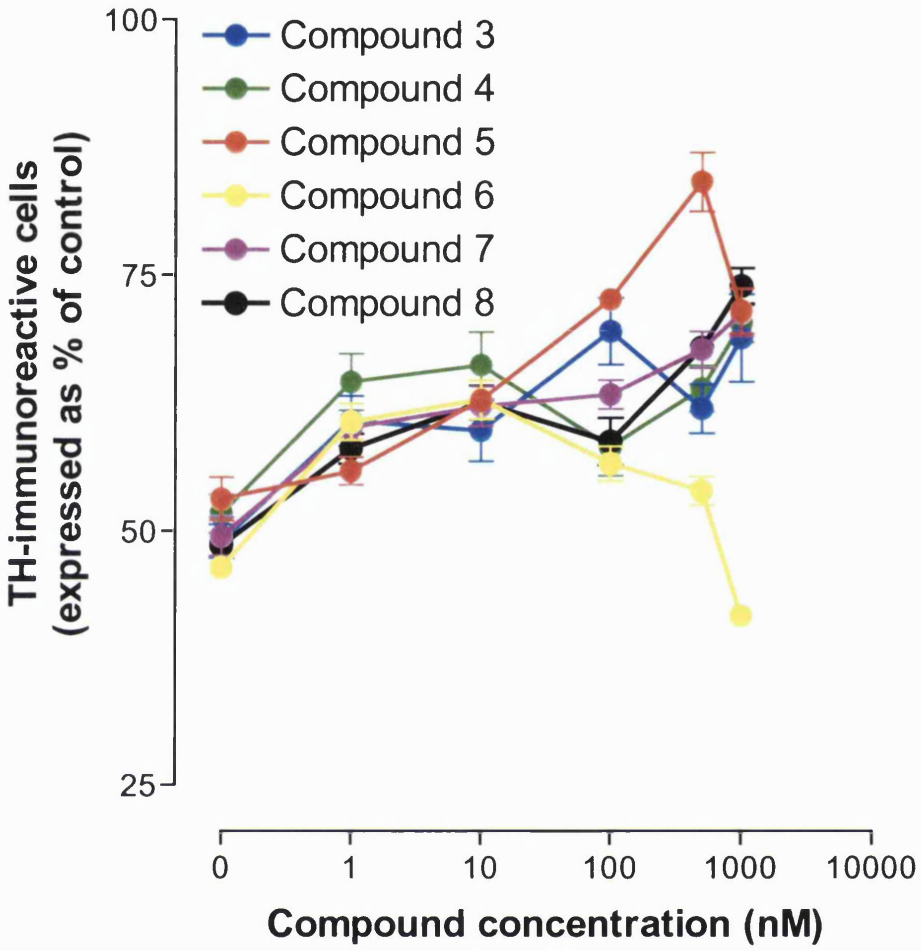
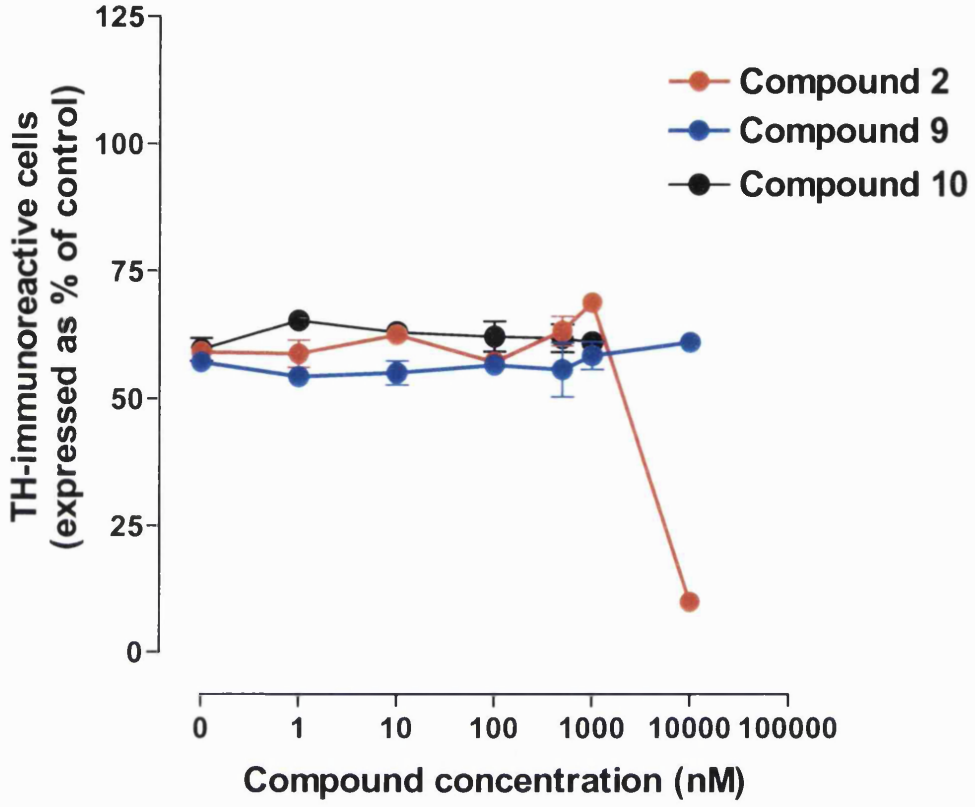


*Figure 5.3. The direct JNK/p38 inhibitor Compound 1 promotes survival of mesencephalic TH-immunoreactive neurones. Compound 1 and MPP^+ were coadministered to mesencephalic cultures 5 days following plating for 48 hours; significant attenuation of MPP^+ mediated toxicity was observed with Compound 1 concentrations of 0.01μM and above (*p<0.05, **p<0.01 by one-way ANOVA followed by Dunnett's test comparing all groups to MPP^+ alone). Data shown are the mean ± s.e.m. of four independent wells from one sample experiment (at 500nM, Compound 1 was tested 6 times; a significant partial neuroprotection was observed in each experiment - not shown).*



Concentration (+MPP+ 10μM)	Compound 3		Compound 4		Compound 5		Compound 6		Compound 7		Compound 8	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	49.00	1.66	51.69	1.90	53.14	2.14	46.36	0.88	49.45	1.91	48.60	0.53
1nM	60.74	2.42	64.55 *	2.77	55.85	1.35	60.66 **	1.79	60.09 **	0.84	58.03 **	1.46
10nM	59.77	2.97	66.16 **	3.24	62.74	0.41	62.81 **	1.89	62.19 **	1.93	62.55 **	1.65
100nM	69.48 **	3.25	58.20	2.82	72.60 **	0.90	56.55 **	1.71	63.30 **	1.40	58.73 **	2.33
500nM	61.90	2.40	63.86 *	2.27	84.07 **	2.89	53.87 **	1.39	67.69 **	1.79	67.86 **	0.94
1μM	68.81 **	4.30	70.21 **	1.81	71.48 **	2.19	41.63	0.66	71.28 **	2.31	73.92 **	1.73

*Figure 5.4. Effects of a range of inhibitors of JNK/p38 on survival of TH-immunoreactive neurones exposed to MPP⁺. All of the inhibitors tested had significant neuroprotective effect from MPP⁺ toxicity (*p<0.05, **p<0.01 by one-way ANOVA followed by Dunnett's test comparing all groups to MPP⁺ alone); the protection with each of these inhibitors was only partial, and was similar in extent to that observed with CEP-1347 and Compound 1. Two of the inhibitors caused toxicity at the higher concentrations which was not confined to the dopaminergic population. Each data point represents the mean ± s.e.m. of four independent wells from one experiment per compound.*



Concentration	Compound 2		Compound 9		Compound 10	
(+MPP+ 10μM)	Mean	SEM	Mean	SEM	Mean	SEM
0	59.08	1.39	57.04	1.26	59.57	2.26
1nM	58.72	2.67	54.20	1.46	65.26	1.31
10nM	62.41	1.05	54.87	2.38	62.99	0.40
100nM	57.09	0.91	56.46	1.11	62.12	2.97
500nM	63.18	2.86	55.50	5.34	61.76	2.73
1μM	68.76 **	0.90	58.35	2.79	60.99	0.95
10μM	9.84 **	1.98	60.90	1.04		

*Figure 5.5. Selective inhibitors of p38 have little neuroprotective effect on MPP⁺ treated TH-immunoreactive neurones. Two of the inhibitors tested had no neuroprotective effect at any concentration tested; compound 2 had a neuroprotective effect at only 1μM (**p<0.01 by one-way ANOVA followed by Dunnett's test comparing all groups to MPP⁺ alone). Each data point represents the mean ± s.e.m. of four independent wells from one experiment; two full concentration response curve experiments were carried out using Compound 2 and Compound 10; Compound 9 was tested once. Compound 2 was tested at 500nM in several other experiments, always with a lack of significant neuroprotection.*

5.4); all of these compounds exerted neuroprotective effect, at similar concentrations to both CEP-1347 and Compound 1. As all of the compounds tested other than CEP-1347 were combined p38/JNK inhibitors, experiments were undertaken to evaluate the neuroprotective effects of selective p38 inhibitors. Three selective p38 inhibitors were tested. All of the p38 inhibitors were less potent than the combined p38/JNK inhibitors in protecting TH-immunoreactive neurones (Figure 5.5). Two of the inhibitors, Compounds 9 and 10 had no significant effect at any concentration tested; Compound 2 caused neuroprotection at the 1μM concentration only, a concentration higher than the JNK IC_{50} for this compound.

Compound 1 and Compound 2 were also tested for ability to increase ^{3}[H]-DA uptake in primary cultures of mesencephalic neurones exposed to MPP^{+}. No increase in ^{3}[H]-DA uptake was observed when either of the compounds was added together with MPP^{+} for 48 hours (not shown). When Compound 1 was added 24 hours prior to MPP^{+} addition at 0.1μM, there was a significant increase in ^{3}[H]-DA uptake by the cultures, an effect which was not observed with the selective p38 inhibitor Compound 2. When Compound 1 was pre-applied, MPP^{+} added for 24 hours, and compound reapplied for a further 24 hours, significant increases in ^{3}[H]-DA uptake were observed, though the relative increases declined with increasing recovery time; this decline appeared to correlate with a recovery of ^{3}[H]-DA uptake in cells treated with MPP^{+} 0.1μM alone (Figures 5.6 and 5.7).

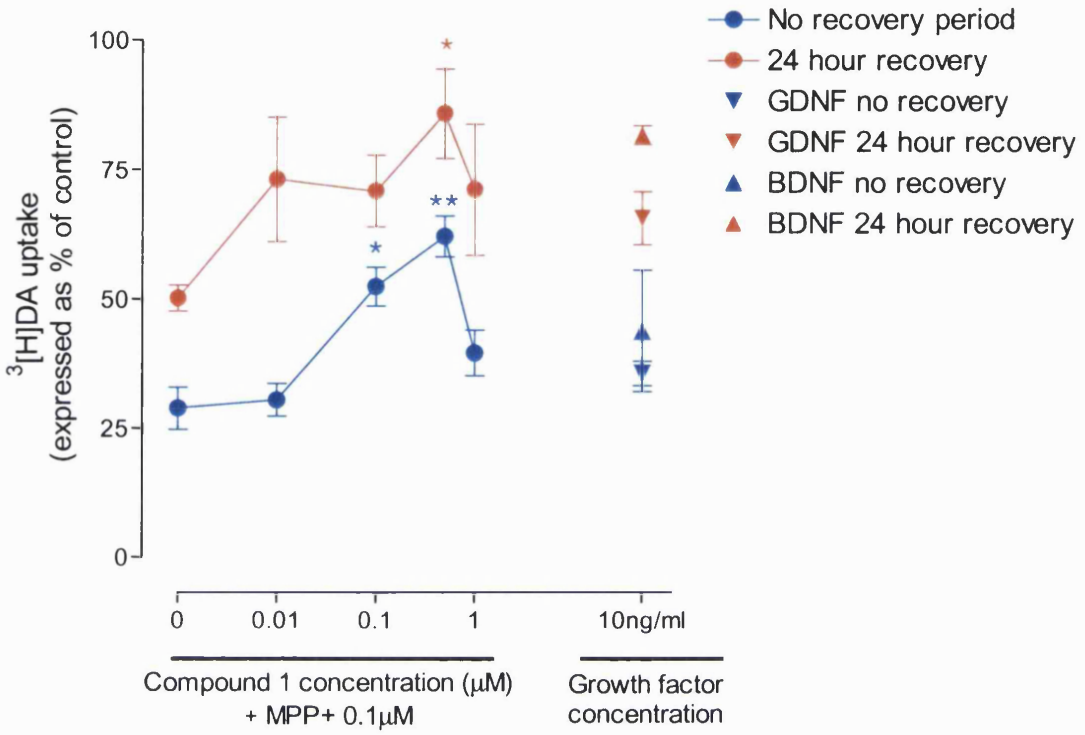


*Figure 5.6. The dual JNK/p38 inhibitor Compound 1 increases uptake of ^{3}[H]DA in primary mesencephalic cultures exposed to MPP$^+$ 0.1μM. The compound was pre-applied for 24 hours prior to MPP$^+$ exposure; MPP$^+$ was applied either for 48 hours prior to assay, or was applied for 24 hours and the cells allowed to recover in the presence of Compound 1 for a further 24 hours. In both of these treatment groups, there was an increase in the uptake of ^{3}[H] DA by the cultures. Results shown are the counts per minute with blank counts subtracted, expressed as percentage of untreated control. Each data point is the mean ± s.e.m. of four independent wells from one sample experiment of two performed. Significant differences (*p<0.05, **p<0.01 by one way ANOVA followed by Dunnett's test) were observed with Compound 1 at 100nM and 500nM with no recovery period, and at 500nM with 24 hour recovery.*

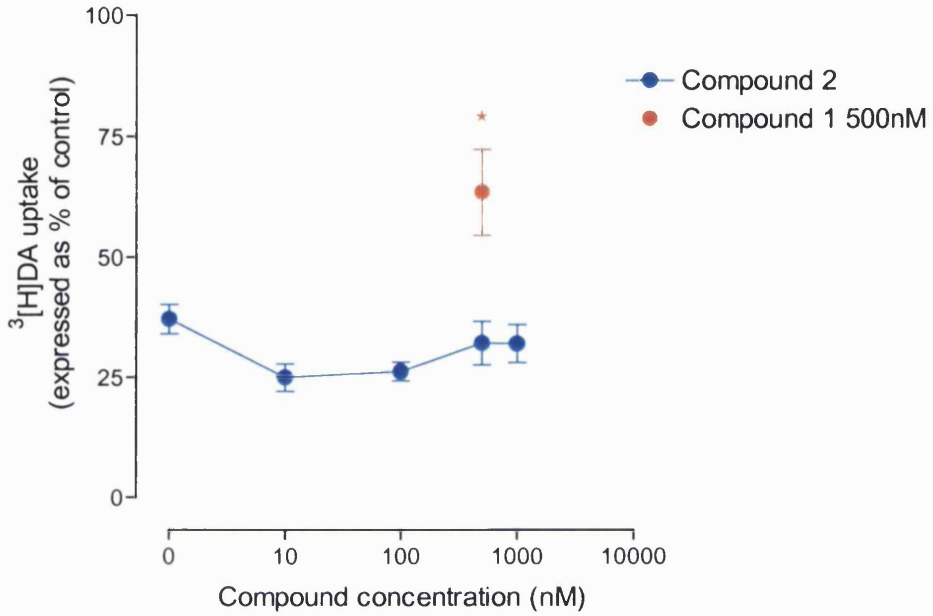


*Figure 5.7. The selective p38 inhibitor compound 2 does not restore ^{3}[H]DA uptake in primary mesencephalic cultures exposed to MPP$^+$ 0.1μM for 48 hours with no recovery period. Compound 1, the dual JNK/p38 inhibitor was included at 500nM and significantly (*p<0.05) increased uptake of ^{3}[H]DA. Data points shown are mean ± s.e.m. of four independent wells from one experiment; two experiments were carried out.*

In order to further evaluate the neuroprotective effects of JNK inhibition, experiments were undertaken to examine expression of phosphorylated c-jun and apoptotic morphology in MPP^+ treated dopaminergic neurones.

5.2.2. MPP^+ increases expression of phosphorylated c-jun.

Primary cultures of dopaminergic neurones were exposed to MPP^+ for timepoints up to 48 hours, then were fixed and double immunostained using primary antibodies raised against TH and phosphorylated c-jun. Nuclei were counterstained using Hoechst 33342 for observation and quantification under fluorescence. Photomicrographs of double immunostained control and MPP^+ treated TH-immunoreactive neurones are shown in Figure 5.8. MPP^+ increased expression of phosphorylated c-jun in TH-immunoreactive neurones in a time-dependent manner compared with control cultures fixed at the same timepoints. MPP^+ also increased the number of apoptotic nuclei in dopaminergic neurones in a time dependent manner.

In order to quantify expression of phosphorylated c-jun and apoptotic morphology in TH-immunoreactive neurones, fluorescent staining was quantified. Ten fields of view were visualised from each well from each of two wells per condition; fields of view were scored for the number of TH-immunoreactive cells, the number of TH-immunoreactive cells with apoptotic nuclei, the number of phosphorylated c-jun immunoreactive cells, the number of phosphorylated c-jun immunoreactive cells with apoptotic nuclei, and the number of phosphorylated c-jun expressing TH-immunoreactive cells. The results from this quantification are

shown in Figure 5.9. At the three hour timepoint, there was little difference between control and MPP^+ treated cultures, with around 3 – 5% of TH-immunoreactive cells co-expressing phosphorylated c-jun; at all timepoints after this, however, the number of TH/phospho-c-jun immunoreactive cells increased in the MPP^+ treated group. The maximal expression of phospho-c-jun was at the 24 hour timepoint, where over 30% of TH-immunoreactive neurones also expressed phospho-c-jun; the number declined thereafter, to 16% at the 48 hour timepoint. The number of control cells co-expressing TH and phospho-c-jun remained constant at around 5% for all timepoints. When the number of TH-immunoreactive neurones expressing apoptotic nuclear morphology was quantified, there was no increase over control at all timepoints up to 24 hours, with again around 2 - 5% of TH-immunoreactive cells being apoptotic. At 30 hours, however, the number of apoptotic cells in MPP^+ treated cells had risen to 16%, and by 48 hours over 50% of TH-immunoreactive neurones had apoptotic nuclear morphology. The control levels remained relatively constant at all timepoints. Slightly increased numbers of non-TH-immunoreactive cells expressing phospho-c-jun were observed at all timepoints in the MPP^+ treated group compared to the control group, indicating some effect of MPP^+ on non-dopaminergic cells (Figure 5.9.B). Very few phospho-c-jun expressing cells (4 cells of 876 counted) also expressed apoptotic morphology; none of these were TH-immunoreactive, indicating perhaps a transient expression of phosphorylated c-jun prior to onset of apoptotic execution. Thus, MPP^+ leads to phosphorylation of c-jun, which is maximal prior to the onset of increased apoptotic morphology.

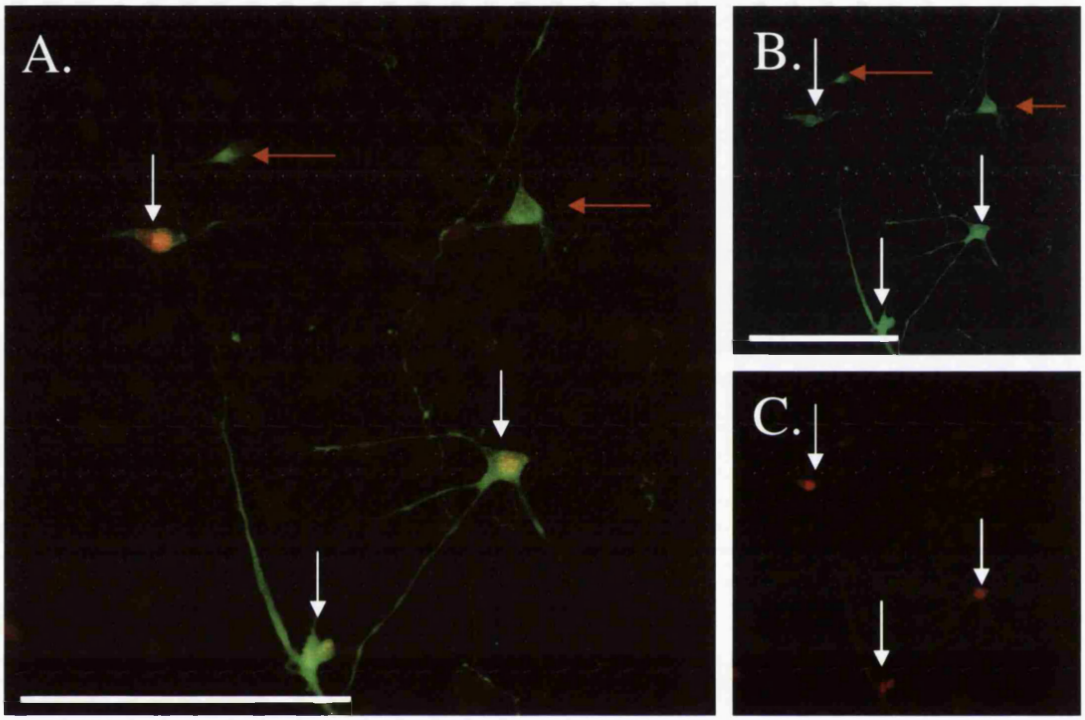


Figure 5.8. MPP^+ induces expression of phosphorylated c-jun in TH-immunoreactive cells in primary cultures of mesencephalic neurones. Cultures were maintained for 5 days following plating and then treated with MPP^+ 10μM for 24 hours. Cultures were double immunostained with antibodies raised against TH (green) and serine 63 phosphorylated c-jun (red), and nuclei counterstained with Hoechst 33342 (not shown). Panel A shows double TH/phosphorylated c-jun immunoreactivity, and panels B and C show TH and phosphorylated c-jun respectively. It is apparent that there are several cells within the field of view co-expressing TH and phosphorylated c-jun (white arrows), and two TH cells which do not express phosphorylated c-jun (red arrows). Scalebar = 100μm.

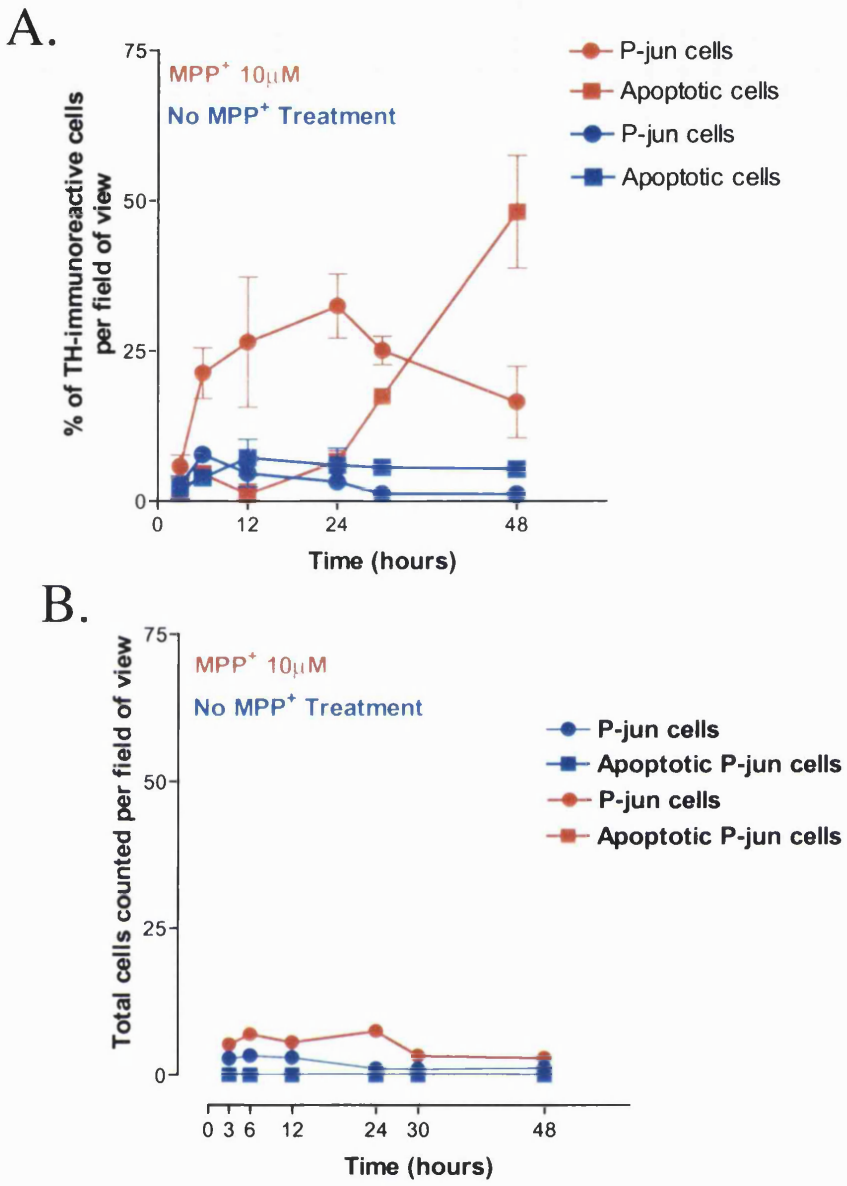


Figure 5.9. Time course of induction of phosphorylated c-jun (serine 63) immunoreactivity and nuclear chromatin condensation in control and MPP+ treated TH-immunoreactive neurones (A) and total cell population (B). Increased expression of phosphorylated c-jun was observed in TH-immunoreactive neurones at all timepoints after 6 hours (A); the maximal expression at 24 hours preceded the increase in apoptotic nuclei, which began at 30 hours. Increased numbers of non TH-immunoreactive cells expressing phosphorylated c-jun were observed at all treatment timepoints. Very few phosphorylated c-jun expressing neurones also expressed apoptotic nuclei. Effects of MPP+ on expression of phosphorylated c-jun and coexpression of phosphorylated c-jun with apoptotic nuclear morphology in the total mesencephalic cell population are shown in (B). MPP+ caused an approximate twofold increase in the numbers of P-jun immunoreactive neurones, the majority of which were TH-immunoreactive neurones. Very few apoptotic P-jun neurones were detected (4 cells of 876 scored) in both the control and MPP+ treated groups. Data shown are the mean ± standard deviation of observations from ten fields of view in two independent wells.

Using this triple labelling technique, the effects of the dual JNK/p38 inhibitor Compound 1, the p38 inhibitor Compound 2, the caspase inhibitors M-791 and zVAD-fmk, and the growth factors BDNF and GDNF on expression of phosphorylated c-jun and apoptotic morphology were examined. JNK, p38 and caspase inhibitors were co-administered with MPP^+ 10μM for 24 or 48 hours; BDNF and GDNF were preadministered for 6 hours prior to MPP^+ administration. Cells were then fixed and triple labelled as described above, using antibodies raised against TH and P-jun, and the nuclei counterstained for evaluation of apoptotic nuclear morphology. Confocal photomicrographs of these cultures are shown in Figure 5.10. All photomicrographs shown are from cultures fixed at the 24 hour timepoint. In control cultures, few P-jun expressing TH-immunoreactive or non-TH-immunoreactive neurones are present; in MPP^+ treated cultures, however, there are increased numbers of P-jun expressing TH-immunoreactive neurones. In cultures treated with the p38 inhibitor Compound 2, there is little change in the number of TH-immunoreactive neurones also expressing P-jun, whereas the JNK inhibitor Compound 1 abolishes the expression of P-jun. In cultures treated with the caspase inhibitor zVAD-fmk, however, there is an increase both in the number of TH/P-jun cells and in the number of non-TH/P-jun cells.

Expression of TH, P-jun and apoptotic markers were quantified as described above. MPP^+ treatment for 24 or 48 hours resulted in increased expression of phosphorylated c-jun in TH-immunoreactive neurones (Figure 5.11.A); this increase was greater at the 24 hour timepoint, where around 20% of dopaminergic

cells were apoptotic. No increase was observed in the number of apoptotic TH-immunoreactive cells with 24 hour MPP^+ treatment; at the 48 hour timepoint, however, the number of apoptotic TH-immunoreactive cells increased to almost 40% (Figure 5.11.B). The p38 inhibitor Compound 2 had no effect on the number of TH-immunoreactive cells expressing phosphorylated c-jun, and was also without effect on the number of apoptotic nuclei at the 48 hour timepoint. The JNK inhibitor Compound 1 reduced expression of phosphorylated c-jun to around 2% of TH-immunoreactive cells at the 24 hour timepoint, and abolished expression at the 48 hour timepoint; this was not sufficient to abolish expression of apoptotic nuclei at the 48 hour timepoint, though the number of apoptotic nuclei was decreased by approximately 50% compared to cultures treated with MPP^+ alone. Both of the caspase inhibitors tested, the broad-spectrum inhibitor zVAD-fmk and the caspase 3 selective compound M-791 increased the number of P-jun expressing cells, but reduced the number of apoptotic cells to control levels. Both BDNF and GDNF decreased the number of P-jun expressing cells at both timepoints, and also decreased the number of apoptotic profiles in the cultures, with BDNF being the more potent of the two in preventing apoptosis. The increase in the number of P-jun-immunoreactive cells observed with the caspase inhibitors was not confined to the TH-immunoreactive population; Figure 5.12 shows the effects of compounds on expression of P-jun in the total cell population (5.12.A), and on the population of non-TH-immunoreactive cells (5.12.B). Both zVAD-fmk and M-791 increased the number of non-dopaminergic neurones expressing P-jun in the cultures, with zVAD-fmk being the more potent of the

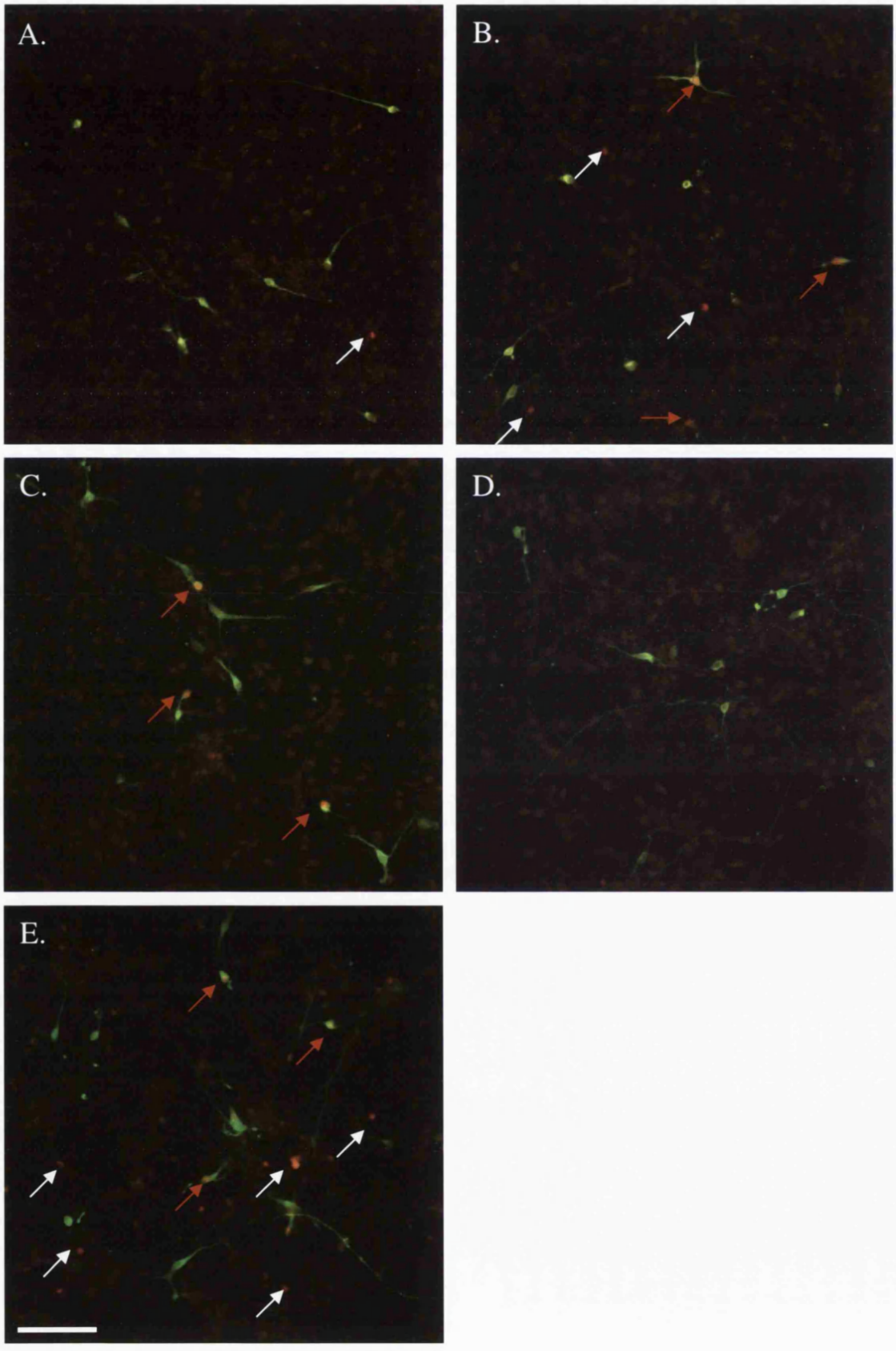


Figure 5.10. Photomicrographs of mesencephalic neurones treated with MPP^+ in the presence and absence of inhibitors of JNK, p38 or caspases for 24 hours. Cultures were double immunolabelled using antibodies to TH (green) and serine 63 phosphorylated c-jun (red). Control cultures are shown in A, MPP^+ 10μM treated cultures in B, and cultures treated with MPP^+ 10μM in the presence of Compound 2 (C), Compound 1 (D) or M-791 (E). Red arrows show TH/P-jun cells, white arrows non-TH/P-jun cells. Scalebar = 100μm.

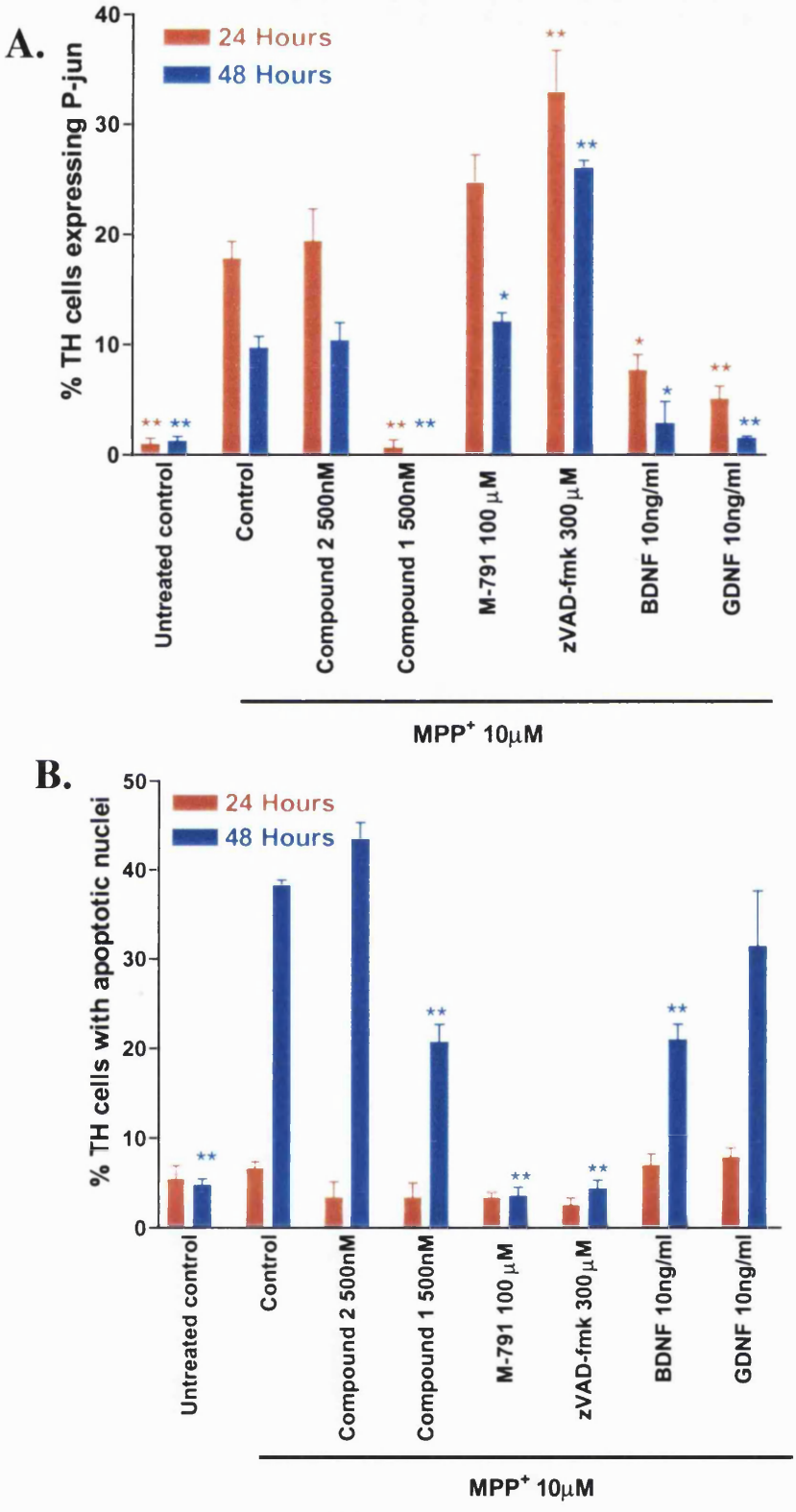


*Figure 5.11. Quantification of P-jun immunoreactivity and apoptotic nuclear morphology in TH-immunoreactive neurones exposed to MPP⁺ 10μM for 24 or 48 hours in the presence or absence of JNK inhibitor, p38 inhibitor, caspase inhibitors or growth factors. 10 fields of view were scored for each of four independent wells per data point (*p<0.05, **p<0.01 by one way ANOVA followed by Dunnett's test comparing all groups at each time to MPP⁺ alone). A shows the expression of P-jun in TH-immunoreactive neurones, B shows the expression of apoptotic nuclear morphology in these neurones.*

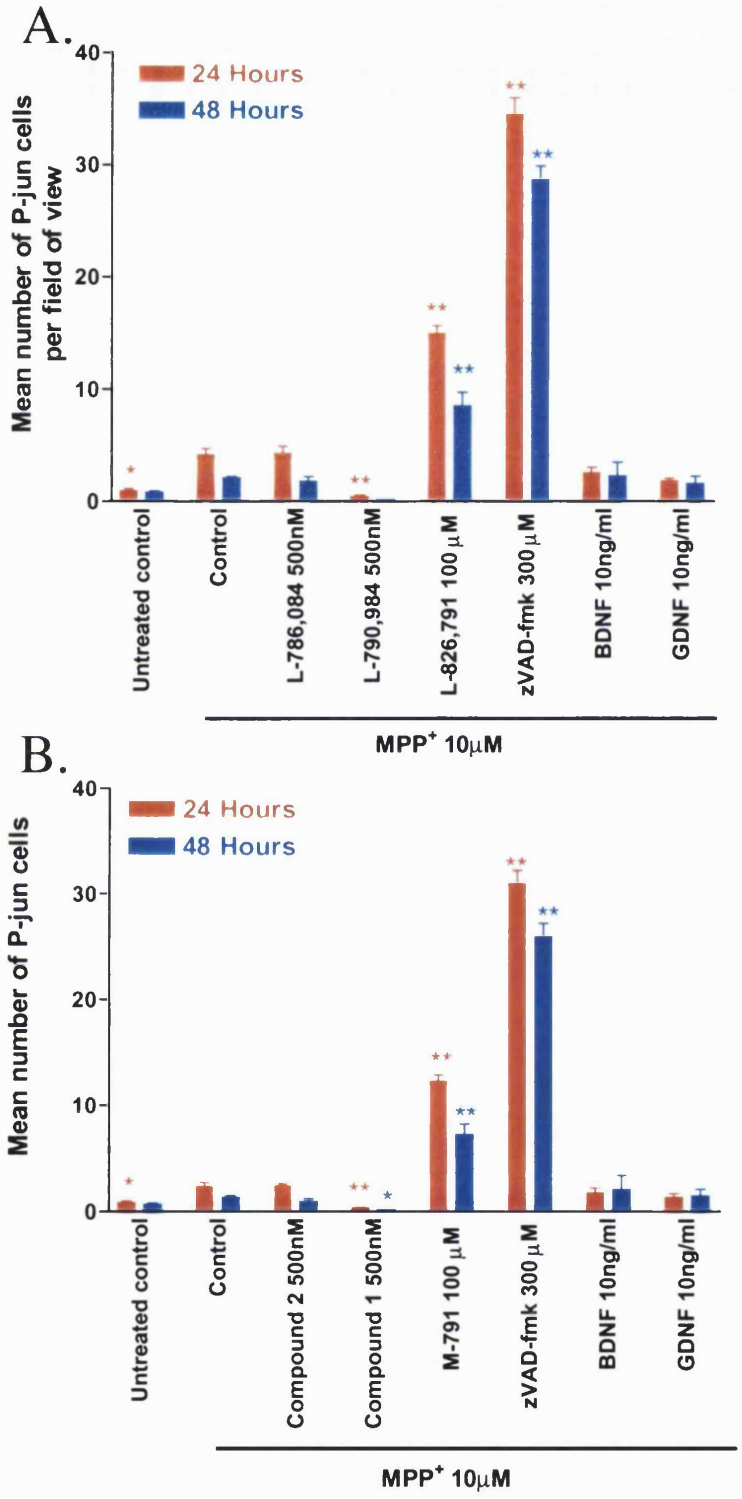


*Figure 5.12. Quantification of P-jun immunoreactivity and apoptotic nuclear morphology in mesencephalic neurones exposed to MPP⁺ 10μM for 24 or 48 hours in the presence or absence of JNK inhibitor, p38 inhibitor, caspase inhibitors or growth factors. 10 fields of view were scored for each of four independent wells per data point (*p<0.05, **p<0.01 by one way ANOVA followed by Dunnett's test comparing all groups at each time to MPP⁺ alone). A shows the expression of P-jun immunoreactivity in the total cell population, B shows the expression of P-jun in non-TH-immunoreactive neurones.*

two. The maximal effect with zVAD-fmk increased the number of P-jun expressing non-TH-immunoreactive neurones from around 2 cells to around 30 cells per field of view. The total number of cells in every field of view was not quantified; typically, though, there were around 300-500 cells in those fields of view which were quantified (data not shown), indicating that the number of cells amounts to around 10% of the total cell population.

5.2.3 JNK activation is upstream of caspase 3 activation.

Chapter 4 demonstrated that caspase 3 inhibition using M-791 was sufficient to restore numbers of MPP^+ treated dopaminergic neurones to near control levels. The data presented in this chapter show that JNK inhibition with a range of pathway and direct inhibitors causes partial neuroprotection. In order to evaluate whether the neuroprotection observed with inhibition of JNK was mediated through decreased activation of caspases downstream, the effects of JNK and p38 inhibitors on expression of active caspase 3 in MPP^+ treated dopaminergic neurones were evaluated. Cultures were exposed to MPP^+ for 24 or 48 hours in the presence or absence of the dual JNK/p38 inhibitor Compound 1 or the selective p38 inhibitor Compound 2. Control cultures were treated with neither compound nor MPP^+. Cultures were fixed and double immunostained using antibodies raised against the active form of caspase 3 and TH. Cultures were scored for TH-immunoreactive cells, active caspase 3 immunoreactive cells and double labelled cells as described above. The results are shown in Figure 5.13. At the 24 hour timepoint, there was a slight increase in the number of active caspase 3 expressing TH-immunoreactive neurones with MPP^+ treatment over

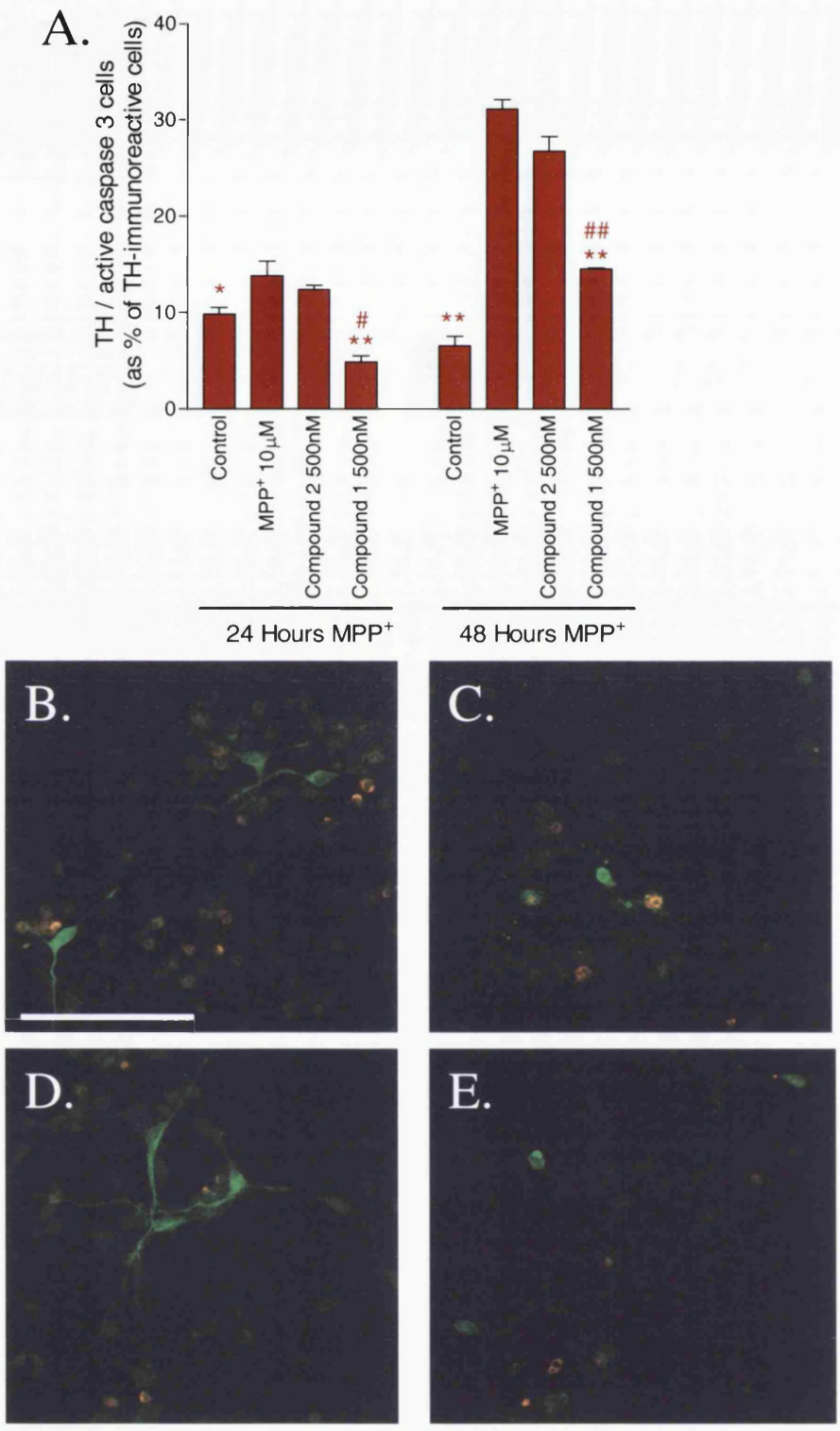


*Figure 5.13 Effects of JNK and p38 inhibitors on expression of active caspase-3 in TH-immunoreactive neurones exposed to MPP+ 10µM for 24 or 48 hours. A shows quantification of the number of TH/active caspase 3 cells. 10 fields of view were scored from each of four independent wells for each data point; no effects were observed with the p38 inhibitor, but inhibition of JNK decreased the number of TH/active caspase 3 cells by around 50% (*p<0.05, **p<0.01 compared to MPP+ group, #p<0.05, ##p,0.01 compared to control group) Photomicrographs of TH (green)/active caspase 3 (red) labelled cultures are shown in B - E; B shows control cultures, C shows cells treated with MPP+ 10µM alone, and D and E show the effects of Compound 1 plus MPP+ and Compound 2 plus MPP+ respectively. Scalebar =100µm*

control; this was not affected by p38 inhibition, but was decreased slightly by JNK/p38 inhibition. By 48 hours, MPP^+ treatment caused a marked increase in the number of active caspase 3 expressing TH-immunoreactive neurones, as previously described in Chapter 4. This increase was not affected by p38 inhibition alone. The dual JNK/p38 inhibitor, however, decreased the number of active caspase 3 expressing TH-immunoreactive neurones by approximately 50%. This accords well with the data previously described, where JNK inhibition increased survival of TH-immunoreactive neurones relative to MPP^+ treated cultures alone by approximately 50% and decreased apoptotic profiles by a broadly similar degree. The number of apoptotic cells were not quantified; many of the active caspase 3 expressing cells, however, also showed evidence of apoptotic nuclei (data not shown).

5.3. Discussion

5.3.1 Results summary

The data presented here confirm the neuroprotective effect of JNK inhibition for dopaminergic neurones treated with MPP^+. CEP-1347 and a number of direct inhibitors of JNK partially protected dopaminergic neurones from MPP^+ toxicity; this effect was likely to be mediated through JNK inhibition as direct inhibition of p38 using specific inhibitors was ineffective at sparing TH-immunoreactive cells. JNK inhibition caused a small but significant increase in ^{3}[H]-DA uptake in MPP^+ treated dopaminergic cultures, which was not observed with p38 inhibition. MPP^+ was shown to increase the expression of P-jun in TH-immunoreactive neurones; the time of maximal P-jun expression preceded the onset of apoptotic morphology. A number of compounds were examined for effects on expression of P-jun and on apoptotic nuclear morphology; JNK inhibition abolished the expression of P-jun but only partially inhibited apoptotic morphology, p38 inhibition was without effect, and caspase inhibition abolished the apoptotic morphology as previously demonstrated, but increased the number of P-jun expressing TH- and non-TH-immunoreactive neurones. The growth factors BDNF and GDNF both decreased the number of P-jun expressing dopaminergic neurones and caused a partial neuroprotection. JNK inhibition but not p38 inhibition significantly decreased the number of TH-immunoreactive neurones which expressed active caspase 3; activation of the JNK pathway therefore appears to be upstream of caspase pathway activation. JNK inhibition was also shown to cause a short term increase in the number of dopaminergic neurones in

the absence of toxin, when inhibitor was applied either immediately after plating or to the cell suspension prior to plating. JNK inhibition was more potent in increasing numbers of dopaminergic neurones than BDNF, GDNF or the caspase inhibitor zVAD-fmk.

5.3.2. General discussion

The previous chapter demonstrated the importance of the caspase family of proteases in mediating MPP^+ toxicity for dopaminergic neurones *in vitro*, and this has also been demonstrated by a number of other groups both *in vivo* and *in vitro* (discussed in detail in Chapter 4, above). As has been discussed, the upstream pathways which may lead to caspase activation are unclear. Recent studies have implicated activation of the stress activated MAP kinases in MPP^+/MPTP toxicity; *in vivo*, several reports have demonstrated neuroprotective effects of JNK pathway inhibition against MPTP toxicity. MPTP treatment *in vivo* results in phosphorylation of JNK and the upstream kinase MKK-4 (Saporito et al., 2000). CEP-1347, which inhibits the JNK pathway at the level of mixed lineage kinase, prevents degeneration of mesencephalic dopaminergic neurones and loss of striatal innervation in MPTP treated mice and primates (Saporito et al., 1999, 2000). Mice expressing the JNK binding domain of JNK inhibitory protein-1 following adenoviral gene transfer are also resistant to MPTP toxicity *in vivo*, and have increased numbers of nigral dopaminergic neurones, increased levels of striatal dopamine and metabolites and behavioural benefit; decreased levels of caspase 3 activation were also observed in the nigra (Xia et al., 2001).

As well as the JNK pathway, a number of models of cellular stress activate the p38 pathway. Recently, it has been demonstrated that inhibition of this pathway protects MPTP treated mouse NT2a cells (DeGirolamo et al., 2002); another report has implicated the p38 pathway in MPP^+ toxicity, though increased generation of H_2O_2 (Du et al., 2001). In this chapter the contribution of the JNK and p38 pathways to MPP^+ toxicity *in vitro* were evaluated using selective pathway inhibitors, to evaluate the extent of the neuroprotective effect observed with these inhibitors, and to determine whether these pathways are linked to downstream caspase activation.

Activation of the JNK signalling pathway has been reported to lead to caspase activation and apoptosis in a number of paradigms of cellular stress. Evidence for a role of JNK activation in MPP^+ toxicity for dopaminergic neurones in this culture system comes from the demonstration of increased levels of phosphorylated c-jun, a transcription factor phosphorylated by JNK but not p38, in MPP^+ treated dopaminergic neurones. These data confirm previous observations in mesencephalic neurones exposed to MPP^+ (Gearan et al., 2001); conflicting data have, however, been reported in the dopaminergic cell line MN9D (Choi et al., 1999, 2001). That the maximum increase in P-jun levels preceded the onset of expression of apoptotic morphology indicates that c-jun phosphorylation precedes apoptotic execution. The decreased levels at timepoints where apoptosis increases may reflect a loss of neurones which have activated P-jun; the persistence of increased numbers of P-jun immunoreactive neurones in cultures co-treated with caspase inhibitor and MPP^+ reinforces this hypothesis.

Both CEP-1347 and the JNK/p38 inhibitors evaluated gave significant partial neuroprotection from MPP^+ toxicity in mesencephalic dopaminergic neurones; in this culture system, then, the JNK pathway is clearly involved in the dopaminergic degeneration. When a selective p38 inhibitor was used, the results were markedly different.

In this culture system, p38 inhibition using selective inhibitors was not sufficient to spare dopaminergic neurones. The three p38 inhibitors described had little effect on the number of remaining TH-immunoreactive neurones following MPP^+ 10μM treatment. Compound 2, the most extensively tested of the selective p38 inhibitors had no effect on the number of apoptotic TH-immunoreactive neurones, nor on the expression of active caspase 3 in TH-immunoreactive neurones at 500nM concentration, significant neuroprotection was observed above this concentration, in the range where some JNK inhibition might be expected. Thus there is little evidence that p38 inhibition spares dopaminergic neurones, indicating that activation of the JNK pathway and consequent caspase activation is of more importance in mediating MPP^+ toxicity within these purified neuronal cultures.

As previously discussed, there are a number of well defined intracellular signalling pathways which can activate the caspase cascade; these include death receptor ligation, release of mitochondrial pro-apoptotic factors into the cytoplasm either through opening of the mitochondrial permeability transition pore or through pores formed by pro-apoptotic Bcl-2 family members, and direct activation of initiator caspases (discussed in Chapter 1 and Chapter 4, above).

The JNK pathway has been shown to be involved in activation of pathways through at least two of these mechanisms. Activation of the JNK pathway is reported to induce expression of the cell death inducing cytokine fas ligand (Le-Niculescu et al., 1999); fas ligand in the cell membrane binds to the death receptor fas, which recruits a number of intracellular transducer proteins and activates a caspase cascade involving activation of caspase 8 and ultimately caspase 3. There is evidence *in vivo* that MPTP treatment causes activation of caspase 8, and inhibition of this pathway is neuroprotective (Hartmann et al., 2001, Viswanath et al., 2001). The JNK pathway has recently been shown to induce apoptosis in β-amyloid peptide (Morishima et al., 2001), and a novel neurotoxic amyloid fragment β-amyloid 17-40 (Wei et al., 2002), treated cortical neurones through induction of fas ligand – this route may also be a candidate pathway for coupling activation of JNK to the caspase cascade. The JNK pathway also interacts with the Bcl-2 family of pro- and anti-apoptotic factors. Activation of the JNK pathway is reported to cause translocation of Bid and Bim, pro-apoptotic BH3 domain only members of the Bcl-2 family, from the cytoplasm to the mitochondrial membrane (Harris and Johnson, 2001). Once located in the mitochondrial membrane, these factors act as accessory factors to facilitate the pore formation by factors such as Bax, allowing cytochrome c release. Transgenic mice lacking Bax show reduced susceptibility to MPTP/MPP^+ toxicity (Vila et al., 2001). This pore forming activity of Bax is prevented by anti-apoptotic Bcl-2 (Antonsson et al., 1997), and neurones from Bcl-2 overexpressing mice are resistant to MPTP/MPP^+ toxicity *in vivo* and *in vitro* (Yang et al., 1998, Offen et

al., 1998). Cytochrome c released from mitochondria forms a complex with APAF-1 and dATP, which activates caspase 9 and then caspase 3 (discussed in detail in Chapter 1 and Chapter 4). Cytochrome c has been shown to be released from mitochondria in cerebellar granule neurones treated with MPP^+ (Du et al., 1997), and it was demonstrated in the previous chapter that inhibition of caspase 9 *in vitro* partially attenuates MPP^+ toxicity *in vitro*. Furthermore, overexpression of X-linked inhibitor of apoptosis (IAP) protein, an endogenous inhibitor of caspase 9, attenuates MPTP toxicity *in vivo*. Thus, this cascade is another possible pathway coupling JNK activation to caspase cleavage. It will be of interest to further examine the relative contribution to MPP^+ mediated apoptosis made by such JNK activated pathways, though it seems likely that these are not the only pathways activated by MPP^+.

In this regard, it is interesting that both of the JNK inhibitors tested caused only a partial sparing of dopaminergic neurones. The JNK/p38 inhibitor abolished the expression of P-jun at both 24 and 48 hour timepoints, yet decreased the number of apoptotic nuclei in those TH-immunoreactive neurones remaining in the culture after 48 hours by only half. Similar results were observed when the expression of active caspase 3 was evaluated, with JNK/p38 inhibition causing only a 50% decrease in the MPP^+ mediated increase in active caspase 3 expression in TH-immunoreactive neurones at 48 hours. These data would indicate that under these culture conditions, inhibition of the JNK pathway alone is not sufficient to spare all dopaminergic neurones from MPP^+ toxicity, nor to prevent all the cleavage and activation of caspase 3. Together these results imply that imply that another

mechanism leading to caspase activation may be involved. Whether this alternative route to apoptosis is normally activated concurrently with JNK phosphorylation or is a compensatory mechanism activated upon JNK inhibition is not clear. An obvious candidate pathway for the alternative route to caspase activation and apoptosis in MPP^+ treated neurones is mitochondrial permeability transition pore opening and subsequent release of pro-apoptotic factors into the cytoplasm. This was discussed in some detail in Chapter 4, above. This is an interesting area for future research.

Although the data presented here appear to indicate that JNK inhibition may be of limited effectiveness in preventing all MPTP/MPP^+ related cell death, it may have therapeutic benefit in Parkinson's disease. CEP-1347 is currently undergoing clinical trials for Parkinson's disease. There is evidence that human PD nigrae express phosphorylated c-jun. In a very interesting recent paper, another possible link to JNK activation in PD was postulated. In this paper (Hashimoto et al, 2002) it was shown that α-synuclein overexpression protected a neuronal cell line from oxidative stress through inactivation of the JNK pathway; synuclein inhibited JNK pathway activity by increasing expression and activity of JIP-1. These data are fascinating, given that there are mutations in the α-synuclein gene which are associated with early onset Parkinson's disease; it will be of great interest to see whether mutant synuclein has reduced JNK pathway inhibitory activity. Another potentially interesting area for further research is the potential for JNK inhibitors to increase survival of primary dopaminergic neurones prior to transplantation; both caspase (Schierle et al., 1999) and the p38/JNK inhibitor

SB203580 (Zawada et al., 2001) increase survival of dopaminergic neurones in transplantation models. *In vitro*, Compound 1 increased survival of mesencephalic dopaminergic neurones either when applied continuously from plating for up to 7 days, or when cultures were pretreated for timepoints up to 2 hours prior to plating (data not shown). This then may represent another potential use for JNK inhibition in PD.

Thus, the data in this chapter demonstrate that in this culture model, treatment with MPP^+ induces phosphorylation of c-jun which appears to precede apoptotic cell death; this cell death can be inhibited by inhibition of JNK, of both JNK and p38, but not by p38 inhibition alone. The activation of the JNK pathway is upstream of caspase activation, and this activation can be inhibited by JNK inhibition, but not by p38 inhibition alone. The JNK pathway, however, is unlikely to be the only pathway activated by MPP^+ treatment, as complete inhibition of c-jun phosphorylation using a JNK inhibitor decreased but did not abolish either caspase 3 activation, TH-immunoreactive cell loss or expression of apoptotic profiles.

Thus, there is strong evidence that the JNK pathway is involved in the apoptotic response to MPTP/MPP^+ in both *in vitro* and *in vivo* models. The next chapter describes a series of experiments carried out to evaluate whether the JNK pathway might be implicated the loss of TH-immunoreactive neurones in another *in vivo* model of Parkinson's disease, 6-hydroxydopamine toxicity.

Chapter 6.

Unilateral 6-OHDA administration to adult rats increases c-jun phosphorylation in dopaminergic neurones.

6.1. Introduction

6.1.1. In-vivo models of Parkinson's Disease

One of the major obstacles to research into Parkinson's Disease is the lack of a robust and accurate animal model of the disease. While there are a number of toxin and mechanical models which cause a loss of the nigral dopaminergic neurones and the nigrostriatal projection in a number of species, the damage typically does not resemble that of idiopathic PD either in the presence of the pathological hallmarks of the disease such as Lewy bodies or in the temporal progression of the cell loss (reviewed in Blum et al., 2001, Betarbet at el., 2002). Furthermore, idiopathic Parkinson's Disease typically involves more neuronal populations than merely the mesencephalic dopaminergic neurones; models which affect merely this population cannot be said to truly mimic the disease. A number of transgenic mouse models of Parkinson's Disease have been created. α-synuclein is a ubiquitous component of Lewy bodies, a pathological hallmark of degenerating nigral neurones in PD, and rare familial forms of PD are associated with mutations in α-synuclein. Transgenic mice expressing human α-synuclein (Masliah et al., 2000), and mice expressing mutant α-synuclein (reviewed in Betarbet et al., 2002) have nigral pathologies similar to PD. While these may prove extremely useful in providing a model of PD more closely resembling the idiopathic condition, they still do not model idiopathic PD in the way that the Huntingtin mutant mouse models Huntingdon's Disease, for example (reviewed in Menalled and Chesselet, 2002).

The most commonly used models of PD involve treatment of animals with factors toxic for mesencephalic dopaminergic neurones. As has been discussed, two of the most widely used of these toxins are MPP^+/MPTP and 6-OHDA; others which have been used include the pesticide rotenone, which is reported to induce cell death recapitulating some of the features of idiopathic PD (Heikkila et al., 1985, Betarbet et al., 2000), and isoquinolines (reviewed in Collins and Neafsey, 2002). The toxicity of MPTP/MPP^+ has been reviewed extensively in Chapter 1; accordingly, it will not be discussed in detail here, save to note that the previously discussed species differences in MPTP limit its usefulness in species other than mouse and primate, and that MPP^+ does not readily cross the blood brain barrier. Another toxin commonly used to model the nigral degeneration of PD is 6-OHDA.

6.1.2. The 6-OHDA model of Parkinson's Disease

6-OHDA is a Parkinsonian neurotoxin, which does not cross the blood brain barrier and must be surgically administered into the nigrostriatal dopaminergic system. One advantage of the use of this toxin is that it can be used in rat, which is resistant to toxicity of MPTP. Timecourse and extent of the lesion is dependent upon the site of administration of the compound. The three main sites of administration into the nigrostriatal dopaminergic system are direct injection into the nigra, injection into the median forebrain bundle (MFB), or injection into the striatum (reviewed in Betarbet et al., 2002). Of these three toxin injection paradigms, the most 'complete' lesion (removing greater than 90% of dopaminergic neurones) is observed with MFB 6-OHDA injection. The toxin is

taken up into the axons of the dopaminergic neurones in both the nigra and the ventral tegmental area, resulting in a profound loss of these neurones. Direct injection of the toxin into the nigra also results in a profound loss of dopaminergic neurones in the nigra; the extent of this lesion, however, is circumscribed by the diffusion of the compound. Typically, little damage is observed in the VTA in this injection paradigm. The final administration paradigm is intrastriatal injection of 6-OHDA. 6-OHDA is taken up by the terminals of the nigrostriatal dopaminergic fibres, resulting in retrograde degeneration and death of the cell bodies in the nigra. This model typically results in a partial lesion, the extent of which depends upon the injection site. Variations of this model use one or several injection sites to vary the extent and severity of the lesion.

The extent of lesion following unilateral 6-OHDA infusion can be evaluated by counting of the number of remaining ipsilateral nigral dopaminergic neurones, and comparing to the contralateral side. Additionally, rotation behaviour can be used to evaluate the extent of lesion. Two rotation models are commonly used, in response to amphetamine or apomorphine respectively (Hefti et al., 1980). Amphetamine administration results in release of monoamines, including dopamine; asymmetry in striatal dopaminergic innervation results in increased rotation behaviour ipsilateral to the site of injection. Apomorphine binds to dopamine receptors in the striatum; dopamine D2 receptors in the denervated ipsilateral hemisphere become supersensitive, so binding of apomorphine to these receptors results in increased rotation behaviour contralateral to the site of injection. The receptor supersensitivity induced by striatal dopaminergic

depletion occurs only when the lesion is large; this model is therefore more useful in models where major damage to the nigrostriatal pathway is apparent. ~~In both models a large lesion is required before rotation becomes apparent; one drawback is that such a large lesion may be difficult to prevent to an extent where effects on rotation are apparent.~~

6.1.3. Aims of this chapter

The primary aim of this chapter is to evaluate activation of the JNK pathway in the 6-OHDA model of Parkinson's disease, using c-jun phosphorylation as a marker. The extent and timecourse of cell death will be evaluated, along with the timecourse of c-jun phosphorylation, and double immunolabelling used to determine whether phosphorylated c-jun is expressed selectively within dopaminergic neurones.

6.2 Results

Initial experiments were undertaken to evaluate the extent of lesion induced by 6-OHDA when administered into the medial forebrain bundle. Animals were unilaterally treated with 6-OHDA, then allowed to recover for 14 days. Animals were perfusion fixed, and the brains removed and processed for paraffin embedding. 6μm thick paraffin sections were cut throughout the nigra, and immunostained for TH expression.

Following fixation, sectioning and immunostaining for TH, dopaminergic cell survival was quantified by counts of TH-immunoreactive neurones. A graph demonstrating the loss of nigral and ventral tegmental area TH-immunoreactive neurones is shown in Figure 6.1. Using this lesion paradigm, after 14 days there was typically a decrease of greater than 80% in the number of TH-immunoreactive cells in the nigra and ventral tegmental area in the ipsilateral hemisphere compared to the contralateral hemisphere. Nigrae and VTA appeared normal in sham lesioned animals (not shown). In another experiment, the toxin was administered unilaterally directly into the nigra; the lesions in this paradigm were variable, and so the median forebrain bundle model was selected for further experiments.

The next series of experiments were undertaken to examine whether 6-OHDA administered into the MFB caused activation of the JNK pathway, as demonstrated by phosphorylation of c-jun. Animals were treated with 6-OHDA as described above and in Chapter 2, and perfused with 10% formal saline at 4

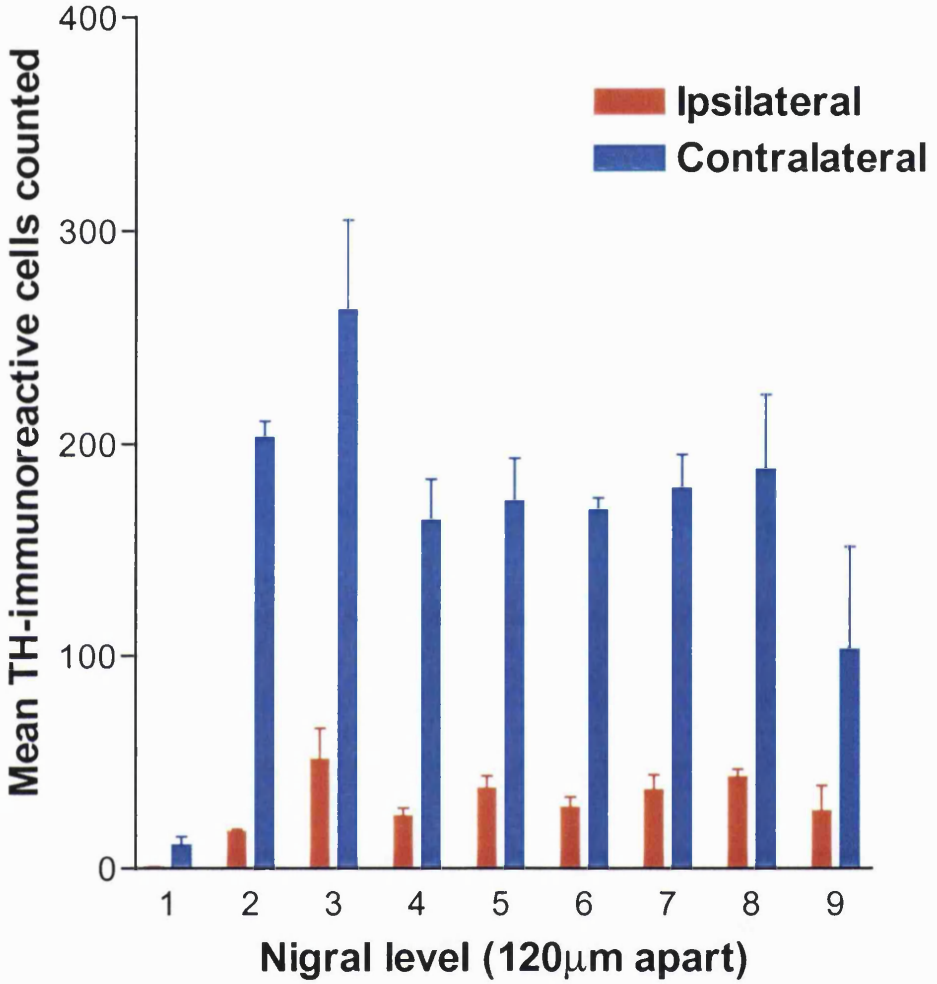


Figure 6.1. Effects of unilateral 6-OHDA infusion into the medial forebrain bundle on the number of nigral TH-immunoreactive neurones. Animals were perfused 14 days following lesion, and 6μm sections cut through the substantia nigra. Two sections were stained per level through the nigra; levels taken were approximately 120μm apart. Counts were made of the contralateral and ipsilateral TH immunoreactive neurones on each section. For meaning of data, sections were aligned to the first section for each animal with a number of dopaminergic neurones greater than 100. Results shown are the mean ± s.e.m. of four animals.

hours, 8 hours, 24 hours, 48 hours or 7 days following lesion. Brains were processed and paraffin embedded, and 6μm thick sections cut through the extent of the substantia nigra. Sections were dewaxed and immunostained for expression of phosphorylated c-jun. In initial experiments, the immunoreactivity for phosphorylated c-jun was very weak, with several phospho-c-jun specific antibodies. Antigen retrieval, however, increased the immunoreactivity for phosphorylated c-jun. The antigen retrieval method chosen was 15 minute microwaving in citrate buffer; this approach has been demonstrated to increase signal in *in situ* hybridisation studies in paraffin sections (Oliver et al., 1997). The increase in P-jun immunoreactivity was selective, with P-jun immunoreactive cells apparent only in the ipsilateral substantia nigra and VTA. A montage of photomicrographs demonstrating the localisation of P-jun immunoreactivity in the ipsilateral substantia nigra and VTA of an animal perfused at 7 days post lesion is shown in Figure 6.2. Counts of P-jun expressing cells in the nigra and VTA of 6-OHDA lesioned rats are shown in Figure 6.3. At all timepoints tested, few P-jun expressing cells were apparent in the contralateral nigra and VTA. There was an increase over time in the numbers of P-jun expressing cells in the ipsilateral nigra and VTA; the maximal increase was observed at the 7 day timepoint, where greater than 200 P-jun expressing cells were observed in the these regions.

To evaluate whether the increased P-jun immunoreactivity observed was in the dopaminergic neurones themselves, a double immunolabelling strategy was used. Paraffin sections from a rat perfused 7 days following 6-OHDA injection into the MFB were cleared and rehydrated, and antigen retrieval was carried out. The

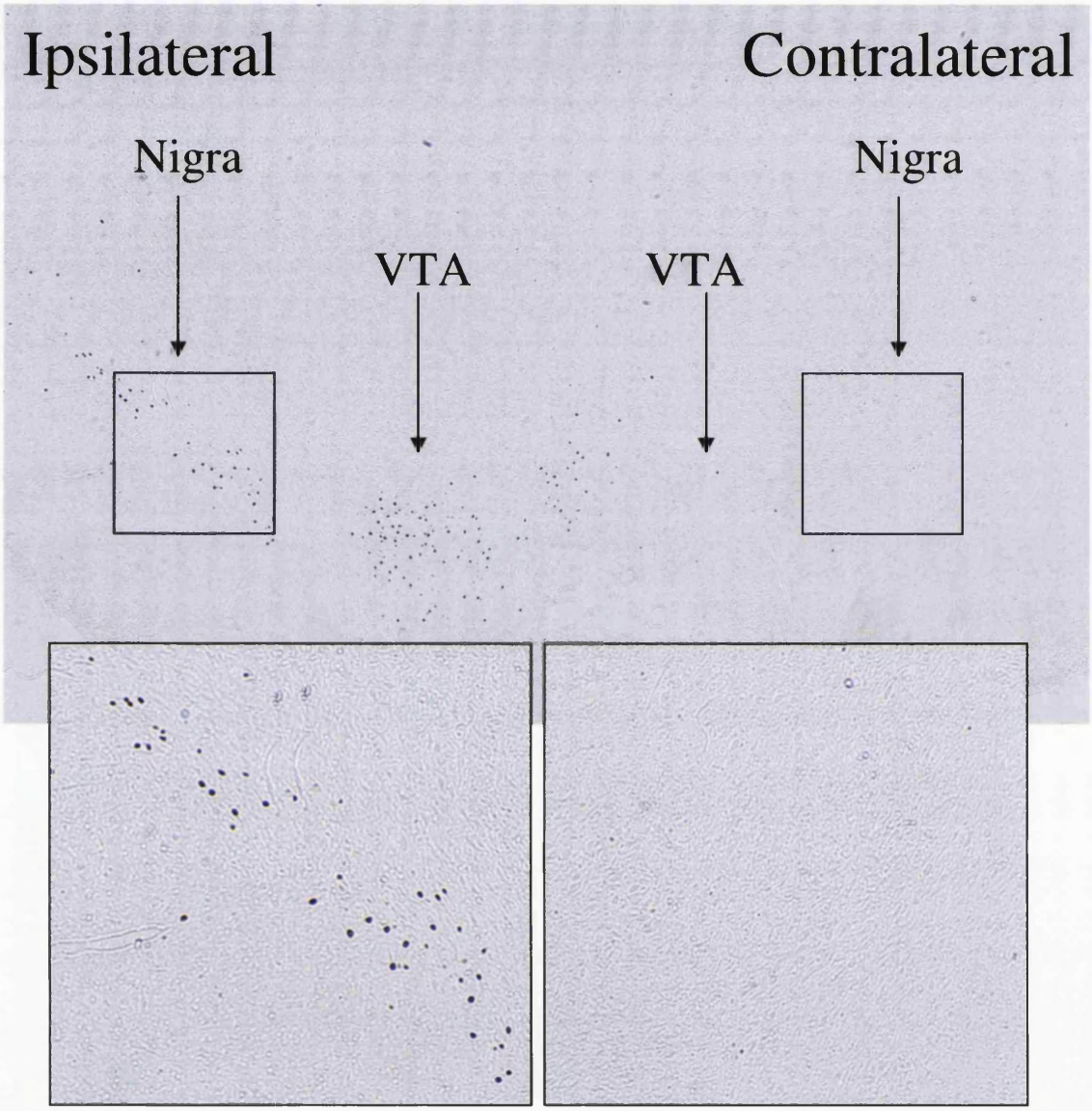


Figure 6.2. Photomontage of induction of expression of phosphorylated c-jun (serine 63) in the ipsilateral substantia nigra and ventral tegmental area 7 days following unilateral 6-OHDA injection. Robust phosphorylated c-jun immunoreactivity is observed in the ipsilateral nigra and VTA (dark spots), with little expression in the contralateral areas. Inset are details of the ipsilateral and contralateral nigrae, in which the increased expression of phosphorylated c-jun can clearly be observed.

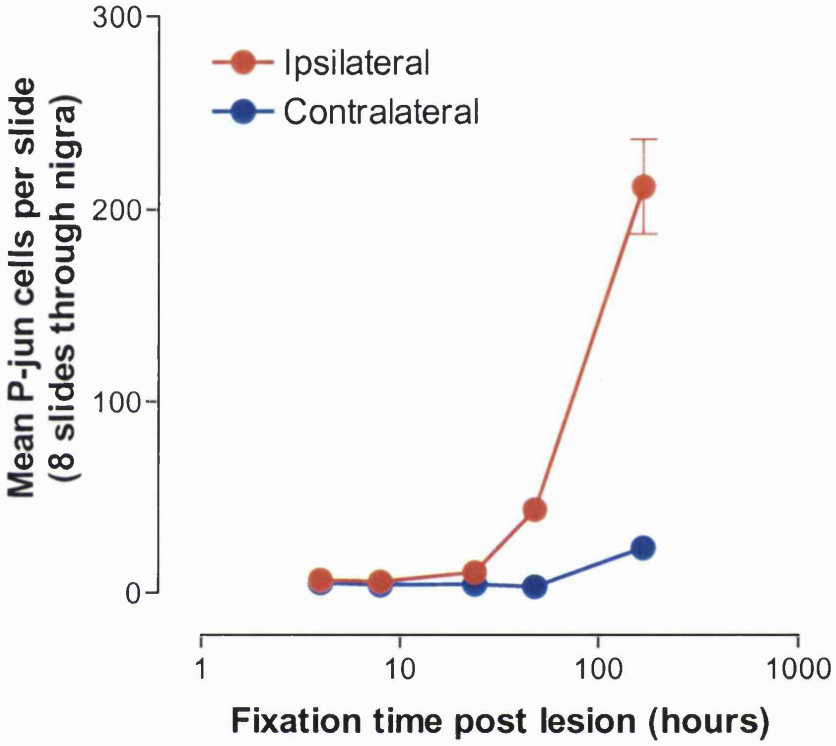


Figure 6.3. Quantification of timecourse of c-jun induction in substantia nigra and ventral tegmental area in ipsilateral and contralateral hemispheres following unilateral 6-OHDA injection. Animals were perfused at 4, 8, 24 or 48 hours, or 7 days following 6-OHDA administration. Data points shown are the mean of all cells counted across regions through the nigrae of one animal per data point; error bars are shown for all data points but in many cases are smaller than the symbols.

slides were stained using a primary antibody for P-jun, and the staining visualised using Vector SG insoluble peroxidase substrate; this gives a dense black precipitate. Following P-jun staining, the slides were washed in PBS, placed into distilled water, and microwaved on full power for 5 minutes to denature the antibodies. The slides were placed in running water for one minute, then washed again in PBS and immunostained for TH using the one day Optimax staining protocol described in Chapter 2 and used for the immunostaining experiments described above. To visualise the TH staining, light DAB staining was employed, using half the recommended reagents in the Biogenix DAB kit; this enhanced the contrast between the black nuclear P-jun staining obtained with Vector SG and the light brown TH staining pattern obtained with the DAB. Control slides were stained only with antibodies to P-jun or to DAB. Sections were lightly counterstained using haematoxylin, cleared, dehydrated and mounted. Photomicrographs of the double immunostaining obtained are shown in Figure 6.4. In the ipsilateral nigra (6.4.B) and VTA (6.4.C) it is apparent that many, though not all, of the TH-immunoreactive cells express phosphorylated c-jun in their nuclei; it is also apparent that the P-jun is expressed primarily within the TH-immunoreactive cells. In the contralateral nigra (6.4.D) and VTA (6.4.E), few cells colocalise TH and phosphorylated c-jun. Quantification of these data are shown in Figure 6.5.A. Counts were made of the total number of TH cells, and of those cells co-expressing TH and P-jun. The results show that in the ipsilateral hemisphere, around 50-70% of TH-immunoreactive neurones also express P-jun, while less than 1% of TH-immunoreactive cells in the contralateral nigra are

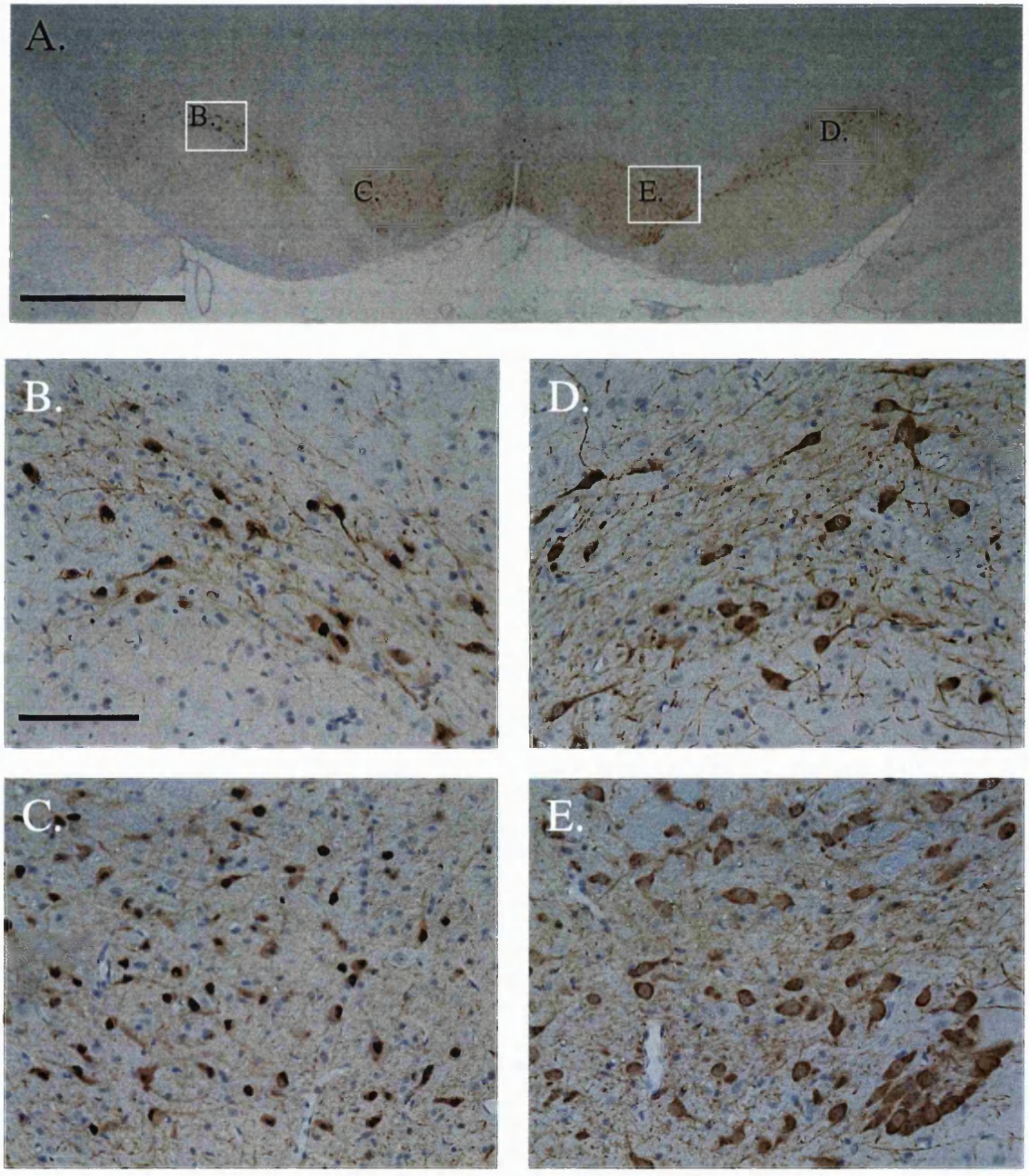


Figure 6.4. Phosphorylated c-jun (serine 63) is expressed in TH-immunoreactive cells in the ipsilateral substantia nigra and VTA 7 days following unilateral 6-OHDA injection into the medial forebrain bundle (A). Phosphorylated c-jun staining (black) is localised in the nuclei of TH-immunoreactive neurones (brown) in the substantia nigra and VTA of the ipsilateral hemisphere (B and C respectively) but not in the contralateral hemisphere (D and E respectively). Scalebars (A) = 1mm, (B) - (E) = 100μm.

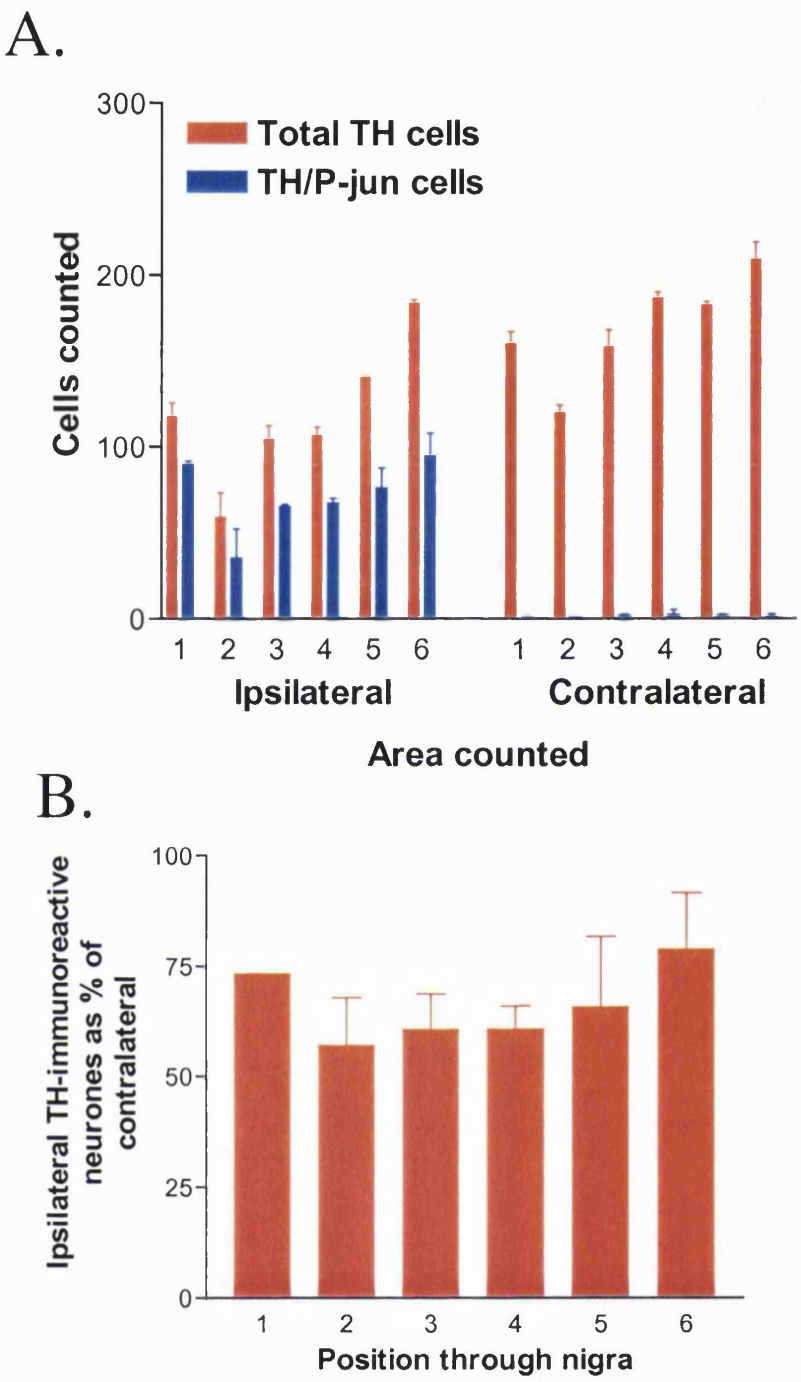


Figure 6.5. Quantification of surviving and phosphorylated c-jun expressing TH-immunoreactive neurones in the substantia nigra and VTA of 6-OHDA lesioned rat brain 7 days following unilateral 6-OHDA administration. Two sections were immunostained at each level through the nigra, and levels were approximately 120μm apart. Increased expression of phosphorylated c-jun in TH-immunoreactive neurones is apparent in the ipsilateral nigra and VTA compared to the contralateral hemisphere (A). In the ipsilateral hemisphere, around 50% to 70% of remaining TH-immunoreactive neurones coexpress P-jun. Data are from sections taken from one animal. (B) shows the number of remaining TH-immunoreactive neurones in the ipsilateral nigra as a percentage of the contralateral counts. Data are the mean ± s.e.m. of sections from two animals.

double labelled. Figure 6.5.B shows the extent of cell loss in the nigrae of 6-OHDA lesioned animals after 7 days. At this timepoint, around 60-70% of nigral TH-immunoreactive neurones yet remain, indicating that the majority of dopaminergic cell loss has yet to occur in this area.

Thus, 6-OHDA injection into the median forebrain bundle results in phosphorylation of c-jun in the substantia nigra and VTA, with maximal activation of c-jun at 7 days preceding the onset of neuronal loss in these areas.

6.3 Discussion

The data in this chapter demonstrate that unilateral injection of 6-OHDA into the medial forebrain bundle of adult rats leads to pronounced cell death in the substantia nigra and VTA, which is preceded by increased expression of phosphorylated c-jun. The increased P-jun expression is localised in the dopaminergic neurones, demonstrated by double immunolabelling. The 6-OHDA model was chosen over the MPP^+/MPTP model for these experiments for two reasons; firstly, many published studies have examined the JNK pathway *in vivo* following MPTP toxicity, and less is known about the relevance in the 6-OHDA model. Secondly, due to safety considerations for animal care staff it was not possible to carry out *in vivo* MPTP or MPP^+ experiments. Thus, the 6-OHDA model was used as an *in vivo* model of PD instead.

The data indicate that the cell death induced by 6-OHDA following medial forebrain bundle administration may be mediated by activation of the JNK pathway. The cell death induced by this lesion paradigm has been shown to induce apoptotic changes in the substantia nigra (Zuch et al., 2000), and previous studies have demonstrated that 6-OHDA induces c-jun phosphorylation in the substantia nigrae of rats when administered intrastriatally (Vaudano et al., 2001); the current data demonstrate that injection of the toxin into the median forebrain bundle also results in c-jun phosphorylation with a similar timecourse. Increased expression (Leah et al., 1993) and activation (Winter et al., 2000) of c-jun has also been demonstrated in rats following median forebrain bundle axotomy. Thus in

axotomy models and in toxin models c-jun is phosphorylated in the nigra following cellular injury.

The timecourse of c-jun phosphorylation is interesting; maximal increases in c-jun phosphorylation were observed at 7 days post toxin administration, a timepoint where extensive cell death has not yet occurred. One might imagine that those cells which express P-jun at earlier timepoints are those which have already degenerated by the 7 day timepoint; further experiments may clarify this. It is interesting that these data in the 6-OHDA *in vivo* model parallel those observed in the MPP^+ model *in vitro* (discussed in Chapter 5); in the *in vitro* model, P-jun expression was observed only very rarely in cells with apoptotic nuclei. It seems likely that c-jun phosphorylation is an early event in both models, which sets in motion a chain of pro-apoptotic events but which may not be sustained over long periods of time.

The importance or otherwise of the JNK pathway in mediating 6-OHDA toxicity will only be addressed by inhibition of the JNK pathway in this model. To date, no published studies have addressed this issue. Attempts were made to test this hypothesis using the JNK/p38 inhibitor Compound 1. Despite encouraging pharmacokinetic data with twice daily oral administration of the compound, no sparing of dopaminergic neurones was observed with this compound; this corresponded to a failure to prevent c-jun phosphorylation in the nigra and VTA at 7 days post compound administration. It is unclear why the compound failed to inhibit c-jun phosphorylation *in vivo*. Some possible reasons may be that the compound might not be stable enough to inhibit JNK continuously; furthermore,

the concentration required to inhibit JNK *in vivo* is not known. As the compound binds to the ATP site of the JNK enzyme, it may be that very high concentrations are required for stable inhibition of the enzyme *in vivo*. Given the lack of effect at inhibiting c-jun phosphorylation, the hypothesis that JNK inhibition may protect from 6-OHDA toxicity *in vivo* was not tested and these data are not included here.

Although there are no published studies in which JNK inhibition has proved neuroprotective in the 6-OHDA model, JNK pathway inhibition is neuroprotective in other *in vivo* PD models. Adenovirus mediated expression of dominant negative c-jun prevents MFB axotomy induced nigral degeneration (Crocker et al., 2001b), and adenoviral gene transfer of JIP-1 protects nigral dopaminergic neurones from MPTP toxicity (Xia et al., 2001). Thus, in different *in vivo* models of nigral degeneration inhibition of the JNK pathway is protective; given the pronounced increase in c-jun phosphorylation in the 6-OHDA model with both intrastriatal (Vaudano et al., 2001) and MFB administration it seems likely that this may also be implicated in the cell death in this model of PD. It is, however, possible that JNK pathway inhibition is not capable of sparing dopaminergic neurones in the 6-OHDA model; the lesions observed with 6-OHDA treatment, particularly when administered into the MFB, are very severe, and it may be that JNK inhibition alone is not sufficient to prevent cell death.

There have been published studies examining the effects of inhibition of other components of the apoptotic cascade in the 6-OHDA model *in vivo*. Inhibition of caspases using zVAD-fmk has been shown to protect nigral neurones following intrastriatal 6-OHDA administration (Cutillas et al., 1999); adenoviral NAIP

expression also protect rat substantia nigra from intrastriatal 6-OHDA toxicity (Crocker et al., 2001a). A number of studies have examined 6-OHDA toxicity for dopaminergic neurones *in vitro*. There is evidence that inhibition of caspases protects PC12 cells from 6-OHDA toxicity (Ochu et al., 1998, Takei et al., 1998), though 6-OHDA also induced a caspase independent necrosis at higher concentrations (Ochu et al., 1998). In primary dopaminergic neurones, caspase inhibition prevents cell death induced by 6-OHDA (Lotharius et al., 1999, von Coelln et al., 2001); similar results are reported in the dopaminergic cell line MN9D, where JNK activation is also observed following 6-OHDA treatment (Choi et al., 1999).

Thus, 6-OHDA toxicity *in vivo* also appears to be mediated by many of the factors which are implicated in MPTP toxicity. It will be of interest to determine whether JNK inhibition, shown to protect dopaminergic neurones in the MPTP model, can also spare these neurones in the 6-OHDA model of PD.

Chapter 7:

General discussion

7.1 General discussion

The primary aims of this study were to examine apoptotic pathways activated in MPP^+ toxicity in primary cultures of mesencephalic dopaminergic neurones. These pathways were examined using commercially available inhibitors and also with a range of novel and selective inhibitors. One of the problems encountered with primary mesencephalic cultures is the heterogenous nature of the cultures; dopaminergic neurones comprise only a small subpopulation of the neurones, making analysis of the signalling pathways activated in toxin models difficult. In this study, double immunocytochemical staining was used to observe MPP^+ induced changes in expression of pro-apoptotic factors. The data indicate that MPP^+ treatment activates pathways involving c-jun-N-terminal kinase, but not p38, which culminate on caspase 3 activation.

Chapter 3 described the validation of the culture system used in this study. The use of the Sato serum substitute allowed mesencephalic cultures to be maintained in the absence of significant numbers of astrocytes. The cultures thus maintained were shown to be susceptible to MPP^+ toxicity as quantified by counts of TH-immunoreactive neurones and by evaluation of ^{3}[H]-DA uptake. The responses of the cultures to the well defined neuroprotective factors GDNF and BDNF, when co-administered with MPP^+, were similar to previous reports in the literature; there was a sparing of both TH-immunoreactive cell number and uptake of ^{3}[H]-DA. This culture system, then, was used to examine the apoptotic pathways

activated by MPP^+. The initial question was to what extent the caspase pathway was involved in the toxicity of MPP^+.

In order to answer this question, a number of caspase inhibitors were tested; these data comprise Chapter 4. Initial experiments confirmed the finding of Dodel and coworkers (1998) that the peptide poly-caspase inhibitor z-VAD-fmk increased numbers of TH-immunoreactive cells in MPP^+ treated primary mesencephalic cultures. These data were expanded by the testing of a number of further peptide inhibitors based on the substrate specificity of individual caspases; the results of these experiments indicated that inhibitors based on the preferred cleavage sites of caspases 2, 3, and 9 were neuroprotective and that an inhibitor based on the cleavage sequence of caspase 1 was not. The involvement of caspases 2, 3 and 9, along with caspase 8, has since been demonstrated in *in vivo* models, though there is some debate over the role of caspase 1 (discussed in Chapter 4). A major problem with the interpretation of these data is the specificity of the inhibitors; none of the peptide inhibitors have much selectivity for individual caspases, and in the case of the caspase 2 inhibitor there is likely to be no specificity of the compound for caspase 2 over caspase 3. In order to address these issues, a number of more selective inhibitors from the Merck-Frosst Canada caspase programme were tested. The data obtained with these inhibitors provided strong evidence for the crucial role of caspase 3 in mediating MPP^+ toxicity. Selective caspase 3 inhibitors protected dopaminergic neurones from MPP^+ toxicity, with potencies reflecting their caspase 3 IC_{50} values. The most potent compound was as effective as broad-spectrum caspase inhibition in protecting dopaminergic

neurones, whereas a structurally related control compound with little caspase inhibitory activity was ineffective. These data were confirmed by double immunolabelling experiments demonstrating activated caspase 3 in MPP^+ treated dopaminergic neurones. The protection afforded by caspase inhibitors was almost total when TH-immunoreactive cell numbers were quantified; this did not, however, correlate to a functional sparing of the neurones as quantified by uptake of ^{3}[H]-DA. Both broad spectrum inhibitors and a selective caspase 3 inhibitor had considerably less potency in preventing MPP^+ mediated decreases in ^{3}[H]-DA uptake. Further analysis demonstrated that the sparing of cell bodies by caspase inhibition did not fully preserve neurites, suggesting that this may underlie this lack of effect.

These data demonstrated both that MPP^+ treatment activated a caspase pathway culminating in activation of caspase 3 like proteases, and that inhibition of this pathway was neuroprotective though did not restore full functionality. Experiments were undertaken to evaluate pathways upstream of caspase activation, and these data are shown in Chapter 5. The Cephalon MLK inhibitor CEP-1347 was reported to protect dopaminergic neurones from MPTP toxicity (Saporito et al., 1999, 2000). As an initial experiment CEP-1347 was tested, and shown to have partial neuroprotective effect on mesencephalic TH-immunoreactive neurones. CEP-1347 was not used for further experiments; the experiments were carried out under a collaborative agreement restricting the work which could be carried out using the compound. Instead, novel direct inhibitors of JNK/p38 and p38 alone were employed. These inhibitors had several

advantages. Firstly, the JNK/p38 inhibitor is a direct inhibitor at the ATP binding site on JNK and p38, and has very little activity at other kinases (Scapin et al., manuscript submitted, Pollack et al., manuscript in preparation). CEP-1347, by contrast, inhibits upstream of JNK and has activity at a number of other kinases. Secondly, both of the compounds tested have considerably higher potency for their targets than any commonly used commercially available inhibitors, such as the Signal compound SP600125 or the p38 inhibitors SB203180 or SB202190 (discussed in Chapter 5). Using these compounds, and a number of other inhibitors, it was demonstrated that inhibition of JNK/p38 partially protected mesencephalic dopaminergic neurones from MPP^+ toxicity, but that inhibition of p38 was ineffective. These data provide the first strong evidence that the p38 pathway is not involved in MPP^+ mediated apoptosis. The JNK/p38 inhibitor protected neurones; this effect may be mediated by inhibition of the JNK pathway or both the JNK and p38 pathways. Given the protective effect was similar to that observed with CEP-1347, which has no activity at p38, it is likely that the protective effect observed with this compound results from inhibition of the JNK pathway. Thus, the data suggest that the JNK pathway and not the p38 pathway is involved in MPP^+ mediated toxicity. The next series of experiments examined whether the partial neuroprotection afforded by JNK inhibition resulted from partial inhibition of JNK or a partial involvement of the JNK pathway in the toxicity. Double immunocytochemical experiments demonstrated that the JNK/p38 inhibitor abolished the expression of phosphorylated c-jun, a marker of JNK activation, in TH-immunoreactive cells, but that this abolition of JNK

activity reduced but did not abolish the expression of apoptotic TH-immunoreactive cells or the expression of active caspase 3 in these cells. p38 inhibition was without effect on all parameters. Thus, inhibition of the JNK pathway is not sufficient to fully spare cells, and another pathway must be activated either concurrently with the JNK pathway or as a result of JNK pathway inhibition.

The final series of experiments, shown in Chapter 6, evaluated activation of JNK in the 6-hydroxydopamine model of nigral cell death *in vivo*. The model chosen was one unilateral injection of 6-OHDA into the median forebrain bundle of rats, intranigral injection having produced variable lesions. This model provided pronounced loss of TH-immunoreactive neurones in the substantia nigra pars compacta and VTA by 14 days post injection. Analysis of the expression of phosphorylated c-jun by immunocytochemistry showed a pronounced upregulation of P-jun expression in the nigra and VTA on the ipsilateral side by 7 days post 6-OHDA administration. This timecourse of induction was similar to that observed with intrastriatal toxin administration (Vaudano et al., 2001) and was maximal before the majority of dopaminergic cell death had occurred. Thus, the JNK pathway may play a role in mediating death of nigral neurones in the 6-OHDA model of Parkinson's disease. A later set of experiments (not shown) using the JNK/p38 inhibitor failed to protect nigral neurones from 6-OHDA toxicity, though there was also no decrease in P-jun expression at the 7 day timepoint and thus the hypothesis was not tested. Future experiments with

another inhibitor may shed light on the neuroprotective potential of JNK inhibition in this model of nigral degeneration.

The *in vitro* data generated in this study suggest that the toxicity of MPP^+ in purified neuronal cultures involves activation of the JNK pathway and activation of a caspase cascade culminating in activation of caspase 3. My data indicate that there may be involvement of caspases 2, 9 and especially 3, but not 1. In the literature, *in vitro* studies have also implicated caspases 3, 8, 9, with caspase 1 proving ineffective. In *in vivo* studies, there has been evidence for involvement of caspase 1, caspase 2, caspase 3, caspase 8 and caspase 9 in MPTP toxicity, though there is some debate over the role of caspase 1. There is likely to be more than one route culminating in activation of caspases, and likely to be more than one caspase pathway activated; these pathways are also likely to have a degree of crosstalk and to feedback one into another. A wide range of other factors have been demonstrated to protect against MPP^+/MPTP toxicity. These include, but are not restricted to, growth factors, immunophilin ligands, cyclooxygenase inhibitors, and calpain. Some of these factors could well interact with components of the apoptotic cascade; growth factors, for example, have been shown to upregulate antioxidant levels (Mattson et al., 1995) and increase the ratio of Bcl-2 to Bax; activation of Akt by BDNF has also been shown to lead to serine phosphorylation and inactivation of caspase 9 in human cells (Cardone et al., 1998), though this serine residue is not present in rodent cells (Fujita et al., 1999). A wide array of growth factors have been shown to prevent MPTP/MPP^+ toxicity, some of which were mentioned in Chapter 1. Cyclooxygenase inhibition

has been shown to protect dopaminergic neurones from MPP^+/MPTP toxicity *in vivo* and *in vitro* (Teismann et al., 2001, Feng et al., 2002, Carrasco et al., 2002); cyclooxygenase inhibitors have also been shown to decrease activity of caspase 3 in trophic factor deprived differentiated PC12 cells (McGinty et al., 2000), indicating that they may link into the caspase cascade. This is an interesting area for further research. With regard to immunophilin ligands, there has been a great deal of interest in these compounds as potential therapies for Parkinson's Disease. The immunophilin ligand GPI-1046 entered clinical trials for Parkinson's Disease, and FK506 has been demonstrated to attenuate toxicity of MPTP/MPP^+. We saw no efficacy with GPI-1046 in the MPP^+ model *in vitro* and limited efficacy in the 6-OHDA model *in vivo* in a previously reported study (Harper et al., 1998), and there has been an ongoing debate in the literature with respect to the efficacy of the compound (Steiner et al., 1997, Winter et al., 2000, Ross et al., 2001, Bocquet at al., 2001, Zhang et al., 2001, Guo et al., 2001, Eberling et al., 2002); GPI-1046 failed to progress past Phase 2 clinical trials. The data obtained with FK506 appear more robust, sparing neurones and increasing their functionality. A recent study has demonstrated that FK506 inhibits JNK1 activation in H2O2 exposed neuronal cell lines, though this did not underlie the neuroprotection with the compound (Klettner et al., 2001); additionally, FK506 was shown to upregulate MAP kinase phosphatase-1 in surviving mamillary but not degenerating nigral neurones following axotomy, indicating another possible level of interaction with the stress activated MAP kinase pathways (Winter et al., 1998). There may thus be some interaction with the apoptotic pathways described

above, but it is likely that other mechanisms are of importance in mediating immunophilin ligand neuroprotection. Another factor which has been shown to be involved in MPTP/MPP^+ toxicity is calpain (Chera et al., 2002), a calcium regulated pro-apoptotic protease. In the *in vitro* model described in this study, inhibition of calpain was partially neuroprotective (data not shown; Crocker et al., 2001); calpain inhibition also protected nigral dopaminergic neurones from MPTP toxicity in mouse *in vivo* in this study. Calpain is activated concurrently with JNK and caspases in a number of models of cellular stress; there is evidence that calpain activation, as well as causing cellular damage in its own right, lies upstream of the caspase pathway. Calpain can activate caspase 12 in the endoplasmic reticulum during cellular stress (Nakagawa and Yuan, 2000), which can in turn activate the caspase cascade and also degrade the anti-apoptotic Bcl-2 family member Bcl-X_L, thereby linking into the mitochondrial route to apoptosis. Calpain can itself also cleave Bid to cause mitochondrial translocation, in a similar fashion to caspases, and may also regulate mitochondrial cytochrome c release at this level (Chen et al., 2001, 2002). A further level of possible interaction comes from the observation that calpain can itself facilitate cleavage of caspase 3 into its active form, though this is not necessary for calpain induced neurotoxicity following neuronal UV irradiation (McCollum et al., 2001). Direct evidence for calpain involvement in MPP^+ toxicity *in vitro* has been presented by Choi and coworkers (2001), who demonstrated that calpain activation was induced by MPP^+ by caspase dependent and independent mechanisms in the dopaminergic cell line MN9D, and led to cleavage of Bax; these authors,

however, found no evidence of apoptosis, in keeping with their previous work in primary culture models (Choi et al., 1999).

Thus, there are putative links into the JNK/caspase apoptotic cascade for a number of other factors which protect dopaminergic neurones in PD models, though it must be stressed that many of these links are speculative. It is clear that there are no shortage of factors which are interact with the apoptotic cascade and which can protect dopaminergic neurones from MPTP/MPP^+ toxicity. It is unclear to what extent these factors provide a functional recovery, and whether they are valid therapeutic targets. In particular, the potential of caspase inhibitors as therapeutic agents is unclear. Caspase inhibition has been shown to be neuroprotective and to improve behavioural performance following cerebral ischaemia *in vivo* even with long delays post insult (discussed in Chapter 4). *In vitro*, however, there is evidence that caspase inhibition may not fully protect cells. Inhibition of caspases in apoptotic models *in vitro* is reported to cause a switch to necrotic death in cerebellar granule neurones (Harada and Sugimoto, 1998), and to lead to activation of forms of cell death distinct from both apoptosis and necrosis in nerve growth factor deprived sympathetic neurones (Xue et al., 1999, Xue et al., 2001, Tolkovsky et al., 2002). A recent publication demonstrates that caspase activation can lead directly to necrosis through cleavage and inactivation of the plasma membrane calcium pump (Schwab et al., 2002), indicating that there may be links between apoptosis and necrosis at the level of caspases. The evidence that caspase inhibition can switch the type of cell death from apoptosis to another mechanism in neurodegenerative conditions is reviewed

by Leist and Jaatella (2001). One further possible drawback is the functionality of cells protected by caspase inhibitors; the cells spared must be functional and innervate their target. A recent review has examined the question of functional recovery with caspase inhibition, and raises the precautionary note that sparing a population of 'undead' but non-functional cells in the brain may have adverse consequences (Nicotera et al., 2000). It may be possible that cells spared by caspase inhibitors might require additional trophic support for long term survival and functionality. It is, however, also the case that there are no caspase inhibitors available with which the hypothesis may be tested, even in animal models. Most of the available inhibitors have poor blood brain barrier permeability, and all of the peptide inhibitors generally used are polycaspase inhibitors with poor specificity and short half lives. A further complication is that it is unclear to what extent the activity of caspases must be inhibited to prevent all cell damage. It is possible that a fractional inhibition of caspase activity may be sufficient to prevent enough of the substrate cleavage to 'save' the cell, but not be sufficient to prevent some damage and loss of functionality (Donald W Nicholson, personal communication). Development of further inhibitors with better blood brain barrier permeability, and increased understanding of caspase pathways in neurodegenerative disease, will shed light on the therapeutic potential of these compounds.

As was discussed at length in the individual chapter discussions, the functional recovery observed with caspase inhibitors in MPTP/MPP^+ models *in vivo* or *in vitro* is less than the neuronal sparing. This is reflected in the reduced level of

neurite sparing *in vitro* and in the partial sparing of striatal innervation reported in the MPTP model *in vivo*; furthermore a fundamental question in the MPTP model which remains unanswered is to what extent prevention of apoptosis can protect hypoenergetic cells which are not generating ATP. At face value, the lack of functional recovery indicates that caspase inhibition may not be a valid therapeutic target in Parkinson's disease models, and, by extension, in Parkinson's disease itself. The nature of Parkinson's disease is another confounding factor – apoptosis is a fundamental physiological process, and a successful anti-apoptotic strategy in PD would have to block the activity of caspases for many years.

With regard to the therapeutic potential of inhibitors of JNK, the question may soon be answered by the progress of CEP-1347 through the clinic. At the time of writing this compound is in Phase 2 clinical trials, and the results are eagerly awaited. It will be interesting to see whether there is a clinically relevant improvement in the condition over the extended periods of time required for a Parkinson's disease therapy and without adverse effects. It is possible that non-subtype selective inhibition of JNK for such a period may cause complications in immune system function. JNK 1 and JNK 2 are implicated in maturation of T cells to Th1 cells and Th2 cells respectively, and Th2 cells from JNK2 null mutant animals show reduced cytokine production (reviewed in Rincon et al., 2000, Hommes et al., 2003). Only the results of these studies are likely to answer these questions; if complications with immune system function should arise, then an alternative strategy may be to design inhibitors with selectivity at the JNK3

isoform, which is selectively expressed in brain and so should not compromise immune system function.

To my mind, it seems likely that the major obstacles to a successful anti-apoptotic strategy in chronic neurodegenerative conditions are a combination of the progressive nature of the conditions, with a gradual loss of cells over a period of years, and the potential adverse effects of blocking apoptosis over such a protracted period. Added to this is the pronounced loss of nigral neurones in Parkinsonian patients at the time of clinical presentation; spared neurones would likely exist in an environment with severely disrupted trophic support through loss of neurones and increased inflammation. These same issues are also likely to be important in defining the efficacy of anti-apoptotic strategies in other chronic neurodegenerative conditions also, and it is possible that acute neurodegenerative conditions such as cerebral ischaemia, where a more 'normal' trophic environment is likely to be restored with time, might prove more amenable to anti-apoptotic therapies (Holtzmann and Deshmukh, 1997). In PD, any anti-apoptotic strategy would likely need to be administered in conjunction with existing therapies such as L-DOPA; it is possible that co-administration of an anti-apoptotic agent with a trophic factor may one day prove of therapeutic benefit.

In general, when looking at cell death pathways, one cannot extrapolate from the results in Parkinson's Disease models to the idiopathic condition itself. No models of the condition replicate all features of the condition in animals; Complex I inhibitors such as MPTP or rotenone are widely used models of the condition,

but here, again, the dosing regimen and timecourse of the condition are very different from the idiopathic condition. In idiopathic PD there are other features such as Lewy bodies which are observed in the toxin models only with long term, low dose treatments, dosing paradigms which are infrequently used by researchers. The models commonly used merely replicate the nigrostriatal neuronal loss, while other populations are lost in the idiopathic condition; furthermore, it is always likely to be difficult to model a condition which develops over decades in an animal which lives for only two years. Expanding knowledge of the role of genetic factors such as mutations in α-synuclein may allow for the development of more robust animal models of the condition, and identification of animal models such as the AS/AGU rat which have age related deficits in the dopaminergic system may also prove valuable. Until such times, the data obtained in animal models using dopaminergic toxins is perhaps best viewed as elucidating the intracellular pathways activated by the toxin rather than as an insight into Parkinson's disease. As such, the data included in this thesis, and in the many publications on the pathways activated by MPTP/MPP^+ *in vivo* and *in vitro*, provide an interesting insight into the complexities of the intracellular pathways activated in a compromised dopaminergic cell. The relevance of the data to the human condition has yet to be established, though it is to be hoped that in time the relevance of these and other cell death mechanisms will be elucidated in Parkinson's disease.

7.2 Future directions

One of the main aims in continuing this work would be the elucidation of the upstream pathways leading to executioner caspase activation by MPP^+. In the course of this work I attempted to visualise redistribution of SMAC/DIABLO and cytochrome c from mitochondria to the cytoplasm in MPP^+ treated dopaminergic neurones, but without success. This area is of great interest, and should be continued – the use of higher throughput imaging systems such as the Atto Pathway confocal based system would allow for visualisation of these changes in small populations of cells. Alternatively, the use of a dopaminergic cell line such as MN9D or IRBN27 cells would allow for techniques such as western blotting to be used to quantify changes in protein expression in the mitochondrial and cytoplasmic fractions.

Another interesting and linked area for future research is the alternative mechanism leading to caspase 3 activation and apoptosis in JNK inhibitor and MPP^+ treated neurones. This alternative pathway may be through release of mitochondrial pro-apoptotic factors, or effects on Bcl-2 family members, or upregulation of pro-apoptotic factors such as fas ligand; also of interest is whether this pathway is activated concurrently with JNK activation, or is a result of JNK inhibition.

One major problem with determining the relative contributions of caspase family members is the lack of selective inhibitors for individual family members. Future work could utilise the emerging siRNA technology – which has been shown to

function in cultured postmitotic mammalian neurones - to selectively knock down expression of individual caspases. This would allow the caspase cascade to be dissected and would provide more information on the pathways activated than is possible with any of the currently available inhibitors.

Finally, it has not yet been demonstrated to what extent inhibition of caspases or JNK is protective in the rat 6-OHDA model of Parkinson's disease, and this study failed to address this. These experiments would be interesting to carry out with more effective JNK inhibitors, and with brain penetrant caspase 3 inhibitors as these become more widely available.

7.3 Final conclusion

The data presented in this study confirm that MPP^+ toxicity for dopaminergic neurones in primary mesencephalic neuronal cultures is through caspase mediated apoptosis. This caspase activation is mediated in part through the JNK pathway, but does not appear to involve the p38 pathway. The data obtained in this simple *in vitro* model provide an insight into the complexity of pathways activated by MPP^+ toxicity, and provide a solid platform for further analysis of the intracellular pathways activated in this PD model; the *in vivo* data indicate that the JNK pathway may also be of interest in the 6-OHDA model, and further work should clarify this.

Reference list

Abramova NA, Cassarino DS, Khan SM, Painter T, Bennett JPJ (2002) Inhibition by R(+) or S(-) pramipexole of caspase activation and cell death induced by methylpyridinium ion or beta amyloid peptide in SH- SY5Y neuroblastoma. J Neurosci Res 67: 494-500.

Adrain C, Martin SJ (2001) The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. Trends Biochem Sci 26: 390-397.

Agid Y, Ruberg M, Raisman R, Hirsch E, Javoy-Agid F (1990) The Biochemistry of Parkinson's Disease. Parkinson's Disease. Ed. G.M. Stern. Chapman and Hall Medical.

Akaneya Y, Takahashi M, and Hatanaka H (1995) Involvement of free radicals in MPP^+ neurotoxicity against rat dopaminergic neurons in culture. Neurosci Lett 193: 53-56.

Alnemri ES, Livingstone DJ, Nicholson DW, Salveson G, Thornberry NA, Wong WW, Yuan J (1996) Human ICE/CED-3 protease nomenclature. Cell 87: 171

Andersen JK (2001) Does neuronal loss in Parkinson's disease involve programmed cell death? Bioessays 23: 640-646

Anderson AJ, Su JH, Cotman CW (1996) DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. J Neurosci 16: 1710-1719

Anglade P, Vyas S, Javoy-Agid F, Herrero MT, Michel PP, Marquez J, Mouatt-Prigent A, Ruberg M, Hirsch EC, Agid Y (1997) Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. Histol Histopathol 12: 25-31

Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R, Martinou JC (1997) Inhibition of Bax channel-forming activity by Bcl-2. Science 277: 370-372.

Ashwell S (2001) Caspases: recent advances in small molecule inhibitors. Exp Opin Ther Pat 11: 1593-1603.

Avery L, Horvitz HR (1987) A cell that dies during wild-type C. elegans development can function as a neuron in a ced-3 mutant. Cell 51: 1071-1078

Ballard PA, Tetrud JW, Langston JW (1985) Permanent human parkinsonism due to 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP): seven cases. Neurology 35: 949-956.

Banati RB, Daniel SE, Blunt SB (1998) Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. Mov Disord 13: 221-227

Beck KD, Knusel B, Pasinetti G, Michel PP, Zawadzka H, Goldstein M, Hefti F (1991) Tyrosine hydroxylase mRNA expression by dopaminergic neurons in culture: effect of 1-methyl-4-phenylpyridinium treatment. J Neurochem 57: 527-532.

Beck KD, Knusel B, Winslow JW, Rosenthal A, Burton LE, Nikolics K, Hefti F (1992) Pretreatment of dopaminergic neurons in culture with brain-derived neurotrophic factor attenuates toxicity of 1-methyl-4-phenylpyridinium. Neurodegeneration 1: 27-36.

Behrens A, Sibilia M, Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. Nat Genet 21: 326-329

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 98: 13681-13686

Betarbet R, Sherer TB, Greenamyre JT (2002) Animal models of Parkinson's disease. Bioessays. 24: 308-318.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3: 1301-1306.

Bilsland J, Rigby M, Young L, Harper S (1999) A rapid method for semi-quantitative analysis of neurite outgrowth from chick DRG explants using image analysis. J Neurosci Meth 92: 75-85.

Bilsland J, Roy S, Xanthoudakis S, Nicholson DW, Han Y, X, Grimm E, Hefti F, Harper SJ (2002) Caspase inhibitors attenuate 1-methyl-4-phenylpyridinium toxicity in primary cultures of mesencephalic dopaminergic neurons. J Neurosci 22: 2637 -2649.

Bilsland J, Harper S (2003) CEP-1347 promotes survival of NGF responsive neurones in primary DRG explants. Neuroreport (in press)

Blum D, Torch S, Lambeng N, Nissou M, Benabid AL, Sadoul R, Verna J (2001) Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. Prog Neurobiol 65: 135-172.

Bocquet A, Lorent G, Fuks B, Grimée R, Talaga P, Daliers J, Klitgaard H (2001) Failure of GPI compounds to display neurotrophic activity *in vitro* and *in vivo*. Eur J Pharmacol 415: 173-180.

Borasio GD, Horstmann S, Anneser JM, Neff NT, Glicksman MA (1998) CEP-1347/KT7515, a JNK pathway inhibitor, supports the *in vitro* survival of chick embryonic neurons. Neuroreport 9: 1435-1439

Bossy Wetzel E, Newmeyer DD, Green DR (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J 17: 37-49.

Bottenstein JE, Sato GE (1979) Growth of a rat neuroblastoma cell line in serum free supplemented medium. Proc Natl Acad Sci USA 76: 514-517.

Boyce S, Kelly E, Reavill C, Jenner, P, and Marsden CD (1984) Repeated administration of N-methyl-4-phenyl 1,2,5,6- tetrahydropyridine to rats is not toxic to striatal dopamine neurones. Biochem Pharmacol 33, 1747-1752.

Bradbury AJ, Costall B, Domeney AM, Jenner P, Kelly ME, Marsden C, and Naylor RJ (1986) 1-methyl-4-phenylpyridine is neurotoxic to the nigrostriatal dopamine pathway. Nature 319, 56-57.

Bratton SB, Cohen GM (2001) Apoptotic death sensor: an organelle's alter ego? Trends Pharmacol 22: 306-315.

Braun JS, Tuomanen EI, Cleveland JL (1999) Neuroprotection by caspase inhibitors. Exp Opin Invest Drugs 8: 1599-1610

Brecht S, Simler S, Vergnes M, Mielke K, Marescaux C, Herdegen T (1999) Repetitive electroconvulsive seizures induce activity of c-Jun N-terminal kinase and compartment-specific desensitization of c-Jun phosphorylation in the rat brain. Brain Res Mol Brain Res 68: 101-108

Brewer GJ, Cotman CW (1989) Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. Brain Res 494: 65-74

Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin I (1983) A primate model of Parkinsonism: selective destruction of dopaminergic neurons in the pars

compacta of the substantia nigra by N-methyl-4- phenyl-1,2,3,6-tetrahydropyridine. Proc Natl Acad Sci 80: 4546-4550.

Butterworth NJ, Williams L, Bullock JY, Love DR, Faull RL, Dragunow M (1998) Trinucleotide (CAG) repeat length is positively correlated with the degree of DNA fragmentation in Huntington's disease striatum. Neuroscience 87: 49-53

Cai J, Yang J, Jones DP (1998) Mitochondrial control of apoptosis: the role of cytochrome c. Biochim Biophys Acta 1366: 139-149.

Cain K, Bratton SB, Cohen GM (2002) The Apaf-1 apoptosome: a large caspase-activating complex. Biochimie 84: 203-214

Carboni L, Tacconi S, Carletti R, Bettini E, Ferraguti F (1997) Localization of the messenger RNA for the c-Jun NH2-terminal kinase kinase in the adult and developing rat brain: An in situ hybridization study. Neuroscience 80: 147-160.

Carboni L, Carletti R, Tacconi S, Corti C, Ferraguti F (1998) Differential expression of SAPK isoforms in the rat brain. An in situ hybridisation study in the adult rat brain and during post-natal development. Mol Brain Res 60: 57-68.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC (1998) Regulation of cell death protease caspase-9 by phosphorylation. Science. 282: 1318-1321.

Carletti R, Tacconi S, Bettini E, Ferraguti F (1995) Stress activated protein kinases, a novel family of mitogen-activated protein kinases, are heterogeneously expressed in the adult rat brain and differentially distributed from extracellular-signal-regulated protein. Neuroscience 69: 1103-1110.

Carrasco E, Werner P (2002) Selective destruction of dopaminergic neurons by low concentrations of 6-OHDA and MPP(+): protection by acetylsalicylic acid aspirin. Parkinsonism Relat Disord. 8: 407-411.

Cassarino DS, Fall CP, Smith TS, Bennett JP Jr (1998) Pramipexole reduces reactive oxygen species production *in vivo* and *in vitro* and inhibits the mitochondrial permeability transition produced by the parkinsonian neurotoxin methylpyridinium ion. J Neurochem 71: 295-301

Cassarino DS, Parks JK, Parker WDJ, Bennett JPJ (1999) The Parkinsonian neurotoxin MPP^+ opens the mitochondrial permeability transition pore and releases cytochrome c in isolated mitochondria via an oxidative mechanism. Biochim Biophys Acta 1453: 49-62.

Cecconi F, Alvarez Bolado G, Meyer BI, Roth KA, Gruss P (1998) Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell 94, 727-737.

Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, Van Ness K, Greenstreet TA, March CJ, Kronheim SR, Druck T, Cannizzaro LA, Huebner K, Black RA (1002) Molecular cloning of the interleukin-1 beta converting enzyme. Science 256: 97-100

Chalmers-Redman RM, Fraser AD, Carlile GW, Pong A, Tatton WG (1999) Glucose protection from MPP+-induced apoptosis depends on mitochondrial membrane potential and ATP synthase. Biochem Biophys Res Commun 257: 440-447

Chaudhary P, Ahmed F, Quebada P, Sharma SC (1999) Caspase inhibitors block the retinal ganglion cell death following optic nerve transection. Brain Res Mol Brain Res 67: 36-45

Che Y, Yu YM, Han PL, Lee JK (2001) Delayed induction of p38 MAPKs in reactive astrocytes in the brain of mice after KA-induced seizure. Brain Res Mol Brain Res 94: 157-165

Chen JY, Hsu PC, Hsu IL, Yeh GC (2001) Sequential up-regulation of the c-fos, c-jun and bax genes in the cortex, striatum and cerebellum induced by a single injection of a low dose of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in C57BL/6 mice. Neurosci Lett 314: 49-52.

Chen M, He H, Zhan S, Krajewski S, Reed JC, Gottlieb RA (2001) Bid is cleaved by calpain to an active fragment *in vitro* and during myocardial ischemia/reperfusion. J Biol Chem 276: 30724-30728.

Chen M, Won DJ, Krajewski S, Gottlieb RA (2002) Calpain and mitochondria in ischemia/reperfusion injury. J Biol Chem 277: 29181-29186.

Cheng Y, Deshmukh M, DCosta A, Demaro JA, Gidday JM, Shah A, Sun YL, Jacquin MF, Johnson EM, Holtzman DM (1998) Caspase inhibitor affords neuroprotection with

delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. J Clin Invest 101: 1992-1999.

Chera B, Schaecher KE, Rocchini A, Imam SZ, Ray SK, Ali SF, Banik NL (2002) Calpain upregulation and neuron death in spinal cord of MPTP-induced parkinsonism in mice. Ann N Y Acad Sci 965: 274-80.

Chinnaiyan AM, O Rourke K, Tewari M, Dixit VM (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81: 505-512.

Chinnaiyan AM, Orth K, O'Rourke K, Duan H, Poirier GG, Dixit VM (1996) Molecular ordering of the cell death pathway. Bcl-2 and Bcl-xL function upstream of the CED-3-like apoptotic proteases. J Biol Chem 271: 4573-4576

Choi DW (1996) Ischemia-induced neuronal apoptosis. Curr Opin Neurobiol 6: 667-672

Choi WS, Lee EH, Chung CW, Jung YK, Jin BK, Kim SU, Oh TH, Saido TC, Oh YJ (2001) Cleavage of Bax is mediated by caspase-dependent or –independent calpain activation in dopaminergic neuronal cells: protective role of Bcl-2. J Neurochem 77: 1531-1541.

Choi WS, Yoon SY, Oh TH, Choi EJ, O'Malley KL, Oh YJ (1999) Two distinct mechanisms are involved in 6-hydroxydopamine- and MPP^+- induced dopaminergic neuronal cell death: role of caspases, ROS, and JNK. J Neurosci Res 57: 86-94.

Chun HS, Gibson GE, DeGiorgio LA, Zhang H, Kidd VJ, Son JH (2001) Dopaminergic cell death induced by MPP^+, oxidant and specific neurotoxicants shares the common molecular mechanism. J Neurochem 76: 1010-1021.

Cobb MH (1999) MAP kinase pathways. Prog Biophys Mol Biol 71: 479-500

Collins MA, Neafsey EJ (2002) Potential neurotoxic "agents provocateurs" in Parkinson's disease. Neurotoxicol Teratol 24: 571-577.

Crocker SJ, Lamba WR, Anisman H, Slack RS, Grimm E, Han Y, Harper S, Bilsland J, Merali Z, Robertson GS, Park DS (2001) Calpain inhibition prevents MPTP-induced dopamine neuron loss and behavioural impairment in mice. Soc Neurosci Abstr 27: 749.17

Crocker SJ, Wigle N, Liston P, Thompson CS, Lee CJ, Xu D, Roy S, Nicholson DW, Park DS, MacKenzie A, Korneluk RG, Robertson GS (2001a) NAIP protects the nigrostriatal dopamine pathway in an intrastriatal 6-OHDA rat model of Parkinson's disease. Eur J Neurosci. 14: 391-400.

Crocker SJ, Lamba WR, Smith PD, Callaghan SM, Slack RS, Anisman H, Park DS (2001b) c-Jun mediates axotomy-induced dopamine neuron death *in vivo*. Proc Natl Acad Sci USA. 98:13385-13390.

Cutillas B, Espejo M, Gil J, Ferrer I, Ambrosio S (1999) Caspase inhibition protects nigral neurons against 6-OHDA-induced retrograde degeneration. Neuroreport 10: 2605-2608.

Darmon AJ, Ehrman N, Caputo A, Fujinaga J, and Bleackley RC (1994) The cytotoxic T cell proteinase granzyme B does not activate interleukin-1 beta-converting enzyme. J Biol Chem 269: 32043-32046.

Darmon AJ, Nicholson DW, and Bleackley RC (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell- derived granzyme B. Nature 377: 446-448.

Deckwerth TL, Adams LM, Wiessner C, Allegrini P, Rudin M, Sauter A, Hengerer B, Sayers RO, Rovelli G, Aja T, May R, Nalley K, Linton S, Karanewsky DS, Wu JC, Roggo S, Schmitz A, Contreras PC, Tomaselli KJ (2001) Long-term protection of brain tissue from cerebral ischemia by peripherally administered peptidomimetic caspase inhibitors. Drug Devel Res 52: 579-586.

De-Girolamo L-A, Hargreaves AJ, Billett EE (2001) Protection from MPTP-induced neurotoxicity in differentiating mouse N2a neuroblastoma cells. J Neurochem 76: 650-660.

Degli EM (1998) Inhibitors of NADH-ubiquinone reductase: an overview. Biochimica et Biophysica. Acta. 1364: 222-235.

Denner L (1999) Caspases in apoptotic death. Exp Opin Invest Drugs 8: 37-50.

Dipasquale B, Marini AM, Youle RJ (1991) Apoptosis and DNA degradation induced by 1-methyl-4-phenylpyridinium in neurones. Biochem Biophys Res Comm 137: 1442-1448

Dodel RC, Du Y, Bales KR, Ling ZD, Carvey PM, Paul SM (1998) Peptide inhibitors of caspase-3-like proteases attenuate 1- methyl-4- phenylpyridinum-induced toxicity of cultured fetal rat mesencephalic dopamine neurons. Neuroscience 86: 701-707.

Dodel RC, Du Y, Bales KR, Ling Z, Carvey PM, Paul SM (1999) Caspase-3-like proteases and 6-hydroxydopamine induced neuronal cell death. Brain Res Mol Brain Res 64: 141-148.

Dragunow M, Beilharz E, Sirimanne E, Lawlor P, Williams C, Bravo R, Gluckman P (1994) Immediate-early gene protein expression in neurons undergoing delayed death, but not necrosis, following hypoxic-ischaemic injury to the young rat brain. Brain Res Mol Brain Res 25: 19-33

Dragunow M, Young D, Hughes P, MacGibbon G, Lawlor P, Singleton K, Sirimanne E, Beilharz E, Gluckman P (1993) Is c-Jun involved in nerve cell death following status epilepticus and hypoxic-ischaemic brain injury? Brain Res Mol Brain Res 18: 347-352

Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a Mitochondrial Protein that Promotes Cytochrome c–Dependent Caspase Activation by Eliminating IAP Inhibition. Cell 102: 43-53

Du Y, Dodel RC, Bales KR, Jemmerson R, Hamilton Byrd E, Paul SM (1997) Involvement of a caspase-3-like cysteine protease in 1-methyl-4- phenylpyridinium-mediated apoptosis of cultured cerebellar granule neurons. J Neurochem 69: 1382-1388.

Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, Triarhou LC, Chernet, Perry KW, Nelson DL, Luecke S, Phebus LA, Bymaster FP, Paul S (2001) Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proc Natl Acad Sci USA 98: 14669-14674.

Eberhardt O, Coelln R, V, Kugler S, Lindenau J, Rathke HS, Gerhardt E, Haid S, Isenmann S, Gravel C, Srinivasan A, Bahr M, Weller M, Dichgans J, Schulz JB (2000) Protection by synergistic effects of adenovirus-mediated X-chromosome- linked inhibitor of apoptosis and glial cell line-derived neurotrophic factor gene transfer in the 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine model of Parkinson's disease. J Neurosci 20: 9126-9134.

Eberling JL, Pivirotto P, Bringas J, Steiner JP, Kordower JH, Chu Y, Emborg ME, Bankiewicz KS (2002) The immunophilin ligand GPI-1046 does not have neuroregenerative effects in MPTP-treated monkeys. Exp Neurol 178: 236-242.

Eilers A, Whitfield J, Babij C, Rubin LL, Ham J (1998) Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic neurons. J Neurosci 18:1713-1724

Eilers A, Whitfield J, Shah B, Spadoni C, Desmond H, Ham J. (2001) Direct inhibition of c-jun N-terminal kinase in sympathetic neurones prevents c-jun promoter activation and NGF withdrawal-induced death. J Neurochem 76: 1439-1454

Enari M, Hug H, Nagata S (1995) Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature 375: 78-81.

Enari M, Talanian RV, Wong WW, Nagata S (1996) Sequential activation of ICE-like and CPP32-like proteases during Fas- mediated apoptosis. Nature 380: 723-726.

Enslen H, Raingeaud J, Davis RJ (1998) Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J Biol Chem 273: 1741-1748.

Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R, Johnson EM Jr. (1994) Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J Cell Biol 127: 1717-1727

Fall CP, Bennett Jr JP (1998) MPP^+ induced SH-SY5Y apoptosis is potentiated by cyclosporin A and inhibited by aristolochic acid. Brain Res 811: 143-146

Fan D, Ogawa M, Ikeguchi K, Fujimoto K, Urabe M, Kume A, Nishizawa M, Matsushita N, Kiuchi K, Ichinose H, Nagatsu T, Kurtzman GJ, Nakano I, Ozawa K (1998) Prevention of dopaminergic neuron death by adeno-associated virus-vector mediated GDNF gene transfer in rat mesencephalic cells *in vitro*. Neurosci Lett 248: 61-64

Fawcett JW, Barker RA, Dunnett SB (1995) Dopaminergic neuronal survival and the effects of bFGF in explant, three dimensional and monolayer cultures of embryonic rat ventral mesencephalon. Exp Brain Res 106: 275-282.

Feng ZH, Wang TG, Li DD, Fung P, Wilson BC, Liu B, Ali SF, Langenbach R, Hong JS (2002) Cyclooxygenase-2-deficient mice are resistant to 1-methyl-4-phenyl1, 2, 3, 6-

tetrahydropyridine-induced damage of dopaminergic neurons in the substantia nigra. Neurosci Lett 329: 354-358.

Fernandes Alnemri T, Litwack G, and Alnemri ES (1994) CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta- converting enzyme. J Biol Chem 269, 30761-30764.

Fernandes Alnemri T, Litwack G, and Alnemri ES (1995) Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. Cancer Res 55: 2737-2742.

Ferrer I, Blanco R, Carmona M, Puig B, Barrachina M, Gómez C, Ambrosio S (2001) Active, phosphorylation-dependent mitogen-activated protein kinase (MAPK/ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p38 kinase expression in Parkinson's disease and Dementia with Lewy bodies. J Neur Trans 108: 1383-1396.

Ferrer I, Planas AM, Pozas E (1997) Radiation-induced apoptosis in developing rats and kainic acid-induced excitotoxicity in adult rats are associated with distinctive morphological and biochemical c-Jun/AP-1 (N) expression. Neuroscience 80: 449-458.

Fink K, Zhu JM, Namura S, ShimizuSasamata M, Endres M, Ma JY, Dalkara- T, Yuan JY, Moskowitz MA (1998) Prolonged therapeutic window for ischemic brain damage caused by delayed caspase activation. J Cer Blood Flow Met 18: 1071-1076.

Friedlander RM, Gagliardini V, Hara H, Fink KB, Li W, Macdonald G, Fishman MC, Greenberg AH, Moskowitz MA, Yuan J (1997) Expression of a dominant negative

mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. J Exp Med 185: 933-940.

Fujita E, Jinbo A, Matuzaki H, Konishi H, Kikkawa U, Momoi T (1999) Akt phosphorylation site found in human caspase-9 is absent in mouse caspase-9. Biochem Biophys Res Commun. 264: 550-555.

Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, and Thornberry NA (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem: 32608-32613.

Gearan T, Castillo OA, Schwarzschild MA (2001) The parkinsonian neurotoxin, MPP^+ induces phosphorylated c-Jun dopaminergic neurones of mesencephalic cultures. Parkinsonism and Related Disorders 8: 19-22.

Gerlach G, Riederer P, Przuntek H, Youdim MBH (1991) MPTP mechanisms of toxicity and their implications for Parkinson's disease. Eur J Pharmacol 208, 273-286.

Gillardon F, Kiprianova I, Sandkuhler J, Hossmann KA, Spranger M (1999) Inhibition of caspases prevents cell death of hippocampal CA1 neurons, but not impairment of hippocampal long-term potentiation following global ischemia. Neuroscience 93: 1219-1222.

Glicksman MA, Chiu AY, Dionne CA, Harty M, Kaneko M, Murakata C, Oppenheim RW, Prevette D, Sengelaub DR, Vaught JL, Neff NT (1998) CEP-1347/KT7515 prevents

motor neuronal programmed cell death and injury-induced dedifferentiation in vivo. J Neurobiol 35: 361-370

Gómez C, Reiriz J, Piqué M, Gil J, Ferrer I, Ambrosio S (2001) Low concentrations of 1-methyl-4-phenylpyridinium ion induce caspase- mediated apoptosis in human SH-SY5Y neuroblastoma cells. J Neurosci Res 63: 421-428.

Gorman AM, Orrenius S, and Ceccatelli S (1998) Apoptosis in neuronal cells: role of caspases. Neuroreport. 9, 49-55.

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309-1312.

Grutter MG (2000) Caspases: key players in programmed cell death. Curr Opin Struct Biol 10: 649-655

Gunn-Moore FJ, Tavare JM (1998) Apoptosis of cerebellar granule cells induced by serum withdrawal, glutamate or β-amyloid, is independent of Jun kinase or p38 mitogen activated protein kinase activation. Neurosci Lett 250: 53-56

Guo X, Dawson VL, Dawson TM (2001) Neuroimmunophilin ligands exert neuroregeneration and neuroprotection in midbrain dopaminergic neurons. Eur J Neurosci 13: 1683-1693.

Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard B, Davis RJ (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 15: 2760-2770.

Hadjiconstantinou M, Fitkin JG, Dalia A, Neff NH (1991) Epidermal growth factor enhances striatal dopaminergic parameters in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mouse. J Neurochem 57: 479-482

Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS, Elia A, de la Pompa JL, Kagi D, Khoo W, Potter J, Yoshida R, Kaufman SA, Lowe SW, Penninger JM, and Mak TW (1998) Differential requirement for caspase 9 in apoptotic pathways *in vivo*. Cell 94, 339-352.

Hallman H, Lange J, Olson L, Stromberg I, Jonsson G (1985) Neurochemical and histochemical characterization of neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on brain catecholamine neurones in the mouse. J Neurochem 44: 117-127.

Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL (1995) A c-jun dominant negative mutant protects sympathetic neurones against programmed cell death. Neuron 14: 927-939

Han BH, Xu D, Choi J, Han Y, Xanthoudakis S, Roy S, Tam J, Vaillancourt J, Colucci J, Siman R, Giroux A, Robertson GS, Zamboni R, Nicholson DW, Holtzmann DM (2002) Selective, reversible caspase-3 inhibitor is neuroprotective and reveals distinct pathways of cell death after neonatal hypoxic-ischemic brain injury. J Biol Chem 277: 30128-30136

Hara H, Friedlander RM, Gagliardini V, Ayata C, Fink K, Huang ZH, Shimizu Sasamata M, Yuan JY, Moskowitz MA (1997a) Inhibition of interleukin 1 beta converting enzyme

family proteases reduces ischemic and excitotoxic neuronal damage. Proc Natl Acad Sci USA 94: 2007-2012.

Hara H, Fink K, Endres M, Friedlander RM, Gagliardini V, Yuan JY, Moskowitz MA (1997b) Attenuation of transient focal cerebral ischemic injury in transgenic mice expressing a mutant ICE inhibitory protein. J Cer Blood Flow Met 17: 370-375.

Harada J, Sugimoto M (1998) Inhibitors of interleukin-1 beta-converting enzyme-family proteases (caspases) prevent apoptosis without affecting decreased cellular ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in cerebellar granule neurons. Brain Res 793: 231-243.

Harada J, Sugimoto M (1999) An inhibitor of p38 and JNK MAP kinases prevents activation of caspase and apoptosis of cultured cerebellar granule neurons. Jpn J Pharmacol 79: 369-378

Harding TC, Xue L, Bienemann A, Haywood D, Dickens M, Tolkovsky AM, Uney JB (2001) Inhibition of JNK by overexpression of the JNL binding domain of JIP-1 prevents apoptosis in sympathetic neurons. J Biol Chem 276: 4531-4534

Harper S, Bilsland J, Young L, Bristow L , Boyce S, Mason G, Rigby M, Hewson L, Smith D, O'Donnell R, O'Connor D, Hill RG, Evans D, Swain C, Williams B, Hefti F (1999) Analysis of the neurotrophic effects of GPI-1046 on neuron survival and regeneration in culture and *in vivo*. Neuroscience 88: 257-267.

Harper SJ, Lograsso P (2001) Signalling for survival and death in neurones: the role of stress-activated kinases, JNK and p38. Cell Signal 13: 299-310.

Harper SJ, Saporito MS, Hewson L, Young L, Smith D, Rigby M, Jackson P, Curtis N, Swain C, Hefti F, Vaught JL, Sirinathsinghji D (2000) CEP-1347 increases ChAT activity in culture and promotes cholinergic neurone survival following fimbria-fornix lesion. NeuroReport 11: 2271-2276

Harris CA, Johnson EMJ (2001) BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. J Biol Chem 276: 37754-37760.

Hartmann A, Hunot E, Michel P, Muriel M, Vyas S, Faucheux B, Mouatt-Prigent A, Turmel H, Srinivasan A, Ruberg M, Evan G, Agid Y, Hirsch E (2000) Caspase 3: a vulnerability factor and a final effector in the apoptotic death of dopaminergic neurones in Parkinson's Disease. Proc Natl Acad Sci USA 97: 2875-2880

Hartmann A, Michel PP, Troadec JD, Mouatt-Prigent A, Faucheux BA, Ruberg M, Agid Y, Hirsch EC (2001) Is Bax a mitochondrial mediator in apoptotic death of dopaminergic neurons in Parkinson's disease? J Neurochem 76: 1785-1793

Hartmann A, Troadec JD, Hunot S, Kikly K, Faucheux BA, Mouatt, Ruberg M, Agid Y, Hirsch EC (2001) Caspase-8 is an effector in apoptotic death of dopaminergic neurons in Parkinson's disease, but pathway inhibition results in neuronal necrosis. J Neurosci 21: 2247-2255.

Hashimoto M, Hsu LJ, Rockenstein E, Takenouchi T, Mallory M, Masliah E (2002) alpha-Synuclein protects against oxidative stress via inactivation of the c-Jun N-terminal kinase stress-signaling pathway in neuronal cells. J Biol Chem 277: 11465-11472

Hassouna I, Wickert H, Zimmermann M, Gillardon F (1996) Increase in bax expression in substantia nigra following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment of mice. Neurosci Lett 204: 85-88

Hayashi T, Sakai K, Sasaki C, Zhang WR, Warita H, Abe K (2000) c-Jun N-terminal kinase (JNK) and JNK interacting protein response in rat brain after transient middle cerebral artery occlusion. Neurosci Lett 284: 195-199

Hefti F, Melamed E, Sahakian BJ, Wurtman RJ (1980) Circling behavior in rats with partial, unilateral nigro-striatal lesions: effect of amphetamine, apomorphine, and DOPA. Pharmacol Biochem Behav 12: 185-188.

Hefti F, Araujo D, Beck KD, Knusel B, Lapchak PA, Michel PP, Ohsawa F (1993). Experimental systems to study neurotrophic factor effects on rat brain cells. Neuromethods Vol. 25: Neurotrophic factors Eds. A. Boulton, G. Baker and F. Hefti. Humana Press.

Heikkila RE, Hess A, Duvoisin RC (1984) Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6- tetrahydropyridine in mice. Science 224: 1451-1453.

Heikkila RE, Nicklas WJ, Vyas I, Duvoisin RC (1985) Dopaminergic toxicity of rotenone and the 1-methyl-4-phenylpyridinium ion after their stereotaxic administration

to rats: implication for the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity. Neurosci Lett. 62: 389-394.

Hengartner MO, Ellis RE, Horvitz HR (1992) Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. Nature 356: 494-499

Hengartner MO, Horvitz HR (1994) Activation of C. elegans cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. Nature 369: 318-320

Hengartner MO, Horvitz HR (1994) C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell 76: 665-676

Herdegen T, Claret FX, Kallunki T, Martin-Villalba A, Winter C, Hunter T, Karin M (1998) Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. J Neurosci 18: 5124-5135

Hirsch EC, Hunot S, Faucheux B, Agid Y, Mizuno Y, Mochizuki H, Tatton WG, Tatton N, Olanow WC (1999) Dopaminergic neurons degenerate by apoptosis in Parkinson's disease. Mov Disord 14: 383-385

Holtzman DM, Deshmukh M (1997) Caspases: A treatment target for neurodegenerative disease? Nat Med 3: 954-955.

Hommes DW, Peppelenbosch, MP, van Deventer SJH (2003) Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. Gut 52: 144-151

Horger BA, Nishimura MC, Armanini MP, Wang L-C, Poulsen, KT Rosenblad C, Kirik D, Moffat B, Simmons L, Johnson Jr E, Millbrandt J, Rosenthal A, Bjorklund A, Vandlen RA, Hynes MA, Phillips HS (1998) Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurones. J Neurosci 18: 4929-4937

Horstmann S, Kahle PJ, Borasio GD (1998) Inhibitors of p38 mitogen-activated protein kinase promote neuronal survival *in vitro*. J Neurosci Res 52: 483-490

Horvitz HR, Sternberg PW, Greenwald IS, Fixsen W, Ellis HM (1983) Mutations that affect neural cell lineages and cell fates during the development of the nematode Caenorhabditis elegans. Cold Spring Harb Symp Quant Biol 48: 453-463

Hotchkiss RS, Chang KC, Swanson PE, Tinsley KW, Hui JJ, Klender, Xanthoudakis S, Roy S, Black C, Grimm E, Aspiotis R, Han Y, Nicholson DW, Karl IE (2000) Caspase inhibitors improve survival in sepsis: a critical role of the lymphocyte. Nat Immunol 1: 496-501.

Hou JG, Lin LF, Mytilineou C (1996) Glial cell line-derived neurotrophic factor exerts neurotrophic effects on dopaminergic neurons *in vitro* and promotes their survival and regrowth after damage by 1-methyl-4-phenylpyridinium. J Neurochem 66: 74-82.

Ichas F, Mazat JP (1998) From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high- conductance state. Biochim Biophys Act -Bioenergetics 1366: 33-50.

Jackson-Lewis V, Jakowec M, Burke RE, Przedborski S (1995) Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurodegen 4: 257-269.

Jacobson MD (1997) Apoptosis: Bcl-2-related proteins get connected. Curr Biol 7: 277-281.

Jacobson MD, Weil M, Raff MC (1997) Programmed cell death in animal development. Cell 88: 347-354.

Jeon SH, Kim YS, Bae CD, Park JB (2000) Activation of JNK and p38 in rat hippocampus after kainic acid induced seizure. Exp Mol Med 32: 227-230

Jordan J, Galindo MF, Miller RJ (1997) Role of calpain- and interleukin-1 beta converting enzyme-like proteases in the beta-amyloid-induced death of rat hippocampal neurons in culture. J Neurochem 68: 1612-1621

Kaneko M, Saito Y, Saito H, Matsumoto T, Matsuda Y, Vaught JL, Dionne CA, Angeles TS, Glicksman MA, Neff NT, Rotella DP, Kauer JC, Mallamo JP, Hudkins RL, Murakata C (1997) Neurotrophic 3,9-bis[(alkylthio)methyl]-and-bis(alkoxymethyl)-K-252a derivatives. J Med Chem 40: 1863-1869.

Kang CD, Jang JH, Kim KW, Lee HJ, Jeong CS, Kim CM, Kim SH, Chung BS (1998) Activation of c-jun N-terminal kinase/stress-activated protein kinase and the decreased ratio of Bcl-2 to Bax are associated with the auto- oxidized dopamine-induced apoptosis in PC12 cells. Neuroscience 256: 37-40.

Kawasaki H, Morooka T, Shimohama S, Kimura J, Hirano T, Gotoh Y, Nishida E (1997) Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. J Biol Chem 272: 18518-18521

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-257.

Kiessling M, Stumm G, Xie Y, Herdegen T, Aguzzi A, Bravo R, Gass P (1993) Differential transcription and translation of immediate early genes in the gerbil hippocampus after transient global ischemia. J Cereb Blood Flow Metab 13: 914-924

Kim HE, Yoon SY, Lee JE, Choi WS, Jin BK, Oh TH, Markelonis GJ, Chun SY, Oh YJ (2001) MPP(+) downregulates mitochondrially encoded gene transcripts and their activities in dopaminergic neuronal cells: protective role of Bcl-2. Biochem Biophys Res Commun 286: 659-665

Kinemuchi H, Arai Y, Toyoshima Y (1985) Participation of brain monoamine oxidase B form in the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: relationship between the enzyme inhibition and the neurotoxicity. Neurosci Lett 58: 195-200

King TD, Bijur GN, Jope RS (2001) Caspase-3 activation induced by inhibition of mitochondrial complex I is facilitated by glycogen synthase kinase-3beta and attenuated by lithium. Brain Res 919: 106-114.

Klettner A, Baumgrass R, Zhang Y, Fischer G, Burger E, Herdegen T, Mielke K (2001) The neuroprotective actions of FK506 binding protein ligands: neuronal survival is

triggered by de novo RNA synthesis, but is independent of inhibition of JNK and calcineurin. Brain Res Mol Brain Res 97: 21-31.

Klevenyi P, Andreassen O, Ferrante RJ, Schleicher JRJ, Friedlander RM, Beal MF (1999) Transgenic mice expressing a dominant negative mutant interleukin-1beta converting enzyme show resistance to MPTP neurotoxicity. Neuroreport 10: 635-638.

Kluck RM, Bossy Wetzel E, Green DR, Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis (see comments). Science 275, 1132-1136.

Kuan CY, Yang DD, Roy D-RS, Davis RJ, Rakic P, Flavell RA (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. Neuron 22: 667-676.

Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P, Flavell RA (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell 94, 325-337.

Kummer JL, Rao PK, Heidenreich KA (1997) Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. J Biol Chem 272: 20490-20494

Kyriakis JM, Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81: 807-869

Langston JW, Ballard P (1984) Parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): implications for treatment and the pathogenesis of Parkinson's disease. Can J Neurol Sci 11: 160-165.

Langston JW, Ballard P, Tetrud JW, Irwin I (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219: 979-980.

Langston JW, Forno LS, Rebert CS, Irwin I (1984a) Selective nigral toxicity after systemic administration of 1-methyl-4- phenyl-1,2,5,6-tetrahydropyrine (MPTP) in the squirrel monkey. Brain Res 292: 390-394.

Langston JW, Irwin I, Langston EB, Forno LS (1984b) 1-Methyl-4-phenylpyridinium ion (MPP^+): identification of a metabolite of MPTP, a toxin selective to the substantia nigra. Neurosci Lett 48: 87-92.

Langston JW, Irwin I (1986) MPTP: current concepts and controversies. Clin Neuropharmacol 9: 485-507.

Langston JW, Irwin I, Langston EB, Forno LS (1984c) Pargyline prevents MPTP-induced parkinsonism in primates. Science 225: 1480-1482.

Lawler S, Fleming Y, Goedert M, Cohen P (1998) Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases *in vitro*. Curr Biol 8: 1387-1390.

Leah JD, Herdegen T, Murashov A, Dragunow M, Bravo R (1993) Expression of immediate early gene proteins following axotomy and inhibition of axonal transport in the rat central nervous system. Neuroscience 57: 53-66.

Lee D, Long SA, Adams JL, Chan G, Vaidya KS, Francis TA, Kikly K, Winkler JD, Sung C-M, Debouck C, Richardson S, Levy MA, Dewolf Jr WE, Keller PM, Tomaszek T, Head MS, Ryan MD, Haltiwanger RC, Liang P-H, Jansen CA, McDevitt PJ, Johanson K, Concha NO, Chan W, Abdel-Meguid SS, Badger AM, Lark MW, Nadeau DP, Suva LJ, Gowen M, Nutall ME (2000) Potent and selective nonpeptide inhibitors of caspases 3 and 7 inhibit apoptosis and maintain cell functionality. J Biol Chem 275: 16007-16014

Leist M, Jaattela M (2001) Four deaths and a funeral: From caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2: 589-598.

Le-Niculescu H, Bonfoco E, Kasuya Y, Claret FX, Green DR, Karin M (1999) Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. Mol Cell Biol 19: 751-763.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91: 479-489.

Li WP, Chan WY, Lai HW, Yew DT (1997) Terminal dUTP nick end labeling (TUNEL) positive cells in the different regions of the brain in normal aging and Alzheimer patients. J Mol Neurosci 8: 75-82

Lisnock JM, Griffin P, Calaycay J, Frantz B, Parsons J, O'Keefe SJ, LoGrasso P (2000) Activation of JNK3alpha1 requires both MKK4 and MKK7: Kinetic characterization of *in vitro* phosphorylated JNK3alpha1. Biochemistry 39: 3141-3148.

Liu XH, Kwon D, Schielke GP, Yang GY, Silverstein FS, Barks J-DE (1999) Mice deficient in interleukin-1 converting enzyme are resistant to neonatal hypoxic-ischemic brain damage. J Cer Blood Flow Met 19: 1099-1108.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86: 147-157.

Liverton NJ, Butcher JW, Claiborne CF, Claremon DA, Libby BE, Nguyen KT, Pitzenberger SM, Selnick HG, Smith GR, Tebben A, Vacca JP, Varga SL, Agarwal L, Dancheck K, Forsyth AJ, Fletcher DS, Frantz B, Hanlon WA, Harper CW, Hofsess SJ, Kostura M, Lin J, Luell S, O'Neill EA, Orevillo CJ, Pang M, Parsons J, Rolando A, Sahly Y, Visco DM, O'Keefe SJ. (1999) Design and synthesis of potent, selective and orally bioavailable tetrasubstituted imidazole inhibitors of p38 mitogen-activated protein kinase. J Med Chem 42: 2180-2190

Loddick SA, MacKenzie A, Rothwell NJ (1996) An ICE inhibitor, (Z)-VAD-DCB attenuates ischaemic brain damage in the rat. Neuroreport 7: 1465-1468.

Loetscher H, Niederhauser O, Kemp J, Gill R (2001) Is caspase-3 inhibition a valid therapeutic strategy in cerebral ischemia? Drug Discovery Today 6: 671-680.

Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc Natl Acad Sci USA 90: 7951-7955

Los M, Van de Craen M, Penning LC, Schenk H, Westendorp M, Baeuerle PA, Droge W, Krammer PH, Fiers W, Schulze-Osthoff K (1995) Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis. Nature 375: 81-83.

Lotharius J, Dugan LL, Malley KL (1999) Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. J Neurosci 19: 1284-1293.

Luo Y, Umegaki H, Wang X, Abe R, Roth GS (1998) Dopamine induces apoptosis through an oxidation-involved SAPK/JNK activation pathway. J Biol Chem 273: 3756-3764.

Ma J, Endres M, Moskowitz MA (1998) Synergistic effects of caspase inhibitors and MK-801 in brain injury after transient focal cerebral ischaemia in mice. Br J Pharmacol 124: 756-762.

MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston E (1993) Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. Neurosci Lett 164: 89-92

Margolin N, Raybuck SA, Wilson KP, Chen W, Fox T, Gu Y, Livingston DJ (1997) Substrate and inhibitor specificity of interleukin-1 beta- converting enzyme and related caspases. J Biol Chem 272: 7223-7228.

Markey SP, Johannessen JN, Chiueh CC, Burns RS, Herkenham MA (1984) Intraneuronal generation of a pyridinium metabolite may cause drug- induced parkinsonism. Nature 311: 464-467.

Maroney AC, Finn JP, Connors TJ, Durkin JT, Angeles T, Gessner G, Xu Z, Meyer SL, Savage MJ, Greene LA, Scott RW, Vaught JL (2001) CEP-1347 (KT7515), A synthetic inhibitor of the Mixed Lineage Kinase family. J Biol Chem 276: 25302-25308.

Marsden CD (1990) Neurophysiology. Parkinson's Disease. Ed. G.M. Stern. Chapman and Hall Medical.

Martin LJ, Price AC, Kaiser A, Shaikh AY, Liu Z (2000) Mechanisms for neuronal degeneration in amyotrophic lateral sclerosis and in models of motor neuron death. Int J Mol Med 5: 3-13

Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, Takeda A, Sagara Y, Sisk A, Mucke L (2000) Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science 287: 1265-1269.

Mattson MP, Lovell MA, Furukawa K, Markesbery WR (1995) Neurotrophic factors attenuate glutamate-induced accumulation of peroxides, elevation of intracellular Ca2+ concentration, and neurotoxicity and increase antioxidant enzyme activities in hippocampal neurons. J Neurochem. 65: 1740-1751.

McCollum AT, Nasr P, Estus S (2002) Calpain activates caspase-3 during UV-induced neuronal death but only calpain is necessary for death. J Neurochem 82: 1208-1220.

McGinty A, Chang YW, Sorokin A, Bokemeyer D, Dunn MJ (2000) Cyclooxygenase-2 expression inhibits trophic withdrawal apoptosis in nerve growth factor-differentiated PC12 cells. J Biol Chem. 275: 12095-12101.

Menalled LB, Chesselet MF (2002). Mouse models of Huntington's disease. Trends Pharmacol Sci. 23: 32-39.

Michel PP, Dandapani BK, Knusel B, Sanchez Ramos J, Hefti F (1990) Toxicity of 1-methyl-4-phenylpyridinium for rat dopaminergic neurons in culture: selectivity and irreversibility. J Neurochem 54: 1102-1109.

Michel PP, Dandapani BV, Sanchez-Ramos J, Efange S, Pressman BC, Hefti F. (1989) Toxic effects of potential environmental toxins related to 1-methyl-4-phenylpyridinium on cultured rat dopaminergic neurons. J Pharmacol Exp Ther 248: 842-850

Mielke K, Brecht S, Dorst A, Herdegen T (1999) Activity and expression of JNK1, p38 and ERK kinases, c-Jun N-terminal phosphorylation, and *c-jun* promotor binding in the adult rat brain following kainate-induced seizures. Neuroscience 91: 471-483

Mielke K, Damm A, Yang DD, Herdegen T (2000) Selective expression of JNK isoforms and stress-specific JNK activity in different neural cell lines. Mol Brain Res 75: 128-137

Mielke K, Herdegen T (2000) JNK and p38 stresskinases - degenerative effectors of signal- transduction-cascades in the nervous system. Prog Neurobiol 61: 45-60.

Minden A, Karin M. Regulation and function of the JNK subgroup of MAP kinases (1997) Biochim Biophys Acta. 1333: 85-104

Mochizuki H, Goto K, Mori H, Mizuno Y (1996) Histochemical detection of apoptosis in Parkinson's disease. J Neurol Sci 37: 120-123

Mochizuki H, Hayakawa H, Migita M, Shibata M, Tanaka R, Suzuki A, Shimo-Nakanishi Y, Urabe T, Yamada M, Tamayose K, Shimada T, Miura M, Mizuno Y (2001) An AAV-derived Apaf-1 dominant negative inhibitor prevents MPTP toxicity as antiapoptotic gene therapy for Parkinson's disease. Proc Natl Acad Sci USA 98: 10918-10923

Mochizuki H, Nakamura N, Nishi K, Mizuno Y (1994) Apoptosis is induced by 1-methyl-4-phenylpyridinium ion (MPP^+) in ventral mesencephalic-striatal co-culture in rat. Neurosci Lett 170: 191-194.

Moore JD, Rothwell NJ, Gibson RM (2002) Involvement of caspases and calpains in cerebrocortical neuronal cell death is stimulus-dependent. Br J Pharmacol 135: 1069-1077

Morishima Y, Gotoh Y, Zieg J, Barrett T, Takano H, Flavell R, Davis R, Shirasaki Y, Greenberg ME (2001) Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. J Neurosci 21: 7551-7560.

Munday NA, Vaillancourt JP, Ali A, Casano FJ, Miller DK, Molineaux SM, Yamin TT, Yu VL, Nicholson DW (1995) Molecular cloning and pro-apoptotic activity of ICErelII and ICErel III, members of the ICE/CED-3 family of cysteine proteases. J Biol Chem 270: 15870-15876.

Murakata C, Kaneko M, Gessner G, Angeles TS, Ator MA, O'Kane TM, McKenna BA, Thomas BA, Mathiasen JR, Saporito MS, Bozyczko-Coyne D, Hudkins RL (2002) Mixed lineage kinase activity of indolocarbazole analogues. Bioorg Med Chem Lett 12: 147-150

Muzio M, Salvesen GS, and Dixit VM (1997) FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. J Biol Chem 272: 2952-2956

Mytilineou C, Cohen G (1984) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine destroys dopamine neurons in explants of rat embryo mesencephalon. Science 225: 529-531.

Mytilineou C, Cohen G, Heikkila RE (1985) 1-Methyl-4-phenylpyridine (MPP^+) is toxic to mesencephalic dopamine neurons in culture. Neurosci Lett 57: 19-24.

Nakagawa T, Yuan J (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol 150: 887-894.

Namgung U and Xia Z. Arsenite induced apoptosis in cortical neurones is mediated by c-Jun N-terminal protein kinase 3 and p38 mitogen-activated protein kinase. J Neurosci 20: 6442-6451

Nath R, Probert A Jr, McGinnis KM, Wang KK (1998) Evidence for activation of caspase-3-like protease in excitotoxin- and hypoxia/hypoglycemia-injured neurons. J Neurochem 71: 186-195

Neame SJ, Rubin LL, Philpott KL (1998) Blocking cytochrome c activity within intact neurons inhibits apoptosis. J Cell Biol 142: 1583-1593.

New L, Han J (1998) The p38 MAP kinase pathway and its biological function. Trends Cardiovasc Med 8: 220-228.

Ni B, Wu X, Du Y, Su Y, Hamilton-Byrd E, Rockey PK, Rosteck Jr P, Poirer GG, Paul SM (1997) Cloning and expression of a rat brain interleukin-1β-converting enzyme (ICE)-related protease (IRP) and its possible role in apoptosis of cultured cerebellar granule neurones. J Neurosci 17: 1561-1569

Nicholson DW, Thornberry NA (1997) Caspases: killer proteases. Trends Biochem Sci 22: 299-306.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt, JP, Ding C, Gallant M, Gareau Y, Griffin PR, Labelle M, and Lazebnik YA (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376: 37-43.

Nicotera P, Leist M, Fava E, Berliocchi L, Volbracht C (2000) Energy requirement for caspase activation and neuronal cell death. Brain Pathol 10: 276-282.

Nicotra A, Parves SH. (2000) Cell death induced by MPTP, a substrate for monoamine oxidase B. Toxicol 153: 157-166.

Nishi K (1997) Expression of c-Jun in dopaminergic neurons of the substantia nigra in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. Brain Res 771: 133-141.

Ochu EE, Rothwell NJ, Waters CM (1998) Caspases mediate 6-hydroxydopamine-induced apoptosis but not necrosis in PC12 cells. J Neurochem 70: 2637-2640.

Offen D, Beart PM, Cheung NS, Pascoe CJ, Hochman A, Gorodin S, Melamed E, Bernard R, Bernard O (1998) Transgenic mice expressing human Bcl-2 in their neurons are resistant to 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine neurotoxicity. Proc Natl Acad Sci USA 95: 5789-5794.

Olanow CW, Tatton WC (1999) Etiology and pathogenesis of Parkinson's disease. Annu Rev Neurosci 23: 123-44

Oliver KR, Heavens RP, Sirinathsinghji DJ (1997) Quantitative comparison of pretreatment regimens used to sensitize in situ hybridization using oligonucleotide probes on paraffin-embedded brain tissue. J Histochem Cytochem 45: 1707-1713.

Oo TF, Henchcliffe C, James D, Burke RE. (1999) Expression of c-fos, c-jun, and c-jun N-terminal kinase (JNK) in a developmental model of induced apoptotic death in neurons of the substantia nigra. J Neurochem 72: 557-564.

Otto D, Unsicker K (1990) Basic FGF reverses chemical and morphological deficits in the nigrostriatal system of MPTP-treated mice. J Neurosci 10: 1912-1921

Pan G, Humke EW, Dixit VM (1998) Activation of caspases triggered by cytochrome c *in vitro* (published erratum appears in FEBS Lett 1998 May 29; 428(3): 309). FEBS Lett 426: 151-154.

Park H, Lee J, Huh S, Seo J, Choi E (2001) Hsp-72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. EMBO J 20: 446-456

Paroni G, Henderson C, Schneider C, Brancolini C (2001) Caspase-2-induced apoptosis is dependent on caspase-9, but its processing during UV- or tumor necrosis factor-dependent cell death requires caspase-3. J Biol Chem 276: 21907-21915

Petronilli V, Penzo D, Scorrano L, Bernardi P, Di Lisa F (2001) The mitochondrial permeability transition, release of cytochrome c and cell death - Correlation with the duration of pore openings in situ. J Biol Chem 276: 12030-12034.

Pifl C, Giros B, and Caron MG (1993) Dopamine transporter expression confers cytotoxicity to low doses of the parkinsonism-inducing neurotoxin 1-methyl-4-phenylpyridinium. J Neurosci 13: 4246-4253.

Pirvola U, Xin-Qun L, Virkkala J, Saarma M, Murakata C, Camoratto AM, Walton KM, Ylikoski J (2000) Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. J Neurosci 20: 43-50

Rabuffetti M, Sciorati C, Tarozzo G, Clementi E, Manfredi AA, Beltramo M (2000) Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone induces long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines. J Neurosci 20: 4398-4404.

Rano TA, Timkey T, Peterson EP, Rotonda J, Nicholson DW, Becker JW, Chapman KT, Thornberry NA (1997) A combinatorial approach for determining protease specificities: application to interleukin-1beta converting enzyme (ICE). Chem Biol 4: 149-155.

Ransom BR, Kunis DM, Irwin I, Langston JW (1987) Astrocytes convert the parkinsonism inducing neurotoxin, MPTP, to its active metabolite, MPP^+. Neuroscience 75: 323-328

Raymon HK, Celeridad MT, Sakata ST, Bennett BL, Satoh Y, Bhagwat SS, Manning AM (2000) The JNK inhibitor SPC0009766 reverses neurotoxin-induced damage in cultures of rat dopaminergic neurons. Soc Neurosci Abstr 26: 700.10

Reed JC (1997) Double identity for proteins of the Bcl-2 family. Nature 387: 773-776.

Ricaurte GA, Langston JW, DeLanney LE, Irwin I, Brooks JD (1985) Dopamine uptake blockers protect against the dopamine depleting effect of 1-methyl-4-phenyl-tetrahydropyridine (MPTP) in the mouse striatum. Neurosci Lett 59: 259-264

Rincon M, Flavell R, Davis RA (2000) The JNK and p38 MAP kinase signaling pathways in T cell-mediated immune responses. Free Radical Biol Medicine 28: 1328-1337

Ross DT, Guo H, Howorth P, Chen Y, Hamilton GS, Steiner JP (2001) The small molecule FKBP ligand GPI 1046 induces partial striatal re- innervation after intranigral 6-hydroxydopamine lesion in rats. Neurosci Lett 297: 113-116.

Sahgal A, Andrews JS, Biggins JA, Candy JM, Edwardson JA, Keith A, Turner JD, Wright C (1984) N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) affects locomotor activity without producing a nigrostriatal lesion in the rat. Neurosci Lett 48: 179-184.

Salvesen GS and Dixit VM (1997) Caspases: Intracellular signaling by proteolysis. Cell 91, 434-446

Sanchez Ramos J, Barrett JN, Goldstein M, Weiner WJ, Hefti F (1986) 1-Methyl-4-phenylpyridinium (MPP^+) but not 1-methyl-4-phenyl-1,2, 3,6- tetrahydropyridine (MPTP) selectively destroys dopaminergic neurons in cultures of dissociated rat mesencephalic neurons. Neurosci Lett 72: 215-220.

Sanchez Ramos JR, Michel P, Weiner WJ, Hefti F (1988) Selective destruction of cultured dopaminergic neurons from fetal rat mesencephalon by 1-methyl-4-phenylpyridinium: cytochemical and morphological evidence. J Neurochem 50: 1934-1944.

Saporito MS, Brown EM, Miller MS, Carswell S (1999) CEP-1347/KT-7515, an inhibitor of c-jun N-terminal kinase activation, attenuates the 1-methyl-4-phenyl tetrahydropyridine-mediated loss of nigrostriatal dopaminergic neurons *in vivo*. J Pharmacol Exp Ther 288: 421-427.

Saporito MS, Thomas BA, Scott RW (2000) MPTP activates c-Jun NH2-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons *in vivo*. J Neurochem 75: 1200-1208.

Schapira AH (1998) Human complex I defects in neurodegenerative diseases. Biochim Biophys Acta 1364: 261-270

Schauwecker PE (2000) Seizure-induced neuronal death is associated with induction of c-Jun N-terminal kinase and is dependent on genetic background. Brain Res 884: 116-128

Schendel SL, Montal M, Reed JC (1998) Bcl-2 family proteins as ion-channels. Cell Death Diff 5: 372-380.

Schielke GP, Yang GY, Shivers BD, Betz AL (1998) Reduced ischemic brain injury in interleukin-1 beta converting enzyme- deficient mice. J Cer Blood Flow Met 18: 180-185.

Schierle GS, Hansson O, Leist M, Nicotera P, Widner H, Brundin P (1999) Caspase inhibition reduces apoptosis and increases survival of nigral transplants. Nat Med 5: 97-100

Schlegel J, Peters I, Orrenius S, Miller DK, Thornberry NA, Yamin TT, Nicholson DW (1996) CPP32/apopain is a key interleukin 1 beta converting enzyme-like protease involved in Fas-mediated apoptosis. J Biol Chem 271: 1841-1844.

Schmidt CJ, Matsuda LA, and Gibb JW (1984) *In vitro* release of tritiated monoamines from rat CNS tissue by the neurotoxic compound 1-methyl-phenyl-tetrahydropyridine. Eur J Pharmacol 103: 255-260.

Schulz JB, Gerhardt E (2001) Apoptosis: its relevance to Parkinson's disease. Clin Neur Res 1: 427-433

Schwab BL, Guerini D, Didszun C, Bano D, Ferrando-May E, Fava E, Tam J, Xu D, Xanthoudakis S, Nicholson DW, Carafoli E, Nicotera P (2002) Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis. Cell Death Diff 9: 818-831

Seaton TA, Cooper JM, Schapira AH (1998) Cyclosporin inhibition of apoptosis induced by mitochondrial complex I toxins. Brain Res 809: 14-17

Shimoke K, Chiba H (2001) Nerve growth factor prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced cell death via the Akt pathway by suppressing caspase-3-like activity using PC12 cells: relevance to therapeutical application for Parkinson's disease. J Neurosci Res 63: 402-409

Shimoke K, Kudo M (2002) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine has a transient proliferative effect on PC12h cells and nerve growth factor additively promotes this effect: possible involvement of distinct mechanisms of activation of MAP kinase family proteins. Dev Brain Res 133: 105-114.

Simons M, Beinroth S, Gleichmann M, Liston P, Korneluk RG, MacKenzie AE, Bahr M, Klockgether T, Robertson GS, Weller M, Schulz JB (1999) Adenovirus-mediated gene transfer of inhibitors of apoptosis protein delays apoptosis in cerebellar granule neurons. J Neurochem 72: 292-301

Sirinathsinghji DJ, Whittington PE, Audsley AR (1986) Neurochemical changes in the substantiae nigrae and caudate nuclei following acute unilateral intranigral infusions of N-methyl-4- phenyl-1,2,3,6-tetrahydropyridine (MPTP). Brain Res 399: 339-345.

Srinivasula SM, Ahmad M, Fernandes Alnemri T, Alnemri ES (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell 1: 949-957.

Srinivasula SM, Ahmad M, Fernandes Alnemri T, Litwack G, Alnemri ES (1996) Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. Proc Natl Acad Sci USA 93: 14486-14491.

Srinivasula SM, Datta P, Fan XJ, Fernandes-Alnemri T, Huang Z, Alnemri ES (2000) Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. J Biol Chem. 275: 36152-36157.

Stefanis L, Troy CM, Qi H, Shelanski ML, Greene LA (1998) Caspase-2 (Nedd-2) processing and death of trophic factor-deprived PC12 cells and sympathetic neurons occur independently of caspase-3 (CPP32)-like activity. J Neurosci 18: 9204-9215

Steiner JP, Hamilton GS, Ross DT, Valentine HL, Guo H, Connolly MA, Liang S, Ramsey C, Li JH, Huang W, Howorth P, Soni R, Fuller M, Sauer H, Nowotnik AC, Suzdak PD (1997) Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. Proc Natl Acad Sci U S A. 94: 2019-2024.

Stennicke HR, Salvesen GS (1998) Properties of the caspases. Biochim Biophys Acta 1387, 17-31.

Stennicke HR, Salvesen GS (2000) Caspases - controlling intracellular signals by protease zymogen activation. Biochim Biophys Acta 1477: 299-306

Sundstrom E, Goldstein M, Jonsson G (1986) Uptake inhibition protects nigro-striatal dopamine neurons from the neurotoxicity of 1-methyl-4-phenylpyridine (MPP^+) in mice. Eur J Pharmacol 131, 289-292.

Susin SA, Zamzami N, Kroemer G (1998) Mitochondria as regulators of apoptosis: doubt no more. Biochim Biophys Acta 1366: 151-165.

Swerdlow RH, Parks JK, Miller SW, Tuttle JB, Trimmer PA, Sheehan JP, Bennett Jr JP, Davis RE, Parker Jr. WD (1996) Origin and functional consequences of the complex I defect in Parkinson's disease. Ann Neurol 40: 663-671.

Talanian RV, Quinlan C, Trautz S, Hackett MC, Mankovich JA, Banach D, Ghayur T, Brady KD, Wong WW (1997) Substrate specificities of caspase family proteases. J Biol Chem 272: 9677-9682.

Tatton NA, Kish SJ (1997) In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. Neuroscience 77: 1037-1048.

Tatton WG, Chalmers-Redman RME, Rideout HJ, Tatton NA (1999) Mitochondrial permeability in neuronal death: possible relevance to the pathogenesis of Parkinson's disease. Park Rel Dis 5: 221-229.

Tatton WG, Olanow CW (1999) Apoptosis in neurodegenerative diseases: the role of mitochondria. Biochim Biophys Acta 1410: 195-213

Teismann P, Ferger B (2001) Inhibition of the cyclooxygenase isoenzymes COX-1 and COX-2 provide neuroprotection in the MPTP-mouse model of Parkinson's disease. Synapse. 39: 167-174.

Tewari M, Quan LT, O Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, and Dixit VM (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA- inhibitable protease that cleaves the death substrate poly(ADP- ribose) polymerase. Cell 81: 801-809.

Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, Elliston KO, Ayala JM, Casano FJ, Chin J, Ding GJ.-F, Egger LA, Gaffney EP, Limjuco G, Palyha OC, Raju SM, Rolando AM, Salley JP, Yamin T-T, Lee TD, Shively JE, MacCross M, Mumford RA, Schmidt JA,

Tocci MJ (1992). A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. Nature 356: 768-774

Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia CM, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 272: 17907-17911.

Tolkovsky AM, Xue LZ, Fletcher GC, Borutaite V (2002) Mitochondrial disappearance from cells: a clue to the role of autophagy in programmed cell death and disease? Biochimie 84: 233-240.

Tompkins MM, Basgall EJ, Zamrini E, Hill WD (1997) Apoptotic-like changes in Lewy-body-associated disorders and normal aging in substantia nigral neurons. Am J Pathol 150: 119-131

Touzani O, Boutin H, LeFeuvre R, Parker L, Miller A, Luheshi G, Rothwell N (2002) Interleukin-1 influences ischemic brain damage in the mouse independently of the interleukin-1 type I receptor. J Neurosci 22: 38-43.

Troy CM, Rabacchi SA, Friedman WJ, Frappier TF, Brown K, Shelanski ML (2000) Caspase-2 mediates neuronal cell death induced by beta-amyloid. J Neurosci 20: 1386-1392

Turmel H, Hartmann A, Parain K, Douhou A, Srinivasan A, Agid Y, Hirsch EC (2001) Caspase-3 activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. Mov Disord 16: 185-189.

Usha R, Muralikrishnan D, Thomas B, Ghosh S, Mandal C, Mohanakumar KP (2000) Region-specific attenuation of a trypsin-like protease in substantia nigra following dopaminergic neurotoxicity by 1-methyl-4-phenyl-1,2, 3,6-tetrahydropyridine. Brain Res 882: 191-195

Vaudano E, Rosenblad C, Björklund A (2001) Injury induced c-Jun expression and phosphorylation in the dopaminergic nigral neurons of the rat: correlation with neuronal death and modulation by glial-cell-line-derived neurotrophic factor. Eur J Neurosci 13: 1-14.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a Mammalian Protein that Promotes Apoptosis by Binding to and Antagonizing IAP Proteins. Cell 102: 43-53

Vila M, Jackson L, V, Vukosavic S, Djaldetti R, Liberatore G, Offen D, Korsmeyer SJ, Przedborski S (2001) Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl- 4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. Proc Natl Acad Sci USA 98: 2837-2842.

Vincenz C, Dixit VM (1997) Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. J Biol Chem 272: 6578-6583.

Viswanath V, Wu Y, Boonplueang R, Chen S, Stevenson FF, Yantiri F, Yang L, Beal MF, Andersen JK (2001) Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease. J Neurosci 21: 9519-9528.

Von Coelln R, Kugler S, Bahr M, Weller M, Dichgans J, Schulz J (2001) Rescue from death but not from functional impairment: caspase inhibition protects dopaminergic cells against 6-hydroxydopamine-induced apoptosis but not against the loss of their terminals. J Neurochem 77: 263-273

Walker N-PC, Talanian R, Brady KD, Dang LC, Bump NJ, Ferenz CR, Franklin S, Ghayur T, Hackett MC (1994) Crystal structure of the cysteine protease interleukin-1-beta- converting enzyme: A (p20/p10)-2 homodimer. Cell 78: 348-352

Walton KM, DiRocco R, Bartlett BA, Koury E, Marcy VR, Jarvis B, Schaefer EM, Bhat RV (1998) Activation of p38MAPK in microglia after ischemia. J Neurochem 70: 1764-1767

Watson A, Eilers A, Lallemand D, Kyriakis J, Rubin LL, Ham J (1998) Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. J Neurosci 18: 751-762

Wei W, Norton DD, Wang X, Kusiak JW (2002) Abeta 17-42 in Alzheimer's disease activates JNK and caspase-8 leading to neuronal apoptosis. Brain 125: 2036-2043

Widmann C, Gerwins P, Johnson NL, Jarpe MB, Johnson GL (1998) MEK kinase 1, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. Mol Cell Biol 18: 2416-2429.

Wiessner C, Sauer D, Alaimo D, Allegrini PR (2000) Protective effect of a caspase inhibitor in models for cerebral ischemia *in vitro* and *in vivo*. Cell Mol Biol 46: 53-62.

Wilson KP, Black-Jo AF, Thomson JA, Kim EE, Griffith JP, Navia MA, Murcko MA, Chambers SP, Aldape RA, Raybuck SA, Livingston DJ (1994) Structure and mechanism of interleukin-1-beta converting enzyme. Nature 370: 272-275

Winter C, Schenkel J, Burger E, Eickmeier C, Zimmermann M, Herdegen T (2000) The immunophilin ligand FK506, but not GPI-1046, protects against neuronal death and inhibits c-Jun expression in the substantia nigra pars compacta following transection of the rat medial forebrain bundle. Brain Res 801: 198-205.

Winter C, Schenkel J, Zimmermann M, Herdegen T (1998) MAP kinase phosphatase 1 is expressed and enhanced by FK506 in surviving mamillary, but not degenerating nigral neurons following axotomy. Brain Res 801: 198-205.

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi DK, Ischiropoulos H, Przedborski S (2002) Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. J Neurosci 22: 1763-1771

Wullner U, Kornhuber J, Weller M, Schulz JB, Loschmann PA, Riederer P, Klockgether T (1999) Cell death and apoptosis regulating proteins in Parkinson's disease--a cautionary note. Acta Neuropathol (Berl) 97: 408-412

Xia XG, Harding T, Weller M, Bieneman A, Uney JB, Schulz JB (2001) Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease. Proc Natl Acad Sci USA 98: 10433-10438.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 70: 1326-1331

Xu DG, Bureau Y, McIntyre DC, Nicholson DW, Liston P, Zhu Y, X, Fong W, Crocker SJ, Korneluk RG, Robertson GS (1999) Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. J Neurosci 19: 5026-5033.

Xu DG, Crocker SJ, Doucet JP, StJean M, Tamai K, Hakim AM, Ikeda JE (1997) Elevation of neuronal expression of NAIP reduces ischemic damage in the rat hippocampus. Nat Med 3: 997-1004.

Xu Z, Maroney AC, Dobrzanski P, Kukekov N, V, Greene LA (2001) The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. Mol Cell Biol 21: 4713-4724.

Xue L, Fletcher GC, Tolkovsky AM (2001) Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis. Curr Biol 11: 361-365.

Xue LZ, Fletcher GC, Tolkovsky AM (1999) Autophagy is activated by apoptotic signalling in sympathetic neurons: An alternative mechanism of death execution. Mol Cell Neurosci 14: 180-198.

Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, Rakic P, Flavell RA (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature 389: 865-870

Yang L, Matthews RT, Schulz JB, Klockgether T, Liao AW, Martinou J, Penney JBJ, Hyman BT, Beal MF (1998) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyride neurotoxicity is attenuated in mice overexpressing Bcl-2. J Neurosci 18: 8145-8152.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked (see comments). Science 275: 1129-1132.

Ylikoski J, Xing-Qun L, Virkkala J, Pirvola U (2001) Blockade of c-jun N-terminal kinase pathway attenuates gentamycin-induced cochlear and vestibular hair cell death. Hear Res 3783: 1-11

Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM Mak TW (1998) Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 94: 739-750.

Yoshinaga N, Murayama T, Nomura Y (1998) Death by a dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium ion (MPP+) and protection by EGF in GH3 cells. Brain Res 794: 137-142.

Yoshinaga N, Murayama T, Nomura Y (2000) Apoptosis induction by a dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium ion (MPP(+)), and inhibition by epidermal growth factor in GH3 cells. Biochem Pharmacol 60: 111-120.

Young L, Bilsland J and Harper S (1999) A rapid method for determination of cell survival in primary neuronal DRG cultures. J Neurosci Meth 93: 81-89

Yuan JY, Horvitz HR (1990) The Caenorhabditis elegans genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. Dev Biol 138: 33-41

Yuan J, Horvitz HR (1992) The Caenorhabditis elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. Development 116: 309-320

Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR (1993) The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 75: 641-652.

Zawada WM, Meintzer MK, Rao P, Marotti J, Wang X, Esplen JE, Clarkson ED, Freed CR, Heidenreich KA (2001) Inhibitors of p38 MAP kinase increase the survival of transplanted dopamine neurons. Brain Res 891: 185-196

Zhang C, Steiner JP, Hamilton GS, Hicks TP, Poulter MO (2001) Regeneration of dopaminergic function in 6-hydroxydopamine-lesioned rats by neuroimmunophilin ligand treatment. J Neurosci 21: RC156.

Zhou Q, Snipas S, Orth K, Muzio M, Dixit VM, Salvesen GS (1997) Target protease specificity of the viral serpin CrmA: Analysis of five caspases. J Biol Chem 272: 7797-7800

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90: 405-413.

Zuch CL, Nordstroem VK, Briedrick LA, Hoernig GR, Granholm AC, Bickford PC (2000) Time course of degenerative alterations in nigral dopaminergic neurons following a 6-hydroxydopamine lesion. J Comp Neurol 427: 440-454.

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Caspase Inhibitors Attenuate 1-Methyl-4-Phenylpyridinium Toxicity in Primary Cultures of Mesencephalic Dopaminergic Neurons

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Parkinson's disease is characterized by a loss of dopaminergic nigrostriatal neurons. This neuronal loss is mimicked by the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ toxicity is mediated through inhibition of mitochondrial complex I, decreasing ATP production, and upregulation of oxygen radicals. There is evidence that the cell death induced by MPP^+ is apoptotic and that inhibition of caspases may be neuroprotective. In primary cultures of rat mesencephalic dopaminergic neurons, MPP^+ treatment decreased the number of surviving dopaminergic neurons in the cultures and the ability of the neurons to take up [^{3}H]dopamine ([^{3}H]DA). Caspase inhibition using the broad-spectrum inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) spared MPP^+-treated dopaminergic neurons and increased somatic size. There was a partial restoration of neurite length in zVAD-fmk-treated cultures, but little restoration of [^{3}H]DA uptake. Peptide inhibitors of caspases 2, 3, and 9, but not of caspase 1, caused significant neuroprotection. Two novel caspase inhibitors were tested for neuroprotection, a broad spectrum inhibitor and a selective caspase 3 inhibitor; both inhibitors increased survival to >90% of control. No neuroprotection was observed with an inactive control compound. MPP^+ treatment caused chromatin condensation in dopaminergic neurons and increased expression of activated caspase 3. Inhibition of caspases with either zVAD-fmk or a selective caspase 3 inhibitor decreased the number of apoptotic profiles, but not expression of the active caspase. We conclude that MPP^+ toxicity in primary dopaminergic neurons involves activation of a pathway terminating in caspase 3 activation, but that other mechanisms may underlie the neurite loss.

Key words: Parkinson's disease; apoptosis; MPP^+; caspase; neuroprotection; dopaminergic neurons

Parkinson's disease is a neurodegenerative condition characterized by rigidity and akinesia. A major pathological hallmark of Parkinson's disease is the degeneration of nigrostriatal dopaminergic neurons (Marsden, 1990), which is mimicked *in vivo* by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The toxicity of MPTP is mediated through the toxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+). The mechanism by which MPP^+ kills dopaminergic neurons is unclear. MPP^+ is known to inhibit mitochondrial complex I, decreasing cellular metabolism and increasing generation of oxygen radicals (Akaneya et al., 1995; Degli, 1998; Schapira, 1998). Evidence has emerged recently that MPP^+ treatment may lead to apoptosis.

After MPTP or MPP^+ treatment, apoptotic nuclei have been detected *in vivo* (Tatton and Kish, 1997) and *in vitro* (Mochizuki et al., 1994, Dodel et al., 1998; Eberhardt et al., 2000). Transgenic mice overexpressing anti-apoptotic Bcl-2 are resistant to MPP^+ toxicity *in vitro* and MPTP toxicity *in vivo* (Offen et al., 1998, Yang et al., 1998). Inhibition of caspases, mediators of the apoptotic response, has been reported to prevent MPP^+-mediated cell death *in vitro* (Du et al., 1997; Dodel et al., 1998). Mice overexpressing dominant negative caspase 1 have been shown to be resistant to MPTP toxicity *in vivo* (Klevenyi et al., 1999), and activation of caspases 3, 8, and 2 has been reported in the substantia nigra of MPTP-treated mice (Yang et al., 1998; Hartmann et al., 2001; Turmel et al., 2001). Both caspase inhibition and overexpression of inhibitor of apoptosis protein (IAP) have been shown to protect dopaminergic neurons from MPP^+ *in vivo* and *in vitro* (Eberhardt et al., 2000).

Although these data indicate that MPP^+ toxicity is mediated by caspase activation and subsequent apoptosis, reports conflict regarding the mechanism of MPP^+ toxicity *in vitro* and the efficacy of caspase inhibition. Lotharius and coworkers (1999) found no evidence of phosphatidylserine externalization, a marker of apoptosis, after MPP^+ treatment of mesencephalic neurons, and they reported that the toxicity was not inhibited by treatment with a broad-spectrum caspase inhibitor. Hartmann and coworkers (2001) reported that caspase inhibition potentiated MPP^+-mediated cell death *in vitro* by increasing necrosis, unless neurons were maintained in elevated glucose levels.

Thus, the mechanism of MPP^+ toxicity *in vitro*, and the role of caspases, is unclear. In this study we have tested a number of peptide caspase inhibitors for neuroprotective effects against MPP^+ toxicity in rat mesencephalic dopaminergic neurons *in vitro*, together with two novel caspase inhibitors and an inactive analog. MPP^+-treated dopaminergic neurons show apoptotic profiles and express activated caspase 3. Caspase inhibition restores the number of surviving dopaminergic neurons and increases somatic size and neurite length in these neurons but is less effective in restoring [^{3}H]DA uptake. Broad-spectrum caspase inhibitors caused survival of dopaminergic neurons to >90% of untreated control, as did a novel caspase 3 inhibitor. These data suggest that the pathways activated by MPP^+ in this culture system converge during caspase 3 activation and that

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inhibition of caspase 3 is sufficient to prevent the MPP^+-mediated death of these neurons.

MATERIALS AND METHODS

Materials. Pregnant Sprague Dawley rats were purchased from Harlan Seralabs. DMEM, HBSS, and trypsin were purchased from Invitrogen (Paisley, UK). Fetal bovine serum (FBS), mazindol, antibiotic/antimycotic solution, Cy-3-conjugated goat anti-rabbit IgG, extravidin-FITC, and tetramethylrhodamine isothiocynate-conjugated anti-rabbit IgG were purchased from Sigma-Aldrich Co. (Poole, UK). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (zDEVD-fmk), benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (zLEHD-fmk), benzyloxycarbonyl-Tyr-Val-Ala-Asp-chloromethylketone (zYVAD-cmk), and the FITC-FragEL apoptosis detection kit were all purchased from Calbiochem/Novabiochem (Nottingham, UK). Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). MPP^+ iodide was purchased from RBI. Vectastain Elite ABC kits, Vector SG insoluble peroxidase substrate, and normal goat serum were obtained from Vector Laboratories (Peterborough, UK). Rabbit polyclonal anti-tyrosine hydroxylase (TH) antiserum was purchased from the Institut Jacques Boy (Reims, France). Mouse monoclonal anti-TH was purchased from Chemicon. Rabbit anti-cleaved caspase 3 was purchased from New England Biolabs. Sato serum substitute (Bottenstein and Sato, 1979) was made in-house (final concentration in medium: 4.3 mg/ml bovine serum albumin, 0.77 μg/ml progesterone, 20 μg/ml putrescine, 0.49 μg/ml L-thyroxine, 0.048 μg/ml selenium, and 0.42 μg/ml tri-iodo-thyronine). All components of this serum substitute were purchased from Sigma-Aldridge Co. [^{3}H]DA was purchased from Amersham Biosciences.

Mesencephalic cultures. The ventral mesencephalon was dissected from 14 d gestation Sprague Dawley rat embryos (Harlan Ltd.). Tissues were incubated with 0.25% trypsin in HBSS for 20 min at 37°C/5% CO_2, then mechanically dissociated using a flame-polished Pasteur pipette. For cell survival assays, cells were plated at a density of 200,000 cells per well onto poly-D-lysine-coated eight-well chamber slides (Invitrogen) in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution and incubated for 2 hr. This medium was then aspirated and replaced with DMEM supplemented with Sato serum substitute. Cultures were incubated for a further 5 d before experimental procedures.

Treatment with compounds. zVAD-fmk, zDEVD-fmk, zLEHD-fmk, and zYVAD-cmk were prepared in DMEM supplemented with Sato and added to the cultures 15 min before MPP^+ exposure at concentrations ranging from 0.1 to 300 μM. Each compound was added to four independent wells at each concentration tested. Control cultures were returned to DMEM/Sato in the absence of compounds. MPP^+ iodide was prepared at a concentration of 110 μM, then added directly to the medium in the wells to give a final concentration in each well of 10 μM; control cultures were treated with tissue culture medium in the absence of MPP^+. Cultures were incubated at 37°C/5% CO_2 for a further 48 hr, then were fixed using 4% paraformaldehyde in PBS and immunostained for TH.

Determination of TH-immunoreactive neuronal survival. To determine the number of surviving dopaminergic neurons, immunocytochemistry was performed using a rabbit polyclonal antibody raised against TH. Nonspecific binding sites were blocked using 10% normal goat serum in PBS, then primary antibody was added at 4°C overnight. The next day, the cells were washed and treated with biotin-conjugated goat anti-rabbit IgG for 1 hr, followed by peroxidase-conjugated avidin–biotin complex, both made up from the Vectastain Elite ABC kit according to the manufacturer's instructions. Staining was visualized using Vector SG insoluble peroxidase substrate according to the manufacturer's instructions. After staining, the gaskets were removed from the chamber slides, and the slides were mounted using aqueous mountant. Slides were blinded by another investigator before quantification of TH-immunoreactive cell survival.

To determine TH-immunoreactive cell survival, cells were observed under transmitted light on a Zeiss Axiovert inverted microscope using a 10× objective. Counts were made of all the TH-immunoreactive cells present in each well. The culture conditions described here typically produce a yield of ~0.5–1% TH-immunoreactive cells, or ~1500 cells in a control well. For each compound tested, three independent experiments were performed, each consisting of four independent wells. Each compound was also tested in the absence of MPP^+ to detect any nonspecific neuroprotective or toxic effects (data not shown).

[^{3}H]DA uptake assays. Primary cultures of mesencephalic dopaminergic neurons were prepared as described above and plated at a density of 2.5×10^5 cells per well in poly-D-lysine-coated 48-well tissue culture clusters. Cultures were maintained for 5 d at 37°C/5%CO_2 in DMEM supplemented with Sato. After 5 d, medium was aspirated and replaced with either MPP^+ at concentrations ranging from 0.01 to 100 μM or with zVAD-fmk at concentrations ranging from 1 to 300 μM in the presence of 1 or 10 μM MPP^+. In both cases compounds were prepared in DMEM/Sato. Four independent wells were treated for each condition in each experiment; three independent experiments were performed for each data point. Cultures were incubated for a further 48 hr, then [^{3}H]DA uptake was evaluated.

To determine [^{3}H]DA uptake, the medium was aspirated from each well and replaced with DMEM supplemented with 5.6 mM glucose, 1.3 mM EDTA, 0.2 mg/ml ascorbic acid, and 0.5 μCi/ml [^{3}H]DA. Control cultures were treated with the above medium with the addition of the dopamine uptake blocker mazindol (10 μM). Cultures were incubated for 30 min, then washed twice and lysed using 95% ethanol at 37°C for 30 min. Lysates were transferred to aqueous scintillant, and the activity was quantified. Results were expressed as percentage of untreated control culture response.

Visualization of apoptotic nuclei. For determination of apoptotic nuclei, cells were plated as described above into eight-well chamber slides. After 5 d *in vitro*, the medium was aspirated and replaced with DMEM/Sato or zVAD-fmk 300 μM. Cultures were returned to the incubator for 15 min, after which MPP^+ iodide was added as described above to give a final concentration in each well of 10 μM. Control cultures were treated with DMEM/Sato only. Cultures were fixed using 4% paraformaldehyde at 24 and 48 hr after MPP^+ exposure and immunostained for TH. This was followed by determination of apoptotic nuclei using the nuclear stain Hoechst 33342 to evaluate chromatin condensation.

Quantification of somatic area and neurite length. Microcomputer imaging device (MCID) image analysis (Brock University, Ontario, Canada) was used to evaluate the somatic area of TH-immunoreactive neurons. Area quantification was made from dopaminergic neurons in one experiment, from untreated control cultures, from cultures treated with 10 μM MPP^+ for 48 hr, and from cultures treated with 10 μM MPP^+ in the presence of 100 or 300 μM zVAD-fmk. One hundred cells were measured from random fields of view throughout each of four wells for each treatment group. To quantify area in micrometers squared, the image analysis system was first calibrated in micrometers using a graticule. The area of immunostained soma were then established using the Autoscan tool. For each neuron, a control density was set outside the area of the stained soma; the stained area of the soma was then established. Neurites were excluded from each measurement. Mean areas for soma within each area were then established, and the results were presented as the mean area across four wells.

For neurite length measurements, MCID image analysis was used to quantify the length of the longest neurite for each of 100 TH-immunoreactive neurons in four wells per treatment group. The image analysis system was calibrated as described above. Neurite length measurements were taken from control cultures, cultures exposed to 10 μM MPP^+ for 48 hr, or cultures exposed to 10 μM MPP^+ in the presence of 300 μM zVAD-fmk. To determine neurite length, a sample tool was used to draw manually along the length of the longest visible neurite. Results were expressed both as mean neurite length for each group and as a percentage of cells with only rudimentary processes; rudimentary processes were defined as being ≤10 μm in length.

Statistical analyses. All statistical analyses that were performed used one-way ANOVA followed by Dunnett's test comparing all groups with cultures treated with 10 μM MPP^+ alone; for control MPP^+ experiments, all groups were compared with untreated control results. Significance was reached at $p < 0.05$.

RESULTS

Toxic effects of MPP^+ on dopaminergic neurons

MPP^+ was added at concentrations ranging from 0.001 to 100 μM to primary cultures of mesencephalic dopaminergic neurons (Fig. 1). Significant decreases in the number of TH-immunoreactive neurons were observed with MPP^+ concentrations of 0.1 μM and above. At 10 μM, MPP^+ reduced the number of surviving TH-immunoreactive neurons to ~50% of control (Fig. 1*A*), and this concentration was selected for further experiments. MPP^+ was more potent at decreasing [^{3}H]DA uptake than at decreasing the number of TH-immunoreactive neurons, reflecting the loss of

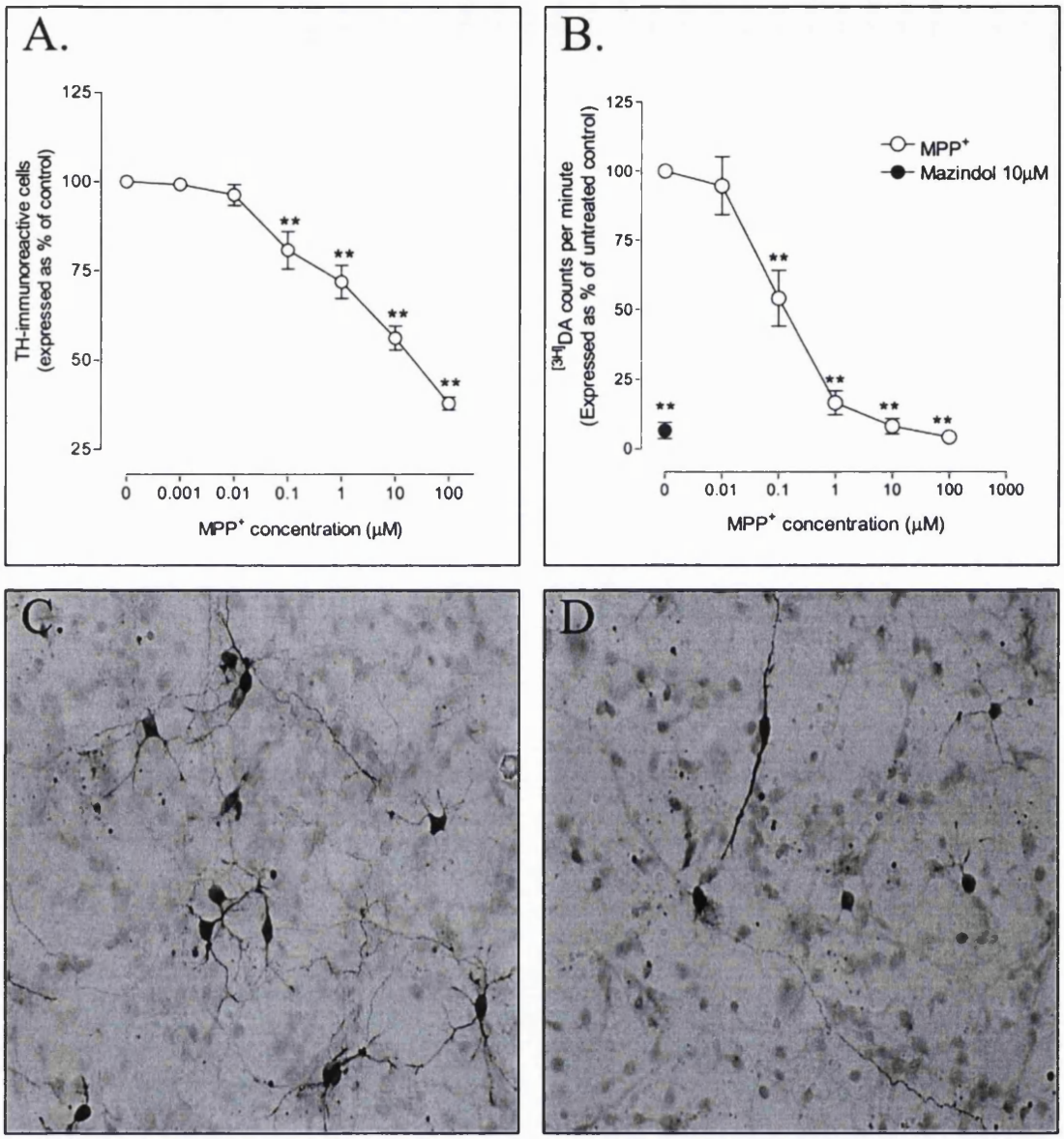
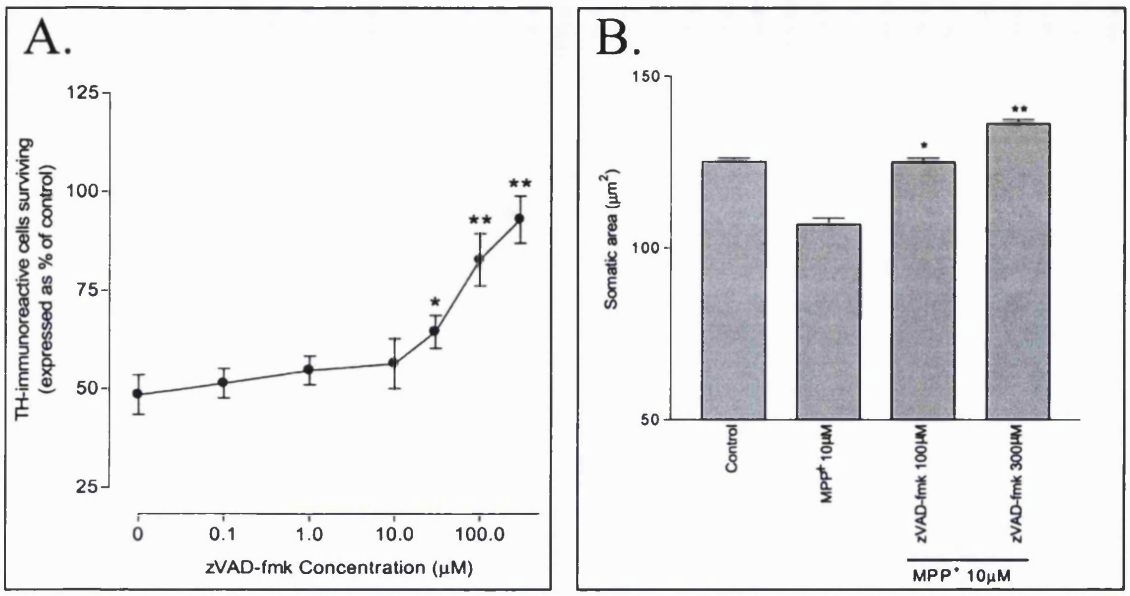


Figure 1. Effects of MPP+ on survival (*A*) and [^{3}H]DA uptake (*B*) in primary cultures of mesencephalic dopaminergic neurons. MPP+ was added at concentrations ranging from 0.01 to 100 μM for 48 hr. Cultures were then either fixed and immunostained for TH, and the surviving TH-immunoreactive cells were counted, or [^{3}H]DA uptake was assayed. Data shown in each case are the mean ± SEM of three independent experiments and are expressed as percentage of untreated control cultures (**$p < 0.01$; established by one-way ANOVA followed by Dunnett's test). Representative photomicrographs of control (*C*) or 10 μM MPP+-treated (*D*) TH-immunoreactive neurons are shown. Cultures were treated for 48 hr, then fixed and immunostained for TH.

dopamine transporter sites on the neurite terminals. Again, significant decreases were observed at 0.1 μM MPP+ and above, but the response was decreased to ~20% of control with MPP+ concentrations of 1 μM and above. Photomicrographs of control cultures (Fig. 1*C*) and cultures treated with 10 μM MPP+ for 48 hr (Fig. 1*D*) show a loss of dopaminergic neurons in the MPP+-treated cultures. The cell bodies of the MPP+-treated TH-immunoreactive neurons are also smaller, and there are fewer neurites.

The broad-spectrum caspase inhibitor zVAD-fmk protects dopaminergic cell bodies against MPP+ toxicity but does not restore [^{3}H]DA uptake

To determine the role of caspases in mediating toxicity of MPP+, we tested the broad-spectrum caspase inhibitor zVAD-fmk for neuroprotective effects. Figure 2 shows the effects of zVAD-fmk on the toxicity induced by 10 μM MPP+. Treatment of cultures with 10 μM MPP+ resulted in a loss of ~50% TH-immunoreactive neurons in the cultures; zVAD-fmk treatment resulted in a concentration-dependent sparing of these neurons, with the maximal effect restoring dopaminergic neuronal number to >90% of control cultures (Fig. 2*A*). Photomicrographs of these cultures are shown in Figure 2*C–E*. Control cultures are shown in Figure 2*C*; Figure 2, *D* and *E*, shows cultures treated with 10 μM MPP+ and 300 μM zVAD-fmk plus 10 μM MPP+, respectively. The cultures treated with MPP+ alone have reduced numbers of dopaminergic neurons, and those surviving neurons have smaller cell bodies. Speckled staining is apparent around the neurons, which may reflect the remains of degenerated neurites. In the cultures



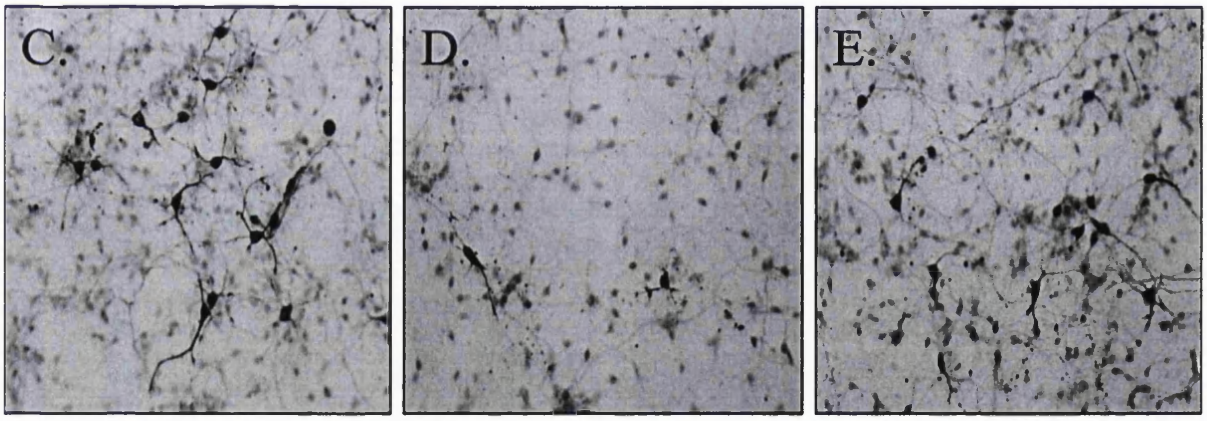


Figure 2. zVAD-fmk attenuates 10 μM MPP^+ toxicity and increases somatic size in mesencephalic dopaminergic neurons. For survival quantification (*A*), cultures were exposed to 10 μM MPP^+ for 48 hr in the presence of various zVAD-fmk concentrations. Cultures were then fixed and immunostained for TH. Slides were blinded, and TH-immunoreactive cells were counted. Data points shown are from three independent experiments, each consisting of four independent wells, and are expressed as percentage of untreated control cultures ($^*p < 0.05$, $^{**}p < 0.01$; established by one-way ANOVA followed by Dunnett's test). Somatic size measurements (*B*) were made from each of four wells from one representative experiment. Random fields of view were visualized using MCID image analysis, and densitometry was used to establish the area occupied by the soma of TH-immunoreactive neurons. One hundred cells per well were measured for each data point. Photomicrographs of control (*C*), 10 μM MPP^+-treated (*D*), and 10 μM MPP^+- and 300 μM zVAD-fmk-treated (*E*) mesencephalic cultures are shown. Cultures were immunostained for TH, and representative photomicrographs were taken.

treated with both MPP^+ and zVAD-fmk, there is a restoration of cell number; those neurons remaining have larger cell bodies, and a restoration of neurite number can also be seen, although some speckled staining is also apparent that may reflect a loss or remodeling of neurites.

Quantification of the somatic area of the dopaminergic neurons is shown in Figure 2*B*. Treatment with 10 μM MPP^+ resulted in a significant decrease in the somatic area of the surviving TH-immunoreactive neurons. This decrease in somatic area was attenuated by treatment with zVAD-fmk at 100 and 300 μM. At 300 μM zVAD-fmk, the somatic area was significantly greater than that observed in control cultures. The effects of zVAD-fmk on MPP^+-mediated neurite loss and the decrease in [^{3}H]DA uptake are shown in Figure 3. To establish whether caspase inhibition could increase [^{3}H]DA uptake in MPP^+-treated primary mesencephalic cultures, zVAD-fmk was coadministered with MPP^+ concentrations of either 1 or 10 μM (Fig. 3*A*). zVAD-fmk was tested at concentrations ranging from 1 to 300 μM. The results for both MPP^+ concentrations show a significant increase in [^{3}H]DA uptake only with a zVAD-fmk concentration of 300 μM. The increase observed was relatively small in comparison with the increases observed with counts of TH-immunoreactive neurons, indicating that those neurons spared by caspase inhibition may be compromised in their ability to take up [^{3}H]DA. This limited effect may be mediated by degeneration of neurites in the dopaminergic neurons. MPP^+ treatment causes a marked decrease in

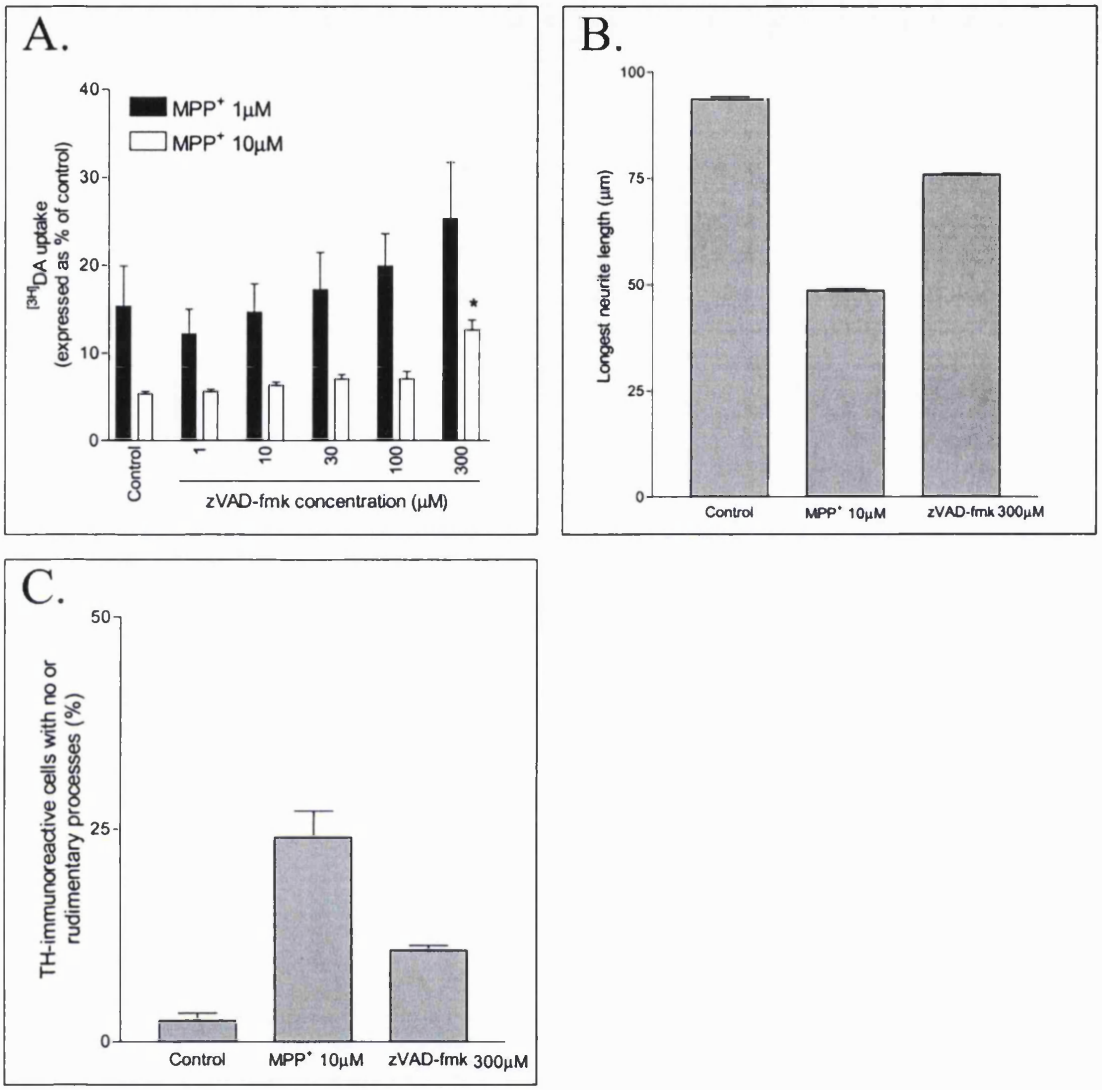


Figure 3. Effects of zVAD-fmk on [^{3}H]DA uptake in primary mesencephalic dopaminergic neurons exposed to 1 or 10 μM MPP$^+$ and effects of 300 μM zVAD-fmk treatment on neurite length of dopaminergic neurons. For [^{3}H]DA uptake assays (Fig. 3*A*), cultures were exposed to 1 or 10 μM MPP$^+$ in the presence of various concentrations of zVAD-fmk for 48 hr, and then the ability of the cells to take up [^{3}H]DA was assayed. Each data point is the mean ± SEM of three independent experiments, each consisting of four independent wells, and is expressed as percentage of untreated control cultures (*$p < 0.05$, **$p < 0.01$; established by one-way ANOVA followed by Dunnett's test). Neurite length measurements were made from TH-immunoreactive neurons in control cultures, cultures exposed to 10 μM MPP$^+$ for 48 hr, and cultures treated for 48 hr with MPP$^+$ and 300 μM zVAD-fmk. MCID image analysis was used to quantify the length of the longest neurite in each of 100 TH-immunoreactive neurons in four independent wells per treatment group. *B* shows the mean neurite length of TH-immunoreactive neurons. *C* shows the percentage of TH-immunoreactive cells in each treatment group with no, or only rudimentary, neurites; this was defined as a longest process of <10 μm in length.

neurite length, which is only partially restored by zVAD-fmk treatment (Fig. 3*B*). Similarly, MPP$^+$ caused an increase in the percentage of neurons with no or rudimentary processes (Fig. 3*C*), and this was only partially restored by 300 μM zVAD-fmk. Thus, the limited effects of zVAD-fmk in restoring [^{3}H]DA uptake are likely to be attributable to a degeneration of processes and thus of dopamine transporter sites.

Peptide inhibitors of caspases 2, 3, and 9, but not of caspase 1, partially protect dopaminergic neurons from MPP$^+$ toxicity

The effects of a range of peptide inhibitors based on the preferred cleavage sites of specific caspases are shown in Figure 4. Four specific inhibitors were tested: zDEVD-fmk, zVDVAD-fmk, zLEHD-fmk, and zYVAD-cmk. These inhibitors are based on the cleavage sites of caspases 3, 2, 9, and 1, respectively, and act by binding to and inhibiting the respective enzymes. Although zVDVAD-fmk is an inhibitor based on the preferred cleavage site for caspase 2, it is unlikely to be absolutely specific for caspase 2. The presence of an Asp residue in the P4 position of the inhibitor is a requirement for peptide inhibitors of caspases 3 and 7, and the VDVAD sequence has also been shown to inhibit these enzymes (Thornberry et al., 1997). Thus, the neuroprotection observed with this inhibitor may be attributable in part to an inhibition of caspase 3. Neither zLEHD-fmk nor zYVAD-cmk is likely to significantly inhibit caspase 3-like proteases; neither of these sequences has the required Asp in the P4 position. The

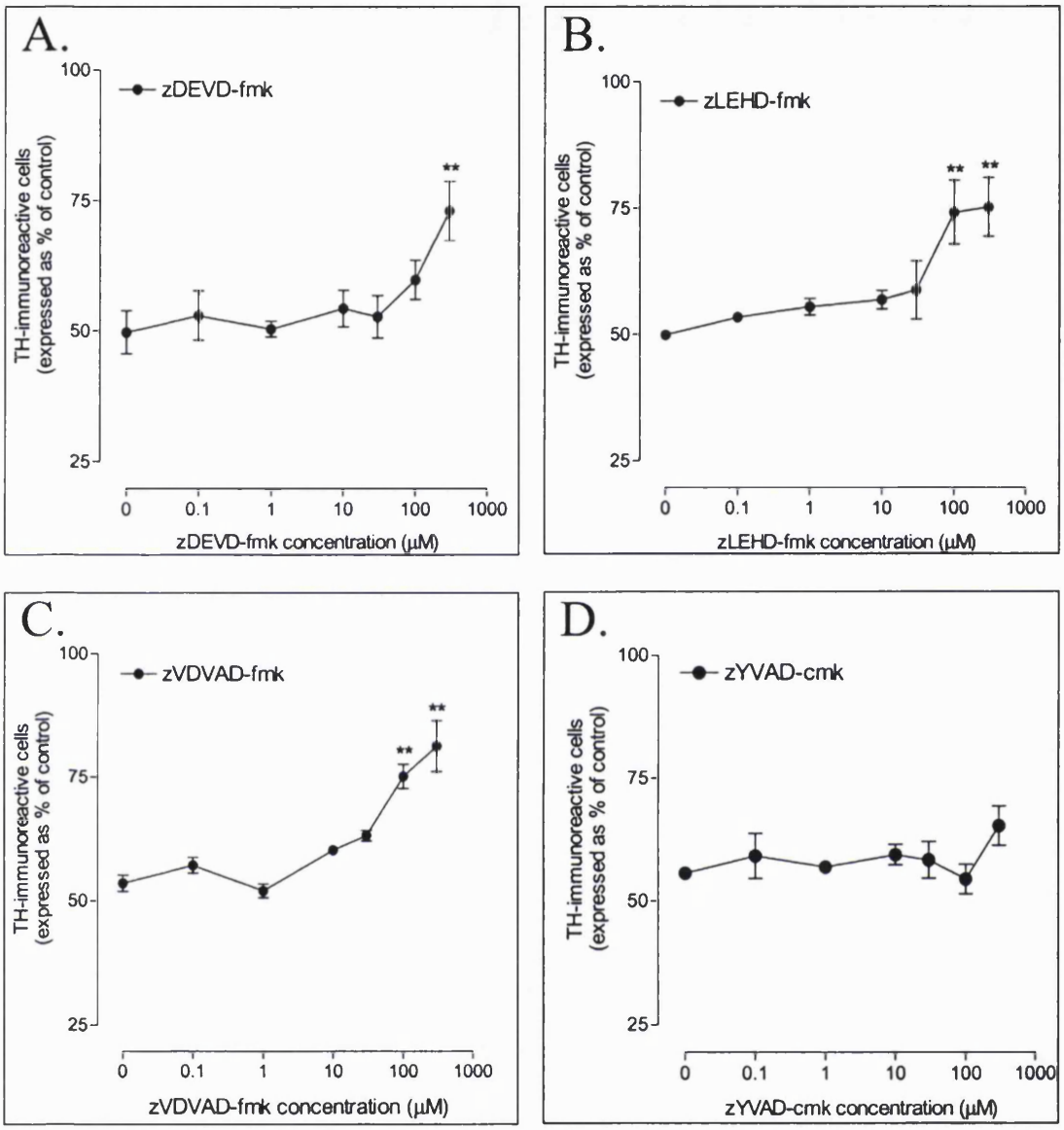


Figure 4. Effects of peptide caspase inhibitors on survival of TH-immunoreactive neurons after 10 μM MPP^+ treatment. Primary cultures of mesencephalic dopaminergic neurons were exposed to 10 μM MPP^+ for 48 hr in the presence of zDEVD-fmk (*A*), zLEHD-fmk (*B*), zVDVAD-fmk (*C*), or zYVAD-cmk (*D*). Cultures were fixed and immunostained for TH. Slides were then blinded, and the number of surviving TH-immunoreactive neurons was counted. Each data point represents the mean ± SEM of three independent experiments, each consisting of four independent wells, and is expressed as percentage of untreated control cultures (**$p < 0.01$; established by one-way ANOVA followed by Dunnett's test).

YVAD sequence is ~10,000-fold more selective for caspase 1 than for caspase 2, 3, or 7 and ~1000-fold more selective for caspase 1 than for caspase 9 (Garcia et al., 1998). The LEHD sequence does resemble the cleavage sites of caspases 4 and 5; thus there may be some inhibition of these caspases.

Concentration-dependent increases were observed with three of the inhibitors, zDEVD-fmk (caspase 3), zLEHD-fmk (caspase 9), and zVDVAD-fmk (caspase 2) (Fig. 5*A–C*), but no significant increases were observed with the caspase 1 inhibitor zYVAD-cmk (Fig. 5*D*). Significant increases in TH-immunoreactive cell number were observed with zLEHD-fmk and zVDVAD-fmk concentrations of 100 μM and above. The caspase 3 inhibitor zDEVD-fmk caused significant increases in dopaminergic neuronal survival only at 300 μM, whereas no significant increases were observed with zYVAD-cmk at any concentration tested.

Effects of novel caspase inhibitors on survival of mesencephalic dopaminergic neurons exposed to MPP^+

Two novel inhibitors of caspases were tested for neuroprotective effects in dopaminergic neurons exposed to 10 μM MPP^+: M-920, a nonspecific inhibitor of caspases, and M-791, a selective caspase 3 inhibitor. M-725, an inactive analog of M-920, was also tested. These inhibitors are described in a model of sepsis by Hotchkiss and coworkers (2000). The results of these experiments are shown in Figure 5*A*. Both of the active caspase inhibitors caused significant increases in the number of surviving TH-immunoreactive neurons. Significant neuroprotection was observed with M-920 concentrations of 10 μM and above; at concentrations of 10 μM and above, the survival was similar to that observed in untreated control cultures. Treatment of dopaminergic neurons

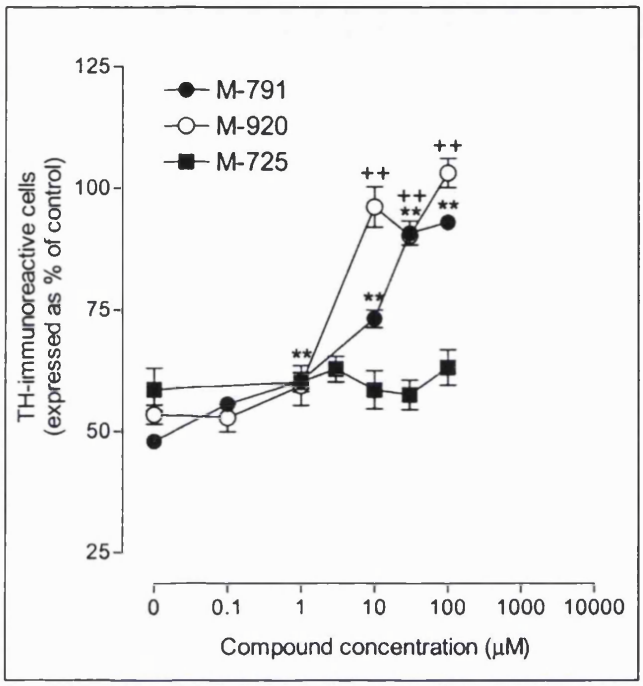


Figure 5. Effects of novel caspase inhibitors on survival of dopaminergic neurons treated with MPP^+. Two novel caspase inhibitors were tested for survival-promoting effects in primary cultures of dopaminergic neurons, M-920 and M-791, together with an inactive analog, M-725. Compounds were coadministered with 10 μM MPP^+ for 48 hr, and then surviving TH-immunoreactive cells were quantified. Results shown are the mean ± SEM of four independent wells per treatment group (M-791 response, $^{**}p < 0.01$; M-920 response, $^{++}p < 0.01$; both established by one-way ANOVA followed by Dunnett's test).

with M-791 caused significant neuroprotection at concentrations of 1 μM and above; the maximal response observed with this inhibitor increased the number of surviving TH-immunoreactive neurons to >90% of untreated control. The significant neuroprotective effects that were observed with M-791 at 1 μM indicate that the neuroprotection is likely to be mediated by inhibition of caspase 3-like proteases. The survival response with this caspase 3 inhibitor is considerably higher than that observed with zDEVD-fmk, the peptidergic caspase 3 inhibitor, which might indicate limited cell permeability of the peptide caspase inhibitor. The magnitude of the survival effect of M-791 is equivalent to the effects observed with both zVAD-fmk and M-920, the broad spectrum caspase inhibitors. This indicates that inhibition of caspase 3 alone is sufficient to prevent almost all the toxicity of MPP^+ in this culture system. When cultures were treated with the inactive compound M-725, no neuroprotective effects were observed at any of the concentrations tested.

With regard to the specificity of the inhibitors, M-920 is reported to have an IC_{50} value of 0.002 μM for caspase 3 in sepsis models and submicromolar IC_{50} values for caspases 1, 4, 7, and 8. The IC_{50} values for caspases 5 and 6 are 2 and 1.5 μM, respectively. M-791 has an IC_{50} value of 0.008 μM for caspase 3 and 0.23 μM for caspase 7 in the sepsis model; the IC_{50} for caspase 8 is 4 μM, and for other caspases it is in the mid-micromolar range (Hotchkiss et al., 2000). IC_{50} values on a range of caspases and in two whole-cell *in vitro* models for these three compounds are shown in Table 1.

Table 1. IC_{50} values for novel caspase inhibitors for various caspases, together with the IC_{50} values obtained in whole-cell apoptosis assays in cerebellar granule neurons (CGN) and hNT-2 cells

	Cell free assays		
	M-920	M-791	M-725
Caspase			
1	0.01	11	15
2	>50	>450	
3	0.002	0.008	45
7	0.02	0.23	>100
8	0.04	4	68
9	>50	>450	
Cell type	Whole cell apoptosis IC_{50}		
hNT2	0.12 μM	0.49 μM	
1° mCGN	0.2 μM	1.0 μM	

M-920 potently inhibits a number of members of the caspase family, whereas M-791 is a potent inhibitor of caspase 3 and also inhibits caspase 7. M-725 has little potency at any caspase tested. IC_{50} values presented are micromolar.

MPP^+ causes apoptotic features and activated caspase 3 expression in degenerating dopaminergic neurons; effects of caspase inhibition

Nuclear morphology was assessed in dopaminergic neurons after MPP^+ exposure to determine whether the induced cell death was apoptotic. Photomicrographs of mesencephalic cultures stained for TH and counterstained with Hoechst 33342 to visualize nuclei are shown in Figure 6. TH-immunoreactive neurons are stained *green*, and Hoechst stained nuclei fluoresce *blue*. Double exposures were also taken to confirm localization of TH-immunoreactive cell nuclei. TH-immunoreactive neurons are shown in Figure 6, *A*, *D*, and *F*, Hoechst 33342-stained nuclei are shown in Figure 6, *B*, *E*, and *H*, and double-exposed images to show colocalization are shown in Figure 6, *C*, *F*, and *I*. Figure 6*A–C* shows control cultures. TH-immunoreactive neurons have large cell bodies and extensive neurites; the nuclear morphology of these neurons shows no chromatin condensation, illustrated by the *yellow arrows*. Figure 6*D–F* photomicrographs are of a field of view from cultures exposed to 10 μM MPP^+ for 48 hr. Within the field, a number of degenerating TH-immunoreactive neurons can be observed (*white arrows*). The nuclei of these neurons show chromatin condensation when stained with Hoechst 33342, a characteristic feature of apoptosis. Also within the well are a number of TH-immunoreactive neurons that do not appear to have degenerated; the nuclei of these neurons do not show chromatin condensation (*yellow arrow*). Figure 6*G–I* shows cultures exposed to 10 μM MPP^+ for 48 hr in the presence of 300 μM zVAD-fmk. The TH-immunoreactive neurons within the culture do not appear to have degenerated, and their nuclei do not show chromatin condensation (*yellow arrow*). Also within each well, there is a population of cells that exhibit chromatin condensation but are not TH immunoreactive; these are highlighted by the *red arrows*. Such nuclei are observed in control, MPP^+-treated, and MPP^+- and zVAD-fmk-treated cultures. These profiles may reflect a population of non-dopaminergic cells in the culture that are undergoing cell death, perhaps as a result of a change in the medium on the cultures.

To visualize activated caspase 3 in dopaminergic neurons after MPP^+ treatment, double-immunolabeling studies were performed using primary antibodies to activated caspase 3 and to TH. Cultures were grown for 5 d, then returned to culture

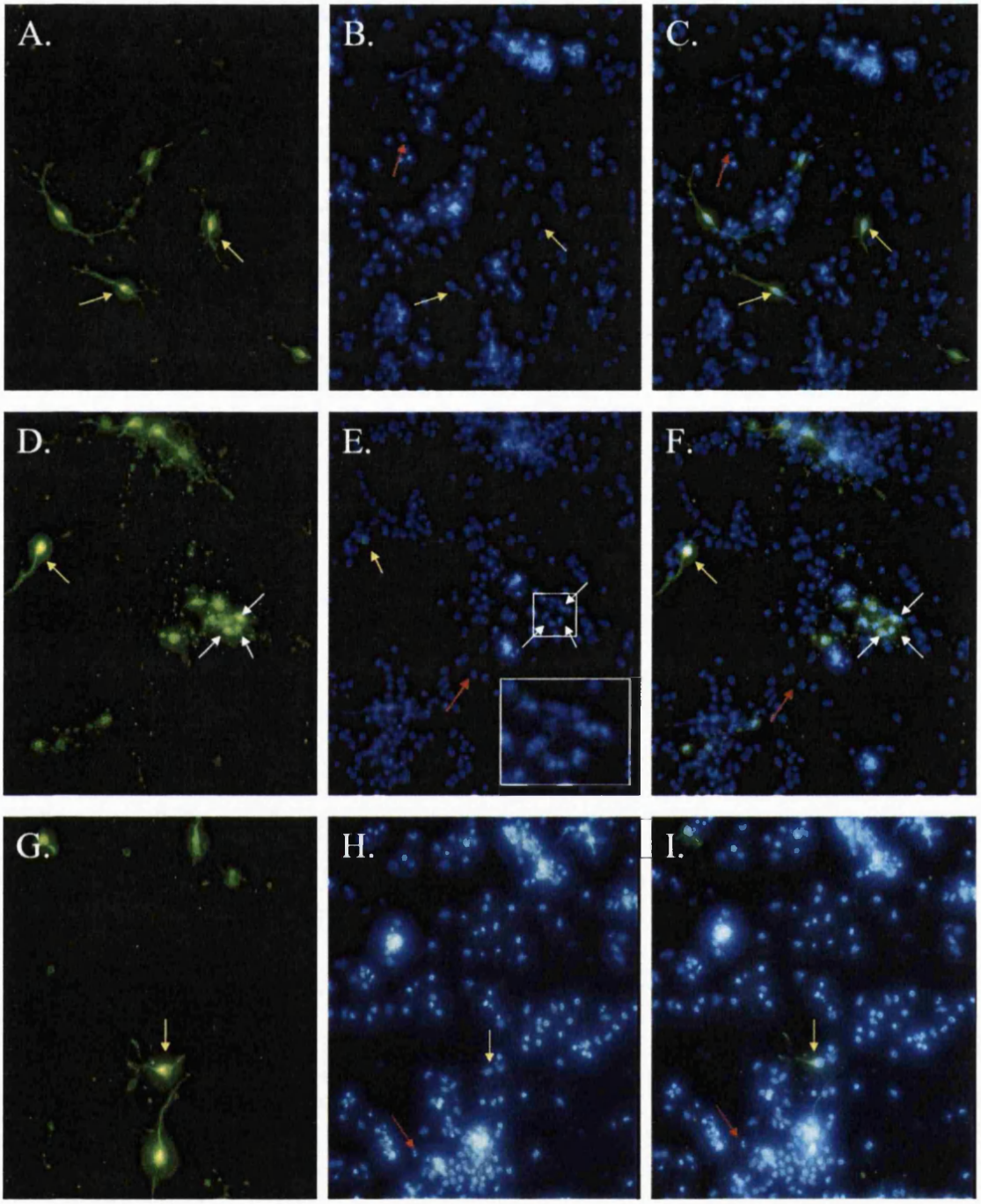


Figure 6. Colocalization of apoptotic nuclei with TH immunoreactivity in primary mesencephalic cultures after MPP^+ exposure. Cultures were stained with a primary antibody raised against TH and visualized using FITC. Nuclei were visualized by counterstaining using Hoechst 33342. *A–C*, Control cultures; *D–F*, cultures exposed to 10 μM MPP^+ for 48 hr; *G–I*, cultures exposed to 10 μM MPP^+ in the presence of 300 μM zVAD-fmk. Each of the photomicrographs within a condition is of the same field of view, stained with tyrosine hydroxylase (*A*, *D*, *G*) or Hoechst 33342 (*B*, *E*, *H*) or dual exposed to show colocalization (*C*, *F*, *I*). Apoptotic dopaminergic nuclei are shown by *white arrows* (and magnified in *E*, *inset*). Representative non-apoptotic dopaminergic nuclei are indicated by *yellow arrows*, and non-dopaminergic apoptotic nuclei by *red arrows*.

medium alone (Fig. 7*A–C*) or treated with 10 μM MPP^+ for 24 hr (Fig. 7*D–F*) or 48 hr (Fig. 7*G–I*). Figure 7, *A*, *D*, and *G*, shows TH immunoreactivity. Figure 7, *B*, *E*, and *F*, shows activated caspase 3 immunoreactivity in the same field of view, and Figure 7, *C*, *F*, and *G*, shows colocalization of caspase 3 with TH immunoreactivity.

In control cultures, a number of TH-immunoreactive neurons can be observed (Fig. 7*A*), along with a population of cells expressing activated caspase 3 (Fig. 7*B*); however, there is little coexpression of activated caspase 3 with TH in these cultures (Fig. 7*C*), indicating that caspase 3 is not active in dopaminergic neurons. In cultures treated with MPP^+ for 24 or 48 hr, however, a population of dopaminergic neurons that coexpress TH and caspase 3 is apparent (Fig. 7*F*,*I*). In all treatment groups, a

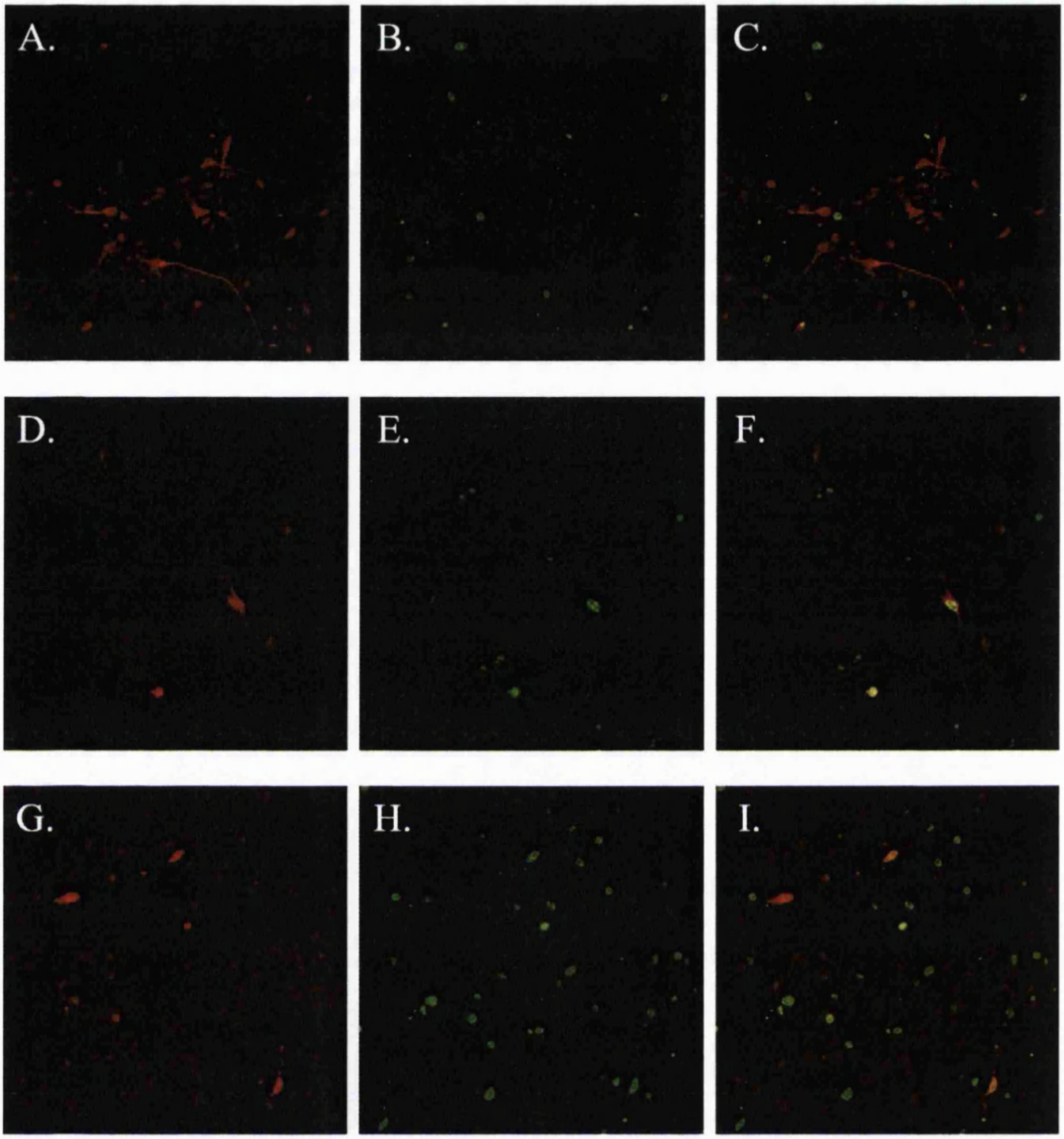


Figure 7. TH-immunoreactive neurons exposed to 10 μM MPP^+ for 24 or 48 hr express activated caspase 3. Cultures were treated with MPP^+ for the required time, then fixed and double immunostained using a monoclonal tyrosine hydroxylase antibody and a polyclonal antibody raised against active caspase 3. *A–C*, Control cultures; *D–F*, cultures treated with 10 μM MPP^+ for 24 hr; *G–I*, cultures exposed to MPP^+ for 48 hr. *A*, *D*, and *F* show immunostaining using an antibody to TH; *B*, *E*, and *H* show the same field of view stained with the activated caspase 3 antibody. Colocalization of these antibodies is shown in *C*, *F*, and *I*.

population of non-dopaminergic neurons that express activated caspase 3 is apparent, indicating that there is a population of cells within the cultures undergoing apoptosis; this is in accordance with the presence of apoptotic profiles in a population of non-dopaminergic neurons observed in Figure 6. Thus, MPP^+ treatment of primary cultures of dopaminergic neurons for 24 or 48 hr causes activation of caspase 3 in these neurons.

To quantify the MPP^+-induced caspase activation and chromatin condensation, a triple-labeling experiment was performed. Cultures were treated with MPP^+ for 48 hr in the presence or absence of 300 μM zVAD-fmk or the caspase 3 inhibitor M-791, then fixed and double immunostained for active caspase 3 and TH. Nuclei were counterstained using Hoechst 33342, and quantification was performed. Ten fields of view containing at least three TH-immunoreactive cells were quantified in each of three independent wells. The total number of nuclei was established, and the number of these that showed apoptotic features was established. The number of TH-immunoreactive neurons and the number of active caspase-3 neurons were also counted. Each field of view was quantified for the number of neurons coexpressing TH/active caspase 3 and TH/condensed chromatin. These data are shown in Figure 8. In Figure 8*A*, the number of apoptotic cells and the number of active caspase 3-immunoreactive cells in each treatment group are shown, expressed as a percentage of the total number of cells within the cultures. In control cultures there is a population of ~20% of cells that express apoptotic morphology, likely as a result of stress through changing the medium or a natural attrition of cells within the culture. There is a slight

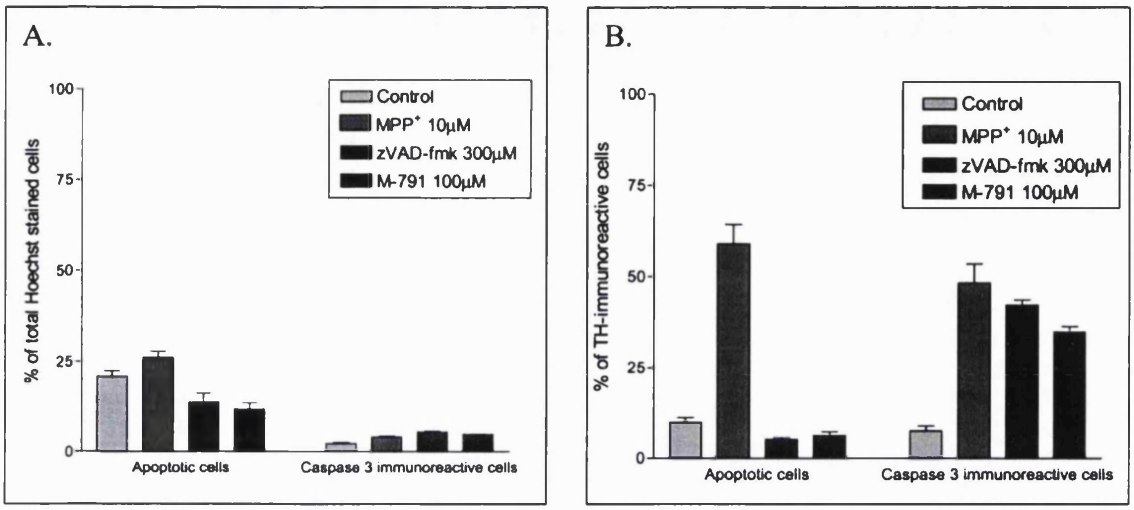


Figure 8. Effects of MPP^+ treatment in the presence and absence of caspase inhibition on the number of apoptotic profiles and active caspase 3 immunoreactive cells. Cells were double labeled for TH and active caspase 3, and the nuclei were counterstained with Hoechst 33342. Cells were visualized using a 20× objective, and the total number of nuclei, the number of apoptotic nuclei, the number of TH-immunoreactive cells, and the number of active caspase 3-immunoreactive cells were quantified, together with the number of cells coexpressing TH and apoptotic nuclei, and TH and active caspase 3. Ten fields of view were quantified in each of three wells per treatment group. Cultures that were quantified were untreated control cultures, cultures exposed to 10 μM MPP^+ for 48 hr, or cultures exposed to 10 μM MPP^+ for 48 hr in the presence of either 300 μM zVAD-fmk or 100 μM M-791. *A* shows the expression of apoptotic profiles and active caspase 3 as a percentage of the total cell population. *B* shows the cells coexpressing either activated caspase 3 or apoptotic profiles with TH expressed as a percentage of the total number of TH-immunoreactive cells.

increase in the number of apoptotic cells in the MPP^+-treated group that is reduced by the caspase inhibitors. There is also a small population (<10%) of active caspase 3-immunoreactive cells within control cultures. This is increased by MPP^+ treatment, but this increase is not reversed by the caspase inhibitors. Figure 8*B* shows the expression of apoptotic nuclei and active caspase 3 in TH-immunoreactive neurons. Approximately 10% of TH-immunoreactive neurons have apoptotic nuclei in control cultures; this is markedly increased by MPP^+ treatment, which increases the number of apoptotic nuclei in the remaining TH-immunoreactive neurons to ~60%. Both of the caspase inhibitors that were tested completely reverse the increase in apoptotic nuclei induced by MPP^+. When coexpression of TH and activated caspase 3 was examined, there was again a marked increase in the number of coexpressing cells, from ~10% in control cultures to ~50% in 10 μM MPP^+-treated cultures. When MPP^+ was coadministered with the caspase inhibitors, however, there was little decrease in the expression of activated caspase 3 in TH-immunoreactive neurons. This lack of decrease with the caspase inhibitors is likely attributable to the mode of action of the inhibitors, which bind to the cleavage site of the active caspase and prevent cleavage of cellular substrates rather than preventing formation of the active caspase from the inactive zymogen. Thus, in the inhibitor and MPP^+-treated dopaminergic neurons, the caspase appears to be activated as in cultures treated with MPP^+ alone, but inhibition prevents it from executing the apoptotic response; this leads to the decreased evidence of chromatin condensation and the increased neuronal number.

Not all MPP^+-treated dopaminergic cells visualized expressed chromatin condensation or active caspase 3; this may reflect a population that has not yet effected the apoptotic response after MPP^+ treatment. At 48 hr treatment, only ~50% of dopaminergic cells remain in the cultures compared with untreated controls. Dopaminergic cells expressing active caspase 3 were also present within the cultures at earlier time points. It is likely that these cells that activate caspase 3 earlier in the time course undergo apoptosis and detach from the substratum, resulting in this decrease in numbers, and that the number of dopaminergic neurons counted with the active enzyme at 48 hr underestimates the number of neurons that express this over the total treatment period.

DISCUSSION

Although MPP^+ is a commonly used model for selective dopaminergic neuronal cell death *in vitro* (Sanchez Ramos et al., 1986; Michel et al., 1989; Michel et al., 1990; Beck et al., 1991), reports conflict regarding the cell death mechanism. Apoptosis has been shown *in vivo* in the substantia nigra of MPTP-treated mice (Tatton and Kish, 1997; Eberhardt et al., 2000), depending on the dosing regimen used (Jackson-Lewis et al., 1995). *In vitro*, apoptosis has been demonstrated after MPP^+ treatment in rat mesencephalic–striatal cocultures (Mochizuki et al., 1994), in dissociated cultures of cerebellar granule cells and mesencephalic dopaminergic neurons (Dipasquale et al., 1991; Du et al., 1997; Dodel et al., 1998), and in the SH-SY5Y neuronal cell line (Fall and Bennet, 1998). In contrast, however, Lotharius and coworkers (1999) found no evidence for apoptosis in MPP^+-treated rat mesencephalic neurons. MPP^+ treatment of dopaminergic MN9D cells also failed to produce evidence of apoptotic markers (Choi et al., 1999). The evidence for and against a role of apoptosis in MPP^+ toxicity is reviewed by Nicotra and Parves (2000); it appears most likely that the differences in types of cell death observed by different groups are dependent on the severity of the insult or the culture conditions that are used.

Here we show that MPP^+ treatment of primary dopaminergic neurons causes apoptosis and that caspase inhibition with zVAD-fmk prevents the MPP^+-mediated loss of dopaminergic neurons. The number of surviving dopaminergic neurons in 10 μM MPP^+-treated cultures decreased to ~50%, with zVAD-fmk restoring numbers to ~90%, confirming several previous reports. zVAD-

fmk has been reported to attenuate MPP^+ toxicity in cerebellar granule neurons (Du et al., 1997) and mesencephalic dopaminergic neurons (Dodel et al., 1998; Eberhardt et al., 2000). In this study, zVAD-fmk increased the number of MPP^+-treated dopaminergic neurons and the somatic size of these neurons after 48 hr; increased TH-immunoreactive cell number was observed up to 5 d after coadministration of the compounds (data not shown). zVAD-fmk was less effective at preventing the MPP^+-mediated loss of [^{3}H]DA uptake, with significant increases only at 300 μM. Only a partial restoration of the neurite length of these neurons was observed, indicating that the dopamine transporter sites may not be spared. These data are similar to reported studies with both MPP^+ (Eberhardt et al., 2000) and 6-OHDA (Von Coelln et al., 2001), in which little neurite or [^{3}H]DA uptake restoration was observed with zVAD-fmk. Although these data and reports from other groups indicate an important role for caspases in mediating MPP^+ toxicity, a number of groups have found conflicting effects. Lotharius et al. (1999) found no protection from MPP^+ toxicity with another broad-spectrum caspase inhibitor, Boc-Asp-fmk, and zVAD-fmk did not protect dopaminergic MN9D cells from MPP^+ toxicity (Choi et al., 1999). Hartmann et al. (2001) reported that MPP^+ treatment induced apoptosis in primary dopaminergic neurons, but that caspase inhibition potentiated cell death by increasing necrosis, an effect that has been reported previously in other cell types (Lemaire et al., 1998); this effect was reversed if cultures were grown in elevated glucose. Thus, reports conflict regarding the efficacy of caspase inhibition in preventing MPP^+ toxicity *in vitro*.

In this study, caspase inhibition clearly promotes survival of dopaminergic neurons. Caspases can be divided into three families on the basis of structure and function; these families typically are involved in the inflammatory response, caspase activation, and execution of apoptosis, respectively (for review, see Nicholson and Thornberry, 1997; Stennicke and Salvesen, 1998). To determine which specific caspases mediate the toxicity, peptide inhibitors of specific caspases were tested; partial neuroprotection was observed with inhibitors of caspases 2, 3, and 9, but not with an inhibitor of caspase 1. A novel caspase 3 inhibitor had neuroprotective effects equivalent to either zVAD-fmk or another broad-spectrum caspase inhibitor, M-920.

Coadministration of dopaminergic neurons with MPP^+ and the selective caspase 3 inhibitor M-791 caused almost complete protection of TH-immunoreactive neurons *in vitro*. The protection obtained with this compound was similar to that obtained with either of the broad-spectrum caspase inhibitors tested, zVAD-fmk or M-920, and greater than with the peptide inhibitor zDEVD-fmk. That the effects were mediated by caspase inhibition is indicated by the lack of effect of M-725, a structural analog of M-920 lacking activity at caspases. These data provide compelling evidence that in dopaminergic neurons exposed to MPP^+ *in vitro*, inhibition of caspase 3 alone is sufficient to protect the neurons. Inhibition of caspase 3 with M-791 also decreased to control levels the number of apoptotic dopaminergic cells, a response similar to that of zVAD-fmk. In contrast, neither of these inhibitors prevented an MPP^+-mediated increase in the number of TH-immunoreactive cells expressing activated caspase 3. A likely explanation for this is that the inhibitors do not prevent cleavage and activation of the caspase zymogen but rather bind to the active site of the activated caspase to prevent substrate cleavage.

Caspase 3 is involved in the execution of apoptosis in a number of neuronal cell types after a range of insults. *In vivo*, caspase 3 inhibition attenuates damage after ischemia (Ma et al., 1998) and axotomy of retinal ganglion neurons (Kermer et al., 1998). *In vitro*, caspase 3 inhibition protects cerebellar granule neurons from K^+ deprivation-induced apoptosis (Ni et al., 1997) and PC12 cells from 6-hydroxydopamine toxicity (Ochu et al., 1998; Lotharius et al., 1999). Caspase 3 is activated by a range of factors, including caspase 9. Caspase 9 is activated during release of cytochrome *c* from mitochondria; the released cytochrome *c* forms a complex with cytoplasmic APAF-1 and caspase 9 in the presence of ATP and activates caspase 9 (Liu et al., 1996; Zou et al., 1997). Activated caspase 9 then cleaves and activates caspase 3, leading to the apoptotic death of the cell (Li et al., 1997; Cai et al., 1998; Pan et al., 1998). Cytochrome *c* release into the cytoplasm of cerebellar granule cells has been shown after MPP^+ treatment (Du et al., 1997). Inhibition of caspase 9 using zLEHD-fmk significantly increased survival of MPP^+-treated TH-immunoreactive neurons, indicating that this pathway may indeed be activated in MPP^+ toxicity.

Because the specificity of the caspase 2 inhibitor is suspect, it is possible that the effects observed with this inhibitor are mediated through inhibition of another caspase such as caspase 3. There is evidence *in vivo* that caspase 2 may be involved in MPTP toxicity; mice overexpressing Bcl-2 are resistant to MPTP toxicity, with decreased expression of active caspase 2 after MPTP treatment compared with wild-type animals (Yang et al., 1998). More selective inhibitors may allow further clarification of the role of this caspase.

No effects were observed with the caspase 1 inhibitor zYVAD-cmk, consistent with previous reports in primary dopaminergic neurons (Dodel et al., 1998). These data, however, conflict with studies in transgenic mice overexpressing dominant negative caspase 1 that were resistant to MPTP toxicity *in vivo* (Klevenyi et al., 1999), and caspase 1 activation was observed in the dopaminergic cell line SN4741 after MPP^+ or oxidant treatment (Chun et al., 2001). Caspase 1 has been implicated as both a downstream target and an activator of caspase 8, and caspase 8 inhibition has been shown to be protective against MPTP toxicity in mice *in vivo*, although not *in vitro* (Hartmann et al., 2001). Both caspase 3 and caspase 8 are expressed in the substantia nigra of Parkinson's disease patients (Hartmann et al., 2000, 2001). An explanation for the apparent conflict of these results may be that MPP^+ is capable of activating multiple caspase pathways depending on cellular conditions and that there may be a redundancy of function of some of these pathways under certain conditions.

The toxicity of MPP^+ for dopaminergic neurons under these conditions appears to be mediated by pathways that converge on activation of caspase 3, and inhibition of caspase 3 is sufficient to spare at least the neuronal somata. The events before the caspase 3 activation are less clear. Caspase 9 inhibition provides partial neuroprotection, indicating that cytochrome *c* release from mitochondria might be important. MPP^+ has been shown to open the mitochondrial permeability transition pore (PTP) *in vitro* (Cassarino et al., 1999), although inhibition of the mitochondrial PTP using cyclosporin A does not protect SH-SY5Y cells (Fall and Bennett, 1998) or mesencephalic dopaminergic neurons (data not shown). Cyclosporin A, however, is toxic at higher concentrations, so any potential neuroprotective effects may be masked. Another route of cytochrome *c* release from mitochondria is through pores formed by pro-apoptotic members of the Bcl-2 family. Mice overexpressing Bcl-2 are resistant to MPTP toxicity *in vivo* and MPP^+ toxicity *in vitro* (Offen et al., 1998; Yang et al., 1998), so this may be a possible mechanism underlying the cell

death. In addition to cytochrome *c*, other pro-apoptotic factors can be released from mitochondria in apoptosis, including second mitochondria-derived activator of caspase (SMAC)/direct IAP binding protein with low pI (DIABLO) and apoptosis-inducing factor (AIF). SMAC/DIABLO inhibits the activity of members of the IAP family, leading to caspase activation (Du et al., 2000; Verhagen et al., 2000). In this regard, it is interesting that adenoviral expression of X-chromosome-linked IAP protects nigral neurons from MPTP toxicity in mice *in vivo* (Eberhardt et al., 2000). No reports have been published as yet showing direct evidence for release of either SMAC/DIABLO or AIF. Further investigation may clarify the roles of mitochondrial factors in MPP^+-induced apoptosis.

In conclusion, we show that caspase inhibition protects dopaminergic neurons from MPP^+ toxicity *in vitro* and that the caspases 2, 3, and 9, but not caspase 1, are involved in the pathway. The pathways activated by MPP^+ appear to converge on activation of caspase 3, because inhibition of caspase 3 alone is sufficient to fully protect cells from MPP^+-mediated cell death. Thus, the caspase cascade, or factors upstream regulating caspase activation, are targets for neuroprotective strategies in models of Parkinson's disease.

REFERENCES

Akaneya Y, Takahashi M, Hatanaka H (1995) Involvement of free radicals in MPP^+ neurotoxicity against rat dopaminergic neurons in culture. Neuroscience 193:53–56.

Beck KD, Knusel B, Pasinetti G, Michel PP, Zawadzka H, Goldstein M, Hefti F (1991) Tyrosine hydroxylase mRNA expression by dopaminergic neurons in culture: effect of 1-methyl-4-phenylpyridinium treatment. J Neurochem 57:527–532.

Bottenstein JE, Sato GH (1979) Growth of a rat neuroblastoma cell line in serum free medium. Proc Natl Acad Sci USA 76:514–517.

Cai J, Yang J, Jones DP (1998) Mitochondrial control of apoptosis: the role of cytochrome *c*. Biochim Biophys Acta 1366:139–149.

Cassarino DS, Parks JK, Parker Jr WD, Bennett Jr JP (1999) The Parkinsonian neurotoxin MPP^+ opens the mitochondrial permeability transition pore and releases cytochrome *c* in isolated mitochondria via an oxidative mechanism. Biochim Biophys Acta 1453:49–62.

Choi W-S, Yoon S-Y, Oh TH, Choi E-J, O'Malley KL, Oh YJ (1999) Two distinct mechanisms are involved in 6-hydroxydopamine and MPP^+ induced dopaminergic neuronal cell death: role of caspases, ROS and JNK. J Neurosci Res 57:86–94.

Chun HS, Gibson GE, DeGiorgio LA, Zhang H, Kidd VJ, Son JH (2001) Dopaminergic cell death induced by MPP^+, oxidant and specific neurotoxicant shares the common molecular mechanism. J Neurochem 76:1010–1021.

Degli EM (1998) Inhibitors of NADH-ubiquinone reductase: an overview. Biochim Biophys Acta 1364:222–235.

Dipasquale B, Marini AM, Youle RJ (1991) Apoptosis and DNA degradation induced by 1-methyl-4-phenylpyridinium in neurons. Biochem Biophys Res Commun 137:1442–1448.

Dodel RC, Du Y, Bales KR, Ling ZD, Carvey PM, Paul SM (1998) Peptide inhibitors of caspase-3-like proteases attenuate 1-methyl-4-phenylpyridinum-induced toxicity of cultured fetal rat mesencephalic dopamine neurons. Neuroscience 86:701–707.

Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. Cell 102:43–53.

Du Y, Dodel RC, Bales KR, Jemmerson R, Hamilton Byrd E, Paul SM (1997) Involvement of a caspase-3-like cysteine protease in 1-methyl-4-phenylpyridinium-mediated apoptosis of cultured cerebellar granule neurons. J Neurochem 69:1382–1388.

Eberhardt O, Coelin R, Kugler S, Lindenau J, Rathke-Hartlieb S, Gerhardt E, Haid S, Isenmann S, Gravel C, Srinivasan A, Bahr M, Weller M, Dichgans J, Schultz J (2000) Protection by synergistic effects of adenovirus-mediated X-chromosome-linked inhibitor of apoptosis and glial cell line-derived neurotrophic factor gene transfer in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's Disease. J Neurosci 20:9126–9134.

Fall CP, Bennett Jr JP (1998) MPP^+ induced SH-SY5Y apoptosis is potentiated by cyclosporin A and inhibited by aristolochic acid. Brain Res 811:143–146.

Garcia CM, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem 273:32608–32613.

Hartmann A, Hunot E, Michel P, Muriel M, Vyas S, Faucheux B, Mouatt-Prigent A, Turmel H, Srinivasan A, Ruberg M, Evan G, Agid Y, Hirsch E (2000) Caspase 3: a vulnerability factor and a final effector in the apoptotic death of dopaminergic neurones in Parkinson's Disease. Proc Natl Acad Sci USA 97:2875–2880.

Hartmann A, Troadec J-D, Hunot S, Kikly K, Faucheux B, Mouatt-Prigent A, Ruberg M, Agid Y, Hirsch E (2001) Caspase-8 is an effector in apoptotic death of dopaminergic neurons in Parkinson's disease, but pathway inhibition results in neuronal necrosis. J Neurosci 21:2247–2255.

Hotchkiss RS, Chang KC, Swanson PE, Tinsley KW, Hui JJ, Klendre P, Xanthoudakis S, Roy S, Black C, Grimm E, Aspiotis R, Han Y, Nicholson DW, Karl IE (2000) Caspase inhibitors improve survival in sepsis: a critical role of the lymphocyte. Nat Immunol 1:496–501.

Jackson-Lewis V, Jakowec M, Burke RE, Przedborski S (1995) Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurodegeneration 4:257–269.

Kermer P, Klocker N, Labes M, Bahr M (1998) Inhibition of CPP32-like proteases rescues axotomized retinal ganglion cells from secondary cell death *in vivo*. J Neurosci 18:4656–4662.

Klevenyi P, Andreassen O, Ferrante R, Schleicher Jr J, Friedlander R, Flint Beal M (1999) Transgenic mice expressing a dominant negative mutant interleukin-1β converting enzyme show resistance to MPTP toxicity. NeuroReport 10:635–638.

Lemaire C, Andreau K, Souvannavong V, Adam A (1998) Inhibition of caspase activity induces a switch from apoptosis to necrosis. FEBS Lett 425:266–270.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome *c* and dATP-dependent formation of APAF-1/caspase 9 complex initiates an apoptotic protease cascade. Cell 91:479–489.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 86:147–157.

Lotharius J, Dugan LL, Malley KL (1999) Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. J Neurosci 19:1284–1293.

Ma J, Endres M, Moskowitz MA (1998) Synergistic effects of caspase inhibitors and MK-801 in brain injury after transient focal cerebral ischaemia in mice. Br J Pharmacol 124:756–762.

Marsden CD (1990) Neurophysiology. In: Parkinson's disease (Stern GM, ed), pp 57–98. London: Chapman and Hall Medical.

Michel PP, Dandapani BK, Sanchez Ramos J, Efange S, Pressman BC, Hefti F (1989) Toxic effects of potential environmental neurotoxins related to 1-methyl-4-phenylpyridinium on cultured rat dopaminergic neurons. J Pharmacol Exp Ther 248:842–850.

Michel PP, Dandapani BK, Knusel B, Sanchez Ramos J, Hefti F (1990) Toxicity of 1-methyl-4-phenylpyridinium for rat dopaminergic neurons in culture: selectivity and irreversibility. J Neurochem 54:1102–1109.

Mochizuki H, Nakamura N, Nishi K, Mizuno Y (1994) Apoptosis is induced by 1-methyl-4-phenylpyridinium ion (MPP^+) in ventral mesencephalic-striatal co-culture in rat. Neurosci Lett 170:191–194.

Ni B, Wu X, Du Y, Su Y, Hamilton-Byrd E, Rockey PK, Rosteck Jr P, Poirer GG, Paul SM (1997) Cloning and expression of a rat brain interleukin-1β-converting enzyme (ICE)-related protease (IRP) and its possible role in apoptosis of cultured cerebellar granule neurons. J Neurosci 17:1561–1569.

Nicholson DW, Thornberry NA (1997) Caspases: killer proteases. Trends Biochem Sci 22:299–306.

Nicotra A, Parves SH (2000) Cell death induced by MPTP, a substrate for monoamine oxidase B. Toxicology 153:157–166.

Ochu EE, Rothwell NJ, Waters CM (1998) Caspases mediate 6-hydroxydopamine-induced apoptosis but not necrosis in PC12 cells. J Neurochem 70:2637–2640.

Offen D, Beart PM, Cheung NS, Pascoe CJ, Hochman A, Gorodin S, Melamed E, Bernard R, Bernard O (1998) Transgenic mice expressing human Bcl-2 in their neurons are resistant to 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. Proc Natl Acad Sci USA 95:5789–5794.

Pan G, Humke EW, Dixit VM (1998) Activation of caspases triggered by cytochrome c in vitro. FEBS Lett 426:151–154.

Sanchez Ramos J, Barrett JN, Goldstein M, Weiner WJ, Hefti F (1986) 1-Methyl-4-phenylpyridinium (MPP^+) but not 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) selectively destroys dopaminergic neurons in cultures of dissociated rat mesencephalic neurons. Neurosci Lett 72:215–220.

Schapira AHV (1998) Human complex I defects in neurodegenerative diseases. Biochim Biophys Acta 1364:261–270.

Stennicke HR, Salvesen GS (1998) Properties of the caspases. Biochim Biophys Acta 1387:17–31.

Tatton NA, Kish SJ (1997) In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. Neuroscience 77:1037–1048.

Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia CM, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 272:17907–17911.

Turmel H, Hartmann H, Parain K, Douhou A, Srinivasan A, Agid Y, Hirsch E (2001) Caspase 3 activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. Mov Disord 16:185–189.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102:42.

Von Coelln R, Kugler S, Bahr M, Weller M, Dichgans J, Schulz J (2001) Rescue from death but not from functional impairment: caspase inhibition protects dopaminergic cells against 6-hydroxydopamine-induced apoptosis but not against the loss of their terminals. J Neurochem 77:263–273.

Yang L, Matthews RT, Schulz JB, Klockgether T, Liao AW, Martinou J, Penney JBJ, Hyman BT, Beal MF (1998) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyride neurotoxicity is attenuated in mice overexpressing Bcl-2. J Neurosci 18:8145–8152.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*-dependent activation of caspase-3. Cell 90:405–413.

Caspases and neuroprotection

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Apoptotic cell death has been implicated in the pathogenesis of both acute and chronic neurodegenerative disorders. The caspase family of cysteine proteases are involved both in the initiation and final execution of apoptosis. Inhibition of the caspase family prevents cell death in a number of models of neurodegenerative cell death in vivo and in vitro. This sparing of neurons does not always correlate with long-term functional recovery, possibly due to the limitations of the available inhibitors. In this review, the evidence for a neuroprotective role of caspase inhibition in models of Parkinson's disease and cerebral ischemia is critically evaluated.

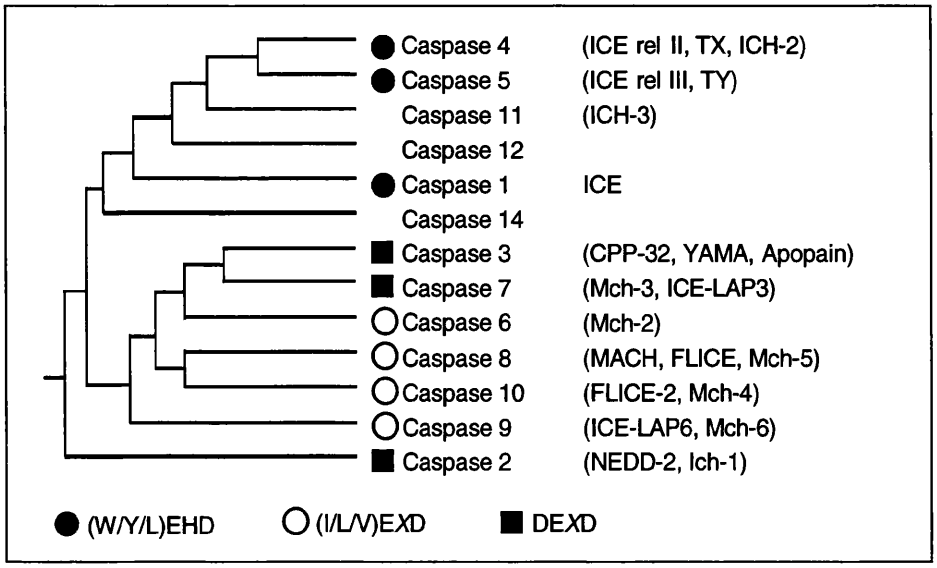
Keywords Apoptosis, caspases, ischemia, neurodegeneration, Parkinson's disease

Introduction

Caspases are a family of cysteine proteases that cleave cellular substrates after aspartate residues and which are implicated in inflammatory responses and the initiation and execution of apoptosis [1-3]. The prototypic caspase is interleukin-1β-converting enzyme (ICE) which was identified in 1993 and shown to have homology to the *Caenorhabditis elegans* death gene *ced-3* [4]. A number of related members of the family were identified, and shown to have a pivotal role in apoptotic cell death [5-7,8••,9,10]. This family of enzymes were designated caspases (Cysteine ASPartASES) in 1995 [11], with ICE being designated caspase 1 and the others named in order of identification; the family has grown to include at least 14 members to date (Figure 1).

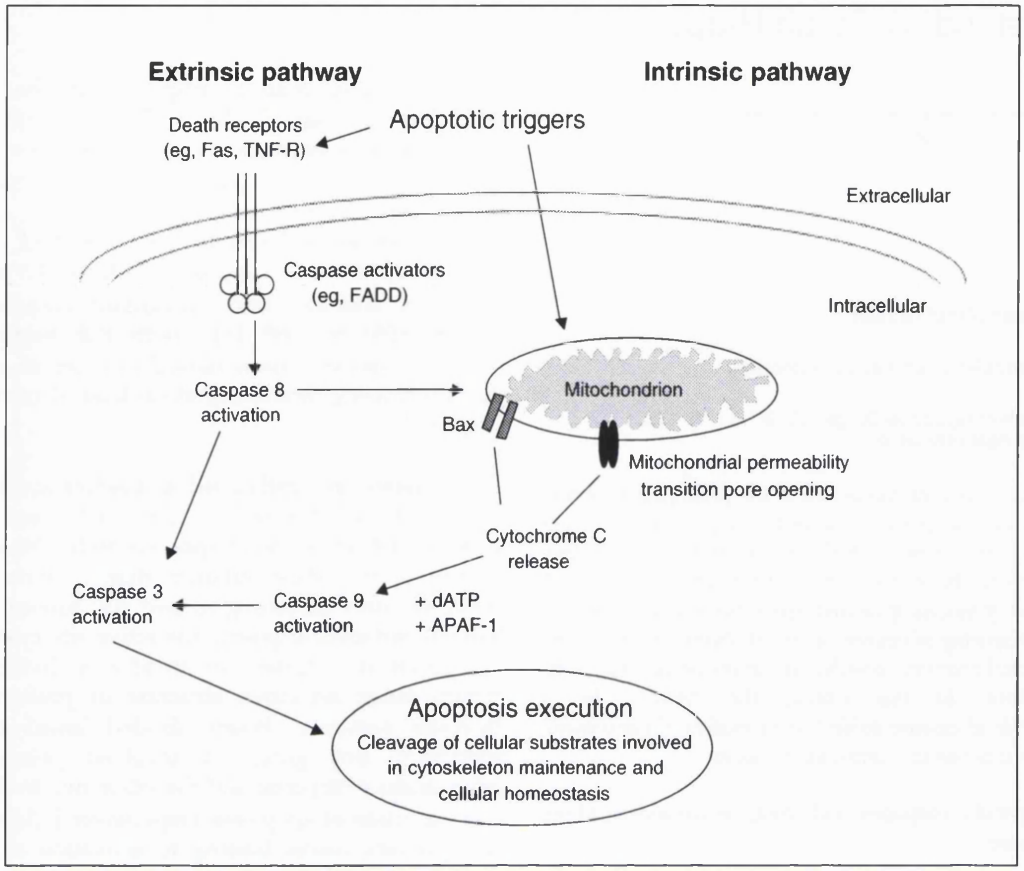
The caspases are synthesized as inactive zymogens, which are activated by cleavage at a conserved aspartate residue to give two subunits, one of approximately p10 and the other of around p20; these subunits then combine to form an active tetramer consisting of two p20 subunits flanked by two p10 subunits, exposing the active site cysteine residue. The family of caspases can broadly be divided into three groups based on either structure or preferred substrate cleavage sequence. When divided based on substrate specificity, one group is involved primarily in the inflammatory response and the other two in the activation and execution of apoptosis, respectively [1,12••]. There are two primary routes leading to activation of the caspase cascade, an extrinsic route involving ligation of death receptors, and an intrinsic route involving release of pro-apoptotic factors from mitochondria; these pathways are shown in simplified form in Figure 2.

Figure 1. The caspase family.



The phylogenetic relationship of caspase family members is shown, together with their preferred substrate sequence (*X* can be any amino acid). By phylogenetic relationship, the caspases divide into three groups, with caspases 1, 4, 5, 11, 12 and 14 in one group, caspases 3, 6, 7, 8, 9 and 10 in another, and caspase 2 in a separate third group. When organized by preferred substrate, the caspases fall into groups which are more aligned with their function. Thus, caspases 1, 4 and 5 form one group and these enzymes are thought to be more involved in the inflammatory response than in apoptotic execution. Caspases 6, 8, 9 and 10 form a second group; caspases 8 and 9 are involved more in the initiation of apoptosis, cleaving and activating other downstream caspases. Caspases 2, 3 and 7 are the executioners of apoptosis, cleaving a wide range of cellular proteins involved in cytoskeleton maintenance and cellular homeostasis to bring about apoptosis.

Figure 2. The caspase cascade.



Primary pathways of caspase activation. Two major pathways are involved in activation of the apoptotic caspase cascade. The first is triggered by signals extrinsic to the cell through ligation of death receptors such as Fas or tumor necrosis factor-α (TNFα) receptor. Ligation of these receptors recruit a number of adaptor molecules to the intracellular domain of the receptor, which culminate in activation of the initiator caspase 8 [5,87]. This caspase, in turn, cleaves executioner caspases such as caspase 3, which cleave a wide range of intracellular substrates to effect the apoptotic response [88-90]. The second major pathway which activates the caspase cascade is an intrinsic cellular response to a range of stressors, and involves release of a number of pro-apoptotic factors from the mitochondrion [91]; there is cross-talk between the two pathways at the level of caspase 8. Mitochondria are involved in apoptotic regulation through the release of pro-apoptotic factors into the cytoplasm. The best characterized of these factors is cytochrome C [92], which can be released either following a decrease in mitochondrial membrane potential and opening of the mitochondrial permeability transition pore [93-95], or through pores formed by pro-apoptotic Bcl-2 family members, such as Bax [96-99]. Upon release into the cytoplasm, cytochrome C forms a complex designated the apoptosome with dATP and apoptosis activating factor-1 (APAF-1). This complex cleaves and activates procaspase 9, which in turn, activates caspase 3 and thus brings about the apoptotic response [100,101]. Both of the caspase cascade pathways converge upon activation of executioner caspases, of which the best characterized is caspase 3.

Caspase inhibitors

The differing substrate specificities of the caspase family members has allowed the development of a number of peptide inhibitors [13-18]. Caspases have a near absolute requirement for an aspartic acid residue in the S1 subsite. Substitutions in the P4 positions confer a degree of specificity, with caspases 1, 4, 5 and 13 preferring a hydrophobic residue, caspases 2, 3 and 7 requiring aspartic acid, and caspases 6, 8, 9 and 10 requiring a branched aliphatic residue (Figure 1). Peptide inhibitors contain a tetrapeptide which binds to the catalytic cysteine of the active caspase tetramer and prevents substrate cleavage. The inhibitors can be separated into reversible or irreversible inhibitors based on modifications to the peptide. Irreversible inhibitors are α-substituted ketones with the structure peptide-CO-CH_2-X, with X being either a halide (eg, fluoromethylketone (FMK), chloromethylketone (CMK)) or similar. Reversible inhibitors are aldehyde, ketone or nitrile modified peptides. The most commonly used caspase inhibitor is benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethylketone (Z-VAD-FMK); this is a poly-caspase inhibitor, though it does not inhibit all members of the family, a notable exception being caspase 2. Inhibitors based on the preferred cleavage sequence for specific caspases include Asp-Glu-Val-Asp (DEVD) for caspase 3, Tyr-Val-Ala-Asp (YVAD) or Trp-Glu-His-Asp (WEHD) for caspase 1, Leu-Glu-His-Asp (LEHD) for caspase 9, and Ile-Glu-Thr-Asp (IETD) for caspase 8. It is essential to note that while these inhibitors are based upon the cleavage sequence for specific caspases, they have little or no selectivity for individual caspases. The LEHD sequence, for example, also inhibits other activator caspases such as 8 and 6, and possibly also some group 1 caspases. At the high concentrations typically used in reported studies, there is likely to be very little selectivity with these inhibitors, certainly across related groups of caspases.

A number of companies have been actively developing small molecule caspase inhibitors. The current situation with regard to development of novel caspase inhibitors has

recently been the subject of two excellent reviews, and so will not be covered in detail in this review [19,20]. Merck Frosst, Warner Lambert and Idun Pharmaceuticals have developed a series of caspase inhibitors, a number of which are claimed to have efficacy in *in vivo* models of neurodegeneration [20••]; GlaxoSmithKline has developed compounds with efficacy in *in vitro* models of neurodegeneration as well as a novel class of non-peptide isatin sulfonamide derivative selective caspase 3/7 inhibitors which do not bind in the S1 aspartic acid binding pocket [21]. These compounds have greater selectivity than the classical peptide inhibitors and thus may be more useful in dissecting the caspase pathways; as yet, however, they have not been tested in models of neurodegeneration. Vertex Pharmaceuticals has developed a number of series of small molecule caspase inhibitors. One of these compounds, VX-740 (Pralnacasan), an orally available caspase 1 inhibitor, is being developed by Aventis Pharma for osteoarthritis and is currently in phase II clinical trials. This is the first caspase inhibitor to enter the clinic [20••]. No published studies have evaluated effects of this compound in neurodegenerative models.

With all classical peptide inhibitors and most of the novel inhibitors, poor brain permeability has limited their usefulness *in vivo*. In most studies, the compounds require administration intracerebroventricularly - development of blood-brain barrier permeable compounds is thus important if caspase inhibitors are to be used *in vivo*. A range of blood-brain barrier permeable compounds has been synthesized by Idun Pharmaceuticals. A further complication in interpreting these studies is the extent to which the caspases must be inhibited to prevent all activity. In both *in vivo* and *in vitro* models, a sustained inhibition of more than 95% of caspase activity is required to fully prevent cleavage of apoptotic substrates; in *in vivo* models, continuous intravenous infusion of caspase inhibitor is required to reach these levels [DW Nicholson, personal communication]. Thus, it is difficult to interpret studies in which one intracerebroventricular administration of caspase inhibitor is made, as it is unclear for how long and to what extent caspases are inhibited, and also for how long and to what extent caspases require to be inhibited for a full functional recovery. This may particularly be a problem with peptide caspase inhibitors given the lack of selectivity associated with these compounds.

A number of endogenous cellular or virally encoded direct caspase inhibitors also exist. Cellular caspase inhibitors include the inhibitor of apoptosis family (IAP) of proteins; six members of this family have been identified to date, including neuronal apoptosis inhibitory protein (NAIP) and X-chromosome linked inhibitor of apoptosis (XIAP). All members of this family inhibit processing of pro-caspase 9 and thus prevent activation of executioner caspases, as well as directly inhibiting caspase 3. Two virally encoded caspase inhibitors are cytokine response modifier A (crm-A) from cowpox virus, which inhibits all caspases apart from 3, 6 and 7, and p35 from baculovirus which inhibits caspases 1, 2, 3 and 4 (reviewed in [22]). Genetic manipulation of these factors has been used in *in vivo* models of neurodegeneration to examine the effects of caspase inhibition. Another means of blocking the activation of the caspase cascade is to target the release of pro-apoptotic factors from the mitochondrion. Release of cytochrome C through pores formed by Bax (see Figure 2) can be blocked by overexpression of anti-apoptotic Bcl-2 family members, and a number of compounds, such as cyclosporin A and bongkrekic acid, block the opening of the mitochondrial permeability transition pore; this provides another means of examining the caspase cascade.

Caspases in neurodegeneration

Apoptotic cell death and caspase activation have been reported in a wide range of neurodegenerative conditions [23,24••], including chronic neurodegenerative conditions such as Alzheimer's disease (AD) [25-27] and Parkinson's disease (PD) [28,29], along with acute conditions such as cerebral ischemia; for most of these conditions, however, there is debate over the mechanisms of cell death. This review will focus on the evidence for neuroprotection by caspase inhibition in PD and cerebral ischemia as chronic and acute neurodegenerative conditions, respectively.

Caspases and Parkinson's disease

PD is a movement disorder characterized by tremor, rigidity and bradykinesia. The pathological hallmarks of PD are a degeneration of dopaminergic neurons in the brain; the movement deficits are primarily caused by the degeneration of the nigrostriatal dopaminergic neurons. Apoptotic cells have been demonstrated within the *substantia nigra* in Parkinsonian brain, and immunocytochemical studies have demonstrated expression of the active forms of caspases 2, 3, 8 and 9 [30,31]. However, conflicting reports have also been published, in which no evidence of apoptotic cell death nor active caspase expression were observed [32••]. These conflicting data are likely due to a combination of factors. By the time PD is diagnosed, the majority of cells in the substantia nigra have already been lost. Apoptosis is a dynamic process and apoptotic cells are rapidly scavenged; identifying a small population of degenerating apoptotic cells within an already reduced population may therefore be problematic. Secondly, the drugs used to treat PD, such as L-DOPA, are themselves suggested to exert toxicity for dopaminergic neurons over prolonged treatment times. Given these problems, data from PD models may give a clearer insight into the protective potential of caspase inhibition in PD.

A number of animal models of PD have been developed. These include toxin models, notably 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [33-37] and its neurotoxic metabolite 1-methyl-4-phenylpyridinium (MPP^+) [38-42] and 6-hydroxydopamine (6-OHDA) treatment. In the MPTP model of PD, immunohistological studies have demonstrated expression of active caspases 3, 8 and 9 in the *substantia nigra* of the mouse [30,43-45]. In MPP^+-treated dopaminergic neurons from the ventral mesencephalon *in vitro*, there is evidence that caspases are activated; inhibition of caspases using the polycaspase inhibitor Z-VAD-FMK is neuroprotective, sparing around 80 to 90% of dopaminergic neurons [46,47]. Peptide caspase inhibitors have been tested in this model; *in vitro*, peptide inhibitors based on the preferred cleavage sites of caspases 2, 3, 8 and 9 have been shown to have neuroprotective effect, though the caspase 8 inhibitor required elevated glucose in the medium [48]. A highly selective non-peptide caspase 3 inhibitor developed by Merck Frosst Canada & Co, M-791 [49], was shown to prevent MPP^+ toxicity *in vitro* [47]. Inhibition of caspase 1-

like proteases using Z-YVAD-FMK was ineffective in two studies [47,50•]. Other groups have, however, failed to find evidence for protection of MPP^+-treated cells by caspase inhibition [51,52], and it is likely that the culture conditions used are critical in determining the response of cells [44]. In 6-OHDA toxicity *in vitro*, caspase inhibition prevents the apoptotic component of toxicity [51,52]. The cells spared with caspase inhibition, however, may not be fully functional in either model. In both toxin models, caspase inhibition failed to fully spare neurites or restore uptake of tritiated dopamine [47,53,54•]. This may reflect either an alternate mechanism causing neuritic degeneration and preventing functional recovery or incomplete blockade of caspase activity. Further studies should clarify this.

In vivo, fewer studies have been carried out with caspase inhibitors. The poly-caspase inhibitor Z-VAD-FMK is reported to attenuate toxicity of 6-OHDA [55] and MPTP [54•] *in vivo*. Much of the evidence that caspase inhibition may be neuroprotective originated from transgenic animals. Dopaminergic neurons from mice overexpressing anti-apoptotic proteins Bcl-2 [56,57], XIAP [54•] or p35 [45], are resistant to MPTP-mediated cell death. XIAP overexpression alone was not sufficient to spare the dopaminergic innervation to the striatum in the absence of additional trophic support [54•]. Cell lines expressing XIAP are also resistant to 6-OHDA toxicity [53] and adenoviral-mediated NAIP expression protects the rat nigrostriatal system from intrastriatal 6-OHDA administration [58]. Caspase 1 null mutant animals demonstrate reduced MPTP toxicity [59]; it is unclear why caspase 1 blockade should be neuroprotective from MPTP toxicity *in vivo* but not MPP^+ toxicity *in vitro*. Mice overexpressing the c-jun-N-terminal kinase inhibitory protein JIP-1 show reduced MPTP toxicity and reduced activation of caspase 3 [60].

Thus, caspases are implicated in both PD and in models of the condition, and caspase inhibition is neuroprotective in these models. There is, however, less effect on sparing of neurites than neuronal somata both *in vivo* and *in vitro* with the currently available inhibitors, and it is unclear how effective caspase inhibition might be as a longer term therapeutic strategy.

Caspases in cerebral ischemia

In cerebral ischemia, there is a central core of necrotic cell death; this is surrounded by a zone, the ischemic penumbra, where the cell death is delayed and may be apoptotic. The first report of neuroprotection by caspase inhibition in cerebral ischemia was published; in this study, the caspase inhibitor Z-VAD-DCB decreased infarct volume following permanent middle cerebral artery occlusion (MCAO) [61••]. Z-VAD-FMK has since been reported to be more potent than other selective peptide caspase inhibitors [62], and it and another polycaspase inhibitor, Boc-Asp-FMK, have also been shown to be effective in a number of models of ischemia [63,64]. Evidence for a role of caspase 1 came from studies in transgenic mice. Both mice lacking the caspase 1 enzyme [65,66] and mice expressing a dominant negative caspase 1 [67,68] showed protection from ischemic damage in permanent MCAO and transient MCAO with 24 h reperfusion, respectively. Inhibition of caspase 1-like proteases using Z-YVAD-CMK in permanent MCAO prevents both apoptosis and inflammatory mechanisms [69]. A novel peptidomimetic caspase 1 inhibitor developed by Warner-Lambert also attenuates lesion size in transient MCAO in mice [20••]. It seems likely that the neuroprotective effect of caspase 1 inhibition may be mediated by both prevention of apoptosis and prevention of interleukin-1β production, as interleukin-1β has been shown to exacerbate ischemic damage [70].

A number of studies have also shown neuroprotection by caspase 3-like protease inhibition. The peptide inhibitor Z-DEVD-FMK has been shown to prevent delayed cell death in the hippocampus following transient global ischemia [63] and MCAO [62]; the effect was greatest when the inhibitor was administered prior to ischemia, but was still apparent when Z-DEVD-FMK treatment began, following ischemia [71]. In addition to peptide inhibitors, novel small molecule caspase inhibitors have been tested in ischemia models. The peptidomimetic caspase inhibitors IDN-5370 and IDN-7866 (Idun Pharmaceuticals Inc) protected cells for up to 28 days following permanent MCAO when administered intracerebroventricularly; IDN-7866 also protected against permanent and transient MCAO after 24 h when administered intravenously [72•]. Peptidomimetic caspase 3 inhibitors developed by Merck Frosst Canada & Co attenuate damage following 45 min regional cerebral ischemia and 3 h reperfusion; attenuation of damage following MCAO was observed with novel non-peptide caspase 3 inhibitors [20••]. This group have also recently demonstrated that caspase 3 inhibition with a highly selective novel inhibitor M-826 (Merck Frosst Canada & Co) blocked caspase 3 activation and delayed cell death in a neonatal hypoxic-ischemic brain injury model, but did not prevent activation of calpain and caspase 2 processing in the early post-lesion period [73].

As well as caspase inhibitory compounds, studies using overexpression of IAPs show neuroprotective effect. Adenoviral overexpression of NAIP [74] or XIAP [75] attenuated neuronal loss following ischemia; in the latter study [75], behavioral deficits were also attenuated. Caspase inhibition may be synergistic with other potential therapies in ischemia, notably the NMDA receptor antagonist MK-801 (Merck & Co Inc) [76].

Thus, the studies using caspase inhibitors indicate a potential therapeutic role for small molecule caspase inhibitors in cerebral ischemia. There is, however, some controversy over caspase inhibition as a valid therapeutic strategy in ischemia. In one study, caspase inhibition spared CA1 neurons of the hippocampus, but did not restore deficits in hippocampal long-term potentiation, indicating a possible lack of functional protection [77], though this may reflect a lack of complete enzyme inhibition by the compound used. Development of more selective and potent compounds should allow for a clearer evaluation of the ability of caspase inhibition to restore functional capability in models of ischemia. For a recent review of the evidence against caspase inhibition as a therapeutic strategy in ischemia, see [78•].

Limitations to caspase neuroprotection

Caspase inhibition is clearly neuroprotective in models of both chronic and acute neurodegenerative processes.

Ultimately, however, the question must be addressed whether caspase inhibitors are likely to be candidate drugs for neurodegenerative conditions. Caspase inhibition has been shown to be neuroprotective and to improve behavioral performance *in vivo* even with long delays post-insult. *In vitro*, however, there is evidence that caspase inhibition may not fully protect cells. Inhibition of caspases in apoptotic models *in vitro* is reported to cause a switch to necrotic death in cerebellar granule neurons [79], and to lead to activation of forms of cell death distinct from both apoptosis and necrosis in nerve growth factor-deprived sympathetic neurons [80-82]. A recent publication, however, demonstrates that caspase activation can lead directly to necrosis through cleavage and inactivation of the plasma membrane calcium pump [83], indicating that there may be links between apoptosis and necrosis at the level of caspases. The evidence that caspase inhibition can switch the type of cell death from apoptosis to another mechanism in neurodegenerative conditions is reviewed in [84]. One further possible drawback is the functionality of cells protected by caspase inhibitors; the cells spared must be functional and innervate their target. A recent review has examined the question of functional recovery with caspase inhibition, and raises the precautionary note that sparing a population of 'undead' but non-functional cells in the brain may have adverse consequences [85]. It may be possible that cells spared by caspase inhibitors might require additional trophic support for long-term survival and functionality, though, as discussed, the limitations of the available inhibitors and the requirement for sustained blockade of caspase activity make interpretation of these studies difficult.

These caveats primarily apply to the situation in chronic neurodegenerative conditions, where the cellular environment is poor and the cells themselves are likely to be compromised and to lack the trophic support of a normal cellular environment. It is less likely to be an issue in acute neurodegenerative conditions, where a normal cellular environment is more likely to be restored with time [86].

Conclusions

Caspases have been implicated in many neurodegenerative conditions, and caspase inhibition is neuroprotective. In this review, the evidence for caspase inhibition as a neuroprotective strategy in a chronic neurodegenerative condition, PD, and an acute condition, cerebral ischemia, has been discussed. It is clear that inhibition of caspases prevents the apoptotic death of neurons in models of both of these conditions; it is not clear from studies with the available inhibitors to what extent caspase inhibition alone will prevent loss of neuronal functionality. Development of novel, more potent and selective caspase inhibitors should allow these questions to be answered. In chronic neurodegenerative conditions, there is typically a loss of neurites as well as neuronal somata; there are also likely to be serious disruptions to the trophic environment of the cells. Furthermore, given the chronic nature of these diseases, a successful caspase inhibitory strategy would have to inhibit caspase function for a period of years; as apoptosis is a fundamental physiological process in all tissues of the body, this may in itself have serious repercussions. In acute neurodegeneration, caspase inhibition may be a more viable strategy; if cells can be maintained until the normal trophic environment is restored, much of the secondary cell loss may be prevented.

Prevention of apoptotic cell death through caspase inhibition is a possible strategy for a wide range of neurodegenerative disorders; it is possible, though, that in chronic conditions it may not in itself be a viable therapeutic strategy. For acute neurodegeneration, on the other hand, caspase inhibition may indeed be a viable approach for preventing neuronal loss.

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References

1. Stennicke HR, Salvesen GS: **Properties of the caspases.** *Biochim Biophys Acta* (1998) **1387**:17-31.

2. Jacobson MD, Weil M, Raff MC: **Programmed cell death in animal development.** *Cell* (1997) **88**:347-354.

3. Salvesen GS, Dixit VM: **Caspases: Intracellular signaling by proteolysis.** *Cell* (1997) **91**:443-446.

4. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR: **The *C elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β-converting enzyme.** *Cell* (1993) **75**:641-652.

5. Muzio M, Chinnaiyan AM, Kischkel FC, O Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M *et al*: **FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex.** *Cell* (1996) **85**:817-827.

6. Muzio M, Salvesen GS, Dixit VM: **FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens.** *J Biol Chem* (1997) **272**:2952-2956.

7. Ni B, Wu X, Du Y, Su Y, Hamilton BE, Rockey PK, Rosteck PJ, Poirier GG, Paul SM: **Cloning and expression of a rat brain interleukin-1β-converting enzyme (ICE)-related protease (IRP) and its possible role in apoptosis of cultured cerebellar granule neurons.** *J Neurosci* (1997) **17**:1561-1569.

8. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA: **Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis.** *Nature* (1995) **376**:37-43.

•• *Demonstration of caspase 3 involvement in apoptosis.*

9. Orth K, O'Rourke K, Salvesen GS, Dixit VM: **Molecular ordering of apoptotic mammalian CED-3/ICE-like proteases.** *J Biol Chem* (1996) **271**:20977-20980.

10. Orth K, Chinnaiyan AM, Garg M, Froelich CJ, Dixit VM: **The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A.** *J Biol Chem* (1996) **271**:16443-16446.

11. Alnemri ES, Livingston DJ, Nicholson DW, Salvesen GS, Thornberry NA, Wong WW, Yuan J: **Human ICE/CED-3 protease nomenclature.** *Cell* (1995) **87**:171.

• *New designation as caspases for ICE-like proteases.*

12. Nicholson DW, Thornberry NA: **Caspases: Killer proteases.** *Trends Biochem Sci* (1997) **22**:299-306.

•• *Good review of caspase structure and substrate specificity.*

13. Zhou Q, Snipas S, Orth K, Muzio M, Dixit VM, Salvesen GS: **Target protease specificity of the viral serpin CrmA. Analysis of five caspases.** *J Biol Chem* (1997) **272**:7797-7800.

14. Talanian RV, Quinlan C, Trautz S, Hackett MC, Mankovich JA, Banach D, Ghayur T, Brady KD, Wong WW: **Substrate specificities of caspase family proteases.** *J Biol Chem* (1997) **272**:9677-9682.

15. Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA: **Inhibition of human caspases by peptide-based and macromolecular inhibitors.** *J Biol Chem* (1998) **273**:32608-32613.

16. Rano TA, Timkey T, Peterson EP, Rotonda J, Nicholson DW, Becker JW, Chapman KT, Thornberry NA: **A combinatorial approach for determining protease specificities: Application to interleukin-1β converting enzyme (ICE).** *Chem Biol* (1997) **4**:149-155.

17. Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW: **A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis.** *J Biol Chem* (1997) **272**:17907-17911.

18. Margolin N, Raybuck SA, Wilson KP, Chen W, Fox T, Gu Y, Livingston DJ: **Substrate and inhibitor specificity of interleukin-1β-converting enzyme and related caspases.** *J Biol Chem* (1997) **272**:7223-7228.

19. Denner L: **Caspases in apoptotic death.** *Exp Opin Invest Drugs* (1999) **8**:37-50.

20. Ashwell S: **Caspases: Recent advances in small molecule inhibitors.** *Exp Opin Ther Patents* (2001) **11**:1593-1603.

•• *Excellent review of caspase patent literature up to April 2001.*

21. Lee D, Long SA, Adams JL, Chan G, Vaidya KS, Francis TA, Kikly K, Winkler JD, Sung C-M, Debouck C, Richardson S *et al*: **Potent and selective nonpeptide inhibitors of caspases 3 and 7 inhibit apoptosis and maintain cell functionality.** *J Biol Chem* (2000) **275**:16007-16014.

22. Nicholson DW, Thornberry NA: **Caspases: Killer proteases.** *Trends Biochem Sci* (1997) **22**:299-306.

23. Nicotera P: **Caspase requirement for neuronal apoptosis and neurodegeneration.** *IUBMB Life* (2000) **49**:421-425.

24. Braun JS, Tuomanen EI, Cleveland JL: **Neuroprotection by caspase inhibitors.** *Exp Opin Invest Drugs* (1999) **8**:1599-1610.

•• *Good, broad-based review of the evidence for caspase inhibitor neuroprotection in a number of neurodegenerative conditions.*

25. Cotman CW, Su JH: **Mechanisms of neuronal death in Alzheimer's disease.** *Brain Pathol* (1996) **6**:493-506.

26. Su JH, Zhao M, Anderson AJ, Srinivasan A, Cotman CW: **Activated caspase-3 expression in Alzheimer's and aged control brain: Correlation with Alzheimer pathology.** *Brain Res* (2001) **898**:350-357.

27. Su JH, Anderson AJ, Cummings BJ, Cotman CW: **Immunohistochemical evidence for apoptosis in Alzheimer's disease.** *Neuroreport* (1994) **5**:2529-2533.

28. Mochizuki H, Goto K, Mori H, Mizuno Y: **Histochemical detection of apoptosis in Parkinson's disease.** *J Neurol Sci* (1996) **137**:120-123.

29. Andersen JK: **Does neuronal loss in Parkinson's disease involve programmed cell death?** *Bioessays* (2001) **23**:640-646.

30. Hartmann A, Hunot S, Michel PP, Muriel MP, Vyas S, Faucheux BA, Mouatt-Prigent A, Turmel H, Srinivasan A, Ruberg M, Evan GI *et al*: **Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease.** *Proc Natl Acad Sci USA* (2000) **97**:2875-2880.

31. Viswanath V, Wu YQ, Boonplueang R, Chen S, Stevenson FF, Yantiri F, Yang LC, Beal MF, Andersen JK: **Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease.** *J Neurosci* (2001) **21**:9519-9528.

32. Schulz JB, Gerhardt E: **Apoptosis: Its relevance to Parkinson's disease.** *Clin Neurosci Research* (2001) **1**:427-433.

•• *Good review of the evidence for apoptosis in Parkinson's disease.*

33. Ballard PA, Tetrud JW, Langston JW: **Permanent human parkinsonism due to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): Seven cases.** *Neurology* (1985) **35**:949-956.

34. Heikkila RE, Hess A, Duvoisin RC: **Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) in the mouse: Relationships between monoamine oxidase, MPTP metabolism and neurotoxicity.** *Life Sci* (1985) **36**:231-236.

35. Heikkila RE, Manzino L, Cabbat FS, Duvoisin RC: **Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and several of its analogues on the dopaminergic nigrostriatal pathway in mice.** *Neurosci Lett* (1985) **58**:133-137.

36. Langston JW, Langston EB, Irwin I: **MPTP-induced parkinsonism in human and non-human primates - clinical and experimental aspects.** *Acta Neurol Scand Suppl* (1984) **100**:49-54.

37. Langston JW, Irwin I, Langston EB, Forno LS: **Pargyline prevents MPTP-induced parkinsonism in primates.** *Science* (1984) **225**:1480-1482.

38. Irwin I, Langston JW: **Selective accumulation of MPP^+ in the *substantia nigra*: A key to neurotoxicity?** *Life Sci* (1985) **36**:207-212.

39. Langston JW, Irwin I, Langston EB, Forno LS: **1-Methyl-4-phenylpyridinium ion (MPP^+): Identification of a metabolite of MPTP, a toxin selective to the *substantia nigra*.** *Neurosci Lett* (1984) **48**:87-92.

40. Mochizuki H, Nakamura N, Nishi K, Mizuno Y: **Apoptosis is induced by 1-methyl-4-phenylpyridinium ion (MPP^+) in ventral mesencephalic-striatal co-culture in rat.** *Neurosci Lett* (1994) **170**:191-194.

41. Mytilineou C, Cohen G, Heikkila RE: **1-Methyl-4-phenylpyridine (MPP+) is toxic to mesencephalic dopamine neurons in culture.** *Neurosci Lett* (1985) **57**:19-24.

42. Sanchez Ramos J, Barrett JN, Goldstein M, Weiner WJ, Hefti F: **1-Methyl-4-phenylpyridinium (MPP^+) but not 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) selectively destroys dopaminergic neurons in cultures of dissociated rat mesencephalic neurons.** *Neurosci Lett* (1986) **72**:215-220.

43. Turmel H, Hartmann A, Parain K, Douhou A, Srinivasan A, Agid Y, Hirsch EC: **Caspase-3 activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice.** *Mov Disord* (2001) **16**:185-189.

44. Hartmann A, Troadec JD, Hunot S, Kikly K, Faucheux BA, Mouatt-Prigent A, Ruberg M, Agid Y, Hirsch EC: **Caspase-8 is an effector in apoptotic death of dopaminergic neurons in Parkinson's disease, but pathway inhibition results in neuronal necrosis.** *J Neurosci* (2001) **21**:2247-2255.

45. Viswanath V, Wu Y, Boonplueang R, Chen S, Stevenson FF, Yantiri F, Yang L, Beal MF, Andersen JK: **Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease.** *J Neurosci* (2001) **21**:9519-9528.

46. Dodel RC, Du Y, Bales KR, Ling ZD, Carvey PM, Paul SM: **Peptide inhibitors of caspase-3-like proteases attenuate 1-methyl-4-phenylpyridinum-induced toxicity of cultured fetal rat mesencephalic dopamine neurons.** *Neuroscience* (1998) **86**:701-707.

47. Bilsland J, Roy S, Xanthoudakis S, Nicholson DW, Han Y, Grimm E, Hefti F, Harper SJ: **Caspase inhibitors attenuate 1-methyl-4-phenylpyridinium toxicity in primary cultures of mesencephalic dopaminergic neurons.** *J Neurosci* (2002) **22**:2637-2649.

48. Hartmann A, Troadec JD, Hunot S, Kikly K, Faucheux BA, Mouatt-Prigent A, Ruberg M, Agid Y, Hirsch EC: **Caspase-8 is an effector in apoptotic death of dopaminergic neurons in Parkinson's disease, but pathway inhibition results in neuronal necrosis.** *J Neurosci* **21**:2247-2255.

49. Hotchkiss RS, Chang KC, Swanson PE, Tinsley KW, Hui JJ, Klender P, Xanthoudakis S, Roy S, Black C, Grimm E, Aspiotis R *et al*: **Caspase inhibitors improve survival in sepsis: A critical role of the lymphocyte.** *Nat Immunol* (2000) **1**:496-501.

50. Dodel RC, Du Y, Bales KR, Ling ZD, Carvey PM, Paul SM: **Peptide inhibitors of caspase-3-like proteases attenuate 1-methyl-4-phenylpyridinum-induced toxicity of cultured fetal rat mesencephalic dopamine neurons.** *Neuroscience* (1998) **86**:701-707.

• *First demonstration that peptide caspase inhibitors protect mesencephalic dopaminergic neurons from MPP^+ toxicity.*

51. Lotharius J, Dugan LL, O'Malley KL: **Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons.** *J Neurosci* (1999) **19**:1284-1293.

52. Choi WS, Yoon SY, Oh TH, Choi EJ, O'Malley KL, Oh YJ: **Two distinct mechanisms are involved in 6-hydroxydopamine- and MPP$^+$-induced dopaminergic neuronal cell death: Role of caspases, ROS, and JNK.** *J Neurosci Res* (1999) **57**:86-94.

53. von Coelln R, Kugler S, Bahr M, Weller M, Dichgans J, Schulz JB: **Rescue from death but not from functional impairment: Caspase inhibition protects dopaminergic cells against 6-hydroxydopamine-induced apoptosis but not against the loss of their terminals.** *J Neurochem* (2001) **77**:263-273.

54. Eberhardt O, Coelln RV, Kugler S, Lindenau J, Rathke-Hartlieb S, Gerhardt E, Haid S, Isenmann S, Gravel C, Srinivasan A, Bahr M *et al*: **Protection by synergistic effects of adenovirus-mediated X-chromosome-linked inhibitor of apoptosis and glial cell line-derived neurotrophic factor gene transfer in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease.** *J Neurosci* (2000) **20**:9126-9134.

• *Demonstration that caspase inhibition by XIAP overexpression spares cell somata in the MPTP mouse PD model, but additional trophic support is required to spare striatal innervation.*

55. Cutillas B, Espejo M, Gil J, Ferrer I, Ambrosio S: **Caspase inhibition protects nigral neurons against 6-OHDA-induced retrograde degeneration.** *Neuroreport* (1999) **10**:2605-2608.

56. Yang L, Matthews RT, Schulz JB, Klockgether T, Liao AW, Martinou JC, Penney JB Jr, Hyman BT, Beal MF: **1-Methyl-4-phenyl-1,2,3,6-tetrahydropyride neurotoxicity is attenuated in mice overexpressing Bcl-2.** *J Neurosci* (1998) **18**:8145-8152.

57. Offen D, Beart PM, Cheung NS, Pascoe CJ, Hochman A, Gorodin S, Melamed E, Bernard R, Bernard O: **Transgenic mice expressing human Bcl-2 in their neurons are resistant to 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity.** *Proc Natl Acad Sci USA* (1998) **95**:5789-5794.

58. Crocker SJ, Wigle N, Liston P, Thompson CS, Lee C, Xu DG, Roy S, Nicholson DW, Park DS, MacKenzie A, Korneluk RG, Robertson GS: **NAIP protects the nigrostriatal dopamine pathway in an intrastriatal 6-OHDA rat model of Parkinson's disease.** *Eur J Neurosci* (2001) **14**:391-400.

59. Klevenyi P, Andreassen O, Ferrante RJ, Schleicher JR Jr, Friedlander RM, Beal MF: **Transgenic mice expressing a dominant negative mutant interleukin-1β converting enzyme show resistance to MPTP neurotoxicity.** *Neuroreport* (1999) **10**:635-638.

60. Xia XG, Harding T, Weller M, Bieneman A, Uney JB, Schulz JB: **Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease.** *Proc Natl Acad Sci USA* (2001) **98**:10433-10438.

61. Loddick SA, MacKenzie A, Rothwell NJ: **An ICE inhibitor, (Z)-VAD-DCB attenuates ischaemic brain damage in the rat.** *Neuroreport* (1996) **7**:1465-1468.

•• *First demonstration of neuroprotection by caspase inhibition in an ischemic model.*

62. Hara H, Friedlander RM, Gagliardini V, Ayata C, Fink K, Huang Z, Shimizu-Sasamata M, Yuan J, Moskowitz MA: **Inhibition of interleukin 1β converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage.** *Proc Natl Acad Sci USA* (1997) **94**:2007-2012.

63. Cheng Y, Deshmukh M, D'Costa A, Demaro JA, Gidday JM, Shah A, Sun Y, Jacquin MF, Johnson EM, Holtzman DM: **Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury.** *J Clin Invest* (1998) **101**:1992-1999.

64. Wiessner C, Sauer D, Alaimo D, Allegrini PR: **Protective effect of a caspase inhibitor in models for cerebral ischemia *in vitro* and *in vivo*.** *Cell Mol Biol* (2000) **46**:53-62.

65. Schielke GP, Yang GY, Shivers BD, Betz AL: **Reduced ischemic brain injury in interleukin-1β converting enzyme-deficient mice.** *J Cereb Blood Flow Metab* (1998) **18**:180-185.

66. Liu XH, Kwon D, Schielke GP, Yang GY, Silverstein FS, Barks JD: **Mice deficient in interleukin-1 converting enzyme are resistant to neonatal hypoxic-ischemic brain damage.** *J Cereb Blood Flow Metab* (1999) **19**:1099-1108.

67. Friedlander RM, Gagliardini V, Hara H, Fink KB, Li W, Macdonald G, Fishman MC, Greenberg AH, Moskowitz MA, Yuan J: **Expression of a dominant negative mutant of interleukin-1β converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury.** *J Exp Med* (1997) **185**:933-940.

68. Hara H, Fink K, Endres M, Friedlander RM, Gagliardini V, Yuan J, Moskowitz MA: **Attenuation of transient focal cerebral ischemic injury in transgenic mice expressing a mutant ICE inhibitory protein.** *J Cereb Blood Flow Metab* (1997) **17**:370-375.

69. Rabuffetti M, Sciorati C, Tarozzo G, Clementi E, Manfredi AA, Beltramo M: **Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone induces long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines.** *J Neurosci* (2000) **20**:4398-4404.

70. Touzani O, Boutin H, LeFeuvre R, Parker L, Miller A, Luheshi G, Rothwell N: **Interleukin-1 influences ischemic brain damage in the mouse independently of the interleukin-1 type I receptor.** *J Neurosci* (2002) **22**:38-43.

71. Fink K, Zhu J, Namura S, Shimizu-Sasamata M, Endres M, Ma J, Dalkara T, Yuan J, Moskowitz MA: **Prolonged therapeutic window for ischemic brain damage caused by delayed caspase activation.** *J Cereb Blood Flow Metab* (1998) **18**:1071-1076.

72. Deckwerth TL, Adams LM, Wiessner C, Allegrini P, Rudin M, Sauter A, Hengerer B, Sayers RO, Rovelli G, Aja T, May R *et al*: **Long-term protection of brain tissue from cerebral ischemia by peripherally administered peptidomimetic caspase inhibitors.** *Drug Dev Res* (2001) **52**:579-586.

• *Peripheral administration of a novel caspase inhibitor developed by Idun Pharmaceuticals attenuates cerebral ischemic damage.*

73. Han BH, Xu D, Choi J, Han Y, Xanthoudakis S, Roy S, Tam J, Vaillancourt J, Colucci J, Siman R, Giroux A *et al*: **Selective, reversible caspase-3 inhibitor is neuroprotective and reveals distinct pathways of cell death after neonatal hypoxic-ischemic brain injury.** *J Biol Chem* (2002) **277**(33):30128-30136.

74. Xu DG, Crocker SJ, Doucet JP, St Jean M, Tamai K, Hakim AM, Ikeda JE, Liston P, Thompson CS, Korneluk RG, MacKenzie A, Robertson GS: **Elevation of neuronal expression of NAIP reduces ischemic damage in the rat hippocampus.** *Nat Med* (1997) **3**:997-1004.

75. Xu D, Bureau Y, McIntyre DC, Nicholson DW, Liston P, Zhu Y, Fong WG, Crocker SJ, Korneluk RG, Robertson GS: **Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus.** *J Neurosci* (1999) **19**:5026-5033.

76. Ma J, Endres M, Moskowitz MA: **Synergistic effects of caspase inhibitors and MK-801 in brain injury after transient focal cerebral ischaemia in mice.** *Br J Pharmacol* (1998) **124**:756-762.

77. Gillardon F, Kiprianova I, Sandkuhler J, Hossmann KA, Spranger M: **Inhibition of caspases prevents cell death of hippocampal CA1 neurons, but not impairment of hippocampal long-term potentiation following global ischemia.** *Neuroscience* (1999) **93**:1219-1222.

78. Loetscher H, Niederhauser O, Kemp J, Gill R: **Is caspase-3 inhibition a valid therapeutic strategy in cerebral ischemia?** *Drug Disc Today* (2001) **6**:671-680.

• *Review of evidence against caspase inhibition as a therapeutic strategy in cerebral ischemia.*

79. Harada J, Sugimoto M: **Inhibitors of interleukin-1β-converting enzyme-family proteases (caspases) prevent apoptosis without affecting decreased cellular ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in cerebellar granule neurons.** *Brain Res* (1998) **793**:231-243.

80. Tolkovsky AM, Xue L, Fletcher GC, Borutaite V: **Mitochondrial disappearance from cells: A clue to the role of autophagy in programmed cell death and disease?** *Biochimie* (2002) **84**:233-240.

81. Xue L, Fletcher GC, Tolkovsky AM: **Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis.** *Curr Biol* (2001) **11**:361-365.

82. Xue L, Fletcher GC, Tolkovsky AM: **Autophagy is activated by apoptotic signalling in sympathetic neurons: An alternative mechanism of death execution.** *Mol Cell Neurosci* (1999) **14**:180-198.

83. Schwab BL, Guerini D, Didszun C, Bano D, Ferrando-May E, Fava E, Tam J, Xu D, Xanthoudakis S, Nicholson DW, Carafoli E, Nicotera P: **Cleavage of plasma membrane calcium pumps by caspases: A link between apoptosis and necrosis.** *Cell Death Differ* (2002) **9**:818-831.

84. Leist M, Jaattela M: **Four deaths and a funeral: From caspases to alternative mechanisms.** *Nat Rev Mol Cell Biol* (2001) **2**:589-598.

85. Nicotera P, Leist M, Fava E, Berliocchi L, Volbracht C: **Energy requirement for caspase activation and neuronal cell death.** *Brain Pathol* (2000) **10**:276-282.

86. Holtzman DM, Deshmukh M: **Caspases: A treatment target for neurodegenerative disease?** *Nat Med* (1997) **3**:954-955.

87. Enari M, Hug H, Nagata S: **Involvement of an ICE-like protease in Fas-mediated apoptosis.** *Nature* (1995) **375**:78-81.

88. Muzio M, Salvesen GS, Dixit VM: **FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens.** *J Biol Chem* (1997) **272**:2952-2956.

89. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Litwack G, Alnemri ES: **Molecular ordering of the Fas-apoptotic pathway: The Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases.** *Proc Natl Acad Sci USA* (1996) **93**:14486-14491.

90. Schlegel J, Peters I, Orrenius S, Miller DK, Thornberry NA, Yamin TT, Nicholson DW: **CPP32/apopain is a key interleukin 1β converting enzyme-like protease involved in Fas-mediated apoptosis.** *J Biol Chem* (1996) **271**:1841-1844.

91. Susin SA, Zamzami N, Kroemer G: **Mitochondria as regulators of apoptosis: Doubt no more.** *Biochim Biophys Acta* (1998) **1366**:151-165.

92. Green DR, Reed JC: **Mitochondria and apoptosis.** *Science* (1998) **281**:1309-1312.

93. Yang JC, Cortopassi GA: **Induction of the mitochondrial permeability transition causes release of the apoptogenic factor cytochrome C.** *Free Radic Biol Med* (1998) **24**:624-631.

94. Ichas F, Mazat JP: **From calcium signaling to cell death: Two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state.** *Biochim Biophys Acta* (1998) **1366**:33-50.

95. Petronilli V, Penzo D, Scorrano L, Bernardi P, Di Lisa F: **The mitochondrial permeability transition, release of cytochrome C and cell death - Correlation with the duration of pore openings *in situ*.** *J Biol Chem* (2001) **276**:12030-12034.

96. Bossy-Wetzel E, Newmeyer DD, Green DR: **Mitochondrial cytochrome C release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization.** *EMBO J* (1998) **17**:37-49.

97. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X: **Prevention of apoptosis by Bcl-2: Release of cytochrome C from mitochondria blocked.** *Science* (1997) **275**:1129-1132.

98. Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K *et al.* **Inhibition of Bax channel-forming activity by Bcl-2.** *Science* (1997) **277**:370-372.

99. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD: **The release of cytochrome C from mitochondria: A primary site for Bcl-2 regulation of apoptosis.** *Science* (1997) **275**:1132-1136.

100. Liu X, Kim CN, Yang J, Jemmerson R, Wang X: **Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome C.** *Cell* (1996) **86**:147-157.

101. Pan G, Humke EW, Dixit VM: **Activation of caspases triggered by cytochrome C *in vitro*.** *FEBS Lett* (1998) **426**:151-154.