INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Université d'Ottawa • University of Ottawa

Novel Therapeutic Strategies for the Treatment of Parkinson's Disease

Stephen J. Crocker

This thesis is submitted in partial fulfillment

of the requirement for

the degree Doctor of Philosophy.

Department of Pharmacology.

University of Ottawa

September 22, 2000

Ottawa, Ontario, Canada

The question, O Me! So sad, recurring – What good amid these, O me, O life?

Answer that you are here – that life exists and identity,

That the powerful play goes on, and you may contribute a verse.

— *Walt Whitman (1819 – 1892)*

Dedication

This thesis is dedicated to my parents.

Acknowledgements

Foremost, I am indebted to Dr. George S. Robertson for affording me the opportunity to do this research and study under his guideance. I wish to thank him for taking the chance on me, teaching me when I needed to learn, listening when I had something to say, humbling me when I needed perspective, and advising me on life, love and the pursuit of knowledge.

As with all research, science is never a venture made in isolation, and as I began graduate studies I learned that a good team can mean good fortune. Accordingly, I met and interacted with many fine individuals, who I am grateful for their assistance and professionalism. These people include Dr. R.G. Korneluk, Dr. A.E. MacKenzie, Dr. A. Hakim, Dr. Y. Nakabeppu, Dr. D.G. Xu, Dr. C.S. Thompson, Dr. P. Liston, Dr. M. Holcik, N. Wigle, Y.Zhu, M. St-Jean, F. Ansari, A. Adeeko and N. Earl. I would also like to especially acknowledge the friendship and support of Chris Lee - together we've shared the trials and tribulations that come with experience during the past years.

I would also like to acknowledge the guidance and patience of two people, in particular, who have endured the past months with me while I wrote this thesis. First, Dr. (David) S. Park, your suggestions and advice are always welcome even though it may not always seem that way. And, Rosa Guzzo...were it not for your understanding, support, and invaluable assistance, this endeavour would have been immeasurably more difficult.

The research presented in this thesis was supported by several granting agencies; namely the Medical Research Council of Canada (MRC), the Ontario Neurotrauma (ONF), the Heart and Stroke Foundation of Ontario (HSFO), the Ontario Mental Health (OMH) Foundation and the Parkinson's disease Foundation of the USA.

Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder associated with the loss of dopamine neurons located in the substantia nigra pars compacta. Current pharmacological approaches for the treatment of PD are confounded by development of abnormal involuntary movements called dyskinesias, and offer limited long-term utility because they do not stop the disease progression. Accordingly, this thesis addressed two primary issues limiting the present treatments of Parkinson's disease: the molecular basis of dopamine receptor-related dyskinesias, and attenuation of dopamine neuron death.

Administration of dopamine receptor agonists to rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal pathway produce changes in the denervated striatum that enable subsequent exposure to dopamine agonists to elicit potentiated circling behaviour, a phenomenon called "priming". Priming is used as a model to study the molecular basis of dyskinesias. Here, I demonstrate that dopamine D1-receptor priming is associated with a profound elevation of the nuclear transcription factor FosB in the denervated striatum. Moreover, intrastriatal delivery of an antisense oligonucleotide to *fosB* effectively blocked the induction of striatal FosB by dopamine agonist administration and attenuated the circling response elicited by injection of a selective dopamine D1-receptor agonist 3 days later. These findings suggest that dopamine receptor mediated FosB expression in the striatum plays a role in priming. In addition, the results from this study suggest that *fosB* may be involved the intracellular events which are responsible for the development of dyskinesias following chronic dopamine replacement therapy.

The molecular processes which mediate the loss of nigral dopamine neurons in Parkinson's disease are not known. Recently, a novel family of mammalian proteins, called Inhibitor of Apoptosis (IAP) proteins, were cloned and shown to prevent cell death induced by a variety of cytotoxic factors. In two separate studies, I demonstrate that enhanced neuronal expression of two of these IAP proteins, neuronal apoptosis inhibitor protein (NAIP) and X-linked IAP (XIAP), prevents the death of nigral dopamine neurons following exposure to the dopaminergic neurotoxins. In rats, intrastriatal administration of recombinant adenoviruses containing NAIP (Ad.NAIP) was shown to prevent both the cellular and

behavioural deficits produced by intrastriatal administration of 6-OHDA when compared to adenovirus control (Ad.lacZ) lesioned animals. Secondly, I describe a novel strain of mice engineered to overexpress XIAP in neurons by using a neuron-specific enolase promoter (NSE-*xiap*). Moreover, I demonstrate that NSE-*xiap* mice were profoundly resistant to the deleterious effects of the dopaminergic neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Since administration of MPTP can produce behavioural and neuropathological deficits reminiscent of PD in humans, results from this study suggest that IAP proteins may have utility for the treatment and prevention of cell death in idiopathic PD. Taken together, these studies suggest that neuroprotective strategies based on enhanced neuronal expression of IAP proteins may have novel therapeutic potential for treating neurodegeneration associated with Parkinson's disease.

Table of Contents

List of Abbreviations

6-OHDA	6-hydroxydopamine
Ad	adenovirus
APO	apomorphine
APP	amyloid precursor protein
AS	antisense
CNS	central nervous system
c-Fos	cellular - follicular osteosacroma proto-oncogene
DA	dopamine (3-hydroxytyramine)
DAT	dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
fra	Fos related antigen
GABA	gamma amino butyric acid
HIAP-1	human IAP protein –1 (c-IAP2)
HIAP-2	human IAP protein-2 (c-IAP1)
HPLC	high performance liquid chromatography
HVA	homovanilic acid
IAP	Inhibitor of Apoptosis
IEG	immediate early gene
i.p	intraperitoneal
L-Dopa	L-3,4-dihydroxyphenylalanine/ Levodopa
LI	like-immunoreactivity
MFB	medial forebrain bundle
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTN	medial terminal nucleus of the optic tract
NAcc	Nucleus Accumbens

NAIP	Neuronal Apoptosis Inhibitor Protein
NSE	neuron-specific enolase promoter
PBS	phosphate buffered saline
PD	Parkinson's disease
RN	random oligonucleotide
ROS	reactive oxygen species
s.c.	subcutaneous
SMA	spinal muscular atrophy
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
TH	tyrosine hydroxylase
XIAP	X-linked Inhibitor of Apoptosis Protein

List of Figures

List of Tables

Preface

In accordance with the guidelines established by the School of Graduate Studies and Research (SGSR) of the University of Ottawa, the experimental findings presented in this thesis are comprised of published or submitted journal articles.

The experimental findings of this thesis are preceeded by an overview of the current state of understanding of Parkinson's disease (PD) in Chapter 1. Chapters 2 and 3 introduce and review the neuroanatomy of the basal ganglia and current theories of what causes Parkinson's disease, respectively. The subsequent chapters (4, 5, and 6) are three manuscripts that represent the two novel therapeutic strategies proposed in this thesis: attenuation of dopamine receptor-related motor complications associated with dopamine drug therapy, and the attenuation of nigral dopmaine neuron death in PD. Finally, the implications of the experimental findings in this thesis are discussed in the context of prospective therapies for Parkinson's disease (Chapter 7), and references cited in the introductory and summary sections are Chapter 8.

Each of the articles included in this thesis is prefaced with a Statement of Contributions of Collaborators, as required by the SGSR (University of Ottawa).

Chapter 1. Introduction to Parkinson's Disease

A Brief History of Parkinson's Disease

1817	Dr. James Parkinson described PD in his monograph entitled "An Essay on the Shaking Palsy", therein classifying the symptoms of PD as a single syndrome.
1867	Charcot and Ordenstein, at the Salpetriere in Paris, describe *paralysis agitans* and note macerated substantia nigra - thereby describing the histopathology of PD as distinct from other disease conditions.
1894	Blocq and Marinesco suggest the substantia nigra as the anatomical substrate of PD on basis of post-mortem analyses.
1919	Tretiakoff systematically describes pathology of SNc in PD, including description of Lewy bodies, mild gliosis and "cellular fallout".
1916-1926	*Encephalitis lethargica* pandemic. Emergence of post-encephalic Parkinsonism.
1957	Montagu discovers dopamine and identified dopamine as a neurotransmitter in mammalian brain homogenates
1959	Carlson and others delineate dopamine neuron populations in brain.
1960	Ehringer and Hornykiewicz report profound depletion of dopamine in PD.
1966	Goldstein and colleagues identify nigrostriatal pathway.
1967	Cotzias reports that large oral doses of L-Dopa result in complete sustained or marked amelioration of PD symptoms. However, also describes dyskinetic side-effects of high does L-Dopa therapy.
1984	Langston *et al.*, report MPTP-induced Parkinsonism in humans and animals, providing MPTP model of PD
2000-	Human genome sequenced - implications for Parkinson's disease research?

The aim of the first chapters of this thesis is to provide the reader with a broad overview of Parkinson's disease. In this chapter I will summarize the clinical and neurochemical features of the basal ganglia relevant to the study and treatment of Parkinson's disease.

1.1 A Brief History of Parkinson's disease

The development of our current understanding of Parkinson's disease is the product of almost two centuries of observation, experimentation, and insight as outlined in Table 1. The study of Parkinson's disease began in 1817, when Dr. James Parkinson published a monograph entitled "*An Essay on the Shaking Palsy*" and therein provided the first early documented description of Parkinson's disease. This description of "the shaking palsy" defined Parkinson's disease as a condition distinguisable from forms of palsy or chorea.

Parkinson's analysis focused on the fundamental symptoms of tremor, stooped posture, and a festinating gait, while neglecting the muscular rigidity included in today's descriptions. Remarkably, based on these observations, Parkinson speculated that this disease condition might have a neurological basis, although, at that time, there was only a rudimentary of understanding of the brain. In addition, Parkinson foresaw the importance of further study, pointing out that neuroanatomical differences in Parkinson's disease (PD) patients may yield important insights into the cause of the symptoms of this disease. This notion was furthered in 1862 by a neurologist named Charcot, at the Salpetriere in Paris. His research group examined post-mortem tissues of PD patients and tentatively determined that the substantia nigra appeared "softened" in Parkinsonian brains, although his observations were not taken further. However, in 1919, a doctoral student in Paris named Tretiakoff undertook a systematic examination of the substantia nigra in post-mortem PD brain tissues and concluded that degeneration of the substantia nigra and the presence of Lewy bodies was a constant feature associated with PD. These seminal findings established the first neuropathological basis for Parkinson's disease.

Since the latter part of the 19th century, advances in our understanding, and hence, our treatment of Parkinson's disease have followed (Table 1). While much more is known about the pathology of PD, when

compared to other neurodegenerative conditions, treatment of PD is essentially incomplete, since all available treatments for this disease condition are palliative - not curative.

1.2 Definition of Parkinson's disease

Parkinson's disease (PD) is a movement disorder characterized by progressive dysfunction of the basal ganglia resulting from degeneration of the pigmented dopamine neurons located in the substantia nigra pars compacta (SNc). Loss of nigral dopamine neurons in PD results in a profound depletion in dopamine levels in the striatum, a forebrain structure responsible for the coordination of movement. Parkinson's disease affects an estimated 1% of people over the age of 55, making PD the second most common neurodegenerative disorder following Alzheimer's disease. The symptoms of PD do not appear until more than 50% of nigral dopamine neurons are lost and dopamine levels in the striatum are diminished by about 80% (McGeer et al., 1977; Riederer and Wuketich, 1976).

Parkinsonism is a neurological syndrome clinically characterized by resting tremor, muscular rigidity, lack of movement (akinesia), slowness of movement (bradykinesia), and a stooped postural gait. The clinical state of *Parkinsonism* can be manifested by a variety of diseases and conditions. The current definition of *Parkinson's disease*, as a distinct nosological entity, is purposefully based on succinct clinical and pathological criteria. Clinically, patients presenting at least two of the cardinal symptoms of Parkinsonism accompanied by responsiveness to L-Dopa therapy are diagnosed as Parkinson's disease. Several other disorders have Parkinsonian signs but also have other neurological deficits. Such conditions, namely progressive supranuclear palsy, multiple system atrophy, and Parkinson-amyotrophic lateral sclerosis are referred to as Parkinson-plus syndromes. Since Parkinson-plus syndromes involve several systems other than the nigrostriatal dopaminergic pathway, individuals with these conditions are generally unresponsive to dopamine replacement therapy. Histopathologically, Parkinson's disease is distinguished by the loss of pigmented dopaminergic neurons in the substantia nigra pars compacta of the ventral midbrain. An additional criterion, revealed only on post-mortem analysis, is the presence of ubiquitin-positive eosiniphilic cytoplasmic inclusions called Lewy bodies. These diagnostic criteria, when

considered collectively are indicative of PD.

1.3 Clinical Symptoms of Parkinson's Disease

Symptoms of PD are categorized as primary and secondary manifestations. The cardinal symptoms of resting tremor, rigidity, bradykinesia, and postural instability are considered primary symptoms of PD because they represent the most readily identifiable signs of the disease.

The "pill-rolling" hand tremor is one of the first signs of PD. As the symptoms of PD are generally asymmetrical, this 3-5 Hz, rhythmic tremor is observable initially in the hand and spreads to the arm and then the leg of the affected side as the disease progresses over time. This symptom is referred to as a rest tremor as it subsides during motor tasks, but is frequently variable and ceases during sleep. While the predominant motor dysfunctions of PD can be attributed to reduced dopaminergic neurotransmission resulting from the destruction of the nigrostriatal pathway, the resting tremor is not consistently relieved with L-Dopa therapy.

The muscular rigidity accompanying PD is a result of enhanced muscle tone in both flexor and extensor muscle groupings. This rigidity is distinguished from muscle spasticity as tone is maintained throughout the range of motion and electromyographs (EMGs) are not silent, but resemble tonic voluntary movements. The cause of the dramatic changes in muscle control in PD is unclear but is nevertheless related to the bradykinesia and/or akinesia which are also hallmarks of the Parkinsonian condition. This symptom or reduced voluntary movement is experienced by all PD patients and worsens over the course of the illness. Akinesia develops as a complex set of signs. Early manifestations include a noticeable slowness in manual dexterity (e.g., handwriting), but, as PD progresses, the more disabling signs of delayed or slowness of voluntary movement, freezing, and hypomimia (masked facial expression) predominate. It is now widely accepted that the reduced capacity for voluntary movement in PD is not a consequence of lack of intent, but rather an inability to initiate an action. As the coordination of movement is largely attributed to dopamine signaling, both akinesias and bradykinesia respond well to dopamine replacement therapy.

The fourth identifying clinical sign of Parkinson's disease is a loss of postural reflex and the genesis of a shuffling gait. The quality of movement steadily decreases with disease advancement. The shuffling gait becoming a festinating gait – one in which the patient's pace hastens to sustain the forward momentum of the torso. Interestingly, these same difficulties in movement sometimes culminate into a complete cessation of voluntary movement, known as *freezing*. Alternatively, these symptoms have been also known to abate temporarily, a condition called *kinesia paradoxica*. Perhaps due to the complex underlying causes of the gait and posture, these difficulties in movement are poorly managed with current therapies.

In addition to the four cardinal clinical signs of PD, there are secondary symptoms associated with this neurological condition which can also be classified into two categories; positive symptoms (those which are not seen in unaffected individuals) and negative symptoms (the loss of normal functions). Some of these secondary manifestations related to Parkinsonism include cognitive impairment (dementia), visuospatial deficits, ocular dysfunction, autonomic dysfunction, and abnormalities in sensory function including sensations of pain (Goetz et al., 1986).

The diversity of possible symptoms for clinical presentation can hinder the accurate diagnosis of Parkinson's disease. Hence, a standardized method of evaluating Parkinsonian behaviours was developed - called the Unified Parkinson's Disease Rating Scale. This survey of mental, motor, and social parameters allows for the diagnosis of Parkinsonism and encorporates the Hoehn and Yahr Staging Scale to determine disease severity (from 1, for mild PD, to 6 denoting confinement to a wheelchair or bed).

1.4 Epidemiology of Parkinson's disease

Parkinson's disease is one of the most common neurodegenerative disorders. Disease frequencies in a population are determined by two statistical measures; the number of new cases occuring in a given time for a specific location (incidence), and the total cases at a specified time (prevalence). In general, the incidence of PD increases proportionally with age. Since the availability of detailed medical records limit the longitudinal trends in PD incidence, it is uncertain whether industrialization, for instance, has

influenced the rate of PD in any given population. In Canada, the annual prevalence of PD between 1979 and 1984 revealed (1) a trend of higher prevalence among men, and (2) a national trend of increased prevalences from east to west (Svenson, 1991). The prevalence of PD in Canada is not significantly different from worldwide statistics. For instance, the prevalence of PD globally varies geographically with higher rates in North America and Europe and lower prevalences in Asia and Africa (reviewed by Tanner and Goldman, 1996). Several reasons may explain the reported differences in PD prevalence among races. First, differences in genetic backgrounds may exist which may influence susceptibility to develop PD. Second, distinct differences in climate and/or environment in different areas of the world may relate to the possibility that exposure to an environmental toxin may produce Parkinson's disease (see below). However, disparities in health care systems between countries may confound accurate determination of the incidence and prevalence of PD due to differences in study design. For instance, more rigourous study formats in localized biracial populations has failed to support the finding of racial disparity in PD prevalence (Tanner and Goldman, 1996; Schoenberg et al., 1985). In a pattern similar to prevalence statistics, the *incidence* of Parkinson's disease varies throughout the world, ranging from 18-234/100,000, with lower incidences in Asia (Tanner and Goldman, 1996). Taken together, the results from current epidemiological studies suggest that Parkinson's disease is a prevalent age-related neurological disorder which affects populations worldwide. While present studies do not establish a causal association with either environmental or genetic factors, additional studies may identify risk factors and offer correlative evidence to support current theories of the etiology of PD.

1.5 **Current Treatments for Parkinson's disease**

1.5.1 *Dopamine Replacement Therapy*

Treatment of Parkinson's disease has been based upon the prevailing understanding of this condition. Historically, PD was perceived as a consequence of poor diet and was treated accordingly. This nutriceutical approach was followed in the 1860's by pharmacologic interventions using anticholinergic drugs which became the standard therapy for PD for almost 100 years. While anticholinergic drugs, like

scopolamine, were efficacious for the amelioration of gain-of-function symptoms, such as tremor, they could not improve loss-of function symptoms of PD, like akinesia. Current drug treatments were only realized with the discovery that PD involves striatal dopamine deficiency and the commensurate development of dopamine replacement therapy (Carlsson et al., 1959). Specifically, L-DOPA-based therapies were found to be an effective treatment for PD (Cotzias et al., 1967; Cotzias et al., 1968; McDowell et al., 1970), and remain the mainstay of PD drug therapy today.

1.5.1.1 *L-Dopa Drug Therapies*

Levodopa (L-DOPA) is the metabolic precursor to dopamine. Consistent with the dopamine-deficiency theory of Parkinson's disease, systemic administration of L-DOPA can replenish levels of dopamine in the striatum. Orally administered L-DOPA has a half-life of approximately 1.3 hours (Fabbrini et al., 1987). The pharmacokinetics of L-DOPA are dependent upon several factors. Most importantly, L-DOPA enters the brain via an amino acid transporter and consequently competes with other proteins for transport into the CNS (Nutt et al., 1984). In addition, since dopamine cannot cross the blood brain barrier, the bioavailability of L-DOPA is limited by enzymes in the gastrointestinal tract which convert L-DOPA into dopamine (Nutt et al., 1985). Hence, co-administration of inhibitors of Dopa decarboxylase, such as carbidopa, which themselves do not cross the blood-brain barrier, an L-aromatic amino acid decarboxylase (L-AAD) inhibitor (e.g. Sinemet, Madopar), selegine (Deprenyl), a monoamine oxidase-B inhibitor, or formulations which allow for prolonged slow-release of L-DOPA (e.g. Sinemet CR, Madopar CR) are currently being used to enhance L-DOPA efficacy (Grandas et al., 1998; Parkinson's Study Group, 1998).

The principal objective of enhancing oral availability of L-DOPA is to attenuate undesirable side-effects resulting from increased peripheral dopamine levels. Notable peripheral side-effects of L-DOPA therapy include nausea and hypotension, which is attributed to the conversion of DA to noradrenaline (NA) resulting in cardiovascular side-effects. While the acute benefits of L-DOPA therapy for the symptomatic treatment of PD have widely been reported, experience from the past decades has demonstrated that long-term administration of high oral doses of L-DOPA therapy is confounded by the development of central

side-effects in 60-80% of patients (Cedarbaum et al., 1991). Notable side-effects from L-DOPA which are mediated by the central nervous system include reduced therapeutic efficacy ("wearing-off"), fluctuations in motor responsiveness ("on-off"), and development of persistent involuntary movements, called dyskinesias. The exact nature of these central effects is presently unclear, however, postsynaptic changes in the denervated striatum are thought to contribute to the evolution of these adverse motor complications from L-DOPA therapy (Doucet et al., 1997).

The effect of combination therapies for the treatment of PD has been the aim of two double-blinded, placebo-controlled clinical trials coordinated by the Parkinson's Disease Research Group of the United Kingdom (UK) (1993, 1995), and the North American Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) trials (Parkinson's Study Group, 1989; 1998). The British study has analysed the impact of L-DOPA therapy against L-DOPA in conjunction with the MAO-B inhibitor selegiline (Deprenyl), or the dopamine agonist bromocriptine. Long-term follow-up of patients enrolled in the UK trial led to the observation that the cohort receiving the combination treatment of L-DOPA and deprenyl had a significantly higher mortality than just L-DOPA (Lees et al., 1995). This result from the UK trial, 5.6 years following the initiation of the clinical trial, is not consistent with similarly treated patient groups in the DATATOP study at 8.2 years following beginning of their treatment (Parkinson's Study Group, 1998). Interestingly, whether deprenyl potentiates mortality or enacts no effect on patient survival, these clinical findings are in contrast to accumulating experimental evidence that suggest deprenyl is neuroprotective. This disparity in deprenyl efficacy has increased awareness of the potential toxicity of L-DOPA, since L-DOPA has no impact on disease progression but may accelerate disease pathology (Przedborkski et al., 1993; Shulman, 2000; Weiner, 2000). Presently, the ability of L-DOPA to enhance quality of life for PD patients is thought to supercede any deleterious effects that may presently result from chronic L-DOPA therapy (Grandas et al., 1998).

1.5.2 *Dopamine receptor agonists.*

The development of motor complications arising from L-DOPA therapy has led to the proposal of employing "L-DOPA sparing strategies" or dopamine receptor agonist monotherapies which may have

improved therapeutic profiles for the long-term treatment of PD. Since the development of dyskinesias is associated with the dopamine D1-receptor, selective D2-dopamine receptor agonists have been developed to remedy the onset and severity of dyskinetic side-effects of dopamine therapies. An increasing number of dopamine D2-receptor specific agonists have been reported to enact clinical efficacy for the treatment of motor complications associated with PD. Initially, D2-type dopamine receptor agonists, such as bromocriptine, lisuride, and pergolide, were studied in PD patients (Rinne, 1989; Lieberman et al., 1983; Lang et al., 1982). More recently, dopamine D3-receptor agonists, such as Ropinirole (Requip™) and pramipexole, are emerging as an alternative treatment for Parkinson's disease since they produce abatement of PD symptomology within weeks and a reduced risk of developing dyskinetic side effects (Phillips, 1999). However, dopamine agonist monotherapies are not without drawbacks. For instance, ropinirole has been associated with an increased propensity for hallucinations. To understand the long-term benefit of dopamine receptor therapies, clinical studies, such as the "Comparison of the Agonist Pramipexole vs Levodopa on Motor Complications in Parkinson's Disease" (CALM-PD; North American Parkinson's Study Group), have been initiated.

In spite of the potential for symptomatic benefit with dopamine agonist monotherapies, L-DOPA, because of its immediate efficacy, is still the treatment of choice for most patients. The complications associated with use of dopamine replacement therapy have increased the need for alternate replacement and neuroprotective strategies for treatment of PD.

1.5.3 *Surgical Strategies for the Treatment of Parkinson's disease*

1.5.3.1 *Neuronal transplantation*

Grafting cells that produce dopamine in the brains of patients with neurodegenerative disorders has gained support as a viable treatment for the long-term treatment of neurodegenerative disorders like Parkinson's disease (Gage and Fisher, 1991). For more than 20 years, the transplantation of fetal dopamine neurons into the striatum of lesioned animals have been reported to provide dramatic improvement of motor symptoms in animal models of PD, as well as human patients (Bjorklund and Stenveni, 1979; Perlow et al., 1979). More importantly, transplanted tissues have been reported to reinnervate host tissue and

therein provide a degree of physiological normalization and improved responsiveness to pharmacotherapy (Lindvall et al., 1990). While considerable attention has focused on the application of human fetal neurons for transplantation, several limitations to this therapeutic approach impede the widespread application of neurotransplantation therapy. Disadvantages to the use of fetal tissues include the limited supply of viable tissues, the ethical issues surrounding the use of fetal tissues, low graft survival rates, and host-immune responses. Hence, adaptations to the current experimental protocols are required to facilitate the practical use of this therapeutic strategy. For instance, advances in our understanding of the molecular processes mediating cell death may lead to neuroprotective strategies that enhance neural graft survival. In keeping with this hypothesis, Schierle et al. (1999) have demonstrated that inhibition of cell death proteases (caspases; see Chapter 3 *Cell Death in PD*) during the grafting procedure can enhance the survival of embryonic neurons transplanted into the striatum of dopaminergically denervated rodents. These findings offer the potential of reducing the amount of tissue required per recipient and may also increase the number of neurons surviving the transplant procedure – providing a longer-lasting and more functional graft. In addition, alternative sources of transplantable tissues may avoid the necessity of utilizing human fetal tissues and thereby avoiding the ethical controversies and providing more abundant tissue sources. For instance, autotransplantation of adrenal medullary cells (Rosenthal, 1998), implantation of animal tissues (*xenotransplantation*), or implantation of polymer encapsulated genetically-modified cells, such as fibroblasts, designed to secrete trophic factors (Takayama et al., 1995) or dopamine, are experimental alternatives currently being explored for the treatment of Parkinson's disease (for review of neural transplants see Olanow et al., 1997).

Adaptation of current experimental transplantation protocols include consideration of the site of implantation. While it is widely established that dopamine release in the striatum can ameliorate functional deficits associated with PD, dopamine release in the substantia nigra may also participate in dopamine-mediated motor function (Robertson and Robertson, 1988). Indeed several groups have attempted neuronal grafts in lesioned animals into either the striatum or substantia nigra and established varying degrees of behavioural recovery. Mendez et al. (1996) demonstrated that simultaneous implant of grafts into both the

nigra and striatum can reestablish a rudimentary nigrostriatal anatomical pathway that can promote functional recovery in previously denervated rodents.

Together, these findings suggest that establishing both optimal grafting procedures and intracerebral sites for transplantation may provide the potential of replacing or supplementing the function of the nigrostriatal dopamine pathway to ameliorate the symptomology of PD.

1.5.3.2 *Surgical Ablation*

Pallidotomy is the surgical ablation of the posteroventral aspect of the globus pallidus (Laitinen et al., 1992). As an experimental treatment for advanced Parkinson's disease, pallidotomy has been shown to provide improvement in akinesia and remedy gross motor abnormalities associated with PD, such as postural instability (Lang et al., 1997; Melnick et al., 1999). Drastic, irreversible procedures such as pallidotomy are reserved for PD patients who fail to respond to medication or who have such profound side-effects that continued drug therapies are ineffective (Melnick et al., 1999). Improvement in motor complications following surgical ablation of the globus pallidus has shown variable results depending upon lesion coordinates, volume of lesion, as well as patient profile (age, duration of L-DOPA treatment, etc.). For review of surgical treatments for PD see Vitek and Bakay (1997).

1.6 *Experimental Strategies for Treatment of Parkinson's disease*

1.6.1 *Stem Cells*

Stem cells are populations of pluripotent cells that are a potential source of replenishing terminally-differentiated cells, such as neurons (Weiss et al., 1996). Neurogenesis, once thought to be an event in select species of animals such as songbirds, has recently been demonstrated to occur in the adult primate and human CNS (Eriksson et al., 1998; Gould et al. 1999). These intriguing findings are in contrast to previous dogma which supposed that the mammalian CNS lacked any regenerative capacity. The identification of neural progenitors in the adult brain has led to the proposal that stem cells could be utilized to alleviate demand pressures for the limited supply of fetal dopamine tissues for transplantation (Studer et al., 1998). Theoretically, stem cell technology could be implemented in two ways. First, stem

cells could be harvested and grown in perpetuity, providing a potentially limitless supply of neural tissue. Lee et al. (2000) have recently shown that embryonic stem (ES) cells can be efficiently generated and differentiated into a dopaminergic phenotype, providing a potential source of transplantable tissues for PD. A second theoretical application of ES cells has been the induction and differentiation of stem cell populations *in vivo*, by recapitulating requisite signals which mediate the differentiation of midbrain dopamine neurons during development. In summary, stem cells offer the intriguing potential of providing a sustainable source of transplantable tissues for the treatment of neurodegenerative disorders, such as Parkinson's disease. The practical application of ES technology will follow an increased understanding of the neurochemical environment in the adult as well as during development.

1.6.2 *Trophic Factors*

To date, the only potential neuroprotectant tested for the treatment of Parkinson's disease is glial cell line-derived neurotrophic factor (GDNF). In experimental models, administration of GDNF improved behavioural deficits in primate and mouse models of PD (Tomac et al., 1995; Gash et al., 1996). Functional improvement in mice treated with GDNF was correlated with increased striatal dopamine (Tomac et al., 1995). Interestingly, while GDNF did not appreciably affect neuroamine levels in primates, the duration of symptomatic improvement with direct intracerebral administration of GDNF surpassed the effects of L-DOPA by several weeks (Gash et al., 1996). The increasing volume of studies demonstrating the efficacy of GDNF in animal models of PD has raised interest in the prospect of employing GDNF for treatment in humans (Bohn, 2000). However, recent attempts at employing the trophic factor ciliary neurotrophic factor (CNTF) to humans for the purpose of treating amytrophic lateral sclerosis (ALS) have not produced the same benefits resulting from administration of CNTF in animal models of this disease (ALS CNTF Treatment Group, 1996). While there have not yet been trophic factor-based therapeutic trials for the treatment of Parkinson's disease, the disparity between results from experimental models and the human disease condition may warrant caution and diligence when attempting this approach in human subjects.

1.6.3 *Gene Therapy*

Of the many prospective long-term therapies for the treatment of Parkinson's disease, gene therapy arguably holds the greatest potential benefit (Suhr and Gage, 1993). Simply stated, gene therapy is the procedure of correcting a human disease condition by replacing or augmentating gene expression. Gene therapy can take the form of direct administration of genetic material or the modification of endogenous gene expression using pharmacological agents. The concept of gene therapy has gained attention as a viable means of correcting known genetic diseases, but also offers the potential for treating neurodegenerative disease conditions, such as Parkinson's disease (Horellou *et al.*, 1997).

The potential of gene therapy as a real alternative to treating degenerative diseases by manipulating gene expression is dependent upon several factors. First, a comprehensive understanding of the disease state, or condition will theoretically yield the selection of a therapeutically relevant gene(s). Second, the mode of gene delivery is critically important for the widespread utility of this therapy. For instance, for the treatment of Parkinson's disease, gene therapy may involve (a) the direct delivery of genes into the brains of individuals with PD (*direct in vivo* gene therapy), (b) the administration of genes to facilitate the survival and/or function tissues used for neural transplantation, such as neural tissues or stem cells (*ex vivo* gene therapy), (c) the manipulation of germline cells for the aim of arresting transmission of a disease condition to subsequent generations (*derivative in vivo* gene therapy), or (d) the administration of compounds for the purpose of elevating or impairing gene expression in the brain (*indirect in vivo* gene therapy).

Current approaches to gene therapy are limited by current gene transfer technologies. Treatment of a disease which affects a defined neuronal population, such as Parkinson's disease, may require the establishment of highly specific and localized expression of therapeutic genes therein minimizing the possible impact on adjacent tissues unrelated to the target site. A second limitation of current gene therapies is the relative short duration of foreign gene expression. As exemplified by PD, to treat diseases manifesting over decades, short-duration gene expression may not influence disease progression or clinical

outcome. Therefore, establishing the utility of certain gene products for the treatment of human disease conditions may be provided by current gene therapy strategies but the translation of these experimental findings to humans require improved understanding and technologies to permit gene therapy to reach its therapeutic clinical potential.

In chapters 5 and 6 of this dissertation, I present two studies involving animal models of PD in which I demonstrate the actions of the Inhibitor of Apoptosis (IAP) proteins in preventing dopamine neuronal death *in vivo*. These lines of investigation are the first studies to support the potential utility of IAP proteins as a neuroprotective treatment in models of PD and may provide a novel strategy for the attenuation of Parkinson's disease.

Chapter 2. The Neurochemistry and Anatomy of the Basal Ganglia

2.1 The Neurotransmitter Dopamine

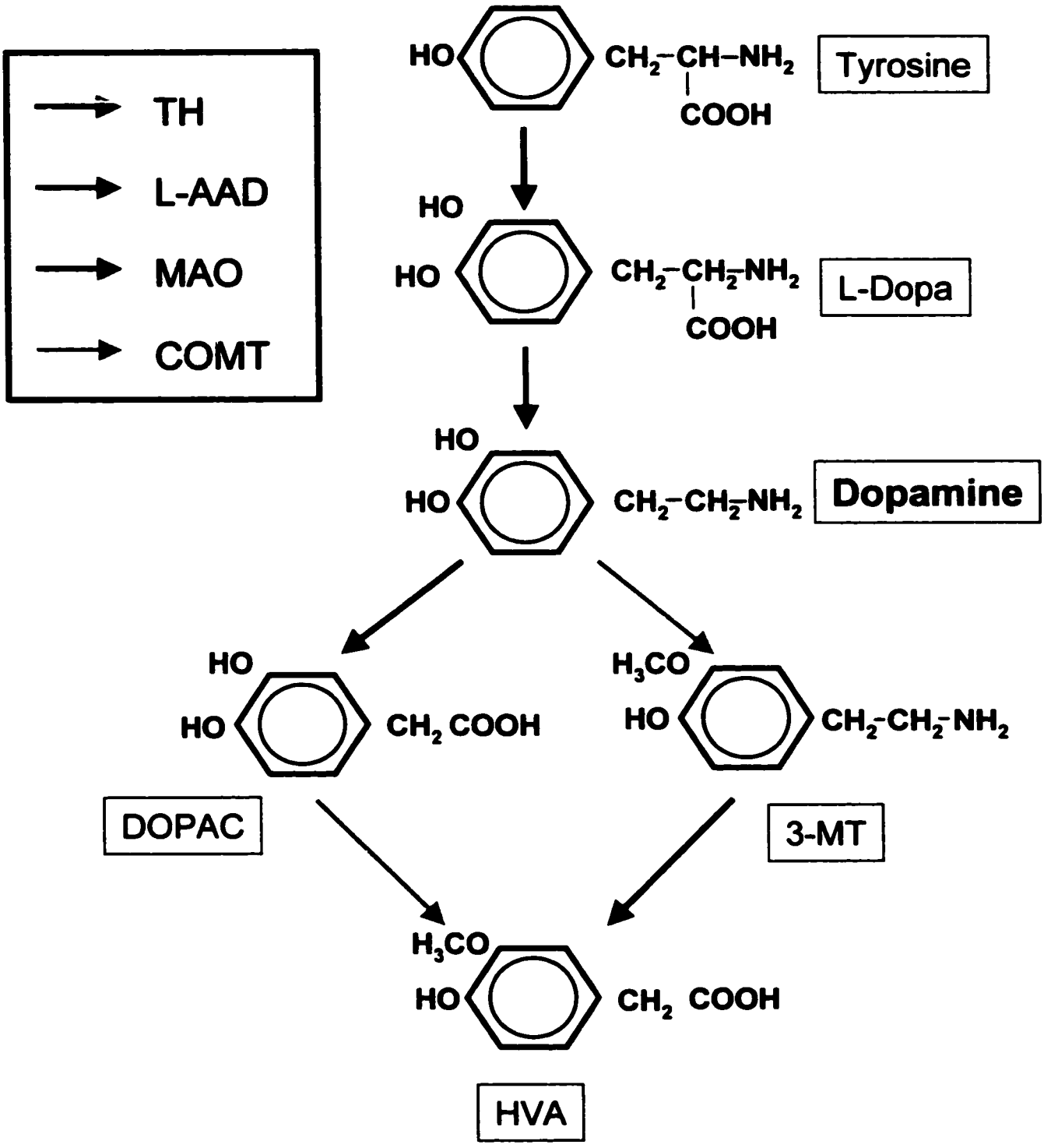
Dopamine is an intermediate in the synthesis of the neuroamines epinephrine and norepinephrine. For this reason, dopamine was not recognized as a neurotransmitter until some time in the 1950s when it was identified as a secreted signaling molecule involved in the gastrointestinal system of ruminent species (Montagu et al., 1957). Subsequently, dopamine was found to also be present in the central nervous system and the existence of dopaminergic populations in the brain was proposed (Carlsson, 1959). This notion was confirmed by Bjorklund et al. (1971) using a formaldehyde condensation reaction to detect dopaminergic neurons histologically. These seminal findings, coupled with the historical descriptions of neuronal dropout in the pars compacta, led to the discovery that PD was associated with a profound depletion of striatal dopamine (Ehringer and Hornykeiwicz, 1960). However, it was not until 1966, that Goldstein and collegues identified the nigrostriatal dopamine pathway. Taken together, these observations became the foundation for the dopamine hypothesis of Parkinson's disease which proposes that Parkinsonism is a syndrome of striatal dopamine deficiency (Hornykiewicz, 1972).

2.2 Dopamine Metabolism

The synthesis of dopamine begins with hydroxylation of the amino acid tyrosine into dihydroxyphenylalanine (L-Dopa) by the enzyme tyrosine-3-monooxygenase (tyrosine hydroxylase) (See *Figure 2.1*). The conversion of the levorotatory enantiomer of tyrosine, L-tyrosine, into L-Dopa is considered the rate-limiting step in dopamine synthesis since (a) the K_m for tyrosine hydroxylase limits the production of L-Dopa and (b) the tyrosine hydroxylase (TH) enzyme is regulated by feedback inhibition (reviewed by Zigmond et al., 1989). Immunohistochemical labelling of TH is widely used as a neuronal marker for the dopaminergic phenotype.

Levodopa, or L-Dopa, is decarboxylated by the enzyme aromatic L-amino acid decarboxylase (L-AAD) to form the neurotransmitter dopamine. Under normal conditions, levels of L-Dopa in the brain are negligible since the metabolism of L-Dopa to dopamine is rapid. Since the enzymatic activity of TH can saturate under normal conditions, the ability to increase production of dopamine by providing excess L-

Figure 2.1 Steps in the enzymatic synthesis and metabolism of dopamine. Chemical formulae are labeled by boxes, with the color of intervening arrows representing the specific enzyme which mediates each step. See text for discussion of dopamine metabolism. Abbreviations: TH – tyrosine hydroxylase; L-AAD – aromatic L-amino acid decarboxylase (or dopa decarboxylase) ; MAO – monoamine oxidase; COMT – catechol-O-methyl transferase; DOPAC – 3,4-dihydroxyphenylacetic acid; HVA – homovanillic acid; 3-MT – 3-methyoxytyramine.


TH
L-AAD
MAO
COMT
HO
CH2–CH–NH2
COOH
Tyrosine
HO
HO
CH2–CH2–NH2
COOH
L-Dopa
HO
HO
CH2–CH2–NH2
Dopamine
HO
HO
CH2 COOH
DOPAC
H3CO
HO
CH2-CH2-NH2
3-MT
H3CO
HO
CH2 COOH
HVA

tyrosine is limited. However, the K_m of L-AAD for L-Dopa is high, making it possible to augment brain dopamine levels by providing excess L-Dopa, which can then be converted into dopamine in the CNS. This concept underlies the current therapeutic use and efficacy of treatment with L-Dopa for Parkinson's disease.

Dopamine is released into the synaptic cleft where cell-surface receptors mediate intracellular responses. The active half-life of dopamine is regulated by two factors; the rate of active reuptake into the presynaptic terminal by the dopamine transporter (DAT), and the rate of enzymatic catabolism by catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO). The principal metabolites of dopamine are homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC). In humans and primates, HVA is the principal dopamine metabolite, while in rodents, DOPAC is the more prevalent (Wilk & Stanley, 1978).

Soon after the identification of dopamine as a prevalent neurotransmitter in the mammalian brain, Hornykiewicz and others (1960) used post-mortem tissues to determine that levels of DA and its principal metabolites were markedly diminished in all regions of the basal ganglia in PD brains (reviewed by Wooten, 1997). Levels of dopamine in the caudate-putamen are reduced up to 97% (Lloyd et al., 1975), while DA levels in the accumbens are 58% less than in brains from non-Parkinson's age-matched tissues (Price et al., 1978). Interestingly, the ratio of reduction in HVA concentrations is not as drastic as the ratio for dopamine. The reasons for this discrepancy in DA and HVA ratios are not well understood, though these may reflect concomitant deficits in the levels of TH and L-ADD or DAT function without significant changes in the activities of either MAO or COMT (Lloyd et al., 1975; Wooten, 1997). Current neurotoxin models of PD, namely systemic administration of MPTP, recapitulate these deficits in dopamine metabolism in mice and primates supporting their use as a practical model for studying the neurochemical changes associated with Parkinson's disease.

Regulation of the duration of dopamine action in the synapse can have significant physiological effects. For instance, the DAT can be blocked by cocaine, where the prolonged actions of dopamine in the synaptic cleft have been associated with the addictive and psychotrophic effects of this compound. Further, MAO type B (MAO-B) inhibitors, such as selegiline (deprenyl), are presently being used to augment L-

Dopa therapy in Parkinson's patients to prolong the actions of dopamine and thereby provide prolonged relief of Parkinsonian symptoms. The long-term effects of MAO inhibitor (MAO-I) therapy in combination with L-Dopa were the focus of a multi-center international study called DATATOP (Deprenyl and tocopherol antioxidative therapy of Parkinsonism).

2.3 Functional Organization of the Basal Ganglia

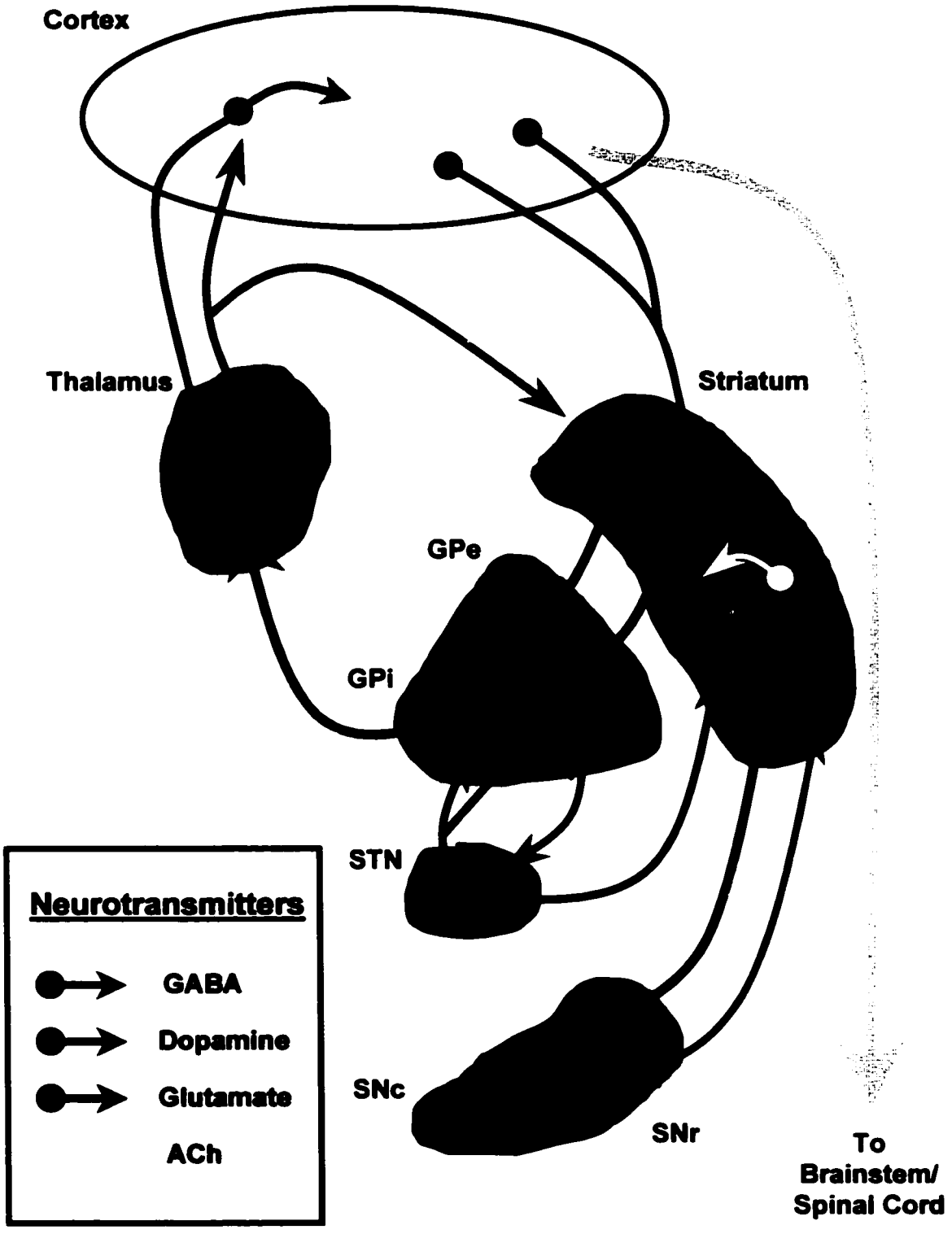
The organization of central motor systems, particularly the basal ganglia, is pivotal to our understanding of the neurological basis of movement. Elucidating the functional connectivity of this area of the brain underlies the foundation of our current understanding of the pathophysiologies in a variety of movement disorders that are associated with dysfunction of the basal ganglia, including PD.

Central control of motor, limbic, and associative function is mediated by parallel neuronal circuits, called *reentrant loops*. These convergent pathways process the information from several areas of the cortex through a series of connections into the basal ganglia. Information is then segregated through the pallidum and thalamus and relayed back to discreet areas of the cortex. Corticospinal projections transmit these motor impulses to elicit coordinated muscle innervations that enact movement. The cortical motor areas and the four subcortical nuclei of the basal ganglia - the striatum, the globus pallidus, the subthalamic nucleus, and the substantia nigra - are the key structures of this motor system (*Figure* 2.2). The following sections will detail the neurochemistry and functional organization of the nuclei comprising the basal ganglia as they pertain to our current understanding of Parkinson's disease.

2.3.1 *The Caudate-Putamen: Composition and Connectivity*

The striatum is a heterogeneous subcortical structure located in forebrain. The striatum is subdivided into three distinct components; the caudate nucleus, the putamen and the nucleus accumbens. The caudate and putamen are separated in higher mammals by fibrous tracts called the internal capsule. These tracts run ventrally to the nucleus accumbens. The putamen is the principal input site to the basal ganglia and receives dopaminergic innervation from the substantia nigra, glutamatergic input from diverse regions of the cortex, as well as serotonergic inputs from the raphe nucleus.

Figure 2.2 Schematic diagram of the connectivity and neurotransmitters of the basal ganglia and thalamo-cortical pathway. Parallel direct and indirect striatal output pathways modulate striatal outflow. The "direct" pathway is represented by projections from the striatum to the GPi and then the output nuclei of the thamalus send glutamatergic projections to cortex. The "indirect" pathway loops through the subthalamic nucleus via the GPe before synapsing on the glutamatergic thalamocortical projection neurons. GPi and SNR are separate in this figure but are closely related, both functionally and anatomically. Efferent projections are indicated by direction of the arrows, with color representing the neurotransmitter phenotype of neurons. Abbreviations: GABA – gamma-amunobutyric acid; ACh – acetylcholine; GPe – external segment of the globus pallidus; Gpi – internal segment of the globus pallidus; STN – subthalamic nucleus; SNc – substantia nigra pars compacta; SNr – substantia nigra pars reticulata. *Adapted from: A.M. Graybiel (1990) TINS 13: 244-254.*


Cortex
Thalamus
Striatum
GPe
GPi
STN
SNc
SNr
To
Brainstem/
Spinal Cord
Neurotransmitters
GABA
Dopamine
Glutamate
ACh

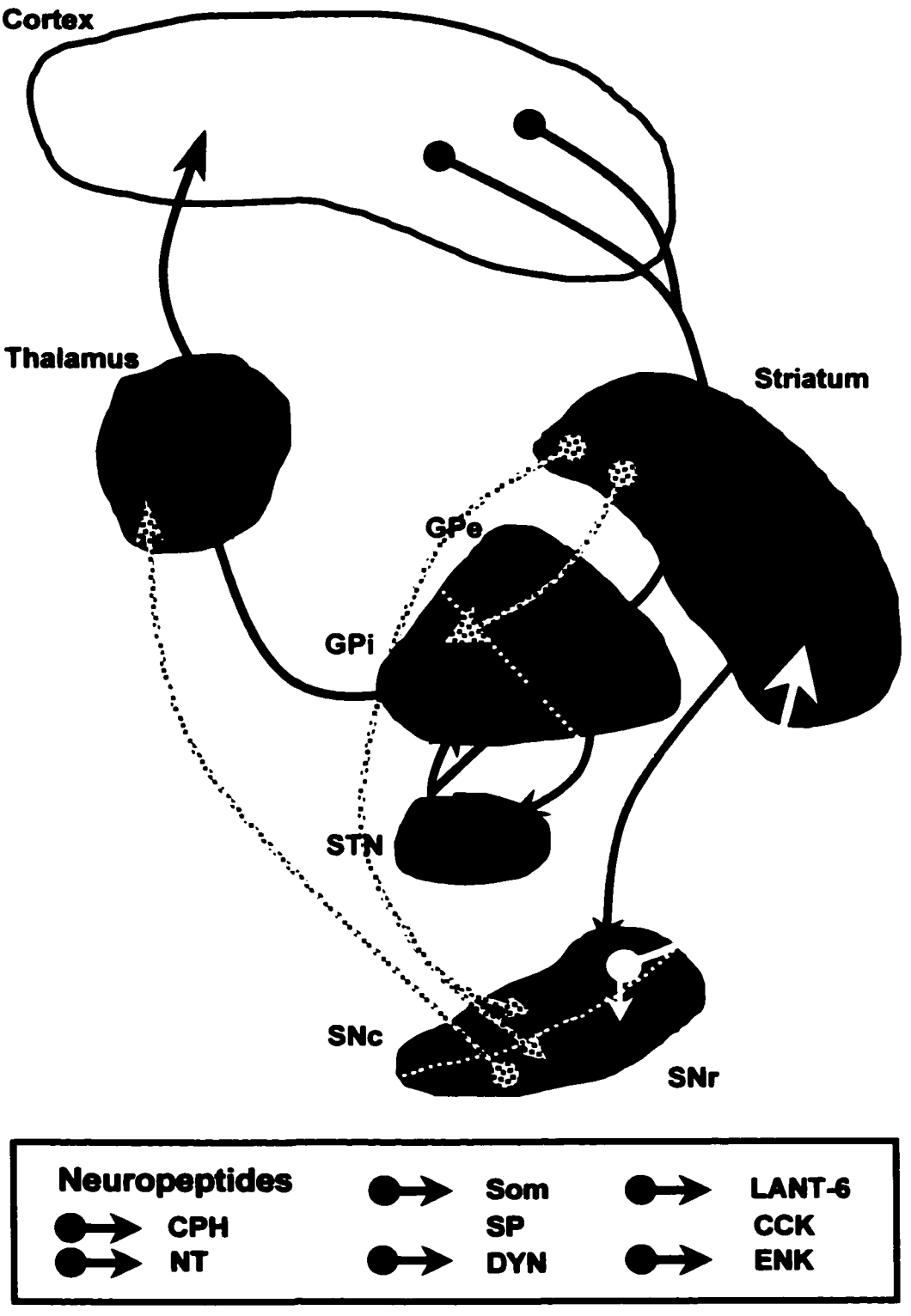
Histologically, the striatum is made-up of two macroscopically distinct compartments, called striosomes (also called patches in rodents) and matrix. The striosomes account for only 10-20% of the striatal volume and are more plentiful in the ventromedial than the dorsolateral part of the striatum. Interestingly, the two compartments of the striatum are differentiated by distinct patterns of neuropeptide expression (*Figure 2.3*). The extrastriosomal matrix is enriched with expression of acetylcholinesterase (AChE) and the neuropeptide somatostatin (Som). In contrast, the striosomal compartments are immunoreactive for the neuropeptides enkephalin and substance P. The neurochemical differences between the matrix and striosomes are extensive and reviewed by Graybiel (1990).

The patch-matrix composition of the striatum is also associated with distinct connections to other basal ganglia nuclei. Medium sized, "spiny" neurons (MSN) which contain the neurotransmitter γ-aminobutyric acid (GABA) make-up 95% of the neurons in the striatum. The remaining striatal neurons are large cholinergic interneurons. These GABA neurons are both the intrinsic targets for cortical afferents and the principal efferent projections from the striatum. Overall, the connections to and from the ventral aspect of the striatum are associated with the limbic system, while the dorsolateral projections appear to be predominantly related to sensorimotor activity. Supporting this notion, work by Gerfen and collegues (1984, 1987) revealed that projections arising from the striosomes of the dorsolateral (putamen) aspect of the striatum innervate the substantia nigra pars compacta while projections from the matrix project to the substantia nigra pars reticulata (SNr). These findings support the hypothesis that normal operation of the basal ganglia is mediated by systems of interconnected but functionally parallel circuits.

2.3.2 *The Globus Pallidus*

The globus pallidus (GP) is a primary site of the output from the basal ganglia. The GP is comprised of external (GPe) and internal segments (GPi), each receiving GABAergic inputs from the putamen. Of note, the substantia nigra pars reticulata (SNr) is essentially a portion of the GPi that has been separated by fibers of the internal capsule. Indeed, the SNr and GPi share common electrophysiology, morphology, connectivity, and embryology. GABA neurons in the internal aspect of the globus pallidus (GPi) project to the thalamus, while the GPe/SNr sends GABA projections to the subthalamic nucleus.

Figure 2.3 Schematic illustration of the neuropeptides that distinguish the different neuronal populations in the basal ganglia. Colored arrows represent the neuropeptide content of the neuronal projection, with stippled coloration representing colocalization of more than one neuropeptide. Neurotransmitter phenotypes can be determined by comparison with Figure 2.2. Populations for which no neuropeptide has been reported are shown in black. Abbreviations are: CPH – carboxypeptidase H; NT – neurotensin; Som – somatostatin and neuropeptide Y; SP – substance P; DYN – dynorphin; LANT-6 – Lys8-Asn9-neurotensin8-13; CCK – cholecystokinin; ENK – enkephalin; GPe – external segment of the globus pallidus; Gpi – internal segment of the globus pallidus; STN – subthalamic nucleus; SNc - substantia nigra pars compacta; SNr – substantia nigra pars reticulata. *Adapted from: A.M. Graybiel (1990) TINS 13: 244-254.*


Cortex
Thalamus
Striatum
GPe
GPi
STN
SNc
SNr
Neuropeptides
CPH
NT
Som
SP
DYN
LANT-6
CCK
ENK

Glutamatergic innervation to the GPi, GPe and the putamen from the subthalamic nucleus can modulate the activity of these inhibitory, GABA neurons. Neurons of the GPe and GPi have similar electrophysiological properties, firing at rates between 60-80 Hz. Changes in the spontaneous discharge rates of either the GPe or GPi/SNr have opposite effects on the activity of the thalamus, the thalamocortical circuit, resulting in modulation of movement.

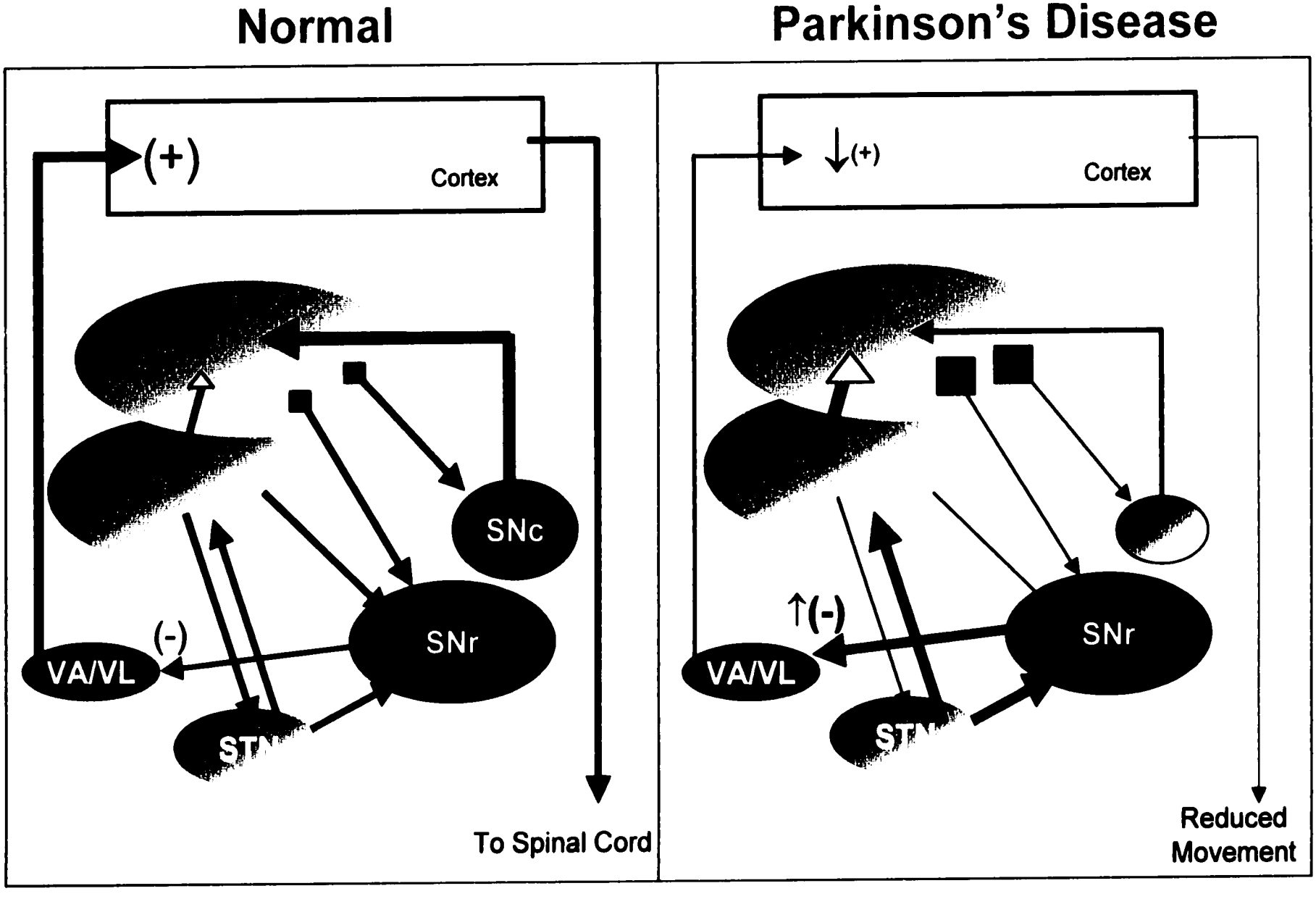
The opposing modulatory influences of the GPi and GPe on movement are a result of two distinct efferent pathways. The *direct pathway* provides reinforcing feedback to the thalamus, and the thalamocortical circuit, resulting from a suppression of the rate of GABA neuron firing, whereas activation of the *indirect pathway* results in repression of thalamic activity. The projection neurons from the striatum to the GPi, which comprise the *direct pathway* contain both GABA and the neuropeptide substance P. The *indirect pathway* is identified by striatal GABA neurons projecting to the external aspect of the GP and contain enkephalin. These neurons synapse on GABAergic neurons in the subthalamic nucleus and thereby modulate the excitatory, glutamatergic output from the STN. Hence, activation of the direct pathway enables movement, while the effect of indirect pathway activation is to restrain movement (*Figure 2.4*)

2.3.4 *The Subthalamic Nucleus*

The striatal efferents from the internal leaflet of the globus pallidus (GPi) project to the ventral thalamus. The frequencies of these GABAergic projections are modulated by glutamatergic innervations from the subthalamic nucleus. Thalamic glutamatergic neurons project to discrete areas of the cortex.

The cardinal motor sign of Parkinsonism, the resting tremor, is believed to be generated by the modified firing pattern of GABAergic neurons of the globus pallidus (GPi) by neurons of the dorsal thalamus (VLA) (Pare et al., 1990). The functional relationship of these neurons is the rationale behind the surgical ablations of either the sensorimotor aspect of the GPi (pallidotomy) or the VLA (thalamotomy) to control this distressing motor complication. These lesioning procedures have proven effective as non-pharmacological approaches for tremor treatment.

Figure 2.4 Schematic representation of the changes in innervation within the basal ganglia in Parkinson's disease. Excitatory D1 dopamine receptors (represented by the green squares) are located on striatopallidal neurons which comprise the "direct" pathway, while inhibitory D2 dopamine receptors (yellow triangles) are located on neurons which project to the GPi, refered to as the "indirect" pathway [Control]. In Parkinson's disease, loss of dopaminergic flow to the striatum results in deregulation of striatal outflow through the direct and increased activity of the indirect pathway. The net effect is increased inhibition of thalamocortical neurons and reduced output to the cortex. See text for further details. Relative thickness of lines is representative of changes in neuronal activity in PD. Similarly, increased size of DA receptor symbols represents heightened sensitivity of these receptors to dopamine following loss of nigrostriatal dopamine input. Abbreviations: GP – globus pallidus; SNr – substantia nigra pars reticulata; SNc – substantia nigra pars compacta; STN – subthalamic nucleus; VA/VL – ventroanterior and ventolateral nuclei of the thalamus, respectively; (+) excitatory innervation; (-) inhibitory innervation. *Adapted from Standaert and Young, "Treatment of Central Nervous System Degenerative Disorders", in* <u>*The Pharmacological Basis of Therapeutics 9th Ed.*</u> *Molinoff and Ruddon, eds. (1996) McGraw-Hill, New York, NY.*


Normal
Parkinson's Disease
(+)
Cortex
↓(+)
Cortex
SNc
SNr
(-)
VA/VL
STN
↑(-)
SNr
VA/VL
STN
To Spinal Cord
Reduced
Movement

2.3.5 *The Substantia Nigra*

The substantia nigra is located in the ventral midbrain. It is comprised of two areas, the pars compacta (SNc) and the pars reticulata (SNr). These two areas contain predominantly dopaminergic and GABAergic neurons, respectively. The GABA neurons in the SNr receive innervation from the matrix component of the striatum. These neurons also provide feedback innervation to the dorsal striatum as well as send projections to the thalamus and neighbouring pars compacta.

Nigral dopamine neurons, and the adjacent dopamine neuron populations in the ventral tegmental area, can be divided into dorsal and ventral tiers (Fallon and Moore, 1978), which reflect the pattern of nigrostriatal projections from each grouping (Gerfen et al., 1987). The dorsal tier, which contains predominantly neurons of the ventral tegmental area (A10) as well as the dorsal nigral neurons (A9), project to both the patches and matrix of the ventral and lateral striatum (nucleus accumbens and ventral caudate). Dopamine neurons in the ventral portion of the nigra pars compacta (A9) project to the patches of the dorsolateral striatum. In addition to site directed projections within the striatum, the nigrostriatal dopaminergic projections also exhibit topographic patterning, in the area of striatal innervation related to the rostrocaudal-dorsoventral distribution neurons within the SNc. Essentially, the striatum is innervated in a dorsolateral to medioventral pattern corresponding to the rostrocaudal location of the nigral dopamine neuron. However, this topographic scheme is not absolute for every neuron, as there are exceptions to this pattern, and there is also evidence for collateral innervation to the SNc of the opposite hemisphere. It is also worth noting that the dendritic processes of SNc dopamine neurons also project into the SNr.

2.4 **Dopamine Receptors and Parkinson's Disease**

The therapeutic relevance of dopamine receptors in the treatment of Parkinson's disease are twofold. First, depleted levels of striatal dopamine in Parkinsonian condition produce adaptive postsynaptic changes resulting from a heightened sensitivity of dopamine receptors to dopamine and dopamine agonists - a condition called "dopamine receptor supersensitivity". Hence, understanding the molecular basis of the postsynaptic changes in the denervated striatum may offer insight and the possibility

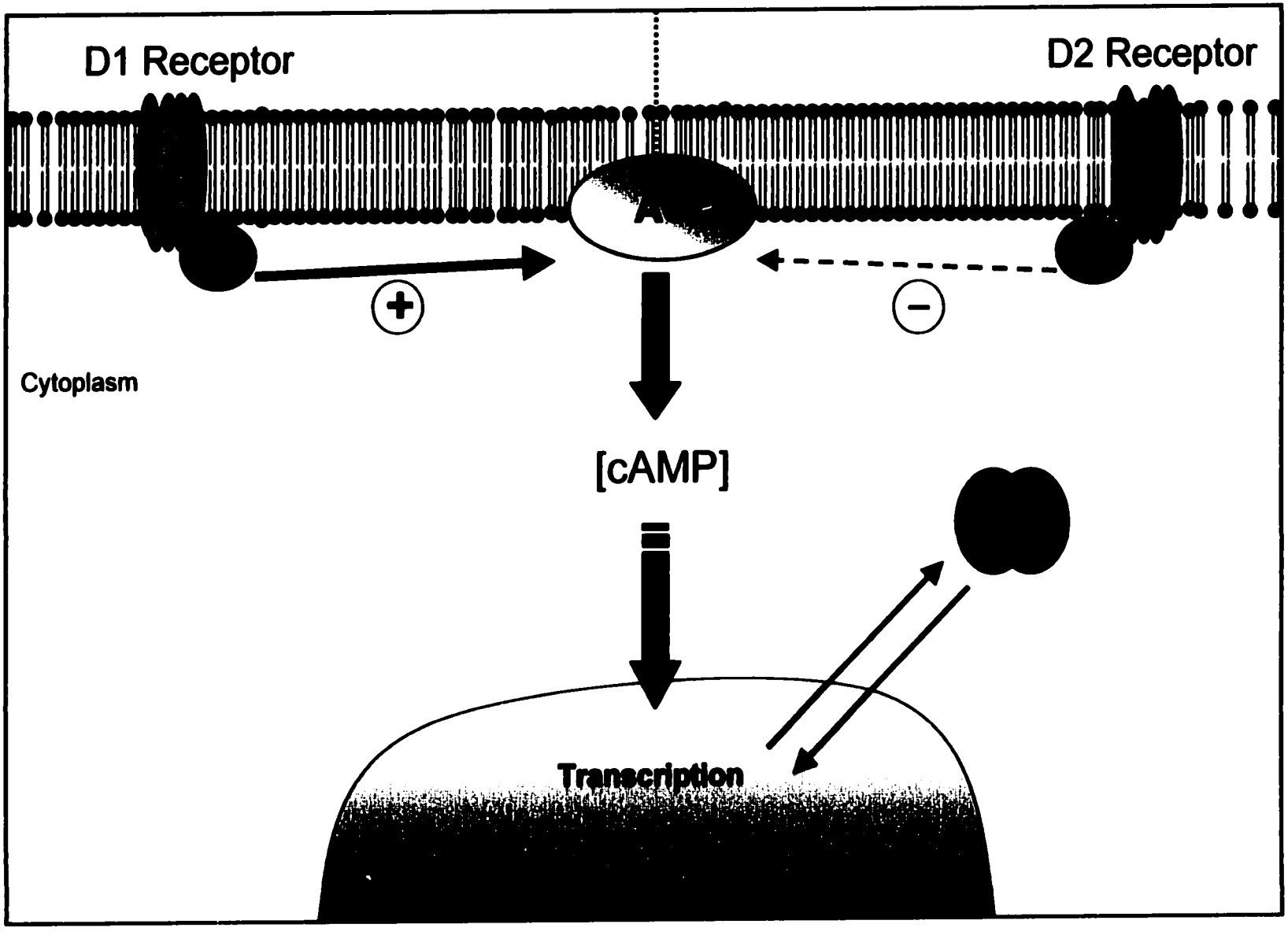
of designing superior therapeutics to alleviate the confounding side-effects of chronic intermittent L-Dopa therapy. Secondly, as PD progresses, the remaining dopamine neurons have a diminished capacity to synthesize dopamine from L-Dopa therein limiting the effectiveness of this therapeutic approach. Consequently, direct acting dopamine-receptor agonists may provide symptomatic relief and, more importantly, bypass the metabolic demands on the remaining nigrostriatal dopamine neurons. Indeed, several clinical studies currently support the notion of dopamine agonist therapies for the alleviation of symptomatic Parkinsonism. Hence, the following section of this chapter will introduce the role of dopamine receptors and dopamine receptors agonists for the symtomatic treatment of Parkinson's disease.

2.3.1 *Structure and Functions of Dopamine Receptors*

The biological actions of dopamine are mediated by G-protein coupled receptor proteins. There are currently five (5) types of dopamine receptors, termed D1-D5, which are characterized by functional and pharmacological properties. Dopamine D1-like receptors, which include the D1 and D5/D1b subtypes, are coupled to Gs proteins and stimulate the formation of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase (AC). In contrast, D2-like dopamine receptors, which include D2, D3 and D4 receptor subtypes, are associated with Gi proteins and retard the formation of cAMP by inhibiting AC activity (Kebabian and Calne, 1979). Amino acid sequence analysis of dopamine receptors has revealed that significant homology exists between all dopamine receptors, particularly within the transmembrane domains of members of the same subtype (Jarvie and Caron, 1993).

All dopamine receptors share common structural characteristics. For instance, D1-like and D2-like dopamine receptors are comprised of seven transmembrane domains with cytoplasmic C-termini. D1-like receptors have longer cytoplasmic carboxy tails and shorter third intracellular loops, characteristic of Gs coupled receptors. In contrast, D2-like dopamine receptors have shorter C-terminal tails and larger third intracellular loops – indicative of their negative influence on AC (reviewed by Missale et al., 1998; See *Figure 2.5*). In addition to influencing the function of AC, dopamine receptors have also been reported to modulate cation homeostasis by affecting calcium channels, potassium channels, the activity of the sodium/potassium exchanger, and the sodium-potassium-ATPase (reviewed by Missale et al., 1998).

Figure 2.5 Schematic representation of the nature of adenylyl cyclase coupling of dopamine D1 and D2 receptor subtypes. Postsynaptic D1 dopamine receptors, through Gs protein coupling, induce the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) by adenylyl cyclase (AC). In contrast, and in separate neurons, Gi-coupled D2 dopamine receptors inhibit the activity of AC. Changes in intracellular concentration of cAMP ([cAMP]) can mediate the regulation of gene expression, including the expression of immediate early genes (IEGs), a family of inducible nuclear transcription factors. See text for detailed discussion and implications of dopamine regulated IEG expression in the pathophysiology of PD.


D1 Receptor
D2 Receptor
Cytoplasm
[cAMP]
Transcription

2.3.2 *Distribution of Dopamine Receptors in the Basal Ganglia*

In the basal ganglia, dopamine receptor subtypes are located on anatomically and neurochemically distinct neuronal populations. In the caudate nucleus, postsynaptic D1 dopamine receptors are predominantly located on GABAergic striatonigral projection neurons that also express the neuropeptides dynorphin and substance P (*Figure 2.3*; Gerfen et al., 1990; Huang et al., 1992; LeMoine et al., 1991). D5 receptors are expressed at post-synaptic sites in the striatum and co-localize with D1 receptors. However, D1 receptors are also found in the substantia nigra pars reticulata where no D5 receptors have been detected (Bergson et al., 1995). D2 dopamine receptors are located on postsynaptic striatal GABAergic neurons which project to the GPi (the striatopallidal neurons) and express enkephalins (Gerfen et al., 1990). The shell of the nucleus accumbens and the Islets of Calleja contain D2-like dopamine receptors (D2 and D3) where increasing experimental evidence supports the notion that inhibition of these D2 receptors mediate the actions of typical antipsychotic medications (Abi-Dargham et al., 2000). Presynaptic D2-dopamine receptors are also found on the dopaminergic nigrostriatal neurons of the pars compacta, where stimulation by dopamine may serve to regulate synaptic release of dopamine (Meador-Woodruff et al., 1989). Hence, stimulation of dopamine receptors results in the parallel activation of striatonigral and striatopallidal neurons by D1 and D2 receptors, respectively. Therefore, motor function is facilitated by the coordinated activity of these opposing neural circuits.

2.3.3 *Dopamine Receptors in Parkinson's Disease*

Central to the application of dopamine receptor agonists for the treatment of motor control in Parkinson's disease is an understanding of the changes in striatal dopamine receptors in PD. Studies using Positron Emission tomography (PET) and [11C]raclopride, a D2-receptor antagonist, binding as a measure of D2-receptor levels revealed that levels of D2 receptors are increased in PD when compared to age-matched controls (Antonini et al., 1997). However, following sustained treatment with L-Dopa, these changes in striatal D2 receptors are reversed, with significantly lower levels in the caudate of PD patients (Antonini et al. 1997).

Activation of D1 and D2 dopamine receptors evoke motor activity in unlesioned animals (Braun and Chase, 1986). Depletion of striatal dopamine levels by ablation of the nigrostriatal dopamine pathway produces a condition of heightened dopamine receptor sensitivity (Arnt, 1985). Since the individual actions of each dopamine receptor subtype are known to exert opposing influences on the production of cAMP and neuropeptide expression, it is therefore all the more remarkable that coincident stimulation of D1-like and D2-like dopamine receptors can elicit synergistic effects on motor function – even though these receptor populations reside on distinct neuronal populations (Robertson and Robertson, 1986; Sonsalla et al., 1988; Robertson, 1992). This finding is reflected in the clinical setting by the observation that L-Dopa therapy in combination with bromocriptine, a D2 agonist, enhances the efficacy of L-dopa (Calne et al., 1974; Rinne et al., 1987). Further, the fact that switching PD patients from oral, intermittent L-Dopa to a continuous administration regime can also diminish motor response fluctuations has facilitated investigation into the role of dopamine receptor supersensitivity and dose scheduling of dopamine agonists for the treatment of PD (Chase et al., 1993; Shoulson et al., 1975). Consistent with the clinical effects of changing dopamine agonist scheduling in PD patients, modification of dopamine agonist administration in a rat 6-OHDA model of PD also influences behavioural outcome through D1 and D2 receptors (Engber et al., 1989).

Dopaminergic regulation of gene expression is dramatically influenced by striatal dopaminergic denervation. Following destruction of the nigrostriatal dopamine pathway in rodents, striatopallidal neurons display increased levels of activity while striatonigral neurons become less active (Pan et al., 1985; Pan et al., 1988; Crossman, 1990). These findings are consistent with the notion that striatal dopamine exerts a tonic inhibitory influence on striatopallidal neurons (Gerfen et al., 1990). Perhaps through changes in neuronal activity, denervation causes sustained changes in gene expression in striatal efferent neurons, such as changes in nuclear transcription factors called immediate early genes (Jian et al., 1993; Engber et al., 1991; Hong et al., 1978).

2.5 Immediate Early Genes

Immediate Early Genes (IEGs) are a class of rapidly inducible proteins, many of which are nuclear transcription factors and protooncogenes. IEG proteins are characterized by their rapid and transient induction without requiring *de novo* mRNA synthesis, short protein half-lives, and the requirement for protein synthesis for cessation of action (Sheng and Greenberg, 1990). Since IEG proteins are readily and rapidly induced by extracellular stimulation, IEG proteins are thought to provide a means by which extracellular events are coupled to intracellular responses (Morgan and Curran, 1989). Many IEG proteins are associated with cellular transformation and cell cycle progression, however, in post-mitotic neurons, these protooncogenes play important functions in the regulation of neuronal adaptation and plasticity.

Two prototypic classes of IEG proteins are the Fos and Jun families. Currently, there are at least four identified Fos-related proteins, c-Fos, FosB, Fra-1, and Fra-2 (Fos-related antigen), and three Jun family proteins, c-Jun, junB and JunD. The transcriptional activation by IEG proteins involves the dimerization of Jun and Fos proteins via tandem leucine repeat domains called the "leucine zipper". Fos proteins can heterodimerize with Jun family proteins, but Jun proteins can also homodimerize. As dimers, IEG complexes regulate gene expression by binding to a DNA motif called the AP-1 site found in the promoter of targeted genes. Interestingly, AP-1-like binding sites are found in the promoters of the neuropeptides enkephalin, neurotensin, substance P, and dynorphin, suggesting that IEGs can regulate expression of neuropeptides and influence postsynaptic neuronal activity (Robertson et al., 1995).

2.5.1 *c-fos and Dopaminergic Neurotransmission*

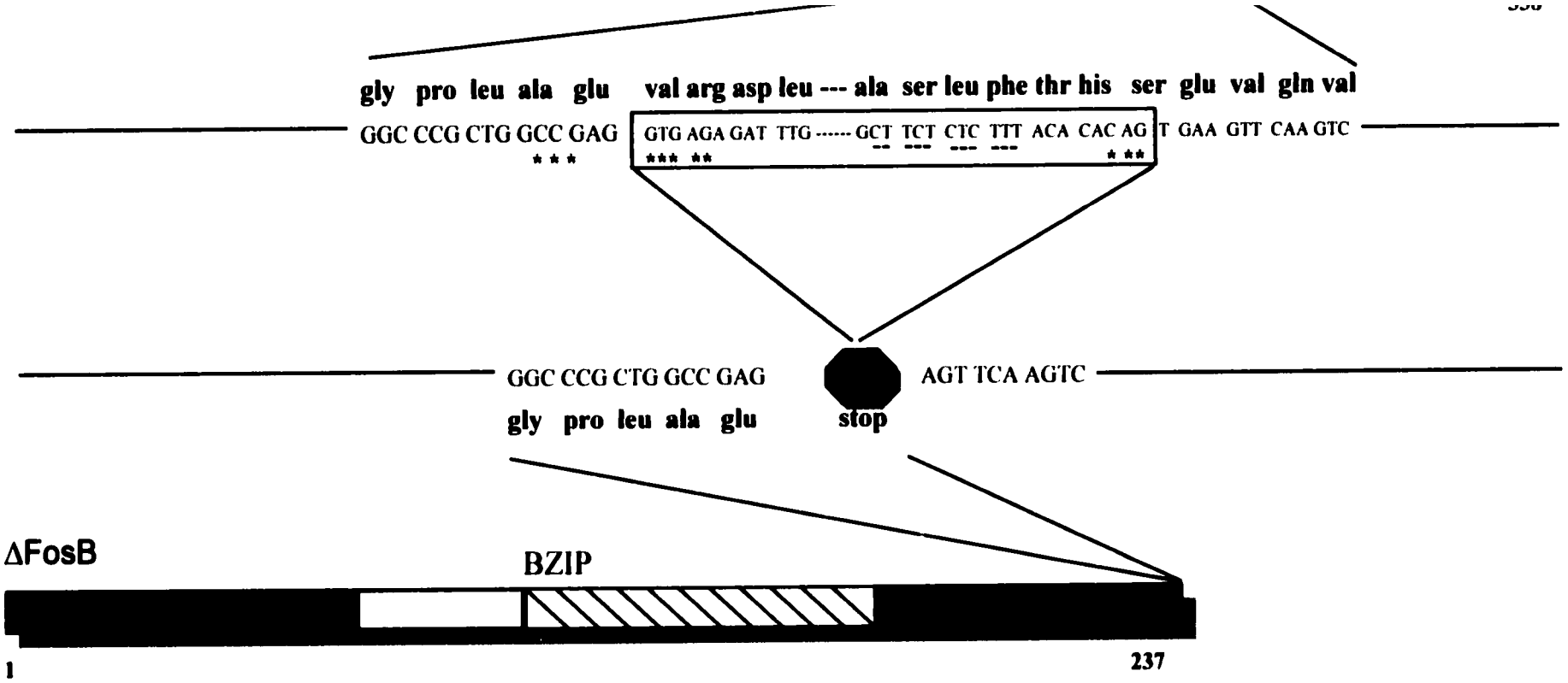
Fos is a nuclear transcription factor and the 55kDa protein product of the IEG *c-fos* (for cellular *f*ollicular *os*teosarcoma). *c-fos* mRNA is readily induced by factors such as growth factors, calcium, and is coordinately regulated by several DNA elements in its promoter region (reviewed by Herdegen and Leah, 1998). Because basal expression of Fos in the brain is very low, and Fos is both highly inducible and transient, detection of Fos can be employed as a marker of neuronal activation (Sagar et al., 1988). For instance, systemic administration of stimulants which greatly enhance extracellular concentrations of dopamine, such as cocaine or amphetamine, induce dramatic elevations in *c-fos* expression in the striatum

(Robertson et al., 1989; Graybiel et al., 1991). In contrast, levodopa and direct-acting D1-like receptor agonists, such as SKF 38393, only weakly increase Fos-like immunoreactivity in the intact striatum (Robertson et al., 1989a, 1989b; Robertson et al., 1992). Destruction of the nigrostriatal pathway, however, endows levodopa and D1-like agonists with the ability to dramatically enhance *c-fos* expression in the denervated striatum (Morelli et al., 1993; Paul et al., 1992; Robertson et al., 1989), which are completely blocked using D1-receptor antagonists (Morelli et al., 1993; Robertson et al., 1989a). Interestingly, D2-receptor agonists induce Fos in the intact striatum but fail to induce *c-fos* in the denervated striatum (Robertson et al., 1989b; Paul et al., 1992; Robertson et al., 1992). However, D2 agonists increase Fos expression in the GP following striatal denervation, suggesting that these changes are mediated by the population of striatopallidal neurons which express D2 receptors (Jian et al., 1993). Interestingly, administration of either D1 or D2 receptor agonists elicit ipsiversive turning behaviour in rats unilaterally lesioned with 6-OHDA. However, coadministration of D1 and D2 receptor agonists evoke a potentiated behavioural response, suggesting that post-synaptic alterations in gene expression modify striatal responsiveness to dopamine agonists. Supporting this notion, sustained increases in striatal expression of the IEG, ΔfosB, have been reported in the denervated striatum of rodents and primates – suggesting that post-synaptic changes in the striatum following chronic dopaminergic denervation may be facilitated by this transcription factor.

2.5.2 *FosB, a marker of chronic neuronal activation*

The protein product of the *fosB* gene, FosB, is a 45 kDa nuclear transcription factor and proto-oncogene which was isolated by virtue of homology in the DNA binding domain of c-Fos (Zerial et al., 1989). FosB, reminiscent of other Fos family proteins, can bind Jun proteins and regulate AP-1 mediated gene expression. Alternative splicing of the *fosB* transcript yields a second FosB protein product, called FosB/SF or ΔFosB (Nakabeppu and Nathans, 1991; Zerial et al., 1991). This shorter version of FosB results from a 140 bp deletion in the *fosB* mRNA and the loss of a C-terminal 47 amino acid proline rich region common to Fos proteins (See *Figure 2.6*). Like other Fos family IEG proteins, ΔFosB protein

Figure 2.6 Alignment and comparison of protein sequences of FosB and ΔFosB proteins. Alternate splicing of *fosB* results in the translation of two FosB proteins, FosB and ΔFosB. A 140 bp deletion results in the creation of a stop codon as indicated by the cDNA sequence in box. The truncated species, ΔFosB, which contains amino acids 1-237 of FosB lacks the domain required for transcriptional transactivation by Fos-Jun heterodimer complexes. See text for details. Abbreviations: BZIP –DNA binding domain including basic (B) and leucine zipper (ZIP) regions; (Pro)7 – hepatoproline sequence; ** - asterisks indicate conserved residues in donor-acceptor sequences found in FosB. (Nakabeppu and Nathans, 1991).



Adapted from: Nakabeppu & Nathans, 1991

heterodimerize with Jun family proteins and can bind DNA at AP-1 sites (Nakabeppu and Nathans, 1991; Yen et al., 1991). However, this truncated form of FosB lacks transcriptional transactivation activity and may even suppress transcriptional activity of c-Fos and full-length FosB (Nakabeppu and Nathans, 1991; Yen et al. ,1991).

FosB is 338 amino acids in length while ΔFosB is 237 amino acids. Since ΔFosB shares complete nucleotide homology with FosB, selective detection of ΔFosB is not possible by immunohistochemical techniques. However, selective immunohistochemical detection of FosB is possible using antibodies directed against the C-terminal region of FosB, which is not found in ΔFosB (Doucet et al., 1996; Chapter 4). Hence, ΔFosB expression can be inferred by examining the differential immunoreactivity of antibodies directed against C- or N-terminal regions of FosB. Basal expression of FosB in the adult CNS is very low (Herdegen et al., 1995).

Sustained alterations in striatal dopamine levels produce prolonged induction of AP-1 DNA binding protein complexes which contain Fos-related proteins, called "chronic FRAs" (Fos related antigens (Moratalla et al., 1996). Chronic administration of cocaine (20 mg/kg, i.p. for 6-7 consecutive days) increases levels of extracellular dopamine in the striatum and induces chronic FRAs (Hope et al., 1992; Hope et al., 1994). Interestingly, chronic FRAs are also induced by administration of dopamine agonists to animals which have sustained depletion of striatal dopamine following lesions of the nigrostriatal pathway (Pennypacker et al., 1992; Doucet et al., 1996). One component of the chronic FRAs which comprise the increased AP-1 activity following chronic dopaminergic stimulation has been identified as ΔFosB (Doucet et al., 1996; Hiroi et al., 1997).

ΔFosB expression has been proposed to mediate changes in gene expression which produce drug addiction (Hiroi et al., 1997), seizures (Chen et al., 1995; Hiroi et al., 1998), and dopamine induced dyskinesias (Doucet et al., 1996; Andersson et al., 1999). Recently, Greenberg and collegues generated a mouse line deficient of *fosB* (Brown et al., 1996). These mutant mice did not exhibit any cognitive deficits, but suffered from a defect in nurturing behaviour attributed to a disruption of gene expression in the preoptic area of the hypothalamus (Brown et al., 1996). In addition, *fosb*$^{-/-}$ mice exhibit sensitization to

chronic cocaine administration, but show a delay in the development of tolerance to electroconvulsive seizure activity (Hiroi et al., 1997; 1998).

Taken together, dramatic and sustained elevations in neuronal ΔFosB expression are associated with changes in behavioural responses to stimuli. ΔFosB has been proposed to mediate long-term changes in gene expression related to the adaptive neuronal reponses which mediate these behaviours. Hence, in the instance of striatal dopamine deficiency accompanying PD, ΔFosB expression may facilitate the development of post-synaptic changes which produce dyskinesias in response to repeated administration of dopamine agonists (Dragonow et al., 1991; Doucet et al. 1996).

In Chapter 4 of this thesis, I present data that describe a functional role for striatal FosB expression in a rat 6-OHDA model of behavioural sensitization to intermittent administration of dopamine receptor agonists (Crocker et al., 1998). Results from this study support the proposal that D1-receptor mediated induction of striatal FosB expression may be causally related to the debilitating behavioural side-effects associated with chronic dopamine drug therapies in PD.

Chapter 3. Etiology of Parkinson's disease

Current Perspectives and Hypotheses

Neurodegeneration and Neuroprotection in Parkinson's disease

Even though the identification that Parkinson's disease is associated with the loss of midbrain dopamine neurons, there continues to be debate over what processes are involved in the demise of this neuronal population. The aim of this chapter will be to summarize the current understanding over the questions of cell death and apoptosis in Parkinson's disease. Further, this chapter will also outline the principal mechanisms underlying neuronal death with reference to the potential scientific and therapeutic opportunities these may offer for the treatment of PD.

3.0 *Neuronal Death and Parkinson's disease*

Cells undergo one of two distinct forms of death, necrosis or apoptosis. Necrosis, is a rapid and uncontrolled form of cell death often associated with traumatic cellular injury. Necrosis is marked by loss of ion control, resulting in osmotic stress and marked cytoplasmic swelling (Johnson and Deckwerth, 1993). Cells that die necrotically lyse, spill their contents into their immediate environment, and frequently elicit humoral or inflammatory responses (Raff, 1998). In contrast, apoptosis, frequently referred to as programmed cell death, is a form of "cell suicide" in which a cascade of intracellular signals culminates in the orderly dismantling of the cell into membrane-enclosed vesicles (Kerr et al., 1972; Wylie et al., 1980). Apoptosis is defined by morphological criteria, namely, membrane blebbing, chromatin condensation, and endonuclease activation resulting in DNA fragmentation seen as laddering by gel electrophoresis (Wylie et al., 1980). As apoptosis is actively executed by the cell itself, these physical transformations require *de novo* mRNA and protein synthesis.

3.0.1 *Caspases and Neuronal Death*

Neuronal death is recognized as a component of normal vertebrate development which is thought to ensure the formation of appropriate cellular connectivity, since cells that do not establish appropriate connections are deprived of necessary trophic support (Oppenheim, 1991; Raff et al., 1993). More recently, inappropriate cell death has been associated with a variety of neurodegenerative disorders,

prompting the notion that inhibition of neuronal death in pathological conditions may prevent loss of function accompanying brain injury or disease (Thompson, 1995).

The initiation and execution of programmed cell death is mediated by a family of cysteine proteases termed caspases. Horvitz and colleagues were the first to implicate caspases in the developmental apoptosis of the nematode *Caenorhabditis elegans* (Ellis et al., 1986). During development, 131 of 1090 cells in *C. elegans* die by apoptosis (Ellis et al., 1991). Subsequent studies determined that several proteins, termed *ced-3*, *-4*, and *–9*, are key determinants in the proper regulation of cell death during *C.elegans* development. Mutation or deletion of *ced-3* results in a loss of developmental death, while *ced-9* functions to antagonize the death promoting actions of both *ced-3* and *ced-4*.

Identification of mammalian homologs of the *ced* genes has fostered the theory that the machinery governing programmed cell death is evolutionarily conserved. For instance, the mammalian CED-9 homolog, BCL-2, is a potent inhibitor of cell death in both developmental and pathological conditions (Allsop et al., 1994; Martinou et al., 1994). The mammalian homolog of the nematode *ced-3* gene is the enzyme, interleukin 1-β converting enzyme (ICE). Yuan et al., (1993) demonstrated that expression of ICE was sufficient to induce cell death, supporting the notion that the machinery mediating cell death in worms (C. elegans) was conserved in mammals. This finding prompted the identification a burgeoning family of ICE-like enzymes of which there are currently at least 14 members. Given the diversity of nomenclature initially, this protease family was renamed "caspase" – denoting the specificity of these enzymes for cysteine residues "c", and the tendency of these enzymes to cleave after aspartic acid residues (Alnemri et al., 1996).

Caspases are classified into three groups, based on their prefered tetrapeptide target sequence and physiological role(s). Group I caspases (caspase-1,-4,-5) are predominantly involved in inflammation and cleave following the tetrapeptide sequence WEHD. Accumulating evidence suggests that group II caspases (caspase-2,-3,-7) are involved in the execution of cell death and exhibit selectivity for the tetrapeptide sequence DexD. Finally, group III caspases (caspase-6,-8,-9) are thought to initiate cell death and prefer the tetrapeptide sequence I/L/VexD (Cohen, 1997; Nicholson and Thornbury, 1997).

3.0.2 *Inhibitor of Apoptosis (IAP) proteins*

Increasing evidence suggests that neuronal death under a variety of physiological and pathological conditions is mediated by caspase-3 (Kuida et al., 1996; Fink et al., 1998; Xu et al., 1999; Gervais et al., 1999). Recent studies have also described caspase-3 activation in several models of neurodegeneration and demonstrated that inhibition of this caspase can enhance neuron survival (Hara et al., 1997; Fink et al., 1998; Xu et al., 1999). Hence, factors capable of blocking caspase-3 may have significant utility for the treatment of neurodegenerative disorders (Robertson et al., 2000).

A family of mammalian proteins which function as potent inhibitors of caspases 3 and 7 have recently been cloned and characterized (Roy et al., 1995; Liston et al., 1996; Devereaux et al., 1997; Roy et al., 1997). Inhibitor of Apoptosis (IAP) proteins were first characterized as proteins encoded by insect viruses which facilitate propagation in host cells by impairing defensive apoptosis responses. The first IAP proteins to be discovered were found in baculoviruses *Cydia pomonella* granulosis virus (CpGV) and *Orgyia pseudotsygata* nuclear polyhedrosis virus (OpMNPV) (Birnbaum et al., 1994; Crook et al., 1993). Subsequent identification of mammalian homologs of viral inhibitor of apoptosis (IAP) proteins revealed that IAPs may represent an evolutionarily conserved mechanism of preventing cell death (Huang et al. 2000).

Presently, six mammalian IAP proteins have been identified, including neuronal apoptosis inhibitor protein (NAIP) (Roy et al., 1995), X-linked IAP protein (XIAP/hILP) (Liston et al., 1996; Duckett et al., 1996), Human IAP proteins-1 and 2 (HIAP-1/c-IAP-2; HIAP-2/c-IAP-1) (Liston et al., 1996; Rothe et al., 1995), survivin (Ambrosini et al., 1997), and BRUCE (Hauser et al., 1998) (*Figure 3.1*). NAIP, the first mammalian IAP to be identified, was found using positional cloning as a candidiate gene for the childhood neuromuscular disorder spinal muscular atrophy (SMA). It it currently thought that NAIP may be associated with disease severity and, not necessarily, causality, since deletions in the *naip* locus are more prevalent in type 1 SMA – the most severe form of this disease (Roy et al., 1995). Supporting this notion,

Xu et al. (1997b) found a correlation between neurons that display NAIP immunoreactivity and those neuronal populations that have reported to suffer pathological alterations in severe forms of SMA.

3.0.2.1 *Structure and Function of IAP Proteins*

IAP proteins are defined by the presence of one or more baculovirus IAP repeat (BIR) domains. BIR domains are conserved sequence of 45-60 amino acids which have been accredited with the ability of IAP proteins to inhibit group II caspases (Devereaux et al., 1998), but may also function to mediate a variety of protein-protein interactions, including regulators of IAP function (Miller, 1999). In support of this latter notion, drosophila IAP proteins (such as DIAP-1) regulate the activation of drosophila caspases (Drice) by sequestering the pro-apoptotic proteins Hid, Grim, and Reaper (Wang et al., 1999; Goyal et al., 2000). By analogy, several recent reports have identified a functional homolog of these proapoptotic-IAP-binding proteins in mammals, and named this protein second mitochondria-derived activator of caspase (Smac; Du et al., 2000) or DIABLO (Verhagen et al., 2000). A subsequent report by Chai et al. (2000) identified the nature of Smac/DIABLO interaction with the mammalian IAP protein, XIAP. Further, this novel mammalian apoptosis regulating protein requires homodimerization to inhibit the ability of XIAP, c-IAP1, or c-IAP2 to inhibit caspase-3 (Chai et al., 2000). In addition, Smac dimers selectively bind the second or third BIR domains (BIR2,3) of XIAP, but not BIR1, and thus impair the ability of XIAP to inhibit caspase-3 mediated proteolysis (Takahashi et al., 1998; Chai et al., 2000). Together with the studies involving Hid, Grim, and Reaper inhibition of dIAP function, these studies support the proposal that regulation of IAP protein function by BIR-interacting proteins has been conserved by evolution.

The second functional domain present in most IAP proteins is a C-terminal RING finger domain. While it has been proposed that this structural motif may facilitate IAP protein interactions with DNA, however, work by Yang et al., (2000) has recently demonstrated that the RING finger domain of XIAP contains E3 ubiquitin ligase activity. Interestingly, the mammalian IAP protein BRUCE contains a ubiquitin conjugating (UBC) domain, though, unlike the other mammalian IAP proteins, BRUCE has not been shown to impair cell death (Hauser et al., 1998; Liston et al., 1996). Hence, IAP-RING finger domains may represent a second important biological function of mammalian IAP proteins - the regulation

of intracellular protein degradation via the ubiquitin-proteosome pathway (reviewed by Ciechanover et al., 2000).

3.0.2.2 *IAPs and Neurodegeneration*

Immunohistochemical studies have shown that there is a striking overlap between neuronal populations lost in SMA and the distribution of NAIP immunoreactivity in the rodent CNS (Xu et al., 1997a). This work also revealed a relative enrichment of NAIP levels in the hind and forebrain neuronal groups known to be relatively resistant to excitotoxic and anoxic damage. For instance, high levels of NAIP immunoreactivity were observed in mesencephalic trigeminal neurons; these sensory neurons are known to be selectively resistant to the injurious effects of kainic acid (Colonnier et al., 1979). In the striatum, NAIP immunoreactivity was confined to cholinergic interneurons and up-regulated in these interneurons by a brief episode of transient forebrain ischemia (Xu et al., 1997b). It is well known that cholinergic neurons in the striatum are considerably more resistant to excitotoxic and ischemic insults than medium-spiny neurons which comprise about 95% of the total populations of neurons in this structure (Bogman et al., 1987; Chesselet et al., 1990). Consequently, the rapid increase in NAIP levels which occur in cholinergic neurons after transient global ischemia may contribute to the relative resistance of these neurons to ischemic and excitotoxic injury. In contrast, neurons of the reticular nucleus of the thalamus, known to be exquisitely sensitive to excitotoxic or ischemic injury, display weak NAIP immunoreactivity. Unlike the majority of thalamic neurons, NAIP levels were not elevated in neurons of the reticular nucleus by a brief period of global ischemia. These findings suggest that there is a positive relationship between the ability of neuorns to increase NAIP expression and resistance to excitotoxic/ischemic injury. These results also suggested that treatments capable of increasing NAIP expression should be neuroprotective.

As a direct test of the *in vivo* neuroprotective effects of NAIP, we used an adenoviral expression system to elevate NAIP levels in the pyramidal CA1 hippocampal neurons (Xu et al., 1997b). This expression system contained a 6-Myc tag that was used to confirm overexpression of the NAIp-Myc tagged construct. Cell counts performed on coronal tissue sections immunohistochemically processed to visualize Myc-labeled CA1 pyramidal neurons suggested that approximately the same number of neurons were

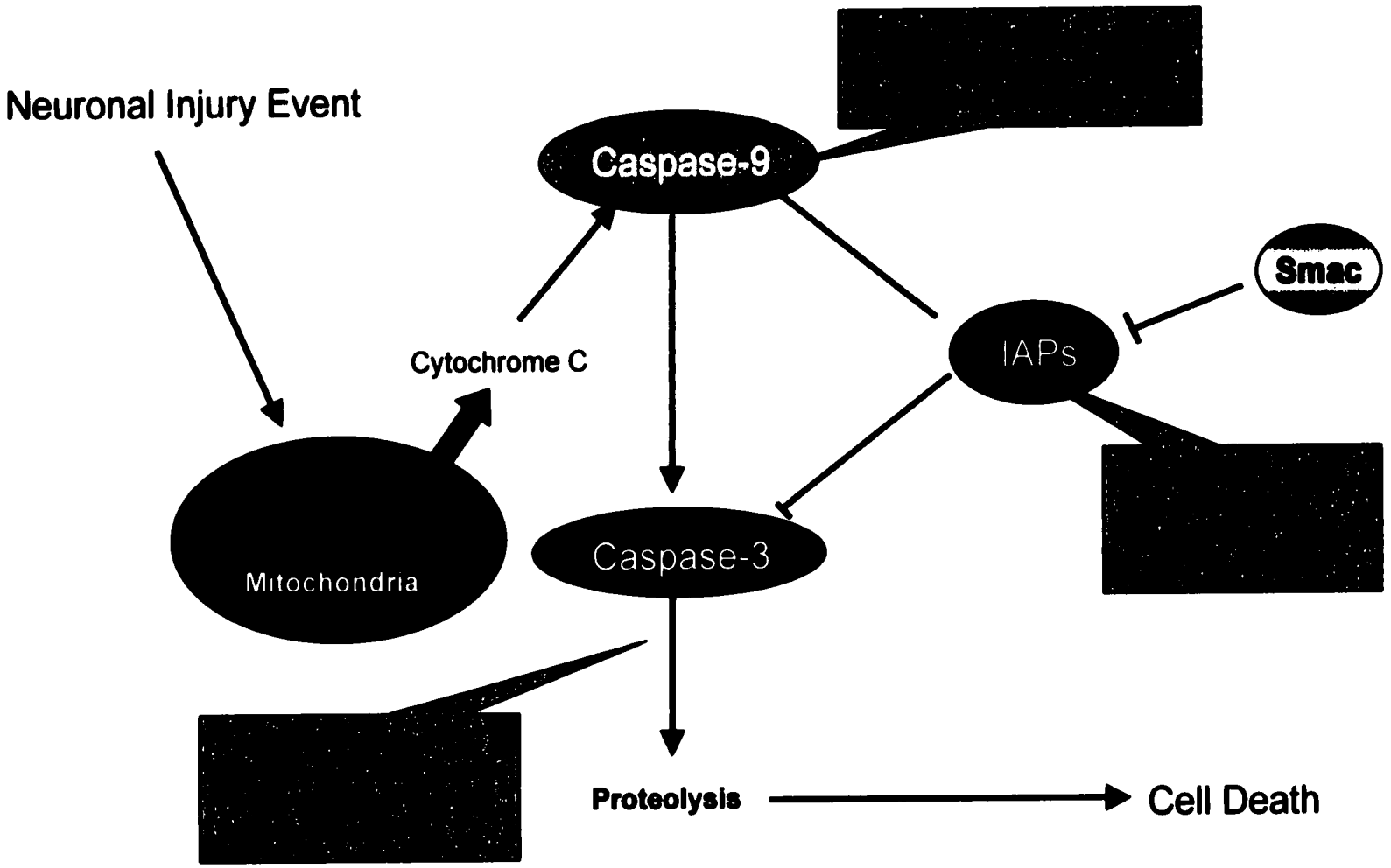
infected as survived the anoxic insult. NAIP overexpression suppressed DNA fragmentation normally evoked by an episode of transient forebrain ischemia suggesting that NAIP overexpression protected against ischemic injury by blocking apoptosis. In support of this hypothesis, adenovirally-mediated overexpression of XIAP, also blocks ischemia-induced increases in conformationally active caspase-3 *and* DNA fragmentation in CA1 neurons (Xu et al., 1999) (See *Figure 3.2*).

While cellular protection by IAP overexpression is profound, a major concern following cerebral ischemia is that blockade of apoptosis may not be sufficient to prevent loss of neurophysiological processes responsible for movement and/or cognition. Spatial memory is sensitive to the loss of CA1 pyramidal neurons following short periods of global cerebral ischemia. The Morris Water maze was therefore employed to determine whether XIAP overexpression in hippocampal neurons could maintain long-term spatial memory. Analysis of neuronal survival 14 days following a 12 min episode of cerebral ischemia revealed a 90% loss of CA1 neurons in the dorsal hippocampus of animals injected bilaterally with control virus encoding the bacterial gene *lacZ*. The XIAP adenovirus increased cell survival by 5-6 fold relative to controls. Moreover, the XIAP adenovirus-treated animals failed to display loss of spatial memory in the water maze test. Furthermore, normalized neuronal function was confirmed using markers of gene expression, verifying the healthly state of the XIAP-protected neurons. Hence, XIAP overexpression maintains the homeostasis of CA1 neurons following transient forebrain ischemia. Indeed, comparable efficacy of IAP-mediated neuroprotection has also been reported in *in vitro* excitotoxic models and *in vivo* axotomy models of neurodegeneration (Simons et al., 1999; Perrelet et al., 2000).

3.0.2.3 *IAP proteins and Parkinson's disease*

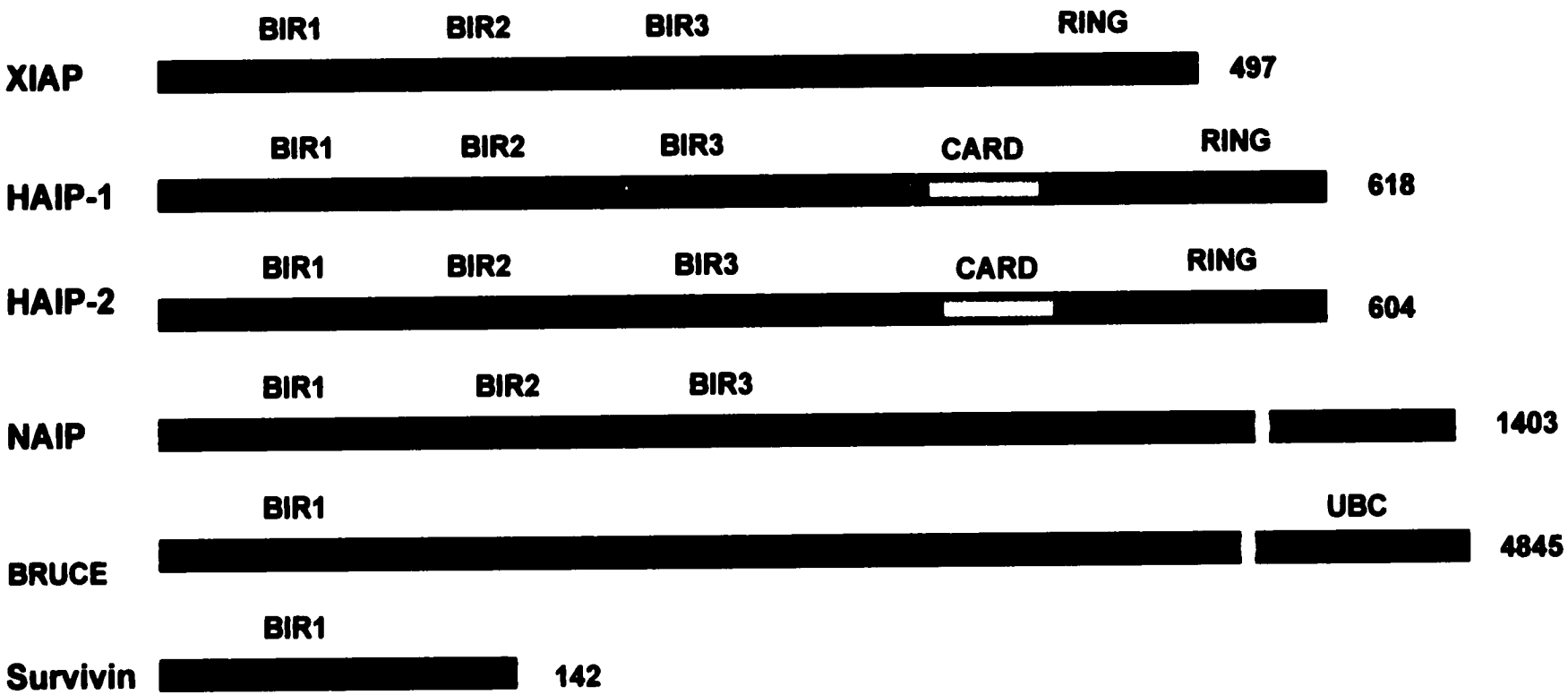
In this thesis, I have examined the neuroprotective effects of IAP overexpression on the survival of dopamine neurons in a 6-OHDA rat model (Chapter 5) and an MPTP mouse model (Chapter 6) of PD. These studies provide the first demonstrations of IAP-mediated functional and cellular neuroprotection in models of PD.

Figure 3.1 Flowscheme depicting the fundamental steps involved in activation of caspases in neuron cell death. A variety of cell death triggers, such as oxidative stress, can produce perturbations in mitochondrial function resulting in the release of cytochrome c and activation of caspase-9. Subsequent activation of caspase-3-mediated proteolysis facilitates the demise of neurons. For example, caspase-3 evokes the degradation of nuclear DNA through the activation of enzymes such as CAD, a deoxyribonuclease, while inactivating proteins involved in DNA repair, such as topoisomerase IIα. As indicated, Inhibitor of Apoptosis (IAP) proteins regulate the cascade of caspase activation in neurons by interrupting the proteolytic activation and enzymatic activity of both "upstream" initiator caspases (caspase-9), and "downstream" executioner caspases (caspase-3). Similarly, IAP proteins can be regulated by the protein Smac - though a role for Smac in neurons has not yet been demonstrated (see text for details).



IAP -Mediated Neuroprotection by Caspase Inhibition

Figure 3.2 Schematic representation of the mammalian Inhibitor of Apoptosis (IAP) family of proteins. IAP proteins are defined by the presence of at least one BIR domain. Functions of the structural motifs are discussed in the text. The CARD domains of HIAP-1/2 are not found in other mammalian IAP proteins and the function of these domains in IAP proteins has not been established. The relative locations of structural features are indicated by colored boxes; baculovirus inhibitor repeat (BIR) domains – red boxes; caspase recruitment (CARD) domains – yellow; RING zinc finger domain – green; ubiquitin-conjugating activity (UBC) domain –blue. Amino acid length is shown to the right of each protein. *Adapted from: Robertson et al. (2000) Brain Pathol.* ***10****: 283-292.*



Members of the Mammalian IAP Protein Family

3.0.3 *Cell Death in Parkinson's Disease*

From the study of apoptosis in development has arisen an increased understanding of the role and actions of cell death in conditions of disease and injury. It is widely held that inappropriate apoptosis is also involved in many neurodegenerative disorders, and that attenuation of cell death in these conditions may offer clinical benefit (Thompson, 1995). A role for apoptosis in neuron death following stroke, traumatic brain injury, and in Alzheimer's and Huntington's diseases is well supported; however, a detailed understanding of the process(es) mediating nigral dopamine neuron loss in PD is presently unclear. Although many neuropathological conditions have been readily shown to involve caspases as well as direct evidence for apoptosis, findings substantiating apoptosis and/or caspase-mediated proteolysis in PD have not yet been demonstrated. In fact, as many reports refute a role for apoptosis in animal models of PD (Jackson-Lewis et al., 1995; Nishi, 1997; Banati et al., 1998; Crocker et al., submitted), as support the claim for apoptotic death in PD (Mukerjee et al., 1997; Tatton and Kish, 1998; Spoonen et al., 1998). Further, analysis of post-mortem human tissues do not resolve this issue of apoptosis in PD (Wullner et al., 1999; Jellinger 2000), since, Anglade et al. (1997) reported evidence of DNA fragmentation, as indicated by TUNEL labeling, in the substantia nigra of normal human tissues, without comparable differences detected in equivalent tissues from PD patients. In contrast, Mochizuki et al. (1997) reported that increased TUNEL labeling was detected in PD tissues, though this amounted to approximately 2.1% of the SNc neurons – ironically, this value is identical to the degree of TUNEL-positive cells reported as a result of aging (Anglade et al., 1997). Clearly, accuracy in the clinical diagnosis of PD aside from the variety of PD-like conditions may dramatically influence the histological outcomes of such studies. Resolution of the nature of cell death in PD requires additional study.

The possible involvement of caspase-3 and the neuroprotective potential of IAP proteins in models of Parkinson's disease have not been previously examined. Accordingly, I have studied the activation of caspase-3 and the effects of IAP-mediated neuroprotection in two animal models of PD; intrastriatal 6-OHDA administration to rats (see below, and Chapter 5) and, administration of MPTP to mice (see below,

and Chapter 6). These studies also analyse caspase-3 activation as a putative mechanism of neuronal loss in these models.

3.1 Theories of Neurodegeneration in Parkinson's disease

The following sections describe the current prevailing hypotheses on the etiology of cell death and neuronal dysfunction in Parkinson's disease.

3.1.1 *Oxidative Stress Hypothesis*

Molecular oxygen is a principal mediator and product of cellular metabolism. The consequence of our dependency on oxygen is the reactive nature of this molecule. It is postulated that dopamine neurons in the substantia nigra pars compacta are particularly sensitive to perturbations in cellular management of oxidative stress. While the reasons for this are presently unknown, several lines of evidence support the notion that abberant production of reactive oxygen species and/or dysfunction of intracellular antioxidant processes manifest neuronal loss in PD.

Free radicals are molecular species which contain unpaired electrons. Oxygen, which is biradical (contains two unpaired electrons), can be activated in the presence of iron (Fe^{2+}) to form several species of reactive oxygen as described by the following equations:

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + OH^{-} + Fe^{3+} \text{ (Fenton Reaction)}$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow HO^{\bullet} + OH^{-} + O_2^{\bullet} \text{ (Haber-Weiss Reaction)}$$

Mitochondria, which employ oxygen as the terminal recipient of electrons to derive ATP, generate reactive oxygen species (ROS) from normal cellular respiration (reviewed by Radi et al., 1997). Accordingly, intracellular antioxidant mechanisms enable cells to combat the potential threat of nucleic acid and protein damage by free radicals. Enzymatic antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GSH), and catalase, as well as non-enzymatic free radical scavengers (principally ubiquinone and glutathione) mediate the neutralization of highly reactive OH species into the lesser reactive forms of

hydrogen peroxide and water, as outlined in the following reactions:

$$2O_2^{\cdot} + 2H^{+} \rightarrow H_2O_2 + O_2 \text{ (via Superoxide Dismutase)}$$

$$2H_2O_2 \rightarrow O_2 + 2H_2O \text{ (via Catalase)}$$

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \text{ (via Glutathione Peroxidase)}$$

In addition to the above reactions, autoxidation and deamination of dopamine also result in the formation of ROS (Cohen, 1978). In the context of Parkinson's disease, it is interesting to note that dopamine can exacerbate free radical mediated damage by participating in the generation of ROS, such as hydrogen peroxide, as described by the following reactions (Olanow and Tatton, 1999):

$$DA + O_2 + H_2O \rightarrow 3,4\ DHPA + NH_3 + H_2O_2$$

$$DA + O_2 \rightarrow SQ^{\bullet} + {}^{\bullet}O_2^{\cdot} + H^{+}$$

followed by, $DA + {}^{\bullet}O_2^{\cdot} + 2H^{+} \rightarrow SQ^{\bullet} + H_2O_2$

Conceptually, there are three means by which oxidative stress could precipitate cellular dysfunction and/or cell death in Parkinson's disease: (1) free radical production is increased; (2) endogenous antioxidant mechanisms are attenuated; and (3) the ability to repair free radical induced damage is impaired. Increased intracellular ROS in dopamine neurons could potentially produce a myriad of consequences on cellular metabolism, and have been reported in PD tissues and experimental models. Physiological consequences of enhanced ROS production in dopamine neurons include: impaired energy production (Berman and Hastings, 1999), changes in mitochondrial membrane potential (Cassarino et al., 1999), modification of intracellular signaling proteins (Cassarino et al., 2000), and inactivation of dopamine synthesis through nitration of tyrosine hydroxylase (Ara et al., 1998).

3.1.1.1 *ROS-mediated Mitochondrial Dysfunction*

Accumulating evidence suggests that dysfunction of mitochondrial function is a common event contributing to the loss of neurons associated with a variety of neurodegenerative conditions (Cassarino and

Bennett Jr., 1999). In Parkinson's disease, the focus of the mitochondrial deficiency has been specifically on complex I (NADH ubiquinone oxidoreductase) of the mitochondrial electron transport chain (ETC). The reasons for this are threefold: (1) deficits in mitochondrial complex I activity have been reported in Parkinson's patients (Schapira et al., 1989; Janetzky et al., 1994), (2) the neurotoxins 6-hydroxydopamine and MPTP generate free radicals and disrupt complex I function (Glinka et al., 1996; Nicklas et al., 1985; Przedborski and Jackson-Lewis, 1998), and (3) cells that have been transformed by mitochondria (termed ρ^0 cell lines, or cybrids) isolated from Parkinson's individuals display a diminished capacity to recover calcium when selectively challenged with complex I inhibitors (MPP+) (Sheehan et al., 1997). Together, these findings suggest that mitochondria may be a physiological point of convergence common to PD caused by genetics, oxidative stressors, or environmental toxins (Schapira et al., 1992).

3.1.1.2 *6-Hydroxydopamine: A dopaminergic neurotoxin.*

6-Hydroxydopamine (6-OHDA) is a neurotoxic derivative of dopamine formed by the autooxidation of dopamine in the presence of ROS. The toxic effects of 6-OHDA are a consequence of interference with mitochondrial respiration (Glinka and Youdim, 1995) and the potentiation ROS formation by nonenzymatic oxidation (Heikkila and Cohen, 1971; Kumar et al., 1995). When injected into the nigrostriatal pathway of rodents, 6-OHDA produces an almost complete destruction of the nigrostriatal dopamine pathway (Ungerstedt et al., 1970), providing an experimental model for the study of the effects of dopamine depletion on the function of the basal ganglia. The active transport of 6-OHDA into dopamine neurons accounts for the selective toxic effects of this neurotoxin on dopamine neurons (Jonsson and Sachs, 1970).

Increasing evidence suggests that formation of 6-OHDA may occur endogenously since detectable levels of 6-OHDA have been reported in both rodent and PD brain tissues (Senoh et al., 1959; Curtuis et al., 1974). Indeed, measurable levels of 6-OHDA are also found in the urine of PD patients on L-Dopa therapy (Andrews et al., 1993). Together, these findings suggest that endogenous oxidative modification of dopamine may produce a toxic DA derivative which may contribute to the pathogenesis of PD (Jellinger et al., 1995).

3.1.1.3 *6-OHDA Models of PD*

Administration of 6-OHDA into the MFB causes an abrupt and profound loss of nigral dopamine neurons resulting in depletion in striatal dopamine levels. For this reason, the MFB-6-OHDA model in rats is widely used to study postsynaptic changes in gene expression in the denervated striatum (Berke et al., 1998). However, because of the severity and rapidity of the cell death resulting from injection of 6-OHDA into the MFB, this model offers limited utility for the study of cell death processes mediating dopamine neuron death. Consequently, Sauer and Oertel (1994) adapted the 6-OHDA model by administering the neurotoxin into the striatum therein producing a terminal lesion of the dopamine neurons of the substantia nigra. In this adapted model, approximately 50% of the tyrosine hydroxylase positive SNc neurons die over a period of several weeks. Due to the delayed and progressive nature of cell loss in this modified application of 6-OHDA, this model is amenable to the study of neurodegeneration and neuroprotection in Parkinson's disease (Sauer and Oertel, 1994; Przedborski et al., 1995). Indeed, an increasing number of studies have employed this model to examine the neuroprotective effects of protein overexpression or administration of neurotrophic factors (Sauer et al., 1995; Choi-Lundberg et al., 1997; Blilang-Bleuel et al., 1997; Yamada et al., 1999).

In this thesis, both the MFB-6-OHDA and intrastriatal 6-OHDA models were used to examine either the effect of dopmaine agonists on striatal gene expression (Chapter 4), or the neuroprotective actions of adenovirally-delivered proteins (Chapter 6), respectively. Further discussion of these models can be found in these sections.

3.1.2 *Environmental Toxins Hypothesis*

Studies on the incidence of Parkinson's disease in agricultural and rural environments have raised interest in the possible neurotoxic effects of pesticides and herbicides. The notion that exposure to chemicals in the environment can manifest a neurological condition has been first put forth by three separate, but related observations. First, case studies reported of individuals who developed a Parkinsonian condition following years of farming, or working in chemical plants (Bocchetta and Corsini, 1986).

Epidemiological studies revealed that rural and argicultural environments, areas of increased pesticide and herbicide use, were correlated with increased risk for the development of Parkinson's disease (reviewed by Tanner et al., 1996). In each instance, exposure to MPTP, or structurally related compounds, resulted in or was associated with an increase in the incidence of Parkinson's disease. In a practical application of these findings, administration of MPTP to mice or non-human primates was found to produce depletions in striatal dopamine levels and selective loss of nigral dopamine neurons in species of mice and non-human primates (Heikkila et al., 1984a; Burns et al., 1983). Interestingly, MPTP-toxicity also results in the formation of intraneuronal inclusions resembling Lewy bodies (Forno et al., 1993), a hallmark pathology of the idiopathic PD condition. These experimental findings support the proposal that the striking similarities between MPTP-induced Parkinsonism may represent a causal link to idiopathic PD. For instance, since MPTP recapitulates both the behavioural and neuropathology of PD, study of MPTP-induced neurotoxicity may yield insights into the pathogenesis of PD. Moreover, these lines of evidence may also support that notion that PD represents a severe form of environmental toxicity.

Environmental factors have also been correlated with increased incidence of PD in Canada. Barbeau et al. (1987) reported on the incidence of PD in the Montreal region of Quebec. Their study revealed that in areas where agriculture was practiced, and hence pesticide useage was increased, the incidence of PD was significantly increased. Similarly, urban Montreal, where exposure to pesticides would, presumably, be less, the incidence of PD was reduced as well. Supporting this notion of an urban-rural setting correlating with PD is another Canadian study by Svenson (1991), wherein the highest incidences of PD, as measured by hospital discharge records, were recorded in Saskatchewan. In fact, the reported incidence of PD in Saskatchewan in this study were higher than anywhere else in Canada (Svenson, 1991). While extensive studies by others have reaffirmed the correlation between argicultural environments and increased PD, a direct correlation of exposure to specific pesticides with PD has not been forthcoming (Rajput and Uittl, 1987). However, the detection of argicultural chemicals in the post-mortem brains of rural PD patients has provided the much needed evidence to support a causal association to PD (Semchuk et al., 1993).

Since the application of MPTP as a model for PD, pesticides and herbicides, which structurally resemble MPP+ (the active metabolite of MPTP), have been investigated for both their neurotoxic and synergistic effects on MPTP-induced dopamine neuron loss. Paraquat is a commerically available herbicide which has been shown to impair mitochondrial function in a manner comparable to MPTP (Shimada et al., 1998)– by inhibition of complex I (Nicklas et al., 1987; Vays et al., 1986). Further, systemic administration of paraquat results in loss of dopamine neurons in the substantia nigra, depletes striatal dopamine levels, and impairs motor function (Brooks et al., 1999). Similar effects from exposure to rotenone, an insecticide, have also been recently presented (Friedrich, 1999). It is worth noting, however, that the reported toxic effects of direct administration of agricultural chemicals on dopaminergic neurons are in contrast to previous work by others using the same compounds (Perry et al., 1986; Ferrante et al., 1997). However, the actions of environmental agents, such as paraquat, may not be a direct toxic effect on dopamine neurons but, rather, the effect of exposure to agricultural chemicals may result in an enhanced or exacerbated the pathogenesis of PD (Corsini et al., 1985).

Whether environmental chemicals are responsible for causing Parkinson's disease or contribute to an increased vulnerability to PD remains to be resolved. Correlative epidemiological studies do not support the notion that exposure to agricultural chemicals, such as paraquat, account for all cases of PD (Rajput and Uittl, 1987). While experimental evidence is presently inconclusive, acute exposure in experimental animals may not produce the same biological consequence as chronic, low-level exposures experienced in rural settings.

3.1.2.1 *N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD*

In 1984, the discovery that a synthetic derivative of heroine used by drug addicts could produce a Parkinson-like state became a serendipitous panacea for Parkinson's disease research. The meperidine derivative, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), when administered systemically, crosses the blood brain barrier and is oxidatively metabolized to 1-methyl-4-phenylpyridium (MPP+) (Markey et al., 1984) by MAO-B (Heikkila et al., 1984b), in astrocytes (Ransom et al., 1987). MPP+ is then actively drawn into dopamine neurons by the dopamine transporter (Javitch et al., 1985; Bezard et al.,

1999) where it is thought to be sequestered by chelating with intraneuronal iron or binding to neuromelanin (D'Amato et al., 1986,1987). While the role of neuromelanin in the nigral dopamine neurons is unclear, pigmentation appears to be related to the selective vulnerability of neuron population (Hirsch et al., 1988).

The MPTP model is widely accepted as a practical and useful model for the study of neuron loss and dopaminegic dysfunction associated with PD. Given the prevalence of illicit drug use in modern society, it would be reasonable to assume that a cohort of PD patients arising from incidental exposure to MPTP may constitute a continuous proportion of PD cases. Consequently, MPTP has been employed to understand the processes mediating cell death in this model and examine the neuroprotective potential of genes or compounds for the potential treatment of PD. Accordingly, I have employed MPTP administration to mice in order to examine the effects of neuronal overexpression of the mammalian IAP protein, XIAP, on dopaminergic neuron survival in a novel mouse line in which the XIAP gene was placed under the control of the neuron-specific enolase promoter (See Chapter 6).

3.1.3 *Genetic Hypotheses*

Evidence for a pattern of genetic inheritance in idiopathic Parkinson's disease is not well supported for a majority of cases, however, clinical studies estimate that a genetic etiology may be responsible for as much as 10% of all PD patients.

Mjones et al. (1949) discovered that 41% of 194 diagnosed cases of PD with affected relatives in a Swedish population brought them to conclude that any candidate gene for Parkinson's disease in their study population would have a dominant negative penetrance. This pattern of inheritance is confirmed with the discovery of two candidate genes from Italian and Japanese families. In another more recent study, however, inheritance among PD affected pedigrees displayed an autosomal dominant pattern of inheritance (Golbe et al., 1990). To further confuse the issue, others have failed to demonstrate a genetic link as surveyed in twin studies (Ward et al., 1983). Though these findings suggest a failed attempt for genetic causality, Elridge and Ince (1984) surmise that low concordance among twins may represent a genetic susceptibility rather than a gene culprit. However, three mutations in two separate genes, and an additional

novel gene have recently been identified as candidates for some heritable forms of PD.

3.1.3.1 *α-synuclein*

In 1996, Polymeropoulos and collegues identified a region of chromosome 4q21-q23 between markers D4S2361 and D4S421 which exhibited linkage in an autosomal dominant fashion and displayed a two-point log odds (lod) score of 6 with a high (85%) penetrance in affected families. The penetrance of this locus in their Mediterranean families suggested that the candidate locus could be sufficient to produce a PD phenotype. This locus, termed PD1, was subsequently found to encode the gene for α-synuclein, a presynaptic nerve terminal protein (Polymeropoulos et al., 1997) which had previously been associated with another neurodegenerative condition, Alzheimer's disease. Interestingly, in the familial PD patients studied, a single point mutation (G209A) results in an alanine to threonine (Ala53Thr) substitution. In another study examining familial PD, Krüger et al. (1998) identified a second type of synuclein mutation (Ala30Pro) in a PD pedigree of German ancestry. While the change in protein function resulting from either of these missense mutations in α-synuclein is presently unclear, it has been speculated that the Ala53Thr modification results in an extension of a β-sheet formation which may promote the aggregation of the mutated synuclein protein with β-amyloid, and amyloid-like deposits. Interestingly, immunohistochemistry from human PD brain has revealed that α-synuclein localizes to neuronal inclusions called Lewy bodies, a hallmark of PD pathology (Spillantini et al., 1997, 1998). Indeed, *in vitro* analysis has shown that familial mutations in α-synuclein can accelerate synuclein fibril formation, supporting the hypothesis that the A53T and A30P mutations may contribute to the generation of Lewy bodies *in vivo* (Conway et al., 1998).

That a single mutation in a gene may produce a PD phenotype has evoked great interest in α-synuclein as the means to better understand the degenerative process(es) involved in idiopathic PD. Accordingly, two groups have recently generated and characterized two recombinant mouse lines which were either deficient of α-synuclein (Abeliovich *et al.*, 2000) or the wildtype human α-synuclein gene was placed under the control of the PDGF-β promoter (Masliah *et al.*, 2000). While each group reported profound deficits in striatal dopaminergic function in these mice, neither line of mice exhibited loss of

nigral dopamine neurons. These findings support the notion that normalized expression of α-synuclein may be an essential component in dopamine neurotransmission. Hence, these findings suggest that α-synuclein may not be a determinant of nigral dopamine neuron survival in PD, but may provide a causal link to the symptomology of this neurological disorder.

Taken together, these results suggest that PD may have a genetic basis. However, analysis of other families with patterns of PD inheritance suggest that the mutations in PD1 reported by Polymeropoulos *et al.* are not observed in other populations of familial PD (Scott *et al.* 1997; Gasser *et al.*, 1997; Brice *et al.*, 1997; Wang *et al.*, 1998). In addition, others have reported that the incidence of inherited PD may only represent 1% of all PD patients. This suggests that the genetic marker identified in the Italian-American and Greek kindred reported by Polymeropoulos may not reflect the etiology in the majority of PD cases. Therefore, an important next step in the understanding of α-synuclein function would be to add a mutated synuclein (either A30P or A53T) in place of the wildtype protein by employing the synuclein null mice, for instance. Only though the generation of a transgenic mouse that selectively expresses the familial mutation(s) could causality to the PD phenotype be substantiated. An enhanced understanding of the rare α-synuclein mutations may therein offer important insights into the underlying etiology of idiopathic PD and other neurodegenerative conditions (Polymeropoulos *et al.*, 1997; Heintz and Zoghbi, 1997).

3.1.3.2 *Parkin*

The novel gene, *parkin*, was identified by Kitada et al. (1998) using PCR screening of Japanese patients with a familial recessive form of Parkinson's disease. Parkin is a 500+ kDa protein with N-terminal ubiquitin-like homology and a cysteine rich C-terminal containing a RING-finger motif. It has been postulated that this protein encodes a novel loss-of-function mutation in the ubiquitin-proteasome degradation process (Kitada et al., 1998; Nussbaum, 1998). The role of parkin in this context is unclear, but the absence of Lewy bodies in autosomal recessive Japanese PD supports this notion.

3.1.3.3 *UCH-L1*

The ubiquitin carboxy-terminal hydroxylase L1 (UCH-L1) gene encodes for one of the most abundant proteins in the central nervous system. UCH-L1 is a deubiquitinating enzyme responsible for the breakdown of polymeric ubiquitin into monomeric subunits. As UCH-L1 is also present in Lewy bodies, a study by Leroy et al. (1998) discovered a missense mutation (Ile93Met) which is correlated with a familial form of PD with incomplete penetrance in a German pedigree. This mutation reduces the efficiency (V_{max}) of the enzyme by half (Leroy et al., 1998), leading to the proposal that reduced catalytic activity of mutated UCH-L1 may promote protein aggregation. Indeed, Lincoln et al. (1999) identified a common polymorphism in the same gene (UCH-L1; Ser18Tyr) which has been correlated with a lower incidence of PD (Maraganore et al., 1999), supporting the notion that I39M mutations in UCH-L1 may slow the ubiquitin-mediated degradation process.

3.1.3.4 *Additional Candidate Genes and Loci*

A fourth candidate gene was mapped to 2q13, and reported to be associated with both inherited *and* sporadic forms of PD (Gasser *et al.*, 1998). However, since this candidate gene has not yet been identified, no further insight is possible at this time. An additional finding reported that a locus located on the q arm of chromosome 17 may be related to susceptibility to another familial form of PD. Additional reports have suggested that polymorphisms in the gene *tau* (AO/AO genotype), which is located near the locus associated with heritable Parkinson's disease, may be associated with PD. This correlation provides a second biochemical connection between the etiology of Parkinson's and Alzheimer's diseases.

The premise underlying the current understanding of candidate gene functions is that dopamine neurons which contain these PD-associated mutations are more vulnerable to perturbances in protein clearance. Consistent with this notion, catabolic deficits resulting from gene mutations may result in the inordinate accumulation of dysfunctional or potentially harmful proteins which are sequestered into intracellular inclusions - resulting in the formation of Lewy bodies. Conceivably, the promotion of aberrant protein aggregation in the form of Lewy Bodies may be facilitated by (a) a reduced effectiveness of mutated UCH-L1 or (b) a proliferation in mutated α-synuclein, which increase propensity for aggregation. Either scenario may result in the accumulation of cytotoxic proteins and the formation of

inclusion bodies, therein leading to the neuronal degeneration associated with Parkinson's disease by a yet, unidentified mechanism. Identification of the relevant functions of PD-associated mutations will facilitate our understanding of the etiology and pathology underlying inherited and idiopathic PD.

3.1.4 *Endogenous Neurotoxins Hypothesis*

It has been suggested that the pathogenesis of PD may be the result of an endogenous toxic factor(s). Several lines of evidence support this proposal. For instance, and as discussed above, increased intracellular generation of reactive oxygen species may facilitate the production of neurotoxic derivatives of dopamine, such as 6-OHDA (Andrews et al. 1993). Secondly, dopamine metabolites resembling the chemical structure of MPP+, the active metabolite of MPTP, have also been reported (Sayre et al., 1991; Kohno et al. 1986; Niwa et al. 1993). Whether endogenous generation of either 6-OHDA or MPP+-like compounds underly the loss of dopamine neurons in Parkinson's disease is presently unclear. However, the possibility that an endogenous neurotoxin may precipitate PD should be acknowledged, especially given the potent and selective toxic nature of these molecules.

3.1.5 *Pathogen Hypothesis*

In 1915-1926, an influenza pandemic resulted in a cohort of survivors who lapsed into a catatonic state reminisent of Parkinson's disease, earning the Parkinsonian condition the monaker *sleeping sickness*. This unusual illness, also as known as van Economo's encephalitis or *encephalitis lethargica*. The nature of the "frozen" state of post-encephalitic patients remained elusive until the 1960s, when the role of dopamine as a neurotransmitter was identified. This discovery yielded the identification of definable neuronal populations in the substantia nigra which lead to the dopamine deficiency hypothesis of Parkinson's disease. Shortly therafter, several neurologists began to report remarkable clinical benefit from large intravenous and oral doses of L-Dopa. Using L-Dopa therapy, an "Awakening" of the post-encephalitic patients from the 1915-1926 pandemic confirmed the similarities of this bizarre post-encephalitic condition to idiopathic PD.

Two conclusions can be reached from this event. First, Parkinsonism may have multiple etiologies, and, secondly, the post-encephalitic nature of this condition has led some to suggest that a latent virus also may be responsible for the etiology of some forms of idiopathic Parkinson's disease. Though no pathogen has yet been identified, a report of infection by the gram-positive bacteria, *Nocardia asteroides*, a genus of anaerobic bacteria can reputedly infect neurons of the central nervous system of animals and humans, without eliciting an immune response (reviewed by Beaman, 1992). Interestingly, Kohbata and Beaman (1991) reported that intravenous infusion of *N. asteroides* produced behavioural impairments and deficits in motor function which were responsive to L-Dopa therapy. Histological examination revealed that the mice which exhibited these spontaneous Parkinsonian-like motor behaviours also developed inclusion bodies and a loss of dopaminergic neurons in the VTA and SNc (Kohbata and Beaman, 1991). Furthermore, an immunological screen of serum from patients diagnosed with Parkinson's disease or age-matched controls revealed that 20/20 PD patients tested positive for exposure to a *N. asteroides*-like bacteria (Kohbata and Shimokawa, 1993). However, 10/14 non-Parkinsonian patients also displayed immunopositive results for the bacterium as well, suggesting that infection by a *N. Asteroides*-like bacterium may be sufficient to produce a PD-like condition. However, exposure to this pathogen may represent a risk factor for developing the disease since non-PD individuals were also exposed to this bacteria. These intriguing results support the proposal that a pathogen can recapitulate both the pathology and clinical symptomology of Parkinson's disease, suggesting that, similar to the 1915 encephalitis pandemic, PD may be the consequence of a latent pathogen infection.

3.1.6 *Additional Theories of PD Etiology*

Since the cause(s) of idiopathic PD remains enigmatic, hypotheses addressing the issue of etiology are increasing and diverse. Therefore, the theories included in the above discussion were selected to describe current animal models of PD and for their direct relevance to the content of this thesis. While additional alternative proposals involving either improper neurodevelopment, neuroinflammatory responses and/or possible autoimmunity in the pathogenesis of PD exist, these theories do not pertain to the specifics

of this doctoral dissertation and are consequently not included in this thesis.

3.2 *Statement of Problem*

Current therapeutic regimes for the treatment of Parkinson's disease are confounded by the development of involuntary movements called dyskineias. Since dyskinesias are considered to be mediated by postsynaptic changes in strital gene expression, I have investigated the effects of inhibiting dopamine agonist-induced FosB expression in the striatum as a potential means to abating the complications associated with current dopamine therapy for PD. However, since dopamine replacement therapy does not stop the underlying neurodegeneration associated with PD, I have also examined the effects of overexpressing IAP proteins to provide functional neuroprotection in two rodent models of Parkinson's disease.

Chapter 4.

Crocker S.J., *et al.* (1998) D1-receptor related priming is attenuated by antisense mediated 'knockdown' of *fosB* expression. *Mol Brain Res.* **53**: 69-77.

This first manuscript demonstrates a behavioural effect of modifying striatal gene expression in a rat 6-OHDA model of Parkinson's disease

The experiments in this report were performed by S.J. Crocker, with the assistance from Mrs. N. Wigle and Mrs. M. St.Jean for the behavioural analysis and immunohistochemistry. Reagents were provided as cited in the text. This manuscript was written by S.J. Crocker with the guidance and editorial assistance of Dr. G.S. Robertson.

D1-RECEPTOR RELATED PRIMING IS ATTENUATED BY ANTISENSE MEDITATED "KNOCKDOWN" OF *fos*B EXPRESSION

S.J. Crocker[1], M. Morelli[2], N. Wigle[1], Y. Nakabeppu[3], G.S. Robertson[1¶]

Department of Cellular and Molecular Medicine[1],
University of Ottawa,
Ottawa, Ontario, Canada, K1H 8M5.

Department of Toxicology[2], University of Cagliari
Viale A Diaz 182, Cagliari 09100, Italy.

Department of Biochemistry[3], Medical Institute of Bioregulation,
Kyushu University 69, Fukuoka 812, Japan.

¶ Correspondence should be addressed to G.S. Robertson, (FAX: 613-562-5456; TELEPHONE: 613-562-5800 ext. 8070)

Key words: antisense oligonucleotide, dopamine receptors, immediate-early genes, 6-hydroxydopamine, striatum.

Abbreviations: AS, antisense oligonucleotide; IEG, immediate-early gene; i.p.,intraperitoneal; LI, Like immunoreactivity; PBS, phosphate buffered saline; RN, random oligonucleotide; 6-OHDA, 6-hydroxydopamine; s.c., subcutaneous.

Abstract

Administration of dopamine receptor agonists to rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal pathway produce changes in the denervated striatum that enable a subsequent injection to elicit more vigorous circling. The molecular basis for this behavioural phenomenon, termed priming, is unknown. D1 receptor-related priming has been associated with a profound elevation of immediate-early gene expression in the denervated striatum. Since immediate early genes encode known transcriptional regulating factors, this observation has led to the suggestion that immediate early gene induction may play a role in the gene signaling pathways which mediate priming.

In the present study, we addressed the role of induction of the immediate early gene *fosB* in dopamine agonist-induced priming by examining whether inhibition of the synthesis of FosB proteins (FosB and ΔFosB) by intrastriatal delivery of an antisense oligonucleotide to *fos*B reduced apomorphine-induced priming. Intrastriatal delivery of an antisense, but not a random, oligonucleotide to *fos*B 18 and 6 hours before apomorphine reduced the ability of this mixed D1\D2-like receptor agonist to prime circling induced by the specific D1-like receptor agonist SKF 38393. Immunohistochemical analysis revealed that only the antisense oligonucleotide blocked apomorphine-induced increases in FosB-like immunoreactivity in the denervated striatum. In contrast, apomorphine-induced increases in JunB-, NGFI-A- and Fos_{2-16}-like immunoreactivities were unaffected by either the antisense or random oligonucleotides, indicating that the antisense oligonucleotide attenuated apomorphine-induced priming by selectively blocking the synthesis of FosB proteins Taken together, these findings suggest that *fos*B induction in the denervated striatum plays a role in mediating D1 receptor-related priming.

Dopamine replacement therapy for Parkinson's disease is often complicated by the development of dyskinetic side effects. Results from the present study suggest that D1 receptor-mediated increases in *fosB* expression may be involved in those intracellular events responsible for the generation of these debilitating side effects.

1. **Introduction**

Rats with unilateral lesions of the nigrostriatal pathway produced by injections of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle serve as a model for Parkinson's disease. In this model, the development of dopamine receptor supersensitivity in the deafferented striatum is thought to be responsible for the ability of antiparkinsonian agents such as levodopa to elicit turning behaviour directed away from the lesioned side [32] . Unlike the dopamine precursor levodopa, which produces robust turning behaviour in drug naive 6-OHDA-lesioned rats, initial administration of the selective D1-like receptor agonist SKF 38393 is strongly potentiated by pretreatment with the mixed D1/D2 dopamine receptor agonist apomorphine [12]. Although it is thought that D1 receptor-related priming is mediated by alterations in synaptic plasticity in the 6-OHDA-denervated striatum, the molecular basis for these changes is presently unclear.

The proto-oncogenes *c-fos* and *c-jun* belong to a class of inducible genes referred to as immediate early genes (IEGs). The protein products of these IEGs, Fos and Jun, are transcriptional regulating factors that couple extracellular signals to alterations in cellular phenotype by regulating the expression of specific target genes [18]. Basal *c-fos* expression in the striatum is very low but is rapidly elevated by systemic administration of cocaine and *d*-amphetamine, stimulants which greatly enhance extracellular concentrations of dopamine [1,6,27]. In contrast, levodopa and directly acting D1-like receptor agonists such as SKF 38393 and CY 208-243 only weakly increase Fos-like immunoreactivity in the intact striatum [26,27,29]. Destruction of the nigrostriatal pathway with 6-OHDA is required to endow levodopa and D1-like agonists with the ability to substantially enhance *c-fos* expression in the denervated striatum [15,23,27]. These increases are completely blocked by the selective D1-like receptor antagonist SCH 23390 indicating that activation of D1-like receptors plays an important role in levodopa- and D1-like agonist-induced *c-fos* expression [15,27]. The dramatic effects of D1-like agonists on *c-fos* expression in the 6-OHDA-denervated striatum coupled with the fact that Fos is a transcriptional regulating factor has led to the hypothesis that IEGs may play a role in the signalling pathways which facilitate D1-receptor sensitivity and priming.

Molecular cloning studies have revealed that *c-fos* belongs to a family of IEGs. Presently, the *fos* family consists of *c-fos*, *fos*B, and the *fos*-related antigens, *fra-1* and *fra*-2 [2,3,22,35]. Two different forms of *fosB* mRNA are generated through alternative splicing of the transcript from a single gene [4,19,20,34]. One transcript encodes a protein 338 amino acids long (FosB) while the other encodes a truncated protein 237 amino acids in length (ΔFosB). FosB can be selectively detected using an antibody (FosB(C)) that recognizes a portion of the C-terminus of FosB that is missing from ΔFosB (amino acids 245-315) [20]. In contrast, both FosB and ΔFosB can be detected using a second antibody (FosB(N)) raised against amino acids 79-131 of the N-terminus of FosB, a region common to both FosB and ΔFosB [20]. Using these, and other antibodies, we have recently demonstrated that compared to Fos and FosB, ΔFosB displays prolonged induction kinetics in the 6-OHDA-denervated striatum after a single administration the D1-like agonist CY 208-243 [5]. Peak increases in Fos occurred at 2h, whereas maximal elevations of FosB and ΔFosB were detected at 4h. By 8h, Fos had returned to near basal levels while FosB and ΔFosB remained elevated. At 24h, FosB levels had declined markedly while ΔFosB levels were still elevated. Time course studies have shown that D1 receptor-related priming is optimal 3 days after administration of the dopamine agonist primer. Hence, the protracted elevation of ΔFosB expression in the 6-OHDA-denervated striatum after D1 agonist administration suggests that *fos*B induction may play a role in mediating priming. In order to address this hypothesis, we examined whether inhibition of apomorphine-induced elevations of FosB proteins (FosB and ΔFosB) by intrastriatal delivery of an antisense oligonucleotide to *fos*B reduced this mixed D1/D2 receptor agonist to prime SKF 38393-induced circling.

2. **Materials and Methods**

All animals were treated in strict accordance with procedures outlined in the *Guide for Care and Use of Experimental Animals* endorsed by the Medical Research Council of Canada [7].

2.1. 6-OHDA lesions and behavioural assessment of nigrostriatal damage.

Adult male Wistar rats (275-325g; Charles Rivers, Montréal) were housed 4 per cage in a temperature-controlled environment with a 12 h light/12 h dark cycle and were given free access to water and Purina laboratory chow. Animals were injected with desmethylimipramine (25 mg/kg, i.p.) and then anesthetized 30 min later with sodium pentobarbital (50 mg/kg, i.p.). Unilateral medial forebrain bundle (MFB) lesions were made by injection of 11.4 µg of 6-hydroxydopamine HBr (Sigma) dissolved in 4 µl of saline containing 0.05% ascorbic acid. The solution was injected over 10 min into the right MFB at the coordinates (in mm) AP 4.0, ML 1.3 and DV 1.6 from interaural zero according to the atlas of Paxinos and Watson [25]. Unilateral 6-OHDA-induced degeneration of the nigrostriatal pathway produces a profound deviation in movements and posture to the lesioned side as well as contralateral sensory neglect [10,32,33]. Animals that have sustained only partial lesions of the nigrostriatal pathway fail to reliably display these behavioural abnormalities [9]. Consequently, only animals that displayed a strong postural bias towards the lesioned side coupled with a failure to orient toward tactile stimulation (whisker brushing) of the contralateral side one week after 6-OHDA surgery were selected for experimentation. Approximately 20% of 6-OHDA-lesioned rats were not used for subsequent experimentation because they failed to meet these behavioural criteria.

2.2. Experimental protocol for priming

In a first experiment, we confirmed that pretreatment with apomorphine (0.1 mg/kg, s.c.) enhanced the circling response to the D1-like receptor agonist 1-phenyl-2,3,4,5-tetrahydro-1H-3-benzaepine-7,8-diol (SKF 38393; 3.0 mg/kg, s.c.) three days later. Two groups, containing six drug naive 6-OHDA-lesioned rats each, were injected with either saline (0.9%, 1.0 ml/kg, s.c.) or apomorphine (0.1 mg/kg, s.c.) 14 days after 6-OHDA surgery. Contralateral rotational behavioural was monitored after administration of saline or apomorphine using an automated rotometer. Three days later, all of the animals were injected with the SKF 38393 (3.0 mg/kg, s.c.) and contralateral turning was measured for 30 mintues beginning 30 minutes after the injection. In a second experiment, we determined whether apomorphine-induced increases in FosB(N)-like immunoreactivity (LI) were reduced by intrastriatal administrations of an antisense

oligonucleotide to *fos*B. Three groups, composed of six 6-OHDA-lesioned rats each, that had received two intrastriatal administrations of either saline, antisense oligonucleotide (AS) or random oligonucleotide (RN) were injected with apomorphine (0.1 mg/kg, s.c.). Apomorphine-induced contralateral rotation was recorded for 30 minutes in consecutive 1 min periods using an automated rotometer. Only those animals that turned at an average rate of at least seven quarter turns/min were used in the study. Two hours after the injection, all animals were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) immediately prior to intracardial perfusion with saline (150 ml) followed by phosphate buffer (0.1 M; 200 ml) containing 4% paraformaldehyde. Brains were rapidly removed, postfixed overnight and sections (30 μm thick) cut from the striatum using a vibratome. Adjacent sections were processed for FosB(N)-, JunB-, NGFI-A- and Fos_{2-16}-LI. In a third experiment, we compared the effects of intrastriatal injections of either saline or AS or RN on the ability of apomorphine to prime SKF 38393-induced turning. Three groups, composed of six 6-OHDA-lesioned rats each, that had received two intrastriatal administrations of either saline or AS or RN were injected with apomorphine (0.1 mg/kg, s.c.). Three days after administration of apomorphine (0.1 mg/kg, s.c.), all of the animals received a single administration of SKF 38393 (3.0 mg/kg, s.c.). Rotational behaviour was recorded in 1 min intervals for 30 min beginning 30 min after injection of SKF 38393 (3.0 mg/kg). Two hours after the SKF 38393 injection, all of the animals were intracardially perfused with fixative, and adjacent sections were processed for FosB(N)-, JunB-, NGFI-A- and Fos_{2-16}-LI. This was done to ensure that intrastriatal infusions of antisense oligonucleotide 3 days previously had not produced a long-lasting impairment in the ability of th 6-OHDA-lesioned striatum to express D1 receptor-mediated increases in IEG immunoreactivity.

2.3. Antisense oligonucleotide design and synthesis.

Both oligonucleotides used in this study were synthesized by Oligos Etc. (Wilsonville, OR, USA). Sequences underwent an extraction/precipitation process of primer-grade purification to greater than 90% purity as verified by HPLC analysis (described as Level 1 purification by Oligos Etc.). Oligonucleotides were rendered more resistant to nuclease degradation by phosphorothioate modifications (replacement of a nonbridging oxygen with sulphur) of the first two (5') and last two (3') phosphodiester linkages. The

sequence of the *fosB* antisense (AS) and control oligonucleotide (RN) were 5'-CTT-gAA-ACA-TTT-CCC-Tgg-3' and 5'-AgA-TCT-TCT-CAA-gTg-CTT-3', respectively. The AS sequence, complementary to bases 1194-1121 of *fosB*, covered the initiation codon and did not display significant homology with any other sequences registered in current databases (GenBank). The RN sequence was a random assortment of bases that composed AS and failed to exhibit significant homology with any sequences found in current databases.

2.4. Intrastriatal injection of oligonucleotides.

Rats with unilateral lesions of the nigrostriatal pathway were implanted with a 10 mm guide cannula (22 gauge; Plastics One, Roanoke, VA) in the denervated striatum at the coordinates (in mm) AP 1.0, ML -2.7, DV -4.0 from bregma at the skull surface [25]. Animals were permitted one week recovery and then randomly allocated to three groups, composed of six animals each, that received intrastriatal injections of one of the following: saline, antisense oligonucleotide or random oligonucleotide. Each animal received two injections (2 µl infused at a rate of 1 µl/min) of either saline (0.9%; vehicle), AS (5.0 µg/µl in saline) or RN (5.0 µg/µl in saline) into the striatum. The injections occurred 18 and 6 hours before administration of the mixed D1/D2 receptor agonist apomorphine (0.1 mg/kg, s.c.). Two injections of antisense oligonucleotide were administered at 18 and 6 hours before apomorphine (1.0 mg/kg, s.c.) To ensure that sufficient antisense oligonucleotide was present in the striatum to effectively "knockdown" apomorphine-induced *fosB* expression.

2.5. Antisera and immunohistochemistry.

Fos_{2-16}-LI was detected using sheep polyclonal antisera purchased from Cambridge Research Biochemicals (CRB OA-11-823). FosB(N)-LI was detected using an affinity-purified rabbit polyclonal antibody raised against amino acids 79-131 of the N-terminus of FosB [20,21]. NGFI-A- and JunB-LI were detected using antisera generously provided by R. Bravo. These antibodies were generated in rabbits by immunization with bacterially expressed fusion proteins [8]. The following sequences were used for fusion: NGFI-A, amino acids 15-322; JunB, amino acids 46-344. NGFI-A was expressed and fused to MS2 polymerase. JunB was expressed in bacteria fused to ß-galactosidase. The specificity of these

antibodies has been established by immunoprecipitation and Western blot experiments [8]. Immunohistochemistry was performed using a modified avidin-biotin protocol previously described by Robertson and Fibiger [28]. Briefly, sections were washed in 0.01M phosphate buffered saline (PBS) for 10 min followed by washing in PBS containing 0.03% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then washed three times in PBS for 10 min and incubated for 48 hours at 4°C in PBS containing 0.3% Triton X-100, 0.02% sodium azide and either FosB© (1:1500), FosB(N) (1:1500), NGFI-A (1:20 000), JunB (1:2000), or $Fos_{2\text{-}16}$ (1:2500) primary antisera. Next, sections were washed three times with PBS and incubated at 4°C overnight in affinity purified biotin-labeled donkey anti-rabbit or biotin-labeled donkey anti-sheep secondary antisera (Jackson laboratories; 1:200). Sections were then washed three times with PBS and incubated at room temperature for 3 hours in PBS containing 0.3% Triton X-100 and streptavidin-horseradish peroxidase (Amersham; 1:200). After three washes in PBS, sections were transferred to 0.1 M acetate buffer (pH 6.0). The reaction was visualized using a glucose oxidase-diaminobenzidine-nickel method described previously [31]. The reaction was terminated by washing in acetate buffer and sections mounted on chrome-alum-coated slides. After drying, sections were dehydrated through a graded series of alcohols and two changes of xylene, and coverslipped for microscopic observation.

2.6. Quantification of immunoreactive cells.

FosB(N)-, NGFI-A-, JunB- and $Fos_{2\text{-}16}$-LI nuclei in the denervated striatum were counted using an image analysis system equipped with Image 1.47 software (Wayne Rasband, NIMH) according to Robertson et al. [30]. Cell counts were performed by sampling an area 660 X 800 μm in the dorsolateral striatum [30].

2.7. Statistical analyses

A one-way analysis of variance was performed on cell count data for each of the four IEG antibodies. If the analysis was significant, multiple comparisons were performed using the Student Newman-Keuls test. All other statistical comparisons were made with an unpaired t-test.

3. **Results**

3.1. Apomorphine-induced priming of the turning response to SKF 38393.

We examined the effects of pretreatment with either saline (1.0 ml/kg, s.c.) or apomorphine (0.1 mg/kg, s.c.) on the turning response to SKF 38393 (3.0 mg/kg, s.c.) 3 days later. Consistent with previous work [11,14], 6-OHDA-lesioned animals which had received apomorphine (0.1 mg/kg, s.c) 3 days earlier turned significantly more to SKF 38393 (3.0 mg/kg, s.c.) than those which had been pretreated with saline (1.0 ml/kg, s.c.) (Table 1).

3.2. Effects of intrastriatal administrations of an antisense oligonucleotide to fosB on apomorphine-induced increases in IEG immunoreactivity.

The object of this experiment was to determine if intrastriatal administration of an antisense oligonucleotide to *fos*B selectively reduced increases in FosB(N)-LI in the 6-OHDA-denervated striatum observed 2 h after administration of apomorphine (0.1 mg/kg, s.c.) (Figs. 1 and 2A). Intrastriatal delivery of the antisense, but not the random, oligonucleotide 18 and 6 hours before apomorphine (0.1 mg/kg, s.c.) reduced the ability of this mixed D1/D2-like receptor agonist to elevate FosB(N)-LI in the denervated striatum (Figs. 1 and 2A). Cells counts indicated that compared to the random oligonucleotide, the antisense oligonucleotide reduced the number of neurons which displayed apomorphine (0.1 mg/kg, s.c.)-induced FosB(N)-LI by about 65%. A third group received intrastriatal infusions of saline in order to assess potential toxic effects of the random oligonucleotide. Animals that had received intrastriatal infusions of either saline or the random oligonucleotide displayed the same number of FosB(N)-LI neurons indicating that the random oligonucleotide did not alter the ability of striatal neurons to express FosB(N)-LI. Lastly, similar increases in $Fos_{2\text{-}16}$-, JunB- and NGFI-LI 2 h after apomorphine (0.1 mg/kg, s.c.) were displayed in animals in all treatment groups.

3.3. Effects of intrastriatal administrations of an antisense oligonucleotide to fosB 3 days previously on SKF 38393-induced increases in IEG immunoreactivity.

In order to determine whether intrastriatal infusions of the antisense oligonucleotide had any long lasting effects on the ability of striatal neurons to express FosB(N)-LI, we examined the effects of SKF 38393 (3.0 mg/kg, s.c.) on FosB(N)-LI in animals that had previously received intrastriatal infusions of either random oligonucleotide, antisense oligonucleotide or saline prior to systemic administration of apomorphine (0.1 mg/kg, s.c.). Infusions occurred 18 and 6 h before apomorphine (0.1 mg/kg, s.c.) which were administered 3 days prior to SKF 38393 (3.0 mg/kg, s.c.). FosB(N)-LI was examined 2 h after SKF 38393 (3.0 mg/kg, s.c.). In contrast to the reduction in apomorphine-induced FosB(N)-LI observed in antisense treated animals in the previous experiment, levels of SKF 38393-induced FosB(N)-LI were similar in antisense oligonucleotide, random oligonucleotide, and saline treated animals (Fig. 2B) Furthermore, similar levels of SKF 38393-induced Fos_{2-16}-, JunB- and NGFI-A-LI were detected in these three groups (Fig. 3B).

3.4. Effects of intrastriatal administrations of an antisense oligonucleotide to fosB on apomorphine-induced priming.

Prior to immunohistochemical analysis of SKF 38393-induced IEG expression in the previous experiement, we examined the effects of intrastriatal administrations of the antisense oligonucleotide, random oligonucleotide or saline on the ability of apomorphine (0.1 mg/kg, s.c.) to enhance the turning response to SKF 38393 (3.0 mg/kg, s.c.) (D1-receptor related priming). All animals that had received intrastriatal infusions of either oligonucleotide or saline rotated at similar rates after administration of apomorphine (0.1 mg/kg, s.c) (Table 2). However, administration of SKF 38393 (3.0 mg/kg, s.c.) 3 days later produced significantly fewer rotations in animals treated with the antisense oligonucleotide as compared to other treatments.

4. Discussion

Administration of the selective D1-like agonist SKF 38393 to drug naive 6-OHDA-lesioned rats 14-21 days after stereotaxic injection of 6-OHDA into the medial forebrain bundle elicits weak contralateral rotation [12]. However, pretreatment with the mixed D1\D2-like agonist apomorphine 3 days prior to SKF 38393 administration dramatically enhances the rotational response to this D1-like agonist [12]. As expected, in this study, administration of apomorphine (0.1 mg/kg, s.c.) 14 days after lesioning of the nigrostriatal pathway with 6-OHDA enhanced the rotation response to SKF 38393 (3.0 mg/kg, s.c.) given three days later. This facilitation of the circling response to SKF 38393 by apomorphine pretreatment, termed D1 receptor-related priming, is considered to be a form of behavioural sensitization [16]. Coincident with this behavioural potentiation, apomorphine administration produces large increases in *c-fos*, *c-jun*, *jun*B and *ngfi*-A expression in the denervated striatum of drug naive 6-OHDA-lesioned rats, suggesting that these IEGs may play a role in the mediation of D1 receptor-related priming.

4.1. Effects of the fosB antisense oligonucleotide on priming

In order to establish whether increased *fosB* expression was necessary for D1 receptor-related priming, we utilized an antisense oligonucleotide to "knockdown" the expression of this IEG [30]. A first series of studies examined the effects of intrastriatal infusions of either an antisense or random oligonucleotide on increases in FosB(N)-LI 2h after injection of apomorphine (0.1 mg/kg, s.c.). Only the antisense treatment reduced apomorphine-induced elevations of FosB(N)-LI in the 6-OHDA-denervated striatum. In addition, neither antisense nor random oligonucleotide treatments altered the facilitatory effects of apomorphine (0.1 mg/kg, s.c.) on $Fos_{2\text{-}16}$-, JunB- and NGFI-A-LI. These results indicate that the antisense oligonucleotide selectively reduced the ability of apomorphine to elevate FosB-like proteins. A third group of animals which received intrastriatal infusions of saline displayed the same number of FosB(N)-, $Fos_{2\text{-}16}$-, JunB- and NGFI-A-LI neurons 2 h after apomorphine (0.1 mg/kg, s.c.) as that observed in animals treated with the random oligonucleotide. This indicates that the random oligonucleotide did not interfere with the ability of striatal neurons to express D1 agonist-induced IEGs.

Although the antisense oligonucleotide decreased the facilitatory effects of apomorphine on FosB(N)-LI in the 6-OHDA-denervated striatum, it did not reduce apomorphine-induced rotation indicating that dopamine receptor-mediated signal transduction mechanisms necessary for contralateral turning were unaffected by the oligonucleoide. This is not surprising given that apomorphine produces contralateral turning within minutes of administration while peak increases in FosB and ΔFosB do not occur until 2-4 h, by which time apomorphine-induced turning has long since ended.

The effects of the antisense and random oligonucleotide on both priming and IEG expression were examined in a final experiment. Although intrastriatal infusion of the AS blocked apomorphine-induced FosB(N)-LI, administration of SKF 38393 (3.0 mg/kg, s.c.) 3 days after apomorphine (0.1 mg/kg, s.c.) produced similar increases in the number of FosB(N)-, Fos_{2-16}-, JunB- and NGFI-A-LI neurons in all treatment groups. The recovery of D1 receptor-related increases in FosB(N)-LI 3 days after administration of the antisense oligonucleotide indicated that this treatment did not produce a permanent impairment in the ability of striatal neurons to manufacture FosB-like proteins. Nevertheless, animals treated with the antisense oligonucleotide displayed significantly lower rotation rates to SKF 38393 (3.0 mg/kg, s.c.) than those animals which received intrastriatal infusions of random oligonucleotide or saline. This demonstrates that D1 receptor-related priming was attenuated by intrastriatal administration of the antisense oligonucleotide to *fosB*. Consequently, the fact that the antisense oligonucleotide selectively reduced apomorphine-induced increases in FosB(N)-LI suggests that *fosB* activation contributes to the mediation of D1 receptor-related priming.

Several lines of evidence indicate that the AS reduced SKF 38393-induced rotation by blocking *fosB* expression rather than interfering with the basic neuronal mechanism of D1-dependent turning independently of priming. Firstly, the rotational responses to apomorphine were similar in animals which had received intrastriatal injections of either saline, AS or RN oligonucleotides (Table 2). Since apomorphine-induced rotational behaviour is mediated by both D1- and D2-like receptor activation, it seems improbable that administration of the AS reduced SKF 38393-induced turning by interfering with the basic neural mechanism of D1 dependent turning. Secondly, animals treated with AS and apomorphine

turned considerably more to SKF 38393 (Table 2; 11.3±1.2) than animals which had received saline (Table 1; 2.8±0.6). These results suggest that the AS produced only a partial reduction in priming and did not interfere with the ability of SKF 38393 to produce turning. Thirdly, in contrast to the effects of apomorphine, SKF 38393 produced equivalent elevations of IEG expression (FosB, $Fos_{2\text{-}16}$, NGFI-A and JunB) in animals treated with either saline, AS or RN. This demonstrates that there had been a recovery of D1 receptor-related increases in FosB-LI 3 days after administration of AS and that the striatal response to SKF 38393 was similar in all three treatment groups. Lastly, animals which received intrastriatal injections of either saline or random oligonucleotide turned at equivalent rates to SKF 38393. This result indicated that the ability of AS to attenuate apomorphine-induced priming of the turning response to SKF 38393 was sequence dependent and not a non-specific property of the oligonucleotide.

Since the antisense oligonucleotide was designed to straddle the initiation start site of the *fosB* gene, intrastriatal administrations of this sequence blocked apomorphine-induced increases in both FosB and ΔFosB. This antisense strategy therefore does not permit evaluation of the relative individual contributions of FosB or ΔFosB to D1 receptor-related priming. Nevertheless, differences in the induction time courses for these *fos*B products suggest that they may play distinct roles. For instance, D1 receptor-related increases in striatal ΔFosB expression are long lasting [5], suggesting that this transcriptional regulating factor may maintain those alterations in neuronal gene expression required for priming.

4.2. IEG expression in priming

Since D1 receptor-related priming is blocked by the NMDA receptor antagonist MK-801 [13], a recent study investigated whether blockade of apomorphine-induced priming by MK-801 (0.1 mg/kg, s.c.) produced concordant reductions in the excitatory effects of this mixed D1/D2 dopamine receptor agonist on *c-fos*, *c-jun*, *junB* and *ngfi-A* in the denervated striatum [24]. Administration of a low dose of MK-801 (0.1 mg/kg, s.c.) 30 min prior to apomorphine (0.5 mg/kg, s.c.) did not reduce the circling or IEG response to this dopamine agonist. However, administration of SKF 38393 (2.0 mg/kg, s.c.) 3 days later produced only weak contralateral circling in these animals, indicating that while MK-801 had not reduced apomorphine-induced increases in *c-fos*, *c-jun*, *junB* and *ngfi-A* expression it had significantly blocked the ability of this

agonist to prime the circling response to SKF 38393. These results demonstrate that apomorphine-induced priming can be dissociated from its excitatory effects on the expression of these IEGs in the denervated striatum. It should be noted, however, that this finding does not necessarily preclude the possibility that IEGs such as *c-fos*, *c-jun*, *junB* and *ngfi-A* are involved in the mediation of D1 receptor-related priming. Recently, results conflicting with those of Paul et al. [24] have been reported [17]. Morelli et al. [17] have shown that the ability of a low dose of MK-801 (0.1 mg/kg, s.c.) to disrupt D1 receptor-related priming was correlated with a reduction in apomorphine (0.1 mg/kg, s.c.)-induced Fos-LI in the denervated striatum. The basis for these conflicting results is presently unclear. Nevertheless, the discrepant nature of these reported findings [17,24] suggest that IEGs other than *c-fos* may play a role in the mediation of priming. Indeed, findings from the present study which suggest that *fosB* activation plays an important role in the mediation of priming are consistent with this view.

Currently, Parkinson's disease is treated through dopamine replacement therapy by administration of levodopa. Unfortunately, levodopa therapy is often complicated by the development of dyskinetic side effects. We have recently reported that the development of D1 receptor-mediated dyskinesias in MPTP-lesioned monkeys is associated with increased production of FosB-like protein(s) [5]. This observation led us to speculate that *fosB* activation may be involved in those intracellular events which mediate the generation of dyskinesias. Our hypothesis is supported by findings from the present study which show that *fos*B induction is a necessary step in those intracellular events which mediate the enduring behavioural effects of a dopamine receptor agonist (apomorphine) in an animal model of Parkinson's disease. Determining the precise role of D1 receptor-mediated increases in *fosB* expression may therefore have important implications for the treatment of this disorder.

Acknowledgements

This work was supported by a grant from the Medical Research Council of Canada (MT-11539). SJC was supported by a summer studentship from the Parkinson's Disease Foundation of the USA.

References

[1] Cenci, M.A., Campbell, K., Wictorin, K. and Björklund, A., Striatal *c-fos* induction by cocaine or apomorphine occurs preferentially in output neurons projecting to the substantia nigra. *Eur. J. Neurosci.*, 4 (1992) 376-380.

[2] Cohen, D.R. and Curran, T., *fra-1*: a serum inducible, cellular immediate-early gene that encodes a Fos-related antigen. *Mol. Cell Biol.*, 8 (1988) 2063-2069.

[3] Curran T., Gordon, M.B., Rubino, K.L. and Sambucetti, L.C. Isolation and characterization of the *c-fos* (rat) cDNA and analysis of post-translational modification *in vitro*. *Oncogene* 2 (1987) 79-84.

[4] Dobrzanski, P., Noguchi, T., Kovary, K., Rizzo, P., Lazo, PS. and Bravo, R., Both products of the *fos*B gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. *Mol. Cell Biol.*, 11 (1991) 5470-5478.

[5] Doucet, J.P., Nakabeppu, Y., Bdard, P., Hope, B.J., Nestler, E.J, Jasmin, B.J., Chen, J.-S., Iadarola, M.J., St-Jean, M., Wigle, N., Blanchet, P., Grondin, R. and Robertson, G.S., Chronic alterations in dopaminergic neurotransmission produce a persistent elevation of ΔFosB-like protein(s) in the striatum. *Eur. J. Neurosci.*, 8 (1996) 365-381.

[6] Graybiel, A.M., Moratalla, R. and Robertson, H.A., Amphetamine and cocaine induce drug specific activation of the *c-fos* gene in striosome-matrix compartments and limbic subdivisions of the striatum. *Proc. Natl. Acad. Sci. USA*, 87 (1990) 6912-6916.

[7] *Guide for the Care and Use of Experimental Animals*. Canadian Council of Animal Care, 151 Slater, Ottawa, Ontario, Canada K1P 4H3.

[8] Kovary, K. and Bravo, R., Expression of different JUN and FOS proteins during the G0 to G1 transition in mouse fibroblasts: *in vitro* and *in vivo* associations. *Mol. Cell Biol.*, 11 (1991) 2451-2459.

[9] Ljungberg, T. and Ungerstedt, U., Sensory inattention produced by 6-hydroxydopamine-induced degeneration of ascending dopamine neurons in the brain. *Exp. Neurology*, 53 (1976) 585-600.

[10] Marshall, F.J., Richardson, S. and Teitelbaum, P., Nigrostriatal bundle damage and the lateral hypothalamic syndrome. *J. Comp. Physiol. Psychol.*, 87 (1974) 808-830.

[11] Morelli M., Fenu,S. and Di Chiara, G., Behavioural expression of D-1 receptor supersensitivity depends on previous stimulation of D-2 receptors. *Life Sci.* 40 (1987) 245-251.

[12] Morelli, M. and Di Chiara, G., Agonist-induced homologous and heterologous sensitization to D-1 and D-2-dependent contraversive circling. *Eur. J. Pharmacol.*, 141 (1987) 101-107.

[13] Morelli, M and Di Chiara, G., Stereospecific blockade of N-methyl-D-aspartate transmission by MK-801 prevents priming of SKF 38393 induced turning. *Psychopharmacol.*, 101 (1990) 287-288.

[14] Morelli, M., Fenu, S., Garau, L. and Di Chiara, G., Time and dose dependence of the 'priming' of the expression of dopamine receptor supersensitivity. *Eur. J. Pharmacol.*, 162 (1989) 329-335.

[15] Morelli, M., Cozzolino, A., Pinna, A., Fenu, S., Garau, L. and Di Chiara, G., L-dopa stimulates *c-fos* expression in dopamine denervated striatum by combined activation of D-1 and D-2 receptors. *Brain Res.*, 623 (1993) 334-336.

[16] Morelli, M., Fenu, S., Pinna, A., Cozzolino, A., Carta, A. and Di Chiara, G., 'Priming' to dopamine agonist-induced contralateral turning as a model of non-associative sensitization to the expression of the postsynaptic dopamine message. *Behav. Pharmacol.*, 4 (1993) 389-397.

[17] Morelli, M., Fenu, S., Carta, A. and Di Chiara, G., Effect of MK 801 on priming of D1-dependent contralateral turning and its relationship to *c-fos* expression in the rat caudate-putamen. *Behavioural Brain Res.*, 79 (1996) 93-100.

[18] Morgan, J.I. and Curran, T., Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. *Annu. Rev. Neurosci.*, 14 (1991) 421-451.

[19] Mumberg, D., Lucibello, F.C., Schuermann, M. and Müller, R., Alternative splicing of *fos*B transcripts results in differentially expressed mRNAs encoding functionally antagonistic proteins. *Genes and Dev.*, 5 (1991) 1212-1223.

[20] Nakabeppu, Y. and Nathans, D., A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. *Cell*, 64 (1991) 751-759.

[21] Nakabeppu, Y., Oda, S. and Sekiguchi, M., Proliferative activation of quiescent Rat 1A cells by ΔFosB. *Mol. Cell Biol.*, 13 (1993) 4157-4166.

[22] Nishina, H., Sato, H., Suzuki, T., Sato, T. and Iba, H., Isolation and characterization of *fra-2*, an additional member of the *fos* gene family. *Proc. Natl. Acad. Sci. USA*, 87 (1990) 3619-3623.

[23] Paul, M.L., Graybiel, A.M., David, J.C. and Robertson, H.A., D1-like and D2-like dopamine receptors synergistically activates rotation and *c-fos* expression in the dopamine-depleted striatum in a rat model of Parkinson's disease. *J. Neurosci.*, 12 (1992) 3729-3742.

[24] Paul, M.L., Currie, R.W. and Robertson, H.A., Priming of a D1 dopamine receptor behavioral response is dissociated from striatal immediate-early gene activity. *Neuroscience*, 66 (1995) 347-359.

[25] Paxinos, G. and Watson, C. (1986) *The rat brain in stereotaxic coordinates*, 2nd ed. Academic Press, New York.

[26] Robertson, G.S., Herrera, D.G., Dragunow, M. and Robertson, H.A., L-Dopa activates *c-fos* in the striatum ipsilateral to a 6-hydroxydopamine lesion of the substantia nigra. *Eur. J. Pharmacol.*, 159 (1989) 99-100.

[27] Robertson, H.A., Peterson, M.R., Murphy, K. and Robertson, G.S., D1-dopamine receptor agonists selectively activate *c-fos* independent of rotational behaviour. *Brain Res.*, 503 (1989) 346-349.

[28] Robertson, G.S. and Fibiger, H.C., Neuroleptics increase *c-fos* expression in the forebrain: contrasting effects of haloperidol and clozapine. *Neuroscience*, 46 (1992) 315-328.

[29] Robertson, G.S., Vincent, S.R. and Fibiger, H.C., D1 and D2 dopamine receptors differentially regulate *c-fos* expression in striatonigral and striatopallidal neurons. *Neuroscience*, 49 (1992) 285-296.

[30] Robertson, G.S., Tetzlaff, W., Bedard, A., St-Jean, M. and Wigle, N., *c-fos* mediates antipsychotic-induced neurotensin gene expression in the rodent striatum. *Neuroscience*, 67 (1995) 325-344.

[31] Shu, S., Ju, G. and Fan, L., The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci. Lett.*, 85 (1988) 169-171.

[32] Ungerstedt, U. and Arbuthnott, G., Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res.*, 24 (1970) 485-493.

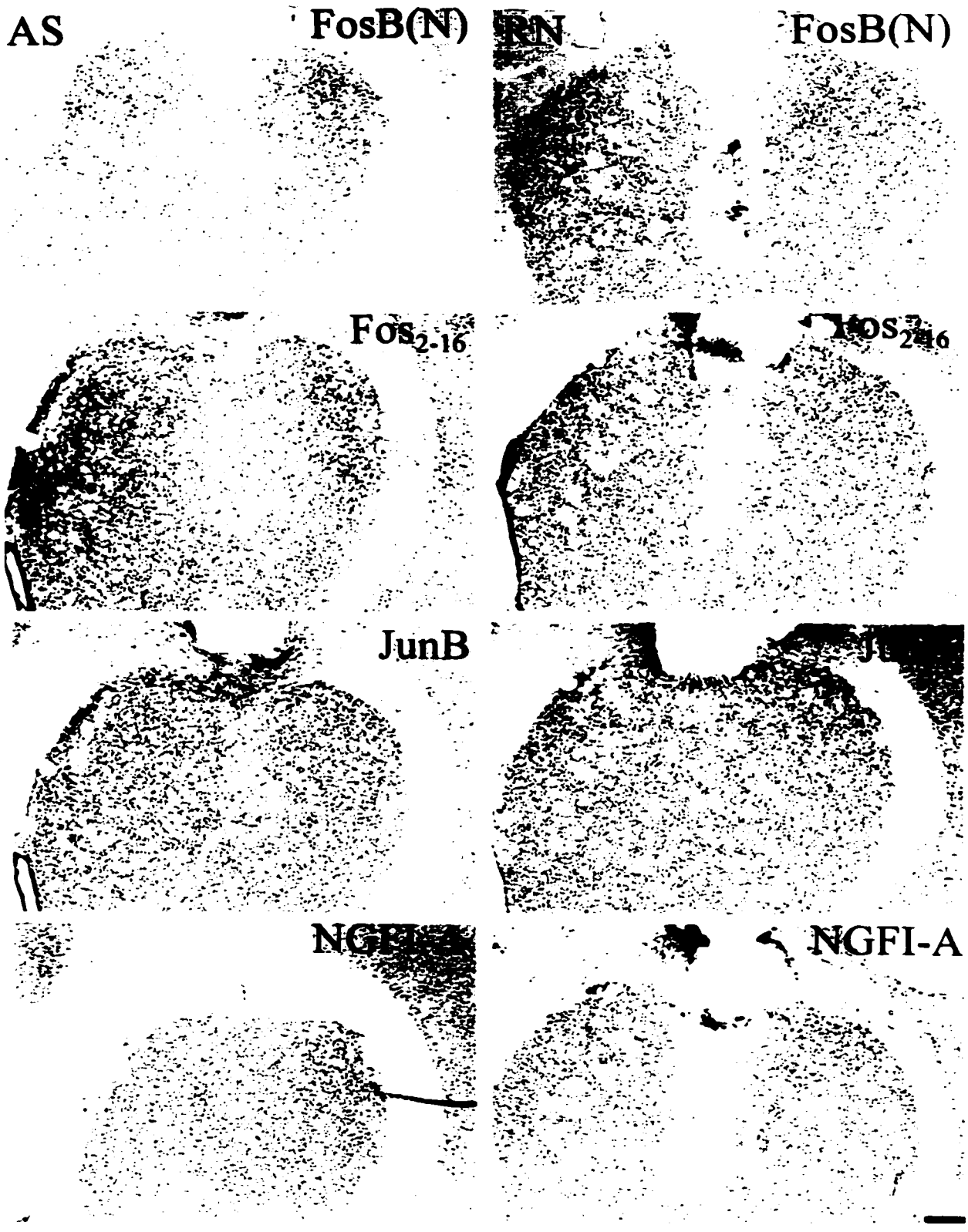
[33] Ungerstedt, U., Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. *Acta phys. Suppl.* 367 (1971) 69-93.

[34] Yen, J., Wisdom, R.M., Tratner, I. and Verma, I.M., An alternative spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins. *Proc. natn. Acad. Sci. USA*, 88 (1991) 5077-5081.

[35] Zerial, M., Toschi, L., Ryseck, R.-P., Schuermann, M., Müller, R. and Bravo, R., The product of a novel growth factor activated gene, *fos*B interacts with Jun proteins enhancing their DNA binding activity. *EMBO J.*, 8 (1989) 805-813.

Fig. 1. Photomicrographs illustrating the effects of intrastriatal injection of either the antisense oligonucleotide (AS) or random oligonucleotide (RN) to *fos*B on increases in Fos_{2-16}-, FosB(N)-, JunB- and NGFI-A-like immunoreactivity detected 2 h after administration of apomorphine (0.1 mg/kg, s.c.). Scale bar = 500 mm.

Fig. 2. Effects of intrastriatal injection of saline, random or antisense oligonucleotides to *fos*B on apomorphine (0.1 mg/kg, s.c.) - and SKF 38393 (3.0 mg/kg, s.c.)-induced increases in Fos_{2-16}-, FosB(N)-, JunB- and NGFI-A-like immunoreactivity in the 6-OHDA-denervated striatum. (A) Levels of immediate-early gene immunoreactivity in the 6-OHDA-denervated striatum 2 h after apomorphine (0.1 mg/kg, s.c.) in animals injected with either the antisense or random oligonucleotide. Note that the antisense oligonucleotide selectively reduced the number of neurons which expressed apomorphine-induced FosB(N)-like immunoreactivity. (B) SKF 38393-induced immediate-early gene immunoreactivity in the 6-OHDA-denervated striatum of apomorphine-primed animals that had been injected with either the antisense or random oligonucleotide 3 days previously. Each bar represents the average of 6 animals. A cell count datum for each animal represents the average of 3 determinations, each performed on a separate serial section. Cell counts were performed in area (660 X 800 mm) located in the dorsolateral striatum using computer assisted image analysis. Asterisk, significantly different from saline treated animals ($p<0.01$), Newman-Keuls test.


AS
FosB(N)
RN
FosB(N)
Fos2-16
Fos2-16
JunB
NGFI-A
NGFI-A

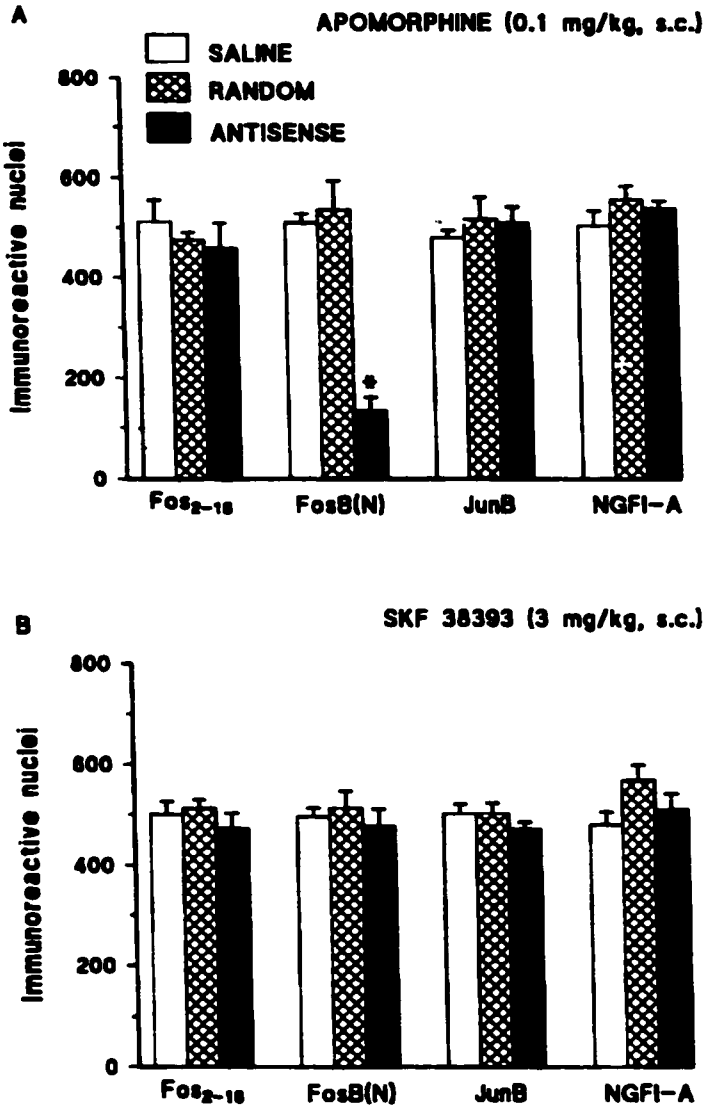

A
APOMORPHINE (0.1 mg/kg, s.c.)
SALINE
RANDOM
ANTISENSE
Immunoreactive nuclei
800
600
400
200
0
*
Fos2-16
FosB(N)
JunB
NGFI-A
B
SKF 38393 (3 mg/kg, s.c.)
Immunoreactive nuclei
800
600
400
200
0
Fos2-16
FosB(N)
JunB
NGFI-A

Table 1.

Apopmorphone priming of SKF38393-induced turning in 6-OHDA lesioned rats

Primer		Day 1 (Primer)	Day 4 (SKF38393)
Saline	(1.0 ml/kg, s.c.)	0.6±0.2	2.8±0.6
Apomorphine	(0.1 mg/kg, s.c.)	2.8±0.6[a]	20.9±3.7[a]

Values represent total number of ¼ rotations for 30 min beginning immediately after injection of primer (apomorphine or saline) on Day 1 and 30 min after injection of SKF38393 (3.0 mg/kg, s.c.) on Day 4.

Data are expressed as means±S.E.M. for 6 rats per group.

[a]P< 0.01 compared to rats administered saline.

Table 2.

Effects of intrastriatal injection of saline, RN or AS to *fosB* on D_1-receptor-related Priming of 6-OHDA lesioned rats.

	Day 1 (Apomorphine)	Day 4 (SKF38393)
Saline	6.9±1.6	20.9±3.4
RN	7.5±1.1	21.8±4.0
AS	6.9±1.3	11.3±1.2[a]

Values represent total nulber of ¼ rotations for 30 min beginning immediately after injection of apomorphine (0.1 mg/kg, s.c.) on Day 1 and 30 min after injection of SKF38393 (3.0 mg/kg, s.c.) on Day 4.

Data are expressed as means±S.E.M. for 6 rats per group.

[a] $P < 0.05$ compared to rats administered saline.

Chapter 5

Crocker et al. (2000) Neuronal Apoptosis Inhibitor Protein Protects Nigrostriatal Dopamine Pathway from Intrastriatal 6-OHDA-induced Degeneration in the Adult Rat. (submitted)

This manuscript demonstrated the functional neuroprotective effects of adenovirus-mediated gene transfer of Neuronal Apoptosis Inhibitor Protein (NAIP) in the nigrostriatal pathway *in vivo*.

All surgical and immunohistochemistry in this experiments described in this manuscript were performed by S.J. Crocker, with the occassional assistance of Mrs. N. Wigle and Mr. C.J. Lee. In situ hybridization was performed by Dr. C.S. Thompson. Reagents were provided as cited in the text. This manuscript was written by S.J. Crocker, under the guidance of Dr. G.S. Robertson and editorial assistance of Dr. D.S. Park.

C.E. HENDERSON

Receiving Editor, Neuronal Development and Cell Death

Neuronal Apoptosis Inhibitor Protein Protects Nigrostriatal Dopamine Pathway from Intrastriatal 6-OHDA-induced Degeneration in the Adult Rat.

S.J. Crocker[1,2], N. Wigle[1], P. Liston[3,4], C.S. Thompson[3], C.J. Lee[1], D.G. Xu[5], S. Roy[6], D.W. Nicholson[6], D.S. Park[2], A. MacKenzie[3,4], R.G. Korneluk[3,4] and G.S. Robertson[1,5]

Departments of Cellular and Molecular Medicine[1] and Neuroscience Research Institute[2]

Faculty of Medicine, University of Ottawa,

451 Smyth Road, Ottawa, Ontario K1H 8M5, Canada

Apoptogen Inc.[3], Ottawa, Ontario K1H 8M5, Canada

Solange Gauthier-Karsh Molecular Genetics Laboratory[4]

Children's Hospital of Eastern Ontario,

401 Smyth Road, Ottawa, Ontario K1H 8L1, Canada

Departments of Pharmacology[5] and Biochemistry and Molecular Biology[6]

Merck Frosst Canada Inc. P.O. Box 1005

Pointe-Claire – Dorval, Quebec H9R 4P8, Canada

Running Title: *NAIP attenuates 6-OHDA-induced nigral degeneration.*

Correspondence to:

S.J. Crocker
Neuroscience Research Institute
University of Ottawa
451 Smyth Road, Ottawa
Ottawa, ON K1H 8M5
Phone: 613/562-5800 ext. 8346
Fax: 613/562-5403
e-mail: crocker@uottawa.ca

Manuscript Information:

Words in Abstract: 139
Words in Introduction (without refs): 588
Words in Discussion: 1062

Text Pages: 27
Figures: 5
Tables: 1

ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder of the basal ganglia, associated with the inappropriate death of dopaminergic neurons of the substantia nigra. We show that adenovirally-mediated expression of Neuronal Apoptosis Inhibitor Protein (NAIP) ameliorates the loss of nigrostriatal function by attenuating the death of dopamine neurons in the intrastriatal 6-OHDA rat model of PD. Because of evidence supporting the role of IAP proteins as potent blockers of caspase-3, we assessed caspase-3 activity in this adult 6-OHDA model. Although, there was an absence of caspase-3 activity in the nigral dopamine neurons of rats lesioned as adults, caspase-3-like proteolysis was found in two other models of *in vivo* dopamine neuron degeneration, suggesting that caspase-3 can be induced in dopamine neurons. Taken together, these results suggest that therapeutic strategies based on NAIP may have value in the treatment of PD.

Parkinson's disease (PD) is a chronic neurodegenerative disorder affecting approximately 1 in 200 people over the age of 55. Parkinsonism is principally attributed to the selective loss of the pigmented dopaminergic neurons of the substantia nigra pars compacta (SNc), located in the ventral midbrain (Hirsch et al., 1988). This loss of SNc neurons is responsible for a profound reduction in the amount of dopamine released into the striatum, and results in the generation of the cardinal neurological features of PD including resting tremor, bradykinesia, muscular rigidity and a postural gait. Current drug therapies for PD aim to replenish the levels of striatal dopamine by administration of the dopamine precursor, L-Dopa. While this approach has proven clinical efficacy, L-Dopa does not arrest the underlying disease process and thus dopamine replacement strategies have been shown to have limited long-term utility. A neuroprotective treatment which attentuates the progressive death of nigral dopamine neurons may therefore provide an effective and long-term therapy for this neurodegenerative condition (Thompson, 1995; Dunnett and Bjorklund, 1999).

Accumulating evidence suggests that neuron death in PD may be a consequence of aberrant generation of cytotoxic free radicals. Reports describing an increased production of reactive oxygen species (ROS) (Hunot et al., 1997), mitochondrial complex I deficiencies (Schapira et al., 1990), and increased indicators of oxidative protein modifications (Good et al., 1998) in postmortem analyses of PD patients support this proposal. Interestingly, dopaminergic neurotoxins have been shown to generate ROS (Heikkila and Cohen, 1971; Kumar et al., 1995; Sriram et al., 1997) and inhibit mitochondrial respiration (Cleeter et al., 1992; Glinka et al., 1996). While four genes which are associated with familial PD have recently been identified (Polymeropoulos et al., 1997; Gasser et al., 1998; Kitada et al., 1998; Leroy et al., 1998), the significant majority of PD is sporadic (Johnson et al., 1990; Maraganore et al., 1991; Scott et al., 1997), suggesting that idiopathic PD is likely triggered by other factors (Rajput et al., 1987; Koller et al., 1990).

Intrastriatal administration of the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) elicits a progressive degeneration of nigral dopamine neurons over several weeks (Sauer and Oertel, 1994). Several studies have demonstrated that the delayed degeneration of dopamine neurons in this model of PD can be attenuated by the direct application of trophic factors (Sauer et al., 1995; Rosenblad et al., 1999) or through the introduction of virally-mediated gene expression (Bilang-Bleuel et al., 1997; Choi-Lundberg et

al., 1997; Mandel et al., 1997; Yamada et al., 1999). Neuronal Apoptosis Inhibitor Protein (NAIP), a member of the human Inhibitor of Apoptosis (IAP) protein gene family, was identified by virtue of its homozygous deletion in infants with the most severe form of the neurodegenerative disorder spinal muscular atrophy (Roy et al., 1995). IAP proteins have been shown to attenuate cell death induced by a variety of triggers *in vitro* (Liston et al., 1996) as well as enhance hippocampal neuron survival following transient global ischemia in the rat (Xu et al., 1997; Xu et al., 1999). Since ROS have been associated with PD and NAIP has been shown to reduce cell death in response to menadione-induced free radical generation *in vitro*, we decided to investigate the effects of NAIP overexpression on nigrostriatal degeneration in a progressive rat model of PD.

The cysteine protease, caspase-3, is an important mediator of developmental neuronal death (Kuida et al., 1996) as well as a key participant in cell death pathways responsible for a variety of neurodegenerative conditions (Marks and Berg, 1999). Mammalian IAP proteins contain domains of amino acid sequence identity with the baculovirus proteins (Roy et al., 1995; Liston et al., 1996), OpIAP and CpIAP (Clem and Miller, 1993) called baculovirus IAP repeat (BIR) domains. The BIR domains of the IAPs have been shown to be essential for IAP inhibition of select members of the caspase family of cysteine proteases (Deveraux et al., 1997; Roy et al., 1997; Takahashi et al., 1998). This suggests that the neuroprotective actions of IAP proteins *in vivo* may be a consequence of inhibiting caspase-3 activity (Xu et al., 1999). While recent reports suggest that caspase-3 proteolysis may participate in the delayed death of dopamine neurons *in vitro* (Dodel et al., 1998; Dodel et al., 1999; Jeon et al., 1999), activation of caspase-3 in an adult animal model of PD has not yet been reported. Accordingly, we have also examined whether caspase-3 activation occurs in dopaminergic neurons in a progressive rat model of PD. We present evidence which indicates that NAIP overexpression protects nigral neurons in a caspase-3 independent manner.

MATERIALS AND METHODS

Animals. All animals were treated in strict accordance with the Guidelines for the Use and Treatment of Animals set out by the Animal Care Council of Canada and endorsed by the Medical Research Council of Canada.

Recombinant Adenoviruses. Construction of recombinant type 5 adenoviral vectors containing the human cDNA for NAIP (Ad-NAIP) or lacZ (Ad-lacZ) in which expression was driven by the chicken β-actin promoter was performed as previously described (Rosenfeld et al., 1992; Liston et al., 1996). The application of these constructs *in vivo* has also been previously demonstrated (Xu et al., 1997). Briefly, a 3.7 kb fragment of NAIP tagged with the Myc epitope was cloned into the Swa1 site of the adenovirus expression cosmid pAdex1Cawt (Fujita et al., 1995), and adenoviruses were raised in the human kidney 293 cell line. Viruses were purified by CsCl density gradients and titered by plaque assay (Graham and Eb, 1973). High titer stocks were resuspended in 10% glycerol in 0.15 M PBS and maintained at -80 C prior to use. Aliquots of each adenoviral construct were prepared at a working concentration of 1×10^7 pfu/μl immediately before *in vivo* administration.

Animal Surgeries. Male Wistar rats were used for these experiments (see Table 1: Experimental Summary). Animals were housed in a temperature and light controlled environment and given free access to food and water. For stereotaxic surgeries, animals were deeply anesthetized using halothane gas (4% in oxygen) and placed into a small animal stereotaxic frame (David Kopf Instruments). Anesthesia was maintained during all surgical procedures using halothane (2%) delivered through a specially designed nose cone fitted to the animal frame.

Adenoviral Administration. A single stereotaxic injection of viral suspension (3μl total volume at a rate of 0.5 μl/min) was made into the right dorsal striatum at 0.5 mm anterior, 2.5mm to the right and 5.2 mm ventral to bregma, as measured from the skull surface according to Paxinos and Watson (Paxinos G., 1986). One week following adenovirus administration, groups of animals (n=3 per treatment), were used to assess increased adenovirally-mediated gene expression (Xu et al., 1997).

Intrastriatal 6-OHDA. The progressive 6-OHDA lesion model of PD in the rat has been described previously by Sauer and Oertel (1994). In a first experiment, animals (n=8 per group) received a single injection of intrastriatal 6-OHDA (20 μg measured as free base) at the same stereotaxic coordinates as the

viral injections. For each experiment, 6-OHDA was dissolved in 2.8 µl of water containing 0.05% ascorbic acid and maintained on ice to minimize oxidation. 6-OHDA was infused at a rate of 0.7 µl/min and the cannula remained in situ for two minutes following injection. Animals pretreated with adenovirus were assessed for amphetamine-induced circling four weeks later and then used for histological determination of the effects of Ad-NAIP overexpression on SNc neuronal survival. In a second experiment, additional groups of animals (n=3 per group) were decapitated at varying times following an intrastriatal 6-OHDA lesions to assess caspase-3 activation following 6-OHDA administration (see Table 1).

Juvenile 6-OHDA. Postnatal day 7 (PND7) rat pups were lesioned with 6-OHDA (9 µg measured as free base) as outlined by Marti *et al.* (1997) and decapitated at varying post-lesion survival times. (See Table 1).

Medial Forebrain Bundle (MFB) Transection. Mechanical transection of the nigrostriatal tract results in the delayed degeneration of neurons in the substantia nigra (Hagg and Varon, 1993; Brecknell et al., 1995). Adult male Wistar rats (PND42) were axotomized using a wire knife (Scouten et al., 1981) (David Kopf Instruments) lowered 8.0 mm ventral and 2.4 mm to the right and 3.8 mm caudal to bregma. The wire knife was then extruded 2 mm from the end of the blade carrier and raised 2.5 mm. The blade was then returned to its original position, retracted and the blade assembly withdrawn. Animals (n=3 per group) were decapitated at varying post-lesion survival times (See Table 1).

[Insert Table 1]

Behavioural Assessment. Four weeks following intrastriatal 6-OHDA lesioning, Ad-NAIP and Ad-lacZ-treated animals were assessed for amphetamine-induced rotational behavior using an automated rotometer (Crocker et al., 1998). Animals were placed into tethered harnesses and allowed 10 minutes to acclimatize to the rotometer chambers. Rats were then administered D-amphetamine (2 mg/kg, s.c.) and rotational behavior was measured over a 90 minute period. Net rotational asymmetry was calculated for minutes 31-60 for each of the subjects (Ungerstedt and Arbuthnott, 1970).

Histology and Immunohistochemistry. Three hours following D-amphetamine administration, adenovirus-treated animals were anaesthetized with sodium pentobarbital and intracardially perfused with saline (0.9%) followed by an equal volume of paraformaldehyde (4% in 0.1M phosphate buffer). Brains were postfixed overnight and then cryoprotected in 10% sucrose (in 0.1M phosphate buffer). Free-floating

coronal cryostat sections (12 μm thick) were collected through the striatum and the substantia nigra. A mouse monoclonal antibody directed against tyrosine hydroxylase (TH; 1:1000; IncStar), a phenotypic marker enzyme for dopaminergic neurons, was used for immunohistochemical analysis of dopamine cell survival following 6-OHDA lesions (4-5 sections per animal per group). To ensure reliable quantitative comparisons of neurons which received both adenovirus and 6-OHDA, only sections which contained the medial terminal nucleus (MTN), level -4.5 mm according to Paxinos and Watson (Paxinos G., 1986), were used for quantification of SNc neuron survival between treatment groups.

Caspase-3 activation was assessed in brain tissue sections by three separate methods. First, an antibody specific for the catalytically active tetramer $(p17/p12)_2$ (Rasper et al., 1998) was used to detect the presence of active caspase-3 in tissue sections (Xu et al., 1999) (1:2000, Merck-Frosst). In a second approach, we incubated the slide-mounted tissue sections using a biotinylated tetrapeptide caspase-3 inhibitor, acetyl-aspartyl-glutamyl-valyl-aspart-1-aldehyde (DEVD-CHO; 1:1000, Biomol) (Xu et al., 1999). A caspase-3 cleaved form of amyloid precursor protein (APP) was detected using an antibody which recognizes the neoepitope produced by caspase-3 cleavage of APP (Gervais et al., 1999) (ΔC-APP; 1:200, Merck-Frosst). Labeling was visualized using either FITC-conjugated avidin or CY3-conjugated affinity purified donkey-anti-rabbit secondary antibodies (1:200, both Molecular Probes, Inc.). For all models tested, the success of the lesioning procedure was confirmed by increased expression of the immediate early gene protein, c-Jun, using a rabbit polyclonal antibody (1:250, gift from Y. Nakabeppu).

FluoroJade staining was performed as described by Schmued et al. (1997) (Schmued et al., 1997). This fluorescent anionic dye technique has been shown to be a reliable and highly sensitive histological stain for degenerating neurons in brain tissue sections.

In Situ End-labeling (ISEL). Nuclei containing fragmented DNA were visualized using FITC fluoresence in midbrain tissue sections adapted from Fliss and Gattinger (1996) (Fliss and Gattinger, 1996) as previously described (Xu et al., 1997).

In Situ Hybridization Histochemistry. The template for the riboprobe was generated by RT-PCR from rat hippocampal RNA using a primer directed against nucleotide sequences conserved in mouse and human *naip*. A 227 bp cDNA fragment corresponding to sequences within exons 4-5 of the human *naip* gene was ligated into the EcoR1 site of the plasmid pcDNA3 (Invitrogen). Both sense and antisense ^{33}P-labelled

riboprobes were transcribed from the linearized template using the MAXIscript *in vitro* transcription kit (Ambion Inc.). Specific activities ranged from 0.6 to $1.2x10^7$ c.p.m. per µl. Cryostat sections (12 µm thick) were fixed with 4% paraformaldehyde, rinsed, dehydrated and incubated in hybridization buffer containing either a sense or antisense *naip* riboprobe (2×10^6 c.p.m. per 150 µl per slide) overnight at 50°C. Sections were then treated with ribonuclease A (10 µg/ml) at 35°C for 30 minutes and washed in 2X SSC for 30 minutes. After drying, slides were placed on Kodak BIOMAX MR imaging film (Eastman Kodak) for 3 days. After developing the film, slides were dipped in Kodak NTB-2 photographic emulsion, diluted 1:1 in distilled water at 42°C, dried and exposed for three weeks at 4°C. The slides were then developed in Kodak D-19, fixed in Kodak fixer, rinsed and counterstained with 0.5% Toluidine blue in 1% sodium tetraborate, diluted 1:100 in distilled water. After dehydratation and coverslipping, developed silver grains were quantified using a computer assisted image analysis subroutine (Empix Imaging) employing optical densities and silver grain counts (Richardson and Verge, 1989). While it is recognized that absolute quantities of mRNA cannot be determined with confidence by this method, changes in relation to control (endogenous) levels of expression can be determined reliably (Gerfen and Young, 1988). Measurements from both Ad.NAIP and control (untreated or Ad.lacZ) nigra were made using a constant circular field (Gramolini et al., 1997) of 25µm diameter. Semi-quantitative analysis of silver grain densities overlaying counterstained cell bodies were made under oil-immersion (100x) using brightfield microscopy by a party unaware of experimental treatments. A minimum of twenty SN neurons per section from six sections per animal were used for each condition (n=3).

Image Analysis. Digital photomicrographs were captured on a Zeiss Axioskop using a Sony 3-CCD camera and Northern Eclipse software (Empix Imaging Inc.). Counts of TH-positive cell bodies in the substantia nigra pars compacta of both lesioned and contralateral sides were performed manually on at least 5 midbrain sections per animal (n=6 per group). Only sections containing the medial terminal nucleus (MTN) were used for quantitative assessment of neuron survival since it was this aspect of the SNc that were both infected with adenovirus and exposed to the intrastriatal 6-OHDA. Striatal densitometry measurements were performed on at least 6 sections per animal using a computer assisted image analysis subroutine which measures the proportion of area covered by (courtesy of N.Banner, Empix Imaging). An area 660µm x 800µm in the dorsolateral striatum of sections containing the anterior commissure (levels 1.7

to 0.2 mm rostral to bregma (Paxinos G., 1986) were used for striatal measurements. Cell count and striatal terminal staining were calculated as a percent of the unlesioned (contralateral) SNc. All quantification in this study was performed by an individual unaware of the experimental treatments.

Statistical Analysis. A one way analysis of variance was performed on rotational behaviour and cell counts, with significance determined using an unpaired *t*-test.

[Insert Figure 1]

RESULTS

Attenuation of amphetamine-induced rotational behaviour by NAIP is correlated with enhanced nigral dopamine neuron survival.

We first examined the expression of adenovirally-mediated gene expression one week following intrastriatal administration to assess the amount of NAIP increase in nigral dopamine neurons (Figure 1). Using a riboprobe directed against human *naip*, densitometric analysis revealed Ad-NAIP enhanced *naip* mRNA expression four-fold in nigral neurons (9.61±2.0 particles/cell) when compared to the contralateral, uninjected hemisphere (2.46±0.6 particles/cell) (Figure 1B). Tissues from Ad-LacZ treated animals did not exhibit any relative changes in *naip* expression (2.74±0.8 particles/cell) (Figure 1A), suggesting that the enhanced mRNA expression detected was not a generalised response to the adenoviral transfection. Further, immunohistochemistry using antibodies directed against either β-galactosidase or the Myc-tag of adenovirally-derived NAIP confirmed the expression of these adenovirally-derived gene products (Figure 1C,D respectively). Transfected cells in the nigral region represented approximately 59% of TH positive neurons at the level of the MTN.

To determine the effect of increased expression of NAIP on nigrostriatal dopamine function following intrastriatal 6-OHDA administration, adenovirus-injected 6-OHDA-lesioned animals were challenged with D-amphetamine (2 mg/kg, s.c) and stereotypic circling behaviour was recorded for 90 minutes by automated rotometer. Analysis of net ipsilateral turning revealed that animals which had received Ad-LacZ turned an average of 13.6 ± 5.4 ispsilateral quarter turns per minute while Ad-NAIP

treated animals exhibited only 2.5 ± 2.0 (Figure 2.). This difference in amphetamine-induced behaviour was statistically significant ($p<0.05$).

[Insert Figure 2]

Next we examined whether the reduced amphetamine-induced circling behaviour in Ad-NAIP treated animals was correlated with enhanced nigral neuron survival. To assess the degree of neuroprotection achieved by administration of Ad-NAIP, we performed a quantitative analysis of tyrosine hydroxylase (TH) immunoreactive neuronal soma in the substantia nigra pars compacta at the level of the MTN (Figure 3A). Cell counts revealed that animals treated with Ad-LacZ had 55.8 ± 3.3% of the neurons when compared to their unlesioned contralateral side (Figure 3B). However, animals that received the Ad-NAIP construct had 77 ± 5.5% of dopamine cell bodies in the lesioned side (Figure 3C), when calculated as a percent of the unlesioned control SNc. This difference represents a 38% increase in neuronal survival in Ad-NAIP treated animals when compared with Ad-LacZ treated animals (Figure 3D).

[Insert Figure 3]

Previous studies have shown that infusion of GDNF into an intrastriatal 6-OHDA lesioned rat can ameliorate dopamine agonist-induced circling behaviour independent of enhanced dopamine release into the striatum (Bowenkamp et al., 1995). To determine whether the observed attenuation in amphetamine-induced turning in Ad-NAIP treated animals may have been through protection of striatal dopamine nerve terminals, we analysed sections from Ad-NAIP and Ad-LacZ treated animals for TH-immunoreactivity. Consistent with the behavioural analysis, Ad-LacZ-treated animals exhibited a pronounced loss of striatal TH fibrous staining (Figure 4A). Analysis of the density of TH-fibre staining in Ad-NAIP treated animals revealed a significant preservation of dopamine terminal staining (Figure 4B). When calculated as a percent of the unlesioned hemisphere, this difference was significant ($p<0.02$) (Figure 4C).

[Insert Figure 4]

Caspase-3 is not activated in adult rat nigral neurons following intrastriatal 6-OHDA administration. Accumulating evidence suggests that programmed cell death in many neurodegenerative conditions may be mediated by the cysteine protease caspase-3. Given that several members of the IAP gene family, including NAIP (Maier et al., 1999), are a potent endogenous inhibitors of caspases-3 and 7 (Deveraux et al., 1997; Roy et al., 1997), this raised the possibility that the ability of NAIP to attenuate dopamine neuron

death in this 6-OHDA model may be attributable to caspase-3 inhibition. Indeed, recent reports have suggested the involvement of caspase-3 *in vitro* and in 6-OHDA lesioned juvenile rat pups, supporting the proposal that this protease may be a mediator of neuron death in these embyronic and early post-natal situations. To ascertain whether caspase-3 was activated in this adult rat model of PD, we investigated the possible contribution of caspase-3 in the adult intrastriatal 6-OHDA model of PD.

Tissue from the midbrain of adult 6-OHDA animals was processed for immunohistochemical detection of activated caspase-3 using an antibody which selectively recognises the conformation of the activated $(p17/p12)_2$ caspase-3 tetramer (Rasper et al., 1998). Activated caspase-3 was not detected in 6-OHDA treated adult animals at any of the time points tested (data not shown). In addition, active caspase-3 was not detected at any of the time points following 6-OHDA using DEVD-biotin histochemical labelling in adjacent tissue sections. For both of these staining methods, tissue from rats with ischemic injury were used as positive controls for antibody immunoreactivity (Xu et al., 1999).

These results suggested either that caspase-3 may not be activated by 6-OHDA in adult animals, or that caspase-3 may be developmentally downregulated in dopamine neurons and activation may not be possible in adult nigral neurons. To address this issues, we assayed for activated caspase-3 in tissue from juvenile rats lesioned with intrastriatal 6-OHDA, as well as in tissues from adult rats in which the nigrostriatal tract had been transected at the MFB. We then used tissues from these animals to examine for expression of the activate tetramer conformation of caspase-3. Again, we were unable to detect any caspase-3 activity in dopamine neurons by either of these methods (data not shown).

Amyloid Precursor Protein (APP) is cleaved by caspase-3 in nigral neurons of juvenile 6-OHDA lesioned and adult MFB axotomized rats but not in adult 6-OHDA lesioned animals.

Since the active half-life of caspase-3 is approximately 35 minutes (D.W. Nicholson, personal communication), the interval time used for the time courses following 6-OHDA administration or MFB axotomy may have been too long for the detection of the activated enzyme using the $(p17/p12)_2$ casapse-3 tetramer antibody. We therefore decided to use an antibody that selectively detects the truncated end of the amyloid precursor protein (ΔC-APP) when cleaved by caspase-3 (Gervais et al., 1999) as a histochemical marker for caspase-3 activity. In tissues from both juvenile 6-OHDA and adult MFBx, ΔC-APP immunoreactivity was observed 72 hours following the lesion (Figure 5B,D). These results suggest that in

each of these two models of nigral neuron degeneration, caspase-3 was activated but not detected directly by the DEVD or caspase-3 tetramer antibody. However, ΔC-APP was not detected in either the unlesioned, contralateral substantia nigra (Figure 5A) or in tissue from adult 6-OHDA lesioned animals at any of the time points tested (Figure 5C).

[Insert Figure 5]

This absence of detectable caspase-3 in the adult lesion model suggested either an absence of cell death or a form of delayed neuronal death in which caspase-3 is not activated. To clarify the situation, we performed *in situ* end labeling (ISEL) and FluroJade staining (Schmued et al., 1997). DNA fragmentation, as indicated by ISEL labeling, was only detected in juvenile 6-OHDA lesioned animals (Figure 5F), and not in tissues from either the 6-OHDA or MFB axotomy adult lesion paradigms (Figure 5G,H). Nevertheless, FluoroJade staining revealed pronounced labelling in all three lesion models (Figure 5J,K,L). In juvenile animals, the FluoroJade fluorescent stain was predominantly cellular (Figure 5J) while in both adult lesion models, the pattern of staining appeared largely diffuse and fibrous (Figure 5K,L), though neuronal soma were definable (Figure 5K, inset). Loss of nigral neurons was also confirmed by hemotoxyalin and eosin staining in all three lesion paradigms (data not shown). These findings are consistent with previous work by others (Marti et al., 1997; Herdegen et al., 1998) demonstrating that nigral dopamine neurons degenerate following intrastriatal 6-OHDA as indicated by a loss of neuronal Fluorogold labelling (Choi-Lundberg et al., 1997; Yamada et al., 1999). Thus nigral neuronal tissue in both juvenile and adult rats undergo changes consistent with neurodegeneration, suggesting that the observed differences in caspase-3 proteolytic activity and DNA fragmentation were likely attributed to divergent modes of death with respect to age and treatment.

DISCUSSION

Several previous studies have demonstrated the utility of viral vectors for gene transfer and protection of dopamine neurons in the intrastriatal 6-OHDA model (Bilang-Bleuel et al., 1997; Choi-Lundberg et al., 1997; Mandel et al., 1997; Yamada et al., 1999). The present study investigated the effect of NAIP overexpression on dopamine neuron survival in a 6-OHDA lesion model of PD in the adult rat. Enhanced expression of NAIP in the nigrostriatal tract reduced amphetamine-induced ipsilateral turning and loss of tyrosine hydroxylase positive neurons in the substantia nigra pars compacta four weeks following the 6-OHDA lesion. Interestingly, adenovirus-mediated expression of NAIP also attenuated the loss of dopamine fibres in the striatum. While detection of adenovirus-mediated expression in SNc neurons supports the protection of the nigral cell bodies, the results of the present study do not preclude the possiblity that a "bystander" effect may account for the dramatic sparing of striatal fibres in Ad.NAIP treated animals. Together, these results suggested that NAIP was capable of attenuating the delayed death of dopamine neurons following intrastriatal 6-OHDA. However, the issue of whether the delayed death of this neuronal population is actually apoptosis was also addressed.

Apoptosis has been suggested to underlie neuron death in PD (Tatton and Kish, 1997; Hirsch et al., 1999) though this possibility is currently under debate (Burke and Kholodilov, 1998; Wullner et al., 1999). While it has not been conclusively demonstrated that the death of dopamine neurons following 6-OHDA administration in adult animals is apoptotic (Marti et al., 1997), inhibition of dopamine neuron death by the apoptotic inhibitor protein, NAIP, could be interpreted to support the notion of apoptosis in PD. However, in this study, we present two findings which suggest that intrastriatal 6-OHDA induces a delayed neuronal death that is *not* classic apoptosis (Kerr et al., 1972). First, we assessed DNA fragmentation in dopamine neurons following 6-OHDA lesions in either early postnatal or adult 6-OHDA lesioned animals. Consistent with previous findings by others (Marti et al., 1997), we observed ISEL-positive cells in juvenile but *not* adult animals. These findings are in agreement with the view that the mode of cell death following intrastriatal injury is influenced by developmental age.

Secondly, we present findings which suggest that the cysteine protease, caspase-3, is not activated in adult 6-OHDA lesioned rats. This protease is widely implicated as an executioner of apoptotic cell death in a variety of neurodegenerative paradigms, including *in vitro* models of PD (Dodel et al., 1998; Ochu et

al., 1998; Dodel et al., 1999). However, we have shown that caspase-3 is not activated in the dopamine neurons of adult rats following 6-OHDA administration although caspase-3 activity was present in the juvenile 6-OHDA or adult MFB axotomy models. These results are consistent with those of Joen et al. (1999). Taken together, these findings suggest that in the adult 6-OHDA model of PD, the death of SNc neurons is neither caspase-3 dependent nor classic apoptotic death.

Interestingly, the detection of *in vivo* caspase-3 activity in juvenile animals is consistent with reports of protection of embryonic and early post-natal neuronal cultures using peptide inhibitors of caspase-3 (Du et al., 1997; Dodel et al., 1998; Dodel et al., 1999). However, since we have also shown that caspase-3 is not activated in adult 6-OHDA lesioned animals, it is therefore likely that the physiological response of dopamine neurons to a neurotoxic insult may differ significantly depending upon the stage of neuronal development. Consequently, and inasmuch as neurotoxins may recapitulate the etiology of PD, adult animal models of PD may have similarities to the nature of this late onset neurodegenerative condition which may not be mimicked in early developmental settings. In addition, the neuroprotective effects of NAIP in this model suggest that IAP proteins may have cytoprotective properties exclusive of their role as endogenous caspase inhibitors.

There is evidence for suppression of cell death by IAP proteins independent of caspase inhibition. A possible alternate mechanism of neuroprotection may be attributed to the baculovirus IAP repeat (BIR) domains, a distinguishing feature of all IAP family proteins. BIR domains have been reported to be important for caspase inhibition by XIAP, but these homology domains may also provide a mechanism of physiologically relevant protein-protein interactions. For instance, the BIR domains of the *Drosophila* IAP, DIAP-1, have been reported to suppress cell death (Hay et al., 1995) through the sequestration of a number of apoptosis-inducing proteins, including Reaper (RPR), HID and Grim (Vucic et al., 1998). Interestingly, ectopic expression of RPR in mammalian cells induces cell death that is inhibited by the mammalian IAP proteins, c-IAP-1/HIAP-2 and c-IAP-2/HIAP-1 (McCarthy and Dixit, 1998). The ability of mammalian IAP proteins to functionally substitute for their *Drosophila* IAP homologs suggests that there may be multiple cell death pathways which can be blocked by IAP proteins.

While it is tempting to speculate on possible non-caspase dependent mechanisms of NAIP-mediated neuroprotection in this model of PD, it is equally likely that a novel caspase or caspase-like

enzyme may reside in adult dopaminergic neurons. This enzyme may be also be inhibited by NAIP and may therefore account for the potent neuroprotective effects of NAIP in this model. Interestingly, this notion is supported by the observation that the broad spectrum caspase inhibitor zVAD.fmk may rescue dopaminergic neurons in the adult 6-OHDA lesion model (Cutillas et al., 1999). However, the reported absence of detectable caspase-7 in brain (Juan et al., 1997), also suggests that identified group II caspases are not likely mediators of neuronal death in this model. Hence, NAIP may either sequester upstream "activator" proteins or act to inhibit downstream "executioner" components of the cell death pathway(s) in dopamine neurons.. In this regard, the elucidation of factors which may be modulated by NAIP in this 6-OHDA model of PD may serve to expand our understanding of the physiological actions of NAIP but more importantly may identify elements which contribute to the etiology of PD.

In summary, we have shown that adenovirally-mediated overexpression of NAIP in the nigrostriatal pathway can protect this neuronal population from the degeneration and loss of function induced by the dopaminergic neurotoxin, 6-OHDA. In addition, we provide evidence which supports the notion that dopamine neuron death in this model is not mediated by caspase-3. Since IAP proteins are reputed endogenous caspase-3 inhibitors, the mechanism by which NAIP elicits neuroprotection in this model is likely through an additional, caspase-independent mechanism. Consequently, strategies that enhance expression of NAIP, such as gene therapy (Karpati et al., 1996; Barkats et al., 1998), may have therapeutic benefit for the abatement of neuronal death in PD and provide a novel therapeutic strategy for the treatment of this neurodegenerative condition.

ACKNOWLEDGEMENTS

We would like to thank Dr. D.M. Gash, University of Kentucky for his useful comments and suggestions. We gratefully acknowledge A. Adeeko for expert technical assistance. This work was supported by grants from the Medical Research Council of Canada and the Ontario Neurotrauma Foundation (GSR).

ABBREVIATIONS

PD, Parkinson's disease; IAP, Inhibitor of Apoptosis, NAIP, Neuronal Apoptosis Inhibitor Protein; SNc, substantia nigra pars compacta; APP, amyloid precursor protein; MFB, medial forebrain bundle.

REFERENCES

Barkats, M., Bilang-Bleuel, A., Buc-Caron, M.H., Castel-Barthe, M.N., Corti, O., Finiels, F., Horellou, P., Revah, F., Sabate, O. & Mallet, J. (1998) Adenovirus in the brain: recent advances of gene therapy for neurodegenerative diseases. *Prog Neurobiol*, **55**, 333-41.

Bilang-Bleuel, A., Revah, F., Colin, P., Locquet, I., Robert, J.J., Mallet, J. & Horellou, P. (1997) Intrastriatal injection of an adenoviral vector expressing glial-cell- line-derived neurotrophic factor prevents dopaminergic neuron degeneration and behavioral impairment in a rat model of Parkinson disease. *Proc Natl Acad Sci U S A*, **94**, 8818-23.

Bowenkamp, K.E., Hoffman, A.F., Gerhardt, G.A., Henry, M.A., Biddle, P.T., Hoffer, B.J. & Granholm, A.C. (1995) Glial cell line-derived neurotrophic factor supports survival of injured midbrain dopaminergic neurons. *J Comp Neurol*, **355**, 479-89.

Brecknell, J.E., Dunnett, S.B. & Fawcett, J.W. (1995) A quantitative study of cell death in the substantia nigra following a mechanical lesion of the medial forebrain bundle. *Neuroscience*, **64**, 219-27.

Burke, R.E. & Kholodilov, N.G. (1998) Programmed cell death: does it play a role in Parkinson's disease? *Ann Neurol*, **44**, S126-33.

Choi-Lundberg, D.L., Lin, Q., Chang, Y.N., Chiang, Y.L., Hay, C.M., Mohajeri, H., Davidson, B.L. & Bohn, M.C. (1997) Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science*, **275**, 838-41.

Cleeter, M., Cooper, J. & Schapira, A. (1992) Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: evidence for free radical involvement. *J Neurochem*, **58**, 786-9.

Clem, R.J. & Miller, L.K. (1993) Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus. *J Virol*, **67**, 3730-8.

Crocker, S.J., Morelli, M., Wigle, N., Nakabeppu, Y. & Robertson, G.S. (1998) D1-Receptor-related priming is attenuated by antisense-meditated 'knockdown' of fosB expression. *Brain Res Mol Brain Res*, **53**, 69-77.

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.

Deveraux, Q.L., Takahashi, R., Salvesen, G.S. & Reed, J.C. (1997) X-linked IAP is a direct inhibitor of cell-death proteases. *Nature*, **388**, 300-4.

Dodel, R.C., Du, Y., Bales, K.R., Ling, Z., Carvey, P.M. & Paul, S.M. (1999) Caspase-3-like proteases and 6-hydroxydopamine induced neuronal cell death. *Brain Res Mol Brain Res*, **64**, 141-8.

Dodel, R.C., Du, Y., Bales, K.R., Ling, Z.D., Carvey, P.M. & Paul, S.M. (1998) Peptide inhibitors of caspase-3-like proteases attenuate 1-methyl-4- phenylpyridinum-induced toxicity of cultured fetal rat mesencephalic dopamine neurons. *Neuroscience*, **86**, 701-7.

Du, Y., Dodel, R.C., Bales, K.R., Jemmerson, R., Hamilton-Byrd, E. & Paul, S.M. (1997) Involvement of a caspase-3-like cysteine protease in 1-methyl-4- phenylpyridinium-mediated apoptosis of cultured cerebellar granule neurons. *J Neurochem*, **69**, 1382-8.

Dunnett, S. & Bjorklund, A. (1999) Prospects for new restorative and neuroprotective treatments in. *Nature*, **399**, A32-9.

Fliss, H. & Gattinger, D. (1996) Apoptosis in ischemic and reperfused rat myocardium. *Circ Res*, **79**, 949-56.

Fujita, A., Sakagami, K., Kanegae, Y., Saito, I. & Kobayashi, I. (1995) Gene targeting with a replication-defective adenovirus vector. *J Virol*, **69**, 6180-90.

Gasser, T., Muller-Myhsok, B., Wszolek, Z., Oehlmann, R., Calne, D., Bo, V., Bereznai, B., Fabrizio, E., Vieregge, P. & Horstmann, R. (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nat Genetics*, **18**, 262-265.

Gerfen, C. & Young, W. (1988) Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an in situ hybridization histochemistry and fluorescent retrograde tracing study. *Brain Res*, **460**, 161-7.

Gervais, F.G., Xu, D., Robertson, G.S., Vaillancourt, J.P., Zhu, Y., Huang, J., LeBlanc, A., Smith, D., Rigby, M., Shearman, M.S., Clarke, E.E., Zheng, H., Van Der Ploeg, L.H., Ruffolo, S.C., Thornberry, N.A., Xanthoudakis, S., Zamboni, R.J., Roy, S. & Nicholson, D.W. (1999) Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid- beta precursor protein and amyloidogenic A beta peptide formation. *Cell*, **97**, 395-406.

Glinka, Y., Tipton, K. & Youdim, M. (1996) Nature of inhibition of mitochondrial respiratory complex I by 6-Hydroxydopamine. *J Neurochem*, **66**, 2004-10.

Good, P., Hsu, A., Werner, P., Perl, D. & Olanow, C. (1998) Protein nitration in Parkinson's disease. *J Neuropathol Exp Neurol*, **57**, 338-42.

Graham, F.L. & Eb, A.J.v.d. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, **52**, 456-67.

Gramolini, A., Dennis, C., Tinsley, J., Robertson, G., Cartaud, J., Davies, K. & Jasmin, B. (1997) Local Transcriptional Control of Utrophin Expression at the Neuromuscular Synapse. *Journal of Biological Chemistry*, **272**, 8117-8120.

Hagg, T. & Varon, S. (1993) Ciliary neurotrophic factor prevents degeneration of adult rat substantia nigra dopaminergic neurons in vivo. *Proc Natl Acad Sci U S A*, **90**, 6315-9.

Hay, B.A., Wassarman, D.A. & Rubin, G.M. (1995) Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell*, **83**, 1253-62.

Heikkila, R. & Cohen, G. (1971) Inhibition of biogenic amine uptake by hydrogen peroxide: a mechanism. *Science*, **172**, 1257-8.

Herdegen, T., Claret, F.X., 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**, 5124-35.

Hirsch, E., Graybiel, A.M. & Agid, Y.A. (1988) Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature*, **334**, 345-8.

Hirsch, E.C., Hunot, S., Faucheux, B., Agid, Y., Mizuno, Y., Mochizuki, H., Tatton, W.G., Tatton, N. & Olanow, W.C. (1999) Dopaminergic neurons degenerate by apoptosis in Parkinson's disease [letter; comment]. *Mov Disord*, **14**, 383-5.

Hunot, S., Brugg, B., Ricard, D., Michel, P.P., Muriel, M.P., Ruberg, M., Faucheux, B.A., Agid, Y. & Hirsch, E.C. (1997) Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease. *Proc Natl Acad Sci U S A*, **94**, 7531-6.

Jeon, B.S., Kholodilov, N.G., Oo, T.F., Kim, S.Y., Tomaselli, K.J., Srinivasan, A., Stefanis, L. & Burke, R.E. (1999) Activation of caspase-3 in developmental models of programmed cell death in neurons of the substantia nigra. *J Neurochem*, **73**, 322-33.

Johnson, W., Hodge, S. & Duvoisin, R. (1990) Twin studies and the genetics of Parkinson's disease—a reappraisal. *Mov Disord*, **5**, 187-94.

Juan, T., McNiece, I., Argento, J., Jenkins, N., Gilbert, D., Copeland, N. & Fletcher, F. (1997) Identification and mapping of Casp7, a cysteine protease resembling CPP32 beta, interleukin-1 beta converting enzyme, CED-3. *Genomics*, **40**, 86-93.

Karpati, G., Lochmuller, H., Nalbantoglu, J. & Durham, H. (1996) The principles of gene therapy for the nervous system. *Trends Neurosci*, **19**, 49-54.

Kerr, J.F., Wyllie, A.H. & Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, **26**, 239-57.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. & Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, **392**, 605-8.

Koller, W., Vetere-Overfield, B., Gray, C., Alexander, C., Chin, T., Dolezal, J., Hassanein, R. & Tanner, C. (1990) Environmental risk factors in Parkinson's disease. *Neurology*, **40**, 1218-21.

Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. & Flavell, R.A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32- deficient mice. *Nature*, **384**, 368-72.

Kumar, R., Agarwal, A. & Seth, P. (1995) Free radical-generated neurotoxicity of 6-hydroxydopamine. *J Neurochem*, **64**, 1703-7.

Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M., Jonnalagada, S., Chernova, T., Dehejia, A., Lavedan, C., Gasser, T., Steinbach, P., Wilkinson, K. & Polymeropoulos, M. (1998) The ubiquitin pathway in Parkinson's disease. *Nature*, **395**, 451-452.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J.E., MacKenzie, A. & Korneluk, R.G. (1996) Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature*, **379**, 349-53.

Maier, J., Roy, S., Besner-Johnston , A., Nicholson, D. & MacKenzie, A. (1999) The neuronal apoptosis inhibitory protein (NAIP) is a specific caspase-3 and caspase-7 inhibitor: "Symposium on Neuronal Apoptosis." Tubingen, Germany:, pp P11.

Mandel, R.J., Spratt, S.K., Snyder, R.O. & Leff, S.E. (1997) Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. *Proc Natl Acad Sci U S A*, **94**, 14083-8.

Maraganore, D.M., Harding, A.E. & Marsden, C.D. (1991) A clinical and genetic study of familial Parkinson's disease. *Mov Disord*, **6**, 205-11.

Marks, N. & Berg, M.J. (1999) Recent advances on neuronal caspases in development and neurodegeneration. *Neurochem Int*, **35**, 195-220.

Marti, M.J., James, C.J., Oo, T.F., Kelly, W.J. & Burke, R.E. (1997) Early developmental destruction of terminals in the striatal target induces apoptosis in dopamine neurons of the substantia nigra. *J Neurosci*, **17**, 2030-9.

McCarthy, J.V. & Dixit, V.M. (1998) Apoptosis induced by Drosophila reaper and grim in a human system. Attenuation by inhibitor of apoptosis proteins (cIAPs). *J Biol Chem*, **273**, 24009-15.

Ochu, E.E., Rothwell, N.J. & Waters, C.M. (1998) Caspases mediate 6-hydroxydopamine-induced apoptosis but not necrosis in PC12 cells. *J Neurochem*, **70**, 2637-40.

Paxinos G., W.C. (1986) *The Rat Brain in Stereotaxic Coordinates*., Academic Press, New York.

Polymeropoulos, M., Lavedan, C., Leroy, E., Ide, S., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W., Lazzarini, A., Duvoisin, R., Di Iorio, G., Golbe, L. & Nussbaum, R. (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*, **276**, 2045-7.

Rajput, A.H., Uitti, R.J., Stern, W. & Laverty, W. (1987) Early onset Parkinson's disease and childhood environment. *Adv Neurol*, **45**, 295-7.

Rasper, D.M., Vaillancourt, J.P., Hadano, S., Houtzager, V.M., Seiden, I., Keen, S.L., Tawa, P., Xanthoudakis, S., Nasir, J., Martindale, D., Koop, B.F., Peterson, E.P., Thornberry, N.A., Huang, J., MacPherson, D.P., Black, S.C., Hornung, F., Lenardo, M.J., Hayden, M.R., Roy, S. & Nicholson, D.W. (1998) Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. *Cell Death Differ*, **5**, 271-88.

Richardson, P.M. & Verge, V.M.K. (1989) Quantitative Radioautography for NGF Receptors. In (Rush RA, ed*Quantitative Radioautography for NGF Receptors*. John Wiley & Sons Ltd..

Rosenblad, C., Kirik, D., Devaux, B., Moffat, B., Phillips, H. & Bjorklund, A. (1999) Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or the lateral ventricle. *Eur J Neurosci*, **11**, 1554-1566.

Rosenfeld, M.A., Yoshimura, K., Trapnell, B.C., Yoneyama, K., Rosenthal, E.R., Dalemans, W., Fukayama, M., Bargon, J., Stier, L.E., Stratford-Perricaudet, L. & et al. (1992) In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell*, **68**, 143-55.

Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S. & Reed, J.C. (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *Embo J*, **16**, 6914-25.

Roy, N., Mahadevan, M.S., McLean, M., Shutler, G., Yaraghi, Z., Farahani, R., Baird, S., Besner-Johnston, A., Lefebvre, C., Kang, X. & et al. (1995) The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy [see comments]. *Cell*, **80**, 167-78.

Sauer, H. & Oertel, W.H. (1994) Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience*, **59**, 401-15.

Sauer, H., Rosenblad, C. & Bjorklund, A. (1995) Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. *Proc Natl Acad Sci U S A*, **92**, 8935-9.

Schapira, A., Mann, V., Cooper, J., Dexter, D., Daniel, S., Jenner, P., Clark, J. & Marsden, C. (1990) Anatomic and disease specificity of NADH CoQ1 reductase (complex I). *J Neurochem*, **55**, 2142-5.

Schmued, L.C., Albertson, C. & Slikker, W., Jr. (1997) Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. *Brain Res*, **751**, 37-46.

Scott, W.K., Staijich, J.M., Yamaoka, L.H., Speer, M.C., Vance, J.M., Roses, A.D. & Pericak-Vance, M.A. (1997) Genetic complexity and Parkinson's disease. Deane Laboratory Parkinson Disease Research Group [letter; comment]. *Science*, **277**, 387-8; discussion 389.

Scouten, C.W., Cegavske, C.F. & Rozboril, L. (1981) A versatile carrier for simply constructed wire knives and microsyringes. *Physiol Behav*, **26**, 1115-9.

Sriram, K., Pai, K., Boyd, M. & Ravindranath, V. (1997) Evidence for generation of oxidative stress in brain by MPTP: in vitro. *Brain Res*, **749**, 44-52.

Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G.S. & Reed, J.C. (1998) A single BIR domain of XIAP sufficient for inhibiting caspases. *J Biol Chem*, **273**, 7787-90.

Tatton, N.A. & Kish, S.J. (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**, 1037-48.

Thompson, C. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456-62.

Ungerstedt, U. & Arbuthnott, G.W. (1970) Quantitative recording of rotational behavior in rats after 6-hydroxy- dopamine lesions of the nigrostriatal dopamine system. *Brain Res*, **24**, 485-93.

Vucic, D., Kaiser, W.J. & Miller, L.K. (1998) Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by Drosophila proteins HID and GRIM. *Mol Cell Biol*, **18**, 3300-9.

Wullner, U., Kornhuber, J., Weller, M., Schulz, J.B., Loschmann, P.A., Riederer, P. & Klockgether, T. (1999) Cell death and apoptosis regulating proteins in Parkinson's disease—a cautionary note [see comments]. *Acta Neuropathol (Berl)*, **97**, 408-12.

Xu, D., Bureau, Y., McIntyre, D.C., Nicholson, D.W., Liston, P., Zhu, Y., Fong, W.G., Crocker, S.J., Korneluk, R.G. & Robertson, G.S. (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**, 5026-33.

Xu, D.G., Crocker, S.J., Doucet, J.P., St-Jean, M., Tamai, K., Hakim, A.M., Ikeda, J.E., Liston, P., Thompson, C.S., Korneluk, R.G., MacKenzie, A. & Robertson, G.S. (1997) Elevation of neuronal expression of NAIP reduces ischemic damage in the rat hippocampus. *Nat Med*, **3**, 997-1004.

Yamada, M., Oligino, T., Mata, M., Goss, J.R., Glorioso, J.C. & Fink, D.J. (1999) Herpes simplex virus vector-mediated expression of Bcl-2 prevents 6- hydroxydopamine-induced degeneration of neurons in the substantia nigra in vivo. *Proc Natl Acad Sci U S A*, **96**, 4078-83.

FIGURE LEGENDS

Figure 1. Analysis of gene expression in the substantia nigra (SNc) one week following intrastriatal adenoviral administration. Representative brightfield digital micrographs of nigral neurons processed for detection of *naip* mRNA by *in situ* hybridization histochemistry from Ad.lacZ (A) and Ad.NAIP (B) treated animals. β-Gal and Myc immunohistochemistry confirmed adenovirus-mediated protein expression in region of the SNc (C,D). Bars = 30μm (A,B) and 100 μm (C,D).

Figure 2. Enhanced expression of NAIP significantly reduces amphetamine-induced ipsiversive rotational behaviour four weeks following intrastriatal 6-OHDA administration when compared to Ad.lacZ treated animals (*, $p<0.05$). Values are reported as mean ± SEM of net ipsilateral quarter turns per minute.

Figure 3. Percent survival of nigral TH-immunoreactive neurons four weeks following intrastriatal 6-OHDA in Ad.lacZ and Ad.NAIP treated animals. When compared to the contralateral hemisphere (A), animals which had received Ad.lacZ had significantly fewer TH-positive neurons (B). In contrast, Ad.NAIP treatment resulted in an attenuation of intrastriatal 6-OHDA-induced death of dopamine neurons (C). Values are reported as mean ± SEM of data obtained from six animals (D) (*, $p<0.01$). Scale bar = 120 μm.

Figure 4. Ad.NAIP treatment reduces the loss of striatal dopaminergic nerve terminals. Representative photomicrographs of TH immunostaining in coronal brain sections from Ad.lacZ (A) and Ad.NAIP (B) treated rats four weeks following intrastriatal 6-OHDA administration. Densitometric analysis of TH-positive fibres in the dorsal striatum of lesioned (right side) compared to the contralateral (left side) revealed a significant difference in striatal TH-positive fibrous staining in the Ad.lacZ treated animals (C) ($p<0.02$). Values are reported as mean ± SEM.

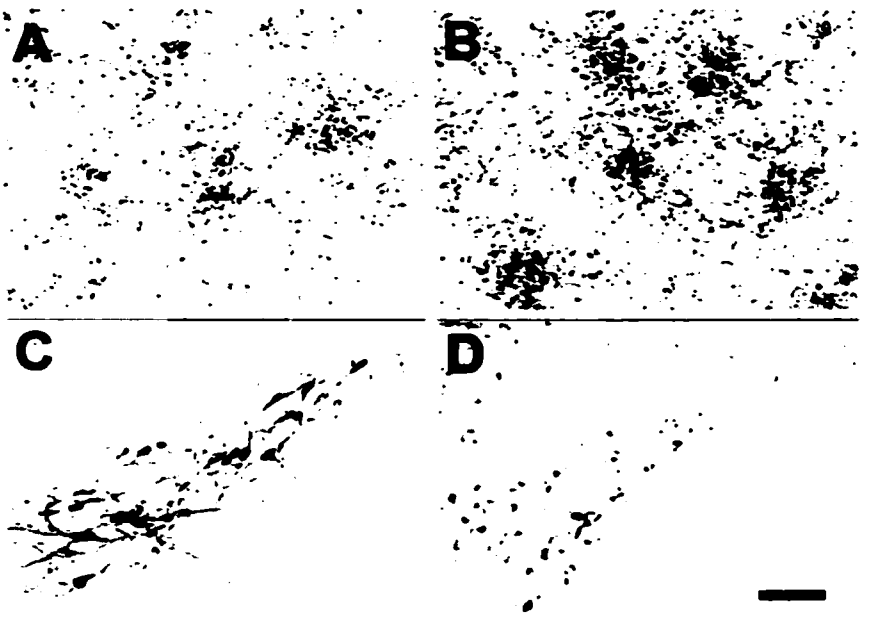
Figure 5. The delayed death of dopamine neurons following intrastriatal 6-OHDA is not accompanied by activation of caspase-3 in the adult rat. Representative sections from adult control (A,E,I),

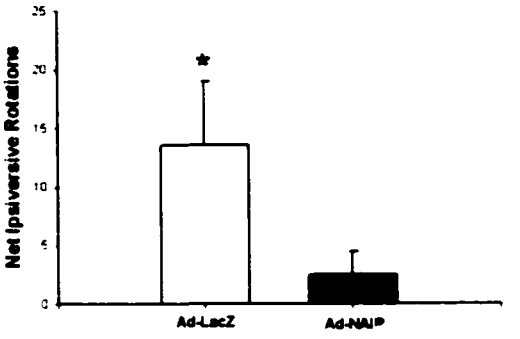
juvenile 6-OHDA lesioned (B,F,J), adult 6-OHDA lesioned (C,G,K) or an adult rat following transection of the medial forebrain bundle (MFB) (D,H,L). Tissues were analyzed for immunohistochemical detection of caspase-3 cleaved amyloid precursor protein (ΔC-APP) (A,B,C,D), DNA framentation (E,F,G,H) and the neuronal degeneration stain, FluoroJade (I,J,K,L). Evidence for caspase-3 activity (ΔC-APP) was only detected in nigral neurons of juvenile-6-OHDA lesioned and adult MFB axotomized animals 72 hours following the lesion (B,D). ISEL-positive neurons were only detected in juvenile 6-OHDA lesioned rats (F), which also displayed apoptotic morphology (inset). FluoroJade staining was detected in the area of the substantia nigra in all three lesion models at 72 hours (J), 14 days (K) or 7 days (L) post-lesion, confirming the degeneration of nigral neurons in each of these models. Bars = 200 μm for juvenile tissues (J), 150 μm for adult tissues (L), and 30 μm for insets (F,K).

Table 1. Summary of Experimental Surgeries and Endpoints

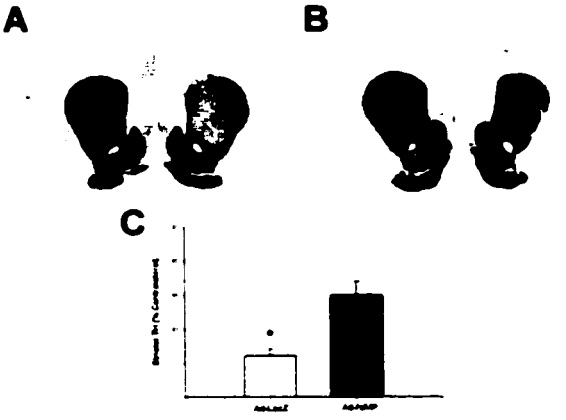
		Survival Time Post-Lesion (days)									
	Age	1	2	3	4	5	7	10	14	21	28
Adenovirus											
Ad.NAIP-6-OHDA	PND 42										•
Ad.LacZ-6-OHDA	PND 42										•
Caspase-3											
Juvenile-6-OHDA	PND 7	•	•	•	•	•	•	•	•	•	•
Adult-6-OHDA	PND 42	•	•	•	•	•	•	•	•	•	•
Adult-MFB Axotomy	PND 42	•	•	•	•	•	•	•	•	•	•

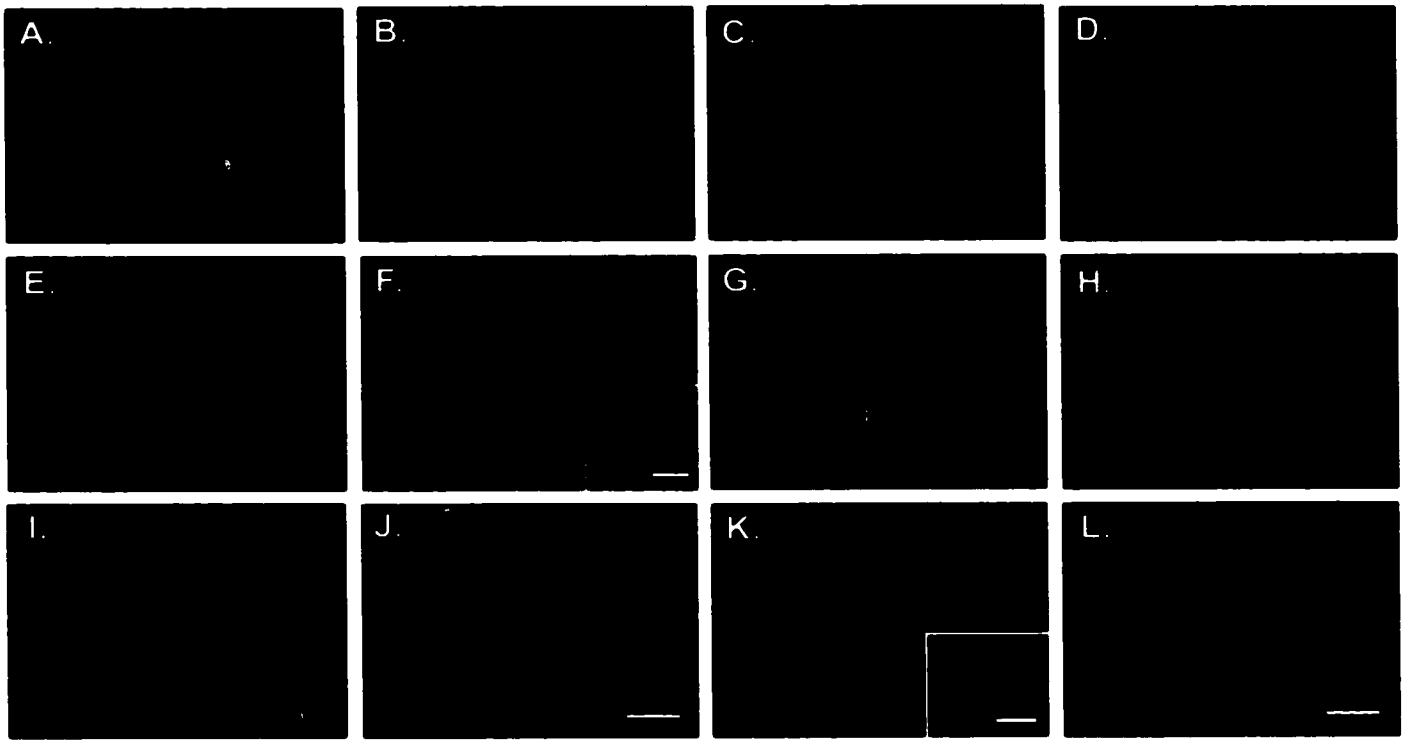
Dots indicate time of experiment endpoint. Ages are reported as postnatal day (PND).
6-OHDA, intrastriatal 6-hydroxydopamine; MFB, medial forebrain bundle.
For descriptions of each experiment refer to *Materials and Methods*.


A
B
C
D


Net Ipsiversive Rotations
25
20
15
10
0
Ad-LacZ
Ad-NAIP


A
B
C
D
*


A
B
C


A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
K.
L.

Chapter 6

Crocker et al. (2000) Mice Overexpressing XIAP are Resistant to MPTP-induced Neurotoxicity. (submitted)

This manuscript describes the generation of transgenic mice which overexpress the human X-linked inhibitor of apoptosis protien (XIAP) under the control of the neuron-specific enolase (NSE) promoter. In addition, this study demonstrated that the neurotoxic effects of the dopaminergic neurotoxin, MPTP, is blocked in this line of transgenic (NSE-*xiap*) mice.

This study was principally a combined effort of the first two authors, as denoted in the manuscript. The development of the NSE-*xiap* construct and initial breeding of the NSE-*xiap* is credited to Dr. P. Liston. Maintenance of the NSE-*xiap* mouse colony, including genotyping was performed by N. Earle (Apoptogen, Inc.). Characterization of the NSE-*xiap* mice, including western blot and immunohistochemical analysis was performed by S.J. Crocker. All analysis of MPTP neurotoxicity in this study involving immunohistochemistry was performed by S.J. Crocker. HPLC analysis of neuroamine concentrations was perfomed by Dr. H. Anisman (Carleton University, Ottawa) on tissue samples collected and prepared by S.J. Crocker. This manuscript was written by S.J. Crocker under the guidance of Dr. G.S. Robertson, with editorial assistance from Drs. D.S. Park and Dr. R.G. Korneluk.

MICE OVEREXPRESSING XIAP ARE RESISTANT TO MPTP-INDUCED NEUROTOXICITY

Stephen J. Crocker[1*], Peter Liston[3,4,*], Hymie Anisman[5], Chris J. Lee[2], Nathalie Earl[3], Charlie S. Thompson[1], David S. Park[1], Robert G. Korneluk[3,4], and George S. Robertson[2,‡]

Neuroscience Research Institute[1], and Department of Cellular and Molecular Medicine[2]

University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

[3]AEgera Therapeutics, Inc., 451 Smyth Rd., Ottawa, Ontario K1H 8L1, Canada

[4]Solange Gauthier-Karsh Molecular Genetics Laboratory,

Children's Hospital of Eastern Ontario, Ottawa, Ontario, K1H 8L1 Canada

[5]Institute of Neuroscience, Carleton University,

Ottawa, Ontario K1S 5B6, Canada

*These authors contributed equally to this study.

‡ Current address: Department of Pharmacology
Merck-Frosst Centre for Therapeutic Research
P.O. Box 1005,
Pointe-Claire – Dorval
Quebec H9R 4P8 Canada

Correspondence to: R.G. Korneluk; bob@mgcheo.med.uottawa.ca

Manuscript Information:

Words in Abstract	114	Figures	5
Words in Introduction	671	Tables	1
Characters (with spaces)	34,852	Text Pages	19

Abstract

Administration of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces the death of dopamine neurons in the substantia nigra pars compacta resulting in a Parkinsonian-like syndrome in both mice and humans. XIAP is a member of the Inhibitor of Apoptosis (IAP) gene family. Here, we describe a novel mouse model in which expression of the human XIAP gene is under the control of the neuron specific enolase promoter (NSE-*xiap*). In addition, we show that the neurodegenerative effects of the dopaminergic neurotoxin, MPTP, are significantly attenuated in NSE-*xiap* mice when compared to wildtype littermate controls. These results suggest that strategies to enhance neuronal expression of XIAP may be of therapeutic benefit in the treatment of Parkinson's disease.

Neuronal cell death is a necessary event during the normal development of the mature central nervous system[1]. Inappropriate neuronal death, however, contributes to many neurological disorders, including amyotrophic lateral sclerosis, stroke and Parkinson's disease[2]. Accumulating evidence suggests that inhibition of neuronal cell death by Inhibitor of Apoptosis (IAP) proteins can ameliorate the loss of function associated with neurodegenerative conditions, such as stroke[3,4]. However, it is not yet known whether IAP proteins can reduce dopamine neuron loss in animal models of Parkinson's disease.

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopamine neurons in the substantia nigra pars compacta (SNc)[5]. Loss of the nigral dopaminergic innervation of the striatum results in a profound impairment of movement and promotes cognitive decline[6]. L-Dopa alleviates the symptoms of PD by increasing production of dopamine in surviving neurons, but does not abate the underlying progressive degeneration of SNc neurons. Hence, therapeutic strategies that promote the survival of SNc neurons in PD may provide a more effective long-term treatment for this neurodegenerative condition. Administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a synthetic meperidine derivative, produces a clinical syndrome which resembles PD in humans, non-human primates and mice[7-9]. Because MPTP can mimic the behavioral and neuropathology deficits observed in idiopathic PD, administration of MPTP to animals is considered a useful experimental model for the study of cell death and neuroprotection in PD.

XIAP is a member of the IAP family of evolutionarily conserved genes found in insect viruses to vertebrates[10-12]. XIAP, and related mammalian IAP proteins, can potently prevent cell death induced by a wide variety of triggers[13,14]. The intracellular functions of XIAP have been associated with two structurally conserved domains; the BIR (Baculovirus IAP Repeat) and RING Zinc-finger domains (Figure 1A). The BIR domain, named for the archetypal baculoviral IAP proteins, OpIAP and CpIAP, is a 70 amino acid motif that mediates protein-protein interactions. The presence of one or more BIR domains is a defining characteristic of all IAP proteins[15]. The BIR domains of XIAP are responsible for mediating the suppression of apoptosis

by the selective inhibition of caspases 3, 7 and 9[16,17]. In addition, BIR domains may function in the induction of c-Jun N-terminal kinase (JNK1) activity[18] and in the promotion of the nuclear translocation of the transcription factor, nuclear factor kappa B (NFκB)[19].

Some of the actions of the XIAP-BIR domains also appear to be dependent upon the C-terminal RING finger domain of XIAP. For instance, truncation of the COOH-terminal RING domain of XIAP can abrogate BIR-mediated inhibition of procaspase-9[17], and eliminate the ability of XIAP to activate NFκB[19]. RING finger domains are found in a diverse array of proteins and typically mediate protein-DNA interactions[20], though at present, no XIAP-dependent transcriptional activity has been reported. However, two functions that have been attributed to XIAP-RING motif are the induction of bone morphogenic protein (BMP2/4) activity through direct interactions with TAB1 and TAK1 signaling proteins[21], and the enhancement of zinc-dependent ubiquitin-ligase activity[22].

We have previously demonstrated that *in vivo* overexpression of XIAP, and the related mammalian inhibitor of apoptosis protein, neuronal apoptosis inhibitor protein (NAIP)[23], can attenuate the loss of CA1 hippocampal neurons induced by transient forebrain ischemia[3,4]. Further, we have also shown that loss of nigral dopamine neurons induced by intrastriatal administration of the dopaminergic toxin 6-hydroxydopamine can be attenuated by overexpression of these proteins[24]. Since these previous studies were performed using viral vectors, the singular effects of enhanced neuronal expression of IAP proteins could not be distinguished from adenoviral infection of non-neuronal cell types. Hence, to investigate the effects of increased neuronal expression of XIAP on the survival of dopamine neurons following administration of MPTP, we have generated transgenic mice in which expression of *xiap* is driven by the neuron-specific enolase (NSE) promoter[25]. Here, we describe the generation and characterization of NSE-*xiap* mice and demonstrate that NSE-*xiap* mice are profoundly resistant to the injurious effects of MPTP-induced dopamine neuron degeneration.

Results

Enhanced Neuronal Expression of XIAP in NSE-XIAP mice.

Previous northern blot analysis of wild-type animals has revealed *xiap* expression in a variety of tissues, with comparatively lower expression in brain[13]. To examine the potential effects of increased neuronal expression of XIAP *in vivo*, we generated a mouse line in which the human *xiap* gene was placed under the control of the neuron specific enolase (NSE) promoter. Transmission of XIAP in successive generations of NSE-*xiap* mice was verified by Southern blot analysis (Figure 1B), while Western blotting revealed increased levels of XIAP protein in NSE-*xiap* mice (tg), relative to wild-type (wt) littermates (-) (Figure 1C).

To characterize the pattern of neuronal XIAP expression in the NSE-*xiap* mice relative to expression in wt brain, we examined XIAP expression by immunohistochemistry. The distribution of XIAP immunoreactivity in the NSE-*xiap* mice was analyzed in a variety of neuroanatomical areas and compared with the patterns of endogenous expression from wild-type littermate brain tissues. In the cerebral cortex of wt mice, endogenous XIAP expression is predominantly in layers 3 and 4 (Figure 1D). In contrast, NSE-*xiap* mice exhibited robust expression of XIAP throughout the cortical strata (Figure 1H). Similarly, expression of XIAP was more intense in pyramidal CA1 hippocampal neurons (Figure 1I) and the lateral habenular nucleus (LHb) (Figure 1J) in NSE-*xiap* mice when compared to wt brain (Figure 1 E,F). In the ventral midbrain, endogenous XIAP in wt brains was pervasive, with expression in a variety of areas, including the lateral hypothalamus (LH) and substantia nigra (SN) (Figure 1G). Consistent with enhanced XIAP expression in other neuronal populations, XIAP expression in NSE-*xiap* mice was noticeably increased in these brain areas as well (Figure 1K).

Germline disruption of caspase-3 or -9 results in profound disturbances in neuronal proliferation during development and only survive for short postnatal periods[26,27]. Since XIAP is a potent endogenous inhibitor of caspases 3, 7 and 9[10], we examined whether the NSE-*xiap* mice exhibited a similar neuronal phenotype to caspase-null mice. First, NSE-*xiap* mice did not display any gross phenotypic abnormalities which were observed in both the caspase-3 and caspase-9 null mice. Second, litters from NSE-*xiap* mice exhibited predicted Mendelian

proportions. Furthermore, a survey of independent neuron populations in the NSE-*xiap* brain revealed no increases in the density of neurons in cortical, CA1 hippocampal (data not shown), or substantia nigra populations compared to wildtype mice (Table 1).

The observation that neuron-specific expression of XIAP did not elicit any overt morphological abnormality in NSE-*xiap* mice, in spite of the role of this protein as a caspase-inhibitor, is possibly due to the late developmental stage (E12.5) at which the NSE promoter induces gene expression in the brain[28]. At this stage, potential critical control of developmental neuronal death has past (E8-E11.5)[1]. These findings are consistent with the reports of others that have used the NSE promoter to drive expression of cell death regulating proteins[29-32].

MPTP-induced degeneration of nigral dopamine neurons is blocked in* NSE-XIAP *mice

Immunohistochemical detection of tyrosine hydroxylase (TH) has been shown to be a sensitive and reliable marker for the dopaminergic neuron phenotype in animal models of PD[33]. Initially, in order to determine whether there were comparable numbers of dopamine neurons in NSE-*xiap* mice and their wild-type littermates, we surveyed the number of TH positive neurons in the SNc of both groups. Counts of estimated total nigral neurons established that there was no significant difference between saline-treated NSE-*xiap* mice or comparably treated wild-type littermates (Figure 2 A,B; Table 1). Administration of MPTP (80 mg/kg, i.p. cumulative) resulted in a profound (45%) loss of TH positive neurons in the SNc of wild-type mice (Figure 2C; Table 1). In contrast, mice that overexpressed XIAP showed no deficit in the number of nigral dopamine neurons following MPTP treatment (Figure 2D; Table 1).

***NSE-xiap* mice are protected from MPTP-induced striatal denervation.**

Since no appreciable loss of nigral dopamine neurons was observed in MPTP-treated NSE-*xiap* mice, we examined the effects of XIAP on dopamine nerve terminals in the striatum. Immunohistochemical analysis of TH+ terminal density in the striatum revealed no differences in the proportion of fibrous labeling in saline-treated wild-type mice when compared to NSE-*xiap*

mice (Figure 3A,B). Following MPTP, wild-type mice exhibited a significant (62%) loss in the proportion of TH+ terminal labeling in the dorsal striatum (Figure 3C), whereas comparably treated NSE-*xiap* mice displayed a modest (35%) reduction in the density of tyrosine hydroxylase positive terminal labeling (Figure 3D). This difference represented a doubling of the striatal terminal fibre density in MPTP-treated NSE-*xiap* mice when compared to MPTP-treated wild-type littermates.

To evaluate the protective effects of XIAP on nigrostriatal dopaminergic function, we also assessed levels of dopamine and its metabolites in NSE-*xiap* mice with wild-type (littermate) animals. In saline-treated NSE-*xiap* or wild-type mice, the concentrations of striatal dopamine (DA), and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were not significantly different (Figure 4). These results support the notion that NSE-*xiap* mice did not have altered dopaminergic physiology and likely owe reduced vulnerability to MPTP to enhanced neuronal expression of XIAP. However, administration of MPTP resulted in comparable reductions in levels of striatal DA in both NSE-*xiap* and wild-type littermate mice (Figure 4). Similarly, MPTP-induced depletion in striatal levels of the dopamine metabolite, DOPAC, which were significantly greater in MPTP-treated wt mice than NSE-*xiap* mice (73% vs. 55% depletion, respectively). No notable differences in HVA levels were detected in any group tested (Figure 4).

Increasing evidence suggests that partial lesions of the nigrostriatal dopamine pathway evoke compensatory changes in neurons spared by the injury[34,35]. Adaptive changes in dopamine synthesis and release following dopaminergic lesions is reflected in increased levels of DA metabolites relative to levels of striatal dopamine[36,37]. To address whether nigral dopamine neurons in NSE-*xiap* exhibit compensatory changes in dopamine turnover, and hence striatal dopaminergic function, we also analyzed the ratio of dopamine to its principal metabolite, DOPAC. Saline treated mice revealed DOPAC:DA ratios of 0.193 and 0.207 for NSE-*xiap* and wild-type littermates, respectively, while MPTP treatment significantly decreased this ratio to 0.075 in wild-type mice. Interestingly, NSE-*xiap* mice exhibited a trend toward normalization of dopamine metabolism by this measure, with a DOPAC:DA ratio of 0.126. These results suggest

that while MPTP induced a significant depletion in striatal dopamine levels in NSE-*xiap* mice, the functionality of the nigrostriatal dopamine pathway in NSE-*xiap* mice was modestly preserved. Taken together, these results suggest that enhanced neuronal expression of XIAP significantly diminished the impact of MPTP-induced neurotoxicity.

Nigral glial response following MPTP administration is attenuated in *NSE-xiap* mice

Accumulating evidence implicates reactive gliosis in the pathogenesis of PD and MPTP-induced dopamine neurotoxicity[38-40]. If gliosis is a generalized response to MPTP intoxication, increased GFAP may be expected in MPTP-treated NSE-*xiap* mice. To ascertain whether XIAP-mediated neuroprotection is also accompanied by astroglial activation, we performed immunohistochemistry for GFAP on nigral tissues from NSE-*xiap* and wt mice following MPTP administration. Consistent with previous reports by others, MPTP induced a dramatic elevation in GFAP-immunoreactivity which was pronounced in the region of the substantia nigra (Figure 5A, inset). In contrast, MPTP did not induce an astrocytic response in NSE-*xiap* mice (Figure 5B). GFAP immunoreactivity in saline treated mice was not notably different from NSE-*xiap* mice (data not shown).

Discussion

We provide the first description of transgenic mice that overexpresses a human IAP protein in neurons. Despite the role of XIAP as a potent endogenous inhibitor of caspase-3, NSE-*xiap* mice do not display any overt neurological phenotype, as reported in mice deficient in caspase-3[26]. A survey of neuronal populations in several discrete areas of the mature mouse brain did not reveal any differences between wildtype and NSE-*xiap* mice, indicating that elevated expression of XIAP controlled by the NSE promoter did not influence CNS development. Indeed, similar results have been reported for other cell death regulating proteins in strains of NSE-transgenic mice[29,31,32]. This observation was likely due to the onset of NSE driven expression

in the developing nervous system[28], which temporally follows the attrition of the majority of neurons in the developing central nervous system[1].

Increased expression of XIAP can prevent cell death induced by a myriad of factors, including, TNF-α, menadione and caspases[10,14]. Enhanced neuronal expression of IAP proteins, including XIAP, can also prevent neuronal death induced by transient cerebral ischemia in rats[3,4] and delay neuronal death following excitotoxic injury *in vitro*[41,42] as well as axotomy *in vivo*[41]. Accumulating evidence suggests that caspase-3 may be a principal mediator of DA cell death in PD[43-45]. However, direct evidence for a causal role of caspase-3 in an *in vivo* MPTP neurodegenerative model is presently lacking. Given the established function of IAP proteins, and XIAP in particular, as potent inhibitors of caspases, the results of the present study may imply a caspase component in the demise of dopamine neurons in this MPTP model. However, we have recently reported an absence of caspase-3 involvement in an adult 6-OHDA model of PD[24]. Moreover, we have been unable to detect active caspase-3 in nigral dopamine neurons following MPTP administration in mice (S.J. Crocker, G.S. Robertson and D.S. Park, unpublished observations), using established methods for detecting activated caspase-3 in other neurodegenerative models[4,46]. Mechanisms of IAP-mediated neuroprotection, independent of caspase inhibition, have been recently reported[47]. Hence, the neuroprotection against MPTP toxicity in NSE-*xiap* mice, together with an absence of detectable caspase-3 activity in this MPTP model, suggest that XIAP may have multiple cell death regulating properties.

The ability of XIAP to prevent MPTP-induced loss of nigral dopamine neurons is associated with a histological protection of striatal dopamine fibres but not represented by a preservation of dopamine levels in the striatum. This result is intriguing since the absolute amounts of dopamine in NSE-*xiap* are diminished following MPTP administration but the ratios of dopamine to its metabolite, DOPAC, revealed compensatory changes in striatal dopamine metabolism. This disparity may also reflect hyperactivity and dysfunction of the nigrostriatal dopamine pathway in NSE-*xiap* mice resulting from MPTP toxicity[34,35]. Alternatively, XIAP expression may afford cellular protection but may also require a prolonged period of

convalescence to regain normal levels of function. In fact, several reports by others have also documented a dissociation between nigral dopamine neuron survival and striatal dopamine[38,48], suggesting that protection of nigral neurons may not be sufficient to attenuate MPTP-induced striatal dysfunction.

The possibility that reactive astrocytes are responsible for MPTP-induced neurotoxicity is supported by the finding that the neurotoxic effects of MPTP are mediated by the conversion of MPTP to its active metabolite, MPP+, in astrocytes[49]. MPP+ is then actively transported into dopamine neurons where it interrupts complex I of the mitochondrial respiration chain, precipitating the formation of cytotoxic reactive oxygen species[50,51]. Recently, several studies have implicated microglial nitric oxide synthase (NOS) as a principal mediator of MPTP-neurotoxicity[38]. In these studies, inhibition of NOS with pharmacological antagonists, or employing mice deficient in NOS enzymes (*nos*$^{-/-}$), results in reduced nigral neuron death and an absence of MPTP-induced reactive gliosis. Interestingly, comparable attenuation of GFAP staining was also observed in MPTP-treated NSE-*xiap* mice. Conceivably, the neuroprotective actions of XIAP in this MPTP-model of PD may involve direct (or indirect) interaction of XIAP with intraneuronal NOS signaling molecule(s), such as NFκB[52]. These possibilities warrant further investigation.

In summary, we have generated and characterized a novel mouse strain in which expression of the human inhibitor of apoptosis gene, XIAP, is driven by the neuron-specific enolase promoter. In addition, we demonstrate that NSE-*xiap* mice exhibit a profound resistance to the neurotoxic effects of MPTP. Taken together, these results suggest that neuroprotective strategies based on the enhancement of XIAP expression may have therapeutic potential for the treatment of Parkinson's disease.

Experimental Procedures

Generation of NSE-*xiap* mice

The cDNA sequences corresponding to the XIAP ORF were amplified by PCR using primers to add BamH1 and Xho1 sites to the 5' and 3' end respectively, cloned and sequenced in their entirety. The *xiap* sequence was then excised and blunt end ligated into the pNSE plasmid[28]. Insert orientation was confirmed by sequencing. NSE-*xiap* plasmids were microinjected into C57Bl/6 ES cells by electroporation and injected into donor blastocysts which were implanted into pseudopregnant foster mothers to generate founders as described[53]. Litters were screened for transmission of the xiap transgene by Southern blot (Figure 1A) and confirmed by PCR analysis (data not shown). Hemizygous transgenic male xiap mice, denoted as Line 727, were used for this study while male wild-type littermates were used as controls.

Western Blot Analysis

Brains from wild-type and *NSE-XIAP* mice were removed on ice and cerebellum, cortex, forebrain and ventral midbrain were dissected. Tissues were dounce homogenized in buffer (0.3M sucrose, 1mM EGTA, 25mM NaCl, 15 mM Tris-HCl (pH 6.8) containing 1 mM PMSF, 50 μg/μl leupeptin and 10μg/μl aprotinin), and then centrifuged at 8000 g for 20 seconds. Pellets were resuspended in buffer, sonicated and centrifuged (8000g/ 20sec). Supernatants were removed and then centrifuged at 13000 g for 10 minutes at 4°C. Protein concentrations were estimated using BioRad reagent, 20 μg of protein from each sample was loaded into a 10% gradient SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with antibodies directed against human XIAP (1:1000)[54] or β-actin (1:3000; Sigma) (Figure 1B).

MPTP Treatment

Male C57Bl/6 NSE-*xaip* mice 8-12 weeks of age were used for this study. Mice were administered MPTP (40 mg/kg in 0.9% saline, i.p.; Sigma) on two consecutive days (80 mg/kg, cumulative)[55]. Additional groups of mice were administered equivalent volumes of sterile saline

(0.9%, i.p.). Following MPTP administration, gross body weights for every animal were monitored throughout the course of the study. This was performed to confirm the health of each subject and ensure that poor health did not confound the experimental outcome. Any animal which lost more than 15% initial body weight was excluded from the study. There were no measurable differences in body weight between any of the treatment groups or genotypes during the course of this study (data not shown).

Immunohistochemistry

For the analysis of CNS XIAP expression, wild-type and NSE-*xiap* mice were perfused with saline (0.9%) followed by 4% paraformaldehyde (in 0.1M phosphate buffer) and brain tissues postfixed in the same fixative overnight at 4°C. Tissues were then cyroprotected using phosphate buffer containing 10% sucrose. Free floating coronal cryostat sections (20 μm thick) were collected. An antibody generated against XIAP (1:1000), described previously[54], was then used to detect endogenous and NSE-driven XIAP expression in the murine brain (Figure 1 C-J). Immunoreactivity was visualized using a glucose-oxidase-diaminobenzidine reaction[3]. Incubation of tissues with only secondary antibodies resulted in a loss of immunoreactivity.

Two weeks following the last injection MPTP, all animals were perfused with 10 ml of saline followed by an equivalent volume of paraformaldehyde (4% in 0.1M phosphate buffer). Brains were postfixed overnight at 4°C in the same fixative and then cryoprotected using phosphate buffer with 10% sucrose. Coronal cryostat sections (20 μm thick) were collected from forebrain and midbrain regions. Specifically, +1.1 to +0.38 mm rostral, and –2.7 to –3.88 mm caudal from bregma, respectively, according to Franklin and Paxinos[56]. Dopaminergic neuron cell bodies (SNc) and striatal fibrous staining were detected using a monoclonal antibody for tyrosine hydroxylase (TH, 1:1000; IncStar), the rate limiting enzyme required for the synthesis of dopamine.

Neuroamine Analysis

NSE-xiap or wild-type littermate mice were treated with either MPTP (40 mg/kg/day for 2 consecutive days, i.p.) or saline (equivalent volume) (n = 4-6 per group) and decapitated two weeks after the second injection. Brains were removed with the caudal aspects of the striata quickly extracted over ice and then rapidly frozen in isopentane cooled over dry ice. Tissues were placed in 0.5 ml homogenizing solution (5 ml methanol with 500 ml water which contained 14.17 g monocholoracetic acid and 0.018 g EDTA) and stored at -80°C until analysis. Striatal concentrations of dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured using high performance liquid chromatography (HPLC) with electrochemical detection as described previously[57].

Morphological Analyses

Digital photomicrographs were captured on a Zeiss Axioskop using a Sony 3-CCD camera and Northern Eclipse software (Empix Imaging Inc.). Striatal densitometry was performed using brightfield light microscopy (20x objective magnification) and a macro subroutine for Northern Eclipse (courtesy N.Banner, Empix Imaging). Briefly, an area of the cerebral cortex from each forebrain section was converted to grayscale (256 shades) and a threshold value for background staining was established. An "average gray" for positive immunolabeling in the striatum was then used to determine the number of pixels in the field of view equal to or greater than the threshold value in the dorsal striatum[58,59]. The number of positive pixels in the striatum was then used to establish the proportion and area covered (μm^2) by immunoreactive terminals. Equivalent sections from each experimental group were developed by immunohistochemistry in parallel, and threshold (average gray) values for each analyzed section did not differ among sections or experimental groups. Six striatal sections per animal (from levels 1.1 to 0.38mm from bregma) were analyzed (n=5 per group) by an individual unaware of the experimental treatments.

The number of nigral dopamine neurons on one side in the substantia nigra was estimated by counting the total number of TH-positive neurons in at least 12 sections distributed along the rostral-caudal axis of the substantia nigra. Since, in this instance, the average neuron

diameter was very similar to the section thickness, the total number of neurons per section could be used to accurately estimate the SN (A9) population according to Abercrombie's correction [60] as described by others[61,62].

Statistical Analysis

Quantitative data are expressed as mean ± standard error. Data were analysed by two-way ANOVA, and when significant, Newman-Keuls post-hoc analyses where performed to compare data set means. Differences were considered significant when $P < 0.05$.

Acknowledgements

We thank A. Adeeko and Y. Zhu for expert technical assistance. S.J.C. was supported by an Ontario Neurotrauma Fellowship. P.L. was supported by a Medical Research Council (MRC) of Canada Centennial Fellowship. R.G.K. is a Howard Hughes Medical Institute International Research Scholar and MRC of Canada Senior Scientist. D.S.P. is an MRC of Canada Scholar. This work was supported by grants from the Medical Research Council of Canada (G.S.R., R.G.K.) and the Ontario Neurotrauma Foundation (G.S.R., D.S.P.).

Figure 1. **Neuronal expression of XIAP in NSE-*xiap* mice.** (A) Schematic representation of the *xiap* protein. Structural domains are indicated by hatched or clear boxes, for BIR and RING finger domains, respectively, with amino acid locations denoted. (B) Transmission of the XIAP transgene was determined by Southern blot analysis. EcoR1 and BamH1 digestion of the targeting vector (+) produced a 400kb fragment as predicted, which was not detected when DNA was not added to reaction buffers (-). Representative DNA samples from NSE-*xiap* mouse line 727 in which a transgenic (tg) exhibits presence of the transgene but a wildtype (wt) littermate does not. (C) Western blot analysis revealed elevated levels of XIAP protein expression in extracts from ventral midbrain region of NSE-*xiap* (tg) or wildtype littermate (wt). Immunohistochemical detection of XIAP in brain tissue sections indicated low levels of this proteins in wt mice in a variety of neuronal populations, including layers III and IV of the cerebral cortex (D), CA1 pyramidal neurons of the hippocampus (E), the lateral habenular nucleus of the thalamus (F) and the ventral midbrain, including the substantia nigra (SN) and lateral hypothalamus (LH) (G). Higher levels of XIAP expression were detected in corresponding regions of NSE-*xiap* mice (H-K). Scale bar = 500 μm (D,G,H,K) and 200μm (E,F,I,J).

Figure 2. **MPTP-induced loss of nigral dopamine neurons is blocked in NSE-*xiap* mice.** Representative photomicrographs of tyrosine hydroxylase immunoreactivity in midbrain sections from wildtype (A,C) or NSE-*xiap* (B,D) mice 2 weeks following administration of saline (A,B) or MPTP (80 mg/kg, s.c. cumulative) (C,D). Scale bar = 150 μm.

Figure 3. **Striatal dopamine fibres are preserved following MPTP in NSE-*xiap* mice.** No difference in striatal TH^+ fibre density was observed between saline treated wt or NSE-*xiap* animals (A,B). TH immunohistochemistry indicated that MPTP-reduced striatal fiber labeling in the striatum of wt (62 ± 1.6%) (C) but was attenuated in NSE-*xiap* mice (35 ± 7.0%) (D). Scale bar = 500μm.

Figure 4. **MPTP-induced depletion of striatal dopamine is not mitigated in NSE-*xiap* mice.** HPLC analysis of striatal dopamine, and its metabolites, DOPAC, and HVA, was performed on striatal extracts from wild-type (black bars) or NSE-*xiap* mice (hatched bars) two weeks following saline or MPTP treatment (40 mg/kg/day, s.c. for two consecutive days; 80 mg/kg cumulative dose). No differences in striatal dopamine content or metabolism were observed in saline treated NSE-*xiap* mice (n=3) or wildtype littermates (n=3) ($p<0.31$). MPTP treatment produced reductions in levels of striatal dopamine, and DOPAC concentrations in NSE-*xiap* and wiltype mice. All animals were of C57Bl/6 background. ANOVA, $p<0.03$ for DA and DOPAC, $p<0.88$ for HVA. $^{*}p<0.05$; $^{**}p<0.01$ compared to controls.

Figure 5. **MPTP-induced reactive astrocytic response is absent in NSE-*xiap* mice.** Glial-acidic fibrillary protein (GFAP) immunoreactivity in the region of the SNc from wild-type (A) or *NSE-xiap* mice (B) 14 days following MPTP administration. Higher magnification of cells in the vicinity of the pars compacta (insets) reveals significant attenuation in nigral astrocytic response to MPTP treatment in NSE-*xiap* mice. Scale bar = 150 μm (40 μm for insets).

References

1. Oppenheim, R.W. *Annu. Rev. Neurosci.* **14**, 453-501 (1991).
2. Thompson, C. *Science* **267**, 1456-62 (1995).
3. Xu, D.G., *et al. Nat Med* **3**, 997-1004 (1997).
4. Xu, D., *et al. J Neurosci* **19**, 5026-33 (1999).
5. Hirsch, E., Graybiel, A.M. & Agid, Y.A. *Nature* **334**, 345-8 (1988).
6. Huber, S.J., Paulson, G.W. & Shuttleworth, E.C. *J. Neurol. Neurosurg. Psychiatry* **51**, 855-8 (1988).
7. Langston, J.W., Ballard, P., Tetrud, J.W. & Irwin, I. *Science* **219**, 979-80 (1983).
8. Heikkila, R.E., Hess, A. & Duvoisin, R.C. *Science* **224**, 1451-3 (1984).
9. Eldridge, R. & Rocca, W.A. *N Engl J Med* **313**, 1159-60 (1985).
10. Deveraux, Q.L. & Reed, J.C. *Genes Dev* **13**, 239-52 (1999).
11. Huang, Q., *et al. Proc. Natl. Acad. Sci.* **97**, 1427-1432 (2000).
12. Miller, L.K. *Trends Cell Biol* **9**, 323-328 (1999).
13. Liston, P., *et al. Nature* **379**, 349-53 (1996).
14. LaCasse, E.C., Baird, S., Korneluk, R.G. & MacKenzie, A.E. *Oncogene* **17**, 3247-59 (1998).
15. Uren, A.G., Coulsen, E.J. & Vaux, D.L. *Trends Biochem Sci* **23**, 159-162 (1998).
16. Deveraux, Q.L., Takahashi, R., Salvesen, G.S. & Reed, J.C. *Nature* **388**, 300-4 (1997).
17. Deveraux, Q.L., *et al. Embo J* **18**, 5242-5251 (1999).
18. Sanna, M.G., Duckett, C.S., Richter, W.M., Thompson, C.B. & Ulevitch, R.J. *Proc. Natl. Acad. Sci.* **95**, 6015-6020 (1998).
19. Hofer-Warbinek, R., *et al. J Biol. Chem.* (2000).
20. Borden, K.L. & Freemont, P.S. *Curr Opin Struct Biol* **6**, 395-401 (1996).
21. Yamaguchi, K., *et al. EMBO J.* **18**, 179-187 (1999).
22. Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M. & Ashwell, J.D. *Science* **288**, 874-877 (2000).
23. Roy, N., *et al. Cell* **80**, 167-78 (1995).

24. Crocker, S., *et al. (submitted)* .

25. Schmecel, D.E. & Marangos, P.J. in *Current Methods in Cellular Neurobiology* (eds. Barker, J. & McKelvey, J.) 1-62 (J.W. Wiley and Sons, New York, 1983).

26. Kuida, K., *et al. Nature* **384**, 368-72 (1996).

27. Kuida, K., *et al. Cell* **94**, 325-337. (1998).

28. Forss-Petter, S., *et al. Neuron* **5**, 187-197 (1990).

29. Bernard, R., Dieni, S., Rees, S. & Bernard, O. *J. Neurosci. Res.* **52**, 247-259 (1998).

30. Martinou, J.C., *et al. Neuron* **13**, 1017-1030 (1994).

31. Offen, D., *et al. Proc Natl Acad Sci U S A* **95**, 5789-94 (1998).

32. Viswanath, V., *et al. Proc. Natl. Acad. Sci. USA* **97**, 2270-2275 (2000).

33. Jackson-Lewis, V., Jakowec, M., Burke, R.E. & Przedborski, S. *Neurodegeneration* **4**, 257-69 (1995).

34. Agid, Y., Javoy, F. & Glowinski, J. *Nature* **245**, 15-16 (1973).

35. Zigmond, M.J. & Hastings, T.G. *Adv. Pharmacol* **42**, 788-92 (1998).

36. Hefti, F., Melamed, E. & Wurtman, R.J. *Brain Res.* **195**, 95-122 (1980).

37. Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K. & Seiterberger, F. *J. Neurol. Sci.* **20**, 415-455 (1973).

38. Liberatore, G.T., *et al. Nat Med* **5**, 1403-9 (1999).

39. Phillips, P. *JAMA* **282**, 929-931 (1999).

40. Reinhard, J.F., Jr., Miller, D.B. & JP, O.C. *Neurosci Lett* **95**, 246-51 (1988).

41. Perrelet, D., *et al. Eur. J. Neurosci.* **12**, 2059-2067 (2000).

42. Simons, M., *et al. J. Neurochem* **72**, 292-301 (1999).

43. Dodel, R.C., *et al. Neuroscience* **86**, 701-7 (1998).

44. Dodel, R.C., *et al. Brain Res Mol Brain Res* **64**, 141-8 (1999).

45. Hartmann, A., *et al. Proc. Natl. Acad. Sci.* **97**, 2875-2880 (2000).

46. Gervais, F.G., *et al. Cell* **97**, 395-406 (1999).

47. Mercer, E.A., *et al. EMBO J.* **19**, 1-11 (2000).

48. Masliah, E., *et al. Science* **287**, 1265-1269 (2000).

49. Ransom, B.R., Kunis, D.M., Irwin, I. & Langston, J.W. *Neurosci Lett* **75**, 323-8 (1987).

50. Nicklas, W.J., Vyas, I. & Heikkila, R.E. *Life Sci* **36**, 2503-8 (1985).

51. Mizuno, Y., Suzuki, K., Sone, N. & Saitoh, T. *Neurosci Lett* **91**, 349-53 (1988).

52. Hunot, S., *et al. Proc Natl Acad Sci U S A* **94**, 7531-6 (1997).

53. Hogan, B., Beddington, R., Constantini, F. & Lacy, E. *Manipulating the Mouse Embryo: A Laboratory Manual.* (Cold Spring Harbour Laboratory, Cold Spring Harbour, New York, 1994).

54. Li, J., *et al. Endocrinology* **139**, 1321-1328 (1998).

55. Tomac, A., *et al. Nature* **373**, 335-339 (1995).

56. Franklin, K.B.J. & Paxinos, G. *The Mouse Brain in Stereotaxic Coordinates* (Academic Press, Toronto, 1997).

57. Hayley, S., Brebner, K., Lacosta, S., Merali, Z. & Anisman, H. *J. Neurosci* **19**, 5654-5665 (1999).

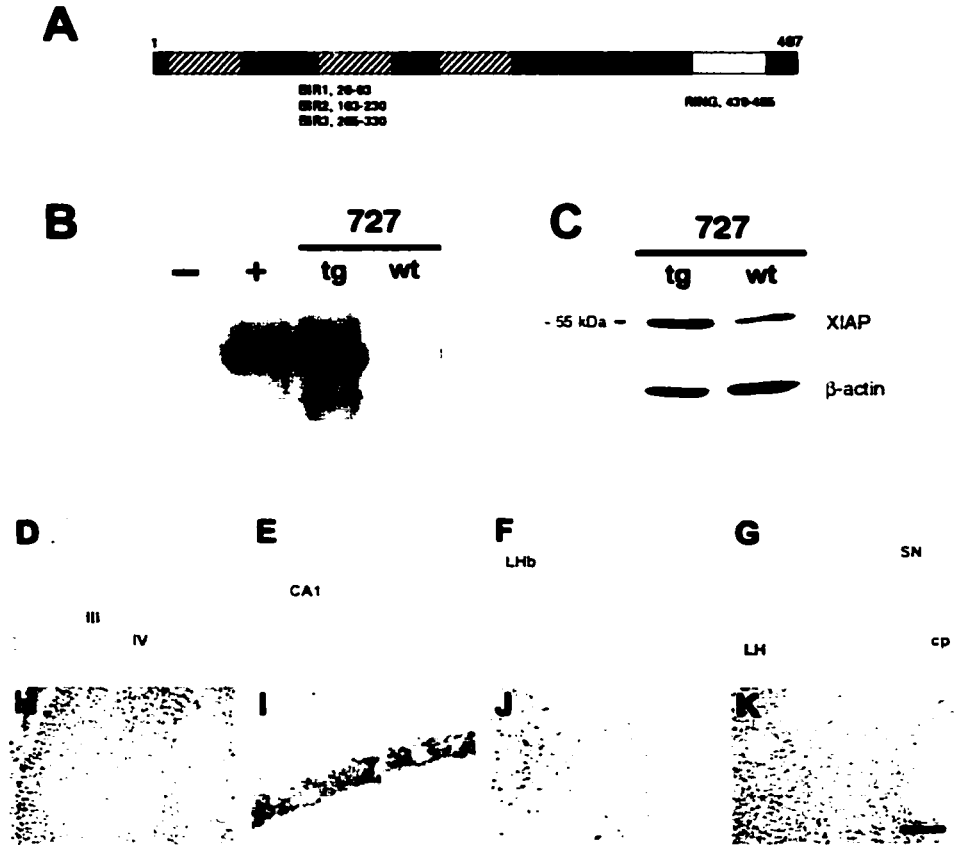
58. Gash, D.M., *et al. Nature* **380**, 252-255. (1996).

59. Brooks, A.I., Chadwick, C.A., Gelbard, H.A., Cory-Slechta, D.A. & Federoff, H.J. *Brain Res.* **823**, 1-10 (2000).

60. Abercrombie, M. *Anat. Rec.* **94**, 239-247 (1946).

61. Hagg, T. & Varon, S. *Proc Natl Acad Sci U S A* **90**, 6315-9 (1993).

62. Tseng, J.L., Baetge, E.E., Zurn, A.D. & Aebischer, P. *J. Neurosci* **17**, 325-333 (1997).


A
1
487
BIR1, 26-93
BIR2, 160-230
BIR3, 265-330
RING, 439-485
B
727
–
+
tg
wt
C
727
tg
wt
- 55 kDa –
XIAP
β-actin
D
III
IV
E
CA1
F
LHb
G
SN
LH
cp
H
I
J
K

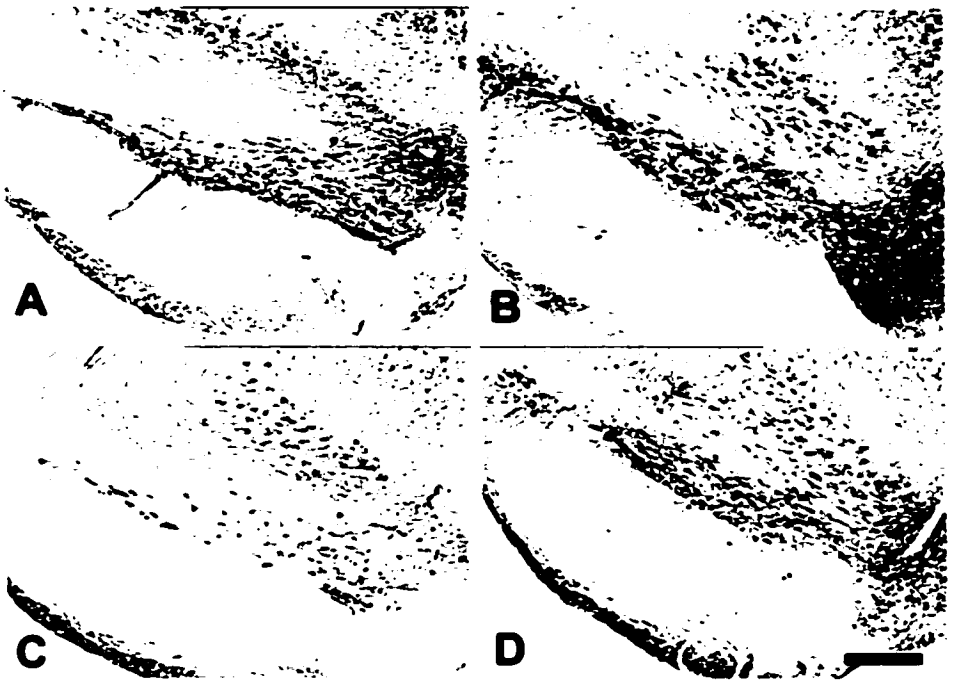
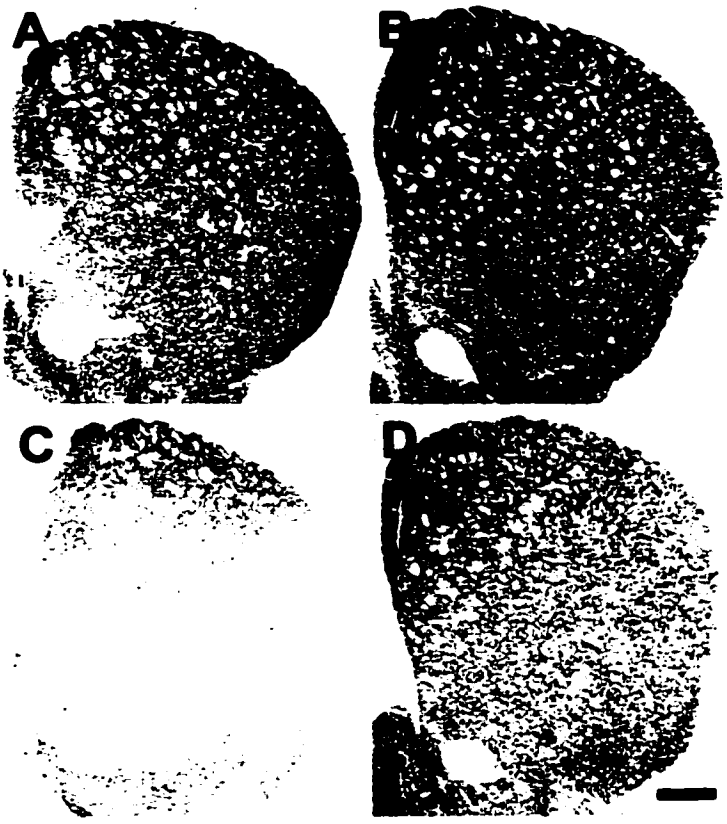
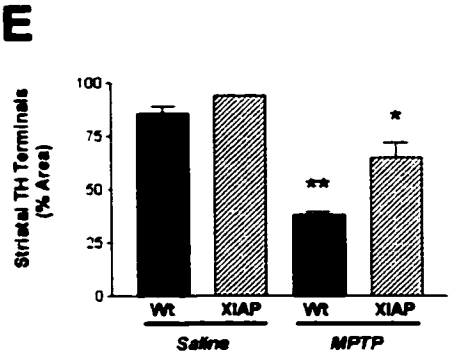

A
B
C
D

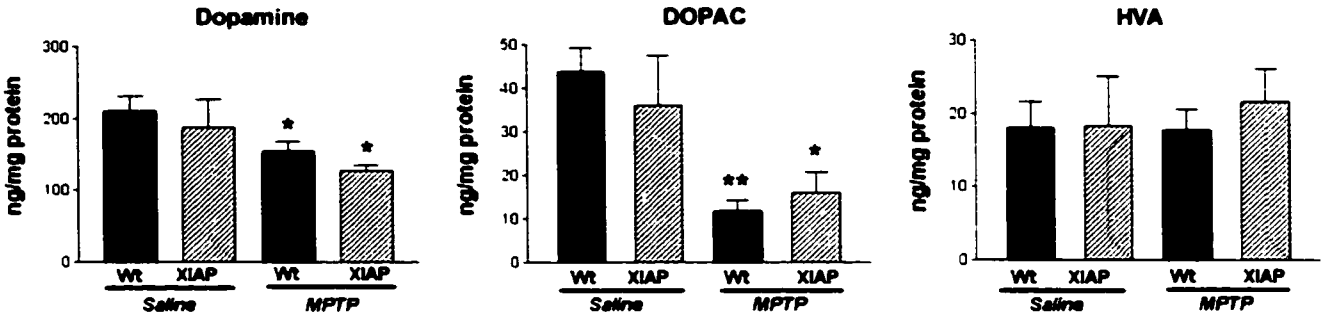
Table 1. Quantification of TH+ Nigral Neuron Populations[a]

Treatment	Genotype	
	Wildtype	NSE-*xiap*
Saline	7081.3 ± 703.8	6016.1 ± 483.8
MPTP	3902.5 ± 604[b]	6363.6 ± 335.5

[a] mean ± S.E.M., n=5-6 per group
[b] statistically significant, P<0.02


A
B
C
D


E
Striatal TH Terminals (% Area)
100
75
50
25
0
**
*
Wt
XIAP
Wt
XIAP
Saline
MPTP


Dopamine
ng/mg protein
300
200
100
0
*
*
Wt
XIAP
Wt
XIAP
Saline
MPTP
DOPAC
ng/mg protein
50
40
30
20
10
0
**
*
Wt
XIAP
Wt
XIAP
Saline
MPTP
HVA
ng/mg protein
30
20
10
0
Wt
XIAP
Wt
XIAP
Saline
MPTP


A
B

Chapter 7. Summary

Implications of Reported Findings

7.0 *Summary*

The content of this thesis was divided into two distinct areas of therapeutic value for the treatment of Parkinson's disease. First, postsynaptic changes in gene expression in the striatum resulting from dopaminergic denervation, including increased expression of FosB/ΔFosB, facilitate the development of dyskinetic complications associated with chronic, phasic dopamine replacement therapy. Secondly, degeneration of nigral dopamine neurons in animal models of PD can be blocked by enhanced neuronal expression of Inhibitor of Apoptosis (IAP) proteins. Since the results of each of the studies encorporated into this thesis have been discussed in each of the respective chapters, the following segment will expand upon these experimental findings and convey the potential therapeutic implications of this work.

7.1 *FosB and Neural Plasticity*

The observation that antisense-mediated knockdown of dopamine agonist-induced FosB expression can result in modified behaviour, suggests that plastic postsynaptic changes in the denervated striatum are mediated, in part, by FosB-mediated gene expression. In support of this proposal are several recent studies demonstrating that alterations in FosB function can produce profound changes in responsiveness to dopaminergic stimulation. For instance, Hiroi et al., (1997) describe upregulation of chronic FRAs and ΔFosB in mice treated with cocaine, in much the same way that Doucet et al., (1996) observed increased expression of ΔFosB following DA agonist treatment in the denervated striatum of rats and primates. However, Hiroi et al., (1997) also tested the impact of cocaine and locomotor activity in mice which lack *fosB* (Brown et al., 1996) and observed that *fosB-/-* mice exhibited exaggerated responses to cocaine, but also failed to become sensitized to repeated administration. More recently, Kelz et al., (2000) reported that the use of an inducible transgenic model to enhance neuronal expression of ΔFosB in the nucleus accumbens promotes sensitization to chronic cocaine administration. That knock-out of *fosB* or overexpression of ΔFosB can alter neural adaptation to chronic stimulations supports a model of neuronal plasticity involving FosB-mediated gene transcription.

Central to the hypothesis that FosB is a mediator of long-term adaptation in brain lies the question of which genes are regulated by FosB? Interestingly, Nestler and collegues have reported FosB associated changes in glutamate receptor subunits, namely GluR2 (Kelz et al., 2000) and NMDAR1 (Hiroi et al.,

1998). These findings suggest that FosB could be implemented as a tool to elucidate those downstream genes critical for neural adaptations. For instance, one could examine the changes induced by overexpression of FosB, or conversely, study the differential induction of genes in neurons lacking FosB (*fosB-/-*). A combinatorial approach, such as adapting recent gene chip technologies could be used to address this issue. Alternatively, Berke et al. (1998) employed differential display PCR techniques to examine the effects of dopamine agonist administration on gene expression in the denervated striatum, and reported induction of 30 genes by the D1 receptor agonist SKF 38393. In support of the results reported above in Chapter 4, FosB was among the genes induced. Though the report of Burke and collegues (1998) illustrates the feasibility of a combinatorial approach to study striatal gene expression in PD, this study also stresses the potential drawback of interpreting diverse changes in gene expression without a detailed of the functional changes of these changes in gene expression.

Further studies on the regulation of ΔFosB and the genes regulated by this transcription factor may provide increased understanding of the molecular mechanisms underlying striatal adaptation, but, more importantly, may yield significant insights into the basic processes mediating neural plasticity (Nestler et al., 1999).

7.1.1 *Antisense Strategies for Modulation of Striatal FosB Expression for the Treatment of Dopamine-Receptor Associated Dyskinesias.*

The study presented in Chapter 4 of this thesis described the use of antisense oligonucleotides for the selective inhibition of dopamine-agonist induced striatal FosB expression. The antisense sequence used in this study recognized both the long (*fosB*) and the short (Δ*fosB*) mRNA sequences, antisense administration in this study would attenuate expression of both FosB and ΔFosB proteins. However, this caveat of the current study could be addressed by designing an antisense sequence which spans the splice site in the *fosB* mRNA and is complementary to the sequences on either side of the splice site. Since an antisense designed to complement the sequences on either side of the splice site would preferentially bind the spliced *fosB* mRNA species, this strategy would selectively impair expression of ΔFosB protein.

Recent advances in antisense technology have improved both the half-life of antisense and reduced the concentration of material needed to achieve a biological effect by efficiently inhibiting translation of mRNA. For the antisense knockdown of *fosB* expression described in this thesis,

phosphotioate modified oligonucleotides were used. This class of antisense was selected because, compared to unmodified DNA, phosphothioate modifications increase resistance to RNaseH-mediated degradation and modestly prolong the inhibitory actions of the antisense when compared to unmodified sequences. The therapeutic application of phosphothioate modified antisense oligonucleotides, however, has been confounded by non-specific protein-binding, reduced binding affinities and seqeuence independent toxicity (Stein, 1995). More recently, researchers have discovered that modifying the chemistry of antisense DNA can profound enhance the binding affinity for targeted sequence and resistance to nuclease-mediated breakdown. For instance, phosphoramidate (NP) antisense has been reported to be up to 10 fold more efficient than isosequential phosphorothioate modified oligomers (Skorski et al., 1997; Faria et al., 2001). Hence, it could be proposed that an NP antisense designed to target *ΔfosB* may provide a more efficacious approach to resolving the experimental and therapeutic roles of dopamine agonist-induced striatal ΔFosB expression.

Finally, the results of the study described in Chapter 4 suggested that induction of striatal *fosB* may be associated with the development of the dyskinetic side-effects resulting from chronic dopaminergic therapy for the treatment of Parkinson's disease (Crocker et al., 1998). Subsequent work by others have confirmed our hypothesis and conclusions – by demonstrating that abnormal involuntary movements resulting from chronic administration of l-Dopa to rodents with unilateral nigrostriatal lesions is accompanied by increased expression of striatal *fosB* (Andersson et al., 1999). Consistent with our findings, Andersson and collegues reported that administration of antisense to *fosB* reduced striatal prodynorphin mRNA expression and ameliorated dyskinesia motor complications.

7.2 *Neuroprotective & Neurorestorative Strategies*

7.2.1 *Upregulation of Neuronal IAP Expression*

Enhancing dopamine neuron survival for the treatment of Parkinson's disease using elevated IAP expression could theoretically be achieved in several ways. First, pharmacological upregulation of the IAP protein, NAIP, has been previously demonstrated, suggesting that this approach may be biochemically possible (Xu et al., 1997). However, a caveat of this strategy is a clear comprehension of the biochemical processes governing IAP expression *in vivo*, since these facets of IAP regulation are presently unclear.

However, the observation that the alkaloid K252a can increase NAIP expression may provide a starting point from which investigations into the intracellular regulations of IAP protein expression could begin.

A second strategy by which IAP proteins could be introduced into dopamine neurons to ameliorate Parkinson's disease would be to use gene therapy. Theoretically, viral vectors could be employed as vectors for gene transfer into genes into neurons of the adult CNS. Such an *in vivo* approach would require advances over current vector technologies in order to reduce or significantly minimize the potential risks, increase the specificity and duration of vector derived genes. However, *ex vivo* gene therapy could be adopted to facilitate the survival of transplanted neural grafts, either through the direct application of gene vectors to graft tissues or through the production of transgenic animals capable of generating tissue suitable for xenotransplantation.

7.2.2 *IAPs and Neuronal Transplantation*

Since caspase inhibitor can enhance neural graft survival, it is plausible that genetically modified animals may provide a suitable source of xenografts for Parkinson's disease transplants. Results presented in Chapters 5 and 6 of this thesis demonstrate that the inhibitor of apoptosis (IAP) proteins, NAIP and XIAP, can provide functional protection to dopaminergic neurons against neurotoxic injury. An extension of this work, and other work from our lab, would support the notion that neuronal grafts which overexpress IAP proteins may exhibit increased graft survival. The reasons for this are twofold. First, IAP gene expression can enhance neuron resistance to ischemic injury, and given that the rigours of the transplant procedure are believed to cause a majority of the cell death during grafting, IAPs may reduce the number of cell required per transplant and enhance the longevity of the graft efficacy. The second reason why IAP-based graft augmentation may be beneficial is that transplanting neurons into the Parkinsonian brain may be putting the grafted cells into the same toxic environment which produced the cell death of the endogenous nigrostriatal dopamine neurons. As IAP proteins can prevent cell death induced by either 6-OHDA or MPTP, the application of IAPs may also enhance graft survival by fortifying the transplanted neurons in the potentially toxic environment of the PD brain.

7.2.3 *Mechanisms of Dopamine Neuron Death in PD*

Given the central role for caspases in the initiation and execution of neuronal cell death it was surprising to find a paucity of detectable caspase-3 activity in adult models of dopamine neurodegeneration. While reports by others contend a possible contribution of caspases to the demise of dopamine neurons (as discussed above), a conclusive demonstration refuting or supporting caspase involvement has not yet been reported. However, results from the studies included in Chapters 5 and 6 of this thesis suggest that caspase-3 may not participate in the loss of dopamine neurons in vivo. In addition, the results presented here also suggest that IAP proteins may have multiple cell death inhibiting properties. Hence, a more detailed understanding of the functions and/or interactions of BIR domains (Mercer et al., 2000), and extra-BIR domain regions of IAP proteins may provide important insights into the cytoprotective actions of IAP proteins as well as fundamental processes regulating cell death and survival.

Experimental results presented in this thesis suggest that the loss of dopamine neurons in animal models of PD may not involve caspase-3 mediated proteolysis. Ostensibly there are two experiments which could be performed to confirm these findings; pharmacological inhibitors and recombinant mouse technologies. First, most commercially available caspase-3 inhibitors are not highly selective for caspase-3, but rather can inhibit other proteases, rendering conclusions based on these pharmacological inhibitor studies unconclusive. Therefore, mice lacking caspase-3 may provide a more dircect test for the role of this cyteine protease for the loss of nigral dopamine neurons *in vivo*, with the caveat of developmental compensation for the loss of caspase-3 may confound these studies. Previous work by others reported that caspase-3 null mice exhibited poor post-natal survival (Kuida et al., 1997). However, when bred onto the C57Bl/6 background mice deficient of caspase-3 *can* develop into adulthood (D.W. Nicholson, personal communication). Assessment of dopamine neuron survival following administration of MPTP to caspase-3-/- mice may support the experimental results reported in this dissertation.

7.3 *Concluding Remarks*

In summary, our current understanding of the etiology of PD supports the notion that there are many potentially viable therapeutic avenues which may improve the treatment of this prevalent neurodegenerative condition. The studies included in this thesis represent two future fields of investigation into the pathophysiology of PD. First, determination of the role of adpative changes in neuronal changes in gene expression play in the development of motor-related dyskinesias. Secondly, elucidation of signal transduction pathways responsible for dopamine neuron death *in vivo*. Modulation of these processes, based upon results of the studies included in this thesis, may form the basis for the development of novel therapeutic strategies for the treatment of Parkinson's disease.

Chapter 8. Bibliography

References Cited in Introductory and Summary Chapters

Abeliovich, A., Schmitz, Y., Farinas, I, Choi-Lundberg, D., Ho, W-H., Castillo, P.E., Shinsky, N, Verdugo, J.M.G., Armanini, M., Ryan, A, Hynes, M., Phillips, H., Sulzer, D and A. Rosenthal (2000) Mice lacking α-synuclein Display Functional Deficits in the Nigrostriatal Dopamine System. *Neuron* **25**: 239-252.

Abi-Dargham, A., Rodenhiser, J., Printz, D., Zea-Ponce, Y., Gil, R., Kegeles, L.S., Weiss, R., Cooper, T.B., Mann, J.J., Van Heertum, R.L., Gorman, J.M. and M. Laruelle (2000) Increased baseline occupancy of D2 receptors by dopamine in schizophrenia. *Proc. Natl Acad Sci USA.* **97**: 8104-9.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. and J. Yuan (1996) Human ICE/CED-3 protease nomenclature. *Cell* **87**(2) 171.

ALS CNTF Treatment Study Group (1996) A double-blind placebo-controlled clinical trial of subcutaneous recombinant human ciliary neurotrophic factor (rHCNTF) in amyotrophic lateral sclerosis. *Neurology* **46**: 1244-9.

Ambrosini, G., Adida, C. and D.C. Altieri (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med.* **3**: 917-21.

Andersson, M., Hilbertson, A. and M.A. Cenci (1999) Striatal fosB expression is causally linked with l-DOPA-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. *Neurobiol. Dis.* **6**(6): 461-474.

Andrews, R., Watson, D.G., Best, S.A., Midgley, J.M., Wenlong, H. and R.K. Petty (1993) The determination of hydroxydopamines and other trace amines in the urine of parkinsonian patients and normal controls. *Neurochem Res* **18**(11): 1175-7.

Anglade, P., Vyas, S., Hirsch, E.C. and Y. Agid (1997) Apoptosis in dopaminergic neurons of the human substantia nigra during normal aging. *Histol. Histopathol.* **12**(3): 603-610.

Antonini, A., Leenders, K.L., Vontobel, P., Maguire, R.P., Missimer, J., Psylla, M. and I Gunther (1997) Complementary PET studies of striatal neuronal function in the differential diagnosis between multiple system atrophy and Parkinson's disease. *Brain* **120**: 2187-95.

Ara, J., Przedborski, S., Naini, A.B., Jackson-Lewis, V., Trifiletti, R.R., Horwitz, J. and H. Ischiropoulos (1998) Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc. Natl. Acad. Sci. U.S.A.* **95**(13): 7659-7663.

Arnt, J. (1985) Behavioural stimulation is induced by separate dopamine D-1 and D-2 receptor sites in reserpine pre-treated but not normal rats. *Eur J. Pharmacol* **113**: 79.

Asanuma, M., Hirata, H. and J.L. Cadet (1998) Attenuation of 6-hydroxydopamine-induced dopaminergic nigrostriatal lesions in superoxide dismutase transgenic mice. *Neuroscience* **85**: 907-917.

Banati, R.B., Daniel, S.E. and S.B. Blunt (1998) Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. *Mov. Disord.* **13**(2): 221-227.

Barbeau, A., Roy, M., Bernier, G., Campanella, G. and S. Paris (1987) Ecogenetics of Parkinson's disease: prevalence and environmental aspects in rural areas. *Can J.Neurol. Sci.* **14**: 36-41.

Beaman, B.L. (1992) Nocardia as a pathogen of the brain: mechanisms of interactions in the murine brain – a review. *Gene*. **115**: 213-217.

Berke, J.D., Paletzki, R.F., Aronson, G.J., Hyman, S.E and C.R. Gerfen (1998) A complex program of striatal gene expression induced by dopaminergic stimulation. *J Neurosci* **18**(14): 5301-10.

Berman, S.B. and T.G. Hastings (1999) Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria: implications for Parkinson's disease. *J. Neurochem.* **73**: 1127-1137.

Birnbaum, M.J., Clem, R.J. and L.K. Miller (1994) An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. *J Virol* **68**: 2521-8.

Bjorklund, A., Nobin, A. and U. Stenevi (1971) Acid catalysis of the formaldehyde condensation reaction for a sensitive histochemical demonstration of tryptamines and 3-methoxylated phenylethylamines. 2. Characterization of amine fluorophores and application to tissues. *J Histochem Cytochem.* **19**(5): 286-98.

Bjorklund, A. and U. Stenevi (1979) Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. *Brain Res.* **177**(3): 555-60.

Bocchetta, A. and G.U. Corsini (1986) Parkinson's disease and pesticides. *Lancet* **2**(8516): 1163.

Bohn, M.C. (2000) Parkinson's disease: a neurodegenerative disease particularly amenable to gene therapy. *Mol Ther.* **1**(6): 494-6.

Braun, A.R. and T.N. Chase (1986) Obligatory D-1/D-2 receptor interaction in the generation of dopamine agonist related behaviours. *Eur J. Pharmacol* **131**: 301.

Brooks, A.I., Chadwick, C.A., Gelbard, H.A., Cory-Slechta, D.A. and H.J. Federoff (1999) Paraquat elicited neurobehavioural syndrome caused by dopaminergic neuron loss. *Brain Res.* **823**(1-2): 1-10.

Brown, J.R., Ye, H., Bronson, R.T., Dikkes, P. and M.E. Greenberg (1996) A defect in nurturing in mice lacking the immediate early gene fosB. *Cell* **86**: 297-309.

Calne, D.B., Teychenne, P.F., Leigh, P.N., Bamji, A.N. and J.K. Greenacre (1974) Treatment of Parkinsonism with bromocriptine. *Lancet* **2**: 1355-6.

Carlson, A. (1959) The occurance, distribution and physiological role of catecholamines in the nervous system. *Pharmacol Rev.* **11**: 490-493.

Cassarino, D.S. and J.P. Bennett Jr. (1999) An evaluation of the role of mitochondria in neurodegenerative diseases: mitochondrial mutations and oxidative pathology, protective nuclear responses, and cell death in neurodegeneration. *Brain Res Reviews.* **29**: 1-25.

Cassarino, D.S., Parks, J.K, Parker Jr., W.D. and J.P. Bennett Jr. (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.

Cassarino, D.S., Halvorsen, E.M., Swerdlow, R.H., Abramova, N.N., Parker Jr., W.D., Sturgill, T.W and J.P. Bennett Jr. (2000) Interaction Among Mitochondria, Mitogen-Activated Protein Kinases, and Nuclear Factor-kB in Cellular Models of Parkinson's disease. *J. Neurochem.* **74**: 1384-1392.

Cedarbaum, J.M., Gandy, S.E. and F.H. McDowell (1991) "Early" initiation of levodopa treatment doea not promote the development of motor response fluctuations, dyskinesias, or dementia in Parkinson's disease. *Neurology* **41**: 622-629.

Chabrier, P.E., Demerle-Pallardy, C. and M. Auguet (1999) Nitric oxide synthases: targets for therapeutic strategies in neurological diseases. *Cell Mol. Life Sci.* **55**: 1029-1035.

Chai, J., Du, C., Wu, J.W., Kyin, S., Wang, X. and Y. Shi (2000) Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* **406**: 855-862.

Chase, T.N., Mouradian, M.M. and T.M. Engber (1993) Motor complications and the function of striatal efferent systems. *Neurology* **43**: S23-S27.

Chen, J., Nye, H.E., Kelz, M.B., Hiroi, N., Nakabeppu, Y., Hope, B.T. and E.J. Nestler (1995) Regulation of ΔFosB and FosB-like proteins by electroconvulsive seizure and cocaine treatments. *Mol. Pharm.* **48**: 880-889.

Chen, T.S., Koutsilieri, E. and W.D. Rausch (1995) MPP+ selectively affects calcium homeostasis in mesencephalic cell cultures from embryonal C57/Bl6 mice. *J Neural Trans Gen Sect* **100**: 153-163.

Choi-Lundberg, D.L., Lin, Q., Chang, Y.N., Chiang, Y.L., Hay, C.M., Mohajeri, H., Davidson, B.L. and M.C. Bohn (1997) Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* **275**: 838-841.

Ciechanover, A., Orian, A. and A.L. Schwartz (2000) Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* **22**(5): 442-451.

Cohen, G. (1978) The generation of hydroxyl radicals in biologic systems: toxicological aspects. *Photochem. Photobiol.* **28**(4-5): 669-675.

Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem J* **326**: 1-16.

Conway, K.A., Harper, J.D and P.T. Lansbury (1998) Accelerated *in vitro* fibril formation by a mutant α-synuclein linked to early-onset Parkinson disease. *Nat. Med.* **4**(11): 1318-1320.

Corsini, G.U., Pintus, S., Chiueh, C.C., Weiss, J.F. and I.J. Kopin (1985) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice is enhanced by pretreatment with diethyldithiocarbamate. *Eur J Pharmacol* **119**: 127-8.

Cotzias, G.C. (1968) L-Dopa for Parkinsonism. *N Engl J Med.* **278** (11): 630.

Cotzias, G.C., Papavasiliou, P.S. and R. Gellene (1969) L-dopa in parkinson's syndrome. *N Engl J Med.* **281**(5): 272.

Crocker, S.J., Morelli, M., Wigle, N., Nakabeppu, Y. and G.S. Robertson (1998) D1-receptor related priming is attenuated by antisense-mediated "knockdown" of *fosB* expression. *Mol Brain Res.* **53**: 69-77.

Crook, N.E., Clem, R.J and L.K. Miller (1993) An apoptosis-inhibiting baculovirus gene with a zinc-finger-like motif. *J. Virol* **67**: 2168-74.

Crossman, A.R. (1990) A hypothesis on the pathophysiological mechanisms that underlie levodopa- or dopamine agonist-induced dyskinesia in Parkinson's disease: implications for future strategies in treatment. *Mov Disord* **5**(2): 100-8.

D'Amato, R.J., Alexander, G.M., Schwartzman, R.J., Kitt, C.A., Price, D.L. and S.H. Snyder (1987) Evidence for neuromelanin involvement in MPTP-induced neurotoxicity. *Nature* **327**: 324-6.

D'Amato, R.J., Lipman, Z.P. and S.H. Snyder (1986) Selectivity of the parkinsonian neurotoxin MPTP: toxic metabolite MPP+ binds to neuromelanin. *Science* **231**: 987-9.

Devereaux, Q.L., Takahashi, R., Salvesen, G.S. and J.C. Reed (1997) X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* **388**: 300-304.

Devereaux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S. and J.C. Reed (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J.* **17**(8): 2215-23.

Dexter, D.T., Wells, F.R., Agid, Y., Lees, A.J., Jenner, P and C.D. Marsden (1987) Increased nigral iron content in post-mortem parkinsonian brain. *Lancet* **2**: 1219-1220.

Dexter, D.T., Cater, C.J., Wells, F.R., Javoy-Agid, F., Agrid, Y., Lees, A., Jenner, P. and C.D. Marsden (1989) Basal lipid peroxidation is increased in Parkinson's disease. *J. Neurochem.* **52**: 381-389.

Doucet, J.P., Nakabeppu, Y. Bedard, P.J., Hope, B.T., Nestler, E.J., Jasmin, B.J., Chen, J.S, Iadarola, M.J., St-Jean, M., Wigle, N., Blanchet, P., Grondin, R. and G.S. Robertson (1996) Chronic alterations in dopaminergic neurotransmission produce a persistent elevation of deltaFosB-like protein(s) in both rodent and primate striatum. *Eur J. Neurosci.* **8**(2): 365-81.

Duckett, C.S., Nava, V.E., Gedrich, R.W., Clem, R.J., Van Dongen, J.L., Gilfillan, M.C., Shiels, H., Hardwick, J.M. and C.B. Thompson (1996) A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *EMBO J* **15**(11): 2685-94.

Ehringer, H. and O. Hornykeiwicz (1960) Verteilung von noradrenalin und dopamine (3-hydroxytyramin) im gehrin des menschen. *Klin Wschr.* **38**: 1236-9.

Eldridge, R. and S.E. Ince (1984) The low concordance rate for Parkinson's disease in twins: a possible explanation. *Neurology* **34**(10): 1354-6.

Ellis, H.M. and H.R. Horvitz (1986) Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44**(6): 817-29.

Ellis, R.E., Jacobson, D.M. and H.R. Horvitz (1991) Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans. *Genetics* **129**(1): 79-94.

Engber, T.M., Susel, Z., Juncos, J.L. and T.N. Chase (1989) Continuous and intermittent levodopa differentially affect rotation induced by D1 and D2 dopamine agonists. *Eur J Pharmacol.* **168**: 291-8.

Engber, T.M., Susel, Z., Kuo, S., Gerfen, C.R. and T.N. Chase (1991) Levodopa replacement therapy alters enzyme activities in striatum and neuropeptide content in striatal output regions of 6-hydroxydopamine lesioned rats. *Brain Res.* **552**: 113-118.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, J., Albour, A.M., Nordborg, C., Peterson, D.A. and F.H. Gage (1998) Neurogenesis in the adult human hippocampus. *Nat Med.* **4**(11): 1313-1317.

Fabbrini, G., Juncos, J., Mouradian, M.M., Serrati, C., and T.N. Chase (1987) Levodopa pharmacokinetic mechanisms and motor fluctuations in Parkinson's disease. *Ann Neurol.* **21**(4): 370-6.

Fallon, J.H. and R.Y. Moore (1978) Catecholamine innervation of the basal forebrain. IV. Topography of the dopamine projection to the basal forebrain and neostriatum. *J. Comp Neurol.* **180**(3): 545-80.

Faria, M., Spiller, D.G., Dubertret, C., Nelson, J.S., White, M.R.H., Scherman, D., Helene, C. and C. Giovannangeli (2001) Phosphoamidate oligonucleotides as potent antisense molecules in cells and *in vivo*. *Nat Biotech.* **19**: 40-44.

Ferrante, R.J., Schulz, J.B., Kowall, N.W and M.F. Beal (1997) Systemic administration of rotenone produces selective damage in the striatum and globus pallidus, but not in the substantia nigra. *Brain Res.* **753**(1): 157-62.

Fink, K., Zhu, J., Namura, S., Shimizu-Sasamata, M., Endres, M., Ma, J., Dalkar, T., Yuan, J. and M.A. Moskowitz (1998) Prolonged therapeutic window for ischemic brain damage caused by delayed caspase activation. *J Cereb Blood Flow Metab.* **18**:1071-6.

Forno, L.S., DeLanney, L.E., Irwin, I. and J.W. Langston (1993) Similarities and differences between MPTP-induced parkinsonism and Parkinson's disease. Neuropathologic considerations. *Adv. Neurol.* **60**: 600-608.

Frei, B. and C. Richter (1986) N-methyl-4-phenylpyridine (MPP+) together with 6-hydroxydopamine of dopamine stimulates Ca^{2+} release from mitochondria. *FEBS Lett* **198**: 99-102.

Friedrich, M.J. (1999) Pesticide study aids Parkinson research. *JAMA* **282**(23): 2200.

Gage, F.H. (2000) Mammalian Neural Stem Cells. *Science* **287**: 1433-1438.

Gage, F.H. and L.J. Fisher (1991) Intracerebral grafting: A tool for the neurobiologist. *Neuron* **6**: 1-12.

Gash, D.M., Zhang, Z., Ovadia, A., Cass, W.A., Yi, A., Simmerman, L., Russell, D., Martin, D., Lapchak, P.A., Collins, F., Hoffer, B.J. and G.A. Gerhardt (1996) Functional recovery in parkinsonian monkeys treated with GDNF. *Nature* **380**: 252-5.

Gasser, T., Muller-Myhsok, B., Wszolek, Z.K., Durr, A., Vaughan, J.R., Bonifati, V., Meco, G., Bereznai, B., Oehlmann, R., Agid, Y., Brice, A. and N. Wood (1997) [Letter] Genetic complexity and Parkinson's disease. *Science* **277**: 388-389.

Gasser, T., Muller-Myhsok, B., Wszolek, Z.K., Oehlmann, R., Calne, D.B., Bo, V., Bereznai, B., Fabrizio, E., Vieregge, P. and R.D. Horstmann (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nat Genet* **18**: 262-265.

Gerfen, C.R., Baimbridge, K.G. and J. Thibault (1987) The neostriatal mosaic: III. Biochemical and developmental dissociation of patch-matirx neostriatal systems. *J. Neurosci.* 7(12): 3935-44.

Gerfen, C.R., Engber, T.M., Mahan, L.C., Susel, Z., Chase, T.N., Monsma, F.J. Jr. and D.R. Sibley (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science*. **250**: 1429-32.

Gervais, F.G., Xu, D., Robertson, G.S., Vaillancourt, J.P., Zhu, Y., Huang, J., LeBlanc, A., Smith, D., Rigby, M., Shearman, M.S., Clarke, E.E., Zheng, H., Van Der Ploeg, L.H., Ruffolo, S.C., Thornberry, N.A., Xanthoudakis, S., Zamboni, R.J., Roy, S. & Nicholson, D.W. (1999) Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid- beta precursor protein and amyloidogenic A beta peptide formation. *Cell*, **97**, 395-406.

Glanow, C.W. and W.G. Tatton (1999) Etiology and pathogenesis of Parkinson's disease. *Annu. Rev. Neurosci.* **22**: 123-144.

Glinka, Y., Tipton, K.F. and M.B. Youdim (1996) Nature of inhibition of mitochondrial respiratory complex I by 6-hydroxydopamine. *J. Neurochem* **66**: 2004-2010.

Glinka, Y. and M.B. Youdim (1995) Inhibition of mitochondrial complexes I and IV by 6-hydroxydopamine. *Eur. J. Pharmacol.* **292**(3-4): 329-332.

Goetz, C.G., Tanner, C.M., Levy, M. et al., (1986) Pain in idiopathic Parkinson's disease. *Mov. Disord* **1**:45-50.

Golbe, L.I., Lazzarini, A.M., Schwartz, K.O., Mark, M.H., Dickson, D.W. and R.C. Duvoisin (1993) Autosomal dominant parkinsonism with benign course and typical Lewy-body pathology. *Neurology* **43**: 2222-7.

Gould, E., Reeves, A.J., Graziano, M.S. and C.G. Gross (1999) Neurogenesis in the neocortex of adult primates. *Science* **286**: 548-552.

Goyel, L., McCall, K., Agapite, J., Hartwieg, E. and H. Steller (2000) Induction of apoptosis by Drosophila reaper, hid and grim through inhibition of IAP function. *EMBO J.* **19**(4): 589-597.

Grandas, F., Martinez-Martin, P., and G. Linazasoro (1998) Quality of life in patients with Parkinson's disease who transfer from standard levodopa to Sinemet CR: the STAR Study. The STAR Multicenter Study Group. *J. Neurol.* **245**: S31-3.

Graybiel, A.M. (1990) Neurotransmitters and neuromodulators in the basal ganglia. *Trends Neurosci.* **13**(7): 244-54.

Graybiel, A.M., Moratalla, R. and H.A. Robertson (1990) Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum. *Proc Natl Acad Sci USA* **87**: 6912-6.

Hantraye, P., Brouillet, E., Ferrante, R., Palfi, S., Dolan, R., Matthews, R.T. and M.F. Beal (1996) Inhibition of neuronal nitric oxide synthase prevents MPTP-induced parkinsonism in baboons. *Nat Med.* **2**: 1017-1021.

Hara, H., Friedlander, R.M., Gagliardini, V., Ayata, C., Fink, K., Huang, Z., Shimizu-Sasmata, M., Yuan, J. and M.A. Moskowitz (1997) Inhibition of interleukin-1B converting enzyme family proteases reduced ischemic and excitotoxic neuronal damage. *Proc. Natl. Acad. Sci. USA* **94**: 2007-2012.

Hauser, H.P., Bardroff, M., Pyrowolakis, G. and S. Jentsch (1998) A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. *J Cell Biol* **141**: 1415-22.

Heikkila, R.E. and G. Cohen (1971) Inhibition of biogenic amine uptake by hydrogen peroxide: a mechanism for toxic effects of 6-hydroxydopamine. *Science* **172**: 1257-1258.

Heikkila, R.E., Hess, A. and Duvoisin, R.C. (1884) Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. *Science* **224**: 1451-1453.

Heikkila, R.E., Manzino, L., Cabbat, F.S. and R.C. Duvoisin (1984) Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature*. **311**: 467-9.

Heintz, N. and H. Zoghbi (1997) α-synuclein – a link between Parkinson and Alzheimer diseases? *Nat Genet* **16**: 325-327.

Herdegen, T., Kovary, K., Buhl, A., Bravo, R., Zimmerman, M. and P. Gass (1995) Basal expression of the inducible transcription factors c-Jun, JunB, JunD, c-Fos, FosB and Krox-24 in the adult rat brain. *J. Comp. Neurol.* **354**: 39-56.

Herdegen, T and J.D. Leah (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos, Krox, and CREB/ATF proteins. *Brain Res Brain Res Rev.* **28**: 370-490.

Hiroi, N., Brown, J.R., Haile, C.N., Ye, H., Greenberg, M.E. and E.J. Nestler (1997) FosB mutant mice: Loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 10397-10402.

Hiroi, N., Marek, G.J., Brown, J.R., Ye, H., Saudou, F., Vaidya, V.A., Duman, R.S., Greenberg, M.E. and E.J. Nestler (1998) Essential role of the *fosB* Gene in Molecular, Cellular, and Behavioural Actions of Chronic Electroconvulsive Seizures. *J. Neurosci.* **18**(7): 6952-6962.

Hirsch, E., Graybiel, A.M. and Y.A. Agid (1988) Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature.* **334**: 345-8.

Huang, Q., Devereaux, Q.L., Meada, S., Salvesen, G.S., Stennicke, H.R., Hammock, B.D. and J.C. Reed (2000) Evolutionary conservation of apoptosis mechanisms: lepidoperan and baculoviral inhibitor of apoptosis proteins are inhibitors of mammalians caspase-9. *Proc Natl Acad Sci USA.* **97**(4): 1427-32.

Huang, Q., Zhou, D., Chase, K., Gusella, J.F., Aronin, N. and M. DiFiglia (1992) Immunohistochemical localization of the D1 dopamine receptor in rat brain reveals its axonal transport, pre-and postsynaptic localization, and prevalence in the basal ganglia, limbic system, and thalamic reticular nucleus. *Proc. Natl. Acad. Sci USA* **89**: 11988-92.

Hunot, S., Boissiere, F., Faucheux, B., Brugg, B., Mouatt-Prigent, A., Agid, Y and E.C. Hirsch (1996) Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. *Neuroscience.* **72**: 355-363.

Horellou, P., Bilang-Bleuel, A and J. Mallet (1997) In vivo adenovirus-mediated gene transfer for Parkinson's disease. *Neurobiol Dis.* **4**:280-7.

Ikebe, S., Tanaka, M., and T. Ozawa (1990) Point mutations of mitochondrial genome in Parkinson's disease. *Mol Brain Res.* **28**: 281-295.

Ilic, T.V., Jovanovic, M., Jovicic, A. and M. Tomovic (1999) Oxidative stress indicators are elevated in *de novo* Parkinson's disease patients. *Funct. Neurol.* **14**: 141-147.

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

Janetzky, B., Hauck, S., Youdim, M.B.H., Riederer, P., Jellinger, K., Pantucek, F., Zochling, R., Boissl, K.W. and H. Reichmann (1994) Unaltered aconitase activity but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. *Neurosci Lett.* **169**: 126-128.

Jarvie, K.R. and M.G. Caron (1993) Heterogeneity of dopamine receptors. *Adv. Neurol.* **60**: 325-33.

Javitch, J.A., D-Amato, R.J., Strittmatter, S.M. and S.H. Snyder (1985) Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc Natl Acad Sci USA.* **82**: 2173-7.

Jellinger, K.A. (2000) Cell death mechanisms in Parkinson's disease. *J. Neural. Transm.* **107**: 1-29.

Jellinger, K.A., Linert, L., Kienzl, E., Herlinger, E. and M.B. Youdim (1995) Chemical evidence for 6-hydroxydopamine to be an endogenous toxic factor in the pathogenesis of Parkinson's disease. *J. Neurol. Transm. Suppl.* **46**: 297-314.

Jian, M., Staines, W.A., Iadarola, M.J. and G.S. Robertson (1993) Destruction of the nigrostriatal pathway increases Fos-like immunoreactivity predominantly in striatopallidal neurons. *Brain Res Mol brain Res* **19**: 156-60.

Johnson, E.M. Jr. and T.L. Deckwerth (1993) Molecular mechanisms of developmental neuronal death. *Annu Rev Neurosci.* **16**: 31-46.

Jonsson, G. and C. Sachs (1970) Effects of 6-hydroxydopamine on the uptake and storage of noradrenaline in sympathetic adrenergic neurons. *Eur J. Pharmacol* **9**(2): 141-55.

Kass, G.E., Wright, J.M., Nicotera, P. and S. Orrenius (1988) The mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity: role of intracellular calcium. *Arch Biochem Biophys* **260**: 789-797.

Kebabian, J.W. and D.B. Calne (1979) Multiple receptors for dopamine. *Nature*. **277**: 93-6.

Kerr, J.F., Wyllie, A.H. and A.R. Currie (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**: 239-57.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and N. Shimizu (1998) Mutations in the *parkin* gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**: 605-608.

Kohbata, S. and B.L. Beaman (1991) L-Dopa-responsive movement disorder caused by Nocardia asteroides localized in the brains of mice. *Infect Immun* **59**: 181-191.

Kohbata, S. and K. Shimokawa (1993) Circulating antibody to Nocardia in the serum of patients with Parkinson's disease. *Adv. Neurol* **60**:355-357.

Kohno, M., Ohta, S. and M. Hirobe (1986) Tetrahydroisoquinoline and 1-methyl-tetrahydroisoquinoline as novel endogenous amines in rat brain. *Biochem Ciophys Res Commun* **140**(1): 448-54.

Kostic, V., Gurney, M.E., Deng, H.X., Siddique, T., Epstein, C.J. and S. Przedborski (1997) Midbrain dopaminergic neuronal degeneration in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Ann Neurol.* **41**: 497-504.

Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L. and O. Riess (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. *Nat Genet* **18**: 106-108.

Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and R.A. Flavell (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**: 368-372.

Kumar, R., Agarwal, A.K. and P.K. Seth (1995) Free radical-generated neurotoxicity of 6-hydroxydopamine. *J. Neurochem.* **64**: 1703-1707.

Laitinen, L.V., Bergenheim, A.T. and M.I. Hariz (1992) Leksell's posteroventral pallidotomy in the treatment of Parkinson's disease. *J Neurosurg* **76**: 53-61.

Lang, A.E., Quinn, N. and S. Brincat (1982) Pergolide in late stage Parkinson's disease. *Arch Neurol.* **40**: 785-787.

Lang, A.E., Lozano, A.M., Mongomery, E., Duff, J, Tasker, R and W. Hutchinson (1997) Posteroventral medial pallidotomy in advanced Parkinson's disease. *N. Engl. J. Med.* **337**: 1036-42.

Lee, S-H, Lumelsky, N., Studer, L., Auerbach, J.M. and R.D. MacKay (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotech.* **18**: 675-678.

Lees, A.J. (1995) Comparison of therapeutic effects and mortality data of levodopa combined with selegiline in patients with early, mild Parkinson's disease. Parkinson's Disease Research Group of the United Kingdom. *B. Med. Journal.* **311**: 1602-7.

Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T., Dehejia, A., Lavedan, C., Gasser, T., Steinbach, P.J., Wilkinson, K.D. and M.H. Polymeropoulos (1998) The ubiquitin pathway in Parkinson's disease. *Nature* **395**: 451-452.

Liberatore, G.T., Jackson-Lewis, V., Vukosavic, S., Mandir, A.S., Vila, M., McAuliffe, W.G., Dawson, V.L., Dawson, T.M. and S. Przedborski (1999) Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. *Nat Med.* **5**: 1403-1409.

Lieberman, A.N., Neophytides, A., Leibowitz, M. et al. (1983) Comparative efficacy of pergolide and bromocriptine in patients with advanced Parkinson's disease. *Adv. Neurol.* **37**: 95-108.

Lincoln, S., Vaughan, J., Wood, N., Baker, M., Adamson, J., Gwinn-Hardy, K., Lynch, T., Hardy, J. and M. Farrer (1999) Low frequency of pathogenic mutations in the ubiquitin carboxy-terminal hydroxylase gene in familial Parkinson's disease. *Neuroreport* **10**: 427-429.

Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., Leenders, K.L., Sawle, G., Rothwell, J.C., Marsden, C.D. and A. Bjorklund (1990) Grafts of Fetal Dopamine Neurons Survive and Improve Motor Function in Parkinson's Disease. *Science* **247**: 574-577.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J-E., MacKenzie, A. and R.G. Korneluk (1996) Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* **379**: 349-353.

Maraganore, D.M., Farrer, M.J., Hardy, J.A., Lincoln, S.J., McDonnell, S.K. and W.A. Rocca (1999) Case-control study of the ubiquitin carboxy-terminal hydroxylase L1 gene in Parkinson's disease. *Neurology* **53**(8): 1858-1860.

Markey, S.P., Johannessen, J.N., Chiueh, C.C., Burns, R.S. and M.A. Herkenham (1984) Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. *Nature* **311**: 464-7.

Martinou, J.C., Dubois-Dauphin, M. Staple, J.K., Rodriguez, I., Frankowski, H., Missotten, M., Albertini, P., Talabot, D. and S. Catsicas (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* **13**(4): 1017-30.

Masliah, E., Rockenstein, E., Veinberg, I., Mallory, M., Hashimoto, M., Takedo, A., Sagara, Y., Sisk, A. and L. Mucke (2000) Dopaminergic Loss and Inclusion Body Formation in α-synuclein Mice: Implications for Neurodegenerative Disorders. *Science* **287**: 1265-1269.

Mattson, M. (1997) Neuroprotective signal transduction: relevance to stroke. *Neurosci Behav. Rev.* **21**: 193-206.

McDowell, F., Lee, J.E., Swift, T., Sweet, R.D., Ogsbury, J.S. and J.T. Kessler (1970) Treatment of Parkinson's disease with L dihydrophenylalanine (levodopa). *Ann Intern Med* **72** (1): 29-35.

McGeer, P.L., McGeer, E.G. and J.S. Suzuki (1977) Aging and extrapyramidal function. *Arch Neurol.* **34**: 33-5.

McNaught, K.St.P. and P. Jenner (1999) Altered glial function causes neuronal death and increases neuronal susceptibility to 1-methyl-4-phenylpyridinium- and 6-Hydroxydopamine-Induced Toxicity in Astrocyte/Ventral Mesencephalic Co-cultures. *J. Neurochem* **73**: 2469-2476.

Meador-Woodruff, J.H., Mansour, A., Bunzow, J.R., Van Tol, H.H., Watson, S.J. and O. Civelli (1989) Distribution of D2 dopamine receptor mRNA in rat brain. *Proc. Natl Acad Sci USA* **86**: 7625-8.

Melnick, M.E., Dowling, G.A., Aminoff, M.J. and N.M. Barbaro (1999) Effect of pallidotomy on postural control and motor function in Parkinson's disease. *Arch Neurol* **56**(11): 1361-5.

Mendez, I., Sadi, D. and M. Hong (1996) Reconstruction of the Nigrostriatal Pathway by Simultaneous Intrastriatal and Intranigral Dopaminergic Transplants. *J. Neurosci.* **16**(22): 7216-7227.

Miller, L.K. (1999) An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends Cell Biol* **9**(8): 323-8.

Missale, C., Nash, S.R., Robinson, S.W., Jaber, M. and M.G. Caron (1998) Dopamine receptors: from structure to function. *Physiol. Rev.* **78**: 189-225.

Mochizuki, H., Mori, H. and Y. Mizuno (1997) Apoptosis in neurodegenerative disorders. *J. Neural. Transm. Suppl.* **50**: 125-140.

Montagu, K.A. (1957) Catechol compounds in rat tissues and in brains of different animals. *Nature* **180**:244.

Morgan, J.I. and T. Curran (1989) Stimulus-transcription coupling in neurons: role of cellular immediate early genes. *Trends Neurosci.* **12**(11): 459-62.

Mouatt-Prigent, A., Karlsson, J.O., Agid, Y. and E.C. Hirsch (1996) Increased m-calpain expression in the mesencephalon of patients with Parkinson's disease but not in other neurodegenerative disorders involving the mesencephalon: a role in nerve cell death? *Neuroscience* **73**: 979-987.

Nakabeppu, Y and D. Nathans (1989) The basic region of Fos mediates specific DNA binding. *EMBO J.* **8**(12): 3833-41.

Nakabeppu, Y. and D. Nathans (1991) A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. *Cell* **64**: 751-759.

Nakao, N., Frodl, E.M., Widner, H., Carlson, E., Eggerding, F.A., Epstein, C.J. and P. Brundin (1995) Overexpressing Cu/Zn superoxide dismutase enhances survival of transplanted neurons in a rat model of Parkinson's disease. *Nat. Med.* **1**: 226-231.

Nestler, E.J., Kelz, M.B. and J. Chen (1999) DeltaFosB: a molecular mediator of long-term neural and behavioural plasticity. *Brain Res* **835**: 10-17.

Nicklas, W.J., Vyas, I. and R.E. Heikkila (1985) Inhibition of NADH-linked oxidation in brain mitochonidria by MPP+, a metabolite of the neurotoxin, MPTP. *Life Sci* **36**: 2503-2508.

Nicklas, W.J., Youngster, S.K., Kindt, M.V. and R.E. Heikkila (1987) MPTP, MPP+ and mitochondrial function. *Life Sci.* **40**(8): 721-729.

Nicholson, D.W. and N.A. Thornberry (1997) Caspases: killer proteases. *Trends Biochem Sci.* **22**: 299-306.

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.

Niwa, T., Takeda, N., Keneda, N., Hashizume, Y. and T. Nagatsu (1987) Presence of tetrahydroisoquinoline and 2-methyl-tetrahyrodquinoline in parkinsonian and normal human brains. *Biochem Biophys Res Commun.* **144**: 1084-9.

Niwa, T., Takeda, N., Yoshizizumi, A., Yoshida, M., Dosert, P., Naoi, M. and T. Nagatsu (1993) Presence of tetrahydroisoquinoline-related compounds, possible MPTP-like neurotoxins, in parkinsonian brains. *Adv. Neurol* **60**: 234-7.

Nussbaum, R.L. (1998) Putting the Parkin into Parkinson's. *Nature* **392**: 544-545.

Nutt, J.G., Woodward, W.R., Hammerstead, J.P. et al. (1984) The "on-off" phenomenon in Parkinson' s disease: Relation to levodopa absorption and transport. *N. Eng. J.Med.* **310**: 483-488.

Nutt, J.G., Woodward, W.R., and J.L. Anderson (1985) The effect of carbidopa on the pharmacokinetics of intravenously administered levodopa: the mechanism of action in the treatment of parkinsonism. *Ann. Neurol.* **18**(5): 537-543.

Oestreicher, E., Sengstock, G.J., Riederer, P., Olanow, C.W., Dunn, A.J. and G. Arendash (1994) Degeneration of nigrostriatal dopaminergic neurons increases iron within the substantia nigra: a histochemical and neurochemical study. *Brain Res.* **660**: 8-18.

Olanow, C.W., Freeman, T.B. and J.H. Kordower. (1997) Transplantation Strategies for Parkinson's Disease *in* Movement Disorders: Neurologic Principles and Practice R.L. Watts and W.C. Koller, eds. (McGraw-Hill, Toronto).

Oppenheim, R.W. (1991) Cell death during development of the nervous system. *Annu Rev Neurosci.* **14**: 453-501.

Pan, H.S., Penney, J.B. and A.B. Young (1985) gamma-aminobutyric acid and bezodiazepine receptor changes induced by unilateral 6-hydroxydopamine lesions of the medial forebrain bundle. *J. Neurochem.* **45**: 1396-1404.

Pan, H.S. and J.R. Walters (1988) Unilateral lesion of the nigrostriatal pathway decreases the firing rate and alters the firing pattern of globus pallidus neurons in the rat. *Synapse.* **2**(6): 650-6.

Pare, D., Curro'Dossi, R. and M. Steriade (1990) Neuronal basis of the parkinsonian resting tremor: a hypothesis and its implication for treatment. *Neuroscience.* **35**(2): 217-26.

Parkinson's Disease Research Group of the United Kingdom (1993) Comparisons of therapeutic effects of levodopa, levodopa and selegiline, and bromocriptine in patients with early, mild Parkinson's disease: three years interim report. *B Med Journal.* **307**: 469-472.

Parkinson's Disease Research Group of the United Kingdom (1995) Comparisons of therapeutic effects and mortality data of levodopa and levodopa combined with selegiline in patients with early, mild Parkinson's disease. *B Med Journal.* **311**: 1602-7.

Parkinson's Study Group (1989) DATATOP: a multicenter controlled clinical trial in early Parkinson's disease. *Arch Neurol.* **46**(10): 1052-60.

Parkinson's Study Group (1998) Mortality in DATATOP: a multicenter trial in early Parkinson's disease. Parkinson's Study Group. *Ann Neurol.* **43**(3): 318-25.

Pastor, P., Ezquerra, M., Munoz, E., Marti, M.J., Blesa, R., Tolosa, E. and R. Oliva (2000) Significant association between the tau gene AO/AO genotype and Parkinson's disease. *Ann Neurol* **47**: 242-245.

Perlow, M.J., Freed, W.J., Hoffer, B.J., Seiger, A., Olson, L., and R.J. Wyatt (1979) Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. *Science* **204**: 643-647.

Perrelet, D., Ferri, A., MacKenzie, A.E., Smith, G.M., Korneluk, R.G., Liston, P., Sagot, Y., Terrado, J., Monnier, D. and A.C. Kato (2000) IAP family proteins delay motoneuron cell death *in vivo*. *Eur. J. Neurosci.* **12**: 2059-67.

Perry, T.L., Yong, V.W., Wall, R.A. and K. Jones (1986) Paraquat and two endogenous analogues of the neurotoxic substance N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine do not damage dopaminergic nigrostriatal neurons in the mouse. *Neurosci. Lett.* **69**(3): 285-289.

Phillips, P. (1999) Several classes of new drugs emerging for Parkinson's disease. 282(10): 929-31.

Polymeropoulos, M.H., Higgins, J.J., Golbe, L.I., Johnson, W.G., Ide, S.E., Di Iorio, G., Sanges, G., Stenroos, E.S., Pho, L.T., Schaffer, A.A., Lazzarini, A.M., Nussbaum, R.L. and R.C. Duvoisin (1996) Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. *Science* **274**: 1197-1199.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I. and R.L. Nussbaum (1997) Mutations in the α-synuclein gene identified in families with Parkinson's disease. *Science* **276**: 2045-2047.

Przedborski, S., Kostic, V., Jackson-Lewis, V., Naini, A.B., Simonettie, S., Fahn, S., Carlson, E., Epstein, C.J. and J.L. Cadet (1992) Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J. Neurosci.* **12**:1658-67.

Przedborski, S., Jackson-Lewis, V., Muthane, U., Jiang, H., Ferreira, M., Naini, A.B. and S. Fahn (1993) Chronic levodopa administration alters cerebral mitochondrial respiratory chain activity. *Ann Neurol.* **34**(5): 715-23.

Przedborski, S., Jackson-Lewis, V., Yokoyama, R., Shibata, T., Dawson, V.L. and T. Dawson (1996) Role of neuronal nitric oxide in 1-methyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. *Proc. Natl. Acad. Sci. USA* **93**: 4565-4571.

Przedborski, S., Levivier, M., Jiang, H., Ferreira, M., Jackson-Lewis, V., Donaldson, D. and D.M. Togasaki (1995) Dose-dependent lesions of the dopaminergic nigrostriatal pathway induced by intrastriatal injection of 6-hydroxydopamine. *Neuroscience.* **67**: 631-47.

Przedborski, S. and V. Jackson-Lewis (1998) Experimental developments in movement disorders: update on proposed free radical mechanisms. *Curr. Opin. Neurol.* **11**(4): 335-339.

Radi, R., Castro, L., Rodriguez, M., Caddina, A. and L. Thomson (1997) "Free Radical damage to Mitochondria" in *Mitochondria and Free Radicals in Neurodegenerative Diseases* (Beal, M.F., Howell, N. and I. Bodis-Wollner eds.) Wiley-Liss Inc.

Raff, M. (1998) Cell suicide for beginners. *Nature*. **396**: 119-22.

Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y. and M.D. Jacobson (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science*. **262**: 695-700.

Rajput, A.H. and R.J. Uitti (1987) Paraquat and Parkinson's disease. *Neurology* **37**(11): 1820-1821.

Ransom, B.R., Kunis, D.M., Irwin, I. and J.W. Langston (1987) Astrocytes convert the parkinsonism inducing neurotoxin, MPTP, to its metabolite, MPP+. *Neurosci Lett*. **75**: 323-8.

Riederer, P. and S St. Wuketich (1976) Time course of nigrostriatal degeneration in Parkinson's disease. *J Neural Transm* **38**: 277-301.

Riederer, P., Sofic, E., Rausch, W.D., Schmidt, B., Reynolds, G.P., Jellinger, K. and M.B.H. Youdim (1989) Transition metals, ferritin, glutathione and ascorbic acid in parkinsonian brains. *J. Neurochem*. **52**: 515-520.

Rinne, U.K. (1987) Madopar HBS in the long-term treatment of parkinsonism with fluctuations in disability. *Eur Neurol* **27**: 120-5.

Rinne, U.K. (1989) Lisuride a dopamine agonist in the treatment of early Parkinson's disease. *Neurology* **39**: 336-339.

Robertson, G.S., Crocker, S.J., Nicholson, D.W. and J.B. Schulz (2000) Neuroprotection by the Inhibition of Apoptosis. *Brain Pathol.* **10**: 283-292.

Robertson, G.S. and H.A. Robertson (1986) Synergistic effects of D1 and D2 dopamine agonists on turning behaviour in rats. *Brain Res.* **384**: 387-90.

Robertson, G.S. and H.A. Robertson (1988) Evidence that the substanti nigra is a site of action for L-DOPA. *Neurosci. Lett.* **89**: 204-208.

Robertson, H.A. (1992) Dopamine receptor interactions: some implications for the treatment of Parkinson's disease. *Trends Neurosci* **15**: 201-206.

Robertson, H.A., Peterson, M.R., Murphy, K. and G.S. Robertson (1989) D1-dopamine receptor agonists selectively activate striatal c-fos independent of rotational behaviour. *Brain Res.* **503**: 346-9.

Rosenthal, A. (1998) Auto Transplants for Parkinson's Disease? *Neuron* **20**: 169-172.

Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M. and D.V. Goeddel (1995) The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* **83**: 1243-52.

Roy, N., Mahadevan, M.S., McLean, M., Shutler, G., Yaraghi, Z., Farahani, R., Baird, S., Besner-Johnston, A., Lefebvre, C., Kang, X. & et al. (1995) The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell*, **80**, 167-78.

Roy, N., Devereaux, Q.L., Takahashi, R., Salvesen, G. and J.C. Reed (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J.* **16**: 6914-6925.

Sagar, S.M., Sharp, F.R and T. Curran (1988) Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* **240**: 1328-31.

Sanchez-Ramos, J.R., Song, S., Facca, A., Basit, A and C.J. Epstein (1997) Transgenic murine dopaminergic neurons expressing human Cu/Zn superoxide dismutase exhibit increased density in culture, but no resistance to methylphenylpyridinium-induced degeneration. *J Neurochem.* **68**: 58-67.

Sauer, H and W.H. Oertal (1994) Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience.* **59**(2): 401-15.

Sauer, H., Rosenblad, C. and A. Bjorklund (1995) Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. *Proc Natl Acad Sci USA* **92**(19):8935-9.

Sayre, L.M., Wang, F.J. Arora, P.K., Riachi, N.J., Harik, S.I. and C.L. Hoppel (1991) Dopaminergic neurotoxicity in vivo and inhibition of mitochondrial respiration in vitro by possible endogenous pyridinium-like substances. *J. Neurochem.* **57**(6): 2106-15.

Schapira, A.H. and J.M. Cooper (1992) Mitochondrial function in neurodegeneration and ageing. *Mutat. Res.* **275**(3-6): 133-143.

Schapira, A.H., Cooper, J.M., Dexter, D., Jenner, P., Clark, J.B. and C.D. Marsden (1989) Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* **1**: 1269.

Schierle, G.S., Hannson, O., Leist, M., Nicotera, P., Widner, H. and P. Brundin (1999) Caspase inhibition reduces apoptosis and increases survival of nigral transplants. *Nat Med.* **5**(1): 97-1000.

Schoenberg, B.S., Anderson, D.W. and A.F. Haerer (1985) Prevalence of Parkinson's disease in the biracial population of Copiah County, Mississippi. *Neurology* **35**: 841-845.

Schulman, L.M. (2000) Levodopa toxicity in Parkinson's disease: reality or myth? Reality – practice patterns should change. *Arch Neurol* **57**: 406-7.

Schultz, J.B., Matthews, R.T., Muqit, M.M., Browne, S.E. and M.F. Beal (1995) Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. *J. Neurochem.* **64**: 936-939.

Scott, W.K., Stajich, J.M., Yamaoka, L.H., Speer, M.C., Vance, J.M., Roses, A.D., Pericak-Vance, M.A. and Deane Lab Parkinson Disease Research Group (1997) [Letter] Genetic complexity and Parkinson's disease. *Science* **277**: 387-388.

Semchuk, K.M., Love, E.J. and R.G. Lee (1993) Parkinson's disease: a test of the multifactorial etiologic hypothesis. *Neurology* **43**(6): 1173-1180.

Sengstock, G.J., Olanow, C.W., Dunn, A.J. and G.W. Arendash (1992) Iron induces degeneration of nigrostriatal neurons. *Brain Res Bull* **28**:645-649.

Sheehan, J.P., Swerdlow, R.H., Parker, W.D., Miller, S.W., Davis, R.E. and J.B. Tuttle (1997) Altered calcium homeostasis in cells transformed by mitochondria from individuals with Parkinson's disease. *J. Neurochem* **68**: 1221-1233.

Sheng, M and M.E. Greenberg (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron.* **4**(4): 477-85.

Shimada, H., Hirai, K.-I., Simamura, E. and J. Pan (1998) Mitochondrial NADH-quinone oxidoreductase of the outer membrane is responsible for paraquat cytotoxicity in rat livers. *Arch. Biochem. Biophys.* **351**: 75-81

Shimura-Miura, H., Hattori, N., Kang, D., Miyako, K., Nakabeppu, Y. and Y. Mizuno (1999) Increased 8-oxo-dGTPase in the mitochodria of substantia nigra neurons in Parkinson's disease. *Ann Neurol* **46**: 920-924.

Shoulson, I., Glaubieger, G.A. and T.N. Chase (1975) "On-off" response: clinical and biochemical correlations during oral and intravenous levodopa administration in Parkinsonian patients. *Neurology* **25**: 1147-1148.

Simons, M., Beinroth, S., Gleichmann, M., Liston, P., Korneluk, R.G., MacKenzie, A.E., Bahr, M., Klockgether, T., Robertson, G.S., Weller, M. and J.B. Schulz (1999) Adenovirus-mediated gene transfer of inhibitors of apoptosis protein delays apoptosis in cerebellar granule neurons. *J. Neurochem.* **72**: 292-301

Skorski, T., Perrotti, D., Nieborowska-Skorska, M., Gryaznov, S. and B. Calabretta (1997) Antileukemia effect of c-myc N3'-P5' phosphoramidate antisense oligonucleotides *in vivo*. *Proc Natl. Acad. Sci USA* **94**: 3966-3971.

Sofic, E., Paulus, W., Jellinger, K., Riederer, P. and M.B.H. Youdim (1991) Selective increase of iron in substantia nigra zona compacta in parkinsonian brains. *J. Neurochem.* **56**: 978-982.

Sofic, E., Lange, K.W., Jellinger, K. and P. Reiderer (1992) Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neurosci Lett.* **142**: 128-130.

Sonsalla, P.K., Manzino, L. and H.E. Heikkila (1988) Interactions of D1 and D2 dopamine receptors on the ipsilateral vs. contralateral side in rats with unilateral lesions of the dopaminergic nigrostriatal pathway. *J Pharmacol Exp Ther* **247**: 180-185.

Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R. and M. Goedert (1997) α-synuclein in Lewy bodies. *Nature* **388**: 839-840.

Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., and M. Goegert (1998) α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy Bodies. *Proc. Natl. Acad. Sci USA* **95**: 6569-6473.

Stadler, J., Billiar, T., Curran R., Stuehr, D., Ochoa, J. and R. Simmons (1991) Effects of exogenous and endogenous nitric oxide on mitchondrial respiration of rat hepatocytes. *Am J Physiol* **260**: 910-916.

Stein, C.A. (1995) Does antisense exist? *Nat Med.* **1**: 1119-1121.

Studer, L., Taber, V. and R.D. MacKay (1998) Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. *Nat Neurosci.* **1**: 290-295.

Suhr, S.T. and F.H. Gage (1993) Gene therapy for neurologic disease. *Arch Neurol.* **50**:1252-68.

Svenson, L.W. (1991) Regional disparities in the annual prevalence rates of Parkinson's disease in Canada. *Neuroepidemiology*. **10**(4): 205-10.

Swerlow, R.H., Parks, J.K., Miller, S.W., Tuttle, J.B., Trimmer, P.A., Sheehan, J.P., Bennett Jr., J.P., Davis, R.E. and W.D. Parker Jr. (1996) Origin and functional consequences of the complex I defect in Parkinson's disease. *Ann Neurol* **40**: 663-671.

Swerdlow, R.H., Parks, J.K., Davis, J.N., Cassarino, D.S., Trimmer, P.A., Currie, L.J., Dougherty, J., Bridges, W.S., Bennett Jr., J.P., Wooten, G.F and W.D. Parker (1998) Martilineal inheritance of complex-I dysfunction in a multigenerational Parkinson's disease family. *Ann Neurol.* **44**: 873-881.

Takahashi R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G.S. and J.C. Reed (1998) A single BIR domain of XIAP sufficient for inhibiting caspases. *J. Biol. Chem.* **273**: 7787-7790.

Takayama, H., Ray, J., Raymon, H.K., Baird, A., Hogg, J., Fisher, L.J. and F.H. Gage (1995) Basic fibroblast growth factor increases dopaminergic graft survival and function in a rat model of Parkinson's disease. *Nat Med.* **1**(1): 53-58.

Tanner, C.M. (1989) The role of environmental toxins in the etiology of Parkinson's disease. *Trends Neurosci* **12**: 49-54.

Tanner, C.M. and S.M. Goldman (1996) Epidemiology of Parkinson's disease. *Neurol Clin.***14**(2): 317-335.

Temlett, J.A., Landsberg, J.P., Watt, F. and G.W. Grime (1994) Increased iron in the substantia nigra compacta of the MPTP-lesioned hemiparkinsonian African green monkey: evidence from proton microprobe elemental microanalysis. *J. Neurochem* **62**: 134-146.

Thompson, C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267**: 1456-62.

Tomac, A., Lindqvist, E., Lin, L.F., Ogren, S.O., Young, D., Hoffer, B.J. and L. Olson (1995) Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. *Nature* **373**: 335-9.

Ungerstedt, U. and G. Arbuthnott (1970) Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res.* **24**: 485-493.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Mortiz, R.L., Simpson, R.J. and D.L. Vaux (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102**(1): 43-53.

Vitek, J.L and R.A. Bakay (1997) The role of pallidotomy in Parkinson's disease and dystonia. *Curr Opin Neurol.* **10**(4): 332-9.

Wang, K.K.W. and P.-O. Yuen (1994) Calpain inhibition: an overview of its therapeutic potential. *Trends Pharm Sci* **15**: 412-419.

Wang, S.L., Hawkins, C.J., Yoo, S.J., Muller, H.A. and B.A. Hay (1999) The Drosophila caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* **98**: 453-463.

Wang, W.W., Khajavi, M., Patel, B.J., Beach, J., Jankovic, J and T. Ashizawa (1998) The G209A mutation in the α-synuclein gene is not detected in familial cases of Parkinson disease in non-Greek and/or Italian populations. *Arch Neurol.* **55**: 1521-1523.

Weiner, W.J. (2000) Is levodopa toxic? *Arch Neurol.* **57**:408-10.

Weiss, S., Reynolds, B.A., Vescovi, A.L., Morshead, C., Craig, C.G. and D. van der Kooy (1996) Is there a neural stem cell in the mammalian forebrain? *TINS* **19**(9): 387-393.

Wilk, S. and M. Stanley (1978) Dopamine metabolites in human brain. *Psychopharmacol* **57**: 77-81

Wooten, G.F. (1997) Functional anantomical and behavioural consequences of dopamine receptor stimulation. *Ann NY Acad Sci* **835**: 153-6.

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

Xu, D.G., Korneluk, R.G., Tamai, K., Wigle, N., Hakim, A., MacKenzie, A. and G.S. Robertson (1997a) Distribution of neuronal apoptosis inhibitory protein-like immunoreactivity in the rat central nervous system. *J Comp Neurol.* **382**(2): 247-59.

Xu, D.G., Crocker, S.J., Doucet, J.P., St-Jean, M., Tamai, K., Hakim, A., Ikeda, J.E., Liston, P., Thompson, C.S., Korneluk, R.G., MacKenzie, A. and G.S. Robertson (1997b) Elevation of neuronal expression of NAIP reduced ischemic damage in the rat hippocampus. *Nat Med.* **3**(9): 997-1004.

Xu, D.G., Bureau, Y., McIntyre, D.C., Nicholson, D.W., Liston, P., Zhu, Y., Fong, W.G., Crocker, S.J., Korneluk, R.G. and G.S. Robertson (1999) Attenuation of ischemia-induced cellular and behavioural deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. *J. Neurosci.* **19**: 5026-5033.

Yamada, M., Oligino, T., Mata, M., Gross, J.R., Glorioso, J.C. and D.J. Fink (1999) Herpes simplex virus vector-mediated expression of Bcl-2 prevents 6-hydroxydopamine-induced degeneration of neurons in the substantia nigra *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **96**: 4078-4083.

Yen, J., Wisdom, R.M., Tratner, I. and I.M. Verma (1991) An alternative spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins. *Proc Natl Acad. Sci USA* **88**: 5077-5081.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and H.R. Horvitz (1993) The C.elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta converting enzyme. *Cell.* **75**(4): 641-52.

Zerial, M., Toschi, L., Ryseck, R-P., Schuermann, M., Muller, R. and R. Bravo (1989) The product of the a novel growth factor activated gene, fosB, interacts with JUN proteins enhancing their DNA binding activity. *EMBO J* **8**(3): 805-13.

Zhang, J., Graham, D.G., Montine, T.J. and Y.S. Ho (2000) Enhanced N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity in mice deficient in Cu/Zn-superoxide dismutase or glutathione peroxidase. *J Neuropathol Exp Neurol.* **59**: 53-61.

Appendix A. Letters for Permission to Reprint Published Materials

FCR/jl/may00.011
22 May 2000

Mr Stephen J Crocker
Neuroscience Research Institute
University of Ottawa
451 Smyth Road
Ottawa ON K1H 8M5
Canada

ELSEVIER SCIENCE

Elsevier Science Limited

The Boulevard
Langford Lane
Kidlington
Oxford OX5 1GB
England

Tel: +44 (0) 1865 ...
Fax: +44 (0) 1865 ...

www.elsevier.nl

Dear Mr Crocker

MOLECULAR BRAIN RESEARCH, Vol 53, 1998, pp69-77

As per your letter dated 9 May 2000, we hereby grant you permission to reprint the aforementioned material at no charge **in your thesis** subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made as follows:

   "Reprinted from Journal title, Volume number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier Science".

3. Reproduction of this material is confined to the purpose for which permission is hereby given.

4. This permission is granted for non-exclusive world **English** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.

5. This includes permission for the National Library of Canada to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely

Frances Rothwell (Mrs)
pp Subsidiary Rights Manager

The processing of permission requests for all Elsevier Science (including Pergamon imprint) journals has been centralised in Oxford, UK. Your future requests will be handled more quickly if you write directly to: Subsidiary Rights Department, Elsevier Science, PO Box 800, Oxford OX5 1DX, UK.
Fax: 44-1865 853333; e-mail: permissions@elsevier.co.uk

Université d'Ottawa • University of Ottawa
Institut de recherche en neurosciences
Neuroscience Research Institute

The Editor-in-Chief
European Journal of Neuroscience
Dept of Experimental Psychology
University of Cambridge
Downing Street
Cambridge CB2 3EB
UK

May 31, 2000

Dear Dr. Everitt,

We would like to submit the enclosed manuscript entitled "**Neuronal Apoptosis Inhibitory Protein Protects Nigrostriatal Dopamine Pathway from Intrastriatal 6-OHDA-induced Degeneration in the Adult Rat**" by S.J. Crocker *et al.* to be considered for publication as an original research article in the *European Journal of Neuroscience*. Please find enclosed a copy of the full manuscript with figures.

This work not under review at another journal and has not been previously published. All authors on this study have approved this final version of this manuscript as submitted.

This article represents the first demonstration of the neuroprotective actions of a member of the human Inhibitor of Apoptosis (IAP) proteins in a Parkinson's disease model. This novel group of proteins has gained interest because they have been previously reported by others to be potent endogenous inhibitors of the cell death proteases, caspases. Given the putative central role caspases have been purported to play in neurodegenerative conditions, we have also investigated the involvement of caspase-3 this adult *in vivo* model of PD. We provide evidence which suggest that, in this model, NAIP-mediated neuroprotection is enacted in manner independent of caspase-3.

We would like to suggest the following as possible expert reviewers who we feel are especially qualified to referee this manuscript.

Robert E Burke
Department of Neurology
Columbia University
710 W 168th St,
New York, NY
10032
Fax: 212-305-5450
Phone: 212-305-7374

Anders M Bjorklund
Wallenberg Neurocentrum.
Inst. Fysiologi
Neurovetenskap.
Solvegatan 17
223 62 Lund
Sweden
Fax: 046-222-0559
Phone: 046-222-0541

Faculté de Médecine • Faculty of Medicine
451 Smyth Ottawa ON K1H 8M5
(613) 562-5461 • Téléc./Fax: 562-5403
http://www.nri.on.ca

Martha C. Bohn
Department of Pediatrics
Northwestern University Medical School
Children's Memorial Hospital
2300 Children's Plaza, Box 209
Chicago, IL 60614
Fax: 773-868-8066
Phone: 773-868-8052

Ronald J Mandel
Department of Neuroscience
University of Florida Brain Institute
Gainesville, FL
32610-0244 USA
Fax: 352-392-8347
Phone: 352-294-0446

Thank you for your consideration.

Sincerely,

Stephen J. Crocker

Encl.

Children's Hospital of Eastern Ontario
Hôpital pour enfants de l'est de l'Ontario
A University of Ottawa Teaching Hospital / Un Hôpital d'enseignement affillié a l'Université d'Ottawa
401 SMYTH, OTTAWA, ONT. K1H 8L1 TELEPHONE (613) 737-7600

August 28, 2000

Dr. C. Jennings, Editor
Nature Neuroscience
345 Park Avenue South
New York, NY
USA 10010-1707

Dear Sir:

We would like to submit the enclosed manuscript entitled "**Mice Overexpressing XIAP are resistant to MPTP-induced Neurotoxicity**" by S.J. Crocker *et al.* to be considered for publication as an original research article in *Nature Neuroscience*.

This article represents the first description of a transgenic animal which overexpresses the human Inhibitor of Apoptosis (IAP) protein, XIAP. Of significance, we demonstrate that mice which overexpress XIAP, under the control of the neuron-specific enolase promoter, are profoundly resistant to the deleterious effects of the dopaminergic neurotoxin, MPTP. In light of recent reports on the intracellular interactions of XIAP, we feel this report to be timely. Indeed, XIAP has received an increasing amount of attention with recent publications by Du *et al.* (102: 33-42), and Verhagen *et al.* (102: 43-53) in *Cell*, and more recently by Chai *et al.* (406. 855-862), in *Nature*

This work not under review at any other journal, and has not been previously published. All authors on this study have approved this final version of this manuscript as submitted

Please find four (4) copies of the manuscript, with figures, enclosed As well, attached is a list of suggested reviewers who have expertise in this field of neuroscience

We thank you for your consideration and look forward to hearing from you in due course.

Sincerely.

R.G. Korneluk, Ph.D.
Children's Hospital of Eastern Ontario Research Institute
Phone (613) 738-3281
Fax (613) 738-4833
E-mail: ccc@mgcnec.med.uottawa.ca

Appendix B. Additional Publications

Elevation of neuronal expression of NAIP reduces ischemic damage in the rat hippocampus

D.G. Xu[1], S.J. Crocker[1], J.-P. Doucet[1], M. St-Jean[1], K. Tamai[2], A.M. Hakim[1], J.-E. Ikeda[3], P. Liston[4], C.S. Thompson[5], R.G. Korneluk[4], A. MacKenzie[1] & G.S. Robertson[1]

[1]*Department of Cellular and Molecular Medicine, and Neuroscience Research Institute, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5*

[2]*Medical and Biological Laboratories Co., Ltd., 5F Sumitomoshoji Marunouchi Building, 5-10 Marunouchi 3 chome, Naka-ku, Nagoya 460, Japan*

[3]*The Institute of Medical Science, Tokai University, Kanagawa 259-11, Japan*

[4]*ApoptoGen, Inc., and Molecular Genetics Research Laboratory, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada K1H 8L1*

We show here that transient forebrain ischemia selectively elevates levels of neuronal apoptosis inhibitory protein (NAIP) in rat neurons that are resistant to the injurious effects of this treatment. This observation suggests that increasing NAIP levels may confer protection against ischemic cell death. Consistent with this proposal, we demonstrate that two other treatments that increase neuronal NAIP levels, systemic administration of the bacterial alkaloid K252a and intracerebral injection of an adenovirus vector capable of overexpressing NAIP *in vivo*, reduce ischemic damage in the rat hippocampus. Taken together, these findings suggest that NAIP may play a key role in conferring resistance to ischemic damage and that treatments that elevate neuronal levels of this antiapoptotic protein may have utility in the treatment of stroke.

Transient forebrain ischemia causes damage to certain neuronal populations. Neurons in neocortex, CA1 of the hippocampus, reticular thalamus and medium-sized striatal neurons are among the most vulnerable [1]. Recent morphological and biochemical evidence suggests that these neurons may die by an apoptotic mechanism after transient global ischemia [2]. Consistent with this proposal, transgenic mice that overexpress the antiapoptotic protein Bcl-2 are more resistant to ischemic brain damage [3]. Similarly, delivery into the hippocampus and striatum of a herpes simplex virus vector capable of overexpressing Bcl-2 *in vivo* attenuated the damaging effects of focal ischemia in these structures [4]. These findings suggest that manipulations that elevate expression of antiapoptotic proteins such as Bcl-2 may have utility in the treatment of stroke. However, the fact that Bcl-2 levels are very low in the adult central nervous system, coupled with the observation that Bcl-2 concentrations do not change appreciably after transient cerebral ischemia, indicates that this protein is not a key determinant of susceptibility to neuronal death following ischemia [5].

Recently, we isolated a candidate gene for spinal muscular atrophy (SMA) [6]. This gene, encoding neuronal apoptosis inhibitory protein (NAIP), is homologous to two baculovirus inhibitors of apoptosis proteins (Cp-IAP and Op-IAP) and is partly deleted in individuals with type I SMA. Furthermore, we have demonstrated that overexpression of NAIP protects several different cell lines from apoptotic death induced by a variety of stimuli [7]. These data are consistent with an absence or reduction of NAIP leading to excessive apoptotic death of motor neurons exacerbating the SMA phenotype. In keeping with this proposal, we have demonstrated a close correspondence between the regional distribution of NAIP-like immunoreactivity (NAIP-LI) in the central nervous system (CNS) and the previously reported neurodegenerative alterations in SMA (ref. 10). In the present study we have examined the effects of transient forebrain ischemia on NAIP-LI in the hippocampus, striatum and thalamus of rats to elucidate the functional role of NAIP as an antiapoptotic protein in ischemia-induced neuronal injuries.

A bacterial alkaloid, K252a, has been shown to exert neuroprotective effects both *in vitro* and *in vivo*. Unlike neurotrophic proteins, this compound can readily cross the blood–brain barrier. Systemic administration of K252a has been shown to attenuate the loss of CA3 neurons produced by intrahippocampal injection of kainic acid [8]. Since excitotoxic mechanisms have been implicated in ischemic cell death, we determined whether pretreatment with K252a reduces the loss of CA1 hippocampal neurons in rats after transient global ischemia. We have also examined the effects of K252a administration on NAIP expression in the rat thalamus, hippocampus, striatum and spinal cord. Last, we have assessed the neuroprotective potential of NAIP by determining whether virally mediated NAIP overexpression renders rat CA1 neurons more resistant to the damaging effects of transient cerebral ischemia.

Effects of transient forebrain ischemia on NAIP levels

Initially, we surveyed the forebrain for changes in neuronal NAIP expression following 10 periods of transient cerebral ischemia of rats using the 4-vessel occlusion model [11]. Low levels of NAIP-LI were detected in hippocampal CA1 neurons of sham-treated animals (Fig. 1*a*). The intensity of NAIP-LI in CA1 neurons did not appear to be altered 3 and 24 hours after 10 minutes of transient forebrain ischemia (Fig. 1*b* and

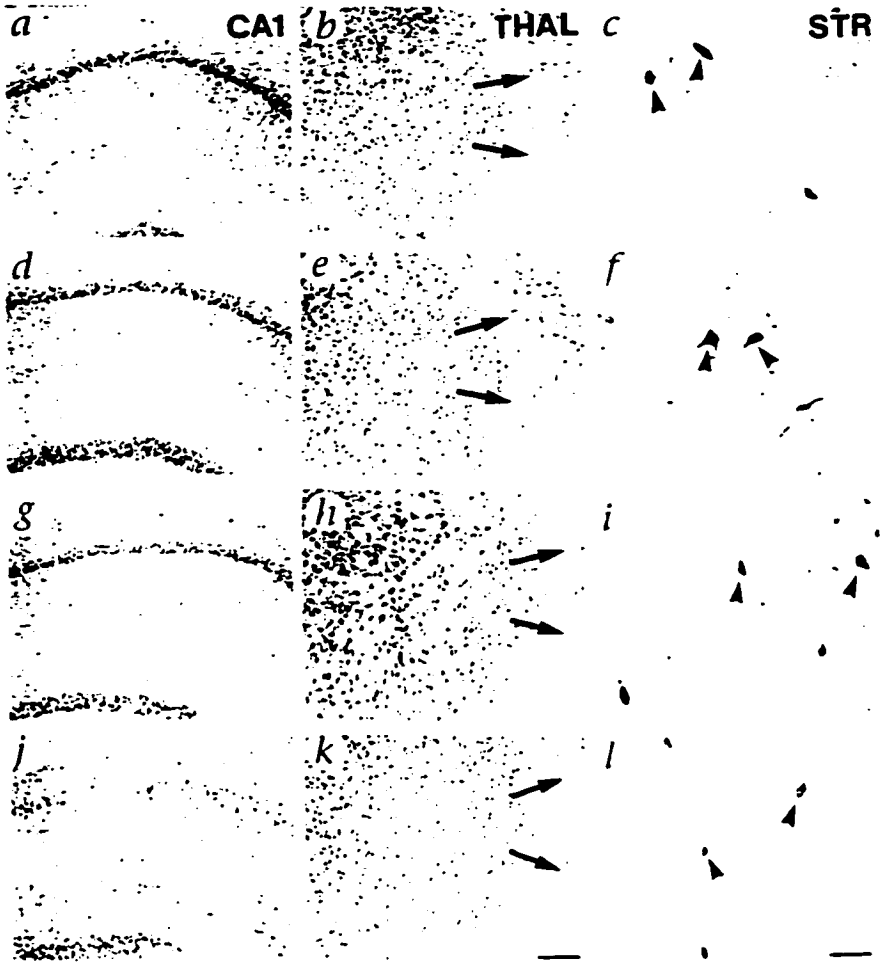


Fig. 1 NAIP-like immunoreactivity in hippocampal CA1 (*a, d, g* and *j*), thalamic (*b, e, h* and *k*) and striatal (*c, f, i* and *l*) neurons of rats subjected to a sham ischemia procedure (*a, b* and *c*) or 10 min of transient forebrain ischemia (*d-l*). Moderate levels of NAIP-LI were present 24 h after the sham procedure in hippocampal CA1 neurons (*a*) and ventrolateral thalamus (*b*). In striatum (*c*), high levels of NAIP-LI were exhibited by large cholinergic neurons (arrowheads). No significant change in NAIP-LI occurred in CA1 neurons at 3 h and 24 h after global ischemia (*d* and *g*), whereas NAIP levels appeared to be decreased at 72 h (*j*). NAIP-LI was dramatically elevated in ventral and lateral aspects of the thalamus 3 h (*e*) and 24 h (*h*) after four-vessel occlusion. In contrast, NAIP-LI was not elevated in the reticular nucleus of the thalamus (arrows) at these time points. At 72 h, NAIP-LI still appeared to be elevated in ventral and lateral regions of the thalamus but was clearly depressed below basal levels in the reticular nucleus (arrows) (*k*). NAIP-LI seemed to be elevated primarily in large-sized striatal neurons (arrowheads) 3 h (*f*) and 24 h (*i*) after global ischemia. By 72 h, the intensity of NAIP labeling in large striatal neurons (arrowheads) appeared to have declined to basal levels (*l*). Scale bars: *a, b, d, e, g, h, j, k*, 300 μm; *c, f, i* and *l*, 50 μm.

g). However, NAIP-LI decreased to below basal levels 72 hours following recirculation (Fig. 1*j*). The majority of neurons in the ventrolateral and ventromedial portions of the thalamus contained NAIP-LI (Fig. 1*b*). In general, these neuronal populations displayed large increases in NAIP-LI 3 and 24 hours after restoration of blood flow (Fig. 1*e* and *h*). A notable exception was the reticular nucleus of the thalamus, which failed to display enhanced labeling at these time points. By 72 hours, NAIP-LI in the reticular nucleus of the thalamus was depressed below basal levels, whereas the intensity of NAIP staining in other regions of the thalamus had returned to normal (Fig. 1*k*). NAIP-LI in the striatum was located in about 1% of the total neuronal population. These neurons were large in size (25–30 μm in diameter) and displayed higher levels of NAIP-LI than hippocampal CA1 neurons (Fig. 1*c*). Consistent with these features, double-labeling immunohistofluorescence has revealed that striatal NAIP-LI is localized exclusively in cholinergic interneurons. Transient forebrain ischemia produced a large elevation of NAIP-LI in striatal cholinergic neurons 3 hours after reperfusion (Fig. 1*f*). NAIP-LI still appeared to be elevated at 24 hours but had returned to basal levels 72 hours after 4-VO (Fig. 1*i* and *l*).

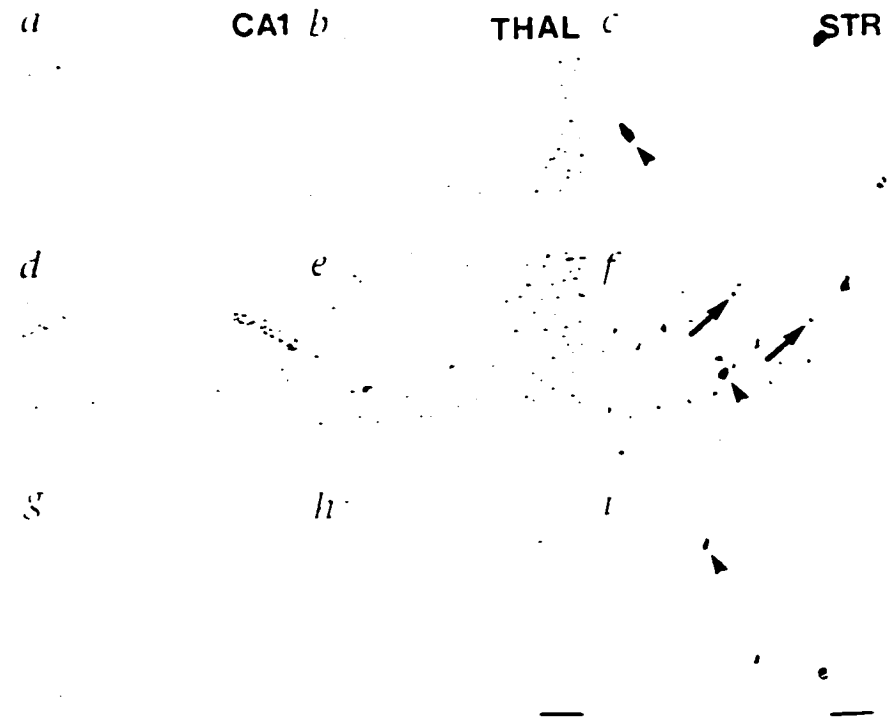


Fig. 2 Effects of a single administration of vehicle (1 ml/kg, s.c.) or K252a (0.1 mg/kg, s.c.) on NAIP-LI in the hippocampus (*a, d* and *g*), thalamus (*b, e* and *h*) and striatum (*c, f* and *i*). In rats treated with vehicle, moderate levels of NAIP-LI were present in hippocampal CA1 (*a*) and thalamic (*b*) neurons in the sham (S) group, high levels of NAIP-LI were exhibited by large striatal (*c*) neurons (arrowheads) in the sham group, 3 h after a 10-min [illegible] ischemia, NAIP-LI was dramatically elevated in the thalamus (*e*) and striatum (*f*) but not in the hippocampus (*d*). 24 h after ischemia, NAIP-LI had returned to basal levels in the hippocampus (*g*), thalamus (*h*) and striatum (*i*). Scale bar: [illegible] and *h*, 300 μm; *c, f* and *i*, 50 μm.

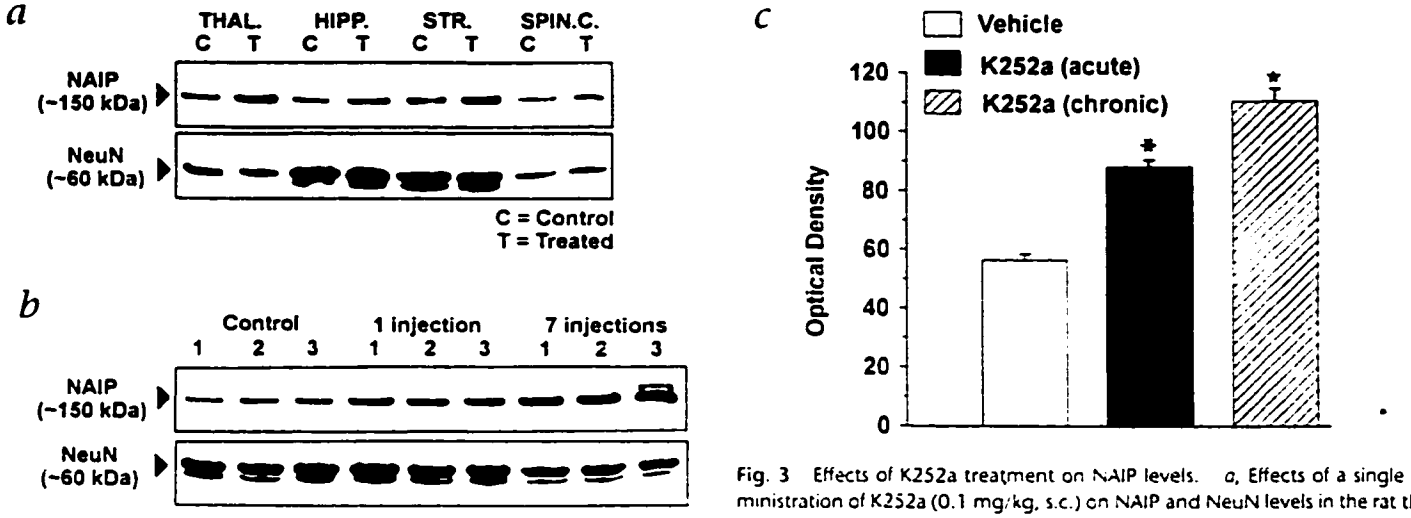


Fig. 3 Effects of K252a treatment on NAIP levels. *a*, Effects of a single administration of K252a (0.1 mg/kg, s.c.) on NAIP and NeuN levels in the rat thalamus, hippocampus, striatum and spinal cord. Western analysis of NAIP and NeuN levels in the thalamus, hippocampus, striatum and spinal cord 3 h after a single injection of vehicle (1 ml/kg, s.c.) (control, C) or K252a (0.1 mg/kg, s.c.) (treated, T). K252a elevated NAIP (150 kDa) levels in all four regions with the largest increases occurring in thalamus and striatum. High levels of NeuN (60 kDa) were present in hippocampus and striatum; lower levels were present in the thalamus and spinal cord. In all four brain regions, NeuN levels were similar in control and treated conditions, indicating that each of these lanes was loaded with an equivalent amount of proteins. The same membrane was used for immunoblotting of NAIP and NeuN. *b*, Comparison of the effects of acute and chronic administration of K252a on NAIP and NeuN levels in the rat hippocampus. Western analysis of NAIP and NeuN levels in the hippocampus after a single injection of vehicle (1 ml/kg, s.c.) or K252a (0.1 mg/kg, s.c.) or chronic administration of K252a (0.1 mg/kg, s.c., once daily for 7 days). By comparison with vehicle-injected rats, a single administration of K252a elevated NAIP levels (150 kDa) in all three animals. Chronic administration of K252a produced a further elevation of NAIP levels in each of the three animals that were examined. NeuN levels remained constant, indicating that each of these lanes was loaded with an equivalent amount of proteins. *c*, Densitometric measurement of the effects of single and repeated administration of K252a on NAIP levels in the hippocampus. A single administration of K252a (0.1 mg/kg, s.c.) elevated NAIP levels by approximately 50%, whereas chronic administration of K252a (0.1 mg/kg, s.c., once daily for 7 days) increased NAIP levels by approximately 100%. Histograms and bars represent means and s.e.m. for three animals. Asterisk, significantly different from vehicle-treated animals ($P < 0.01$, Newman-Keuls test). Star, significantly different from rats that received an acute injection of vehicle or K252a ($P < 0.01$, Newman-Keuls test).

Effects of acute K252a administration on NAIP levels

Three hours after a single administration of K252a [0.1 mg/kg, subcutaneously (s.c.)], NAIP-LI was enhanced in CA1 neurons (Fig. 2, *a* and *d*). By 24 hours, NAIP-LI had returned to preinjection levels in these neurons (Fig. 2*g*). Similarly, NAIP-LI in the ventromedial and ventrolateral regions of the thalamus was elevated 3 hours after a single administration of K252a (0.1 mg/kg, s.c.) (Fig. 2, *b* and *e*). By 24 hours, NAIP-LI had returned to normal levels in these thalamic areas (Fig. 2*h*). NAIP-LI was also increased in striatal neurons 3 hours after K252a (0.1 mg/kg, s.c.) and returned to basal levels by 24 hours (Fig. 2, *c*, *f* and *i*). In the striatum, K252a administration appeared to increase NAIP-LI primarily in medium-sized neurons (Fig. 2*f*). Immunoblotting indicated that the NAIP antibody recognized a single band with a molecular mass of 150 kDa, the predicted molecular mass for NAIP (Fig. 3*a*). Consistent with immunohistochemical results indicating the presence of NAIP within a large number of thalamic neurons, western analysis demonstrated that this antiapoptotic protein is highly enriched in the thalamus (Fig. 3*a*). Despite the fact that NAIP expression appears to be restricted to only 1% of striatal neurons (cholinergic interneurons), levels similar to that observed in the thalamus were found in the striatum. Comparatively smaller, but clearly discernible, levels of NAIP protein were detected in the hippocampus and spinal cord. In agreement with our immunohistochemical findings, immunoblotting revealed that NAIP levels were elevated in the striatum, hippocampus and thalamus 3 hours after a single administration of K252a (0.1 mg/kg, s.c.) (Fig. 3*a*). As a control for protein loading, western analysis of the levels of the neuron-specific marker protein NeuN was performed on the same membrane used to detect NAIP. Immunoblotting demonstrated that high levels of NeuN (60 kDa) were present in the hippocampus and striatum, whereas lower levels are found in the thalamus and spinal

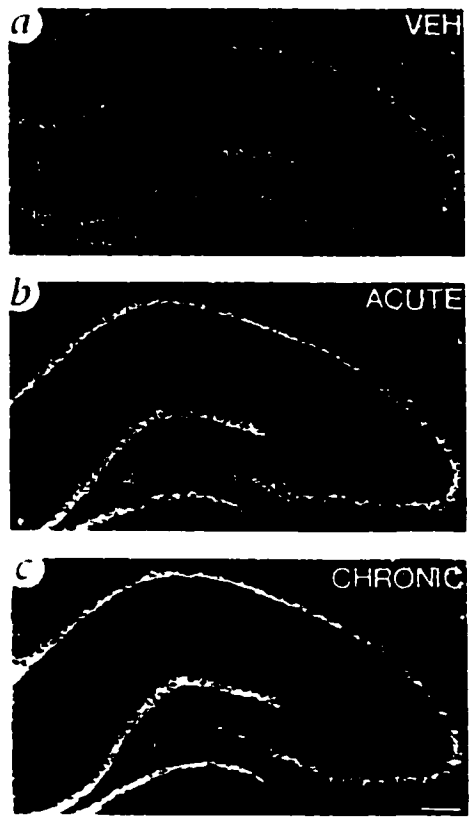


Fig. 4 Effects of acute and chronic administration of K252a on expression of NAIP mRNA in the rat hippocampus as detected by *in situ* hybridization histochemistry. [illegible] levels of NAIP mRNA were present in the hippocampus of animals injected with vehicle (1 ml/kg, [illegible]). A single administration of K252a (0.1 mg/kg, [illegible]) elevated NAIP mRNA levels in the hippocampus. Chronic administration of K252a (0.1 mg/kg, s.c., once daily for 7 days) produced a further elevation of NAIP mRNA levels. All animals were killed 3 h after the last injection.

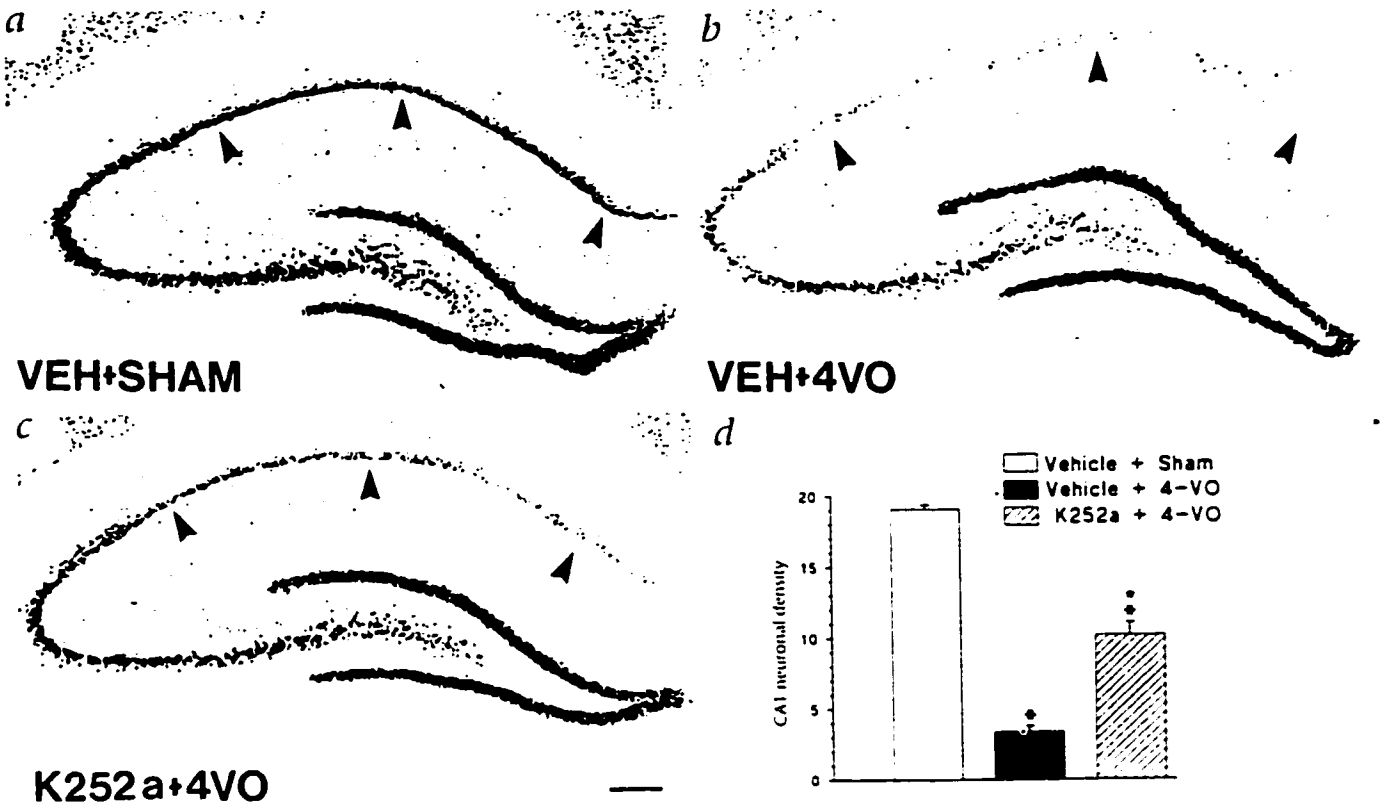


Fig. 5 Effects of chronic pretreatment with K252a on CA1 neuronal loss 5 days after 10 min of four-vessel occlusion (4-VO) *a*, Immunohistochemical detection of neurons with a monoclonal antibody (A60) against the neuron-specific marker NeuN in the hippocampus of sham-operated animals injected with vehicle (VEH + SHAM, arrows indicate CA1 region) *b*, Massive loss of NeuN-like immunoreactive neurons in the CA1 region (arrowheads) of vehicle-treated animals 5 days after recirculation (VEH + 4VO) *c*, Reduction of ischemia-induced neuronal death in the CA1 region (arrowheads) after chronic K252a administration (K252a + 4VO) Scale bar, 500 μm. *d*, The density of CA1 neurons expressing NeuN-LI assessed by computer-assisted image analysis A 70% loss of CA1 neurons was detected in the CA1 region 5 days after 10 min of 4-VO Chronic administration of K252a reduced ischemic damage in the CA1 region by approximately 50% (K252a + 4VO). Histograms and bars represents means and the s.e.m. for six animals Asterisk, significantly different from vehicle-treated shams (VEH + SHAM) ($P < 0.01$, Newman-Keuls test) Star, significantly different from vehicle-treated animals subjected to 4-VO (VEH + 4-VO) ($P < 0.01$, Newman-Keuls test)

cord (Fig. 3*a*). In all four brain regions, NeuN levels were similar in animals treated with vehicle (1 ml/kg, s.c.) or K252a (0.1 mg/kg, s.c.), confirming that each lane was loaded with equivalent amounts of protein.

Effects of chronic K252a administration on NAIP levels

Acute administration of K252a (0.1 mg/kg, s.c.) appeared to elevate NAIP levels in the hippocampi of each of the three animals examined (Fig. 3*b*). Optical density measurements of the 150-kDa NAIP immunoreactive band indicated that acute administration of K252a (0.1 mg/kg, s.c.) elevated NAIP levels in the hippocampus by approximately 50% (Fig. 3*c*). Levels of NAIP were further elevated by chronic administration of K252a (0.1 mg/kg, s.c., once daily for 7 days) (Fig. 3*b* and *c*). By comparison with that in vehicle-injected controls, chronic administration of K252a (0.1 mg/kg, s.c., once daily for 7 days) increased NAIP concentration by approximately [illegible] μg protein loading). Western [illegible] performed on the same [illegible] immunoblotting demonstrated that NeuN levels were not elevated by either acute or chronic administration of K252a, indicating that each lane was loaded with equivalent [illegible]

Effects of K252a administration on NAIP mRNA expression

In situ hybridization histochemistry revealed that low levels of NAIP mRNA are present in the hippocampus of animals injected with vehicle (Fig. 4*a*). Administration of K252a (0.1 mg/kg, s.c.) produced a rapid (3-hour) elevation of NAIP mRNA levels in CA1–CA3 subfields as well as the dentate gyrus (Fig. 4*b*). Chronic administration of K252a (0.1 mg/kg per day for 7 days) appeared to result in a further elevation of NAIP mRNA levels in the hippocampus (Fig. 4*c*).

Table 1 Effects of acute administration of K252a on blood pressure (BP), pH, pO and pCO

Time after K252a treatment (min)	BP (mm Hg)	pH	pO (mm Hg)	pCO (mm Hg)
baseline	114.5 ± 3.8	7.44 ± 0.01	92.2 ± 3.1	35 ± 4
30	120.0 ± 3.8	7.45 ± 0.03	96.3 ± 3.3	33.2 ± 5
60	125.0 ± 3.8	7.46 ± 0.03	89.9 ± 1.7	31.3 ± 4
90	117.3 ± 4.8	7.46 ± 0.02	94.3 ± 4.3	33.1 ± [illegible]
120	113 ± 3	7.47 ± 0.02	93.0 ± 4.7	31 ± [illegible]
150	123.3 ± [illegible]	7.44 ± [illegible]	[illegible] ± 2	31 ± [illegible]
180	122 ± [illegible]	[illegible]	[illegible] ± 1.8	33 ± [illegible]

Data represent the means ± s.e.m. (n = 3 animals).
Acute administration of K252a (0.1 mg/kg, s.c.) failed to alter blood pressure, pH, pO and pCO ($P > 0.05$, one-way analysis of variance). Baseline measurements were performed 30 min before administration of K252a.

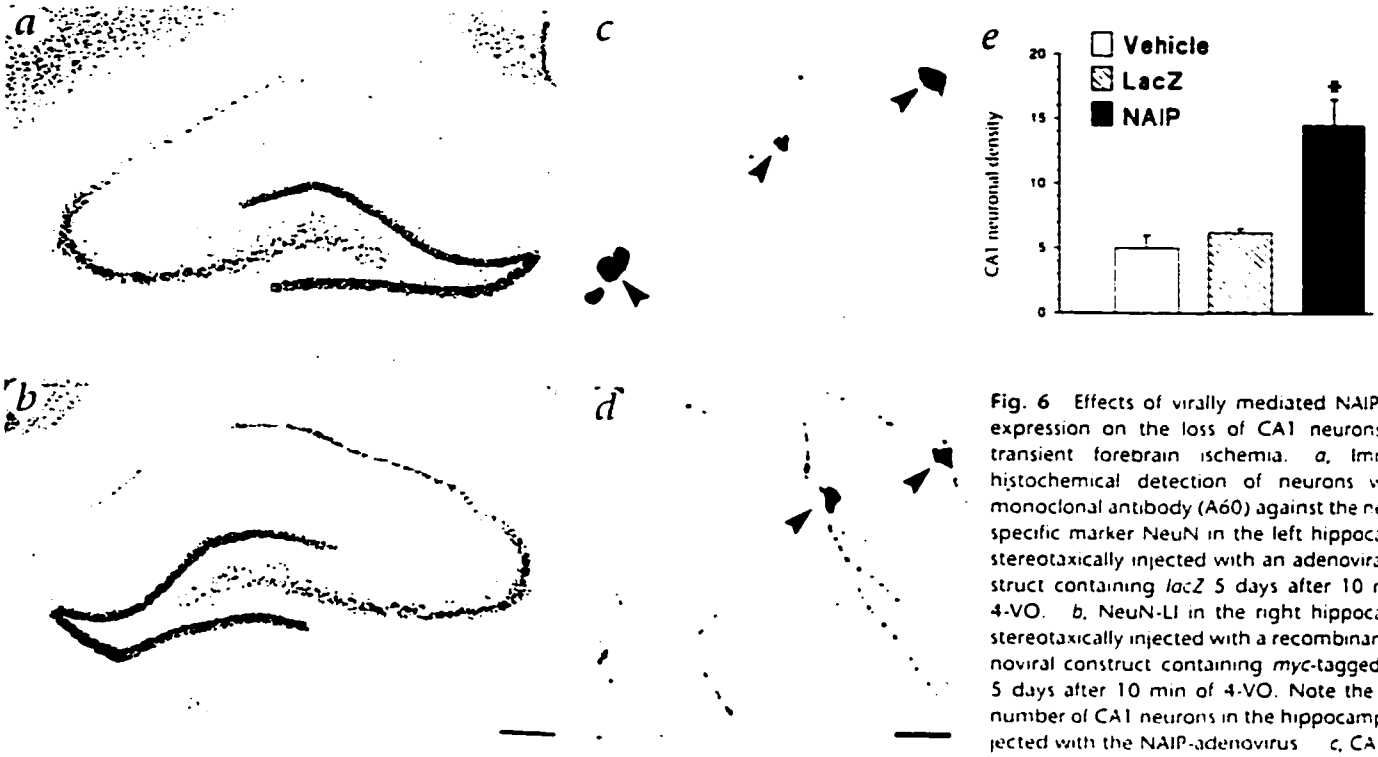


Fig. 6 Effects of virally mediated NAIP overexpression on the loss of CA1 neurons after transient forebrain ischemia. *a*, Immunohistochemical detection of neurons with a monoclonal antibody (A60) against the neuron-specific marker NeuN in the left hippocampus stereotaxically injected with an adenoviral construct containing *lacZ* 5 days after 10 min of 4-VO. *b*, NeuN-LI in the right hippocampus stereotaxically injected with a recombinant adenoviral construct containing *myc*-tagged NAIP 5 days after 10 min of 4-VO. Note the larger number of CA1 neurons in the hippocampus injected with the NAIP-adenovirus. *c*, CA1 neurons (arrowheads) that display X-gal staining after intrahippocampal injection of the *lacZ* construct in an animal that was subjected to sham 4-VO. *d*, CA1 neurons (arrowheads) in the NAIP-adenovirus-injected side labeled with the mAb 9E10, which recognizes *myc*-tagged NAIP. Blank regions in the center of the CA1 field represent damage produced by the injection procedure. Scale bars, *a* and *b*, 500 μm; *c* and *d*, 25 μm. *e*, Quantitative comparisons of the effects of virally mediated NAIP and *lacZ* overexpression on the loss of CA1 neurons after transient forebrain ischemia. The density of CA1 neurons expressing NeuN-LI was assessed by computer-assisted image analysis. Neuronal density represents the number of labeled cells per 100 μm length of the CA1 pyramidal layer. Cell counts indicated that a similar number of neurons were present in CA1 subfields injected with either vehicle or lacZ-adenovirus. By comparison with these treatments, the NAIP-adenovirus produced a doubling in CA1 neuronal survival. Histograms and bars represent means and s.e.m. for six animals. Asterisk, significantly different from either vehicle or *lacZ* vector ($P < 0.01$, Newman Keuls test).

Chronic K252a administration protects neurons from ischemic injury

Transient cerebral ischemia reduced the number of CA1 neurons in the hippocampus by approximately 70% (Fig. 5, *a* and *b*). Systemic administration of K252a (0.1 mg/kg, s.c., once daily) for 7 days before 4-VO and each day afterwards (5 days) significantly reduced the loss of hippocampal CA1 neurons produced by 10 minutes of transient forebrain ischemia (Fig. 5*c*). Cell counts indicated that chronic K252a administration reduced depletion of CA1 neurons by about 50% (Fig. 5*d*).

Effects of K252a on blood pressure, pH, pO_2 and pCO_2

The basal values (means ± s.e.m.) for blood pressure, pH, pO_2 and pCO_2 were 114.5 ± 13.8 mm Hg, 7.44 ± 0.01, 92.2 ± 3.1 mm Hg and 35.6 ± 4.9 mm Hg, respectively (Table 1). These values were not significantly different from those measured at each of the time points noted after administration of K252a (0.1 mg/kg, s.c.) (Table 1).

Neuroprotective effects of virally mediated NAIP overexpression

To test the neuroprotective potential of elevating neuronal NAIP levels directly, we determined whether intrahippocampal injection of a recombinant adenoviral construct capable of overexpressing *myc*-tagged NAIP reduced CA1 neuron loss 5 days after an episode of transient cerebral ischemia. As a control, the recombinant adenoviral construct containing a gene encoding the bacterial enzyme LacZ was injected into the contralateral hip-

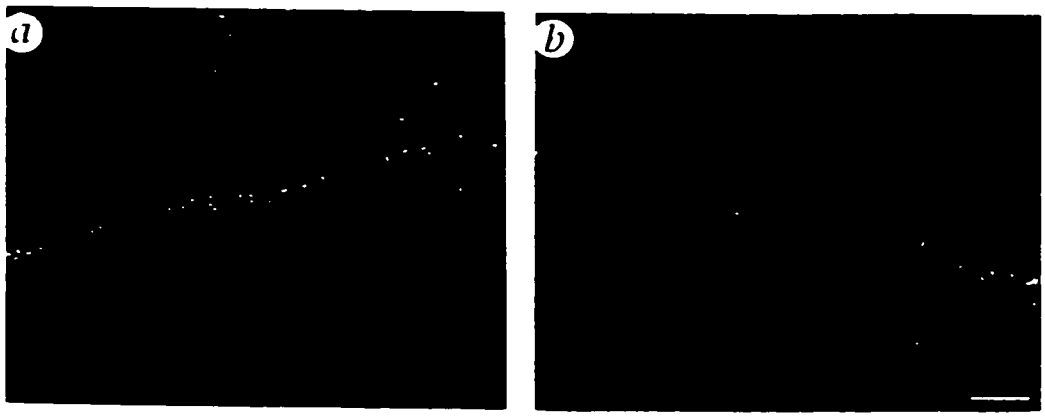


Fig. 7 Effects of intrahippocampal injection of *lacZ*- and NAIP-adenoviral constructs on DNA fragmentation in CA1 neurons 5 days after an episode of transient cerebral ischemia, as demonstrated by TUNEL. A large [illegible] were observed in the [illegible] injected with the NAIP-adenovirus. Bar, 100 μm.

pocampus. Cell counts indicated that by comparison with the *lacZ*-injected side, the NAIP-adenovirus produced a doubling in neuronal survival (Fig. 6, *a*, *b* and *e*). Overexpression of NAIP and *lacZ* was confirmed by immunohistochemical detection of Myc and 5-chromo-4-chloro-3-indoyl-β-galactoside (X-gal) staining, respectively (Fig. 6, *c* and *d*). Cell counts revealed that similar numbers of neurons were infected by the NAIP-adenoviral construct (7 ± 1/100 μm) and the *lacZ*-adenoviral construct (10 ± 2/100 μm) in animals subjected to sham 4-VO. Although a similar number of Myc immunoreactive neurons was observed in animals exposed to an episode of transient cerebral ischemia, few X-gal-stained neurons were detected in the CA1 region after global ischemia (data not shown). As a control for the effects of the *lacZ*-adenovirus on CA1 neuron survival, a second group of rats received intrahippocampal injections of *lacZ*-adenovirus (left side) and the vehicle used to suspend the adenoviral constructs (right side). Cell counts indicated that a similar number of CA1 neurons survived after an episode of transient cerebral ischemia in hippocampi injected with either vehicle or the *lacZ*-adenoviral construct (Fig. 6*e*). In order to establish whether NAIP overexpression may reduce ischemic injury by inhibiting apoptosis, *in situ* end labeling (ISEL) of fragmented DNA was performed 5 days after ischemia. Consistent with previous reports [7], ischemic injury in the *lacZ*-adenovirus-injected side was associated with a large number of ISEL-labeled CA1 neurons (9 ± 2/100 μm). In contrast, considerably fewer ISEL-positive CA1 neurons (3 ± 1/100 μm) were seen in hippocampi injected with the NAIP-adenoviral construct (Fig. 7, *a* and *b*).

Discussion

NAIP expression and resistance to ischemic injury. Transient cerebral ischemia dramatically elevated NAIP-like immunoreactivity in striatal cholinergic neurons, whereas NAIP-LI in medium spiny neurons in the striatum did not appear to be appreciably increased by this insult. With the notable exception of neurons in the reticular nucleus of the thalamus, neuronal labeling in ventral and lateral aspects of the thalamus was also substantially increased by a short period of global ischemia. Consistent with reports of depressed protein synthesis in CA1 neurons after transient global ischemia [8], 4-VO did not elevate NAIP-LI in these neurons in rats; rather, a reduction in immunoreactivity was observed 72 hours after recirculation. Hence, those neuronal populations known to be susceptible to the damaging effects of transient global ischemia, such as striatal medium spiny, thalamic reticular and hippocampal CA1 neurons [1], failed to display elevated NAIP-LI after 10 minutes of 4-VO. In contrast, we observed large increases in levels of this antiapoptotic protein in striatal cholinergic and thalamic neurons, which are resistant to the injurious effects of a transient ischemic attack [1]. It is therefore tempting to speculate that the susceptibility of these neuronal populations to the damaging effects of transient cerebral ischemia may be determined, at least in part, by their ability to increase NAIP levels after an ischemic insult.

K252a reduces ischemic damage and elevates NAIP expression. What initiated the present study was that pretreatment with the neuroprotective compound K252a reduced CA1 loss after 10 minutes of transient cerebral ischemia in rats. Our results indicate that ischemic damage in the hippocampus was decreased by administration of K252a once daily (0.1 mg/kg, s.c.) for 7 days before 4-VO and each day afterwards. Because CA1 neuron loss was examined 5 days after 4-VO, further studies will be required to confirm that the neuroprotective effects of K252a are not transient. Administration of K252a did not alter blood pressure, gases (pCO_2 and pO_2) or pH, indicating that it did not reduce CA1 neuron loss by altering these physiological variables. The fact that chronic treatment with K252a was required to decrease ischemic damage suggests that pretreatment may elevate the expression of protective protein (or proteins) that renders CA1 neurons more resistant to the injurious effects of transient cerebral ischemia. Consistent with this proposal, we observed that acute administration of K252a (0.1 mg/kg, s.c.) elevated both NAIP mRNA and protein levels in the hippocampus. Moreover, chronic administration of K252a (0.1 mg/kg, s.c., once daily for 7 days) produced a larger elevation of NAIP mRNA and protein levels than seen after a single administration of this compound (0.1 mg/kg, s.c.). It is therefore possible that the larger increases in NAIP levels we have observed after chronic compared to acute treatment with K252a may have relevance for reports indicating that repeated K252a administration produces greater neuroprotection than a single administration of this compound [9]. Acute administration of K252a also increased NAIP levels in the rat striatum, thalamus and spinal cord, suggesting that this compound may also exert neuroprotective actions in these regions through enhanced NAIP expression. In keeping with this hypothesis, K252a has been shown to have neurotrophic-like effects on primary cultures of striatal and spinal motor neurons [10].

The pathways by which K252a upregulates NAIP expression remain to be determined. Several lines of evidence suggests that K252-like compounds exert their beneficial effects by activating growth factor receptor-linked tyrosine kinases. K252a possesses some of the biological effects of nerve growth factor [11] and can stimulate tyrosine phosphorylation [12]. Furthermore, the neuroprotective effects of K252-like compounds are blocked by the selective tyrosine kinase inhibitor genistein, but not by its inactive analogue, genistin [13]. Whether the ability of K252a to increase NAIP expression is related to activation of a tyrosine kinase-mediated transduction pathway remains to be clarified.

Virally mediated NAIP overexpression is neuroprotective. An adenoviral construct capable of overexpressing NAIP mRNA was used to determine whether increasing levels of this antiapoptotic protein reduced the damaging effects of transient forebrain ischemia in the hippocampus. As a control, an adenoviral construct containing the gene encoding the bacterial enzyme [illegible] was injected into the contralateral hippocampus. Cell counts revealed that the NAIP-adenovirus increased CA1 neuron survival after an episode of transient forebrain ischemia in rats [illegible]. NAIP generated from the adenoviral construct was Myc-tagged, which made it possible to identify infected cells by immunohistochemistry [illegible] by using an antibody that selectively recognizes the Myc antigen. Immunohistochemical detection of Myc demonstrated that CA1 neurons were infected by the NAIP-adenovirus. Cell counts demonstrated that on average, [illegible] neurons per [illegible] were infected by the NAIP-adenovirus. [illegible] the degree of [illegible] CA1 neuron survival [illegible] the contralateral [illegible] (5 ± 2/100 μm).

[illegible] have addressed [illegible] which NAIP

duces ischemic neuronal damage. Several studies employing *in situ* end labeling (ISEL) have shown that CA1 neurons injured by transient cerebral ischemia display fragmented DNA, a characteristic feature of apoptosis[1,4]. We therefore performed ISEL labeling on hippocampal sections from rats that had received stereotaxic injections of *lacZ*- and NAIP-adenoviral constructs. In agreement with previous reports, a large number of ISEL-positive CA1 neurons were detected 5 days after an episode of transient cerebral ischemia in the hippocampus, after it had received a stereotaxic injection of the *lacZ*-adenovirus. By comparison, considerably fewer ISEL-positive CA1 neurons were observed in the contralateral hippocampus, which had been injected with the NAIP-adenovirus. Inasmuch as ISEL labeling (DNA fragmentation) is indicative of apoptosis, these findings suggest that NAIP reduced ischemic neuronal injury by blocking apoptosis.

In summary, we have demonstrated that rat neuronal survival following a brief episode of global ischemia and the neuroprotective effects of K252a are closely correlated with increased expression of NAIP, suggesting that induction of this novel antiapoptotic gene may confer resistance to ischemic injury. In support of this proposal, virally mediated overexpression of NAIP reduced the injurious effects of transient cerebral ischemia on CA1 neurons in rats. These findings suggest that treatments that upregulate NAIP expression may have therapeutic utility in stroke.

Methods

Experimental protocols. In a first experiment, we examined the effects of transient forebrain ischemia on NAIP-LI in the rat hippocampus, thalamus and striatum. Three groups, composed of five animals each, were subjected to 10 min of 4-VO and killed 3, 24 and 72 h following reperfusion. A fourth group was subjected to sham 4-VO and killed 24 h later. In a second experiment, we determined the effects of acute K252a (0.1 mg/kg, s.c.) administration on NAIP-LI in the hippocampus, thalamus and striatum. Two groups, composed of three animals each, were injected with K252a (0.1 mg/kg, s.c.) and allowed to survive for either 3 or 24 h. A third group served as vehicle controls. These animals were injected with vehicle [1 ml/kg, aqueous solution containing 10% cyclodextrin and 0.5% dimethyl sulfoxide (DMSO)] and killed 3 h later. A third experiment was performed to determine the effects of K252a on NAIP and NeuN levels as measured by western blotting. Two groups, composed of three animals each, were injected with vehicle (1 ml/kg, s.c.) or K252a (0.1 mg/kg, s.c.) and killed 3 h later. In a fourth experiment, we compared the effects of acute and chronic K252a administration on NAIP mRNA and protein levels in the hippocampus by *in situ* hybridization histochemistry and western blotting, respectively. Three groups, composed of six animals each, were injected with vehicle (1 ml/kg, s.c.), K252a (0.1 mg/kg, s.c.) or K252a (0.1 mg/kg, s.c., once daily for 7 days) and killed 3 h after the last injection. In a fifth experiment, we examined the effects of K252a (0.1 mg/kg, s.c.) on blood pressure, gases (O_2 and CO_2) and pH in four animals. The femoral artery was cannulated with a polyethylene catheter for monitoring of blood pressure, gases and pH. In a sixth experiment, we assessed the effects of K252a administration on CA1 neuronal loss after transient global ischemia. Two groups, composed of six animals each, were injected subcutaneously (s.c.) with vehicle (1 ml/kg, s.c.) or K252a (0.1 mg/kg, s.c.) once daily for 7 days. All of the rats were subjected to 4-VO (10 min) 3 h after the last injection and given a second injection as soon as circulation was restored. A third [illegible] described previously and subjected to [illegible] receive either K252a (0.1 mg/kg, s.c.) [illegible] days after the 4-VO or sham procedure. [illegible] h after the last injection and brain samples processed for immunostaining of NeuN to assess cell loss in CA1. In a final experiment, we examined the effects of virally mediated NAIP overexpression on the loss of CA1 neurons produced by an episode of transient forebrain ischemia. Twelve male rats were stereotaxically injected with recombinant adenoviral constructs (Ad5d1X) containing either *lacZ* or *myc*-tagged NAIP into the left and right dorsal hippocampus, respectively. Adenovirus vectors (3 µl injected over 10 min; 1 × 10⁹ particles per microliter) were injected using a 28-gauge needle at the coordinates (in millimeters): AP –3.6, ML ± 2.2 and DV –3.2 from bregma[31]. Viral constructs have been previously described by Liston *et al.*[7]. Seven days later, half of the animals in each of these groups were subjected to 10 min of 4-VO. The other half were subjected to sham 4-VO. All of these animals were killed 5 days later. CA1 neuron loss was assessed histologically by counting NeuN immunoreactive neurons in the CA1 region of the hippocampus. Ischemic-induced DNA fragmentation was assessed using *in situ* end labeling as previously described by Fliss and Gattinger. Neurons infected with *lacZ*- and *myc*-tagged NAIP-adenoviral constructs were identified by X-gal staining[32] and immunohistochemical detection of the Myc protein tag, respectively. Another group of six animals was stereotaxically injected with the *lacZ*-adenoviral construct or vehicle (10% glycerol in 0.15 M Tris-buffered saline) into the left and right dorsal hippocampus, respectively. These animals were also subjected to 10 min of global ischemia, and their brains were examined for CA1 neuronal damage by NeuN immunohistochemistry.

Transient forebrain ischemia model. Adult male Wistar rats (250–275 g, Charles River Laboratories, Montréal) were used for all of the experiments. All animal procedures conformed to the *Guide for the Care and Use of Experimental Animals*[33] endorsed by the Medical Research Council of Canada. Transient forebrain ischemia was produced using published modifications[34] of the 4-VO method[3]. Animals that had convulsions during 4-VO, or after reperfusion, along with those that did not fully develop loss of righting reflex during ischemia were omitted from the study. In the case of sham-treated animals, the carotids were exposed but not occluded.

NAIP riboprobe. The template for a rat NAIP riboprobe was generated by RT-PCR from rat hippocampal mRNA using primers directed against nucleotide sequences conserved in mouse and human NAIP (ref. 8). A 227-bp sequence was ligated into the *Eco*RI site of the plasmid pcDNA3 (Invitrogen, San Diego, CA). Both sense and antisense ³⁵S-labeled riboprobes were generated from linearized plasmid templated using the MAXIscript *in vitro* transcription kit (Ambion, Inc., Austin, TX). Specific activities ranged from 0.6 to 1.2 × 10⁹ c.p.m. per µl.

***In situ* hybridization histochemistry.** Cryostat sections (12 µm thick) were incubated in hybridization buffer containing either a sense or an antisense NAIP riboprobe (1 × 10⁶ c.p.m. per 100 µl per slide) overnight at 56 °C. Sections were then treated with ribonuclease A (10 µg/ml) at 35 °C for 30 min and washed in 2× SSC for 30 min. After drying, the slides were dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled water; Eastman Kodak, Rochester, NY) at 42 °C, dried and exposed for 2 weeks at 4 °C.

Antibodies and immunohistochemistry. The antibody against NAIP was raised in rabbits against exons 7–11 of NAIP (150-amino acid fragment) and affinity-purified. NeuN was detected using a monoclonal antibody (also referred to as A60, generously provided by R.J. Mullen)[17]. *myc*-tagged NAIP was detected using a monoclonal antibody (mAb 9E10), which binds to the Myc protein tag MEQKLISEEDL. Immunohistochemical detection of NAIP (diluted 1:750), NeuN (1:200) or mAb 9E10 (1:50) was performed on free-floating cryostat sections (12 µm thick) using a previously described method[7].

Western blotting. Cytoplasmic extracts were prepared from the appropriate brain tissues and subjected to electrophoresis in a highly porous 10% gel SDS-PAGE system as described previously. The proteins were transferred to nitrocellulose membranes electrophoretically as described [illegible]. Following incubation with the primary antibodies (NAIP [illegible] or A60 1:1000), the membranes were [illegible] with highly purified [illegible] secondary antibodies [illegible] ImmunoResearch Laboratories, West Grove, PA) and [illegible] horseradish peroxidase (1:2000, Amersham, Buckinghamshire, UK). Finally, the blots were developed by enhanced chemiluminescence (ECL, Amersham).

Statistical analysis. A one-way analysis of variance was performed on the cell count and densitometric data. A one-way analysis of variance with [illegible]

peated measures over time was conducted on the blood gases and pH. If the analysis was significant, multiple comparisons were performed by using the Newman-Keuls test.

Acknowledgment

This study was supported by grants from the Heart and Stroke Foundation of Ontario (NA-2938) and Medical Research Council of Canada (MT-11539).

RECEIVED 28 FEBRUARY; ACCEPTED 1 JULY 1997

1. Pulsinelli, W.A., Brierly, J.B. & Plum, F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann. Neurol.* 11, 491–498 (1982).
2. Smith, M.L., Auer, R.N. & Siesjo, B.K. The density and distribution of ischemic brain injury in the rat following 2-10 min of forebrain ischemia. *Acta Neuropathol. (Berl.)* 64, 319–332 (1984).
3. MacManus, J.P., Buchan, A.M., Hill, I.E., Rasquinha, I. & Preston, E. Differences in DNA fragmentation following transient cerebral or decapitation ischemia in rats. *J. Cerebral Blood Flow Metab.* 15, 728–737 (1995).
4. Nitatori, T. *et al.* Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J. Neurosci.* 15, 1001–1011 (1995).
5. Martinou, J.-C. *et al.* Overexpression of *bcl-2* in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* 13, 1017–1030 (1994).
6. Lawrence, M.S., Ho, D.Y., Sun, G.H., Steinberg, G.K. & Sapolsky, R.M. Overexpression of Bcl-2 with herpes simplex virus vectors protects CNS neurons against neurological insults in vitro and in vivo. *J. Neurosci.* 16, 486–496 (1996).
7. Krajewski, S. *et al.* Up regulation of bax levels in neurons following cerebral ischemia. *J. Neurosci.* 15, 6364–6376 (1995).
8. Roy, N. *et al.* The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell* 80, 167–178 (1995).
9. Liston, P. *et al.* Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* 379, 349–353 (1996).
10. Xu, D.G. *et al.* Distribution of NAIP-LI in the rat central nervous system. *J. Comp. Neurol.* 382, 247–259 (1997).
11. Smith-Swintosky, V.L. *et al.* Bacterial alkaloids mitigate seizure-induced hippocampal damage and spatial memory deficits. *Exp. Neurol.* 141, 287–296 (1996).
12. Tsuiman, R., Xie, Y., Kleinges, P. & Kiessling, M. Persistent inhibition of protein synthesis precedes delayed neuronal death in postischemic gerbil hippocampus. *Acta Neuropathol.* 71, 88–93 (1986).
13. Maruno, M. & Yanagihara, T. Progressive loss of messenger RNA and delayed neuronal death following transient cerebral ischemia in gerbils. *Neurosci. Lett.* 115, 155–160 (1990).
14. Widmann, R. *et al.* [^{14}C]Leucine incorporation into brain proteins in gerbils after transient ischemia: Relationship to selective vulnerability of hippocampus. *J. Neurochem.* 56, 789–796 (1991).
15. Glicksman, M.A. *et al.* K252a and staurosporine promote choline acetyltransferase activity in rat spinal cord cultures. *J. Neurochem.* 61, 210–221 (1993).
16. Glicksman M.A., Forbes, M.E., Prantner J.E. & Neff, N.T. K-252a promotes survival and choline acetyltransferase activity in striatal and basal forebrain neuronal cultures. *J. Neurochem.* 64, 1502–1512 (1995).
17. Knusel, B. & Hefti, F. K252 compounds: Modulators of neurotrophin signal transduction. *J. Neurochem.* 59, 1987–1996 (1992).
18. Maroney, A.C. *et al.* K-252a induces tyrosine phosphorylation of the focal adhesion kinase and neurite outgrowth in human neuroblastoma SH-SY5 cells. *J. Neurochem.* 64, 540–549 (1995).
19. Cheng, B., Barger, S.W. & Mattson, M.P. Staurosporine, K-252a, and K-252b stabilize calcium homeostasis and promote survival of CNS neurons in the absence of glucose. *J. Neurochem.* 62, 1319–1329 (1994).
20. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates*, 2nd edn. (Academic Press, New York, 1986).
21. Fliss, H. & Gattinger, D. Apoptosis in ischemic and reperfused rat myocardium. *Circ. Res.* 79, 949–956 (1996).
22. Canadian Council on Animal Care. *Guide to the Care and Use of Experimental Animals* (eds., Olfert, E.D., Cross, B.M. & McWilliam, A.A.) (Canadian Council on Animal Care, Ottawa, 1993).
23. Pulsinelli, W.A. & Buchan, A.M. The four-vessel occlusion rat model: Method for complete occlusion of vertebral arteries and control of collateral circulation. *Stroke* 19, 913–914 (1988).
24. Mullen, R.J., Buck, C.R. & Smith A.M. NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116, 201–211 (1992).
25. Evan, G.I., Lewis, G.K., Ramsy, G. & Bishop, J.M. Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. *Mol. Cell. Biol.* 5, 3610–3616 (1985).
26. Shu, S., Ju, G. & Fan, L. The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci. Lett.* 85, 169–171 (1988).
27. Doucet, J.-P., Murphy, B.J. & Tuana, B.S. Stabilization of a discontinuous highly porous sodium dodecyl sulfate polyacrylamide gel system for protein electrophoresis. *Anal. Biochem.* 190, 209–211 (1990).
28. Towbin, H., Staehelin, T. & Gordon, T. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350–4354 (1979).