# Factors affecting the neurotoxic effects of 6-hydroxydopamine in an animal model of Parkinson's disease

by

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This thesis is dedicated to the memory of Melanie Reuben, whose skilled technical expertise was surpassed only by her quiet strength, intelligence, and immeasurable kindness.

## ABSTRACT

The neurotoxin 6-hydroxydopamine (6-OHDA) is widely used to produce a neurochemically selective and progressive destruction of dopamine pathways. The resulting motoric deficits and histopathology reproduces several key features of Parkinson's disease, a neurodegenerative disorder for which there is no known cure. The objective of the present thesis was to characterize the *in vivo* neurotoxicity of 6-OHDA in dopamine pathways, and to examine the mechanisms underlying dopamine cell death.

Infusion of 6-OHDA into different dopamine pathways revealed that the nigrostriatal projection was more vulnerable than the mesolimbic dopamine pathway. In addition, a considerable portion of cell bodies was preserved despite significant losses of dopamine markers in nigrostriatal and mesolimbic nerve terminal regions. The partial sparing of cell bodies was enhanced by hypothermia associated with surgical anesthesia. This neuroprotective effect was localized to a specific subregion within the mesolimbic dopamine cell body area. The preservation of dopamine cell bodies following 6-OHDA administration may have functional significance since somatodendritic dopamine release has been demonstrated in this brain area.

The studies in Chapter 3 examined the contribution of two proteases, calpain and caspase-3, in mediating the degeneration resulting from 6-OHDA administration. Calpain activation occurred early and remained elevated in nigrostriatal cell body and terminal field subregions, whereas caspase-3 activation was only transient. Adenoviral delivery of the calpain inhibitor, calpastatin,

completely prevented the elevation in calpain activity following 6-OHDA administration. Moreover, calpastatin delivery significantly improved motor deficits associated with nigrostriatal dysfunction. The behavioural protection was not, however, accompanied by a sparing of nigrostriatal dopamine markers, but was unexpectedly associated with a preservation of mesolimbic dopamine markers. These findings suggest that calpain activation contributes to dopamine cell death, but this effect may be brain region dependent.

Collectively, our findings highlight differential vulnerability amongst midbrain dopamine neurons, of possible relevance to Parkinson's disease. In addition, our work suggests that calpains may represent a future therapeutic target for the treatment of Parkinson's disease. However, present drawbacks associated with this approach indicate that additional studies are necessary before such neuroprotective compounds can be employed to treat Parkinson's disease.

# RÉSUMÉ

La neurotoxine 6-hydroxydopamine est grandement utilisée pour causer la destruction neurochimiquement sélective et progressive des voies dopaminergiques. L'histopathologie et les déficits moteurs qui en résultent reproduisent plusieurs des traits caractéristiques de la maladie de Parkinson, un trouble neurodégénératif pour lequel il n'existe toujours aucune cure. L'objectif de cette thèse est de décrire la neurotoxicité produite par la 6-OHDA *in vivo* dans les voies dopaminergiques, et d'examiner les mécanismes sous-jacents à la mort des neurones dopaminergiques.

L'infusion de 6-OHDA dans différentes voies dopaminergiques révèle que les projections nigro-striées sont plus vulnérables que celles de la voie mésolimbique. De plus, une portion considérable des corps cellulaires est préservée malgré la perte significative de marqueurs dopaminergiques dans les terminaisons nerveuses des régions nigro-striatales et mésolimbique. La sauvegarde partielle des corps cellulaires est accentuée par l'hypothermie associée à l'anesthésie chirurgicale. Cet effet neuroprotecteur est localisé dans un site spécifique situé à l'intérieur de la région mésolimbique où l'on retrouve les corps cellulaires dopaminergiques. La préservation des corps cellulaires dopaminergiques suite à l'administration de 6-OHDA pourrait avoir une signification fonctionnelle puisque la sécrétion somatodendritique de dopamine a été démontrée dans cette région cérébrale.

Les études présentées au chapitre 3 évaluent la contribution de deux protéases, calpaïne et caspase-3, dans la médiation de la dégénaration causée par l'administration de 6-OHDA.

L'activation de la calpaïne se produit rapidement et de façon soutenue dans les corps cellulaires nigro-striés et les sous-régions terminales, alors que l'activation de la caspase-3 n'est que temporaire. La présence de calpastatine, un inhibiteur de la calpaïne livrée par un adénovirus, empêche complètement l'activation de la calpaïne suite à l'administration de 6-OHDA. De plus, la présence de calpastatine améliore de façon significative les déficits moteurs associés à la dysfonction du système nigro-striatal. Par contre, la protection comportementale n'est pas accompagnée d'une sauvegarde des marqueurs dopaminergiques du nigro-striatum mais est étonnamment associée avec la préservation des marqueurs dopaminergiques mésolimbiques. Ces résultats suggèrent que l'activation de la calpaïne contribue à la mort des corps cellulaires dopaminergiques mais que cet effet soit spécifique à certaines régions cérébrales.

Collectivement, ces données mettent en évidence la vulnérabilité différentielle parmi les neurones dopaminergiques mésencéphaliques, avec un rapport possible avec la maladie de Parkinson. De plus, nos travaux proposent que les calpaïnes pourraient être des cibles thérapeutiques pour le traitement du Parkinson. Cependant, les désavantages associés avec cette approche indiquent que des études additionnelles sont nécessaires avant que de tels composés neuroprotecteurs puissent être utilisés pour traiter le Parkinson.

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discussions. The tremendous courage, strength, humor, and generosity that they have demonstrated throughout their careers has imparted a model for me to live up to.

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My gratitude extends to my parents, John and Vicki Grant, who have made many sacrifices to give me the educational opportunities and life enhancing experiences that I have been lucky to enjoy.

Finally, a special thanks to Milan Jelaca for his unwavering support, and for inspiring me to persevere in the face of adversity. Without a doubt, this thesis wouldn't have been completed if it were not for his constant encouragement and enduring confidence in me.

## CONTRIBUTION OF AUTHORS

This thesis is written in manuscript format as permitted by the McGill Faculty of Graduate Studies and is composed of two manuscripts. The contributions of each author is outlined below.

**Chapter 2**: R.J. Grant and P.B.S. Clarke (2002). Susceptibility of ascending dopamine projections to 6-hydroxydopamine in rats: effect of hypothermia. Neuroscience 115 (4): p. 1281-1294.

I performed all of the experiments in this manuscript. The techniques included stereotaxic surgeries (lesions and chronic indwelling cannulae), brain temperature monitoring, cardiac perfusions, cryosectioning, autoradiography for [$^{125}$I]RTI-55, immuno-autoradiography for tyrosine hydroxylase, immunohistochemistry for tyrosine hydroxylase, Nissl staining, quantitative autoradiographic analysis on the MCID 4 imaging system, neuronal profile counts using the optical fractionator on the MCID M5+ imaging system, and statistical analyses.

The manuscript was written and prepared by Rebecca Grant and Paul Clarke.

**Chapter 3** : R.J. Grant, L. Sellings, S.J. Crocker, E. Melloni, D.S. Park, and P. B. S. Clarke. Calpain inhibition preserves motor function following intrastriatal 6-hydroxydopamine administration: evidence for a non-nigrostriatal mechanism. *Submitted.*

In Chapter 3, I performed the great majority of the experiments. My contributions were the following: stereotaxic surgeries (lesions and adenovirus infusion), Western blotting for alpha-spectrin, green fluorescent protein, and actin, densitometry using the M4 Imaging system,

cryosectioning, Nissl staining, immunohistochemistry for calpain-specific breakdown product (150kDa), autoradiography for [$^{125}$I]RTI-55, immuno-autoradiography for tyrosine hydroxylase, and behavioural testing (forelimb stepping and forelimb reaching).

The manuscript was written and prepared by Rebecca Grant and Paul Clarke.

Laurie Sellings illustrated the Western blotting technique, set up the computer-aided testing room for behavioural tests, and performed the vertical exploration behavioural test, and the preliminary forelimb stepping and reaching tests.

Our collaborators in the lab of Dr. David Park provided us with the calpastatin- and GFP-expressing adenoviruses. Dr. Stephen Crocker demonstrated the stereotaxic infusion of adenovirus, and gave us with helpful feedback regarding the manuscript.

Dr. E. Melloni provided our collaborators in Dr. David Park's lab with the construct for making the calpastatin-expressing adenovirus.

## CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

In this thesis I have presented the following original results:

**CHAPTER 2:**

- The nigrostriatal dopamine pathway is more susceptible to neurotoxicity induced by 6-hydroxydopamine administration than the mesolimbic dopamine pathway
- Hypothermia reduces the neurotoxicity of 6-hydroxydopamine
- Neuroprotection by hypothermia is regionally selective, occuring in the lateral ventral tegmental area, and in the terminal regions
- Brain temperature is accurately estimated by measuring rectal temperature
- Rectal and brain temperature decrease substantially (30.4-34.6°C) following anesthetic administration

**CHAPTER 3:**

- The proteases, calpain and caspase-3, are activated following intra-striatal 6-OHDA administration
- The activation of calpain and caspase-3 occurs in the striatum and dopamine cell body regions
- Calpain activation remains elevated for approximately 2 weeks, whereas caspase-3 activity is transient
- Adenoviral delivery of the calpain inhibitor, calpastatin, completely prevents calpain activation in the striatum and dopamine cell body regions
- Calpastatin delivery results in significant behavioural recovery in tests of motor skill

## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ad.GFP | adenovirus expressing green fluorescent protein |
| Ad.CALP | adenovirus expressing calpastatin |
| DA | dopamine |
| DAergic | dopaminergic |
| DAT | dopamine transporter |
| 6-OHDA | 6-hydroxydopamine |
| IA | interaural |
| MFB | medial forebrain bundle |
| MDMA | 3,4-methylenedioxymethamphetamine |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| PD | Parkinson's disease |
| PFA | paraformaldehyde |
| SNR | substantia nigra pars reticulatata |
| PBS | phosphate buffered saline |
| RTI-55 | 3β-(4-iodophenyl)tropan-2β-carboxylic acid methylester |
| SBDP | spectrin breakdown product |
| SN | substantia nigra |
| SNC | substantia nigra pars compacta |
| TH | tyrosine hydroxylase |
| TX | Triton X-100 |
| VTA | ventral tegmental area |
| VEH | vehicle |

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## FORWARD: Dopamine, a short history

Despite being synthesized and tested for biological activity in 1910 (Barger and Ewins, 1910), dopamine, and its precursor L-DOPA, were considered to be pharmacologically inactive (Barger and Dale 1910). Sourkes (2000) illuminates the very different experience of Markus Guggenheim, the first director of pharmacological research at Hoffman-LaRoche Company.

> Finding that his animal subjects responded poorly to L-DOPA, Guggenheim swallowed the remainder of his sample, and soon vomited. He later recalled his experience, and how he had interpreted his emesis as a nonspecific irritation of the gastric mucosa caused by L-DOPA. In fact, the amino acid, extracted from legumes that Guggenheim had grown at home, was decarboxylated to yield dopamine, which then stimulated the trigger zone of the emetic centre in the medulla oblongata. This was the first induced, although unrecognized, action of dopamine.

The importance of dopamine was unknown as recently as fifty years ago, during which time dopamine was considered to be merely an intermediate compound in the synthesis of noradrenaline and adrenaline. The Swedish research team led by Arvid Carlsson developed a method for detecting dopamine content (Carlsson and Waldeck, 1958) and then showed that this chemical exists in significant quantities in the brain. Soon after, Carlsson reported the distribution of dopamine the in brain, noting its accumulation in the basal ganglia, structures known to be involved in motor function (Carlsson et al., 1958).

The discovery that dopamine exists in brain led to extensive research examining its role in the central nervous system. In this regard, Carlsson provided the first evidence linking dopamine to the control of movement. Carlsson (1957) gave rabbits the catecholamine-depleting drug, reserpine, and observed the occurrence of a dramatic behavioural rigidity resembling the

symptomatology of Parkinson's disease (Fig. 1). Carlsson (1957) then administered the precursors of serotonin and dopamine/noradrenaline (5-hydroxytryptophan and L-DOPA, respectively) and determined that the reserpine-induced behavioural rigidity was due to a loss of dopamine, but not of serotonin or noradrenaline. These findings prompted Carlsson to propose a therapeutic role for L-DOPA in Parkinson's disease, and he presented these significant findings at the First International Catecholamine Symposium in 1958.

Carlsson's novel findings did not influence the scientific landscape immediately. In the early 1960s, controversy arose regarding the existence of chemical transmission in the brain and in various parts of the peripheral nervous system. At a Ciba Foundation Symposium, Nobel Laureate Henry Dale supported the existence of chemical transmission, but Sir John Eccles argued in favour of electrical transmission across synapses. Carlsson's findings provided perhaps the first clear evidence of chemical transmission in the brain, but alas, the "soup vs. sparks" debate was initially unaffected by these observations. In retrospect, Carlsson suggested that the implications of his work were straightforward, and that he and his collaborators "simply had the advantage of being ignorant and not so much burdened by dogma" (Carlsson 2000 Nobel Lecture).

Within several years of this meeting, the pathways of major chemicals in the brain, including dopamine, were revealed using the novel technique of histofluorescence (And'en et al., 1964). This method reveals the presence of monoamines in cell bodies,

Figure 1. Rabbits treated with intravenous reserpine before DL-DOPA administration (top panel) and after L-DOPA (bottom panel). From Carlsson et al., 1957.

axons, and dendrites based on the fact that monoamines yield a fluorescent compound under blue-violet light when combined with formaldehyde. Accordingly, by the mid-1960s, a paradigm shift had clearly taken place in neuroscience. The idea of chemical transmission had gained general acceptance, and the critical role of dopamine and other amines as chemical mediators was acknowledged.

The contributions of several other scientists shaped our understanding of dopamine's role in the central nervous system. I would like to acknowledge the early works of some of these scientists.

Although difficult to believe in today's world of internet-based communication, parallel findings regarding the role of dopamine in Parkinson's disease were observed independently of Carlsson's shortly thereafter. To this end, the Canadian researcher Theodore Sourkes and his student, G. Murphy gave L-DOPA to animals and reported a transient but significant increase in brain dopamine for approximately eight hours after only a single administration (Sourkes 1961). These observations suggested that, in humans, dopamine loss may underlie motoric symptoms of Parkinson's disease. Accordingly, in collaboration with the Montreal neurologist A. Barbeau, the Canadian researchers reported a reduction of dopamine (but not of other catecholamines) in the urine of Parkinson's disease patients (Barbeau et al., 1961).

But the Canadians were not alone in their quest to investigate the putative role of dopamine in patients suffering from Parkinson's disease. While their latter paper was in press, Viennese researchers (Ehringer and Hornykiewicz, 1960) demonstrated a loss of dopamine directly in

post-mortem brain, thereby confirming the role of central dopamine in this disease. These findings led each research team to independently examine whether L-DOPA administration would be a simple way of restoring depleted dopamine levels in clinical Parkinson's disease. The Canadians administered capsules containing either L-DOPA, D-DOPA, or L-tyrosine (the precursor of L-DOPA) to Barbeau's patients; only those receiving L-DOPA experienced temporary relief from muscular rigidity and hypokinesia (Barbeau et al, 1962). The Viennese research team conducted a similar study and found correspondingly favourable results (Birkmayer and Hornykiewicz, 1961).

Remarkable progress regarding the role of central dopamine was also made by the Japanese researcher Isamo Sano. Surprisingly, however, little attention has been paid to his findings. In 1959, less than a year after Carlsson's presentation, the distribution of dopamine in the human brain was presented for the first time by Sano. Aware of Carlsson's reserpinized rabbits, Sano proposed a role for dopamine in Parkinson's disease, and suggested the basal ganglia as an important site of action. At a meeting shortly thereafter (1960), Sano confirmed his latter prediction, and also reported alleviating rigidity and tremor in a Parkinsonian patient given intravenous DL-DOPA
(Sano, 2000; Foley, 2000). Behavioural normalization occurred within 15-30 minutes after DL-DOPA infusion, but was only transient and therefore thought to be of little therapeutic value. Despite his negative conclusion regarding the usefulness of L-DOPA in Parkinson's disease, Sano's findings may have, in fact, preceded those of his colleagues in Europe and North America.

Soon after the discovery that striatal dopamine was deficient in the brains of Parkinson's patients, the beneficial effect of L-DOPA on Parkinsonian symptomatology was demonstrated directly in patients. However, the implementation of L-DOPA as a routine therapy for Parkinson's disease was initially met with skepticism. Suboptimal doses were used, which led to contradictory reports, but in 1967, Cotzias described continuous beneficial effects when administering higher doses (Cotzias et al., 1967).

Thus, only a few years after Carlsson's initial discoveries, research highlighting the importance of central dopamine was being carried out simultaneously in three different countries. The discovery that dopamine is a neurotransmitter in the central nervous system has opened a massive field of research focused on strengthening our understanding of dopamine's physiological functions (such as reward), but also of pathological conditions relating to addiction, psychosis, and motor disorders. Dopamine research has therefore contributed to the development of drug therapies that relieve diseases such as schizophrenia and Parkinson's disease.

The present experiments contribute to this rapidly growing body of research on central dopamine. In this thesis, I present experiments outlining the biochemical changes that occur in central dopamine pathways following three different lesioning paradigms commonly used by researchers when modeling Parkinson's disease. In addition, I also report the effects of some manipulations which provided neuroprotection and behavioural sparing from neurotoxin-induced losses of dopamine neurons. The neuroprotection afforded by such manipulations sheds light on

the mechanisms underlying dopamine cell death, and these findings are therefore discussed in terms of therapeutic approaches for Parkinson's disease.

The relevance of this body of work is put into perspective by noting that the most popular treatment for Parkinson's disease remains the combination of L-DOPA and the peripheral decarboxylase inhibitor, carbidopa. Unfortunately, problems associated with L-DOPA, such as the increased risk of chorea, dystonia, and the "on/off" phenomenon (ie., a fluctuation between no therapeutic effect and dyskinesias), inevitably occurs. Thus, the negative impact of such adverse effects dramatically reduces the patient's quality of life, and underlies the intense efforts by researchers to find alternative treatment strategies. Specifically, a greater understanding of mechanisms underlying dopamine cell loss would enable the development of therapies aimed at slowing or even stopping cell death, as opposed to treating the clinical symptoms of the disease.

# Chapter 1: General Introduction

# PARKINSON'S DISEASE

## 1.1 Clinical Features

A thesis on the characterization of dopamine loss in the brain would not be complete without recognizing the highly significant contributions of the surgeon James Parkinson, the individual for whom Parkinson's disease is named. Having observed a total of six cases, three of them being only seen from a distance on the street, Parkinson nonetheless described *paralysis agitans* with astonishing accuracy in his 66 page monograph entitled "Essay on the Shaking Palsy" (Parkinson 1817). In this 1817 essay, he discussed the progressive nature, late onset, and symptomatology of the disease. Today, Parkinson's disease is defined by any combination of six specific motoric features: tremor at rest, bradykinesia, rigidity, loss of postural reflexes, flexed posture, and the freezing phenomenon (in which the feet are "glued to the ground") (Fahn, 2003).

The occurrence of Parkinson's disease increases with age and affects more than 2% of the population over 65 years (Tanner and Goldman, 1996). The mean age of onset is 60 years, and the mean duration is 13 years (Hughes et al., 1993). Less frequently, Parkinson's disease occurs below 40 years (Golbe, 1991).

## 1.2 Nigral degeneration

Nothing of the "shaking palsy's" underlying pathology was known when Parkinson lived. Although he was not able to study the brains of his patients, Parkinson believed the disorder was not due to damage of peripheral nerves, but instead due to a problem of the central nervous

system. His speculation, that the cervical cord and medulla oblongata were likely to be the lesion sites, was only a guess. Evidence for the neuroanatomical basis of the disease was not reported until Tretiakoff (1919) discovered a striking loss of pigmented cells from the midbrain region called the substantia nigra. These pigmented cells are dopamine cells that appear dark even when unstained due to the presence of the neuronal pigment, neuromelanin. Substantial loss of these cell occurs in normal aging but is greatly increased in Parkinson's disease (Zecca et al., 2002; Hirsch et al., 1988; Gerlach et al., 2003).

Subsequently, it has been confirmed that the main pathological hallmark of Parkinson's disease is a progressive loss of neuromelanin-containing dopaminergic neurons from the substantia nigra pars compacta at a rate of approximately 5% per year (Fearnley and Lees, 1991). The loss of nigral neurons follows a specific pattern, with a more susceptible area located laterally in the ventral part of the substantia nigra (Fearnley and Lees, 1991; Gibb, 1992). The severe loss of striatal dopamine that accompanies nigral loss is thought to underlie the motor symptoms, especially akinesia (Jellinger, 1999).

Loss of other transmitter systems, although slight relative to that in the nigrostriatal pathway, is also evident (Jellinger, 1991). Degeneration of the dopaminergic neurons in the ventral tegmental area (Uhl et al., 1985), the noradrenergic locus coeruleus (Hornykiewicz and Kish, 1987; Zarow et al., 2003), and the ascending cholinergic pathway from the Meynert basalis nucleus (Gaspar and Gray, 1984) has also been observed. Evidence suggests that these non-nigrostriatal lesions may underlie cognitive and psychological impairments, such as dementia, which are estimated to occur in approximately 30% of Parkinson's disease cases (Korczyn, 2001; Wolters, 2001).

Another histopathological feature of Parkinson's disease was first reported by Frederick Lewy, who described the presence of cytoplasmic inclusions in degenerating neurons. Lewy bodies are spherical, eosinophilic structures (having an affinity for the acidic dye eosin), exhibiting a dense core and peripheral halo. Interestingly, some patients who are diagnosed with Parkinson's disease on the basis of clinical symptomatology and drug responsiveness do not exhibit Lewy bodies (Matsumine et al., 1997).

## 2. ETIOLOGY OF PARKINSON'S DISEASE: Nature *vs.* Nurture

The first clinical signs of Parkinson's disease are apparent only after severe depletion of dopamine has occurred. This corresponds to a 70 – 80% loss of striatal innervation (Fahn, 2003) and 50% loss of nigral cell loss (German et al., 1989). Prior to motor dysfunction, striatal compensation phenomena, such as increased dopaminergic neuronal activity and sensitization of dopaminergic receptors (Zigmond and Hastings, 1998) occur, and could underlie the sub clinical phase of Parkinson's disease. Consequently, it is not possible to determine the time at which degeneration has begun. Thus, the etiology of Parkinson's disease is difficult to establish, and current therapies are symptomatic at best. Parkinson's disease may be the result of cumulative effects of genetic and environmental factors in a given patient (This topic will be discussed further; see next section).

## 2.1 The genetic basis of Parkinson's disease

A genetic component in Parkinson's disease was initially thought to be unlikely given that studies with twins demonstrated a low concordance rate in monozygotic and dizygotic twins (Tanner et al., 1999). However, the notion of a genetic component in this disease was strengthened when the age of disease onset is considered. There is a high rate of concordance in monozygotic twins that display clinical symptoms before the age of fifty (Duvoisin and Johnson, 1992; Tanner et al., 1999). Moreover, regardless of age of onset, concordance rates among monozygotic twins increases considerably if striatal dopamine uptake is used as an indicator of Parkinson's disease in the asymptomatic twin (Burn et al., 1992). Using positron-emission tomography with $^{18}F$ fluorodopa to measure striatal uptake, an increased risk was also observed among first-degree relatives of patients suffering from a rare form of PD (Piccini et al., 1997).

At present, approximately five to ten percent of Parkinson's disease cases represent familial forms of the disease. Recently, specific mutations have been identified in several genes. Based on the properties of these gene products, a unifying pathological mechanism has emerged despite the genetic and clinical heterogeneity. In particular, the data support a critical role for abnormal protein folding and degradation via the ubiquitin proteasome pathway as a common mechanism leading to neuronal death in these familial forms of the disease.

The ubiquitin proteasome pathway is a degradation process used by cells to eliminate normal proteins, as well as those with undesirable conformations. The removal of misfolded proteins involves the tagging of target proteins with small peptide ubiquitin through the stepwise action of three sets of enzymes, namely, ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2, and ubiquitin ligating enzyme E3.

## 2.11 α-Synuclein

Two point mutations in the α-synuclein gene have been identified in a few families with autosomal dominantly inherited Parkinson's disease. The alanine to threonine substitution at position 53 (A53T) was first demonstrated in families of Italian/Greek descent, and an alanine to proline substitution at position 30 (A30P) was identified in a German family (Polymeropoulos et al., 1997; Kruger et al., 1998). The role of wild-type α-synuclein is currently not established. However, mutated α-synuclein promotes the occurrence of α-synuclein adopting a β-pleated sheet structure (Jensen et al., 1998), and this conformation is associated with neuronal toxicity (Conway et al., 2000).

The involvement of α-synuclein in Parkinson's disease is supported by numerous other findings. For example, α-synuclein is a significant component of Lewy Bodies, one of the pathological hallmarks of Parkinson's disease (Spillantini et al., 1997). However, it is unclear whether aggregated α-synuclein that appears in Lewy bodies has a causative role, or is simply a marker for the underlying pathogenic process. To address this question, expression of the human α-synuclein or mutated α-synuclein genes (A53T or A30P) in Drosophila was performed (Feany and Bender, 2000). Transgenic expression of α-synuclein neurons resulted in an age-dependent loss of dopaminergic neurons and Lewy body-like aggregates (Feany and Bender, 2000). In transgenic mice, expression of human wild-type α-synuclein led to motor impairments, loss of striatal dopamine markers, and neuronal inclusions (Masliah et al., 2000). Thus, it appears that α-synuclein in its wild-type or mutated forms may contribute to degenerative changes in the nigrostriatal pathway, albeit by presently unknown mechanisms.

## 2.12 Parkin

Various mutations in the parkin gene were first identified in Japanese families with autosomal recessive juvenile parkinsonism (Kitada et al., 1998). Patients with parkin mutations develop the disease at a much younger age, suffer severe neuronal loss in the substantia nigra and locus coeruleus, but do not develop Lewy bodies (Hattori et al., 2000). Wild-type parkin has an E3 ubiquitin ligase function, ie., it attaches short ubiquitin peptide chains to proteins to tag them for degradation. Mutations result in diminished E3 ligase function in the substantia nigra and striatum, leading to the abnormal accumulation of substrate proteins (Shimura et al., 2000). Several parkin substrates have been identified, including an isoform of α-synuclein. While it is not entirely clear how the substrates of parkin are involved in the pathogenesis of Parkinson's disease, parkin appears necessary for the formation of Lewy bodies.

Autosomal recessive juvenile parkinsonism results from a loss of function of both copies of the parkin gene (Kitada et al., 1998). Interestingly, there have been a few patients with apparently sporadic Parkinson's disease with an adult onset, who have one mutant allele (Farrer et al., 2001). Precisely what role the parkin mutation plays in the majority of cases, and whether this heterozygous state represents a risk factor remains to be established.

## 2.13 C-Terminal ubiquitin hydrolase UCH-L1

Neuron-specific C-terminal ubiquitin hydrolase (UCH-L1) is an enzyme that hydrolyzes small C terminal adducts of ubiquitin to generate ubiquitin monomers, which are recycled and used to

clear other proteins (Leroy et al., 1998). A missense mutation of isoleucine to methionine at position 93 (I93M) was transmitted as an autosomal dominant genotype in two siblings of German descent (Leroy et al., 1998). The mutant form of UCH-L1 has decreased enzymatic activity resulting in impaired protein clearance through the ubiquitin proteasome pathway (Leroy et al., 1998).

In addition to α-synuclein, parkin and UCH-L1, at least five other loci have been proposed for autosomal dominant and autosomal recessive Parkinson's disease (Nussbaum and Ellis, 2003). Genetic analysis of the more common sporadic forms of this disease has revealed a component of heritability at a locus near the α-synuclein gene (Kruger et al., 1999). Thus, identification of these genes may provide important clues to the etiology of Parkinson's disease.

## 2.2 The environmental basis of Parkinson's disease

The common form of Parkinson's disease is sporadic, and at present, the contribution of genetic mutations cannot account for most late-onset cases (Tanner et al., 1999).

The idea of an environmental basis for Parkinson's disease was supported by the identification of the pyridine derivative, MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), that caused a 23 year old college student severe bradykinesia, rigidity and mutism (Langston and Ballard, Jr., 1983; Langston et al., 1983). The abrupt and early onset of symptoms was so atypical that the patient was initially diagnosed as catatonic schizophrenic. However, the subsequent diagnosis of Parkinson's disease was substantiated by a positive response to L-DOPA (Langston and Ballard, Jr., 1983). The patient eventually admitted to having synthesized and taken several illegal drugs (Langston and Ballard, Jr., 1983). Subsequent analysis of the patient's glassware revealed

pyridines as the main components of his injection mixture. Subsequently, MPTP was identified as the neurotoxin responsible for the symptoms (Langston et al., 1984).

Autopsy of the initial MPTP-exposed patient revealed degeneration of the substantia nigra, thus providing evidence for a possible involvement of environmental neurotoxins as a basis for Parkinson's disease (Langston et al., 1984). Several environmental neurotoxins have been investigated for their potential involvement in Parkinson's disease. To this end, the common herbicide 1,1'dimethyl-4,4'-5 bipyridinium (paraquat) coupled with the administration of the fungicide manganese ethylenepistithiocarbamate (maneb) leads to selective degeneration of dopamine neurons (Thiruchelvam et al., 2000). In addition, the widely used insecticide and fish poison, rotenone, produces parkinsonism in rats when administered intravenously or subcutaneously (Betarbet et al., 2000). Although these compounds can produce dopaminergic lesions, none are clearly responsible for the majority of Parkinson's disease cases. Nevertheless, it is possible that endogenous/exogenous toxins may play a role, especially when an individual's genetic background renders him or her more susceptible.

## 3.0 FACTORS ASSOCIATED WITH CELL DEATH IN PARKINSON'S DISEASE

Research on the etiology of Parkinson's disease has focused on the nigrostriatal dopamine pathway, as it is the most strongly affected pathway and its degeneration is principally responsible for motor deficits. Although the primary cause of nigrostriatal damage has not been established, several phenomena have been suggested to mediate, at least in part, dopaminergic neuron degeneration.

### 3.1 Oxidative stress in Parkinson's disease

Electron transfer reactions can convert molecular oxygen ($O_2$) to hydrogen peroxide and the superoxide and hydroxyl free radicals (pathway 1). Oxyradicals are extremely reactive with other compounds, and can induce cell death through multiple mechanisms, including damage to nucleic acids, oxidation of proteins, and lipid peroxidation (Fahn and Cohen, 1992; Halliwell, 2001).

(1) $O_2 \longrightarrow \cdot O_2^- \longrightarrow H_2O_2 \longrightarrow \cdot OH + H_2O$

Superoxide radical — Hydrogen peroxide — Hydroxyl radical

Although hydrogen peroxide is not a free radical, it can lead to the generation of free radicals through various chemical reactions. For example, hydrogen peroxide, in the presence of a reduced metal, forms the highly reactive hydroxyl radical via the Fenton reaction (pathway 2).

(2) $H_2O_2 + Fe^{2+} \longrightarrow \cdot OH + Fe^{3+}$

Oxyradicals and hydrogen peroxide are produced in the cell as by-products of normal cellular metabolism. However, they are maintained at low levels by two types of protective mechanisms. First, cellular antioxidants can react with and stop the proliferation of free radicals (e.g. Vitamins C and E) (Rice, 2000; Halliwell, 2001). Second, antioxidant enzymes such as superoxide dismutases-1 and -2 can catalyze the conversion of superoxide to hydrogen peroxide (Fridovich, 1989). Hydrogen peroxide is then converted to water by the antioxidant enzymes catalase or glutathione peroxidase.

Under normal conditions, a balance is maintained between the formation and removal of oxyradicals and hydrogen peroxide so that cells are protected from damaging effects. However, if this balance is altered by increased production of free radicals or reduced activity of the protective mechanisms, the cell is in a state of oxidative stress.

### 3.11 Sources of free radicals in Parkinson's disease

There are at least three possible processes by which free radicals may be generated in dopaminergic neurons. First, hydrogen peroxide is generated by normal dopamine catabolism via monoamine oxidase. This reaction gives rise to a molecule of hydrogen peroxide per molecule of dopamine, potentially giving rise to the hydroxyl radical via the Fenton reaction (Equation 2). Dopamine can also undergo nonenzymatic oxidation in the presence of molecular oxygen. This auto-oxidation gives rise to hydrogen peroxide and oxyradicals (Graham, 1978). Finally, iron, which exists in high concentration in the substantia nigra, has been reported to take part in the Fenton reaction (Jellinger et al., 1990). Thus, it has been suggested that nigral dopaminergic neurons may be particularly vulnerable to degeneration because of their ability to generate free radicals under normal conditions (Jenner, 1992).

### 3.12 Evidence of oxidative stress in Parkinson's disease

Reductions in the antioxidant glutathione have been reported in tissue from Parkinson's disease patients (Jenner, 1992). Such reductions in glutathione could promote or be a consequence of

oxidative stress, or both. Because glutathione is involved in the detoxification of $H_2O_2$, reduced glutathione levels could result in the increased concentrations of $H_2O_2$, and the highly reactive OH• in the presence of metals, such as iron. Supporting this idea is the observation of increased lipid peroxidation and oxidative DNA damage in Parkinson's disease relative to age-matched control subjects (Jenner, 1992). Moreover, increased total iron has been observed in the substantia nigra (Dexter et al., 1989; Dexter et al., 1991).

### 3.2 Mitochondrial dysfunction in Parkinson's disease

The main mitochondrial impairment observed in nigral neurons occurs in complex I of the mitochondrial respiratory chain. Complex I is located in the inner mitochondrial membrane and forms part of the oxidative phosphorylation system which produces cellular ATP. In Parkinson's disease, a defect in complex I has been reported in the striatum (Mizuno et al., 1989) and substantia nigra, but not in other brain regions, such as the globus pallidus or cerebral cortex (Schapira et al., 1990). A similar defect has been reported for platelets (Krige et al., 1992), in patients with Parkinson's disease.

Although the cause of mitochondrial impairment is not clear, a change in the genes encoding several mitochondrial proteins has been suggested, but has not been demonstrated in Parkinson's disease (Ozawa, 1995). The final outcome of such mitochondrial dysfunction could lead to an impairment in proton pumping and a decrease in the mitochondrial membrane potential, situations that can trigger an active cell death cascade (Desagher and Martinou, 2000).

## 4.0 CELL DEATH IN PARKINSON'S DISEASE : APOPTOSIS *vs.* NECROSIS

Programmed cell death (PCD) is an essential, highly regulated physiological process governing cellular turnover from fetal development to aging. During the developmental phase of a multicellular organism, PCD ensures the coordinated removal of superfluous cells in order to maintain the structural and functional integrity of cellular systems. Later in life, elimination of dysfunctional cells occurs in concert with their replacement. It has been estimated that approximately 60 billion cells are removed and replaced each day in rapidly proliferating tissues (e.g. skin and bone marrow) in an adult human (Reed, 2002).

Programmed cell death occurs in various forms, including apoptosis. Apoptosis is defined as a regulated mode of cell death, functioning to remove unnecessary, aged, or damaged cells (Thompson, 1995). Cells undergoing apoptosis exhibit distinct morphological characteristics, e.g., chromatin condensation, nuclear fragmentation, plasma membrane blebbing, cell shrinkage, cytoplasmic condensation, and apoptotic body formation. Apoptotic cells are phagocytosed by macrophages or microglial cells thereby preventing inflammation that occurs during necrosis.

Apoptotic death is an insular event, concerning single cells rather than groups of adjacent cells. In marked contrast, necrosis is characterized by cytoplasmic and nuclear swelling, loss of plasma membrane integrity, and the release of cellular contents (Clarke, 1990). The latter event hastens an immune response which has effects on neighbouring cells (Nicotera et al., 2000). Thus, unlike apoptosis, necrosis results in a discernable zone of cellular degeneration.

The recognition that cell death can occur as an active, organized process, soon led to the suspicion that a defect in programmed cell death may play a role in neurodegenerative diseases. In this regard, at least two scenarios can be envisaged. On one hand, apoptosis might remove injured or dysfunctional neurons, in which case their removal would represent a defense mechanism. On the other hand, an aberration in the apoptotic machinery itself could lead to "premature" or "erroneous" death of neurons. In both cases, therapeutic strategies aimed at either buying time to allow self-repair, or halting cell death, would clearly be of significant value. To this end, it is important to identify not only the mode of cell death occurring in Parkinson's disease, but also the mechanisms involved.

At present, there is controversy regarding the mode of cell death occurring in Parkinson's disease, since evidence supporting both apoptotic and necrotic forms has emerged. Mochizuki (1996) was the first to demonstrate DNA fragmentation in nigral neurons in post-mortem tissue from Parkinson's patients using the 3'-end terminal staining of DNA (TUNEL method). Since then, morphological signs of apoptosis, such as chromatin clumping, irregular nuclei morphology, cell shrinkage, presence of apoptotic bodies, and their phagocytosis by microglia, have been observed by several groups (Anglade et al., 1996; Anglade et al., 1997; Tatton et al., 1998). One study detected the increased occurrence of both DNA fragmentation and chromatin condensation in melanized nigral neurons in post mortem tissue from Parkinson's patients relative to control subjects (Tatton, 2000). Moreover, the same study revealed changes in biochemical markers of apoptosis, such as increased caspase-3 and Bax immunoreactivity.

Despite these observations, some studies have failed to demonstrate the presence of apoptosis in degenerating substantia nigra. These studies also used DNA end-labeling methods and/or morphological criteria for detecting apoptosis (Kosel et al., 1997; Jellinger, 2000). Thus, the mode of cell death in Parkinson's disease remains controversial.

The contribution of methodological problems inherent in using post-mortem brain tissue and the *in situ* TUNEL method to detect apoptosis may lead to the demonstration of both apoptosis and necrosis. Because in-situ-end labeling has also been shown to label non-apoptotic cells, results must be interpreted with caution. The possibility of "false-positive" errors using in-situ-end labeling methods underscores the importance of using morphological criteria to establish the mode of death. However, even morphological criteria may lead to ambiguous observations due to tissue variations between (and within) studies. For example, the time lag between death and the fixation of tissue can influence the detection of apoptotic neurons (Banati et al., 1998).

Several hypotheses have been proposed to describe the degenerative process of dopaminergic neurons in Parkinson's disease. These theories may account for the apparent discrepancies in observations of apoptotic *vs.* necrotic neurons. For example, it is presently not clear whether nigral cell death occurs as an *event* or a *process*. In the "one hit model for inherited degeneration" (Clarke et al., 2000), an environmental or endogenous factor is thought to cause a single, early, and significant loss of dopaminergic neurons followed by normal ageing. It is unlikely that a substantial number of neurons could be classified as apoptotic at a given time of histopathological analysis because apoptosis is a short process in vivo, and not detectable at the time clinical symptoms emerge.

In contrast to the "one hit model" model of degeneration is the concept of neuronal loss as an extended process (Schulzer et al., 1994) . The protracted loss of neurons may last several decades as an active process throughout a person's life. In this case, the rate of cell death may be so low that only a small number of apoptotic neurons are observable at any given time.

Clearly it is difficult to reliably demonstrate apoptosis in post mortem Parkinson's disease brain. Hence, the present thinking is that active cell death, if it is occurring, may be due to known apoptosis, and/or an as yet undiscovered type of programmed cell death. In pathological conditions, forms of cell death have been described which exhibit features of both apoptosis and necrosis. It has been suggested that apoptotic cell death can become necrotic if the cell is in a state of energy impairment (Nicotera, 2000). A variant of PCD that has been observed in cultured neurons that does not show signs of apoptosis ('paratosis') has been reported (Sperandio et al., 2000). Finally, it has been suggested that the biochemical processes involved in neuronal apoptosis can also occur in synaptic terminals of neurons and, as such, result in the localized degeneration of synapses and neurites ('synaptosis'), without concomitant or immediate death of the whole neuron (Mattson and Duan, 1999).

## 5.0 MODELLING PARKINSON'S DISEASE IN ANIMALS

Considering the difficulty of interpreting data obtained from human post-mortem samples, animal models of Parkinson's disease are a critical aid in studying pathogenic mechanisms and developing therapeutic strategies. As noted earlier, it was through the use of an animal model

that dopamine deficiency was initially associated with Parkinson's disease symptomatology, and led to the use of L-DOPA therapy to ameliorate motor deficits.

Because Parkinson's disease does not appear spontaneously in animals, the characteristic clinical and pathological features of the disease must be experimentally induced. Several compounds have been employed to induce one or more facets of Parkinson's disease (e.g. a progressive loss of dopaminergic neurons, Lewy body-like inclusions, behavioural deficits, and a responsiveness to L-DOPA therapy). The two main approaches to developing a Parkinsonian state in animals include the administration of a toxin, or the production of a genetic mutation.

Animal models are particularly valuable if the human disease state is faithfully imitated in animals. Given the complexity of Parkinson's disease, obtaining an animal model that possesses the complete phenotype of the human disease is difficult. Although some researchers have argued that only primate models are useful, currently there is no consensus that one species is more useful than another in clinical relevance (Schallert 1995). Animal models that have been generated to date have greatly enhanced our understanding of this multifaceted disorder. Characteristics of specific animal models that have been used are listed in Table 1.

**5.11** ***Reserpine.*** The first animal model was developed following the observation that rabbits become akinetic after systemic administration of reserpine (Carlsson et al., 1957). Reserpine-induced motor deficits likely resulted from the reduction of dopamine uptake into release vesicles in nerve terminals (Hartman and Halaris, 1980). The principal drawbacks of this model are that reserpine-induced loss of dopamine is only temporary, and the reduction of striatal

dopamine is not accompanied by degeneration of nigrostriatal neurons. Despite these drawbacks, the reserpine model revealed the importance of dopamine in movement, and was used to first illustrate the effectiveness of L-DOPA (Carlsson et al., 1957).

**5.12** ***Methamphetamine.*** Like reserpine, methamphetamine administration results in a transient loss of nigrostriatal dopamine at the nerve terminals, but not at the cell body level (Ricaurte et al., 1984). Motor impairments that occur in rodents following methamphetamine administration are improved with L-DOPA therapy (Walsh and Wagner, 1992). Recently, methamphetamine administration resulted in the appearance of intracellular inclusions in striatal neurones and cytoplasm of nigral neurons (Fornai et al., 2004). The main disadvantages of the methamphetamine model is the lack of somatic degeneration in the nigrostriatal pathway (Ricaurte et al., 1984).

**5.13** ***MPTP.*** Accidental injection of the synthetic chemical, MPTP, by a drug addict resulted in the emergence of clinical symptoms identical to those observed in Parkinson's disease. Systemic administration of MPTP is the most widely used *mouse* model of Parkinson's disease. In addition to reproducing parkinsonian-like motor deficits, MPTP given over several days results in a progressive, permanent degeneration of the

| *Models* | *Progressive loss of DA neurons* | *Progressive loss of non-DA neurons* | *Motor deficits* | *L-DOPA responsive* | *Lewy body inclusions* |
|---|---|---|---|---|---|
| **Toxin Models** | | | | | |
| Reserpine | – | – | + | + | – |
| Methamphetamine | – | – | + | + | – |
| 6-OHDA | + | + | + | + | – |
| MPTP | + | – | + | + | Nonfibrillar inclusions |
| Rotenone | + | – | + | + | + |
| Paraquat/maneb | + | – | + | ND | Nonfibrillar inclusions |
| | | | | | |
| **Genetic Models** | | | | | |
| Knock-outs: α-synuclein | – | – | – | ND | – |
| Transgenics: mouse α-synuclein | – | Depends on promotor used | + | ND | + or nonfibrillar inclusions |
| **Transgenics:** Drosophila α-synuclein | + | – | + | + | + |

Table 1: Key features of the models used to recapitualate the neuropathology and symptomatology of Parkinson's disease. Abbreviations: ND, not determined (Adapted from Dawson et al, 2002).

nigrostriatal pathway (Jackson-Lewis et al., 1995) and, in monkeys, produces neuronal nonfibrillar inclusions (Forno et al., 1986). Dopamine selectivity of MPTP toxicity stems from the affinity of its toxic metabolite (MPP+) for the dopamine transporter (Mayer et al., 1986). Once inside dopamine neurons, MPP+ is concentrated within mitochondria and inhibits complex 1 of the mitochondrial electron transport chain (Przedborski et al., 2000). These observations led to the discovery of mitochondrial dysfunction in Parkinson's disease and improved grafting of dopamine neuron transplants (Bankiewicz et al., 1990; Bankiewicz et al., 1991). Despite the overall strength of this model, compounds found to be neuroprotective in MPTP-treated mice are not efficacious in Parkinson's patients (Dawson et al., 2002). Thus, while the MPTP model has revealed significant insights into the mechanisms underlying the loss of nigrostriatal dopamine neurons, its value in predicting neuroprotective therapies is questionable.

**5.14** ***Paraquat and maneb***. Based on its structural similarity to MPP+, the common herbicide paraquat is considered a putative risk factor for Parkinson's disease. Paraquat can cross the blood-brain barrier (Shimizu et al., 2001). Systemic administration of paraquat results in the loss of nigrostriatal dopamine neurons and reduced ambulatory movement (Brooks et al., 1999). The agricultural use of paraquat often overlaps with the fungicide manganese ethylenebisdithiocarbamate (maneb). Systemic injection of maneb potentiates the neurotoxicity of paraquat, and thus the combined administration of these chemicals shows promise as an environmentally relevant model of Parkinson's disease (Thiruchelvam et al., 2000). It is noteworthy that an association between agricultural/horticultural work and Parkinson's disease has been found (Tuchsen and Jensen, 2000).

**5.15** ***Rotenone.*** Rotenone is another agricultural toxin; it has been used as an insecticide and fish poison. When given intravenously to rats, rotenone produces behavioural deficits, a progressive loss of nigrostriatal neurons and cytoplasmic inclusions resembling Lewy bodies (Betarbet et al., 2000). Rotenone is a mitochondrial complex 1 inhibitor which acts uniformly in the brain (Betarbet et al., 2000). However, selective degeneration of nigrostriatal neurons occurs following intravenous administration, suggesting that these neurons are particularly vulnerable to complex 1 inhibitors. The rotenone model recapitulates Parkinson's disease extremely well. However, this model suffers from practical aspects such as high morbidity rate and between-rat variability to toxin susceptibility (Dawson et al., 2002).

**5.16** ***Knock-out mice.*** α-Synuclein knock-out mice did not display behavioural deficits, and histological examination revealed a morphologically intact substantia nigra indistinguishable from wild-type mice (Abeliovich et al., 2000). Knock-out mice did, however, display a reduction in striatal dopamine and an attenuated locomotor response to amphetamine. α-Synuclein knock-out mice appeared less susceptible to MPTP-induced neurotoxicity (Dauer et al., 2002), suggesting an association between α-synuclein and mitochondrial function.

**5.17** ***Transgenic mice.*** To date, transgenic mouse models have focused on α-synuclein transgene expression driven by different promoters. Human wild-type α-synuclein expression driven by the platelet derived growth factor-β promotor resulted in some features of Parkinson's disease. For example, these mice exhibited motor deficits, a reduction in striatal tyrosine hydroxylase expression, and contained cytoplasmic inclusions that were immunopositive for α-synuclein. However, no loss of nigral dopaminergic neurons was detected, and the cytoplasmic

inclusions lacked the fibrillar aggregates typical of Lewy bodies (Masliah et al., 2000). A similar phenotype was observed in mice expressing mutant or wild-type α-synuclein expression driven by the Thy-1 promoter (van der et al., 2000). Using the prion promoter to regulate α-synuclein expression, transgenic mice developed motor deficits later in life, whereas numbers of substantia nigra cell bodies were not reduced. Thus, some transgenic models have resulted in neurodegeneration that involves the dopaminergic system, however, a loss of nigrostriatal neurons has not been observed.

**5.18** *Transgenic Drosophila.* Another genetic approach to studying Parkinson's disease involves the expression of normal and mutated α-synuclein in Drosophila (Feany and Bender, 2000). These flies exhibited motor deficits, as measured by a reduction in their ability to fly to the top of their container (ie., a loss of their normal negative geotaxis behaviour). Moreover, transgenic flies showed a reduction in dopaminergic neurons, and intraneuronal inclusions. Perhaps the well-characterized genetics of Drosophila may allow for rapid screening of candidate therapeutic agents, and the characterization of mechanisms underlying neurodegeneration.

## 5.2 The validity of 6-OHDA as an animal model of Parkinson's Disease

Although there are many animal models of Parkinson's disease, the focus of the present thesis is on the use of 6-hydroxydopamine (6-OHDA) as a selective lesioning tool, and its contribution to the understanding of dopaminergic cell loss.

Administration of 6-OHDA along the nigrostriatal pathway in rats reproduces many of the neuropathological features of Parkinson's disease. 6-OHDA treated rats show an acute loss of nigrostriatal dopamine neurons and severe motor impairments that are easily quantifiable and responsive to L-DOPA therapy. However, intracytoplasmic inclusions resembling Lewy bodies are not observed.

Progressive loss of dopaminergic neurons, a phenomenon which may more accurately mimic the human disease, occurs when 6-OHDA is infused acutely, and *unilaterally* into the striatum (Sauer and Oertel, 1994). In this lesioning paradigm, cell body atrophy is detectable in nigral dopamine neurons by one week, but loss of nigral neurons is a relatively slow process that occurs for at least sixteen weeks post-infusion (Sauer and Oertel, 1994). Therefore, the subtotal loss of nigral dopamine neurons produced by intrastriatal lesions resembles early stage Parkinson's disease, when approximately 80% of striatal dopamine and 50% of nigral neurons are lost (Riederer and Wuketich, 1976). Another potential advantage of intrastriatal 6-OHDA administration is that a significant portion of the nigrostriatal pathway remains intact. The latter aspect is particularly important since the remaining nigrostriatal projection may serve as a substrate for both regenerative sprouting and functional recovery following the administration of neurorestorative compounds.

The unilateral aspect of infusion is an important benefit of the model (Schwarting and Huston, 1996) in part because it provides an internal control (ie., the unlesioned side of the brain) for the quantification of dopaminergic cell loss, but also because aphagia is avoided. Moreover, tests of motor deficits have been developed that rest on the aspect of reduced unilateral dopamine. The

dopamine-depleted rats display abnormalities in turning biases, impairments in locomotion and posture, and deficits in skilled limb movements that are analogous to deficits observed in the human disease (Cenci et al., 2002). Thus, this model has proved useful for evaluating the efficacy of cell transplantation (Dunnett et al., 1981), and of compounds that promote the survival of the degenerating dopaminergic neurons or terminals (Bjorklund et al., 2000).

In addition to its usefulness in evaluating the functional recovery of the dopaminergic pathway, 6-OHDA administration in rats has helped to reveal the mechanisms underlying dopaminergic degeneration. Specifically, the role of oxygen free radicals (Grunblatt et al., 2000) and mitochondrial defects (Glinka et al., 1996) in Parkinson's disease has been elucidated in part from studies employing 6-OHDA. Also, the 6-OHDA model has face validity, insofar as this compound has been found in the brain (Curtius et al., 1974) and urine (Andrew et al., 1993) of patients suffering from Parkinson's disease. Thus, 6-OHDA may not only serve to model dopaminergic death experimentally, but it has also been proposed as a putative endogenous neurotoxic factor in the pathogenesis of Parkinson's disease (Jellinger et al., 1995).

### 5.21 Behavioural Deficits following 6-OHDA

Rats with *bilateral* 6-OHDA lesions of the nigrostriatal pathway develop neuropathological changes quite similar to those in patients with Parkinson's disease. The behavioural syndrome following bilateral 6-OHDA administration includes postural abnormalities, and reduced capacity to maintain balance (Wolfarth et al., 1996). Spontaneous movements are rare, and only short steps are taken when walking is induced (Schallert et al., 1978). Parkinsonian-like tremor

has also been observed in the wrist and paw of the rat during non-weight-bearing postures (Buonamici et al., 1986; Lindner et al., 1999). Despite showing all of the essential elements of parkinsonian symptomatology, bilateral 6-OHDA administration is not a commonly used model. This is because lesioned rats also suffer from severe and disabling deficits of eating and drinking, and therefore require extensive nursing care. Thus, most experimental studies in rats have employed *unilateral* 6-OHDA administration.

*Unilateral* administration of 6-OHDA results in a hemiparkinsonian syndrome which includes asymmetries of body posture and contralateral sensorimotor deficits (Schallert and Tillerson, 2000). Behavioural asymmetry has been widely used to monitor the extent of dopaminergic lesions and putative restorative manipulations. The most common test involves recording the number of turns that are completed by a rat challenged with a dopamine agonist (e.g. amphetamine and apomorphine) (Pycock, 1980).

Circling behaviour following systemic administration of a dopamine agonist occurs due to a physiological imbalance between the lesioned and unlesioned striatum. Circling behaviour induced by some dopamine agonists and has become a standard tool for quantitative functional assessment in the rat model of Parkinson's disease (Pycock, 1980). However, the magnitude of the amphetamine-induced circling response is a poor indicator of the overall extent of nigral cell loss or striatal denervation since equally high rates of turning can be observed over a range of cell loss and/or striatal denervation (approximately 50-90%) (Hudson et al., 1993). In addition, control rats that were not lesioned rotated extensively following amphetamine administration (Hudson et al., 1993). A further potential drawback is that amphetamine- and apomorphine-

induced circling are particularly sensitive to losses of dopamine innervation in the dorsomedial striatum (Mandel et al., 1990) and therefore agonist-induced circling is unlikely to be a useful test to monitor less regionally-specific dopamine lesions. Thus, the validity of circling behaviour has been questioned (Lundblad et al., 2002; Metz and Whishaw, 2002).

Alternative behavioural measures of rat akinesia have been reported following unilateral dopamine depletion. For example, *contralateral* limb use is consistently impaired during spontaneous (non-drug induced) movements, such as walking, grooming, and adjusting posture (Olsson et al., 1995; Schallert and Tillerson, 2000). In Parkinson's disease, movement fragmentation and abnormal postural adjustments are main deficits. Thus, measurement of these behaviours in a unilaterally lesioned rat is a potentially valuable approach to evaluate neuroprotective or restorative therapeutic strategies.

In rats, the forelimbs are used to initiate movements that require weight-shifting, much like the legs are used by humans when they walk. Difficulty in initiating steps from a standing posture is one of the primary signs of extensive nigrostriatal degeneration (Langston, 1990). Thus, one clinically relevant motor test examines weight shifting movements initiated by forelimbs when the unilaterally lesioned rat is placed in a transparent cylinder ("cylinder test"). The cylinder encourages vertical exploration, and forelimb use during vertical rearing movement is videotaped. Unilaterally lesioned rats preferentially use the forelimb *ipsilateral* to the lesion; use of the contralateral forelimb is impaired. This test has been used to assess the efficacy of gene therapy (Choi-Lundberg et al., 1998) and grafts of fetal ventral mesencephalic tissue (Johnston and Becker, 1997).

A second test (adjusting steps, or "wheelbarrow" test) assesses the capacity of unilaterally lesioned rats to make adjusting steps in response to an experimenter-imposed lateral movement. The rat while being held by its torso so that only one forelimb bears weight, and is moved slowly sideways. The wheelbarrow test a is a model of postural balance that assesses the capacity to regain postural stability when weight shifts are imposed. Thus, this test is analogous to the "standing pull test" used by neurologists in Parkinson's disease (Schallert and Tillerson, 2000) and has been used successfully to reveal the utility of dopamine agonists in end-stage Parkinson's disease (Olsson et al., 1995).

A third test ("whisker test") assesses asymmetries in forelimb placing following vibrissae-elicited stimulation. Rats are held by their torsos with both forelimbs hanging freely. Each forelimb is tested independently by moving one side of the rat laterally toward the edge of a tabletop until the vibrissae of one side makes contact with the edge. Unilaterally lesioned rats typically fail to place the contralateral forelimb onto the countertop, whereas non-lesioned rats place both forelimbs onto the tabletop (see chapter 3).

Selecting clinically relevant tests to evaluate motor deficits in animal models is crucial to the successful preclinical screening of therapies. While many behavioural methods have been used in different animal models of Parkinson's disease, tests of contralateral impairments in the unilaterally lesioned 6-OHDA rat provides a practical and predictive strategy.

## 6.0 THE EVOLUTION OF CHEMICAL LESIONS

In Montreal (circa 1963), Ted Sourkes had just embarked on a scientifically fruitful collaboration with the neurologist and neuroanatomist Louis Poirier at the University of Montreal. Their research plan was to reproduce the overt motor symptoms of Parkinson's disease in primates, and then determine if the resulting changes in the brain were interdependent via a connection between the substantia nigra and striatum (Poirier and Sourkes, 1965). They began by making an electrolytic lesion between the substantia nigra and striatum, damaging neither structure. They reasoned that lesion effects would be a result of time-dependent anterograde and retrograde degeneration of fibres. Lesions were made unilaterally so that the side contralateral to damage could serve as a control. In addition, the monkeys' behavioural and neurological responses to drug administrations were monitored at different times following the lesion.

The multifaceted approach employed by Sourkes and Poirier in modeling Parkinson's disease in the monkey led to several important observations. The lesioned monkeys displayed several key behavioural features of Parkinson's disease, namely tremor and hypokinesia of the limbs contralateral to the lesion. Histological and neurochemical analyses of brain tissue revealed that lesions of the ventromedial tegmental area resulted in cell loss from the ipsilateral substantia nigra pars compacta, and in dopamine loss from the ipsilateral striatum. Furthermore, a correlation between the degree of nigral cell loss and striatal catecholamine depletion was observed.

## 6.1 6-Hydroxydopamine as a Chemical Lesioning Tool

Eventual progress in identifying transmitter systems in the brain led to the realization that nonselective lesion techniques, such as the electrolytic approach used by Sourkes and Poirier, often resulted in nonspecific damage. Extensive damage has been reported to occur in neurons from different classes of transmitter systems and in non-neuronal cells. It therefore became clear that lesion techniques that specifically target particular classes of transmitter systems would be required in order to obtain accurate information about neurochemical coding of behaviour. The breakthrough in this field was the discovery by Thoenen and Tranzer (1968) that the noradrenaline analog 6-hydroxydopamine (6-OHDA; 2, 4, 5-trihydroxyphenylethylamine) could produce an acute and selective loss of sympathetic adrenergic nerve terminals after peripheral administration. Thus, 6-OHDA was the first chemical agent discovered that had specific neurotoxic effects on catecholaminergic pathways.

Since 6-OHDA does not easily pass the blood-brain barrier, it has to be infused directly into brain tissue (intraparenchymal) to induce degeneration of the central catecholamines (noradrenaline and dopamine). Intracerebral infusion of 6-OHDA was introduced by Ungerstedt (1968; 1971). This technique involves stereotaxic placement of an infusion cannula into a brain region where catecholamine neurons are located. The cannula is connected to an injection device making it possible to infuse microlitre quantities of the 6-OHDA solution. Since its introduction, 6-OHDA has been used extensively as a lesion tool in determining neuroanatomical and functional relationships, and is a standard tool for the experimental production of selective lesions in central catecholamine systems.

## 7.0 MECHANISM OF 6-OHDA SPECIFICITY AND TOXICITY

The selectivity of 6-OHDA toxicity is due to the neurotoxin's high affinity for the amine reuptake transporter which is located exclusively on catecholamine nerve terminals, axons, and cell bodies. Being chemically very similar to noradrenaline and dopamine (Figure 2), 6-OHDA is taken up by transporters (which normally take up released catecholamines). The selective accumulation of 6-OHDA is therefore responsible for the specificity, however the toxicity is thought to be due to at least two actions: (1) oxidative stress following the generation of oxyradicals, and (2) mitochondrial impairment via direct and/or indirect interaction with complex 1 of the respiration chain (Figure 3).

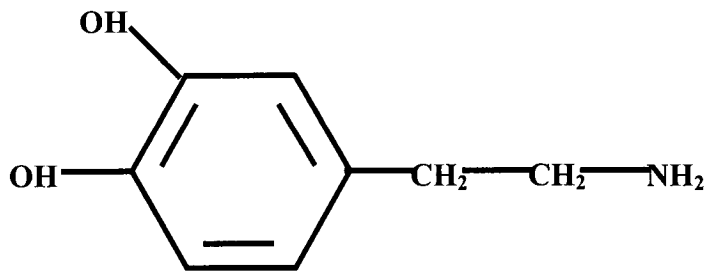
### 7.1 Oxidative Stress

Several studies have confirmed that 6-OHDA produces oxidative stress in vivo (Kumar et al., 1995), as well as in vitro (Tiffany-Castiglioni et al., 1982; Lotharius et al., 1999). In line with this observation antioxidants (Davison et al., 1986; Cadet et al., 1989) as well as overexpression of superoxide dismutase (Asanuma et al., 1998) or glutathione (Bensadoun et al., 1998) have been shown to protect against 6-OHDA-induced degeneration.

Following 6-OHDA administration, oxyradicals may arise from two distinct mechanisms, (1) deamination by monoamine oxidase, or (2) auto-oxidation. Monoamine oxidase (MAO) reacts with 6-OHDA to yield hydrogen peroxide. Reactive oxygen species (ROS) may be subsequently amplified by iron via the Fenton reaction (Equation 2).

Figure 2: The chemical structures of dopamine (A) and 6-hydroxydopamine (B). It is thought that 6-OHDA is taken up by dopamine transporters based on its chemical similarity to dopamine.

**A**




**B**

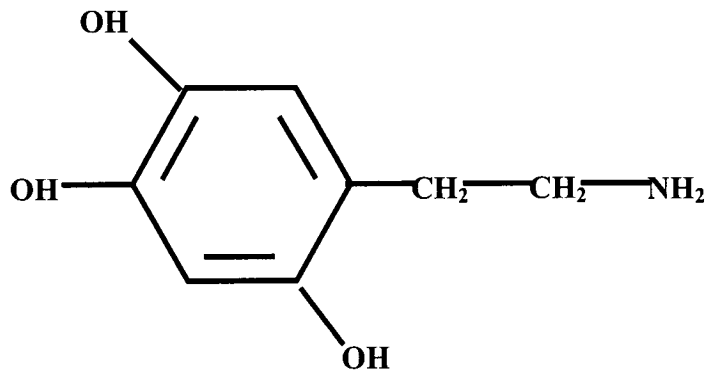
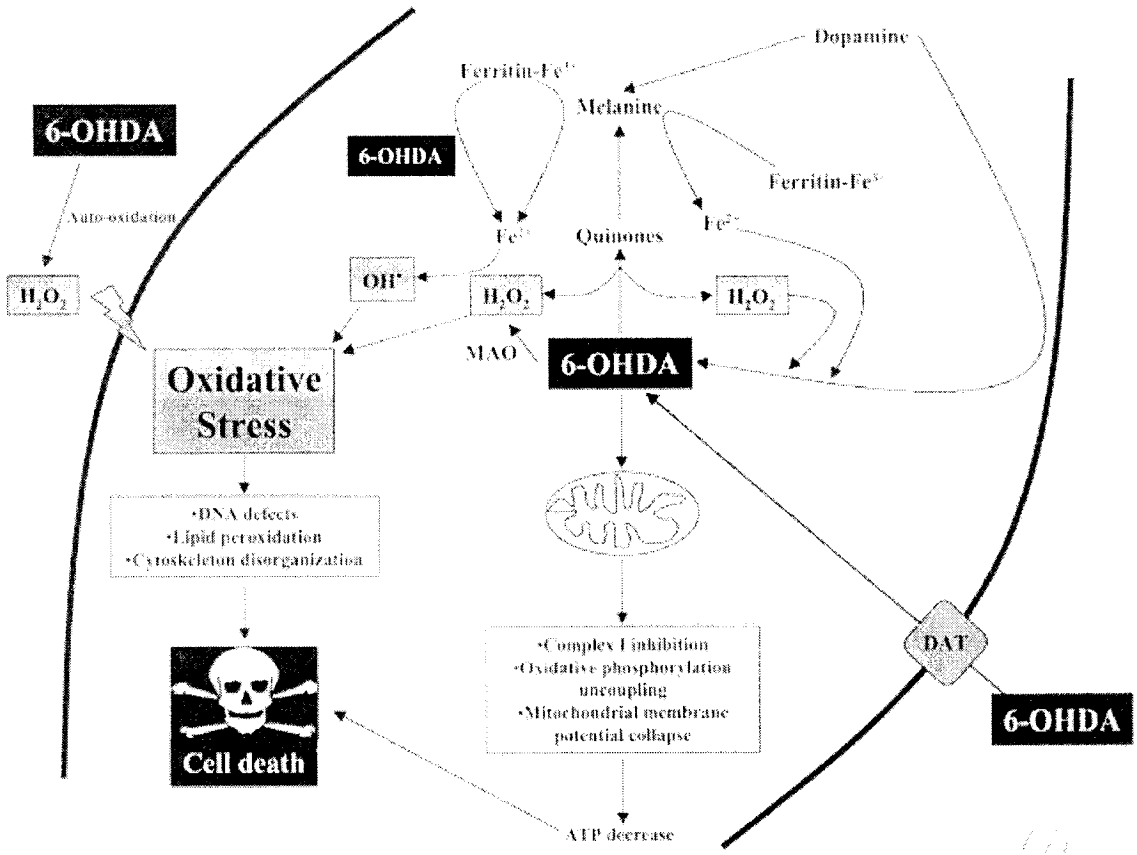


Figure 3: A schematic of the neurotoxic effects resulting from 6-OHDA. 6-OHDA has been proposed to induce catecholaminergic cell death by the production of free radicals, which leads to oxidative stress, and mitochondrial impairment, which leads to decreased ATP availability (Adapted from Blum et al., 2001).


6-OHDA
Auto-oxidation
$H_2O_2$
Oxidative Stress
•DNA defects
•Lipid peroxidation
•Cytoskeleton disorganization
Cell death
6-OHDA
Ferritin-$Fe^{3+}$
$Fe^{3+}$
$OH^{\bullet}$
$H_2O_2$
MAO
Dopamine
Melanine
Ferritin-$Fe^{3+}$
$Fe^{2+}$
Quinones
$H_2O_2$
6-OHDA
•Complex I inhibition
•Oxidative phosphorylation uncoupling
•Mitochondrial membrane potential collapse
ATP decrease
DAT
6-OHDA

In addition to ROS generation by MAO, 6-OHDA readily auto-oxidizes because of its low redox potential. The auto-oxidation of 6-OHDA involves a series of reactions resulting in the formation of several cytotoxic species, such as quinones (Saner and Thoenen, 1971), 5,6-dihydroxyindole (Blank et al., 1972), and the oxygen oxidation products hydrogen peroxide, superoxide, and hydroxyl radicals (Heikkila and Cohen, 1972; Heikkila and Cohen, 1973; Cohen and Heikkila, 1974). In line with this hypothesis, the cytotoxicity of 6-OHDA positively correlates with its rate of auto-oxidation (Soto-Otero et al., 2000).

The formation of ROS generated by MAO and auto-oxidation may be amplified by iron via the Fenton reaction. The contribution of iron in 6-OHDA-induced toxicity is supported by the finding that iron chelating agents partially protected against 6-OHDA induced degeneration (Ben Shachar et al., 1991), and direct administration of iron into the substantia nigra results in neurodegeneration similar to that produced by 6-OHDA (Sengstock et al., 1992). Thus, the combined occurrence of MAO activity and oxidation of 6-OHDA allows for ROS production.

Several hypotheses have been proposed to account for the ensuing degeneration at the molecular level. One proposal is that the oxidation products formed from 6-OHDA may act as alkylation agents, i.e., form irreversible, covalent bonds with SH-groups of proteins in the cytoplasm and membrane (Saner and Thoenen, 1971). In addition, 6-OHDA generated ROS has been shown to cause DNA strand breaks (Bruchelt et al., 1991), mutations (Gee et al., 1992), and impaired glucose uptake (Vroegop et al., 1995).

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### 7.2 Mitochondrial Impairment

6-OHDA has been shown to impair mitochondrial function by several mechanisms. For example, 6-OHDA is able to inhibit complex 1 in isolated brain mitochondria (Glinka and Youdim, 1995). However, in experiments using whole cells (SY5Y neuroblastoma cells), application of 6-OHDA did not result in a reduction of ATP production (Storch et al., 2000). The latter result suggests that inhibition of mitochondrial respiration is not the main mechanism underlying 6-OHDA toxicity. However, Lotharius et al (1999) demonstrated that 6-OHDA induced ROS generation resulted in a collapse of mitochondrial membrane potential. While the details need to be worked out, these data, taken together, suggest that 6-OHDA can impair mitochondrial function, resulting in the impaired energy metabolism within the neuron.

## 8.0 6-HYDROXYDOPAMINE: QUESTIONS UNANSWERED

### 8.1 Characterization of lesioning paradigms: effect of temperature

Researchers have not only infused 6-OHDA into the nigrostriatal pathway of rats to produce an animal model of Parkinson's disease, but also used 6-OHDA to selectively target the *mesolimbic* dopamine pathway, principally to study the rewarding effects of addictive drugs. Such lesions studies have revealed that mesolimbic dopamine cell bodies in the ventral tegmental area (VTA) and their projections to the ventral striatum (ie., nucleus accumbens and olfactory tubercle) mediate the rewarding effects of some addictive drugs (Wise and Rompre, 1989). Despite its wide application as a lesioning tool in the *mesolimbic* pathway, a characterization of 6-OHDA-induced degeneration in dopamine pathways following such lesions has not been reported.

In my own preliminary experiments, 6-OHDA infusion into mesolimbic dopamine terminal regions resulted in an almost complete sparing of DA cell bodies in the VTA. This sparing occurred even after a survival time of two months. Based on these observations, we hypothesized that the mesolimbic cell bodies were more resistant to 6-OHDA neurotoxicity than the nigrostriatal pathway, thus mimicking the selective vulnerability of the nigrostriatal pathway observed in Parkinson's disease (Fearnley and Lees, 1991). In animals, mesolimbic dopaminergic neurons are less susceptible than nigrostriatal neurons to Parkinson-inducing toxins, such as MPTP (Jacobowitz et al., 1984), rotenone (Marey-Semper et al., 1993; Betarbet et al., 2000) and paraquat/maneb (Thiruchelvam et al., 2000).

Loss of nerve terminals without concomitant degeneration of cell bodies is referred to as a terminal axotomy. In dopaminergic pathways, a terminal axotomy has been observed following the administration of high doses of amphetamine and its derivative, methamphetamine (Malberg and Seiden, 1999). Systemic administration of either compound in rodents has resulted in the loss of striatal dopamine (Wagner et al., 1980; Bowyer et al., 1992), decreased tyrosine hydroxylase activity (Ellison et al., 1978; Hotchkiss and Gibb, 1980), a reduction in dopamine transporters (Wagner et al., 1980; Scheffel et al., 1996), and has induced morphological changes in nerve terminals (Ricaurte et al., 1982; Ricaurte et al., 1984) without affecting cell body regions.

The neurotoxicity of amphetamines is significantly influenced by the temperature of the animal. Hyperthermia consistently exacerbates amphetamine-induced dopaminergic degeneration (Miller

and O'Callaghan, 1994), whereas hypothermia is neuroprotective (Ali et al., 1994). The terminal axotomy observed in the mesolimbic pathway following 6-OHDA infusion into mesolimbic nerve terminals was performed under sodium pentobarbital anesthesia. Under this anesthetic, rectal and brain temperature has been reported to decrease (Torbati et al., 2000). Hypothermia exerts a neuroprotective effect after a number of brain insults, including ischemia (for review see Ginsberg et al., 1992; Maher and Hachinski, 1993; Dietrich et al., 1996) and after high doses of 3,4-methylenedioxymethamphetamine ("Ecstasy"; MDMA; for review see Malberg and Seiden, 1999; Colado et al., 1999) in addition to amphetamine and methamphetamine (Bowyer et al., 1992; Bowyer et al., 1994; Albers and Sonsalla, 1995). Temperature was considered as a potential important factor in determining the extent of neurotoxicity following 6-OHDA administration in mesolimbic dopaminergic pathway.

Thus, these observations led to the characterization of dopamine loss in mesolimbic and nigrostriatal subregions following three types of 6-OHDA lesions. In addition, the putative protective effect of hypothermia was examined.

## 8.2 Mechanisms of 6-OHDA cell death

Although the behavioural and neurochemical deficits induced by 6-OHDA have been extensively studied in animal models of Parkinson's disease, the etiology of the disease remains elusive. Advances in the understanding of cell death pathways and their regulation has led to the idea that neuronal degeneration can be halted. In the 6-OHDA animal model of Parkinson's disease, this idea rests on demonstrating that the ensuing degeneration is apoptotic, and thus potentially

regulatable. Therefore, it is important to identify critical components of the pro-death pathways following 6-OHDA administration.

Pro-apoptotic effects of 6-OHDA were first demonstrated in vitro (Walkinshaw and Waters, 1994). Low concentrations of 6-OHDA (<100 μM) resulted in apoptotic morphological changes, such as DNA fragmentation, in differentiated catecholaminergic PC12 cells. Evidence of pro-apoptotic cell death by 6-OHDA was subsequently confirmed in other cell lines, including primary mesencephalic-derived dopaminergic cells (von Coelln et al., 2001), and the mesencephalic-derived dopaminergic MN9D cell line (Oh et al., 1998; Choi et al., 1999).

In vivo administration of 6-OHDA (into the medial forebrain bundle) has resulted in typical apoptotic features in the substantia nigra at various time points following the lesion (Zuch et al., 2000; He et al., 2000). Similarly, intrastriatal 6-OHDA administration resulted in nigral apoptosis in young rats (Marti et al., 1997), but induced both apoptosis and necrosis following the second post-natal week (Burke and Kholodilov, 1998).

In adult rats, evidence from molecular studies suggest that the mode of cell death following 6-OHDA is actively regulated. Genes involved in apoptosis are regulated by p53 (Oren, 2003). While the mechanism underlying p53-associated apoptosis is not well understood, it has been associated with an increase in Bax expression and caspase-3 activation in neurons (Xiang et al., 1998). Following 6-OHDA administration, an increase in p53 has been reported to occur in PC12 cells (Blum et al., 1997) and in the substantia nigra of rats (Qin et al., 1997). The

expression of the apoptotic protein Bax is also increased in PC12 cells following 6-OHDA administration (Blum et al., 1997) which could be related to, or independent of, p53 induction.

Although the roles of Bax and p53 in dopaminergic cell death are not fully characterized, activation of either mediator could ultimately result in apoptotic cell death through caspase activation. While there is evidence that caspase-3 is activated following 6-OHDA administration *in vitro* (Ochu et al., 1998), and in young rats (Jeon et al., 1999), evidence of caspase-3 activation in *adult* rats is equivocal. Indirect evidence of caspase activation has been obtained in adult rats given the pan-caspase inhibitor zVAD.fmk, which partially prevented nigral cell loss (Cutillas et al., 1999). However, using immunohistochemical methods, Crocker et al (2001) failed to detect caspase-3 activation at several time points following intrastriatal 6-OHDA administration. Thus, while evidence supports an apoptotic form of cell death, the mediators of 6-OHDA-induced degeneration are not known.

## 9.0 CALPAINS

Calpains were first reported in rat brain in 1964 (Guroff, 1964), and later identified as $Ca^{2+}$ activated neutral proteases in 1968. To date, the calpain family consists of 14 isoforms, and is divided into two classes according to tissue distribution: ubiquitous and tissue specific. The most extensively studied calpains, calpain I (μ-calpain) and calpain II (m-calpain), are ubiquitously expressed, and can be differentiated on the basis of their in vitro $Ca^{2+}$ sensitivity. Approximately 1 – 20 μM and 250 – 750 μM $Ca^{2+}$ levels are required for half-maximal activity of calpain I and calpain II, respectively (Mellgren, 1987). In brain, calpain I is mainly concentrated in neuronal

cell bodies (Siman et al., 1985), whereas calpain II is predominantly observed in axon tracts and glial cells (Nixon et al., 1986).

The activation of calpains is influenced by several factors. First, calpains must bind $Ca^{2+}$ in order to be active. Since the in vitro $Ca^{2+}$ requirement for calpain activation exceeds intracellular levels (intracellular $Ca^{2+}$ is 50 – 300 nM; (Goll et al., 2003), two mechanisms that lower the intrinsic $Ca^{2+}$ requirements of these enzymes have been reported (Maravall et al., 2000). To this end, association of calpains with membrane phospholipids and calpain autolysis have been reported to occur. Although it remains controversial as to whether autolysis is absolutely required for calpain activation, autolysis has nevertheless been reported to lower the $Ca^{2+}$ requirement for calpain activation, and thereby increase enzyme activity. Finally, calpains coexist in cells with calpastatin, the specific endogenous protein inhibitor, suggesting a role for this inhibitor in the regulation of calpain activity (Averna et al., 2003).

### 9.1 Functions and mechanism of action of calpains

The various functions of calpains are not completely known. However, it is thought that these enzymes function, in general, as bio-modulators of cell physiology since they cleave substrate proteins in a limited manner without degrading them (Goll et al., 2003). Calpain substrates are diverse, and include cytoskeletal and myofibrillar proteins, histones, enzymes, myelin proteins, and receptor proteins (Goll et al., 2003). The numerous substrates and ubiquitous expression of some calpain isoforms suggest important roles for calpains, while impeding the elucidation of possible roles under normal physiological conditions.

The putative activation mechanism of calpains is still under debate. However, it is generally agreed that, upon mild stimulation, calpains translocate to membranes, bind to calpastatin, and are thereby prevented from degrading substrates. However, if the stimulus becomes stronger or is sustained, calpain will degrade its inhibitor, and proceed to cleave other substrates. To this end, calpastatin can be easily degraded by calpains in vitro (Mellgren et al., 1986; Nakamura et al., 1989), even more easily than other endogenous substrates, such as spectrin (Nagao et al., 1994). Thus, insufficient regulation of calpain by calpastatin can lead to cellular demise via calpain-mediated proteolysis of various substrates.

The first *in vivo* demonstration of calpastatin degradation by calpains occurred in neonatal rat brain following transient hypoxic ischemia (Blomgren et al., 1999). In this study, unilateral carotid occlusion induced calpastatin upregulation on the contralateral, nondamaged side of the brain, whereas the ipsilateral side was marked by calpastatin fragmentation, calpain activation, and neuronal degeneration. Moreover, degradation of calpastatin on the ipsilateral side, as evidenced by the appearance of the 50kDa calpastatin fragment, was blocked by the administration of the calpain inhibitor CX295. These findings led the authors to suggest that calpastatin may act as a suicide substrate to calpain under pathological conditions.

### 9.2 Calpains and necrosis

The involvement of calpains in neuronal degeneration was first demonstrated indirectly in a gerbil model of cerebral ischemia, in which administration of the calcium channel blocker,

nilvadipine, prevented substrate degradation (Kuwaki et al., 1989). Traditionally, *in vivo* activation of calpains has been associated with a necrotic type of cell death based on morphological changes such as cytoplasmic swelling and cell fragmentation (Yamashima et al., 2003; Pang et al., 2003). Acute neurological insults such as ischemia and excitotoxicity induce a sustained rise in $Ca^{2+}$ levels, which could then trigger calpain activation (Rami, 2003).

### 9.3 Calpains and apoptosis

In brain, calpains have been implicated in apoptosis based on two types of observations: the activation of calpains during cell death, and (2) the inhibition of neuronal degeneration by calpain inhibitors. Calpain involvement has been observed in numerous forms of experimentally-induced neuronal injury in which apoptosis has been detected. For example, animal models of traumatic brain injury (Kampfl et al., 1997), spinal cord injury (Banik et al., 1998; Wingrave et al., 2003), epilepsy (Sierra-Paredes et al., 1999), Alzheimer's disease (Lee and Tsai, 2003) and Huntington's disease (Gafni and Ellerby, 2002) have all shown evidence of calpain overexpression or activation. In a mouse model of Parkinson's disease, activation of calpains was observed in the substantia nigra of mice after administration of MPTP (Crocker et al., 2003).

Activation or overexpression of calpains has also been reported in human diseases marked by excessive cell death. In Parkinson's disease, overexpression of calpain II was observed in degenerating neurons and fibres that co-expressed tyrosine hydroxylase, and in Lewy bodies (Mouatt-Prigent et al., 1996). More recently, the activation of calpains, as evidenced by

increased accumulation of its substrate, spectrin, was observed in nigral tissue from patients (Crocker et al., 2003). This calpain overexpression and increased activation in post-mortem tissue was not compensated for by a concomitant increase in calpastatin expression (Mouatt-Prigent et al., 2000). In Alzheimer's disease, increased calpain II protein (Grynspan et al., 1997; Tsuji et al., 1998), enhanced autolysis of calpain I (Saito et al., 1993), and a reduction of calpastatin (Nixon et al., 1994) has been observed in post-mortem tissue. Collectively, these observations support the hypothesis that an altered calpain-calpastatin ratio may contribute to the excessive neuronal loss in these degenerative diseases.

While the activation of calpains is suggestive of a role in neuronal degeneration, an essential role for these enzymes in apoptosis has been demonstrated from studies using calpain inhibitors in several animal models of disease. For example, a direct role for calpain involvement in dopaminergic cell death was recently demonstrated in a mouse model of Parkinson's disease. In MPTP-treated mice, inhibition of calpains by the administration of the synthetic calpain inhibitor MDL 28170, or the endogenous calpain inhibitor, calpastatin, resulted in significant neuronal and behavioural protection (Crocker et al., 2003). In traumatic brain injury, calpain inhibitors attenuated motor and cognitive deficits (Saatman et al., 1996) and protected the brain from cortical damage (Posmantur et al., 1997). In ischemia, administration of calpain inhibitors have reduced infarct size (Rami and Krieglstein, 1993; Blomgren et al., 1999; Bartus et al., 1999), and striatal cell death is decreased in models of Huntington's disease (Bizat et al., 2003). Calpain inhibition has also prevented myelin and cytoskeleton degradation characteristic of multiple sclerosis (Banik et al., 1997).

### 9.4 Calpain inhibitors

Interpretation of the effects of calpain inhibitors should be made with caution, since many of the first generation inhibitors are somewhat nonspecific. Thus, it is possible that other proteases, in addition to calpains, were also inhibited by these compounds. The first generation of calpain inhibitors includes the active site inhibitors, leupeptin and E-64. The membrane permeable version of E-64 (E-64c) was initially developed to prevent muscle degradation in patients with muscular dystrophy (Sugita et al., 1980). Despite inhibiting both μ- and m-calpains efficiently, these synthetic compounds exert considerable activity against the proteasome and lysosomal cysteine proteases. More recent active site inhibitors, such as calpain inhibitor I, calpain inhibitor II, and calpeptin are more selective than previous blockers, but also exert effects on lysosomal cysteine proteases and the proteasome (Sasaki et al., 1990; Johnson, 2000). An even more selective inhibitor is PD15606, which interacts with the $Ca^{2+}$ binding sites in the calpain enzymes (Wang et al., 1996).

The most selective calpain inhibitor to date is the endogenous protein, calpastatin. In neurons, the proenzyme calpain is normally associated with calpastatin. It binds reversibly and potently (Ki 3 x $10^{-9}$ M; Johnson, 2000) to calpains and shows no inhibition of other cysteine proteases (Dayton et al., 1976). Calpastatin contains four repeated inhibitory domains, so that each molecule of calpastatin can inhibit four molecules of calpain (Croall and McGrody, 1994; Melloni et al., 1998). In brain, calpastatin is expressed in neurons and glia, and to a lesser extent in axons and myelin (Kamakura et al., 1992). Strong immunostaining for calpastatin was demonstrated in cell membranes, whereas diffuse immunostaining was observed in the

cytoplasm of neurons, in overall accordance with the distribution of calpains. The latter observation supports a regulatory role for calpastatin. Indeed, the intracellular calpain-calpastatin ratio is believed to be critically important in regulating calpain activity (Crawford 1990).

### 9.5 Mechanisms of calpain-mediated cell death

The ability of calpains to promote apoptosis in a variety of neurological models raises the question of molecular mechanisms of its action. Inhibitor studies have demonstrated that the catalytic activity of calpain is required for calpain-mediated cell death, suggesting that cleavage of its specific substrates accounts for the pro-apoptotic effects. A diverse group of substrate proteins have been identified (Chan and Mattson, 1999), making it difficult to establish a clear mechanism. However, some of these substrate proteins are cytoskeletal proteins or proteins that can associate with cell membranes, leading to the speculation that calpains may play a role in the destruction of cellular architecture during apoptosis.

The apoptotic regulatory members, p53, Bax, and procaspases-3, -7, -8, -9, and -12 are also calpain substrates, but the impact of calpain cleavage is conflicting. Available evidence supports a role for calpains in the promotion of apoptosis, by cleaving procaspase-12 to its active form (Nakagawa and Yuan, 2000), and cleavage of procaspase-3 (Blomgren et al., 2001). Calpain-mediated cleavage of Bax results in active pro-apoptotic fragments (Choi et al., 2001), and, moreover, enhances its cytotoxic activity (Wood and Newcomb, 2000). Recently, the pro-apoptotic action of calpains was demonstrated in cortical neurons, by a calpain-mediated activation of p53 (Sedarous et al., 2003). In contrast to the literature outlined above, calpains

have been shown to inactivate caspases-3, -7, -9 (Lankiewicz et al., 2000; Chua et al., 2000). Thus, the effect of calpain cleavage on apoptotic regulatory proteins may vary according to cell type and death stimulus. In addition, the majority of these effects have been reported *in vitro*, thus, the in vivo effects of calpain proteolysis in brain remains to be demonstrated.

## STATEMENT OF PURPOSE

Numerous studies have employed 6-OHDA as a tool to deplete dopamine in the brain, and have subsequently examined the neuropatholgoical and behavioural effect of such lesions. Although 6-OHDA has been used for some time in this regard, several important questions regarding its toxicity and behavioural effects in rats remain unknown. The studies in this thesis were designed to address some of these questions.

The experiments outlined in Chapter 2 were performed to compare the effects of three different 6-OHDA lesioning paradigms that have been commonly used by researchers. In addition, the effect of hypothermia was examined in each lesioning paradigm, given that some anesthetics result in prolonged hypothermia, and hypothermia has been neuroprotective in several models of neurological injury. These experiments address the following specific questions:

1. Are the two main dopamine pathways equally susceptible to 6-OHDA toxicity, or is the pathway which degenerates in Parkinson's disease more vulnerable in the 6-OHDA animal model of the disease?
2. Does a substantially greater number of cell bodies remain intact despite significant losses at the nerve terminals, ie., does an axotomy occur?
3. What effect does hypothermia, which occurs from the surgical anesthetic, have on dopamine neuron degeneration?

Chapter 3 addresses the underlying mechanisms of 6-OHDA-induced cell death. In this study, the time course of *in vivo* activation of two proteases (calpain and caspase-3) following 6-OHDA administration was examined. Next, the effect of adenoviral-mediated delivery of the calpain

inhibitor, calpastatin, on 6-OHDA-induced dopamine cell loss was examined. In addition, tests of motoric deficits resulting from 6-OHDA administration were performed to detect possible protective effects of calpastatin delivery. More specifically, these studies address the following questions:

1. What is the time course of calpain and caspase-3 activation in dopamine subregions following *in vivo* 6-OHDA administration?
2. What is the role of calpain in 6-OHDA-induced cell loss?
3. Is calpain activation associated with behavioural impairments that result from 6-OHDA administration?

The overall relevance of my experimental findings is subsequently discussed with regard to therapeutic approaches to Parkinson's disease (Chapter 4).

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**Intervening Section 1**

6-Hydroxydopamine has been widely used as a lesioning tool in Parkinson's disease research. For this purpose, 6-OHDA is infused into the axon tract, nerve terminal fields, or cell body regions to produce degeneration along the nigrostriatal dopamine pathway. Adjacent to the latter dopamine pathway is the mesolimbic pathway. The mesolimbic dopamine pathway has also been selectively targeted by 6-OHDA lesions. In studies employing lesions of the mesolimbic pathway, this dopamine projection has been shown to play a role in mediating the effects of addictive drugs (Wise and Rompre, 1989). Despite the numerous studies employing 6-OHDA lesions in the mesolimbic pathway, the toxic effects have not been characterized beyond the level of the nerve terminal regions. My own preliminary studies revealed the survival of a significant portion of mesolimbic dopamine cell bodies (in the ventral tegmental region), following nerve terminal lesions. Thus, a terminal axotomy had occurred, and this effect contrasts with the extensive loss of nigrostriatal cell bodies following lesions which target nigrostriatal nerve terminals. These observations led us to systematically test the hypothesis that the mesolimbic dopamine pathway was less vulnerable to the toxic effects of 6-OHDA, and explore several methodological factors that might contribute to the preservation of cell bodies. Thus, three lesioning paradigms were employed in separate groups of rats, and losses of dopamine markers were quantified in several dopaminergic subregions. Among the putative neuroprotective factors, we report the protective effect of hypothermia, which occurred due to the administration of anesthetic at the time of surgery.

# CHAPTER 2: Susceptibility of ascending dopamine projections to 6-hydroxydopamine in rats: effect of hypothermia

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**ABSTRACT**

The aims of this study were to determine (1) whether mesolimbic and nigrostriatal DA cell bodies degenerate to different extents after 6-hydroxydopamine (6-OHDA) is administered into their respective terminal fields and (2) whether hypothermia, associated with sodium pentobarbital anaesthesia, protects DA neurons from the toxic effects of 6-OHDA. To address these questions, 6-OHDA or vehicle was infused into either the ventral or dorsal striatum or into the medial forebrain bundle, under conditions of brain normothermia or hypothermia. Two weeks post-surgery, tyrosine hydroxylase-positive cell bodies were counted in the ventral tegmental area (VTA) and substantia nigra. In addition, autoradiographic labeling of tyrosine hydroxylase protein and dopamine transporter was quantified in dopamine terminal fields and cell body areas. Overall, DA cell bodies in the VTA were substantially less susceptible than those in the substantia nigra to depletion of dopaminergic markers. Hypothermia provided two types of neuroprotection. The first occurred when 6-OHDA was administered into the dorsal striatum, and was associated with a 30-50% increase in residual dopaminergic markers in the lateral portion of the VTA. The second neuroprotective effect of hypothermia occurred when 6-OHDA was given into the medial forebrain bundle. This was associated with a 200-300% increase in residual dopaminergic markers in the mesolimbic and nigrostriatal terminal fields; no significant protection occurred in the cell body regions.

Collectively, these findings show that (1) the dopaminergic somata in the substantia nigra are more susceptible than those in the VTA to 6-OHDA-induced denervation, and (2) hypothermia can provide anatomically selective neuroprotection within the substantia nigra-VTA cell population. The continued survival of mesolimbic dopamine cell bodies after a 6-OHDA lesion

may have functional implications relating to drugs of abuse, as somatodendritic release of dopamine in the VTA has been shown to play a role in the effectiveness of cocaine reward.

## INTRODUCTION

The catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) is commonly infused into the caudate putamen or into the medial forebrain bundle (MFB) in order to produce dopaminergic (DAergic) degeneration (Jonsson, 1983). Behavioural effects of such treatment are almost invariably interpreted in terms of terminal field denervation (Schwarting and Huston, 1996). However, in the nigrostriatal system, a substantial proportion (typically 50%) of cell bodies has been reported to survive despite major terminal dopamine (DA) depletion (Lee et al., 1996; Kirik et al., 1998). The continued survival of DAergic somata may have functional implications given that somatodendritically released DA can modulate DAergic and non-DAergic output pathways from the substantia nigra (SN) (Cheramy et al., 1981; Robertson and Robertson, 1989; Crocker, 1997).

Much less is known about the fate of mesolimbic DA cell bodies after intracerebral 6-OHDA administration. Studies employing 6-OHDA delivered into the MFB indicate that DAergic cell bodies in the ventral tegmental area (VTA) may be less susceptible to degeneration than those in the substantia nigra (SN)(Thomas et al., 1994; Sullivan and Szechtman, 1995). However, infusion of 6-OHDA directly into the nucleus accumbens, has not, to our knowledge, been characterized with respect to DA cell body degeneration. This is perhaps surprising, given that this type of lesion is widely employed in behavioural studies.

In our own preliminary studies, intra-accumbens infusion of 6-OHDA resulted in considerable sparing of cell bodies in the VTA, despite marked depletion of mesolimbic terminal fields. These initial observations were made using sodium pentobarbital anaesthesia, which markedly reduces rectal and brain temperature (Lomax, 1966; Yamaguchi et al., 1986; Torbati et al., 2000).

Hypothermia is known to protect against a number of brain insults, including ischemia (Dietrich et al., 1996), and high doses of 3,4-methylenedioxymethamphetamine ("Ecstasy"; Malberg and Seiden, 1999) and methamphetamine (Bowyer et al., 1992; Albers and Sonsalla, 1995). However, possible effects of temperature on 6-OHDA toxicity have not been reported.

The present study addressed two particular questions. The first was whether nigrostriatal and mesolimbic DA neurons degenerate to different extents after comparable degrees of denervation in their respective terminal fields. The second question was whether anaesthetic-induced hypothermia protects DA cell bodies from 6-OHDA-induced toxicity.

## EXPERIMENTAL PROCEDURES

*Experiment 1: DA cell body loss following 6-OHDA infusion into nigrostriatal vs. mesolimbic terminal fields: effect of temperature*

Rats (n=96) were allocated to four groups (n=24) and were given either 6-OHDA or vehicle into either dorsal or ventral striatum. Each group was further divided into hypothermic and normothermic subgroups (n=12). All rats were anaesthetized with sodium pentobarbital prior to surgery. In the hypothermic subgroups, core temperature was permitted to fall under the influence of the anaesthetic, whereas in the normothermic subgroups, it was thermostatically maintained at 37.0 - 37.4 °C during and following surgery (see below). Fourteen days after surgery, all rats were sacrificed. Within each subgroup, six rats provided perfusion-fixed brains and the remaining six rats provided fresh frozen brains. Degeneration was assessed by tyrosine hydroxylase (TH) immunolabeling of neuronal profiles, Nissl staining (fixed brains), [$^{125}$I]RTI-

55-autoradiography, and [$^{125}$I]TH immunoautoradiography (unfixed brains). Group allocations were random.

*Experiment 2: DA cell body loss following 6-OHDA infusion into MFB: effect of temperature*

In order to achieve an even greater degree of depletion in the terminal fields, 6-OHDA or vehicle (n=12 per group) was infused into the MFB. Six animals in each group were maintained normothermic and the remaining six were allowed to become hypothermic (as in Expt. 1). Fourteen days after surgery, these animals were sacrificed by decapitation, and degeneration was assessed in unfixed brains by [$^{125}$I]RTI-55 autoradiography, TH immunoautoradiography, and Nissl staining. Additional rats, drawn from a separate pool, provided perfusion-fixed brains for TH immunohistochemistry. These rats were randomly allocated to the same four surgery conditions as above (n=4-6 per group).

*Experiment 3: Comparison of rectal vs. brain temperature under normothermic and hypothermic conditions*

To determine whether rectal temperature accurately reflects striatal temperature under conditions similar to those in Expt. 1 and 2, rats (n=7) were implanted with chronic indwelling guide cannulae and were monitored simultaneously for striatal and rectal temperature under sodium pentobarbital anaesthesia. Thus, each rat was monitored in two sessions for striatal and rectal temperatures; these sessions were spaced 7 days apart. In one session, rectal temperature

was maintained at 37.0 – 37.4°C. In the other session, the animals were allowed to become hypothermic. The order of testing was counterbalanced.

*Stereotaxic surgery*

All experimental protocols were approved by the McGill University Animal Care Committee, in accordance with Canadian Council on Animal Care Guidelines. Male Sprague Dawley rats (311-399 g at the time of surgery) were purchased from Charles River, St. Constant, Quebec. Twenty minutes prior to surgery, animals received desipramine HCl (25 mg/kg as salt, i.p.; Merrell Dow Research) in order to inhibit 6-OHDA-induced degeneration of noradrenaline terminals (Kelly and Iversen, 1976). Rats were anaesthetized with sodium pentobarbital (55 mg/kg i.p.) and positioned in a stereotaxic instrument (Kopf, Tujunga, CA, USA). Unless otherwise indicated, stereotaxic coordinates were derived from the mean of two systems based respectively on interaural (IA) zero and bregma (Paxinos and Watson, 1997). Dorsoventral coordinates reflect distance from the skull surface. All infusions were unilateral, and made via a 30-gauge stainless steel cannula attached by polyethylene tubing to a 10-µl Hamilton syringe driven by a syringe pump (MD-1001, BioAnalytical Systems Inc., West Layette, IN, USA). Ambient temperature during surgery and recovery was 22 ± 1 °C.

*Experiment 1 surgery.* In order to achieve a similar degree of depletion in the respective terminal fields, 6-OHDA was given in a higher dose into the dorsal striatum (28 µg base in 4 µl vehicle) than into the ventral striatum (12 µg base in 2 µl). Control rats received vehicle infusions containing 0.3 mg/ml sodium metabisulphite dissolved in 0.9% saline (pH adjusted to 7.4 with dilute NaOH). Sodium metabisulphite was used as an antioxidant instead of the

commonly used ascorbic acid since the latter loses much of its antioxidant effect at neutral pH (M. Louis and P.B.S. Clarke, unpublished observations). During surgery, 6-OHDA was shielded from light to further inhibit oxidation. For dorsal striatal infusions, the cannula was lowered to A +9.2/1.2, L +3.0, V +4.5/-5.5 (toothbar--3.9 mm) corresponding to the middle of the caudate-putamen. For ventral striatal infusions, coordinates were A +10.7/1.7, L +1.5, V +7.8/-2.2 (toothbar –3.9 mm) corresponding to the accumbens-olfactory tubercle border. Infusions were made at a rate of 0.1 µl/min, followed by a 5 minute diffusion period before the cannula was slowly retracted. The wound was sutured with catgut chromic thread (Serag-Weissner, Naila, Germany).

*Experiment 2 surgery.* Rats received unilateral MFB infusions of either 6-OHDA HBr (8 µg base in 3µl; n=23) or vehicle (n=22) given at the level of the lateral hypothalamus. Coordinates were based on interaural zero only: A +5.9, L –2.3, V +2.2 (toothbar –4.2 mm).

*Experiment 3 surgery.* Each rat was chronically implanted with a unilateral 22-gauge stainless steel guide cannula (C313G, Plastics One, Roanoke, VA, USA) with its tip aimed 1 mm dorsal to the nucleus accumbens core. Guide cannulae coordinates were: A 10.7/1.7, L –1.5, V +3.1/-6.9 (toothbar –3.9 mm). The cannula assembly was anchored to the skull by cranioplastic cement and three stainless steel skull screws (Lomir Inc., Quebec). A stainless steel stylet (0.2 mm O.D., C313DC, Plastics One) prevented occlusion of each guide cannula. The stylet protruded 1 mm below the cannula tip.

*Temperature monitoring*

*Rectal Temperature (Experiment 1 and 2).* Following administration of general anaesthesia for surgery, rectal temperature was monitored via a probe attached to an animal blanket control unit (Harvard Apparatus) set to maintain temperature at 37.0 – 37.4°C. Rectal temperature for non-heated rats was monitored via a digital thermometer (FisherBrand, Whitby, ON, Canada). Rectal probes were inserted 6 cm into the rectum (Miyazawa and Hossmann, 1992). Three temperature readings were recorded: ten min following injection of anaesthetic, immediately before infusion of 6-OHDA, and when rectal temperature was lowest.

*Brain vs. rectal temperature (Experiment 3).* A Subminiature K-type thermocouple probe (model KMTSS-010U-6, Omega, Laval, Canada) was connected via a slip-ring commutator (CAY-675-12, Airflyte Electronics Co., Bayonne, NJ, USA) to a thermocouple thermometer unit (DuaLogR® Thermocouple Thermometer, model P-91100-50, Labcor, Anjou, Canada). Ten min following the injection of anaesthetic, the thermocouple probe was manually inserted into the implanted guide cannula. The tip of the thermocouple was aimed at the nucleus accumbens. Brain and rectal temperatures were recorded simultaneously every 10 min until the rat started to recover from anaesthesia. Thermocouples were calibrated against a mercury thermometer marked in 0.1 °C divisions and traceable to NIST standards (Fisher Scientific, Montreal).

*Tissue Processing*

Rats were anaesthetized with pentobarbital and decapitated. Brains were either removed immediately (i.e. unfixed) or were formaldehyde-fixed before removal, depending on the experimental requirements. Unfixed brains were frozen in 2-methyl butane (-50°C for 30 s) and stored at -40°C. Cryostat-cut coronal sections (25 μm thick) were taken at DA terminal levels corresponding to 11.2, 10.7, 10.2, 9.7, 9.2, and 8.7 mm anterior to interaural zero (Paxinos and Watson, 1997). For DA cell bodies, levels corresponding to 3.7, 3.2, and 2.96 mm anterior to interaural zero were collected. Sets of adjacent sections were then taken for [$^{125}$I]RTI-55 autoradiography, [$^{125}$I]TH immunoautoradiography, and Nissl staining with thionin. Sections were thaw-mounted on gelatin-subbed slides, air-dried at RT for 20 min, then stored with desiccant at -40°C.

For protocols requiring fixed tissue (TH immunohistochemistry and cresyl violet staining), rats were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer in 0.1 M NaCl (PBS) and post-fixed for 2-3 hours in the same fixative, then cryoprotected in 20% sucrose in 0.1 M PBS for 36 hours at 4°C. Brains were then frozen as for unfixed brains. Freely floating sections (40 μm) were collected into plastic multiwells containing PBS. Every section between interaural level 2.9 – 4.2 was collected; every fourth section was processed for TH immunoreactivity and adjacent sections were stained with cresyl violet. Since the three DAergic markers had different tissue fixation requirements, TH-positive cell counts were performed on tissue from perfusion-fixed animals, whereas a separate group of animals provided (non-fixed) tissue for $^{125}$I-TH immuno- and [$^{125}$I]RTI-55 autoradiography to be done on adjacent sections.

*TH immunohistochemistry.* Endogenous peroxidase activity was quenched by incubating sections with 0.3% $H_2O_2$ in PBS/0.2 % Triton X-100 (PBS/TX) for 30 min at RT. After rinsing 3x with buffer (PBS/TX), sections were incubated in 10% goat serum (Vector Laboratories, Burlingame, CA) in PBS/TX for 1 hour at RT and then incubated with rabbit polyclonal anti-TH (1:500; Pel Freez, Rogers, AR) in 1% goat serum/PBS/TX for 48 hours at 37°C. Negative controls were included for each rat in each staining batch by omitting the primary antibody. After rinsing sections with buffer, sections were incubated with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories) in 1% goat serum/PBS/TX for 1 hour at 37°C. After a rinse in buffer, sections were incubated with avidin-biotin-peroxidase complex (1:200; Vectastain Standard ABC kit; Vector) for 1 hour at RT. TH immunostaining was visualized by incubating sections in 0.06% 3,3'-diaminobenzidine (Sigma Chemical Co) for 10 min, followed by the addition of 0.01% $H_2O_2$ for approximately 1 min. Sections were mounted onto gelatin-coated glass slides, dehydrated in ascending alcohols, cleared in xylene, then coverslipped with Permount (Fisher Scientific).

*[$^{125}$I]RTI-55 autoradiography.* The extent of 6-OHDA-induced denervation was quantified by autoradiographic labeling of the plasmalemmal DA transporter with [$^{125}$I]RTI-55, which has been shown to bind selectively to DAT provided the serotonin transporter is inhibited (Boja et al., 1992; Cline et al., 1992). Unfixed sections were thawed and incubated for 2 hours at RT with 10 pM [$^{125}$I]RTI-55 (NEN Life Science Products, Inc.; specific activity 2200 Ci/mmol) in 10 mM sodium phosphate buffer containing 0.1 M sucrose and 50 nM citalopram HBr (gift from H. Lundbeck A/S) in order to protect 5-HT transporters. Nonspecific binding was defined by the addition of the DAT inhibitor GBR 12909 (10 μM). Sections were rinsed in ice-cold buffer (1 x

1 min plus 2 x 20 min) and distilled water (5 sec), then blow-dried. Sections were exposed to Kodak X-OMAT X-ray film together with [$^{125}$I]-Microscale autoradiographic standards (Amersham Pharmacia Biotech) for 48 hours. Films were processed with D19 developer and GBX fixer (Kodak).

*Tyrosine hydroxylase immunoautoradiography.* Unfixed sections were post-fixed in an aqueous solution containing 6% paraformaldehyde, 20% absolute alcohol, 20% ethylene glycol, 10% glycerol, and 0.32 M sucrose for 1 hour at -20°C. After washing (2 x 5 min then 1 x 30 min) in buffer (PBS/0.3% Tween-20), sections were incubated in a blocking solution containing 30% skim milk powder (Carnation), 3% rabbit serum (GibcoBRL) and 0.05% $NaN_3$ for 2 hours at RT. After washing with buffer, sections were incubated with mouse monoclonal anti-TH antibody (1:1000; Sigma Chemical Co.) in 1.5% rabbit serum and 0.05% $NaN_3$ overnight at 4°C. After washing with buffer (1x 5 min; 1x 10 min, then 1x 30 min), the secondary antibody, [$^{125}$I]-labeled rabbit anti-mouse IgG (NEN Life Science Products, Inc.; specific activity 1200 Ci/mmol) was applied (8 pM) for 1 hour at RT. This antibody was added to a solution containing 10% skim milk powder, 5% rabbit serum and 0.05% $NaN_3$. Sections were rinsed in buffer, blow-dried, then exposed to Kodak X-OMAT X-ray film together with [$^{125}$I]-Microscale autoradiographic standards (Amersham Pharmacia Biotech) for 6 days. Films were processed with D19 developer and GBX fixer (Kodak).

*TH-positive neuronal profile counts.* The number of TH-positive cell bodies was estimated using the optical fractionator method (West et al., 1991), which is regarded as superior to

assumption- or design-based techniques because it avoids several methodological biases that interfere with estimates of neuron numbers (West, 1999).

Estimates were made using an Olympus BX51 microscope and a motorized X-Y-Z stage controlled by a M5+ MCID computer-based system (Imaging Research, St. Catherines, Canada). Sections from three levels (corresponding to interaural 3.7, 3.2 and 2.9) were first identified under 100X magnification. The VTA was subdivided into medial and lateral regions at each of these rostrocaudal levels; the SN was subdivided into medial, middle, and lateral regions (see Fig.1). A computer-generated counting frame was randomly placed on the counting areas and systematically moved through all regions at 400X magnification. Only the profiles that came into focus within the counting volume were counted. It should be noted that profile counts represent the mean ± SEM number of profiles over the three anterior-posterior levels for each subregion (and not total profiles over the entire anterior-posterior extent of a subregion). Profile quantification was performed in this way so that comparisons of the three markers of DAergic depletion could be made at the same anterior-posterior levels.

Anatomical subdivisions of the SN and VTA in the present study were chosen based on previous delineations (German and Manaye, 1993; McRitchie et al., 1996) (Fig. 1). Depending on the anterior-posterior level, the medial VTA included the central and rostral linear nucleus, interfascicular nucleus, and the paranigral nucleus whereas the lateral VTA included the paranigral nucleus and the parabrachial pigmented nucleus.

It should be emphasized that TH cell counts are a marker of DA phenotype; thus, a reduction in this measure does not necessarily imply cell death.

*Quantitative autoradiographic analysis.* Quantification of DAT and TH was performed at two weeks post-lesion. This time point was based on studies showing that both DAT and TH abundance reach near-maximal depletion within two weeks of 6-OHDA administration either into terminal fields (Sauer and Oertel, 1994; Przedborski et al., 1995) or into the MFB (Gordon et al., 1997). Film autoradiographs were quantified using an M4 MCID computer-based system (Imaging Research, St. Catherines, Canada).

Brain structures were delineated as above for TH cell counts. Autoradiographic images from four rostrocaudal levels of the striatum, corresponding to IA 9.2, 9.7, 10.2, and 10.7 were quantified. For structure identification, each image was aligned with its adjacent Nissl-stained section. Structures were sampled by individually outlining the dorsal striatum (i.e. caudate-putamen) and ventral striatum (i.e. nucleus accumbens core, shell, and olfactory tubercle). Optical density values were converted to wet tissue equivalent values by comparison with [$^{125}$I]-Microscales. To obtain a single value representative of background binding for each autoradiogram, all sections incubated with GBR 12909 ([$^{125}$I]RTI-55), or without the primary antibody ([$^{125}$I] TH immuno), were sampled. Thus, for each film, a mean background value was subtracted from total binding within the same film.

*Statistical analysis*

Data were analyzed using commercial software (Systat 9.0, SPSS Inc., Chicago, IL, USA). Data were subjected to repeated measures ANOVA, with SAMPLE SITE as a within-subject factor and TEMPERATURE (i.e. normothermic *vs.* hypothermic), TOXIN (6-OHDA *vs.* vehicle) and

INJECTION SITE (dorsal *vs*. ventral striatum) as between-subject factors. For repeated measures ANOVA, the Huyhn-Feldt P value is reported. Where appropriate, Student's t-tests were employed. All probability values were two-tailed. In view of the large number of potential group comparisons, a P value of less than 0.01 was considered significant. Data are reported as mean ± SEM.

## RESULTS

*Experiment 1: Dopaminergic markers in non-lesioned brain*

Values of neuronal profile number, autoradiographic labeling of [$^{125}$I]RTI-55 and TH protein in brain areas contralateral to the infusion did not differ significantly between treatment groups ($P>0.5$) and therefore only pooled data are presented here. The mean (± SEM) values for TH+ neuronal profile numbers in the five cell body subregions (medial and lateral VTA, and medial, middle and lateral SN) were, respectively: 189.5 ± 2.0, 185.4 ± 2.1, 174.8 ± 2.2, 94.6 ± 1.4, and 111.1 ± 1.9. [$^{125}$I]RTI-55 labeling (fmol/mg tissue) in the dorsal and ventral striatum, in addition to the five cell body subregions, were, respectively: 0.83 ± 0.02, 0.38 ± 0.01, 0.51 ± 0.01, 1.06 ± 0.02, 1.18 ± 0.02, 0.80 ± 0.01 and 0.68 ± 0.01. Equivalent values (fmol/mg tissue) for [$^{125}$I]TH-immuno autoradiography were: 0.12 ± 0.001, 0.14 ± 0.01, 0.33 ± 0.01, 0.32 ± 0.01, 0.28 ± 0.01, 0.24 ± 0.01, and 0.21 ± 0.01. Since the contralateral hemisphere was not affected by 6-OHDA infusion or temperature in any measure of DAergic depletion, data ipsilateral to the infusion were expressed as a percentage of the contralateral side. Vehicle infusion produced no detectable effects on any measure (all percentage values were close to 100%), and were excluded from analysis.

Table 1 summarizes the precision of the optical fractionator estimates of TH+ neuronal profile numbers. The CE provides an estimate of the within-section variance, and as a general rule, should be less than 0.10 (West 1999; Keuker et al 2001). The mean error variance ($CE^2$) is optimal when it is less than half of the total observed variance ($CV^2$) (Gundersen and Jensen 1987), according to the equation $CV^2 = BV^2 + CE^2$ (where $BV^2$ represents the biological variance). In the present study, the mean $CE^2$ values for each group were always less than one-half, ranging between 23 – 47 %.

*Extent of depletion in dopamine terminal fields following local 6-OHDA infusion*

*[$^{125}$I]RTI-55 and [$^{125}$I]TH immuno- autoradiography.* Infusion of 6-OHDA into dorsal striatum or ventral striatum resulted in a substantial depletion of both DAT and TH labeling in the terminal fields (Fig. 2A and B). The extent of depletion in these terminal fields was not significantly affected by temperature (TEMPERATURE P>0.7 for both markers; Fig. 2A and B). Accordingly, hypothermic and normothermic groups were pooled for subsequent analysis of DAergic markers in terminal fields.

Following infusion of 6-OHDA into the dorsal striatum, both DAT and TH labeling were markedly depleted in this structure (by 88 ± 2.0 % and 95 ± 1.4 %, respectively); both markers, especially DAT labeling, were also reduced in the ventral striatum in these animals (by 64 ± 4.3 % and 17 ± 5.6 %). To examine the possibility that dorsal striatal infusion of 6-OHDA preferentially depleted the core *vs.* shell of the nucleus accumbens, depletion in these two structures was compared. In the normothermic condition, percentage loss of DAergic markers in core *vs.* shell was as follows: 65.5 ± 3.2 % *vs.* 60.1 ± 5.2 % (DAT; t-test, P>0.3); and 23.9 ± 12.3 % *vs.* 14.9 ± 7.1 % ([$^{125}$I]TH immuno autoradiography; t-test, P>0.2). Equivalent values for the hypothermic condition were: 61.8 ± 8.1% *vs.* 60.2 ± 6.4 % (DAT; t-test, P>0.4), and 16.1 ± 10.0 % *vs.* 11.8 ± 7.5 % ([$^{125}$I]TH immuno autoradiography; t-test, P>0.2). Thus, there was no clear evidence of preferential depletion of either the core or shell (Fig. 3A, B, E, and F).

Ventral striatal infusion of 6-OHDA greatly depleted DAT and TH labeling in this structure (84 ± 1.9 % and 88 ± 2.4 %, respectively), with only minimal depletion of the overlying dorsal striatum (11 ± 4.3 % and 6 ± 2.8 % respectively)(Fig. 3C, D, G, and H).

Collectively, these data suggest that 6-OHDA infusion into the dorsal or ventral striatum resulted in substantial terminal loss in each target structure. The extent of *local* depletion was similar, but infusion of 6-OHDA into the dorsal striatum was less anatomically selective.

*Depletion in DAergic cell body regions following 6-OHDA infusion into terminal fields*

Although the VTA and SN were each divided into subregions for image analysis, the effects of 6-OHDA did not appear to be subregion-dependent (Fig. 6A and 6B). Therefore, data were pooled across subregions within either structure. This allowed comparison of overall depletion in the VTA vs. SN.

*TH+ neuronal profiles, [$^{125}$I]RTI-55 and [$^{125}$I]TH-immuno autoradiography.* Although infusion of 6-OHDA into the dorsal striatum and ventral striatum produced similar degrees of local terminal field depletion (see above), the impact at the cell body level was greater when the dorsal striatum was targeted with the toxin. Following 6-OHDA infusion into the dorsal striatum, the percentage loss of DAergic markers in SN was as follows: 50 ± 1.4% (TH+ neuronal profiles), 63 ± 1.4 % (DAT), and 52 ± 2.8 % ([$^{125}$I]TH immuno autoradiography)(Fig. 4A, B, and C). Following toxin infusion into the ventral striatum, the percentage loss of these markers in the VTA was: 33 ± 1.5 % (TH+ neuronal profiles) 30 ± 2.7 % (DAT) and 26 ± 3.1 % ([$^{125}$I]TH immuno autoradiography)(t-test, $P<0.001$ for all markers)(Fig. 4A, B, and C). Overall depletion in the SN was compared to that in the VTA in groups given 6-OHDA into the dorsal or ventral

striatum, respectively. All three DAergic markers were significantly more depleted in the SN than in the VTA (t-test, $P<0.001$) (Fig. 4A, B, C). It should also be noted that DAergic markers in the VTA were more depleted after toxin infusion into the dorsal striatum than into ventral striatum (t-test, $P<0.01$ for all markers).

*Relationship between cell body and terminal field degeneration*

*[$^{125}$I]RTI-55 and [$^{125}$I]TH immuno autoradiography.* To assess the relationship between residual labeling in DAergic cell body regions *vs.* terminal fields, linear regression analysis was performed. In animals that had received 6-OHDA in the dorsal striatum, neither DAT nor TH labeling in the SN was significantly correlated with labeling in the dorsal striatum ($r=0.04$, $P>0.9$ and $r=0.3$, $P>0.5$). However, in animals that had received 6-OHDA in the ventral striatum, DAT labeling in the VTA and ventral striatum was positively correlated ($r=0.83$, $P<0.01$); no significant correlation was found for TH labeling ($r=0.42$, $P>0.1$).

Residual TH labeling in dorsal and ventral striatum was also expressed on a per cell basis, by dividing TH labeling in the dorsal and ventral striatum by TH+ cell counts in the SN or VTA, respectively. Terminal field infusion of toxin resulted in a dramatic reduction in this ratio compared to controls. This was the case for both dorsal striatum ($10.2 \pm 1.2$ vs. $86.3 \pm 3.7$) and ventral striatum ($17.4 \pm 4.4$ vs. $69.8 \pm 1.2$ pmol/mg per neuron). In contrast, a trend ($P<0.03$) towards an *increase* in TH labeling per neuron was observed in the ventral striatum when toxin was infused into the dorsal striatum ($104.3 \pm 1.9$ vs. $67.2 \pm 5.7$ pmol/mg per neuron).

*Effect of temperature on dopamine cell body depletion following a terminal*

*6-OHDA lesion*

Three temperature readings were taken for each rat (see Methods). Since these temperatures were highly inter-correlated (r=0.88 to 0.97), the mean was calculated to provide a single representative temperature for each rat. As expected, mean core temperature was very similar among normothermic groups (mean ± SEM, in °C: Vehicle/DS 37.0 ± 0.1; Vehicle/VS 37.0 ± 0.1; 6-OHDA/DS 37.2 ± 0.1; and 6-OHDA/VS 37.2 ± 0.1). Corresponding values in the hypothermic groups were approximately 5 °C lower (mean ± SEM, in °C: Vehicle/DS 32.1 ± 0.6; Vehicle/VS 32.5 ± 0.4; 6-OHDA/DS 31.3 ± 0.6; and 6-OHDA/VS 31.9 ± 0.4). Temperature did not differ significantly between hypothermic groups (P>0.3). In hypothermic animals, the minimum temperature reached was 2.4 ± 0.2 °C lower than the mean value.

*TH+ neuronal profiles, [$^{125}$I]RTI-55 and [$^{125}$I]TH immuno autoradiography.* All five anatomical subregions of SN and VTA were examined for a possible protective effect of temperature on the three DAergic markers. It should be recalled that DAergic depletion in terminal fields did not differ significantly between normothermic and hypothermic groups (see above). In dorsal striatal-infused animals, a clear protective effect was observed at the cell body level, but only in the lateral VTA (Fig. 5A, B, and C). This was evident for TH+ neuronal profile number (Fig. 6A) and DAT labeling (Fig. 6B) but not for [$^{125}$I]TH immuno autoradiographic labeling (Fig. 6C). Thus, TH+ neuronal profile number decreased significantly less in the hypothermic group than in the normothermic group (37 ± 5.0 % *vs.* 68 ± 5.3 %, respectively: t-test, P<0.001). Corresponding values for DAT labeling were: 40 ± 5.3 % *vs.* 58 ± 4.7 % (t-test, P<0.001). In contrast, no temperature effect was apparent in any brain region when 6-OHDA was infused into the ventral striatum.

To further examine the relationship between temperature and the DAergic markers in the lateral VTA, linear regression analysis was performed on the hypothermic group lesioned in the dorsal striatum. This analysis revealed a highly significant negative relationship between temperature and both TH+ neuronal profile number ($r=0.98$, $P<0.005$) and DAT labeling ($r=0.98$, $P<0.005$) in this VTA subregion (Fig. 7). The regression equations indicated that a 1 °C decrease was associated with a 4.7 % increase in TH+ neuronal profile number and a 3.6 % increase in DAT labeling. There was no significant relationship between temperature and [$^{125}$I]TH immuno autoradiographic labeling in the same VTA subregion ($r=0.58$, $P>0.3$).

*Experiment 2: Extent of depletion in DA terminal fields following 6-OHDA infusion into the MFB*

As in Experiment 1, the three temperature readings taken for each rat. Temperature data is presented separately for rats that provided unfixed *vs.* fixed brain. Temperature readings for rats in the unfixed condition were highly correlated ($r = 0.93$ to $0.97$), and the mean was calculated to provide a single representative temperature for each rat. Among rats that provided unfixed brains, there was virtually no difference between the two normothermic groups (vehicle and 6-OHDA were both $37.2 \pm 0.1$ °C) and between the two hypothermic groups (vehicle $31.6 \pm 0.6$ °C and 6-OHDA $31.6 \pm 0.2$ °C). In the hypothermic groups, the minimum temperature was $1.3 \pm 0.2$ °C lower than the mean temperature. Among rats that provided fixed brains, normothermic group means (°C) were again almost identical: vehicle $37.1 \pm 0.1$ and 6-OHDA $37.1 \pm 0.03$. Corresponding values in the hypothermic groups were: Vehicle $32.6 \pm 0.4$ and 6-OHDA $32.1 \pm$

0.3. Temperatures did not differ significantly between hypothermic groups (P>0.3). In these hypothermic animals, the minimum temperature reached was 1.1 ± 0.2 °C lower than the mean value.

Values of neuronal profile number, [$^{125}$I]RTI-55 and [$^{125}$I]TH immuno autoradiographic labeling in brain areas contralateral to the infusion did not differ significantly between treatment groups (P>0.3). Thus, only pooled data are presented here. Mean (± SEM) values of TH+ neuronal profile numbers in the five cell body regions (medial and lateral VTA, and medial, middle, and lateral SN) were, respectively: 193.7 ±3.2, 205.7 ± 2.6, 188.8 ± 2.8, 103.2 ± 2.2, and 113.5 ± 3.3. Values for [$^{125}$I]RTI-55 (fmol/mg tissue) in the dorsal and ventral striatum, and cell body subregions were, respectively: 0.83 ± 0.03, 0.37 ± 0.01, 0.54 ± 0.01, 1.1 ± 0.02, 1.02 ± 0.02, 0.83 ± 0.02, and 0.71 ± 0.01. Equivalent values (fmol/mg tissue) for [$^{125}$I]TH immunoautoradiography were: 0.1 ± 0.002, 0.09 ± 0.003, 0.3 ± 0.01, 0.31 ± 0.01, 0.27 ± 0.01, 0.24 ± 0.01, and 0.21 ± 0.01. Since the contralateral hemisphere was not significantly affected by 6-OHDA infusion or temperature, data ipsilateral to the infusion were expressed as a percentage of the contralateral side as in Experiment 1. Vehicle infusion produced no detectable effects on any measure (percentage values were always close to 100%; Fig. 8A and 8B), and were excluded from analysis.

Table 2 summarizes the precision of the optical fractionator estimates of TH+ neuronal profile numbers. In the present study, the mean $CE^2$ values for each group were always less than one-half, ranging between 4 – 48 %.

*[$^{125}$I]RTI-55 and [$^{125}$I]TH immuno autoradiography.* Intra-MFB infusion of 6-OHDA resulted in greater depletion in terminal fields than that produced by local infusion in Experiment 1 (compare Fig. 8A and 8B with Fig. 2A and B). Overall, DAT labeling was more depleted in the dorsal striatum than in the ventral striatum ($P<0.001$), whereas TH labeling was depleted to a similar extent in both structures (Fig. 8A and B). Depletion in the core and shell of the nucleus accumbens was compared. In the normothermic condition, DAT depletion was greater in the core than shell (percentage loss 98.8 ± 0.4 % *vs.* 96.1 ± 1.0 %, respectively; $P<0.01$). Equivalent values for [$^{125}$I]TH immuno autoradiography did not approach significance; 96.0 ± 0.7 % *vs.* 97.1 ± 0.1 % ($P>0.3$). In the hypothermic condition, DAT labeling in the core was also significantly more depleted than in the shell (3.1 ± 0.5 % *vs.* 8.2 ± 0.6 %; $P<0.001$). However, the equivalent comparison for [$^{125}$I]TH immunolabeling was nonsignificant (94.8 ± 0.8 % *vs.* 91.5 ±1.8 %, $P>0.1$).

Hypothermia partially protected against depletion of DAT labeling in the terminal fields (TEMPERATURE $P<0.01$) (Fig. 8A). Percentage depletion (mean ± SEM) for normothermic *vs.* hypothermic groups was as follows: 99.6 ± 0.1 % *vs.* 98.4 ± 0.3 % for dorsal striatum (t-test, $P<0.01$), and 98.1 ± 0.5 % *vs.* 93.8 ± 0.5 % for ventral striatum (t-test, $P<0.001$). Visual inspection of the raw data indicated that the relationship between DAT depletion and temperature was likely nonlinear. No protective effect of hypothermia was seen for [$^{125}$I]TH immuno autoradiographic labeling in the terminal fields (Fig. 8B).

*Depletion in cell body regions following 6-OHDA infusion into the MFB*

Intra-MFB infusion of 6-OHDA resulted in substantial depletion of DAergic cell body regions (Fig. 8A and B). The SN exhibited greater loss of TH+ profiles (89 ± 1%), DAT (86 ± 0.7 %) and TH (74 ± 1.9 %) labeling than the VTA (71 ± 1.7%, 71 ± 1.6 % , and 61 ± 1.4 %, respectively). Neither DAT nor TH labeling in the SN or VTA was significantly correlated with residual labeling in the respective terminal field (r=0.01 to r=0.48, P>0.1 for all tests).

*Effect of temperature on DA cell body depletion following MFB infusion of 6-OHDA*

No protective effect of temperature was observed in the DAergic cell body regions for TH+ profiles, DAT or TH labeling (TEMPERATURE P>0.6 and TEMPERATURE x SAMPLE SITE P>0.5 for both measures; Fig. 9A and B). Since Experiment 1 had revealed a localized temperature effect in the lateral VTA, this subregion was analyzed separately. However, no evidence for a protective effect was found either by t-test (P>0.1 for both markers) or by linear regression analysis with temperature as the independent variable (P>0.6).

*Experiment 3: Relation of brain temperature to rectal temperature*

In order to determine whether rectal temperature accurately indicated brain temperature, rats were monitored simultaneously for brain and rectal temperature under surgical anaesthesia. Histological analysis (by Nissl staining) verified that probe placement corresponded to the nucleus accumbens core. In the normothermic condition, brain and rectal temperature did not differ (37.5 ± 0.2 °C *vs.* 37.3 ± 0.1 °C; paired t-test, P>0.5). In the hypothermic condition, both measures decreased gradually and reached a minimum (30.5 to 34.6 °C) within 100-160 min of

anaesthetic administration. Brain temperature was slightly lower than rectal temperature during the descending phase of hypothermia (33.9 ± 0.4 °C *vs.* 34.2 ± 0.4 °C respectively; paired t-test, $P<0.01$), but not during the rewarming phase (paired t-test, $P>0.9$). Full recovery from hypothermia was not observed, even in animals that were monitored for up to 5 hours after anaesthetic administration.

## DISCUSSION

The present study provided several novel findings. First, nigrostriatal DA cell bodies were found to be more susceptible than mesolimbic neurons to loss of DAergic markers after similar degrees of terminal DA denervation by 6-OHDA. Second, we show two types of protective effects of hypothermia. The first occurred after 6-OHDA administration into the terminal regions and was confined to a subregion of the VTA. This appears to be the first evidence for an anatomically selective neuroprotective effect of hypothermia within the SN-VTA DA cell population. The second type of protective effect occurred after 6-OHDA infusion into the MFB. Here, hypothermia inhibited the loss of DAT in nigrostriatal and mesolimbic terminal fields rather than in the cell body regions.

### *Distribution of DAergic markers in the non-lesioned (contralateral) hemisphere*

In the contralateral hemisphere, both TH profile counts and [$^{125}$I]TH immuno autoradiographic labeling revealed a clear mediolateral gradient across the VTA and SN. This pattern of labeling is in broad agreement with previous findings (Blanchard et al., 1994). The divergent patterns of DAT *vs.* TH labeling seen in both DAergic cell body regions and terminal fields have also been reported (Moratalla et al., 1992; Ciliax et al., 1995; Freed et al., 1995; Miller et al., 1997).

In the present study, DAergic markers were unaffected by contralateral infusion of 6-OHDA. In contrast, a small reduction on the contralateral side of a 6-OHDA infusion has been reported previously (Berger et al., 1991; Finkelstein et al., 2000). The lack of contralateral depletion observed in the present study suggests that 6-OHDA leakage was negligible in our rats (perhaps reflecting the slow infusion rate used) and that crossed pathways (Fallon and Loughlin, 1982;

Morgan et al., 1986) were either not depleted or were too sparse to produce a detectable reduction in DAergic markers.

*Differential susceptibility of nigrostriatal vs. mesolimbic DA pathways*

It has been suggested that mesolimbic DA cell bodies are less susceptible than nigrostriatal DA cells to 6-OHDA lesions. Evidence for this notion appears based entirely on the results of toxin infusion into the MFB (Abrous et al., 1990; Abrous et al., 1992; Thomas et al., 1994; Sullivan et al., 1998). In order to test whether differential vulnerability also occurs after terminal field infusion of 6-OHDA, lesion parameters were selected that produced similar degrees of depletion in the dorsal or ventral striatum. Denervation of dorsal striatum resulted in a 50% loss of nigral TH cell counts, consistent with previous reports (Lee et al., 1996; Kirik et al., 1998). In contrast, only 30% of VTA TH-positive cells were lost after ventral striatal denervation. This finding points towards differential susceptibility, but interpretation is complicated by the existence of additional DAergic projections from SN to ventral striatum (Fallon and Moore, 1978; Swanson, 1982; Fallon, 1988; Brog et al., 1993) and from VTA to dorsal striatum (Fallon, 1981; Thomas et al., 1994). Thus, in the present study, toxin infusion into the dorsal striatum resulted in partial depletion of the adjacent ventral striatum, thus encompassing most of the ascending projections from the SN. In contrast, ventral striatal infusion yielded a more focused depletion, which likely spared the projections from the VTA to the overlying dorsal striatum. On balance, it would appear that cell bodies in the nigrostriatal pathway are indeed more susceptible than mesolimbic somata to toxic effects of 6-OHDA administration. However, we cannot exclude the possibility

that residual links between VTA and dorsal striatum may have accounted for some of the difference observed.

TH cell counts in the VTA were reduced not only by the ventral striatal lesion but also by 6-OHDA infusion into the dorsal striatum. The latter finding can probably be explained by the existence of known DA projections from VTA to the ventromedial caudate-putamen and nucleus accumbens (Fallon, 1981; Thomas et al., 1994), both of which were likely depleted by toxin infusion into the dorsal striatum. In contrast, the medial SN, which projects in part to the ventral striatum (Fallon and Moore, 1978; Swanson, 1982; Brog et al., 1993), was not affected by the ventral striatal infusion of 6-OHDA. The latter observation may reflect the high degree of collateralization in this cell population (Fallon, 1981; Loughlin and Fallon, 1982; Prensa and Parent, 2001).

Differential vulnerability has also been observed across terminal fields. In particular, core and caudate-putamen are reported to be more readily depleted by 6-OHDA (Zahm 1991) and methamphetamine (Broening et al 1997) than is the shell. This distinction was also apparent in our quantification of DAT labeling (Expt. 2) despite the use of a near-maximal dose of 6-OHDA. Although TH labeling did not reveal an analogous core/shell difference, this is not surprising since comparable depletions of TH protein have been shown to occur in both core *vs.* shell during the second post-lesion week after midline mesencephalic (Zahm 1991) and MFB (Tan et al 2000) infusions of 6-OHDA.

In the present experiment, DAT expression was generally more depleted than TH expression. Previous studies have shown that following 6-OHDA lesions, percentage losses of DAT (Joyce, 1991a; Joyce 1991b) and [$^3$H]DA uptake (Zigmond et al., 1984; Altar et al., 1987) accurately represent the degree of tissue DA loss. In contrast, the occurrence of TH upregulation may lead

to an underestimation of DA depletion (Zigmond, et al., 1984; Blanchard et al., 1995; Tan et al., 2000). However, the present study revealed further complexities in the regulation of TH. Specifically, a reduction in TH abundance per cell occurred in dorsal and ventral striatum following local 6-OHDA infusion, with no corresponding change in SN and VTA. Factors determining the occurrence and direction of TH regulation are not clear. Possibly, TH downregulation only occurs when a critical threshold of depletion has been reached.

*Neuroprotective effects of hypothermia*

Since monitoring of striatal temperature would have interfered with intrastriatal infusion of toxin or vehicle, brain temperature was indirectly estimated by core temperature. Although brain and core temperatures are usually similar, divergence has been noted after certain pharmacological or behavioural manipulations (Miyazawa and Hossmann, 1992; Colbourne et al., 1993; Schwab et al., 1997; Wass et al., 1998). Furthermore, the relationship between these two measures can change during the cooling and rewarming phases of hyperthermia (Sundgren-Andersson et al., 1998; Wass et al., 1998). In the present study, brain and rectal temperature were found to be very similar during both normothermia and hypothermia. We therefore conclude that rectal temperature provided a satisfactory measure of striatal temperature.

A protective effect of hypothermia has been demonstrated in several animal models of degeneration (see Dietrich et al., 1996; Malberg and Seiden, 1999 for reviews). The present study extended these findings to 6-OHDA-induced degeneration. Two protective effects were observed. The first such effect was observed at the level of DA cell bodies after terminal field infusion of the toxin. Here, neuroprotection showed remarkable anatomical selectivity, since it

was restricted to the lateral VTA and only occurred after dorsal striatal infusion of toxin. The absence of analogous neuroprotection after toxin infusion into the ventral striatum suggests that the neurons in question project to the ventromedial portion of the dorsal striatum. The second neuroprotective effect was observed only at the level of DA terminal fields and occurred after intra-MFB infusion of 6-OHDA. The absence of neuroprotection at the cell body level cannot be readily explained by a floor effect, since appreciable DAT labeling remained in both VTA and SN.

The protective effects of hypothermia observed could potentially occur at all steps of 6-OHDA-mediated neurotoxicity. For example, DAT activity has been shown to decrease by approximately 50% for a 3 °C fall in temperature (Xie et al., 2000). Conversely, the formation of cytotoxic molecular species such as hydroxyl and superoxide radicals, which are produced by auto-oxidation of 6-OHDA (Heikkila and Cohen, 1971; Cohen and Heikkila, 1974), are increased under hyperthermic conditions (Globus et al., 1995; Kil et al., 1996). Why neuroprotection would favor a subpopulation of DA cells is unknown, but likely reflects some aspect of their heterogeneous nature, which has been described at the level of morphology (Phillipson, 1979; Domesick et al., 1983; Prensa and Parent, 2001), neurochemical phenotype (Hokfelt et al., 1980; Gerfen et al., 1985), and electrophysiology (Gardner and Ashby, Jr., 2000).

*Functional implications*

Various degrees of hypothermic neuroprotection have been reported in the literature. In the present study, hypothermia increased DAT labeling (by approx. 30%) and the number of TH+ neurons (by approx. 50%) in lateral VTA after terminal field administration of 6-OHDA. A

comparable level of neuroprotection has been shown in several brain regions after administration of MDMA ("Ecstasy")-treated animals (Colado et al., 2001). In contrast, near-complete protection has been observed in striatal neurons after a slight (2°C) reduction in temperature during ischemia (Minamisawa et al., 1990). However, the degree of protection in ischemic brain varied according to brain region; other structures (i.e. the hippocampus and thalamus) were protected to a lesser extent.

After intra-MFB infusion of 6-OHDA, hypothermia increased residual DAT labeling in the ventral and dorsal striatum by more than 2- and 3-fold, respectively. This degree of neuroprotection may have important functional consequences. In vivo microdialysis studies suggest that DA release remains near normal unless depletion exceeds 95% (Robinson and Whishaw, 1988; Zhang et al., 1988; Castaneda et al., 1990; Parsons et al., 1991). In the present study hypothermia reduced the depletion of DAT from 98% to 93% in the ventral striatum and from 99.7% to 98.5% in the dorsal striatum. Hence, hypothermia may have restored interstitial DA to near-normal levels, at least in the ventral striatum.

The finding that many DA cell bodies survive following terminal field administration of 6-OHDA may also have functional implications. Several studies indicate that somatodendritic release of DA modulates the activity of afferent and efferent pathways not only in the SN (Cheramy et al., 1981; Robertson and Robertson, 1989; Crocker, 1997) but also in the VTA (Ranaldi and Wise, 2001). For example, Ranaldi et al (2001) found that rats trained to self-administer intravenous injections of cocaine modified their responding under the influence of bilateral VTA injections of the D1 dopamine receptor antagonist SCH 23390, suggesting a role for dendritically released DA in the VTA in the effectiveness of cocaine reward. Thus, present findings suggest that studies investigating the effects of 6-OHDA given into the DAergic

terminal fields should take into account the possibility of preserved mesolimbic and nigrostriatal somata.

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*Abbreviations:*

dopamine (DA)

dopamine transporter (DAT)

6-hydroxydopamine (6-OHDA)

interaural (IA)

medial forebrain bundle (MFB)

3,4-methylenedioxymethamphetamine (MDMA)

phosphate buffered saline (PBS)

3β-(4-iodophenyl)tropan-2β-carboxylic acid methyl ester (RTI-55)

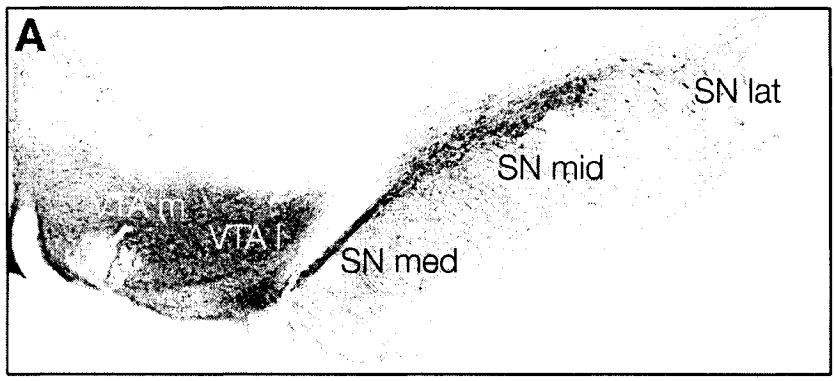
substantia nigra (SN)

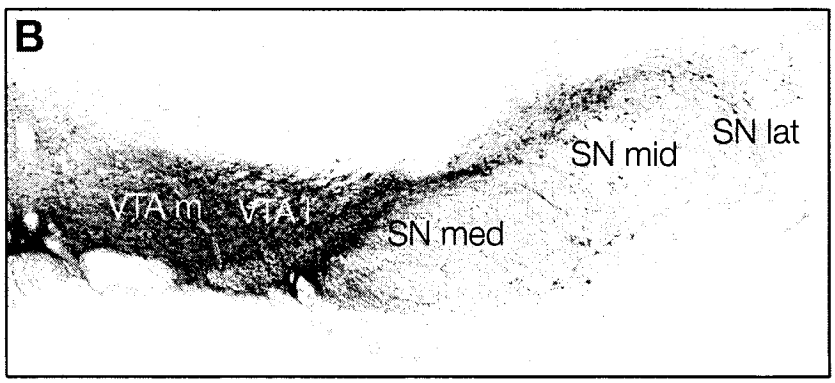
tyrosine hydroxylase (TH)

Triton X-100 (TX)

ventral tegmental area (VTA)

Figure 1: Anatomical subregions within the right substantia nigra and ventral tegmental area indicated on tyrosine hydroxylase-immunostained tissue from a control (vehicle-infused, normothermic) rat. Coronal levels correspond to: 3.7 (A); 3.2 (B); and 2.9 mm (C) anterior to interaural zero (Paxinos and Watson, 1997). Abbreviations: VTA m and VTA l - medial and lateral ventral tegmental area; SN med, mid and lat - medial, middle and lateral substantial nigra. Scale bar = 200 μm.


A
SN lat
SN mid
VTA m
VTA l
SN med


B
SN lat
SN mid
VTA m
VTA l
SN med

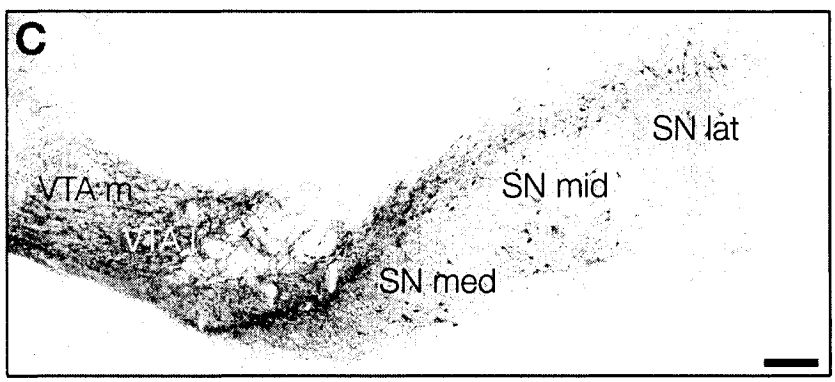

C
SN lat
VTA m
SN mid
VTA l
SN med

**Table 1**

| | Medial VTA | Lateral VTA | Medial SN | Middle SN | Lateral SN |
|---|---|---|---|---|---|
| a. Mean coefficient of error (CE) | | | | | |
| Veh/DS/hypo | 0.050 | 0.044 | 0.074 | 0.116 | 0.068 |
| Veh/VS/hypo | 0.027 | 0.038 | 0.065 | 0.035 | 0.067 |
| 6-OHDA/DS/hypo | 0.096 | 0.048 | 0.116 | 0.078 | 0.068 |
| 6-OHDA/VS/hypo | 0.076 | 0.086 | 0.080 | 0.091 | 0.091 |
| Veh/DS/normo | 0.064 | 0.080 | 0.116 | 0.100 | 0.112 |
| Veh/VS/normo | 0.078 | 0.058 | 0.070 | 0.078 | 0.058 |
| 6-OHDA/DS/normo | 0.115 | 0.088 | 0.073 | 0.078 | 0.097 |
| 6-OHDA/VS/normo | 0.060 | 0.068 | 0.108 | 0.092 | 0.100 |
| b. Mean coefficient of variation (CV) | | | | | |
| Veh/DS/hypo | 0.078 | 0.068 | 0.126 | 0.217 | 0.140 |
| Veh/VS/hypo | 0.047 | 0.060 | 0.128 | 0.063 | 0.108 |
| 6-OHDA/DS/hypo | 0.161 | 0.078 | 0.207 | 0.120 | 0.107 |
| 6-OHDA/VS/hypo | 0.126 | 0.139 | 0.130 | 0.146 | 0.189 |
| Veh/DS/normo | 0.089 | 0.123 | 0.181 | 0.197 | 0.192 |
| Veh/VS/normo | 0.128 | 0.089 | 0.091 | 0.125 | 0.098 |
| 6-OHDA/DS/normo | 0.195 | 0.138 | 0.116 | 0.123 | 0.163 |
| 6-OHDA/VS/normo | 0.096 | 0.102 | 0.185 | 0.150 | 0.187 |

Table 1: Group mean coefficient of error (CE) and coefficient of variation (CV) values for optical fractionator results in Experiment 1. Tyrosine hydroxylase-positive profile numbers were counted from rats (n=6 per group) that had been infused unilaterally with vehicle (Veh) or 6-hydroxydopamine (6-OHDA) into the dorsal striatum (DS) or ventral striatum (VS), while being maintained hypothermic (hypo) or normothermic (normo). Abbreviations: VTA, ventral tegmental area; SN, substantial nigra.

Figure 2: Residual labeling of dopamine transporter and tyrosine hydroxylase (TH) in dopaminergic terminal fields as measured by [$^{125}$I]RTI-55 binding (A) and [$^{125}$I]TH-immunoautoradiography (B), respectively. Rats (n=6 per group) were infused unilaterally with vehicle (Veh) or 6-hydroxydopamine (6-OHDA) into the dorsal striatum (DS) or ventral striatum (VS), while being maintained hypothermic or normothermic. Values (mean ± SEM) are expressed as the percentage of the side contralateral to the infusion. *P<0.001 *vs.* corresponding vehicle control condition.

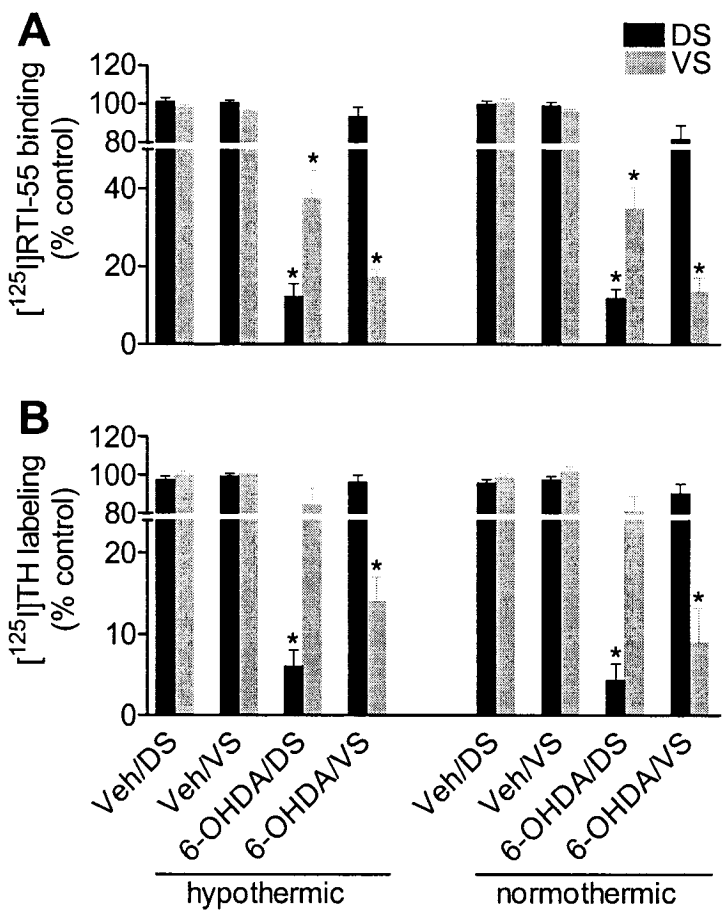

A
DS
VS
[125I]RTI-55 binding (% control)
120
100
80
40
20
0
B
[125I]TH labeling (% control)
120
100
80
20
10
0
Veh/DS
Veh/VS
6-OHDA/DS
6-OHDA/VS
Veh/DS
Veh/VS
6-OHDA/DS
6-OHDA/VS
hypothermic
normothermic

Figure 3: Residual labeling of dopamine transporter and tyrosine hydroxylase (TH) in dopaminergic terminal fields, as measured by [$^{125}$I]RTI-55 binding (A, B, C, and D) and [$^{125}$I]TH-immunoautoradiography (E, F, G, and H). Autoradiograms illustrate depletion of DAT and TH in adjacent brain sections from rats given unilateral infusion of 6-hydroxydopamine (right side) into either the dorsal striatum (DS) or ventral striatum while being maintained normothermic (A, C, E, G) or hypothermic (B, D, F, H). The ventral striatum comprised the nucleus accumbens core (Cor), shell (Sh) and olfactory Tubercle (Tu). Note: In dorsal striatum-infused rats, depletion of the ventral striatum was also apparent but was not restricted to a particular subregion. Arrows indicate the core, whereas arrowheads indicate the shell on the side ipsilateral to the lesion. Images correspond to rostrocaudal level 10.7 mm from interaural zero (Paxinos and Watson, 1997).

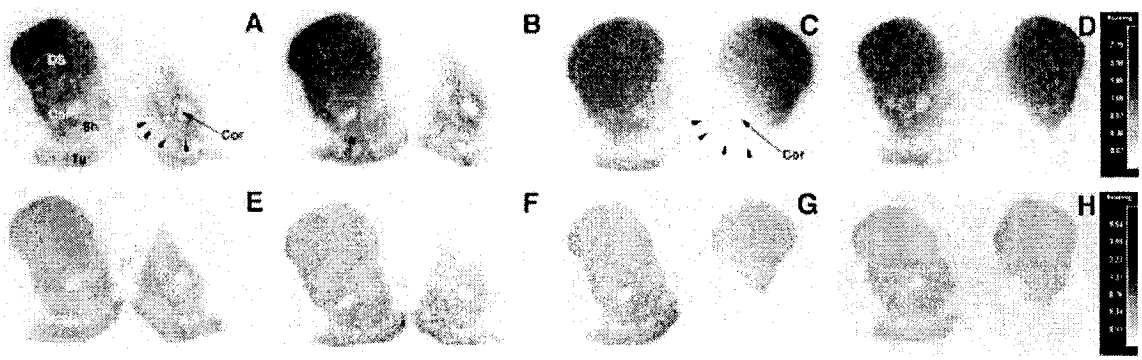

A
B
C
D
E
F
G
H
Cor
Cor

Figure 4: Tyrosine hydroxylase-positive (TH+) profile counts (A), and residual autoradiographic labeling of [$^{125}$I]RTI-55 (B) and TH (C) in the substantia nigra (SN) and ventral tegmental area (VTA). Rats (n=6 per group) were infused unilaterally with vehicle (Veh) or 6-hydroxydopamine (6-OHDA) into the dorsal striatum (DS) or ventral striatum (VS), while being maintained hypothermic or normothermic. Values (mean ± SEM) are expressed as the percentage of the side contralateral to the infusion. *P<0.001 vs. corresponding vehicle control condition; # P<0.001 SN vs. VTA.

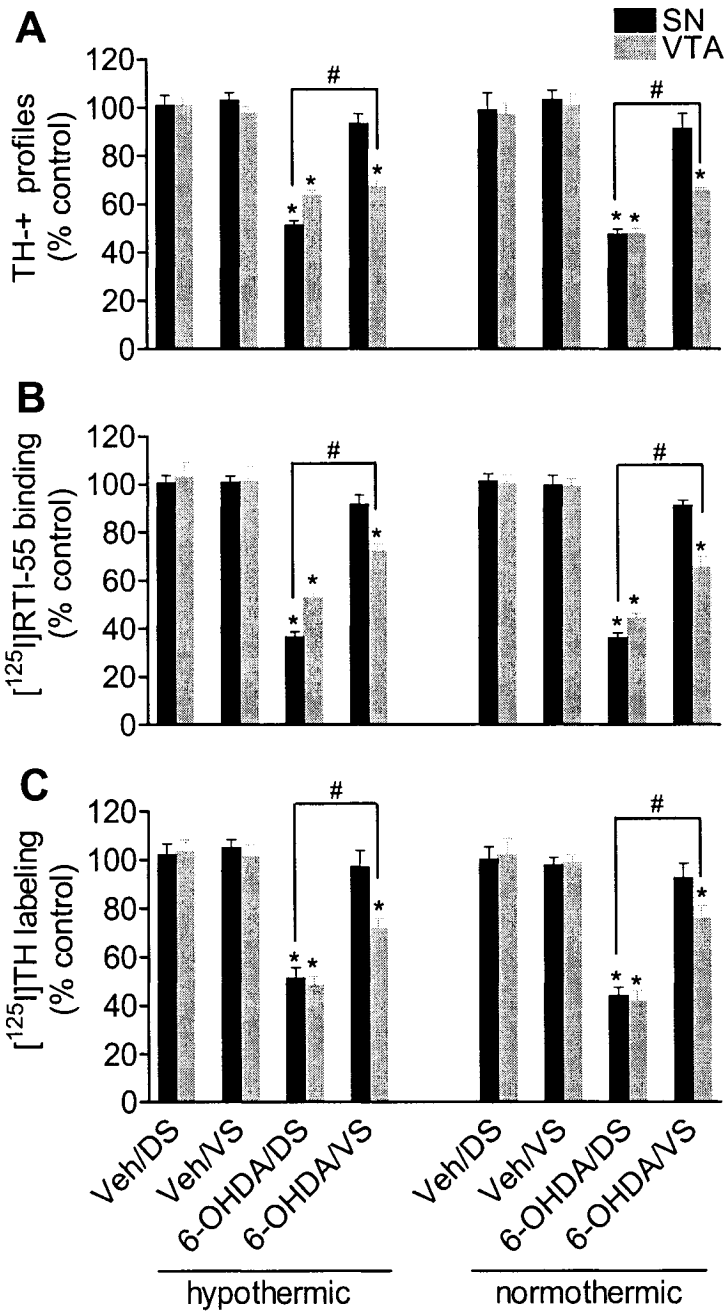

A
SN
VTA
TH-+ profiles (% control)
B
[125I]RTI-55 binding (% control)
C
[125I]TH labeling (% control)
120
100
80
60
40
20
0
#
*
Veh/DS
Veh/VS
6-OHDA/DS
6-OHDA/VS
hypothermic
normothermic

Figure 5: The effect of temperature on tyrosine hydroxylase (TH) immunostaining in ventral tegmental area (VTA) subregions. Rats were infused unilaterally with vehicle (A) or 6-hydroxydopamine (B, C) into the dorsal striatum, while being maintained normothermic (B) or hypothermic (A, C). Note the preservation of TH+ staining in the lateral VTA (VTA l) but not in the medial VTA (VTA m) in the hypothermic condition. Scale bar = 200 μm.

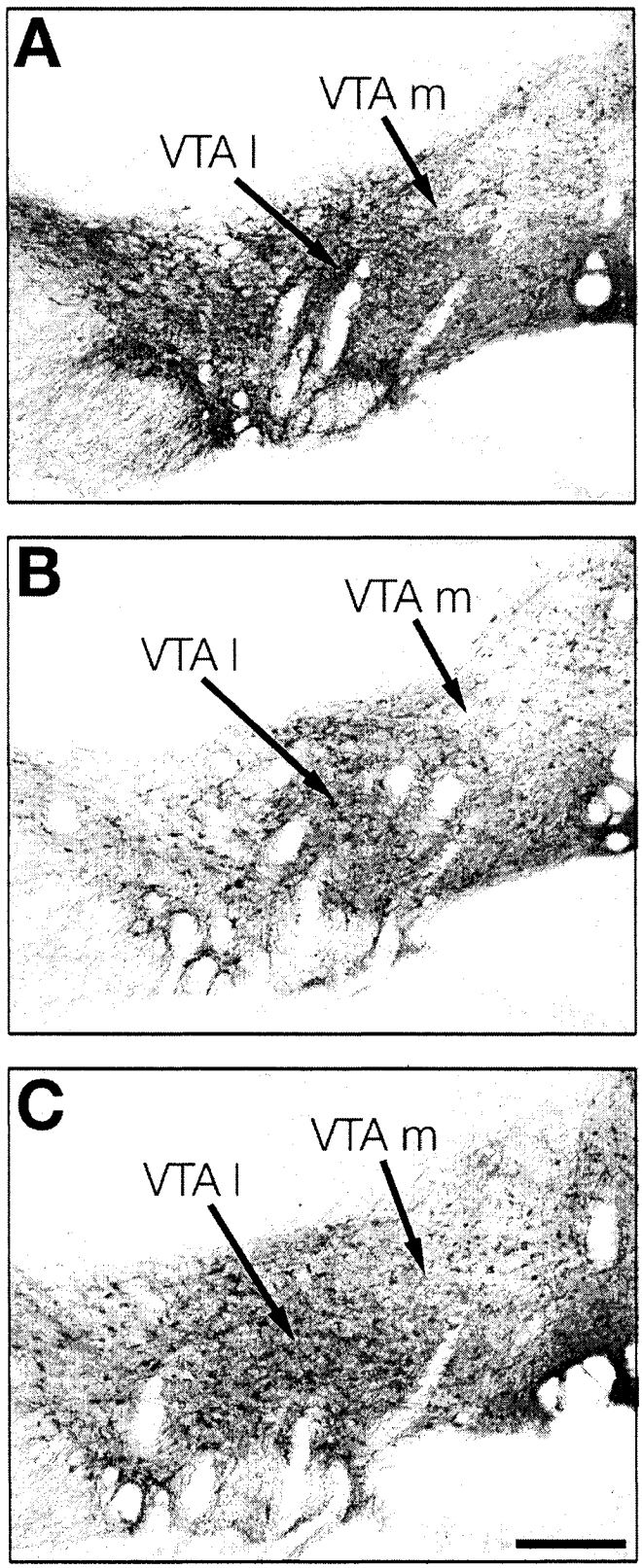

A
VTA m
VTA l
B
VTA m
VTA l
C
VTA m
VTA l

Figure 6: Tyrosine hydroxylase-positive (TH+) profile counts (A), and autoradiographic labeling of [$^{125}$I]RTI-55 (B) and TH (C) in subregions of the substantia nigra (SN) and ventral tegmental area (VTA). Rats (n=6 per group) were infused unilaterally with vehicle (Veh) or 6-hydroxydopamine (6-OHDA) into the dorsal striatum (DS) or ventral striatum (VS), while being maintained hypothermic or normothermic. Values (mean ± SEM) are expressed as the percentage of the side contralateral to the infusion. *P<0.001 hypothermic vs. normothermic. Abbreviations: VTA m and VTA l - medial and lateral ventral tegmental area; SN med, mid and lat - medial, middle and lateral substantial nigra.

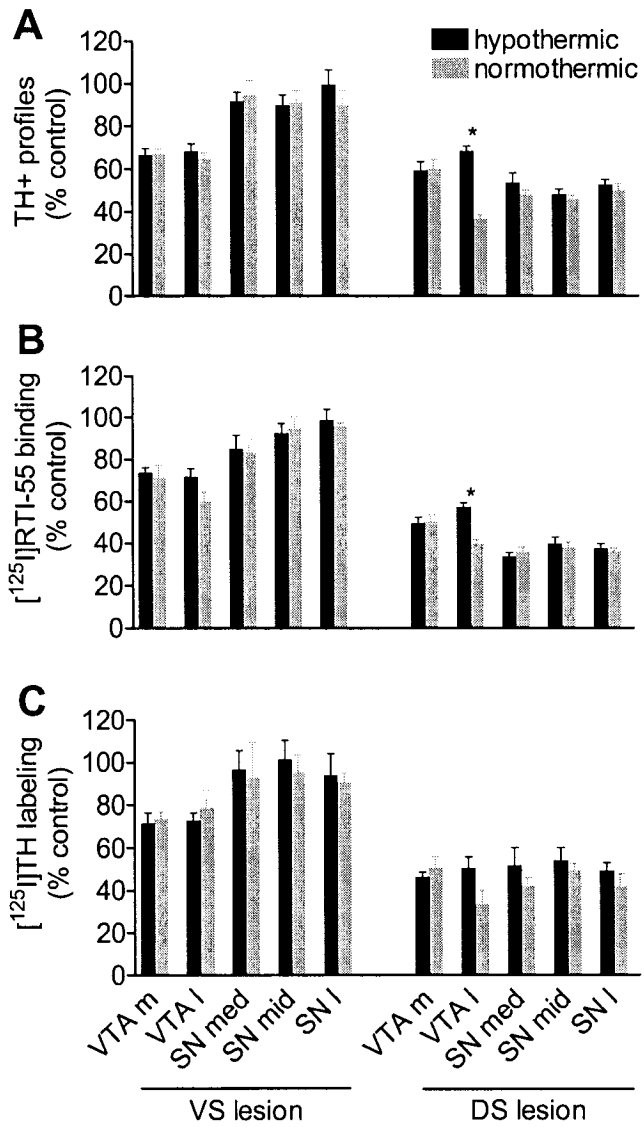

A
hypothermic
normothermic
TH+ profiles (% control)
120
100
80
60
40
20
0
*
B
[125I]RTI-55 binding (% control)
120
100
80
60
40
20
0
*
C
[125I]TH labeling (% control)
120
100
80
60
40
20
0
VTA m
VTA l
SN med
SN mid
SN l
VTA m
VTA l
SN med
SN mid
SN l
VS lesion
DS lesion

Figure 7: Relationship between temperature and tyrosine hydroxylase (TH)-positive cell counts or residual [$^{125}$I]RTI-55 labeling in the lateral ventral tegmental area following unilateral infusion of 6-hydroxydopamine (6-OHDA) into the dorsal striatum (DS). Each point represents a different rat. Significant negative correlations exist between temperature and TH-positive cell counts ($r=0.98$) and [$^{125}$I]RTI-55 binding ($r=0.98$).

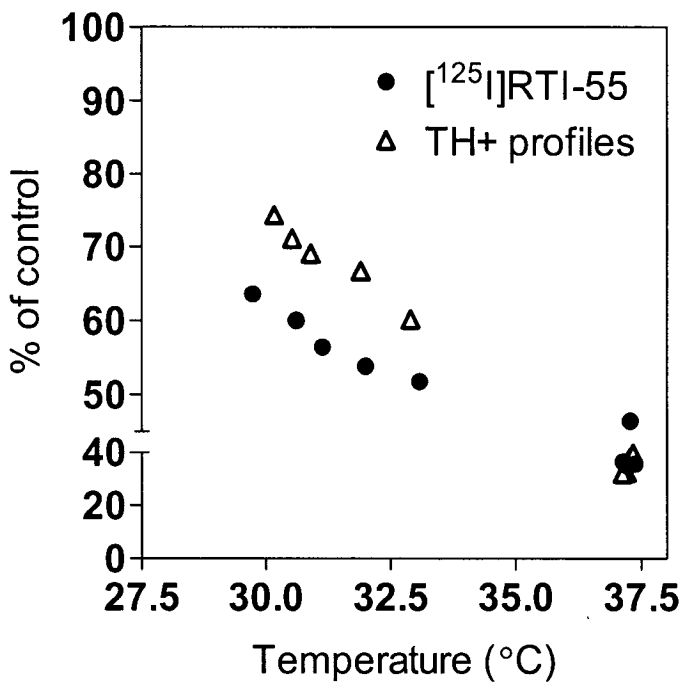

100
90
80
70
60
50
40
20
0
% of control
[125I]RTI-55
TH+ profiles
27.5
30.0
32.5
35.0
37.5
Temperature (°C)

Figure 8: Residual autoradiographic labeling of dopamine transporter and tyrosine hydroxylase (TH) in dopaminergic terminal fields and cell body regions as measured by [$^{125}$I]RTI-55 binding (A) and [$^{125}$I]TH-immunoautoradiography (B). Rats (n=6 per group) were infused unilaterally with vehicle (Veh) or 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB), while being maintained hypothermic or normothermic. Values (mean ± SEM) are expressed as the percentage of the side contralateral to the infusion of vehicle or 6-OHDA. *P<0.01, **P<0.001 *vs.* the corresponding structure in the normothermic condition; #P<0.001 SN *vs.* VTA. Abbreviations: DS, dorsal striatum; VS ventral striatum; SN, substantia nigra; VTA ventral tegmental area.

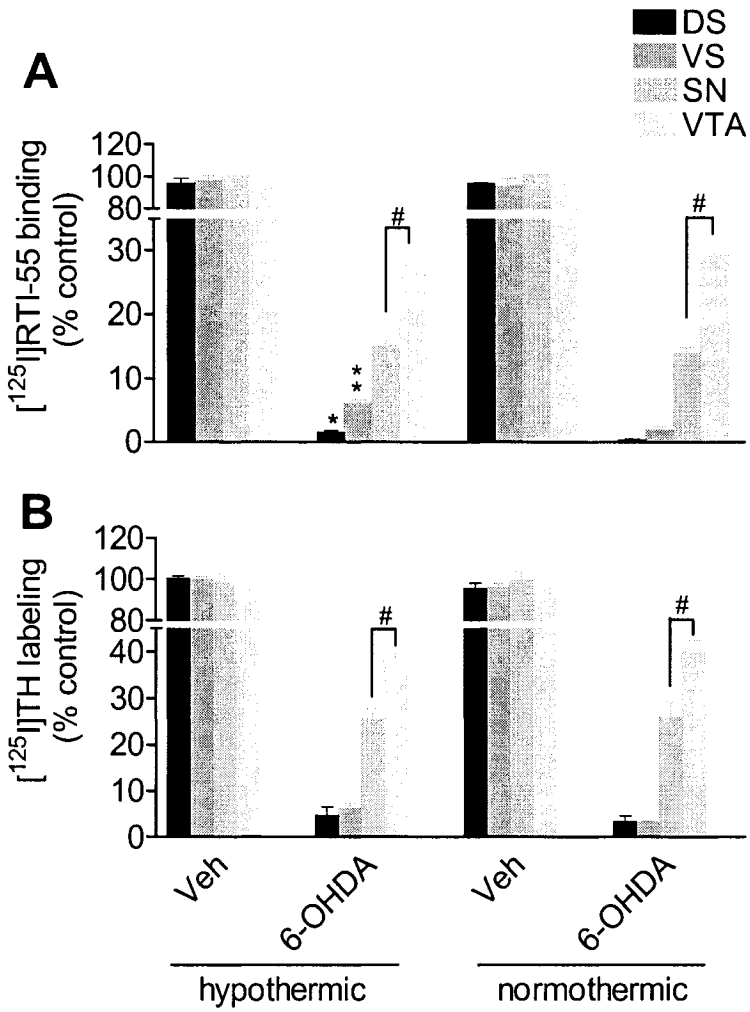
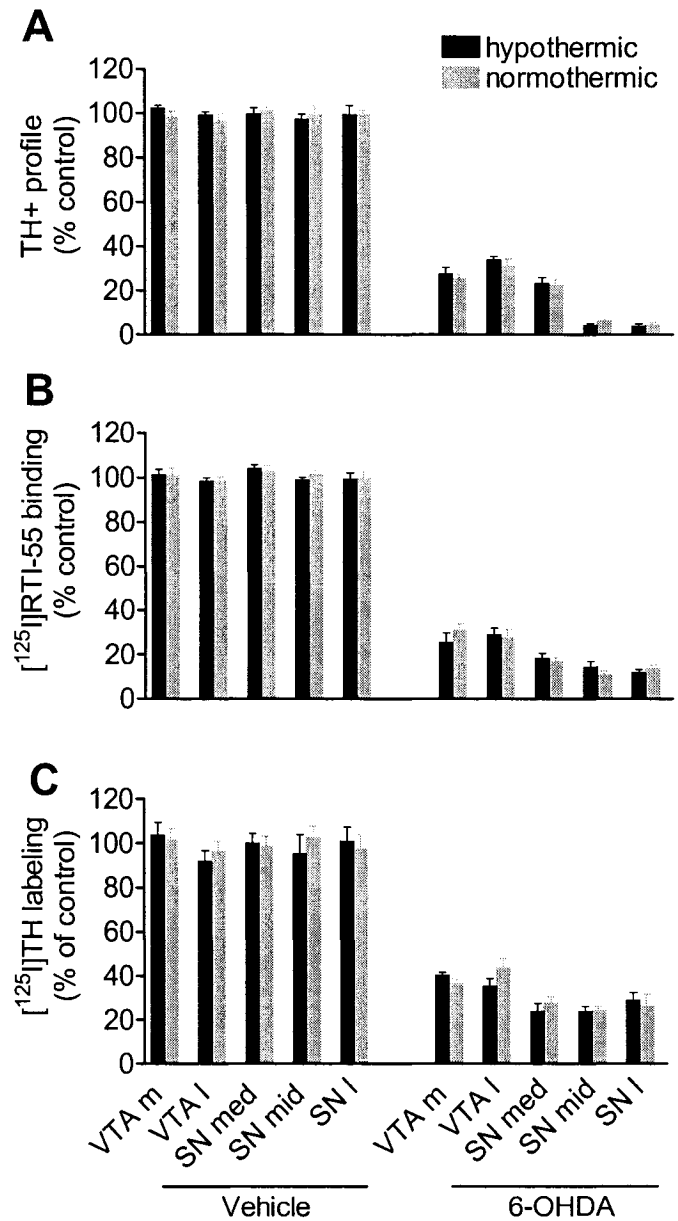

A
DS
VS
SN
VTA
[125I]RTI-55 binding (% control)
120
100
80
30
20
10
0
*
**
#
#
B
[125I]TH labeling (% control)
120
100
80
40
30
20
10
0
#
#
Veh
6-OHDA
Veh
6-OHDA
hypothermic
normothermic

**Table 2**

| | Medial VTA | Lateral VTA | Medial SN | Middle SN | Lateral SN |
|---|---|---|---|---|---|
| a. Mean coefficient of error (CE) | | | | | |
| Veh/hypo | 0.018 | 0.020 | 0.032 | 0.030 | 0.060 |
| 6-OHDA/hypo | 0.088 | 0.058 | 0.106 | 0.106 | 0.116 |
| Veh/normo | 0.025 | 0.040 | 0.018 | 0.035 | 0.018 |
| 6-OHDA/normo | 0.112 | 0.092 | 0.100 | 0.093 | 0.113 |
| b. Mean coefficient of variation (CV) | | | | | |
| Veh/hypo | 0.032 | 0.029 | 0.069 | 0.059 | 0.102 |
| 6-OHDA/hypo | 0.237 | 0.102 | 0.253 | 0.506 | 0.603 |
| Veh/normo | 0.053 | 0.067 | 0.038 | 0.078 | 0.039 |
| 6-OHDA/normo | 0.242 | 0.281 | 0.353 | 0.270 | 0.569 |

Table 2: Group mean coefficient of error (CE) and coefficient of variation (CV) values for optical fractionator results in Experiment 2. Tyrosine hydroxylase-positive profile numbers were counted from rats (n=4-6 per group) that had been infused unilaterally with vehicle (Veh) or 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle, while being maintained hypothermic (hypo) or normothermic (normo). Abbreviations: see Table 1.

Figure 9: Residual Tyrosine hydroxylase-positive (TH+) profile counts (A), and autoradiographic labeling of [$^{125}$I]RTI-55 (B) and tyrosine hydroxylase (C) levels in subregions of the substantia nigra and ventral tegmental area. Rats (n=6 per group) were infused unilaterally with vehicle (Veh) or 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB), while being maintained hypothermic or normothermic. Values (mean ± SEM) are expressed as the percentage of the side contralateral to the infusion of vehicle or 6-OHDA. Note: A protective effect of hypothermia was not observed in any DAergic subregion. Abbreviations: see Fig. 6.


A
hypothermic
normothermic
TH+ profile (% control)
120
100
80
60
40
20
0
B
[125I]RTI-55 binding (% control)
120
100
80
60
40
20
0
C
[125I]TH labeling (% of control)
120
100
80
60
40
20
0
VTA m
VTA l
SN med
SN mid
SN l
VTA m
VTA l
SN med
SN mid
SN l
Vehicle
6-OHDA

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**Intervening Section 2**

The experiments described in the preceding section outline the loss of dopamine markers that occurs following lesions which target the mesolimbic pathway or the nigrostriatal pathway. Overall, it appears that the nigrostriatal pathway, which selectively degenerates in Parkinson's disease, is more susceptible to 6-OHDA toxicity when compared to the adjacent, mesolimbic dopamine projection. Moreover, hypothermia, which occurred during the surgical administration of 6-OHDA, resulted in significant preservation of dopamine neurons, and this effect was dependent on the brain region. In Parkinson's disease, as in this animal model, a significant portion of dopamine neurons are present as clinical symptoms begin to emerge. This point is important because it suggests that the remaining neurons provide a substrate for neuroprotective agents to act on, and that structural *and* functional integrity of these enduring neurons is possible. The desired effect, of course, would be to halt the progression of Parkinson's disease.

In light of these thoughts, the next set of experiments addressed the underlying mechanisms of dopamine cell loss following 6-OHDA. We examined the activation of two proteases implicated in cell death processes, calpain and caspase-3. Following the observation that both proteases were activated after 6-OHDA administration, we then examined the effect of the calpain inhibitor, calpastatin, for its putative neuroprotective effects. While the delivery of calpastatin significantly improved behavioural deficits associated with dopamine loss, neuronal preservation was unexpectedly observed in a dopamine region outside of the nigrostriatal pathway, in the mesolimbic dopamine cell body region.

## CHAPTER 3: Calpain inhibition preserves motor function following intrastriatal 6-hydroxydopamine administration: evidence for a non-nigrostriatal mechanism

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Chapter 3 has been submitted for publication.

## ABSTRACT

The neurotoxin 6-hydroxydopamine (6-OHDA) has been widely used to model Parkinson's disease in rodents, but the mechanisms underlying toxin-induced dopaminergic degeneration and functional impairment have not been fully elucidated. The main aim of the present study was to assess a possible role for calpains in neurochemical and behavioral deficits following unilateral infusion of intrastriatal 6-OHDA in adult rats. Toxin administration resulted in calpain activation in both caudate-putamen and substantia nigra, as measured by the appearance of calpain-specific spectrin breakdown products. These effects peaked at 24 hours after 6-OHDA infusion and remained elevated at later time points. In contrast, caspase-3 activation subsided within 48 hours in both brain areas. In a subsequent experiment, calpain inhibition was achieved by intrastriatal infusion of an adenovirus expressing the endogenous calpain inhibitor, calpastatin. Calpastatin delivery completely prevented the lesion-induced calpain activation and significantly alleviated forelimb asymmetries resulting from unilateral intrastriatal infusion of 6-OHDA. Analysis of dopamine transporter and tyrosine hydroxylase labeling revealed significant neuroprotection in the ventral tegmental area but not in the nucleus accumbens or in the nigrostriatal cell body or terminals regions. The present findings support a role for calpain activation in the occurrence of behavioral deficits accompanying dopaminergic degeneration. However, our results suggest that the primary benefit of calpain inhibition may not occur within the nigrostriatal dopaminergic pathway itself.

## INTRODUCTION

A cardinal feature of Parkinson's disease (PD) is the severe loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNC) (Moore, 2003). The accompanying loss of striatal DA and resultant changes in motor circuitry are thought to underlie the main clinical signs of the disease (Obeso et al., 2000b). Current drug treatments for PD are largely or exclusively symptomatic (Obeso et al., 2000a). Consequently, a greater understanding of the mechanisms underlying degeneration of nigrostriatal DA neurons would be of significant value.

The biochemical events concomitant with DAergic cell death in PD have been partially elucidated. Post-mortem analysis of SNC has revealed enhanced expression of the cysteine proteases, caspases-1, -3, -8 and -9 (Hartmann et al., 2000; Mogi et al., 2000; Andersen, 2001; Hartmann et al., 2001). Although caspases play an important role in some forms of neurodegeneration, their functional importance in DA neuron death is uncertain (Crocker et al., 2001). Activation of calpains, another family of cell death-associated proteases, also occurs in nigral tissue from PD patients (Mouatt-Prigent et al., 1996; Crocker et al., 2003). Calpains appear to play a functional role in neurodegeneration caused by several types of acute and chronic neurological insults (Yokota et al., 1995; Pike et al., 1998; Gafni and Ellerby, 2002; Lee and Tsai, 2003; Wingrave et al., 2003). In mice treated with the DA-selective neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the administration of calpain inhibitors has been shown to be neuroprotective, indicating that calpain activation may indeed promote DAergic cell death (Crocker et al., 2003). However, in this study, protection was selective; calpain inhibition largely prevented MPTP-induced behavioral deficits and the loss of nigral tyrosine hydroxylase positive cells, yet failed to alleviate striatal DA depletion. The latter

findings indicate that the beneficial effects of calpain inhibition may not be restricted to the nigrostriatal pathway.

Like MPTP, the neurotoxin 6-hydroxydopamine (6-OHDA) has been used extensively to model PD. 6-OHDA is particularly intriguing given that it is found in brain and urine samples of PD patients (Curtius et al., 1974; Andrew et al., 1993; Jellinger et al., 1995), suggesting that it may be an endogenous component of PD pathogenesis. The potential role of calpains and their relation to 6-OHDA in the pathogenesis of PD in vivo have not been previously investigated. Hence, the present study sought to characterize the role of calpains in DAergic cell death in 6-OHDA treated rats by examining (1) the time course of calpain activation following 6-OHDA administration, (2) the effect of inhibiting calpains via adenovirally-mediated delivery of the endogenous calpain inhibitor calpastatin (Melloni et al., 1998), and (3) the relationship between behavioral and neuroanatomical indices of neuroprotection.

## *Materials and Methods*

*Experimental plan*

A first experiment determined the time course of calpain and caspase-3 expression following administration of 6-OHDA. Accordingly, groups of rats were sacrificed at different times (1, 3, 6, 18, 24, 48 hours, 7 and 14 days) after stereotaxic infusion of 6-OHDA or vehicle (VEH). A second experiment investigated the effects of calpain inhibition on 6-OHDA toxicity. To this end, rats were randomly allocated to four treatment groups, and received a single stereotaxic infusion of an adenovirus expressing the calpain inhibitor calpastatin or a control virus into the right striatum, followed one week later by infusion of 6-OHDA or VEH into the same site. Animals in each group were either sacrificed one day later (for Western blot analysis of viral

expression and calpain inhibition) or were behaviourally tested 14 days later and then immediately sacrificed (for analysis of DA markers). The latter experiment was performed using two batches of animals; the only procedural difference was that the vertical surface balancing ("cylinder") test was not repeated in the second batch.

*Intrastriatal infusion of 6-OHDA and adenovirus*

All experimental protocols were approved by the McGill University Animal Care Committee in accordance with guidelines set forth by the Canadian Council on Animal Care. Male Sprague Dawley rats (Charles River, St. Constant, Quebec) weighed between 260-300 g at the time of surgery. Rats were anesthetized with ketamine hydrochloride (80 mg/kg, i.p.) and xylazine hydrochloride (16 mg/kg, i.p.). Coordinates for intrastriatal infusion of 6-OHDA or VEH and adenovirus were based on the mean of two systems, based respectively on interaural (IA) zero and bregma (Paxinos and Watson, 1997). The 30-gauge infusion cannula was lowered to A +9.5/1.5, L -3.0, V 4.5/-5.5 (tooth bar 3.9 mm below the ear bars), corresponding to the middle of the caudate putamen (Paxinos and Watson, 1997). 6-OHDA HBr (20 µg/3µl base) was light-protected and maintained on ice throughout the course of surgeries to minimize oxidation. The vehicle was 0.3 mg/ml sodium metabisulphite in 3µl of 0.9% saline (Grant and Clarke, 2002). Adenoviruses (3 µl; 1 x $10^7$ plaque forming units per site) were designed to express either green fluorescent protein (Ad.GFP) alone, or the calpain inhibitor calpastatin (Melloni et al., 1998) and GFP under dual CMV promoters (Ad.CALP). Infusions of toxin or adenovirus were performed at a rate of 0.1 µl/min to minimize the occurrence of non-specific tissue damage. Following infusion, the cannula remained in place for 5 min before being slowly retracted.

Intrastriatal infusion of adenovirus vector has been shown to result in efficient gene transfer to the SNC, mediated by retrograde axonal transport (Kuo et al., 1995; Choi-Lundberg et al., 1998). This delivery method has been used to produce neuroprotection in nigral DAergic cells (Bilang-Bleuel et al., 1997; Mandel et al., 1997; Choi-Lundberg et al., 1998; Crocker et al., 2001; Crocker et al., 2003).

*SDS-PAGE*

Animals were anesthetized with pentobarbital, decapitated, and brains were quickly removed and frozen in 2-methylbutane (-50°C). Using cryostat sectioning to first locate structures, brain tissue was dissected (at -20°C) from the striatum and SNC ipsilateral and contralateral to the 6-OHDA lesion. Tissue samples were homogenized in lysis buffer containing 1% Nonidet P-40, 2 mM EDTA, 50 mM Tris buffer (pH 7.5), and protease inhibitors (Complete Mini; Roche, Mannheim, Germany), and centrifuged at x14000 rpm for 20 min at 4°C. The protein concentration of the resulting supernatant was determined using a Bio Rad kit. Samples (10 μg) were resolved (in duplicate) on an 8% polyacrylamide gel and then transferred to a polyvinylidine difluoride membrane (Immobilon-P, Millipore, Bedford, MA).

*Western Blot Analysis*

Activation of either calpain and caspase-3 by 6-OHDA administration, and their inhibition by calpastatin, was determined by probing blots with a monoclonal antibody against nonerythroid spectrin (1:5000, Chemicon, Temecula, CA). This antibody recognizes the calpain-specific spectrin breakdown product (SBDP) as a band appearing at 145 kDa and a caspase-3 specific signal corresponding to 120 kDa (Wang, 2000). Adenovirus expression was detected with a

polyclonal anti-GFP antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) which recognizes a characteristic GFP band at 27 kDa (Prasher et al., 1992). Immunoblots were developed with a peroxidase-conjugated anti-mouse (for anti-spectrin) or anti-rabbit (for anti-GFP) secondary antibody (Vector, Mississauga, Ontario) and enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA). To normalize for protein loading and transfer, membranes were incubated with a monoclonal antibody against β-actin (1:500 Santa Cruz). The relative optical density of the chemiluminescent signal was quantified using an M4 MCID computer-based imaging system (Imaging Research, Mississauga, Ontario).

*[$^{125}$I]RTI-55 autoradiography and tyrosine hydroxylase immuno-autoradiography*

Quantification of DAergic markers was performed at two weeks post-lesion, at which time DAT and TH abundance are near-maximally depleted (Sauer and Oertel, 1994; Przedborski et al., 1995). We used two histological markers, [$^{125}$I]RTI-55 binding and [$^{125}$I]immunolabeling of TH, which reflect different aspects of the DA phenotype. While up-regulation of the TH enzyme has been suggested following 6-OHDA administration (Zigmond et al., 1984; Barnéoud et al., 1995), DAT binding and [$^{3}$H]DA uptake (Zigmond et al., 1984) can accurately represent residual tissue DA content following a 6-OHDA lesion (Zigmond et al., 1984; Altar et al., 1987; Joyce, 1991a; Joyce, 1991b). In addition, since DA transporters occur on growth cones associated with regenerating neurites (Cerruti et al., 1991), DAT binding should also reflect regenerative sprouting. It should be noted that a loss of these phenotypic markers, whilst suggesting compromised function, does not necessarily indicate DA cell death (Sauer and Oertel, 1994; Bowenkamp et al., 1996).

Loss of DA transporter (DAT) was quantified by autoradiographic labeling of plasmalemmal DAT with [$^{125}$I]RTI-55 (Sp. Act. 2200 Ci/mmol, NEN-Mandel, Guelph, Ontario), as previously described (Grant and Clarke, 2002). Briefly, one coronal section (25 μm) of the striatum and SNC area from unfixed frozen brains was collected from each of eight rostrocaudal levels (10.7, 10.2, 9.7, 9.2, 3.7, 3.7, 3.0, and 2.9 from interaural zero (Paxinos and Watson, 1997) and incubated for 2 h at RT with 10 pM [$^{125}$I]RTI-55 in 10 mM sodium phosphate buffer containing 0.1 M sucrose and 50 nM citalopram HBr (gift from H. Lundbeck A/S). Nonspecific binding was defined by the addition of the DAT inhibitor GBR 12909 (10 μM). Following three rinses in ice-cold buffer, sections were dipped in distilled water (5 s), blow-dried, and exposed to Kodak X-OMAT X-ray film together with [$^{125}$I]-Microscale autoradiographic standards (Amersham; Baie d'Urfe, Quebec). Quantification of DAT and TH (see below) was performed at 14 days post-lesion, by which time depletion is near maximal (Sauer and Oertel, 1994; Przedborski et al., 1995).

Loss of tyrosine hydroxylase (TH) protein was quantified by autoradiographic labeling of TH as previously described (Grant and Clarke, 2002). Briefly, unfixed frozen sections were thawed and immediately immersed in a solution containing 6% paraformaldehyde (PFA), 20% absolute alcohol, 20% ethylene glycol, 10% glycerol, and 0.32 M sucrose for 1 h at -20°C. After rinsing several times at RT in 0.1 M phosphate buffer in 0.1 M saline (PBS/0.3% Tween-20), sections were blocked with 30% skim milk powder (Carnation), 3% rabbit serum and 0.05% $NaN_3$, followed by overnight incubation at 4°C with mouse monoclonal anti-TH antibody (1:1000; Sigma Chemical Co.) in 1.5% rabbit serum and 0.05% $NaN_3$. An [$^{125}$I]-labeled rabbit anti-mouse IgG (NEN Life Science Products; sp. act. 1200 Ci/mmol) was dissolved in a solution containing 10% skim milk powder, 5% rabbit serum and 0.05% $NaN_3$, and applied (8 pM) to sections for 1 h

at RT. Following several rinses, sections were blow-dried, and exposed to Kodak X-OMAT X-ray film together with [$^{125}$I]-Microscale autoradiographic standards (Amersham, Baie d'Urfe, Quebec).

*Immunohistochemical detection of calpain activity*

Calpain activity was detected by an antibody (kind gift from T.C. Saido) that is highly selective for a 150 kDa peptide fragment of α-spectrin that is produced following calpain activation (Saido et al., 1993). Brains from perfusion-fixed animals were processed in parallel. First, they were post-fixed for 3 h in 4% PFA/0.1 M phosphate buffer, cryoprotected overnight in 20% sucrose, and then frozen in 2-methylbutane (-50°C). Every other section (40 μm) from the striatum and SNC was collected in plastic wells for immunostaining. Endogenous peroxidase activity was quenched with 0.3% $H_2O_2$/PBS-Triton, and blocked in 10% goat serum (Vector Laboratories, Burlington, Ontario). Tissue was then incubated overnight (4°C) in goat anti-calpain-mediated spectrin breakdown product (SBDP) antibody (1:400) dissolved in PBS-Triton containing 1% goat serum. After several rinses in buffer, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlington, Ontario) was applied (1:200) for 1 h at RT. Sections were incubated in avidin-biotin-peroxidase complex (1:200; Vectastain Standard ABC kit; Vector Laboratories) for 1 h at RT, followed by several rinses in buffer. Immunostaining was visualized by the addition of 0.01% $H_2O_2$ to 0.06% 3,3'diaminobenzidine solution (Sigma Chemical Co., St. Louis, MI).

*Quantitative image analysis of [$^{125}$I]RTI-55 autoradiography and TH immunoautoradiography*

Film autoradiographs were quantified using an M4 MCID computer-based system (Imaging Research, Mississauga, Ontario). Optical density values were converted to wet tissue equivalent

values using [$^{125}$I]Microscales. For each film, nonspecific binding, as defined by GBR 12909 addition (DAT) or omission of the secondary antibody (TH), was subtracted from total binding.

*Behavioral Testing*

The degree of contralateral sensorimotor neglect was assessed using three previously validated behavioral measures of forelimb placement, namely, the stepping, vibrissae-elicited reaching and vertical surface balancing ("cylinder") tests (Olsson et al., 1995; Schallert et al., 2000). In all three tests, an asymmetry percentage score was calculated as 100 x (Ipsilateral – Contralateral)/ (Ipsilateral + Contralateral). All behavioral testing was done between 10h00 and 17h00 by an experimenter blind to the experimental conditions. Rats were weighed and handled daily for five days prior to commencement of testing. The three tests were performed in a counterbalanced order within each group.

*Stepping test.* Each rat was held with one forepaw and both hindlimbs restrained. The unrestrained forelimb was placed on a flat surface while the rat was moved slowly sideways across the table surface in the forehand direction for 10 sec. The number of adjusting steps taken over the 10 sec period was recorded. The procedure was then repeated for the second forelimb.

*Vibrissae-elicited reaching test.* Rats were held by their torsos so that one forelimb was allowed to hang free. The vibrissae on one side of the animal was brushed against the edge of a table top, and the number of ipsilateral forelimb reaches were recorded. The order of testing was counterbalanced, such that the forelimb ipsilateral to the lesioned striatum was tested first for half of the rats. Each rat was tested 10 times for each forelimb.

*Cylinder test.* Animals were placed in a transparent Plexiglass cylinder (30 cm high x 18 cm diameter), and recorded for 10 minutes with two CCTV cameras (Panasonic) located 180° apart

to view the animals from all sides. Videotapes were played in slow motion to quantify the number of times the rat placed each forelimb on the cylinder wall during vertical explorations (i.e., left, right, or both forelimbs simultaneously). Prior to each trial, the cylinder was cleaned with a 40% ethanol solution, and bedding was replaced. There was no habituation period prior to scoring forelimb use.

*Data analysis*

Statistical analyses were performed using commercial software (Systat 10.0, SPSS Inc., Chicago IL). Data were subjected to analysis of variance, Student's t-test or Mann-Whitney test as appropriate. All probability values were two-tailed; a level of 5% was considered significant. Data are reported as the mean ± SEM.

## RESULTS

In the time course experiment, collection of tissue for Western blotting precluded autoradiographic assessment of DAergic depletion. However, use of identical lesion parameters in the subsequent experiment (see below) resulted in a substantial loss of striatal TH and DAT (>60% and 90%, respectively), at fourteen days post-lesion.

### Time course of calpain activation after intrastriatal 6-OHDA

Calpain activation was assessed in striatum (Fig. 1) and SNC (Fig. 2) by measuring the 145 kDa calpain-specific SBDP band in Western blots. This band was faint in control animals given vehicle in both striatum and SNC (Fig. 1A and 2A). Calpain activity contralateral to the injection side was low and appeared to be unaffected by 6-OHDA or VEH infusion (Fig. 1B and 2B). Following toxin administration, calpain activity was significantly increased beginning at 18 hours post-lesion, and remained elevated throughout the study (Fig. 1B). In the SNC, calpain activation became significant at 18 hours post lesion ($t=5.27$ df 3, $p<0.02$), and was elevated at some later time points as well (Fig. 2B).

Caspase-3 activation following 6-OHDA administration was assessed in the same animals (Fig. 1C and Fig. 2C). In vehicle-infused animals, a weak band corresponding to the caspase-3 mediated SBDP signal (120 kDa) was apparent in both striatum and SNC. Following 6-OHDA treatment, caspase-3 activity ipsilateral to the lesion was significantly elevated in the striatum at 18 hours post lesion ($t=7.74$ df 3, $p<0.01$), but returned to control levels by 48 hours. Caspase-3 activation in the SNC appeared delayed relative to that in the striatum. In this structure, caspase-3 activity was significantly increased only at 24 hours post-lesion ($t=6.11$ df 3, $p<0.01$) and returned to control levels by 48 hours post-lesion.

Anatomical localization of calpain activation was also examined by immunohistochemistry. Twenty-four hours after 6-OHDA administration, dense immunolabeling in the striatum was apparent at the site of toxin infusion, accompanied by a reduction in Nissl staining (Fig. 3). In contrast, vehicle infusion resulted in no positive immunostaining, and Nissl substance was intact.

**Inhibition of calpains by calpastatin overexpression**

Adenovirus expression was confirmed by Western blot for GFP. In both striatum (Fig. 4A) and SNC (Fig. 5A), a single band corresponding to the predicted molecular weight of GFP (27 kDa) was detected in animals given either adenovirus. In both brain structures, however, the densitometric signal was weaker in animals given 6-OHDA *vs.* VEH, presumably because degeneration elicited by the toxin had already begun.

Next, the ability of adenovirally delivered calpastatin to functionally inhibit calpains was examined by Western blot. As expected, the calpain-specific 145 kDa band was increased following 6-OHDA administration in animals given the control adenovirus (Ad.GFP) in both striatum ($t=7.32$ df 2, $p<0.02$; Fig. 4A, B) and SNC ($t=4.97$ df 2, $p<0.05$; Fig. 5A, B). However, no such increase occurred in animals pretreated with calpastatin-expressing virus (Ad. CALP). Activation of caspase-3 was also elevated in lesioned animals receiving the control virus ($p<0.05$ for both structures). This increase appeared somewhat blunted in animals receiving Ad.CALP but caspase-3 activity was still significantly elevated in both structures ($t=4.68$ df 2, $p<0.05$; $t=4.73$ df 2, $p<0.05$ for striatum and SNC respectively; Fig. 4C and Fig. 5C).

**Effect of calpastatin overexpression on behavior**

Rats were subjected to three previously validated behavioral tests in order to assess sensorimotor deficits following unilateral 6-OHDA lesions. These experiments were performed in two stages (see Methods). Since the pattern of results was closely similar in the two batches of animals, the data were combined. Administration of 6-OHDA impaired contralateral forelimb placement in all three tests. In non-lesioned (i.e., vehicle-infused) animals, limb use asymmetry was minimal and was unaffected by calpastatin delivery (Fig. 6).

Analysis of stepping behaviour revealed a significant interaction between toxin and calpastatin factors ($F=4.40$, df 1,40, $p<0.05$; Fig. 6A). Lesioned animals showed a marked deficit in contralateral stepping which was alleviated by calpastatin delivery ($t=3.77$ df 22, $p<0.005$; Fig. 6A). In the reaching test, administration of calpastatin significantly reduced contralateral forelimb neglect in lesioned animals ($U=30.5$, $p<0.02$; Fig. 6B). Cylinder test performance showed a slight ipsilateral (right) forelimb bias in non-lesioned animals. Following 6-OHDA administration, preferential use of the ipsilateral forelimb was significantly increased ($t=4.3$ df 6, $p<0.005$). However, in contrast to the protective effects observed in the other two tests, calpastatin delivery did not reduce contralateral forelimb neglect in this behavioral measure ($t=0.24$ df 9, $p>0.8$) (Fig. 6C).

**Effect of calpastatin overexpression on DAergic markers**

Vehicle infusion did not appreciably alter DAT binding or TH immunolabeling in any brain region, compared to the contralateral side. In animals that received the control virus, 6-OHDA administration almost abolished DAT labeling in striatum (Table 1) with less marked depletion in SNC (Table 1) and VTA (Fig. 7A). DAT labeling was not significantly reduced in the nucleus accumbens core or shell (Table 1). DAT binding in SNR was low and not measured. TH

immunolabeling was reduced in striatum, SNC, SNR, and VTA, but not in core and shell (Table 1; Fig. 7B). Overall, 6-OHDA lesions depleted DAT binding more than TH immunolabeling.

Administration of calpastatin-expressing adenovirus exerted a significant protective effect in the VTA, both in terms of DAT binding ($t=2.47$ df 22, $p<0.025$; Fig. 7A) and TH immunolabeling ($t=3.57$ df 21, $p<0.005$; Fig. 7B). In contrast, no protective effect was observed in the striatum, SNC, SNR, core or shell for either DAergic marker, as shown in Table 1.

## DISCUSSION

This study is the first to describe the time course of calpain expression in a rat model of PD and to demonstrate the effect of calpain inhibition in 6-OHDA lesioned animals. The main novel findings are as follows. First, lesion-induced calpain activation occurred for at least two weeks in the striatum and SNC. In contrast, caspase-3 activation was transient after 6-OHDA administration. Second, calpain inhibition was associated with significant behavioral recovery and a partial sparing of DA markers in the VTA, whereas no such protection was apparent in *nigrostriatal* brain regions. Overall, the present findings suggest that calpain activation may promote VTA DAergic degeneration and hinder functional recovery. In contrast, calpain activation does not appear critical for nigrostriatal degeneration following 6-OHDA.

**Sustained vs. transient activation of calpain and caspase-3 following intrastriatal 6-OHDA**

The sustained activation of calpains in striatum and SNC following intrastriatal infusion of 6-OHDA is in broad agreement with that observed in other forms of neurodegeneration (Roberts-Lewis et al., 1994). In the SNC, there was the appearance of a biphasic effect. Distinct phases of calpain activation have been observed in other experimental models, such as global ischemia (Saido et al., 1993; Roberts-Lewis et al., 1994; Yokota et al., 1995), traumatic brain injury (Saatman et al., 1996) and temporal lobe epilepsy (Bi et al., 1996). However, in these models, both phases occurred earlier (15 to 90 min and 4 hours to 7 days, respectively). These temporal differences presumably depend on the specific insult given, on local cell types, on endogenous calcium buffering capacity (German et al., 1992; Mouatt-Prigent et al., 1994; Tan et al., 1999; Tan et al., 2000), and on the brain regions in question.

Concomitant with calpain activation in 6-OHDA treated animals, an early and transient activation of caspase-3 was also detected in both striatum and SNC. This novel finding contrasts with a previous negative report using immunohistochemical markers (Crocker et al., 2001). This discrepancy may be accounted for by the greater sensitivity conferred by the use of SBDPs to monitor enzyme activity. Thus, the present study detected caspase-3 activity by the appearance of the large (120 kDa) and stable caspase-3 specific SBDP (Vanderklish and Bahr, 2000). By contrast, the active form of the protease has a short half-life of approximately thirty-five minutes (Tawa et al., 2004) which may have precluded its immunohistochemical detection using an antibody that recognized the active caspase-3 conformation (Crocker et al., 2001).

**Neuroprotection associated with calpain inhibition is brain area dependent**

In the present study, Western blot analysis confirmed that gene transfer by the GFP-tagged Ad vectors was successful in both striatum and SNC, in line with previous reports using identical vectors (Crocker et al., 2001; Crocker et al., 2003). Our decision to use a calpastatin-expressing adenovirus was based on considerations of specificity and efficacy. Present evidence indicates that calpastatin possesses high specificity for calpains, whereas synthetic inhibitors of calpains are known to affect other proteases (Wang and Yuen, 1994) and only partially reduce injury-associated calpain activation (Rami et al., 2000; Schumacher et al., 2000; Ray et al., 2001). By contrast, in the present study, adenoviral-mediated calpastatin expression completely prevented calpain activation. A further potential advantage of calpastatin is that it has a higher affinity for calpain under conditions of high intracellular free $Ca^{2+}$ (Kapprell and Goll, 1989), as occurs following 6-OHDA (Frei and Richter, 1986). This characteristic of calpastatin may explain why it inhibited calpain activation but not basal calpain activity.

Intrastriatal infusion of 6-OHDA resulted in a loss of DA markers in the VTA, which was partially prevented by intrastriatal administration of calpastatin-expressing adenovirus. These findings likely reflect the existence of a known DAergic projection from the VTA to ventromedial striatum (Fallon, 1981; Thomas et al., 1994). Thus, it is likely that the adenovirus was retrogradely transported to VTA cell bodies prior to the toxin infusion (Kuo et al., 1995; Choi-Lundberg et al., 1998).

The present study revealed no indication of nigrostriatal sparing following virally-mediated calpain inhibition. These results differ from recent observations using the same virus and a pharmacological calpain inhibitor in MPTP-treated mice (Crocker et al., 2003). In the latter study, calpain inhibition resulted in partial sparing of TH immunostaining in SNC. In addition, although striatal DA content was not preserved, DA transmission in striatum appeared normalized as assessed by fosB expression. The two studies employed lesions of similar size but differed in the toxin and rodent species used. Although 6-OHDA and MPTP share some mechanisms of action (e.g. oxidative stress, calcium dysregulation), they also differ in certain respects (Blum et al., 2001). For example, neurotoxicity following MPTP is primarily associated with the inhibition of complex I of the mitochondrial respiratory chain (Gerlach et al., 1991), whereas 6-OHDA toxicity is primarily due to oxidative stress (Storch et al., 2000). The rodent species is perhaps a less critical factor, inasmuch as calpain inhibition is clearly neuroprotective in other rat brain injury models (Ray and Banik, 2003).

The absence of nigrostriatal neuroprotection following 6-OHDA does not exclude a degenerative role for calpain in this pathway. First, it is possible that the complete block of calpain activation was not maintained. Against this, adenoviral expression is typically sustained for several weeks (Choi-Lundberg et al., 1998). Second, even with calpains inhibited, functional

redundancy may have allowed for cell death via alternative proteolytic enzymes (Lankiewicz et al., 2000; Bizat et al., 2003; Neumar et al., 2003). One such candidate is caspase-3 which remained elevated in the presence of calpastatin. Various forms of cross-talk have been reported to occur between calpains, caspases and calpastatin (Lankiewicz et al., 2000; Reimertz et al., 2001; McCollum et al., 2002; Bizat et al., 2003), and there is a clear need for further investigation of this interplay in dopaminergic lesion models.

**Calpastatin protects against sensorimotor deficits**

The calpain inhibitor MDL 28170 has been reported to alleviate hypolocomotion resulting from MPTP treatment in mice (Crocker et al., 2003). We gauged the extent of lesion-induced functional impairments by analyzing spontaneous forelimb asymmetries. These tests reflect weight-shifting impairments and may thus be particularly relevant to PD (Schallert and Tillerson, 2000; Cenci et al., 2002).

Forelimb asymmetry in all three tests is sensitive to indirect dopamine agonist drugs such as L-DOPA (Schallert et al., 1979; Fleming et al., 2004) and is strongly influenced by DAergic transmission in the caudate-putamen (Schallert and Tillerson, 2000; Kirik et al., 2001). However, DAergic transmission outside the nigrostriatal system also appears to play a role in forelimb use (Ellenbroek et al., 1988; Cools et al., 1994; Winkler et al., 1996; Kirik et al., 2001). Since we observed no neuroprotection in the nigrostriatal pathway, it is likely that behavioral sparing in the stepping and reaching tests occurred either through non-DAergic mechanisms within the striatum or via extra-striatal mechanisms; the lack of sparing in the cylinder test suggests a greater dependence on nigrostriatal DA or on other neural systems not affected by calpastatin.

Within the striatum, sustained postsynaptic changes in gene expression occur following DAergic denervation (Doucet et al., 1996; Winkler et al., 2002). Calpain inhibition has been shown to produce behavioural sparing while normalizing lesion-associated increases in striatal FosB and neurotensin expression (Crocker et al., 2003), but a causal link remains to be established.

If behavioural sparing occurred outside the striatum, the VTA may play a role, since this is the only structure where DA markers were detectably preserved. In this regard, at least three possible mechanisms may be envisaged. First, the VTA sends a major DAergic projection to the nucleus accumbens, a structure reported to modulate forelimb rigidity (Ellenbroek et al., 1988). However, this projection is unlikely to account for the behavioral findings, since DA innervation to nucleus accumbens appeared unaltered by calpastatin delivery. Second, VTA DAergic neurons project to the striatum (Fallon, 1981; Thomas et al., 1994). The bulk of this projection is to the ventromedial portion, which does not appear to influence stepping behaviour (Chang et al., 1999). However, the VTA also weakly innervates more lateral striatal areas (Loughlin and Fallon, 1982; Domesick, 1988) which clearly play a role in forelimb use (Chang et al., 1999). Although DAergic markers in the striatum were not altered by calpastatin delivery, selective sparing of this relatively minor projection may have gone undetected. A third possibility is that preservation of dendritic DA release within the VTA itself was sufficient to maintain behavioral function. Indeed, DA release in the VTA influences non-DAergic projection neurons, an action which is known to modulate motivated behaviour (Ranaldi and Wise, 2001). Functional compensation via non-DAergic mechanisms has also been implicated in preclinical Parkinson's disease (Bezard et al., 2003).

In conclusion, the present study supports the use of calpain inhibitors as a novel therapeutic approach to the alleviation of neurological symptoms associated with PD. However, our results indicate that a primary benefit associated with calpain inhibition occurs outside the nigrostriatal DAergic pathway.

***Acknowledgments***. We thank Dr. A.C. Cuello for demonstrating the substantia nigra tissue dissection, Dr. T. Schallert for expert advice concerning the behavioral tests, K. Bell for help with photomicrography, and Dr. T.C. Saido for providing the calpain antibody. Supported by the Natural Sciences and Engineering Research Council of Canada (NSERC, to PBSC) and the Canadian Institutes of Health Research (CIHR to PBSC and DSP). L.H.L.S holds an NSERC Postgraduate Award and P.B.S.C. is a Chercheur National of the FRSQ.

Figure 1: Time course of calpain and caspase-3 activation in caudate-putamen induced by 6-OHDA. Rats received a unilateral intrastriatal infusion of 6-OHDA or vehicle (VEH) and were sacrificed 1 hour to 14 days later. Panel A shows representative Western blots stained with an antibody recognizing intact spectrin (top band) and spectrin breakdown products (SBDPs). The 145 and 120 kDa protein bands are specific markers for calpain and caspase-3 activity, respectively. These protein bands were quantified by optical densitometry, normalized against the 40 kDa β-actin band, and expressed as mean ± SEM (n=3 to 4 per group). All blots were duplicated. Administration of 6-OHDA resulted in a time-dependent increase in calpain (Panel B) and caspase (Panel C) activity on the lesion side. Calpain activation peaked at 24 hours and remained elevated for the two week period, whereas caspase activation was short-lived. *$p<0.05$, **$p<0.01$, 6-OHDA *vs.* contralateral side. C=vehicle infusion.

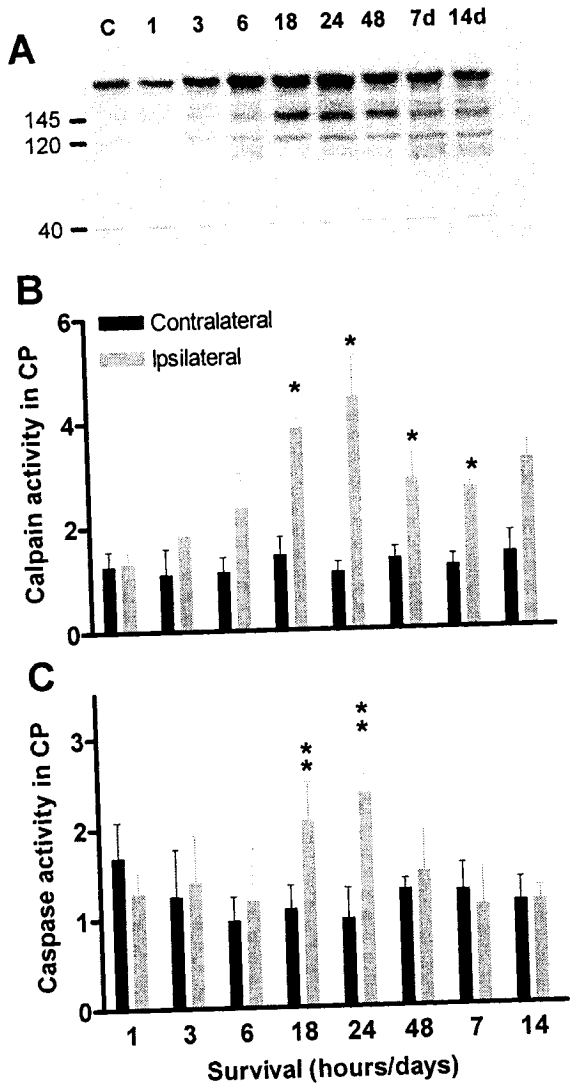
A
C 1 3 6 18 24 48 7d 14d
145
120
40
B
Contralateral
Ipsilateral
Calpain activity in CP
6
4
2
0
*
*
*
*
C
Caspase activity in CP
3
2
1
0
**
**
1 3 6 18 24 48 7 14
Survival (hours/days)

Figure 2: Time course of calpain and caspase-3 activation in substantia nigra induced by 6-OHDA. See Figure 1 for details. Administration of 6-OHDA resulted in a time-dependent increase in calpain (Panel B) and caspase (Panel C) activity on the lesion side. Calpain activation peaked at 24 hours and was also significant at later time points. Caspase activation was transient, as in the caudate-putamen. *$p<0.05$, **$p<0.01$, 6-OHDA *vs.* contralateral side. C=vehicle infusion.

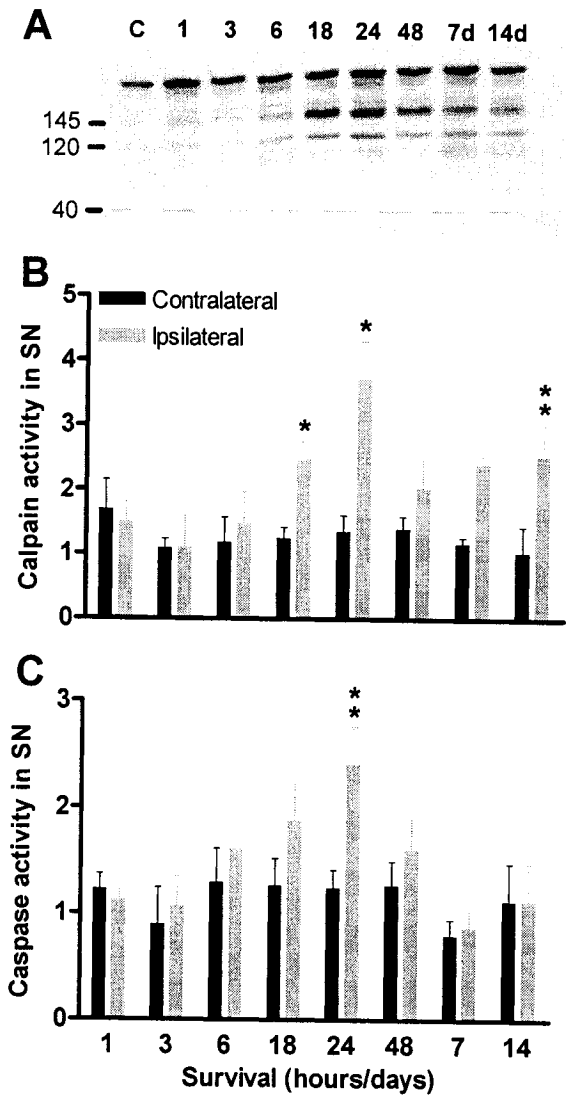
A
C 1 3 6 18 24 48 7d 14d
145
120
40
B
Contralateral
Ipsilateral
Calpain activity in SN
0 1 2 3 4 5
* * **
C
Caspase activity in SN
0 1 2 3
**
1 3 6 18 24 48 7 14
Survival (hours/days)

Figure 3: Photomicrograph of calpain activation in the caudate-putamen induced by 6-OHDA. Twenty-four hours after toxin infusion, rats were sacrificed and tissue sections were incubated with an antibody selective for a calpain-specific 150 kDa peptide fragment of α-spectrin. Visualization was achieved using a horseradish peroxidase-conjugated second antibody. In all four rats examined, immunolabeling was visible in a sphere of approx. 1.5 mm diameter within the caudate-putamen. Scale bar represents 1 mm. CC corpus callosum; LV lateral ventricle.

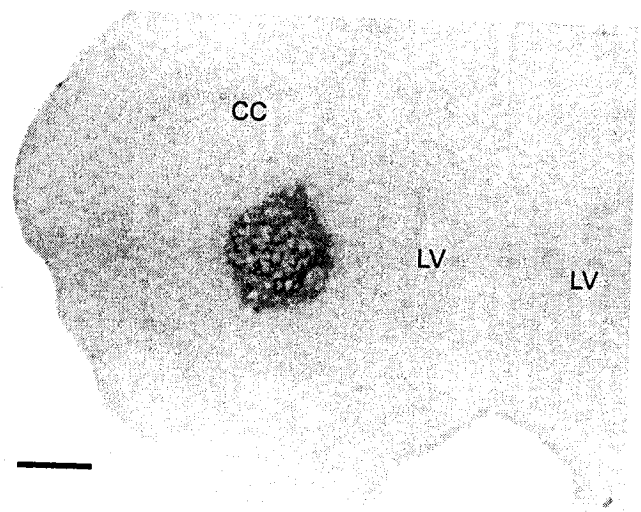
CC
LV
LV

Figure 4: Inhibition of lesion-induced calpain activation in caudate-putamen by adenoviral delivery of calpastatin. Rats received a unilateral intrastriatal infusion of control virus (Ad.GFP) or calpastatin-expressing virus (Ad.CALP), followed one week later by 6-OHDA or vehicle (VEH), and were sacrifice 24 hours later (n=3 to 4 per group). Calpain and caspase-3 activity was quantified in Western blots as before (panel A; see Figure 1 legend). Treatment with the calpastatin-expressing adenovirus completely prevented the lesion-induced activation of calpain (Panel B). Caspase activation remained significantly elevated (Panel C). Green fluorescent protein (GFP) was detected as a 27 kDa band on the adenovirus-infused side only (Panel A). The GFP signal was less abundant in 6-OHDA lesioned animals compared to those that had received vehicle. *$p$ <0.05, 6-OHDA (ipsilateral side) *vs.* contralateral side.

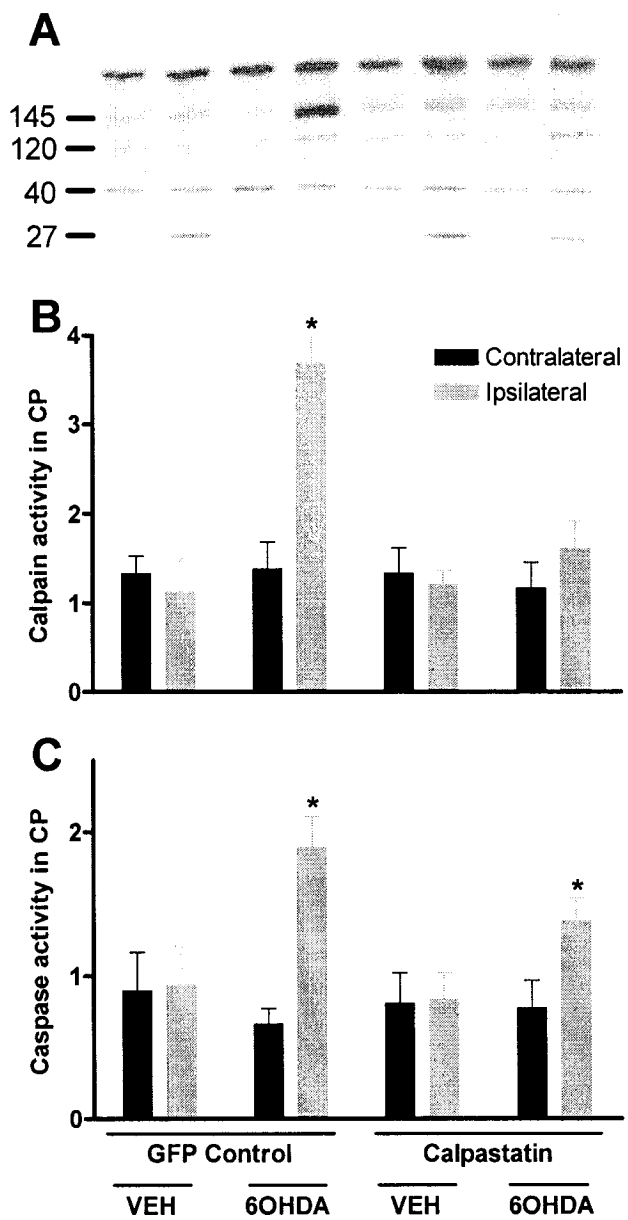
A
145
120
40
27
B
Calpain activity in CP
Contralateral
Ipsilateral
C
Caspase activity in CP
GFP Control
Calpastatin
VEH
6OHDA
VEH
6OHDA

Figure 5: Inhibition of lesion-induced calpain activation in substantia nigra by adenoviral delivery of calpastatin. See Figure 5 for details. Panel A (representative Western blots) show immunostained spectrin breakdown products specific for calpain (145 kDa) and caspase-3 (120 kDa). Treatment with the calpastatin-expressing adenovirus completely prevented the lesion-induced activation of calpain (Panel B). Caspase activation remained significantly increased (Panel C). Green fluorescent protein (GFP) was detected as a 27 kDa band on the adenovirus-infused side only (Panel A). The signal was less abundant in 6-OHDA lesioned animals compared to those that had received vehicle. *$p<0.05$, 6-OHDA (ipsilateral side) *vs.* contralateral side.

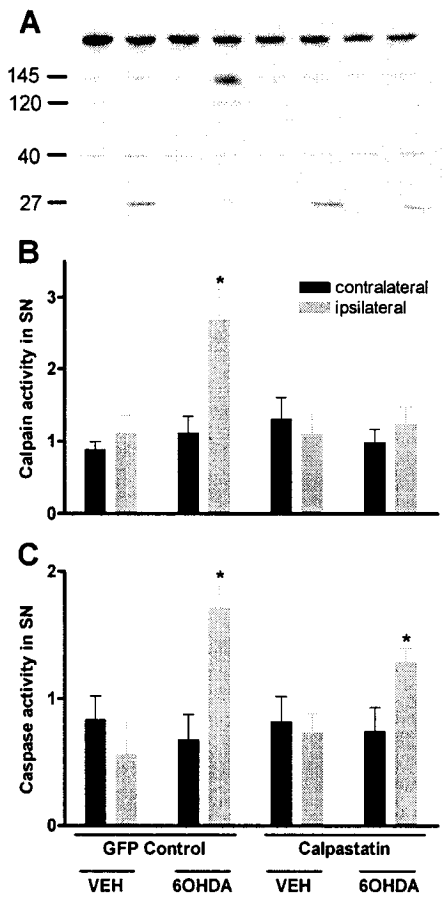
A
145
120
40
27
B
contralateral
ipsilateral
Calpain activity in SN
0
1
2
3
*
C
Caspase activity in SN
0
1
2
*
*
GFP Control
Calpastatin
VEH
6OHDA
VEH
6OHDA

Figure 6: Alleviation of lesion-induced behavioural deficits by adenoviral delivery of calpastatin. Rats received a unilateral intrastriatal infusion of control virus (Ad.GFP) or calpastatin-expressing virus (Ad.CALP), followed one week later by 6-OHDA or vehicle. Two weeks later, each rat received three tests of forelimb placement: (A) stepping, (B) whisker and (C) cylinder. The y axis shows the degree of asymmetry. On this measure, equal use of both forelimbs would score 0%, whereas total neglect of the forelimb contralateral to the lesion would score 100%. Values are expressed as mean ± SEM (n=10-14 rats per group). In the absence of 6-OHDA, limb use asymmetry was minimal and was not significantly affected by calpastatin (e.g. for stepping, t=2.04, df 10, $p$=0.07). Following 6-OHDA treatment, calpastatin produced an alleviation in the stepping and whisker tests only. *$p$<0.02, **$p$<0.005, Ad.CALP virus *vs.* Ad.GFP (control) virus.

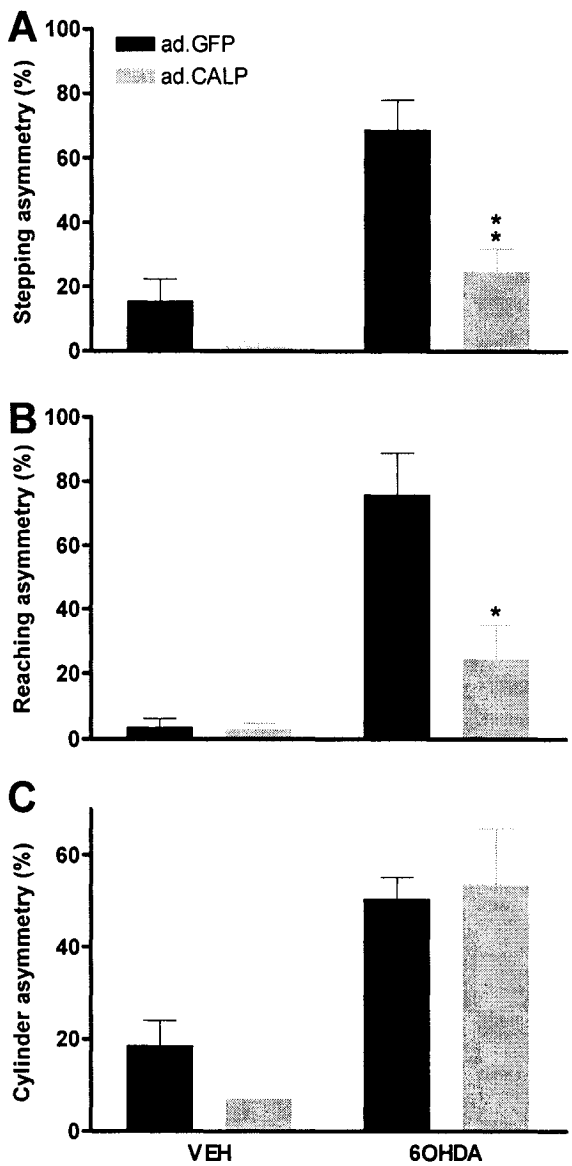
A
ad.GFP
ad.CALP
Stepping asymmetry (%)
100
80
60
40
20
0
**
B
Reaching asymmetry (%)
100
80
60
40
20
0
*
C
Cylinder asymmetry (%)
60
40
20
0
VEH
6OHDA

Figure 7: Partial neuroprotection in ventral tegmental area (VTA) by adenoviral delivery of calpastatin. Following behavioural testing (Figure 7), the abundance of DA transporter (DAT) and tyrosine hydroxylase (TH) was quantified by autoradiography (see Methods). Neither marker was altered contralateral to the 6-OHDA or viral calpastatin treatment. DA transporter (A) and TH (B) labeling on the treated side was therefore expressed as a percent of the contralateral side. The lesion-induced loss of both DAT and TH was partially prevented by virally-delivered calpastatin. *$p<0.05$, **$p<0.005$, Ad.CALP virus *vs.* Ad.GFP (control) virus.

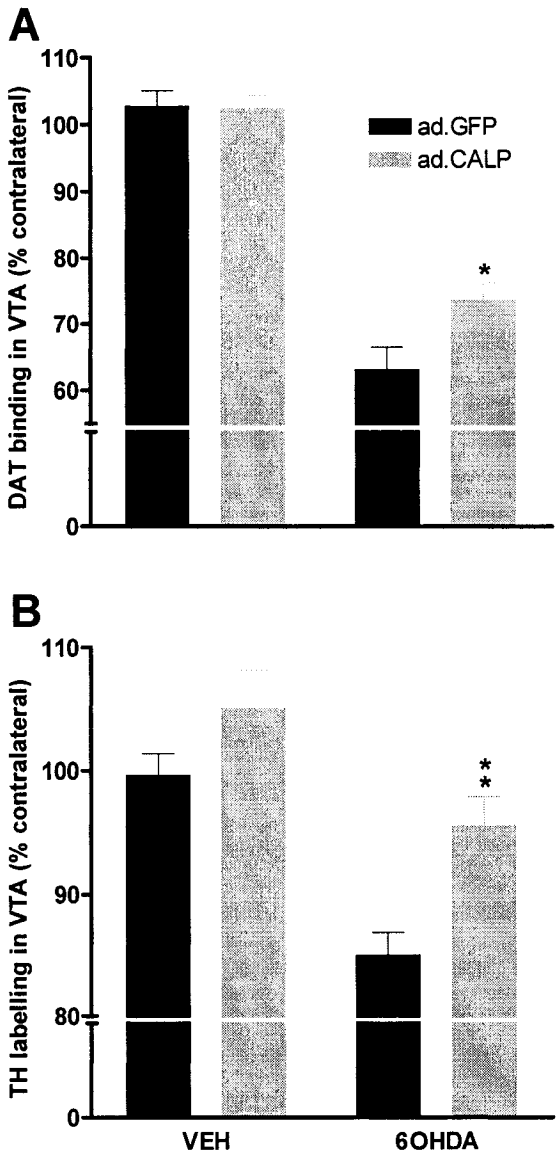
A
110
100
90
80
70
60
0
DAT binding in VTA (% contralateral)
ad.GFP
ad.CALP
*
B
110
100
90
80
0
TH labelling in VTA (% contralateral)
*
*
VEH
6OHDA

**Table 1. DA transporter and tyrosine hydroxylase labeling in animals treated with adenovirus and 6-OHDA**

| | Control virus (Ad.GFP) | | Calpastatin virus (Ad.CALP) | |
|---|---|---|---|---|
| | Vehicle | 6-OHDA | Vehicle | 6-OHDA |
| *n* | 14 | 10 | 10 | 10 |
| DA transporter | | | | |
| CP | 102.8 ± 2.4 | 6.1 ± 0.9 | 104.0 ± 1.8 | 8.5 ± 1.8 |
| SNC | 104.2 ± 3.4 | 57.1 ± 3.9 | 103.6 ± 2.7 | 61.5 ± 3.2 |
| SNR | n.d. | n.d. | n.d. | n.d. |
| Core | 104.1 ± 3.5 | 94.1 ± 3.3 | 106.7 ± 3.6 | 98.3 ± 5.5 |
| Shell | 113.5 ± 4.3 | 110.5 ± 4.2 | 113.5 ± 2.8 | 109.7 ± 4.3 |
| Tyrosine hydroxylase | | | | |
| CP | 100.2 ± 2.4 | 39.9 ± 4.7 | 100.9 ± 2.1 | 38.8 ± 4.4 |
| SNC | 99.8 ± 2.3 | 82.3 ± 2.2 | 103.4 ± 4.0 | 83.3 ± 2.3 |
| SNR | 93.3 ± 2.0 | 69.1 ± 1.5 | 95.0 ± 3.8 | 65.0 ± 2.1 |
| Core | 99.0 ± 1.4 | 101.7 ± 1.3 | 100.9 ± 2.0 | 99.2 ± 0.7 |
| Shell | 102.9 ± 1.4 | 105.1 ± 1.8 | 103.9 ± 2.6 | 100.6 ± 1.8 |

Values are mean ± SEM on the lesion side, expressed as a percentage of the contralateral side. DA transporter labeling in the SNR was low and not determined (n.d.). Corresponding data for the ventral tegmental area (VTA) are shown in Figure 7. Abbreviations: CP caudate-putamen, SNC substantia nigra pars compacta, SNR substantia nigra pars reticulata, nucleus accumbens core and shell.

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# GENERAL DISCUSSION

The neurotoxin 6-OHDA has been widely used by researchers to deplete dopamine in the brain. The targeted disruption of distinct dopamine pathways by 6-OHDA administration has shed light on the critical role of dopamine transmission in several physiological processes. The overall aim of the experiments within this thesis was to characterize the *in vivo* effects of 6-OHDA administration.

The experiments outlined in Chapter 2 revealed that (1) the nigrostriatal dopamine pathway was relatively more susceptible to 6-OHDA toxicity than the mesolimbic dopamine pathway, and (2) that hypothermia, occurring at the time of 6-OHDA administration, resulted in significant preservation of dopamine cell bodies and nerve terminals. The implications of these findings have already been addressed in the Discussion which accompanies Chapter 2.

The experiments in Chapter 3 focused on the mechanisms involved in 6-OHDA-induced cell death. Following the observation that two proteases, calpain and caspase-3, were activated after 6-OHDA administration, the effect of calpain inhibition was assessed by adenoviral delivery of calpastatin. These studies showed that (1) calpain inhibition preserved dopamine neurons in the ventral tegmental area, but not in nigrostriatal regions, and (2) calpain inhibition resulted in significant improvement in motor deficits induced by 6-OHDA. These findings have been previously discussed in the Discussion which accompanies Chapter 3.

The present chapter is a General Discussion which will focus on the implications of determining the mode of cell death following 6-OHDA administration. The relevance of 6-OHDA as an animal model of Parkinson's disease has been addressed in the General Introduction, and will not be reiterated here. In this chapter, the relevance of identifying the mechanisms of cell death will be discussed within the framework of therapeutic approaches to Parkinson's disease, with some examples of anti-apoptotic compounds under development. Finally, some recent scientific discoveries will be considered for their potential therapeutic value as future anti-Parkinson strategies.

**Caspase-3 activation & apoptosis**

Caspases are major mammalian cell death effector proteins during apoptosis (Nicholson, 1999). Caspase-3 is a Group II caspase that preferentially cleaves substrates at aspartic acid residues, which are found in many of the proteins cleaved during apoptosis (Nicholson, 1999). This requirement has led to the classification of caspase-3 as an *executioner* of apoptosis (Nicholson, 1999). Activation of a 'central' executioner, such as caspase-3, has been demonstrated following several types of stimuli, which have induced different apoptotic pathways. In Parkinson's disease, several factors (e.g. oxidative stress, mitochondrial dysfunction, excitotoxicity, and inflammation) are thought to lead to the eventual loss of dopamine neurons (Schapira and Olanow, 2004). Thus, activation of caspase-3 has been put forth as a final common executioner of apoptotic death in Parkinson's disease.

There are two principal pathways leading to apoptotic cell death; the "extrinsic" or death receptor pathway, and the "intrinsic", or mitochondrial pathway (Green, 1998). The present demonstration of caspase-3 activation following 6-OHDA administration suggests that the mode of cell death following 6-OHDA administration includes apoptotic mechanisms. However, the precise role of caspase-3 in apoptotic pathways can not be elucidated from our study.

Support for the notion that 6-OHDA-induced apoptosis occurs through the intrinsic, mitochondrial dependent pathway has been provided by previous studies. For example, 6-OHDA stimulated mitochondrial release of cytochrome c into the cytosol (Han et al., 2003). In another study, primary cultures of neurons from transgenic mice expressing the anti-apoptotic protein, Bcl-2, were more resistant to 6-OHDA treatment (Offen et al., 1998). Moreover, viral delivery of a Bcl-2-expressing vector into the substantia nigra of rats 1 week prior to intrastriatal 6-OHDA administration increased the survival of nigral neurons (Yamada et al., 1999). Since the intrinsic apoptotic pathway is initiated by the release of cytochrome c from the mitochondria, and regulated by the Bcl-2 family members (Heidenreich, 2003), these studies support the involvement of the intrinsic pathway in 6-OHDA-induced apoptosis.

However, evidence for the involvement of the intrinsic apoptotic pathway in 6-OHDA-induced cell death remains equivocal. In one study, overexpression of Bcl-2 did not attenuate 6-OHDA-induced cell death in either the MN9D cell line or in primary dopaminergic neurons (Oh et al., 1995). In another study, deletion of the pro-apoptotic

Bcl-2 family member, Bax, did not rescue dopamine neurons from 6-OHDA toxicity (O'Malley et al., 2003). Finally, microarray analysis of MN9D cells treated with 6-OHDA did not detect the up-regulation of proteins thought to act upstream of the intrinsic pathway, even though downstream caspases were activated (Holtz and O'Malley, 2003). Thus, these data support a model in which 6-OHDA activates apoptosis without inducing the intrinsic pathway.

Another possibility is that 6-OHDA activates the *extrinsic* apoptotic pathway involving death receptors such as Fas and the induction of caspase-8. Activation of the extrinsic pathway requires ligand-mediated death receptor multimerization, adaptor proteins such as FADD, as well as autoproteolysis of caspases-8 and -10 (Wajant, 2002). In the case of 6-OHDA-induced apoptosis, utilization of the extrinsic pathway seems unlikely since the main molecules associated with this pathway (such as Fas, Fas-associated death domain, and tumor necrosis factor receptor 1) were not induced following 6-OHDA treatment on MN9D cells (Holtz and O'Malley, 2003). Thus, it appears that 6-OHDA leads to apoptosis without activating the extrinsic death receptor pathway.

How could 6-OHDA lead to apoptosis if not through the activation of the extrinsic or intrinsic pathways? A novel apoptosis cascade has recently been identified which occurs as a result of endoplasmic reticulum stress (Rao et al., 2002; Morishima et al., 2002). In this pathway, endoplasmic reticulum stress induces apoptosis independent of both extrinsic and intrinsic pathway factors, requiring instead caspase-12 activation (Rao et al., 2002; Morishima et al., 2002). It is possible that apoptosis mediated by 6-OHDA may

occur through this alternative pathway, although the involvement of caspase-12 remains to be determined. The possible involvement of this apoptotic pathway will be addressed again in the next section with respect to calpain activation.

**Calpains & apoptosis**

Increased expression of m-calpain has been identified in post-mortem brain tissue from Parkinson's disease patients (Mouatt-Prigent et al., 1996; Crocker et al., 2003), and in an animal model of the disease (Crocker et al., 2003). A role for calpains in cell death has also been demonstrated in other forms of neuronal injury (Saito et al., 1993; Tsuji et al., 1998; Shields and Banik, 1998). The neuroprotective effect of calpain inhibition in some forms of neuronal injury suggests that this protease may be a novel therapeutic target for neurodegenerative diseases. In our study, administration of the calpain inhibitor, calpastatin, resulted in modest, regionally-selective neuroprotection of dopamine neurons. Thus, our data support a role for calpains in dopamine cell death.

Although the role of caspases in apoptotic cascades has been fairly well characterized, the role of calpains is less clear. Calpain activation has been observed in a number of cell culture (Pike et al., 2000; Nakagawa and Yuan, 2000; Newcomb-Fernandez et al., 2001) and *in vivo* models of apoptosis (Rami et al., 2000; Blomgren et al., 2001; Crocker et al., 2003), but its role appears to vary depending on the cell type being studied, and the type and severity of the apoptotic stimulus.

A role for m-calpain in apoptotic cell death has been demonstrated (Nakagawa and Yuan, 2000). In glial cells that had undergone oxygen and glucose deprivation, m-calpain activation resulted in the formation of active caspase-12 (Nakagawa and Yuan, 2000). In the same study, primary cortical neurons treated with fibrillar amyloid-ß peptide showed caspase-12 cleavage, whereas treatment with calpain inhibitors prevented the cleavage of caspase-12.

The interaction between m-calpain and caspase-12 described above was demonstrated to occur in the endoplasmic reticulum of the cell (Nakagawa and Yuan, 2000). The endoplasmic reticulum plays a central role in protein synthesis and is also the major intracellular organelle involved in calcium storage. Alterations of intracellular calcium homeostasis results in the accumulation of unfolded proteins in the endoplasmic reticulum. These changes in the endoplasmic reticulum leads to the induction of chaperone proteins, an event referred to as the 'unfolded protein response' (Sidrauski et al., 1998).

Recently, the occurrence of the unfolded protein response was demonstrated following the administration of 6-OHDA (Holtz and O'Malley, 2003; Ryu et al., 2002). Gene expression analysis of neuronal PC12 cells or MN9D cells treated with 6-OHDA revealed an increase in transcripts associated with the unfolded protein response (Holtz and O'Malley, 2003; Ryu et al., 2002). Coupled with our observation of calpain activation following 6-OHDA administration, these findings point towards a mechanism of cell death that may be related to endoplasmic reticulum stress. I would have enjoyed

investigating this specific question if I were to continue to characterize the mechanisms of cell death induced by 6-OHDA. This question is particularly interesting to me in light of recent genetic studies (reviewed in Nussbaum and Ellis, 2003) suggesting that nigrostriatal degeneration may occur due to an inability of dopamine neurons to eliminate damaged proteins. Thus, there may be an overall deficit in protein handling by dopamine neurons, which could eventually lead to cellular demise.

Determining the relative roles of calpains and caspases in apoptotic cell death is complicated by the occurrence of cross-talk between the two proteases. A growing body of literature has revealed that calpains regulate caspase activity during apoptosis, and that calpain activity can by regulated by caspases (Neumar et al., 2003 and refs therein). The notion of cross-talk between these proteases led to the speculation that co-administration of calpain *and* caspase inhibitors could provide an enhanced therapeutic effect. In one study, cotreatment with both calpain and caspase inhibitors to animals 60 minutes after the induction of transient forebrain ischemia resulted in a moderate increase in hippocampal neuron protection from apoptotic cell death (Rami et al., 2000).

**Calpain inhibition and behavioural/neurological recovery**

The unilateral loss of dopaminergic neurons following unilateral 6-OHDA infusion results in well-documented behavioural deficits, such as dopamine agonist-induced rotation (Schwarting and Huston, 1996), and forelimb use asymmetries (Schallert and Tillerson, 2000). The role of calpains in mediating behavioural manifestations of cellular

loss has not been extensively studied. In the present study, an improvement in 6-OHDA-induced forelimb asymmetries was observed in animals given the calpain inhibitor, calpastatin. This behavioural sparing was unexpectedly associated with modest protection of the ventral tegmental area dopaminergic neurons, but not with a preservation of the nigrostriatal dopamine pathway. Thus, it appears that a moderate preservation of the ventral tegmental area may be sufficient to normalize forelimb asymmetry, at least in some tests of forelimb use.

Limited neuroprotection was also shown to be sufficient for preserving behaviour in the MPTP mouse model of Parkinson's disease (Crocker et al., 2003). In this study, the MPTP-induced loss of tyrosine hydroxylase-positive cell bodies in the substantia nigra was significantly reduced (by approximately 50%) by intracerebral delivery of the calpain inhibitor, MDL 28170. In addition, markers of striatal postsynaptic activity were normalized by calpain inhibition. However, MDL 28170 did not prevent the loss of striatal dopaminergic markers, an observation which is in agreement with my study. The authors suggested that the normalization of locomotor activity, which was observed following calpain inhibition, could have been mediated by the partial preservation of nigrostriatal cell bodies. A possible contribution of mesolimbic dopamine to behavioural restoration was not examined in this study.

In an animal model of brain injury, the calpain inhibitor, AK295, significantly improved motor and cognitive deficits induced by fluid percussion injury (Saatman et al., 1996). Animals receiving lateral fluid percussion brain injury were given continuous intraarterial

infusion of AK295 fifteen minutes post-injury and were subsequently tested for behavioural improvements. AK295-treated animals showed markedly improved motor and cognitive abilities at 7 days post injury, but not at 48 hours post injury. In a subsequent study by the same authors, the effect of AK295 on histopathology was examined (Saatman et al., 2000). Administration of the calpain inhibitor did not prevent calpain activation in cortical or hippocampal regions at 48 hours and 7 days after injury. Moreover, calpain inhibition failed to reduce cortical lesion size, or the numbers of apoptotic cells in the cortex, subcortical white matter, and hippocampus, at 48 hours after injury. These results suggest that behavioural improvement was not dependent on lesion reduction or calpain activation in the cortical or hippocampal regions.

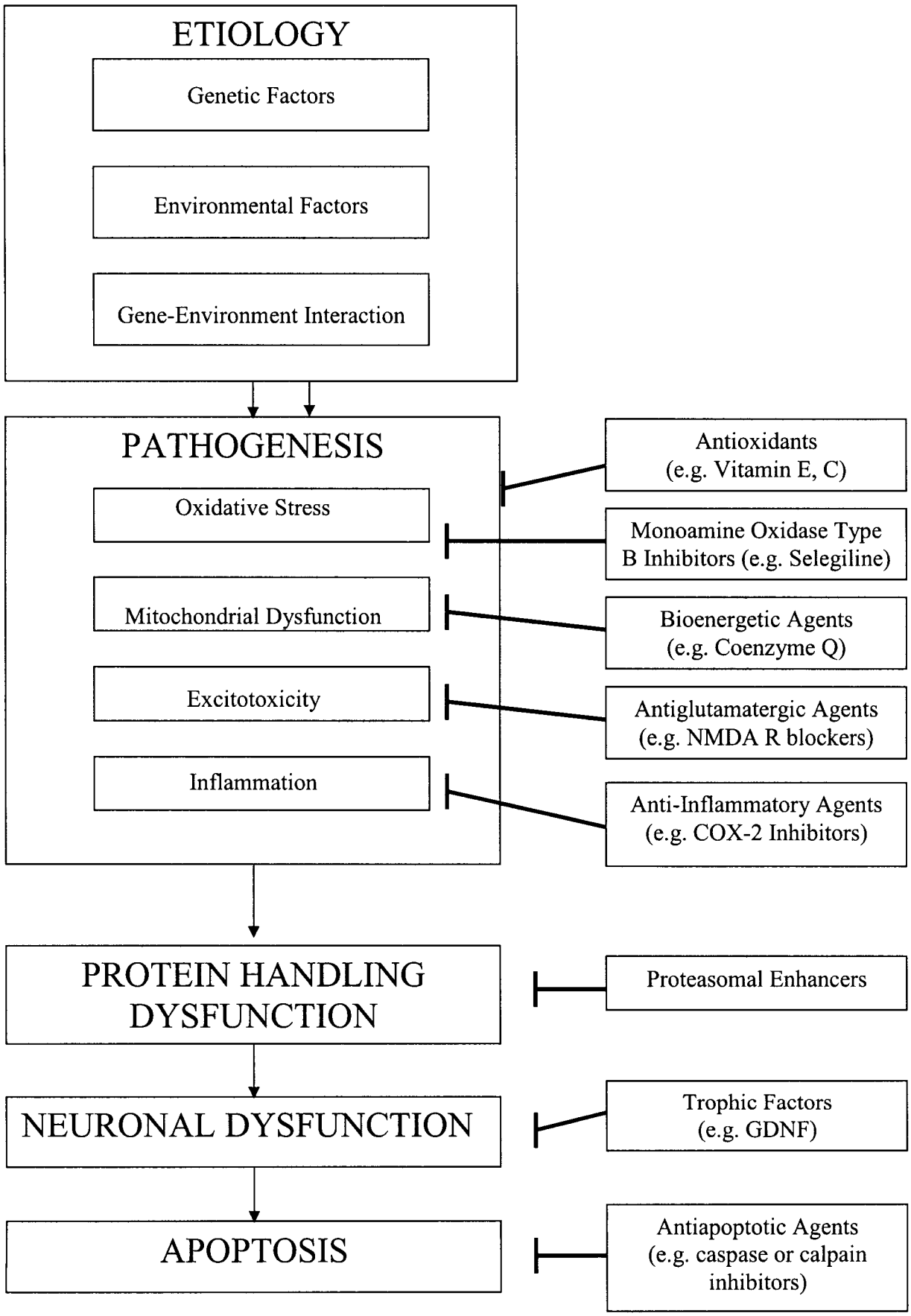
Thus, the mechanisms underlying the behavioural protection following calpain inhibition are not clear. It is possible that calpain inhibition may augment functional recovery of remaining neurons, and this may be sufficient to facilitate some types of behaviours. For example, pretreatment with calpain inhibitors has been shown to preserve long-term potentiation (Lee et al., 1991) and to facilitate recovery of synaptic transmission after CNS injury (Arai et al., 1990; Hiramatsu et al., 1993). Thus, it may not be surprising that robust behavioural improvements can occur without a dramatic preservation of histological markers.

**Apoptotic cell death in Parkinson's disease**

Remarkable progress has been made in the identification of proximal causative factors underlying the progressive loss of dopamine neurons in Parkinson's disease. Evidence has supported the occurrence of at least four specific biochemical events that can lead to nigrostriatal cell death. These different insults, namely, oxidative stress, mitochondrial complex I deficiency, excitotoxicity, and inflammation, appear to result in similar pathology (Schapira and Olanow, 2004). It is generally accepted that the culmination of these factors leads to neuronal dysfunction, and ultimately cell death. Thus, the treatment of Parkinson's disease has benefited from several different therapeutic approaches. Figure 1 summarizes the etiologic and pathogenetic factors that have been implicated in Parkinson's disease, and illustrates some of the therapeutic approaches associated with each theme.

Identifying the mode of cell death in a neurodegenerative disease has significant implications regarding the type of theraputic approaches that may be envisaged. In Parkinson's disease, morphological and biochemical evidence of apoptotic cell death has been obtained in post-mortem substantia nigra (Reed, 2002). While other forms of cell death may occur in Parkinson's disease (Graeber and Moran, 2002), the detection of a type of *programmed* degeneration nonetheless raises hope that therapeutic agents may be designed rationally, with the aim of halting neuronal loss instead of compensating for symptoms. In light of our studies showing a possible role for the pro-apoptotic proteases,

Figure 1: A schema of the etiologic and pathogenic factors that have been implicated in cell death in Parkinson's disease, along with some of the therapeutic approaches which are associated with each factor. (Adapted from Schapira and Olanow, 2004).


ETIOLOGY
Genetic Factors
Environmental Factors
Gene-Environment Interaction
PATHOGENESIS
Oxidative Stress
Mitochondrial Dysfunction
Excitotoxicity
Inflammation
Antioxidants (e.g. Vitamin E, C)
Monoamine Oxidase Type B Inhibitors (e.g. Selegiline)
Bioenergetic Agents (e.g. Coenzyme Q)
Antiglutamatergic Agents (e.g. NMDA R blockers)
Anti-Inflammatory Agents (e.g. COX-2 Inhibitors)
PROTEIN HANDLING DYSFUNCTION
Proteasomal Enhancers
NEURONAL DYSFUNCTION
Trophic Factors (e.g. GDNF)
APOPTOSIS
Antiapoptotic Agents (e.g. caspase or calpain inhibitors)

caspase-3 and calpains in loss of dopamine neurons, the next section will focus on the development of anti-apoptotic compounds for Parkinson's disease.

**Anti-apoptotic compounds under development**

Great interest has emerged in devising therapeutic strategies for modulating the key molecules which determine whether neurons live or die. Among the apoptosis-based drug targets, caspases are at the forefront because of the idea that activation of an executioner caspase (e.g. caspase-3) ultimately results in apoptotic morphology (Nicholson, 1999). Thus, proof of concept data have been obtained in animal models using peptidyl inhibitors of caspases, such as carbobenzoxy-Val-Ala-Asp-fluoromethylketone (zVAD-fmk). Significant protection by zVAD-fmk has been shown in rodent models of Parkinson's disease (Cutillas et al., 1999) and several other neurodegenerative diseases (Li et al., 2000; Endres et al., 1998). Despite the success in rodent models of neurological injury, peptidic compounds may not be useful in human neurodegenerative diseases given the difficulty of targeting these agents to the brain, and the possibility of unwanted side-effects. Thus, such compounds may be viewed as first-generation attempts at exploiting caspases as drug discovery targets.

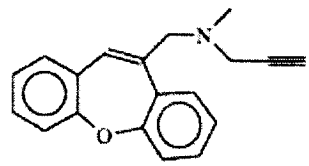
Currently, several brain-penetrating antiapoptotic compounds are under development for the treatment of Parkinson's disease. These compounds are being developed to target components of apoptotic pathways. Given the relative newness of this field, information regarding some of these compounds is difficult to obtain. Some compounds in which

public information is available are described below, and the chemical structure for each compound is displayed in Figure 2.

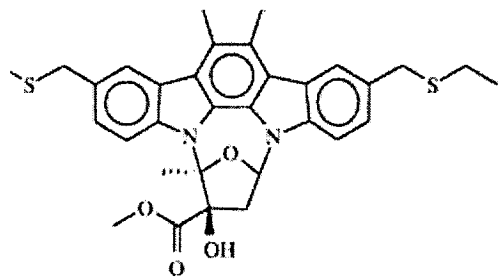
**TCH346.** The deprenyl analogue TCH346 (dibenzo[*b,f*]oxepin-10-yl-methyl-methyl-prop-2-ynyl-amine; labeled previously CGP 3466B) has been tested in a variety of cellular and animal models of Parkinson's disease. In *in vitro* studies, exposure of cultured rat embryonic mesencephalic dopaminergic neurons to TCH346 prevented cell death induced by MPP+ treatment (Waldmeier et al., 2000). In rodent studies, TCH346 administration rescued nigral dopamine neurons following MPTP administration (Waldmeier et al., 2000). In 6-OHDA lesioned rats, systemic administration of TCH346 resulted in enhanced motor function and partially preserved tyrosine hydroxylase immunoreactivity in the substantia nigra (Andringa et al., 2000). Finally, in primate studies, TCH346 significantly prevented the motoric symptoms induced by bilateral MPTP lesions (Waldmeier et al., 2000). A phase II study with TCH346 in de novo Parkinson's disease patients is ongoing at Novartis (Reed 2002).

The mechanism by which TCH346 exerts its therapeutic effects is not known. TCH346 has been shown to bind to and prevent apoptosis-related upregulation and nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Carlile et al., 2000). This enzyme is thought to be involved in p53-dependent apoptosis, in addition to performing housekeeping functions.

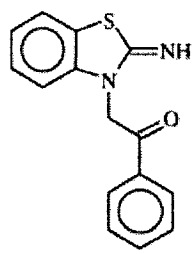
Figure 2: The chemical structures of the antiapoptotic compounds, TCH346 (also called CGP 3466B; top), CEP-1347 (middle), and Pifithrin-α (bottom).



CGP 3466B/TCH346



CEP-1347



pifithrin-α

**CEP-1347.** CEP-1347 is an inhibitor of the mixed-lineage kinase (MLK) family of kinases, and inhibits the c-Jun N-terminal kinase (JNK) pathway (Maroney et al., 2001). The JNK pathway is critical for programmed cell death during development, and may be important for cell death which occurs in neurodegenerative diseases (Saporito et al., 2002). When given to mice prior to and in conjunction with MPTP treatment, CEP-1347 partially prevented the loss of nigral dopamine cell bodies and striatal terminals (Saporito et al., 1999). In contrast, CEP-1347 did not preserve the nigrostriatal pathway when given seven days following the lesion (Saporito et al., 1999). Phase I studies are ongoing at Cephalon Inc under a collaboration with H. Lundbeck A/S.

Recently, the safety, tolerability, pharmacokinetics, and acute symptomatic effects of CEP-1347 was assessed in a study with Parkinson's disease patients (The Parkinson's Research Group, 2004). In this short-term study, CEP-1347 was found to be safe, and well tolerated, suggesting that this compound is well suited for larger and longer studies assessing its therapeutic effects in Parkinson's disease.

**Pifithrin-α.** Pifithrin-α is a synthetic inhibitor of p53-induced transcriptional activation, and was originally designed to protect noncancerous cells from genomic stress induced by cancer therapy (Komarov et al., 1999). Preservation of nigral dopamine neurons and improved motor function was observed in mice given Pifithrin-α and MPTP (Duan et al., 2002). However, p53 plays important roles in cell cycle checkpoint and DNA repair in mitotic cells, therefore the inhibition of p53 may be detrimental to those cells (Bassi et

al., 2002). In addition, because many types of tumor cells have mutations in p53, it is possible that p53 inhibitors may increase the risk of cancer. Thus, further studies are required to develop drug delivery systems that selectively target dying neurons, and to evaluate whether long-term use of Pifithrin-α is safe.

**Calpain as a therapeutic target**

Calpains have been implicated in a number of neurological diseases in which a loss of $Ca^{2+}$ homeostasis in cells is thought to lead to the degradation of proteins that are known calpain substrates. Numerous *in vivo* studies using calpain inhibitors have demonstrated the neuroprotective effects of such compounds, highlighting the calpains as prospective therapeutic targets. To this end, at least one company (Cephalon Inc) has shown interest in developing calpain inhibitors for the treatment of stroke, but clinical trials are not yet underway.

At present, there are several unresolved issues regarding our knowledge of calpains that may preclude the development of these compounds as therapeutic targets. Mu- and m-calpain isoforms are ubiquitously expressed, and are involved in many diverse functions, the details of which are only partially elucidated (Huang and Wang, 2001). Thus, inhibiting a multi-function and widespread enzyme such as calpain could have negative consequences. In addition, the presently available calpain inhibitors have poor membrane permeability, and none are specific for calpains (Wang and Yuen, 1994). These factors present challenges to the development of an inhibitor that is both specific for a particular

calpain isoform and without side-effects. The development of drug delivery strategies that may target specific populations of neurons (e.g. dopamine neurons for Parkinson's disease) may be useful when these unresolved issues are eventually worked out.

Some additional points regarding the interpretation of calpain studies are worth mentioning. First, the ability of calpain inhibitors to prevent protein degradation does not provide conclusive proof that calpains are mediating the subsequent degradation, given that these inhibitors are not specific for calpains. Thus, future studies should focus on the use of calpastatin, which, on present evidence appears to be a specific inhibitor of the calpains (Crawford 1990). Such studies will provide more definitive evidence on the role of these proteases in neurodegenerative diseases.

A second point of contention is that increases in intracellular $Ca^{2+}$ following a given insult is the trigger that activates the calpains. The problem, however, is that the increases in intracellular $Ca^{2+}$ that occur in ischemia, for example, are not sufficient to activate the calpains directly (Goll et al., 2003). Alternatively, it could be that a disruption of $Ca^{2+}$ homeostasis alters the *regulators* of calpain activity, and this latter event results in the pathological activation of calpains. Thus, calcium dysregulation may affect calpastatin, phosphorylation, or additional mechanisms of calpain regulation that are presently unknown.

**General issues regarding the use antiapoptotic compounds in Parkinson's disease**

Although there is evidence for the occurrence of apoptosis in Parkinson's disease, additional research is required before antiapoptotic therapeutic approaches may be implemented. There are several key issues that need to be addressed.

At present, there is a scarcity of information regarding long-term effects of treatment with anti-apoptotic compounds. One possible outcome of anti-apoptotic treatment is that preservation of neurons could occur without concomitant restoration of function. Inhibition of apoptosis by zVAD-fmk has protected SH-SY5Y cells and primary dopaminergic neurons from 6-OHDA induced toxicity (von Coelln et al., 2001). However, this inhibitor did not protect against the loss of neurites and dopamine uptake sites, suggesting that these neurons remained functionally impaired (von Coelln et al., 2001). Intracerebral administration of zVAD-fmk in hemiparkinsonian rats resulted in significant restoration of tyrosine hydroxylase positive neurons in the substantia nigra (Cutillas et al., 1999). However, a concomitant preservation of neuronal function was not examined in this study. Thus, it remains to be demonstrated that *in vivo* administration of zVAD-fmk preserves the structure *and* function of neurons.

Another potential problem with the use of apoptosis inhibitors is that the mode of cell death would simply shift from apoptosis to necrosis. Cellular energy (ATP) levels have been shown to be critical in determining whether a cell will die by necrosis or apoptosis (Nicotera et al., 2000). In one study, no protection of dopamine neurons against $MPP^+$

was observed despite the use of the broad-spectrum caspase inhibitor Boc-(Asp)-fluoromethylketone, and cell death was suggested to occur through nonapoptotic mechanisms (Lotharius et al., 1999). In another study, caspase inhibitors did not protect dopamine cultures against $MPP^+$ toxicity, and the toxic properties of $MPP^+$ were enhanced with regard both to tyrosine hydroxylase cell counts and [$^3$H]dopamine uptake (Hartmann et al., 2001). Because caspase inhibitors did not exert toxic effects in dopamine cultures without the addition of $MPP^+$, it was reasoned that the form of cell death shifted from apoptosis towards necrosis. In studies using 6-OHDA, cotreatment with the caspase inhibitor zVAD-fmk was not associated with a long-term increase in MN9D cell survival, and treatment seemed to redirect cell death to a necrotic form (Han et al., 2003). If apoptosis inhibitors do indeed promote necrotic death, this could worsen the pathology due to inflammation associated with leakage of the extracellular membrane (Nicotera et al., 1999).

Another potential adverse effect of anti-apoptotic agents is cancer. The fear that cancer may be promoted or induced following treatment with apoptosis inhibitors is based on the finding that, in many cancers, there are changes to key regulators of apoptosis (e.g. p53 and Bc2 family members) (Bunz, 2001). Long-term carcinogenicity studies with these inhibitors in animal models will undoubtedly be valuable in this regard.

Finally, it will no doubt be a challenge for pharmaceutical companies to contend with the financial hurdles involved in developing these compounds. Because inhibitors of apoptosis are expected to slow the progression of a chronic disease as opposed to exerting

an acute beneficial symptomatic effect, the clinical trials could be of long duration, costly, and will therefore be risky.

**Is there hope?**

To answer the question 'Is there hope in the treatment of Parkinson's disease?', we must examine the time frame of advancements already accomplished, keeping in mind the advanced technologies that will underlie our future efforts. There is ample reason to be optimistic about improved therapies for this disease. Let's consider that it was within the last 50 years that initial observations linking a loss of central dopamine to motor deficits were made (see Preface). Since then, experiments done in vitro and in animals have been critical in revealing potential mechanisms of cell death, and in the design and evaluation of new therapeutic candidates.

Nevertheless, it is disheartening that L-DOPA, the first drug used to treat Parkinson's disease, remains the best approach to date. This tells us that despite the advances made over the last fifty years, great challenges lie ahead. Three main drawbacks associated with L-DOPA therapy are (1) the emergence of dyskinesias, (2) the "on-off phenomenon", and (3) the continued loss of dopamine neurons despite symptomatic relief. Thus, at least one major challenge is the development of neuroprotective compounds that may be administered during the course of the disease to slow or stop the progression of cell loss.

In the midst of these current challenges, several exciting discoveries regarding the nature of brain cells are being reported by scientists. These findings may provide the basis for future novel therapeutic approaches. One of the foremost examples over the past decade is the finding that the adult brain is capable of generating new neurons. The birth of new neurons, neurogenesis, is not restricted to embryonic development, but normally occurs in the olfactory bulb and the dentate gyrus of the hippocampus (Doetsch et al., 1999). In addition, significant numbers of multipotent neural precursors have been shown in several regions of the brain, including the striatum (Palmer et al., 1995), and substantia nigra (Lie et al., 2002). In addition, the rate of neurogenesis in the substantia nigra was shown to increase after a partial lesion induced by the administration of MPTP (Zhao et al., 2003). One hope is to induce neurogenesis in regions of the brain where it does not normally occur by manipulating the endogenous multipotent precursors. This approach would overcome the limitations currently associated with transplanting exogenous midbrain neurons into the striatum or substantia nigra.

The recent discoveries regarding neurogenesis in the adult brain suggest that an important task is to determine the relevant molecular controls over adult neurogenesis. A striking example of this is the engineering of embryonic stem cells into dopamine neurons *in vitro*, by transfection with the transcription factor *Nurr1* (Kim et al., 2002). These neurons were grafted into a 6-OHDA-denervated host rat, and were shown to not only integrate into host brain but also to normalize behavioural deficits (Kim et al., 2002). In addition, ongoing studies are examining the potential of stem cell-derived neurons isolated from the olfactory bulb as another way of replacing lost dopamine neurons in

Parkinson's disease (Parati et al., 2003). Intrastriatal grafts of these dopaminergic-like neurons resulted in improved motor behaviour in mice given 6-OHDA (Parati et al., 2003).

These studies illustrate the importance of identifying appropriate signals that influence the normal development of dopamine neurons. An ideal situation would be the identification of stem cells from a more accessible source. In this regard, the discovery of multipotent neural stem cells in adult skin has raised the possibility that they may be used one day as an accessible, autologous source of stem cells for transplantation (Toma et al., 2001).

In addition to the current research exploring neurogenesis, there have been exciting advances in our understanding of genetic contributions to Parkinson's disease. Mutations in five different genes (α-synuclein, parkin, UCH-L1, DJ-1, and Nurr-1) have been recently identified (reviewed in Nussbaum and Ellis, 2003), but these mutations account for only approximately 5-10% cases. These mutations have pointed to a dysfunction in the ubiquitin-proteasomal pathway as a final common pathway leading to cellular demise. Future research testing this hypothesis, and identifying key faulty components within the ubiquitin-proteasomal pathway will undoubtedly uncover new therapeutic targets for the treatment of genetic-based cases of Parkinson's disease. Such research may also broaden our understanding of the mechanisms underlying the majority of Parkinson's disease cases which are sporadic.

## Concluding remarks

The studies in this thesis were undertaken to shed light on unresolved issues pertaining to the use of 6-OHDA in the rat model of Parkinson's disease. Because lesion parameters vary amongst different research groups, the characterization of three different 6-OHDA lesion strategies (Chapter 2) may help to explain conflicting reports of overall patterns, extents, and mechanisms of cell losses. Moreover, the observed preservation of a subregion of VTA dopamine cell bodies by hypothermia has underscored the occurrence of dopamine neuron heterogeneity. The aspect of heterogeneity amongst populations of dopamine neurons will likely become increasingly important in future studies that focus on manipulating stem cells into viable dopamine neurons for transplantation purposes. Such heterogeneity may explain the selective vulnerability of some dopamine neuron subregions that has been observed in Parkinson's disease (Damier et al., 1999).

The mechanisms underlying the loss of dopamine neurons in Parkinson's disease remains subject to debate. In the 6-OHDA rat model, I have shown evidence of a regionally-specific role for calpains in degeneration, in addition to the possible involvement of caspase-3 (Chapter 3). Moreover, the inhibition of calpains by its endogenous inhibitor, calpastatin, revealed an important role for calpains in the expression of motor deficits associated with dopamine loss. Taken together, these data suggest that inhibitors of calpains and/or caspases may provide neuroprotection and behavioural improvement in humans suffering from the disease. A careful examination of pathways involved in degeneration may contribute to the development of more effective therapies that promote

the survival *and* functional recovery of dopamine neurons. However, the numerous drawbacks associated with this therapeutic approach suggest that alternative approaches may be advantageous for the treatment of Parkinson's disease. Tremendous hope for finding a cure for Parkinson's disease is offered by recent advancements in our understanding of the brain. With luck, these discoveries will yield promising drug candidates which can then be tested in disease-relevant animal models.

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## Appendix

**I. Use of Animals approval form**

**II. Use of Biohazardous Materials approval form**

**III. Co-author approval forms**