Neural Mechanisms of Levodopa Induced Motor Complications in Parkinson’s Disease

(Evidence for the dissociation between central pharmacokinetics and pharmacodynamics of levodopa)

By

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B.Sc., University of Victoria, 2000

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Graduate Program in Neuroscience)

We accept this thesis as conforming to the required standard:

THE UNIVERSITY OF BRITISH COLUMBIA

March 2004

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Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by a loss of dopamine (DA) neurons. Motor fluctuations and dyskinesia are common complications that appear with long-term Levodopa (L-dopa) therapy.

A rat model of unilateral Parkinsonism was formed using 6-OHDA and treatment with L-dopa was conducted to induce dyskinesia. The abnormal involuntary movement (AIM) rating scale was used for monitoring the time-action curve of the L-dopa response. Striatal DA levels were investigated using in vivo microdialysis. Post-mortem brain sections were taken for autoradiography and in situ hybridization histochemistry.

The validation study (Chapter II) indicates that the AIM rating scale is reproducible across test days and within its four subscores. This offers a reliability that allows for an expanded analysis of the time action curve of dyskinetic movements.

The chronic L-dopa treatment study (Chapter III) demonstrates that AIM magnitude (Emax) correlates negatively with latency to onset and decay time, but positively with rising time. We found that prodynorphin mRNA in the striatum has a significant negative correlation with decay time, and a positive correlation with Emax. This upregulation of prodynorphin mRNA, is also associated with L-dopa-induced dyskinesia and may contribute to the development of motor fluctuations.

The microdialysis experiments (Chapter IV) show that a DA surge response is necessary to cause L-dopa-induced AIM. The time concentration curve of extracellular DA shows a left shift in dyskinetic rats after chronic pulsatile treatment. Also, partially lesioned rats show a smaller increase in extracellular DA levels with greater increase in extracellular DOPAC levels after L-dopa injection, compared to those with severe striatal DA terminal loss. This suggests that the severity of DA terminal loss is a factor determining the level of "DA surge" in the striatum after L-dopa administration.

Experimental observations from this project indicate that both severe DA terminal loss in the striatum and L-dopa treatment contribute to the development
of “DA surge” in the striatum with a therapeutic dose of L-dopa, which is in turn responsible for the development of response fluctuations and dyskinesia in a rat model of Parkinson’s disease.
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Acknowledgements

I would like to thank my supervisor and mentor, Dr. Chong Sik Lee, for his continuing support and help completing this long road. I would also like to thank Dr. Tony Phillips as my often silent but always helpful co-supervisor. Furthermore let me extend my appreciation to my committee members for making time to give me guidance.

The scope of my project crossed into several experimental regions and called upon the support of many people in several labs. Thank you all, I could not have done this without you.

Finally to my family and friends, your love and support even when I had no time to see you was always evident.
Chapter I
The Basal Ganglia and L-dopa Induced Dyskinesia

1.1 Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder, characterized by the loss of nigrostriatal DA neurons and manifestation of tremor, rigidity, postural instability and bradykinesia. The standard pharmaceutical approach to relieve these symptoms is dopaminomimetic treatment with L-dopa. Although L-dopa is an effective therapy for PD, this approach has substantial drawbacks that may outweigh the initial benefit (Hirsch 1997, Marconi 1994, Papa 1994). The motor complications from L-dopa therapy include response fluctuations, and L-dopa-induced dyskinesia (Obeso 2000, Nutt 2002, Nutt 1995). Dyskinesia is a motor problem manifesting as involuntary movements such as chorea. Concern over this problem has led to large amounts of research into the mechanisms underlying the motor complications of L-dopa.

Clinically, about 50% of patients develop motor fluctuations or dyskinesia after 5 years of L-dopa therapy (Marsden 1977, Ahlsgkog. 2001). These complications are difficult to eliminate once they develop (Fahn 2000). Further, dyskinesia coincides with motor fluctuations in ~80% of patients (Obeso, 1991). Motor fluctuations seem to follow a consistent pattern during the course of L-dopa therapy. For example, mild, long-lasting response in the early stage ("stable response"), followed by greater response with shorter duration resulting in waxing and waning between L-dopa doses ("wearing-off") and eventually, abrupt changes in response as if turned on and off by a switch (hence, "on-off") (Duvoisin 1989). Therefore, motor fluctuations are a pharmacodynamic phenomenon, characterized by an L-dopa response with shortening duration and increasing magnitude (Nutt 1996). In contrast, dyskinesia is a motor phenotype, referring to abnormal involuntary movements, including choreiform, dystonic or tic-like movements without stipulating pharmacodynamic characteristics (Nutt 1990). Motor fluctuations and dyskinesia are not mutually exclusive because they
describe different aspects of the L-dopa response. Consistent with this view, dyskinesia is almost an invariable component of “on-off” fluctuations (Nutt 2001).

Many therapeutic approaches have been implemented to combat PD. These include dopaminergic agents, neuroprotective therapies, transplantation strategies and deep brain stimulation; unfortunately these strategies often result in behavioural complications or become less effective over time. The end result is a return to pulsatile stimulation with L-dopa. As this thesis explores L-dopa therapy it is important to define two key terms. Pharmacokinetics is the bodily absorption, distribution, metabolism, and excretion of drugs, whereas pharmacodynamics is the action of a drug on the body over a period of time, such as the resulting behaviour (Webster 2003).

1.2 Functional Anatomy of Dopamine Denervated Striatum

On a functional level the basal ganglia is held in an equilibrium that is created between the direct and indirect pathways and between excitatory and inhibitory inputs. When there is DA denervation to the striatum, the system is compromised and results in upregulated activity of striatopallidal neurons in the indirect pathway and down-regulated activity of striatonigral neurons in the direct pathway. The changes in synaptic transmission also result in an increase in the excitatory input to the internal globus pallidus (GPI). This results in elevated inhibition of the thalamus, which retards movement resulting in the frozen PD state.

It is the diminished dopamine function in PD that is our major concern in attempting to understand the clinical effects. At the striatum, dopamine can have excitatory or inhibitory effects on striatal output neurons depending on the receptors and pathway involved. This results in increased GABA activity to the external globus pallidus (GPE) and a decrease in the activity of the projections to the GPI. The inhibition on the GPE causes decreases in the gabaergic output projections to the sub-thalamic nucleus (STN) and GPI. This reduces the inhibition on the STN, which produces more glutamatergic excitation to the GPI. This summation on the GPI increases the inhibitory projections to the thalamus.
Animals with unilateral nigrostriatal lesions show upregulation of mRNA encoding D2 receptors and enkephalin in striatopallidal neurons on the ipsilateral side to the lesion. There is downregulation of mRNA encoding dynorphin and substance P in the striatonigral neurons on the same side (Gerfen 1991). Pulsatile treatment with L-dopa upregulates expression of mRNA for dynorphin, while expression of enkephalin is not significantly changed (Gerfen 1990, Jolkkonen 1995). Current evidence suggests that while upregulation of enkephalin may play a role in motor recovery, upregulation of dynorphin may contribute to the development of L-dopa-induced dyskinesia (Steiner 1998). Over activity in the GPi and STN appear prior to the appearance of symptoms and therefore might also be related to pre-symptomatic compensation (Bezard 1999).

1.3 Role of Dopamine

The role of dopaminergic transmission in the pathogenesis of L-dopa-induced dyskinesia is evident. There are approximately 15,000 - 20,000 DA cells on each side of the rat brain (Bloom 1996). Areas A8, A9 and A10 of the rat are the key regions of dopamine that project to many locations in the brain including the basal ganglia. These regions include the ventral tegmental area, and the substantia nigra pars compacta (Bloom 1996). They innervate the striatum largely through the medial forebrain bundle. Several studies also suggest that the dopamine neurons of the substantia nigra pars compacta (SNc) innervate in a modest fashion, the GP and the STN (Prensa 2000).

Dopamine originates from the amino acid tyrosine, which is transported into the dopamine neurons. The rate-limiting step once tyrosine reaches the neuron is the conversion to dopa by tyrosine hydroxylase. There is almost no dopa found in the brain as it is immediately converted to dopamine by the enzyme L-aromatic amino acid decarboxylase. L-dopa is used as therapy because it is capable of crossing the blood-brain barrier, and avoids the rate-limiting step in forming dopamine. We administered L-dopa with benserazide because this dopa decarboxylase provides peripheral inhibition of the aromatic amino acid decarboxylase (AADC) in the body so that the L-dopa is not degraded
before it reaches the brain (Nakashima 1996). The co-administration of benserazide significantly enhanced the striatal amount of L-dopa by 6.1 times (Nakashima 1996).

DA enters the synapse through calcium dependent release where it activates the post-synaptic neuron. The DA undergoes reuptake mediated by the dopamine transporter (DAT). The recovered dopamine is broken down by monoamine oxidase (MAO) to dihydroxyphenylacetic acid (DOPAC) (Zetterstrom 1998). DA remaining in the extracellular space is deactivated to primarily DOPAC and homovanillic acid (HVA) by catechol-o-methyltransferase (COMT) and MAO (Bloom 1996).

1.4 Post-synaptic Influences

The striatum is among the brain regions with the highest levels of opioid peptides and receptors (Steiner 1998). Dynorphin and enkephalin are endogenous ligands of the kappa opioid receptors and the delta and mu opioid receptors respectively, which are known to be inhibitory (Steiner 1998). The generalized function of dynorphin and enkephalin are to dampen excessive activation of GABA neurons by dopamine and other transmitters (Steiner 1998). Behavioural consequences of increased opioid peptide function in striatal output pathways may include behavioural sensitization (dynorphin) and recovery of motor function (enkephalin) (Steiner 1998). Evidence suggests that non-DA neurotransmitters in the basal ganglia are directly associated with the manifestation of dyskinesia, as indicated partly in pharmacological studies (Feger 1984, Robertson 1989).

Dynorphin is localized in striatonigral neurons in the rat, which contain predominantly D1 receptors (Gerfen 1990). Stimulation of D1 receptors by pulsatile treatment with dopaminergic drugs increases gene expression and activity of dynorphin in a time-dependent manner (Engber 1991, Gerfen 1990, Li 1986). Dynorphin may contribute to the manifestation of dyskinesia during the DA "on-dose" state -if the gene expression for dynorphin is upregulated (Newman 1997).
L-dopa treatment induces an increase in dynorphin in the denervated striatum as an adaptive or compensatory response. This mechanism seems to dampen the effects of subsequent D1 receptor stimulations (Steiner 1993). The indirect pre-synaptic inhibitory regulation of DA transmission results from the action of released dynorphin on kappa receptors located on DA nerve terminals (Krebs 1994). Although the dynorphin effect may be compensatory at the cellular level, increased negative feedback will affect the dynamics of activity in this pathway and thus alter network properties and consequently behaviour (Steiner 1993).

Met-enkephalin is localized in striatopallidal GABAergic neurons (Aronin 1984; Haber 1981), which predominantly contain D2 receptors (Gerfen 1990). The majority of the changes in enkephalin occur within one week of dopamine denervation and may restore some level of function (Steiner 1993, Nisenbaum 1996). Stimulation of D2 receptors suppresses activity of striatal projection neurons (Calabresi 1993), and inhibits the release of GABA and Met-enkephalin from striatopallidal neurons (Harsing 1997). In the rat, enkephalin likely does not play a significant role in the manifestation of dyskinesia that occurs during the ON-dose state.

The enzyme glutamic acid decarboxylase (GAD67) is responsible for the biosynthesis of GABA. Studies have shown upregulation of GAD67 mRNA in both the striatum and the GP in 6-OHDA lesioned rats (Delfs 1995; Kincaid 1992; Soghomonian 1992) or in MPTP-lesioned primates (Guridi 1996; Soghomonian 1994). Stimulation of D2 receptors disinhibits GP neurons and increases the release of GABA (Filion 1991; Pan 1990). Microinjection of a GABA agonist into the subthalamic nucleus, which receives afferent inputs from the GP, or microinjection of a GABA antagonist into the GP increases locomotor activity in the rat (Austin 1991) and causes contralateral dyskinesia in primates (Crossman 1984). Thus, increased release of GABA from GP neurons during the ON-dose state may contribute to the manifestation of dyskinesia.

The role of glutamate in the striatum cannot be overlooked. In the presence of Mg^{2+} glutamate can stimulate the release of DA by a mechanism that does not use Ca^{2+} dependent exocytosis but instead involves a reversal of DA transporters (Lonart 1991). Lesions cause DA levels to diminish which results
in increased glutamate release in the striatum (Lindefors 1993, Konradi 1998) affirming that DA inhibits glutamate (Shimizu 1990).

1.5 Pre-synaptic Influences

Pre-synaptic factors must also be explored in the understanding of the effects of DA denervation. The vesicular monoamine transporter has been utilized by many studies as an accurate indication of the terminal density remaining in the striatum after dopamine lesions. This is because compensatory mechanisms do not appear to affect this transporter.

The DA transporter (DAT) is designed to reuptake dopamine following release from the synapse. DAT is a 619 amino acid protein that is a member of the Na+/Cl- dependent plasma membrane transporters and can be regulated rapidly (Amara 1993, Zahniser 2001) Using the Na+ gradient DAT is able to recapture dopamine soon after its release or it can be reversed to expel dopamine into the synapse (Vizi 2000, Robinson 2002). The rate of dopamine biosynthesis decreases when reuptake is inhibited. These data indicate a key role of the dopamine transporter in maintaining neurochemical homeostasis in the neuron and synaptic level (Raevskii 2002).

A growing body of evidence indicates that activity of the DAT is down regulated in the striatum with partial DA denervation (Van Home 1992, Gerhardt 1996, Earl 1998). The clinical relevance of this effect is reinforced by a recent finding of in vivo evidence from a PET study, that there is a down-regulation of DAT in patients with early PD (Lee 2000). Under certain pathological conditions in response to restricted physiological events, DA release from varicosities may be mediated by reversal of the DAT from uptake to release mode (Leviel 2001). The concept of reverse transport has been proposed for other neurotransmitters including noradrenaline (Pin 1989).

This atypical mode of release could be evoked directly at the pre-terminal level by multiple endogenous factors involving transient alterations of the sodium gradient. It cannot be excluded that this mode of release participates in firing-induced release, in contrast with the classical exocytosis of a preformed
dopamine pool (Leviel 2001). This adds to the growing body of evidence that reverse transport plays a role in the maintenance of basal extracellular DA concentration in the striatum (Leviel 2001).

1.6 L-dopa Induced Motor Fluctuations

The L-dopa-induced dyskinesia response is manifested by multiple interacting factors including nigrostriatal denervation, pulsatile L-dopa treatment and hyperdopaminergic ON-dose state. The action of L-dopa in the induction of dyskinesia is mediated by two different mechanisms: a short-term and a long-term (sensitization) effect. The short-term effect entails regulatory action on the release of neurotransmitters in the basal ganglia nuclei, which corresponds to the rise and fall of plasma L-dopa levels. The sensitization effect entails regulatory action on gene expression of the neurotransmitters, and their resulting synthesis leading to an improvement over days (Nutt 1997). During the short response there are several types of responses that can manifest: stable (no clinical oscillations), and fluctuating (oscillations can be predictable wearing off or it can be unpredictable on-off) (Nutt 1995, Nutt 2000).

Pulsatile treatment with a subthreshold dose of L-dopa induces dyskinesia after a latent period in most severely lesioned rats. This phenomenon of behavioural sensitization has been well recognized in the rat (Carey 1991, Papa 1994, Sorg 1994) and the primate (Bedard 1986, Crossman 1987). The time course is gradual and prolonged, resulting in an accumulation effect, clearly departing from the time course of 'on-dose' dyskinesia. DA neuronal loss after toxic insult by 6-OHDA is relatively static at least from the second week after the lesion (Altar 1987, Chritin, 1993). There is also no convincing in vivo evidence that chronic L-dopa treatment causes a progressive loss of DA neurons (Kastner 1994; Schneider 1984). Observations that pulsatile treatment with DA receptor agonists also induces behavioural sensitization (Rowlett 1993; Vaughn 1990) support the view that the behavioural sensitization is not directly mediated by pre-synaptic factors.

Therapeutic approaches for motor fluctuations that maximize the stability of dopaminergic levels appear to be of critical importance (Mouradian 1987, Nutt 2002). The pulsatile administration has been shown to cause several key effects
on the time action response. The magnitude of response is less in patients just
starting therapy and increases with treatment (Nutt 1996, Nutt 1997). Also the latency
time to onset and Emax are longer in stable and untreated patients, but treatment
decrease the latency to peak response (Nutt 2002, Contin 1994, Nutt 1992,
Gancher 1988). The slope of the ascending curve also increases indicating a faster
onset of motor complications (Nutt 1996). Pharmacological modeling of the short
duration response indicates that with time the response becomes less graded and small
changes in L-dopa concentration can produce big changes in response (Nutt 1996).

The most common form of motor fluctuations after a dose of L-dopa is 'on-
dose' or peak-dose dyskinesia, which is the form studied in this thesis. L-dopa-
induced dyskinesia is evident in humans, monkeys and rats. This dyskinesia
occurs only in patients with PD or in monkeys, and rats that have DA denervation
(Boyce 1990, Horstink 1990, Schneider 1989). The L-dopa induced dyskinesia
appears on the most affected side first. This indicates that not only the treatment
but also the degree of denervation is important to forming dyskinesia. The
involuntary nature of these L-dopa-induced abnormal movements - both stereotypy and
contraversive rotation – was inferred by their relative lack of suppressibility analogous to L-
dopa-induced dyskinesia in human subjects (Walters 1990), and their self-injuring tendency
in rats.

L-dopa-induced stereotypy in the rat resembles L-dopa-induced
dyskinesia in primates with an MPTP lesion: (i) Both are abnormal involuntary
movements (AIMs) affecting multiple body regions - orofacial dyskinesia, limb
dyskinesia or dystonia (Boyce 1990); (ii) AIMs in both species appear in animals with
 nigrostriatal lesions after chronic pulsatile treatment with L-dopa (Boyce 1990). If the
 nigrostriatal lesion is unilateral, L-dopa-induced AIMs occur on the side contralateral to the
 lesion (Clarke 1989); (iii) In both species, L-dopa-induced AIMs appear in the ON-dose state
 (hyperdopaminergic state following administration of dopaminergic drugs) (Boyce 1990); (iv)
In both species, the severity of AIMs increases gradually with pulsatile treatment with L-
dopa.

In the rat, stereotypy is mediated through the neostriatum and GP, whereas
 locomotor activity is mediated by the nucleus accumbens and ventral palladium (Kelly 1975,
There are also some interspecies differences in L-dopa-induced AIMS between rats and primates. For example, limb dyskinesia occurs mostly in the forelimb in the rat. Such a preferential involvement of limb dyskinesia in the forelimb has not been observed in primates (Boyce 1990, Vidailhet 1994, Barone 1986, Pycock 1980). This interspecies difference may derive from a unique anatomical organization of the rat brain. In the rat, the motor cortex for hindlimbs, unlike the motor cortex for forelimbs, is not differentiated from the sensory cortex (Donoghue 1979). The mechanisms mediating directional bias of locomotion are different from those mediating Parkinsonism or stereotypy of the limb and are poorly understood. The animal model and its pharmacokinetic basis will be explored throughout this thesis.

1.7 Purpose

The information contained within this thesis is a percentage of the work conducted in Dr. Chong Lee’s lab as well as Dr. Tony Phillips lab to further the understanding of treatment-induced changes in L-dopa response by using a rat model of PD and dyskinesia scoring. Three different studies make up the research presented here. These studies have built upon each other to give an insight into neural mechanisms of response changes to L-dopa-induced dyskinesia.

A reproducibility study was conducted to determine if the rat AIM rating scale was reliable between testing days and among its parameters. This information is necessary to allow for an expanded analysis of the time-action curve for AIM in response to L-dopa treatment. The next study was designed to investigate the pre-synaptic and post-synaptic markers and how they relate to the time course of L-dopa-induced AIM response.

Two experiments involve in vivo brain microdialysis with real-time monitoring of L-dopa-induced dyskinesia. These studies had the purpose of exploring the role of DA terminal loss and the effects of L-dopa treatment on the central pharmacokinetics of L-dopa. A pilot experiment was conducted to compare the levels of extracellular DA and its metabolites in the striatum following a therapeutic dose of L-dopa. The rats were assigned to the following
groups: (i) severe DA terminal loss with L-dopa-induced dyskinesia and: (ii) rats with mild to moderate DA terminal loss with no L-dopa-induced dyskinesia. The other study explored the biochemical correlates of response changes to L-dopa by comparing cerebral pharmacokinetics and pharmacodynamics of L-dopa in the rats that received chronic pulsatile L-dopa treatment. Variables describing pharmacokinetics and pharmacodynamics of L-dopa were compared across a range of lesion severities. The results and implications of the experiments that were conducted are discussed in the following chapters.
Chapter II
Validation of the Abnormal Involuntary Movement Scale and Pharmacodynamics

2.1 Introduction

Animal models have played a significant role in L-dopa-induced dyskinesia research. The nature of the movement disorders and their underlying neural mechanisms seem to be remarkably similar across species (Delong 1984). Although the motor repertoires are species-specific, research depends on the validity that can be obtained through careful observation and the animal models' inherent relevance to the clinical setting (Lindner 1996). One of the most prominent has been the 6-OHDA rat model; this effective and inexpensive model has provided key information in the attempts to find therapeutic options for L-dopa-induced dyskinesia (Schwarting 1996) and an understanding of the mechanisms taking place.

The unilateral 6-OHDA model of Parkinson's disease in rats parallels the end point of the human disorder remarkably well. Many forms of behavioural analyses have been used to establish validity between the rat models and human Parkinson's and new more relevant versions continue to appear (Lindner 1996). These claims are supported by the fact that, dyskinetic side effects are attenuated by drugs that are effective clinically (Cenci 1998, Lundblad 2002). However, the behavioural results often have a large variance between testing days, and time points. Therefore there was a need for a more complete behavioural interpretation of the dyskinetic response of “on” phase or peak dose dyskinesia (Obeso 2000) in the 6-OHDA rat model.

The abnormal movements manifested in the rat model through L-dopa treatment can be quantified based on similar characteristics that are used in the clinic (Lee 2000, Hagell 1999). The abnormal involuntary movement (AIM) scoring system (Cenci 1998, Lee 2000) of induced stereotypy is classified into four subtypes based on topographic distribution, and locomotor activity. It can be
used to enhance the sensitivity of detecting behavioural changes resulting from pathology in the neostriatum (Lee 2000).

The purpose of this study was to test the reliability of the AIM scoring system developed for ongoing behavioural dyskinesia experiments. In this chapter we will illustrate the usefulness of this scale by demonstrating its reproducibility and time action curve that can be used to assess multiple features of the behavioural response by the administration of L-dopa.

2.2 Materials and methods

Twenty-six female Sprague-Dawley rats (Vancouver, Canada), weighing 250-275 g at the time of lesion surgery, were given unilateral stereotaxic injections of 6-OHDA (3 μg/μl in 0.02 % ascorbate-saline). The lesions were formed in two locations under isoflurane anaesthesia, the right ascending mesostriatal DA bundle (7.5ug) and the ventral tegmental area (9ug). The stereotaxic coordinates relative to bregma and the dural surface are: (1) A= −4.4, L= 1.2, V= 7.8, tooth bar= −2.4: (2) A= −4.0, L= 0.75, V= 8.0, tooth bar= −3.4. The toxin was infused at a rate of 1 μl/min and the cannula was left in place for one-minute post infusion.

Pulsatile levodopa treatment: Four weeks after the 6-OHDA lesion, 26 rats received L-dopa (Sigma, USA) in a dose ranging paradigm which involved consecutive day doses of 8, 16, 32 and 64 mg/kg mixed with 15mg/kg of benserazide (Sigma, USA) dissolved in saline and injected I.P. This was conducted for 5 weeks and ensured that dyskenisia levels would be stable, as a plateau in AIM response is evident after 3 weeks of treatment. Following this the rats were tested on 3 consecutive days at 8mg/kg using the AIM scale to assess the reproducibility of the scale.

Measurements of L-dopa-induced dyskinesia: L-dopa-induced motor fluctuations were assessed using the AIM scale (Lee 2000, Cenci 1998, Lundblad 2002). This includes four subtypes of movements and stereotypy that are based on topographic distribution: limb dyskinesia, axial dystonia, locomotor activity and masticatory dyskinesia (Table 1). The scoring is based on a scale
from 0 to 4 for each subtype of behaviour with 0 illustrating no dyskinesia and 4 being severe (Lee 2000). These categories were quantified during the reproducibility test period immediately after injection of L-dopa and every 20 minutes thereafter for a total of 140 minutes. Challenging the rat with novel sensory input, such as touching the rat or tilting the cage, tested inhibition of L-dopa-induced AIM. The AIM score is a sum of all measurements from sequential assessments after injection of L-dopa. Two researchers (Bowden G and Kenny J) that were kept blinded to the rat identity scored the AIM by consensus.

**Statistical analysis:** One-Way ANOVA was performed to calculate the standard deviation between and within rats. Confidence intervals and the reliability were then determined so that an accurate indication of the reproducibility of the AIM rat scoring system could be determined. Reliability is the ability of the AIM scoring system to perform its intended application of producing consistent and dependable behavioural information. The reliability coefficients can be used to evaluate sample sizes for future experiments. Magnitude of L-dopa response (Emax) was defined as a mean of three highest consecutive AIM scores in the time response curve.

**Autoradiographic study**

**Tissue preparation:** The animals were sacrificed three days after the last dose of L-dopa. The brains were removed, frozen immediately in cold isopentane (Sigma, USA), and stored at -80°C. Serial sections (16 μm thick) through the basal ganglia were cut coronally on a cryostat and mounted onto superfrost® microscope glass slides (Fisher, USA). The slide-mounted sections were stored at -80 °C.

**Autoradiography:** To label pre-synaptic markers in the dopaminergic nerve terminals in the striatum, one series of sections was incubated with tritiated WIN 35,428 [N-methyl-3H] that binds to the plasma membrane dopamine transporter. A second series was incubated with [3H] DTBZ, which binds the vesicular monoamine transporter. The slide-mounted sections were first washed for 5 min at 4 °C in 50 mM Tris-HCl buffer, pH 7.9, containing 120 mM NaCl and 5mM KCl, and subsequently incubated for 40 min in 50mM Tris-HCl, pH 7.9, 300mM NaCl,
5mM KCl and 10mM [3H] Win 35,428. Control sections were incubated with [3H]WIN 35,428 in the presence of 10 μM nomifensine to determine the non-specific binding. At the end of the incubation, the slide-mounted sections were washed 2 x 1 min in ice-cold 50 mM Tris-HCl, pH 7.9, 300 mM NaCl, and 5mM KCl buffer. Then slides were exposed to tritium-sensitive film (3H Hyperfilm, Amersham) at 4 °C for 4 weeks. The films were developed in Kodak D19, fixed and dried.

Quantitative image analysis: Autoradiographic images were digitized and analysed by the image analysis program, Image (Wayne Rasband, NIMH). The optical density was converted to the intensity of radioactivity using 3H standards (Amersham) for 3H-WIN 35,428 binding. Striatal expression of 3H-WIN 35,428 binding was analysed in 4 sections per brain in the neostriatum, rostrocaudally at the levels of 11 to 18 according to the atlas of Paxinos and Watson (Paxinos 1997).
Table 1. Rating scale for abnormal involuntary movements in the rat, 4 categories of stereotopy and locomotion. These categories are all scored on a 0 to 4 scale. (Lee 2000)

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Limb dyskinesia</strong></td>
<td>repetitive, rhythmic jerky movements or dystonic posturing of the forelimb on the side contralateral to the dopamine-denervated striatum</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Increased appearance of isolated jerky movements of the contralateral forelimb</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Intermittent and frequent repetitive movements of the contralateral forelimb</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Continuous repetitive or dystonic movements; interrupted by sensory distraction</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Compulsive repetitive or dystonic movements; not interrupted by sensory distraction</td>
<td></td>
</tr>
<tr>
<td><strong>Axial dystonia</strong></td>
<td>lateral flexion and axial rotation of the neck and trunk towards the side contralateral to the dopamine-denervated striatum</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Consistent contralateral rotation of the trunk in a bipedal sitting position</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Intermittent, contralateral flexion of the neck superimposed on consistent rotation of the trunk in a bipedal sitting position</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Continuous contralateral flexion and rotation of the neck and trunk; postural balance maintained</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Compulsive contractions of the neck and trunk; postural balance disturbed</td>
<td></td>
</tr>
<tr>
<td><strong>Masticatory dyskinesia</strong></td>
<td>repetitive chewing movements of the jaw ± tongue protrusion</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Increased chewing movements; occasional</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Increased chewing movements; frequent</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Continuous chewing movements with small amplitude</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Compulsive and repetitive biting movements with large amplitude; frequently associated with tongue protrusion</td>
<td></td>
</tr>
<tr>
<td><strong>Contraversive rotation</strong></td>
<td>turning or rotating movements contralateral to the dopamine-denervated striatum</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Contraversive turning with consistent directional bias; locomotor activity may be increased</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Intermittent, contraversive circular rotation</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Continuous contraversive rotation; interrupted by sensory distraction</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Compulsive contraversive rotation; not interrupted by sensory distraction</td>
<td></td>
</tr>
</tbody>
</table>
2.3 Results

The three consecutive days of testing provided several reproducible results. AIM scores were evident only in rats that sustained greater than 80% denervation of the autoradiographic biochemical markers (VMAT2 and DAT). The AIM scoring data was collected over 140 minutes. Abnormal movements have been seen to return to baseline at approximately this time (Lundblad 2002), which corresponds with the pharmacokinetics of the L-dopa in the rat. The 26 rats were observed for the 4 different key features of abnormal movements. The values ranged from a total AIM score of 0 to 94 where the maximum score possible is 128 (16 point maximum scale scored at 8 time points). The final results showed 12 highly (aim >50), 6 moderate (aim <50 >10), and 8 low (aim <10) responding animals.

The overall values for the AIM results had a mean of 33.5. The correlation between the three days of testing reveals a reliability coefficient of 97.8% (SDW-14%, SDB-94%)(Figure 1. and Table 2.). The magnitude of the AIM score (Emax) is obtained by the result of a single time interval. This AIM score component has a maximum value of 16 (4 from each category), and had an average value of 6.551. The Emax across testing days had a reliability coefficient of 98.7% (SDW of 10%, SDB of 89%).

The reproducibility data were separated into the 4 components of stereotypy and rotational measures to assess if any dynamic changes were evident in a particular component of the rating scale. The locomotor (contralateral turning) results were 96.1% (SDW-16%, SDB-178%) reproducible across the test sessions. The Axial (body twisting) was 96.4% (SDW-18%, SDB-95%). The limb and masticulatory movement correlations across tests were 92.5% (SDW-31%, SDB-110%), and 89.3% (SDW-53%, SDB-153%) respectively. In many situations, video rather than online analysis must be conducted. Unfortunately the axial turning often causes the subtle masticulatory movements to be shielded from the camera. Therefore, the result of removing the mouth movements from
the overall AIM score total still provided a reliability of 97.5% (SDW-15%, SDB-90%) across consecutive testing days.

The time action curve of the rats after L-dopa administration is shown in Figure 2. The individual times can be analyzed for their reproducibility. The time grouped test results revealed that the reliability values were 89.9% at 20min, 93.4% at 40min, 93.6% at 60 min, 96.2% at 80min, 95.8% at 100min, 79.6% at 120min, and 62.9% at 140min as seen in Table 2. The deviation was very low during the peak response but had some fluctuation in the last 2 time intervals.
Table 2. One-way variance analysis results for the different measures calculated by the AIM rating scale over 3 consecutive test days. (N=26).

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SDW (% mean)</th>
<th>SDB (% mean)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Totals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIM Total</td>
<td>33.538</td>
<td>31.63 (94%)</td>
<td>4.67 (14%)</td>
<td>97.90%</td>
</tr>
<tr>
<td>Emax</td>
<td>6.551</td>
<td>5.85 (89%)</td>
<td>0.66 (10%)</td>
<td>98.70%</td>
</tr>
<tr>
<td>Components</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locomotion</td>
<td>12.208</td>
<td>9.55 (78%)</td>
<td>1.92 (16%)</td>
<td>96.10%</td>
</tr>
<tr>
<td>Axial</td>
<td>10.359</td>
<td>9.84 (95%)</td>
<td>1.91 (18%)</td>
<td>96.40%</td>
</tr>
<tr>
<td>Mouth</td>
<td>3.551</td>
<td>5.43 (153%)</td>
<td>1.88 (53%)</td>
<td>89.30%</td>
</tr>
<tr>
<td>Limb</td>
<td>7.545</td>
<td>8.28 (110%)</td>
<td>2.35 (31%)</td>
<td>92.50%</td>
</tr>
<tr>
<td>Time</td>
<td>0</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>100%</td>
</tr>
<tr>
<td>20</td>
<td>4.423</td>
<td>4.43 (100%)</td>
<td>1.49 (34%)</td>
<td>89.90%</td>
</tr>
<tr>
<td>40</td>
<td>5.628</td>
<td>5.13 (91%)</td>
<td>1.36 (24%)</td>
<td>93.40%</td>
</tr>
<tr>
<td>60</td>
<td>5.821</td>
<td>5.3 (91%)</td>
<td>1.38 (24%)</td>
<td>93.60%</td>
</tr>
<tr>
<td>80</td>
<td>5.821</td>
<td>5.53 (95%)</td>
<td>1.09 (19%)</td>
<td>96.20%</td>
</tr>
<tr>
<td>100</td>
<td>5.372</td>
<td>5.22 (97%)</td>
<td>1.1 (20%)</td>
<td>95.80%</td>
</tr>
<tr>
<td>120</td>
<td>4</td>
<td>3.7 (92.5%)</td>
<td>1.87 (47%)</td>
<td>79.60%</td>
</tr>
<tr>
<td>140</td>
<td>2.051</td>
<td>2 (97.5%)</td>
<td>1.54 (75%)</td>
<td>62.90%</td>
</tr>
</tbody>
</table>

(SDB-standard deviation between subjects, SDW-standard deviation within subjects, R-reliability)
Figure 1. Comparison of AIM scores assessed over three consecutive days. x-axis: total AIM scores at test 1, y-axis: total AIM scores at test 2 and 3. Regression line: $y = 0.951x + 0.556$ for the test 1 and 2; $y = 0.926x - 0.767$ for the test 1 and 3.
Figure 2. Comparison of AIM scores assessed repetitively every 20 minutes for 140 minutes after injection of L-dopa. Each time point has a maximum value of 16 on the AIM rating scale.
2.4 Discussion

Behavioural scales are essential for measuring the efficacy of treatment, and increasing the understanding of the pathophysiology of dyskinesia. The repeated L-dopa treatment prior to consecutive reproducibility testing gradually induced locomotor rotation and stereotypy in the unilaterally lesioned rats. When the reproducibility was conducted the rats had been dyskinetic for at least 3 weeks, and their behavioural response had plateaued to individual levels. Rats without complete lesions did not develop abnormal movements.

The results of this study indicate that the AIM totals and Emax results were reliable across testing days. The 26 animals studied had individually specific levels of each of the four behavioural categories. Although the rats had very different behavioural characteristics, the motor pattern of each topographic subtype was highly reproducible across all rats, as well as serial observations of the same rat (Lee 2000). This lends credence to the effectiveness of the scoring model.

The masticatory movements observed in this study are easy to score accurately by real-time analysis. However, when video analysis is conducted the rats often turn or obscure the scoring of this movement. Therefore we recalculated the efficacy of this method using only the rotation, axial and limb components, and the correlation remained significant with a mere change across tests from \( r=97.9\% \) to \( r=97.5\% \). By limiting the analysis to specific parameters the richness of the behavioural manifestations can be inevitably reduced. Thus, there appears to be very little difference without the mouth movements in regard to reproducibility.

It is evident from the results that the individual time calculations display highly correlated results over the first 100 min of testing. The reliability of this region of the time action curve makes it useful for peak dose response analysis. However there appeared to be some fluctuation in the descending limb of the behaviours. Possible explanations for this are that the rats may have had a slightly shorter response to the drug with each dose (Fahn 2000, Obeso 2000).
This is because multifactorial changes in the pharmacodynamic curve may have slight variations across treatments, even at what appears to be a stable level. Also, the rapid decline of the abnormal movements may change over a few minutes, which could be enough to alter the values, as our observation for each rat is only one minute per time interval.

The information that can be obtained from the time-action curve generated by the AIM method is quite substantial because L-dopa administration results in a similar curve for peak dose dyskinesia (Lee 2000). This modeling system is able to offer several consistent areas for analysis including Emax, duration, latency, rising time, descending time, area under the curve, and time to Emax. The curves can be analyzed based on amplitude, duration and topographical distribution (Winkler 2002). This allows for the identification and explanation of individual characteristics within the dyskinetic response curve.

This method requires a substantial amount of time to observe the rats during each test. However, the movements can be easily monitored through passive observation of the animals. It is necessary to train the tester to classify consistently the various levels of dyskinesia present. A positive characteristic of the AIM scale is that unlike some methods where testing can not be conducted at high doses as a result of the severe dyskinetic activity affecting the task (Lindner 1996), this method is not affected by this threshold and the behavioural ceiling effect is not created by the scoring system.

In behavioural analysis, variation is inevitable even between days of testing the same animal. However, this testing method reduces this problem and in so doing create more accurate and informative data from fewer animals. The autoradiography results showed that 5 of the low responding rats actually had a partial lesion. This information demonstrates the construct validity of the model in its ability to separate different levels of dyskinesia (Petzinger 2001) and between different sub groups related to the degree of lesion.

The results of this study indicate that the AIM scoring system is reproducible across test days, within individual testing categories and time groupings. The reliability and implications for modelling of the pharmacodynamic
response to L-dopa treatment help make this model a useful tool in the attempt to understand the processes involved in Parkinsonian dyskinesia.

The information about the reliability of the scoring method allowed us to analyse the data of the following studies with more confidence. In the next study we explore the correlations between parameters describing the AIM response to L-dopa and the pre- and post-synaptic markers.
Chapter III
Evidence for a pathogenic role of striatal dynorphin in levodopa-induced response fluctuation: Studies in a rat model of Parkinson's disease

3.1 Introduction
Although treatment-related motor complications have been recognized since the early L-dopa era (Barbeau 1971, Claveria 1973), evidence from pharmacological studies suggests that the pathogenic mechanism may not be the same across all spectrums of motor fluctuations. "Wearing-off" is attributed to a gradual loss of pre-synaptic DA terminals in the striatum (Chase 1989). In contrast, the pathogenesis of "on-off" fluctuations appears to reflect secondary, regulatory changes at the postsynaptic level, resulting from chronic L-dopa therapy, as in the case of L-dopa-induced dyskinesia (Chase 1991). Furthermore, the threshold and time course for dyskinesia and antiparkinsonian effects of L-dopa are similar in patients with motor fluctuations (Mouradian 1988, Metman 1997). These findings suggest that pharmacodynamic changes in dyskinesia response may be mediated by similar mechanisms, at least in part, underlying "on-off" fluctuations.

Despite a substantial amount of clinical and pharmacological data on the motor fluctuations in PD patients, neural mechanisms of motor fluctuations remain to be elucidated. Currently, experimental studies on motor fluctuations in PD use L-dopa-induced rotational behaviour in 6-OHDA-lesioned rats as a behavioural correlate (Engber 1994, Papa 1994, Marin 1996). We recently reported a rat model of L-dopa-induced dyskinesia (Lee, 2000). Using this model, we had explored neural mechanisms of L-dopa-induced dyskinesia (Cenci 1998). In this study, we investigated biochemical correlates of pharmacodynamic changes; in particular, biochemical correlates of variables describing the time course of dyskinesia response. Here we report functional evidence that regulatory changes in dynorphin in the striatonigral neurons play a dual role in
the pharmacodynamic changes in dyskinesia response, which may account for the overlap between dyskinesia and motor fluctuations.

3.2 Methods and materials

Twenty-two female Sprague-Dawley rats (Vancouver, Canada), weighing 200-250 g at the time of lesion surgery, were given unilateral stereotaxic injections of 6-OHDA in the right ascending mesostriatal DA bundle and the ventral tegmental area. Two injections, 7.5 and 9 μg of free-base 6-OHDA (3 μg/μl in 0.02 % ascorbate-saline), were injected over 3-4 min under isoflurane anaesthesia at the following coordinates (in mm), relative to bregma and the dura: (1) A= −4.4, L= 1.2, V= 7.8, tooth bar= −2.4: (2) A= −4.0, L= 0.75, V= 8.0, tooth bar= +3.4. The toxin was infused at a rate of 1 μl/min, and the cannula was left in place another 4 min before being withdrawn.

L-dopa treatment and behavioural measurements. Six weeks after the 6-OHDA lesions, 15 rats were treated with L-dopa (Sigma, USA) at 15 mg/kg i.p. (mixed with 15mg/kg of benserazide) once daily for 28 days. Seven rats assigned to the lesion-control group received saline once daily i.p. for 28 days. L-dopa-induced AIM that includes stereotypy and contraversive rotation, were quantified using the rat AIM rating scale, as reported previously (Ahlskog 2001). Stereotypy was classified into three subtypes based on the topographic distribution: limb dyskinesia, axial dystonia, and masticatory dyskinesia. L-dopa-induced AIM response was assessed twice a week during the treatment period using the rat AIM rating scale (Lee 2000). The time action curve of L-dopa was obtained by assessing the rats before injection of L-dopa and every 20 minutes for 160 min after injection of L-dopa.

Autoradiographic studies and in situ hybridization histochemistry

Tissue preparation: The animals were sacrificed three days after the last dose of L-dopa. The brains were removed, frozen immediately in cold isopentane (Sigma, USA), and stored at −80°C. Serial sections (16 μm thick) through the basal ganglia were cut coronally on a cryostat and mounted onto superfrost®
microscope glass slides (Fisher, USA). The slide-mounted sections were stored at \(-80\, ^\circ\text{C}\).

**Autoradiography:** To label pre-synaptic markers in the dopaminergic nerve terminals, one series of sections was incubated with tritiated ([H\textsuperscript{3}]MTBZ), which binds to the vesicular monoamine transporter (Vander Borght 1995). The slide-mounted sections were first washed for 10 min in 4\% paraformaldehyde. There was a pre-wash for 5 min in potassium buffer, pH 8, containing 137 mM KCl, 3mM NaCl, Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4} 1.5 mM, K\textsubscript{2}HPO\textsubscript{4} 8 mM and EDTA 1 mM. In the same solution the slides were subsequently incubated for 180 min at pH 8 with 10 nM MTBZ. Control sections were incubated with 10 \mu M unlabelled TBZ to determine the non-specific binding. At the end of the incubation, the slide-mounted sections were washed 3 x 3 min in the potassium buffer. Then slides were dried under a cold air stream, and exposed to tritium-sensitive film (\textsuperscript{3}H-Hyperfilm, Amersham) at 4\, ^\circ\text{C} for 4 weeks. The films were developed in Kodak D19, fixed and dried.

**In situ hybridization histochemistry (ISHH):** The probes were oligodeoxyribonucleotides (NAPS, Vancouver) complementary to nucleotides 322-360 of the cloned preproenkephalin cDNA (Howells 1984), nucleotides 934-982 of the cloned prodynorphin gene (Young 1986), and nucleotides coding for amino acids 389-405 of feline glutamic acid decarboxylase (GAD) (Kobayashi 1987). Oligonucleotides (0.5mM) were labelled at the 3' end with 25\, \mu L [a-\textsuperscript{35}S]dATP (9.26M bq/25\, \mu L; Amersham) using 80-100 U of terminal deoxynucleotidyltransferase (TdT; Amersham) for 60-90 min at 37\, ^\circ\text{C}. The labelled probes were purified on Nick\textsuperscript{TM} column nucleic acid purification column (Amersham) to > 10\textsuperscript{5} cpm/\mu L of probe.

All solutions and buffers were prepared with distilled water treated with diethyl-pyrocarbonate (DEPC) of Sigma to inhibit RNAse activity and then autoclaved. The slide-mounted sections were air-dried and fixed in 4 \% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min, then rinsed three times in 1 x PBS (phosphate buffered saline), 5 min each at RT. Equilibrate sections with 10mM DTT in 1 x PBS for 10min at hybridization temperature (42\, ^\circ\text{C}) and followed by incubating sections in 350 mL of blocking solution (10mM
DTT, 18mM iodoacetamide, 0.01mM N-ethylmaleimide and 1 x PBS) at 42°C. Then slides were washed in 2 x 5 min of 1 x PBS and followed by the immersion in 400mL of 0.1M TEA (triethanolamine) buffered at pH 8.0 for 5 min at room temperature. Subsequently, 0.25% acetic anhydride in 0.1M TEA buffer and incubated slides for 10 min at room temperature. The sections were washed in 2 x SSC (300mM NaCl, 30mM sodium citrate and DEPC H₂O) for 2min at room temperature and dehydrated in a series of ascending concentrations of ethanol and air-dried. The slides were then incubated with the hybridization mixture, which comprised 50% formamide (deionized), 4x SSC (1x SSC = 0.15 M NaCl and 0.015 M sodium citrate), 1x Denhardt's solution (0.02 % Ficoll, 0.02 % polyvinyl pyrolidone, 10 mg/ml of RNAse free bovine serum albumine), 1 % sarcosyl, 10 % dextran sulphate, 500 μg/ml sheared and denatured salmon sperm DNA, 200 mM dithiothreitol, and 10⁷ cpm/ml of ³⁵S-labeled oligonucleotide probe. Approximately 50 μl of hybridization cocktail were added to each section. Slides were cover slipped with parafilm and incubated overnight (16-18 hours) at 42 °C in a humid chamber. After hybridization, the parafilm cover slips were floated off in 1x SSC at 55 °C, and the sections were given three washes (15 min each) in 1 x SSC at 55 °C, plus a final wash beginning at 55 °C and cooling to room temperature in 0.1 x SSC. The slides were then rinsed twice in distilled water, dehydrated in a series of ascending concentrations of ethanol and air-dried. They were then exposed to autoradiographic film (Bmax, Amersham, USA) at -20 °C for 10-14 days. The films were developed in Kodak D-19 for 4 minutes and fixed.

**Image analysis:** Autoradiographic images were digitized and analysed using *Image* (Wayne Rasband, NIMH). The optical density was converted to the intensity of radioactivity using ¹⁴C standards (Amersham) for ISHH or [³H] standards (Amersham) for [³H]MTBZ binding exposed on the same film (Miller 1986). Striatal expression of [³H]MTBZ binding or neuropeptide mRNA was analyzed in 4-5 sections of the neostriatum, rostrocaudally at the levels of 10 to 18 according to the atlas of Paxinos and Watson (Paxinos 1997).
Data analysis and statistics: Magnitude of L-dopa response (Emax) was defined as a mean of three highest consecutive AIM scores in the time response curve. Area under curve (AUC) was obtained by the trapezoid rule. The onset (offset) of L-dopa response was defined as the time when the AIM score = 1 in the ascending (descending) phase of L-dopa response. The peak response time was the latency from the administration of L-dopa to the time at Emax. Rising time was the interval from the onset of L-dopa response to the peak response whereas decay time was the interval from the peak response to the offset of L-dopa response.

Repeated measures analysis of covariance was used to examine the association between the magnitude (or latency to the onset) of AIM response and treatment duration, response groups and their interactions.

To analyze the relationship between the Emax and variables describing the time course of AIM response, stepwise regression analysis was employed, and partial correlation coefficients were calculated. An analogous method was used to analyze the associations between pharmacodynamic parameters of the AIM response and striatal expression of mRNAs encoding prodynorphin, GAD$_{67}$ or preproenkephalin.

Statistical significance was set at $p \leq 0.05$. 
3.3 Results

Daily treatment with a therapeutic dose of L-dopa gradually induced contralateral AIM in rats with unilateral 6-OHDA lesions. Seven rats developed AIM in the first week and five rats in the second week. Three rats did not develop AIM by the end of the treatment period. The magnitude of AIM response increased gradually during the first two weeks of treatment with a high individual variance and approached a plateau in the third and fourth week in both high and low response groups. Table 4 summarizes pharmacodynamic parameters of the AIM response to L-dopa throughout the treatment period in both high response and low response groups. Repeated measures ANOVA for the magnitude of AIM response showed significant treatment effects over time (p<0.001) and group difference (p=0.003) with no significant interaction between the two factors (p=0.98). Conversely, latency to the onset of the AIM response was shorter in the high response group than in the low response group, and decreased gradually over time in both response groups. Repeated measures ANOVA for latency to the onset of the AIM response showed significant treatment effects over time (p=0.001) and group differences (p=0.001) with no significant interactions between the two factors (p=0.083). Other variables describing the time course of AIM response (e.g. peak response time, duration, rising time and decay) showed no significant differences between the two groups and no significant differences over time. However, the relationship between the magnitude and variables describing the time course of the AIM response changed with longer duration of treatment. In the second week, stepwise regression analysis of the magnitude on the variables describing the time course of the AIM response showed highly significant negative correlation with latency to the onset (p<0.0001); and after adjustment for latency to the onset, the rising time still showed significant partial positive correlation (p= 0.0011). No further significant correlation with the decay time was found. Similar analysis of the data in the third week showed significant negative correlation with latency to the onset (p<0.0001); after adjustment for the latency to the onset, the rising time showed significant partial positive correlation (p<0.001); and after adjustment for the rising time, the decay time still showed
significant partial negative correlation (p<0.001). Analysis of the data in the fourth week showed analogous findings.

Figure 3 illustrates autoradiographic images of $[^3]$HMTBZ binding and *in situ* histochemistry hybridization for mRNA encoding prodynorphin, GAD$_{67}$ or preproenkephalin in the striatal sections. Results of quantitative analysis for these ligand binding and *in situ* hybridization histochemistry studies were compared between the low response and high response groups (Table 5). There was no significant difference in the striatal $[^3]$HMTBZ binding between the two groups, indicating that the severity of DA terminal loss was not a factor determining the magnitude of L-dopa response (Figure 4). In contrast, striatal prodynorphin and GAD$_{67}$ mRNA levels were significantly higher in the high response group than the low response group. No significant differences were observed in striatal preproenkephalin mRNA. Consistent with these findings, the magnitude of the AIM response showed significant positive correlation with both striatal prodynorphin and GAD$_{67}$ mRNA levels ($r=0.934$, p=0.0006) (Figure 5).

Stepwise regression of $[^3]$HMTBZ binding on variables describing the time course of the AIM response showed significant negative correlation with the rising time ($r=-0.646$, p=0.023); after adjusting for the rising time, latency to the onset still showed significant positive correlation. No further significant correlation with the decay time was found. These findings suggest that those with more severe DA terminal loss showed earlier onset and longer rising time of the AIM response. Stepwise regression of prodynorphin mRNA on variables describing the time course of AIM response showed significant negative correlation with latency to the onset (p=0.002); after adjustment for latency to the onset, the decay time still showed a marginally significant partial negative correlation (p=0.05). No further significant correlation was found. These findings indicate that those with higher expression of prodynorphin mRNA showed greater magnitude, earlier onset and earlier decay of the AIM response. Stepwise regression of striatal GAD$_{67}$ mRNA on variables describing the time course of the AIM response showed significant negative correlation with latency to the onset (p<0.001); after adjustment for latency to the onset, no further significant partial
correlation was found. These findings suggest that those with higher upregulation of striatal GAD$_{67}$ mRNA showed greater magnitude and earlier onset of the AIM response. Finally, stepwise regression of striatal preproenkephalin mRNA on variables describing the time course of AIM response did not show significant correlations with any variables.
Table 3. Pharmacodynamic profile of the AIM response to L-dopa.

<table>
<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Magnitude</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High response</td>
<td>5.70 ± 4.57</td>
<td>10.47 ± 2.37</td>
<td>11.65 ± 2.17</td>
<td>11.43 ± 2.56</td>
</tr>
<tr>
<td>Low response</td>
<td>1.02 ± 0.95</td>
<td>3.62 ± 1.55</td>
<td>4.45 ± 1.64</td>
<td>4.71 ± 1.88</td>
</tr>
<tr>
<td><strong>Latency to onset</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High response</td>
<td>3.75 ± 0.98</td>
<td>2.79 ± 1.11</td>
<td>2.42 ± 0.46</td>
<td>2.34 ± 0.83</td>
</tr>
<tr>
<td>Low response</td>
<td>19.2 ± 4.48</td>
<td>13.5 ± 10.8</td>
<td>9.03 ± 7.84</td>
<td>8.11 ± 5.51</td>
</tr>
<tr>
<td><strong>Peak response time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High response</td>
<td>70.0 ± 1.63</td>
<td>63.4 ± 7.98</td>
<td>63.9 ± 4.08</td>
<td>60.5 ± 8.90</td>
</tr>
<tr>
<td>Low response</td>
<td>74.3 ± 7.61</td>
<td>74.3 ± 17.3</td>
<td>65.6 ± 11.3</td>
<td>64.4 ± 5.27</td>
</tr>
<tr>
<td><strong>Duration (min)</strong></td>
<td></td>
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<tr>
<td>High response</td>
<td>163.7 ± 17.7</td>
<td>156.7 ± 15.0</td>
<td>155.6 ± 7.9</td>
<td>155.6 ± 10.0</td>
</tr>
<tr>
<td>Low response</td>
<td>115.4 ± 20.5</td>
<td>140.0 ± 53.3</td>
<td>155.8 ± 55.8</td>
<td>139.7 ± 24.6</td>
</tr>
<tr>
<td><strong>Rising time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High response</td>
<td>66.2 ± 0.78</td>
<td>60.6 ± 7.43</td>
<td>61.5 ± 4.20</td>
<td>58.1 ± 8.46</td>
</tr>
<tr>
<td>Low response</td>
<td>58.3 ± 3.54</td>
<td>60.8 ± 21.4</td>
<td>56.5 ± 14.6</td>
<td>56.3 ± 7.01</td>
</tr>
<tr>
<td><strong>Decay time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High response</td>
<td>97.5 ± 17.7</td>
<td>96.1 ± 13.8</td>
<td>94.1 ± 7.19</td>
<td>97.5 ± 14.5</td>
</tr>
<tr>
<td>Low response</td>
<td>57.1 ± 17.8</td>
<td>79.3 ± 32.2</td>
<td>99.2 ± 49.1</td>
<td>83.4 ± 19.2</td>
</tr>
</tbody>
</table>

1 All measures are mean ± SD (min). 2 High and Low response groups are divided based on the magnitude of AIM response in the last week of treatment: High response > 8; Low response ≤ 8; 3 The onset and offset of the AIM response are defined as the time when the AIM score = 1 in the ascending and descending phases of the AIM response respectively; 4 Peak response time is when the AIM response is at maximum; 5 (Peak response time – latency to the onset); 6 (Latency to the offset – peak response time)
Figure 3. Autoradiographic images of [3H]MTBZ binding (A1, A2) and in situ hybridization histochemistry for GAD67 mRNA (B1, B2) and prodynorphin (PDyn) mRNA (C1, C2) in striatal sections. **Left column** (A1, B1, C1): representative striatal sections of rats with L-dopa-induced abnormal involuntary movements (AIM); **Right column** (A2, B2, C2): representative striatal sections of rats with no AIM. **Right striatum**: ipsilateral to 6-OHDA lesions; **Left striatum**: contralateral to 6-OHDA lesions.
Table 4. Results of $[^3]H$MTBZ binding and *in situ* histochemistry

*hybridization* (SEM)

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Saline group</th>
<th>Low response</th>
<th>High response</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3]H$MTBZ</td>
<td>.991 ± .098</td>
<td>.038 ± .037</td>
<td>.028 ± .030</td>
<td>.014 ± .014</td>
</tr>
<tr>
<td>PDyn mRNA</td>
<td>1.015 ± .006</td>
<td>.618 ± .137</td>
<td>1.068 ± .136</td>
<td>1.438 ± .228</td>
</tr>
<tr>
<td>GAD$_{67}$ mRNA</td>
<td>1.063 ± .079</td>
<td>1.270 ± .055</td>
<td>1.395 ± .139</td>
<td>1.625 ± .091</td>
</tr>
<tr>
<td>PPE mRNA</td>
<td>1.029 ± .023</td>
<td>1.533 ± .156</td>
<td>1.861 ± .286</td>
<td>1.931 ± .081</td>
</tr>
</tbody>
</table>
Figure 4. Scatter plots showing the relationship between the severity of dopamine terminal loss and mRNA expression of prodynorphin (PDyn) and GAD67 in the striatum. The level of mRNA expression is expressed as the ratio of unlesioned side. Open circle: normal; green diamond: saline group; yellow triangle: low response group; red triangle: high response group.
Figure 5. Scatter plots showing the relationship between mRNA expression and the magnitude of the AIM response to L-dopa in the last week of treatment. *Left*: prodynorphin (PDyn mRNA), $r=0.834$, $p=0.0007$, *Right*: GAD67 mRNA in the striatum, $r=0.795$, $p=0.0020$. Multiple regression of the magnitude of AIM response on both PDyn mRNA and GAD67 mRNA showed tighter correlation ($r=0.934$, $p=0.0006$).
3.4 Discussion

One of the major findings in this study was the fact that L-dopa-induced upregulation in striatal prodynorphin mRNA levels was associated with not only the magnitude but also the time course of L-dopa response: in particular, shortening of the decay time.

L-dopa-induced AIM as a pharmacodynamic marker of L-dopa

As reported in our earlier study (Lee 2000), pulsatile treatment with a therapeutic dose of L-dopa induced AIM in rats with unilateral 6-OHDA lesions. These AIMs, which consist of contraversive rotational response and stereotypy on the side contralateral to the DA denervated striatum, appeared transiently only after administration of L-dopa in those rats with severe DA terminal loss and postsynaptic regulatory changes. Thus, we infer that L-dopa-induced AIM reflects the pharmacological action of exogenous L-dopa in the DA denervated striatum.

In agreement with our earlier study (Lee 2000), we showed that the magnitude of AIM response in the low response group did not change significantly in the third and fourth weeks of treatment at AIM scores less than the maximum attainable scores. A similar “slowing-down” pattern was observed in the L-dopa-induced rotational behaviour in a rat model of PD (Papa 1994). Pulsatile L-dopa treatment changed not only the magnitude but also the time course of AIM response to L-dopa. Although the mean duration of all rats did not show significant changes over time, our consistent findings of negative correlation between the magnitude and decay time in the third and fourth weeks indicate that L-dopa-induced changes in the magnitude and decay time of the AIM response were reciprocal. Similar changes were reported between the magnitude and duration of L-dopa-induced rotational behaviour in a rat model of PD (Engber 1994, Papa 1994, Marin 1996).

Biochemical correlates of L-dopa-induced AIM response

As reported previously (Chesselet 1993, Cenci 1998), expression of GAD_{67} mRNA levels was upregulated in the DA denervated striatum of drug-naive rats in the saline group, and upregulated even further in the DA denervated striatum of treated rats in the L-dopa group. We also observed a significant
correlation between the magnitude of L-dopa response and striatal prodynorphin or GAD$_{67}$ mRNA levels, consistent with our earlier observations (Cenci 1998, Lee 2000). In the present study, we obtained evidence that upregulation of prodynorphin and GAD$_{67}$ mRNA levels in the striatum are also associated with variables of AIM response describing the time course, particularly, latency to the onset, and the decay time. Furthermore, our findings that the rising time correlated with [$^{3}$H]MTBZ binding but the decay time correlated negatively with prodynorphin mRNA levels indicate that the rising time and decay time may be determined differently. Also, the observations that prodynorphin mRNA levels correlated positively with the magnitude, but negatively with the decay time indicates that the increased dynorphin activity in striatonigral neurons may cause opposite effects on the magnitude and decay time of the L-dopa response. Specifically, these findings suggest that the reciprocal changes in the magnitude and decay time of L-dopa response may be accounted for by L-dopa-induced upregulation of prodynorphin mRNA.

**Pharmacological actions of dynorphin**

Dynorphin, the endogenous $k$-opioid receptor agonist, is co-localized with GABA largely in striatonigral neurons that contain D$_{1}$ DA receptors and project to the output regions of the basal ganglia (Chavkin 1982, Gerfen 1990). Stimulation of D$_{1}$ DA receptors releases dynorphin in the output regions of the basal ganglia from striatonigral nerve terminals and also in the striatum from dendrites or collateral axon terminals (You 1994, Maneuf 1995). Microinjection of dynorphin or U-69593, a $k$-opioid receptor agonist into the output regions of the basal ganglia decreases neuronal activity (Lavin 1986, Robertson 1987, Thompson 1990) and induces DA-independent, contraversive rotation in the rat (Herrera 1984, Matsumoto 1988). In contrast, intrastratal injection of dynorphin or k-opioid receptor agonist decrease the release of DA and glutamate, increase DA uptake by the direct action on DA transporter, and finally, inhibits motor activity (Di Chara 1988, Maneuf 1995, Gray 1999). These observations therefore are consistent with the view that the action of dynorphin is distinct between the striatum and the output regions of the basal ganglia (Marin 2003). This dual
action of dynorphin may account for treatment-induced reciprocal changes between the magnitude and decay time of AIM response because dynorphin released in the output regions of the basal ganglia following injection of L-dopa increases the magnitude of AIM response. On the other hand, dynorphin released in the striatum increases DA uptake, and thus, shortens the decay time of AIM response, accounting for the negative correlation between the magnitude and the decay time.

Limitations and implications of the findings on the mechanisms of response fluctuations in PD

The findings in a rat model of PD might not be comparable with those in patients with PD because of potential interspecies differences. However, the anatomical organization of the basal ganglia and its specific neurotransmitters are highly conserved across different species: striatal projection neurons containing dynorphin project to the output regions of the basal ganglia in both rodents and primates (Reiner 1999). Our observation of changes in L-dopa-induced AIM response show similar features to response changes to L-dopa in patients with response fluctuations. First, we demonstrated that the changes in the magnitude and decay time in the rats were reciprocal, and similar changes between the magnitude and decay time are key features of pharmacodynamic changes in response fluctuations (Fabbrini 1987, Contin 1990, Nutt 1990, Colosimo 1996, Contin 1997). Second, we demonstrated that the rising time correlated with the severity of striatal DA terminal loss, whereas the decay time correlated with prodynorphin mRNA levels in the striatum. These findings are consistent with observations in patients with PD, showing a significant correlation of the DA terminal loss with rising time, but not with the decay time, of an L-dopa response, which was estimated by finger tapping (Kumar 2003). It was also reported previously that duration of PD is associated with peak response time (Sohn 1994).

These similar features in L-dopa response suggest that common mechanisms may be responsible for response changes to L-dopa observed in AIM response in a rat model of PD and in response fluctuations in patients with
PD. This view is further supported by the observations that (a) L-dopa-induced
dyskinesia frequently coexists with response fluctuations, especially, on-off
fluctuations (Nutt 1990, Obeso 1991) and, (b) the kinetic-dynamic relationship
becomes similar between antiparkinsonism and dyskinesiogenic actions of L-
dopa in patients with advanced PD (Mouradian 1988, Contin 1997, Metman
1997, Nutt 2001) Therefore, it is proposed that while the 'wearing-off'
phenomenon reflects pre-synaptic DA terminal loss, the "on-off" fluctuations in
advanced PD may reflect additional postsynaptic changes.

In summary, we showed evidence that upregulated activity of dynorphin in
the striatum may account for reciprocal changes in magnitude and duration of
AIM response, which are key features of response fluctuations to L-dopa. These
findings suggest that dynorphin may play a role in both L-dopa-induced response
fluctuations and dynorphin may play a pathogenic role in response fluctuations in
patients with advanced PD.
Chapter IV
Microdialysis as a tool for exploring the cerebral pharmacokinetics of L-dopa

4.1 Introduction

The use of high-pressure liquid chromatography (HPLC) techniques in the understanding of the metabolite and DA levels in the brain opens up a window on the pharmacokinetic interactions of L-dopa. Microdialysis studies have shown that extracellular DA levels in the striatum increase after systemic administration of L-dopa in a dose-dependent manner in both intact and DA-depleted striatum (Jonkers 2000, Abercrombie 1990, Sarre 1994), particularly when the DA terminal density in the striatum is less than 10% (Wachtel 1994). The baseline extracellular DA levels are in the range of 4-20 nM in the striatum (Zetterstrom 1983, Abercrombie 1989).

Nigrostriatal denervation decreases the DA concentration and also changes the metabolism of extracellular DA (Abercrombie 1990). The major metabolite in the rat brain is DOPAC while the human and primate brains have more HVA (Bloom 1996). The metabolites are an indicator of the degree of function seen in the DA turnover rates. Beyond emphasising the breakdown of DA it has been reported that at peak levels these hydrophobic metabolites may modulate the actions of DA on synaptic membranes, causing abnormal movements, at times, resembling the "on-off effects" (Charlton 2000). However other reports indicate that DA-denervated rats did not exhibit a supersensitive response to DOPAC or the other metabolites (Nakazoto 2002).

The purpose of the present microdialysis study was to better understand the mechanisms of response changes to L-dopa, particularly the dyskinesia response induced by therapeutic doses of L-dopa. The real time correlation between the extracellular DA levels, its metabolites, and dyskinesia response will provide insight into how the basal ganglia circuitry responds to striatal DA denervation and drug treatment. This could in turn lead to more rational pharmacological therapy for patients with Parkinson's disease.
4.2.1 Methods and materials (pilot)

Female Sprague-Dawley rats (Vancouver, Canada), weighing 250-275 g at the time of lesion surgery, were given unilateral stereotaxic injections of 6-OHDA (3 μg/μl in 0.02 % ascorbate-saline). The lesions were formed in two locations under isoflurane anesthesia, the right ascending (1) mesostriatal DA bundle (7.5ug) and the (2) ventral tegmental area (9ug). The stereotaxic coordinates relative to bregma and the dural surface were: (1) A= -4.4, L= 1.2, V= 7.8, tooth bar= -2.4: (2) A= -4.0, L= 0.75, V= 8.0, tooth bar= -3.4. The toxin was infused at a rate of 1 μl/min and the cannula was left in place for one-minute post infusion.

Pulsatile L-dopa treatment: Four weeks after the 6-OHDA lesion, 26 rats received L-DOPA (Sigma, USA) in a dose ranging paradigm which involved consecutive day doses of 8, 16, 32 and 64 mg/kg mixed with 15mg/kg of benserazide (Sigma, USA) dissolved in saline and injected I.P. This was conducted for 5 weeks and ensured that dyskenisia levels would be stable, as a plateau in AIM response is evident after 3 weeks of treatment. Subsequently, the rats were tested on 3 consecutive days at 8mg/kg using the AIM scale to assess the reproducibility of the scale. At this time, 8 rats (4 highly dyskinetic and 4 non-dyskinetic) were selected for microdialysis testing.

To prepare for microdialysis, surgery was conducted to implant nitric acid-passivated stainless steel guide cannula (19 gage, 15 mm) bilaterally 1 mm below dura, directly above the striatum (+1.0 mm AP, +/-3.1 mm ML from bregma and -3.0 to -7.0 DV from dura.) Dental acrylic and jeweler's screws were used to form a head cap that held the cannula in place. Following surgery, rats were housed individually in plastic cages. A maintenance dose of 8mg/kg was continued to avoid changes in the AIM response. Ten days post surgery microdialysis was conducted on the rats with simultaneous AIM testing.

Measurements of L-dopa-induced dyskinesia: L-dopa-induced motor fluctuations were assessed using the abnormal involuntary movements (AIM) scale (Lee 2000, Cenci 1998, Lundblad 2002). This includes three subtypes of
movements and stereotypy that are based on topographic distribution: limb
dyskinesia, axial dystonia, and locomotor activity (Table 1). The scoring is based
on a scale from 0 to 4 for each subtype of behaviour with 0 illustrates no
dyskinesia and 4 being severe (Lee 2000). These categories were quantified
during the test period, at injection of L-dopa and every 10 minutes thereafter for
160 minutes. The AIM score is a sum of all measurements from sequential
assessments after injection of L-dopa.

**Microdialysis and HPLC:** Microdialysis probes were concentric in design
with silica inlet/outlet lines. The active surface consisted of a semi-permeable
membrane 4 mm in length (340 um o.d., 65 000 MW cut-off, Filtral 12, Hospal).
Probes were flushed continuously at 1 ul/min with a modified Ringer's solution
(perfusate); 10 mM sodium phosphate, 1.2 mM CaCl$_2$, 3.0 mM KCl, 1.0 mM
MgCl$_2$, 147.0 mM NaCl, pH 7.4) using a 2.5 ml gas tight syringe (Hamilton, Reno,
NV, USA) and a syringe pump (model 22, Harvard Apparatus, South Natick, MA,
USA). Typical *in vitro* probe recoveries of DA conducted at room temperature
were 18±1% of a standard DA solution (Ahn 2002) DA in microdialysates were
separated from other chemical species by high-pressure liquid chromatography
(HPLC) and quantified by electrochemical detection (Antek system). The details
of the HPLC-ED methods have been described previously (Ahn 2002).

Microdialysis sessions were conducted from 08:00–13:00 h in a Plexiglas
chamber (42 cm x 38 cm x 38 cm) fitted with a two-channel liquid swivel (Instech
Laboratories Inc., Plymouth Meeting, PA, USA). Each session involved bilateral
dialysis of the neostriatum. Probes were implanted 14–16 h prior to the collection
of the first microdialysis sample. Implanted animals remained overnight in the
test chamber with their daily ration of food and access to water. Dialysis samples
were collected at 10 min intervals and immediately assayed for DA using HPLC-
ED. Baseline samples were collected until DA levels were stable (less than 5%
variation). The HPLC system used Antek systems.

**Data analyses:** For the purposes of statistical analyses and graphical
representation, neurochemical data were normalised to a baseline value
(calculated by averaging the concentration of DA in the three samples preceding
the final baseline sample). All data were analysed using repeated measures analysis of variance (ANOVA) tests, also stepwise regression was used to correlate relationships between the data. The statistics were conducted using Statview. Statistical significance was set at p ≤ 0.05. Magnitude of L-dopa response (Emax) was defined as a mean of three highest consecutive AIM scores in the time response curve. The peak response time was the latency from the administration of L-dopa to the time at Emax. Rising time was the interval from the onset of L-dopa response to the peak response whereas decay time was the interval from the peak response to the offset of L-dopa response.

Autoradiographic study

**Tissue preparation:** Three days after the last dose of L-dopa, the animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and killed by decapitation. The brains were removed, frozen immediately in cold iso-pentane (Sigma, USA), and stored at -80 °C. Serial sections (16 μm thick) through the basal ganglia were cut coronally on a cryostat and mounted onto superfrost® microscope glass slides (Fisher, USA). The slide-mounted sections were stored at -80 °C. The location of the probes implanted into the brain were calculated during slicing. The microdialysis probes descended into the striatum within the regions of 12-14 (2.5 lateral and -7 from bregma) based on Paxinos and Watson atlas (Paxinos 1997).

**Ligand binding autoradiography for pre-synaptic marker:** To label pre-synaptic markers in the dopaminergic nerve terminals in the striatum, one series of sections was incubated with DTBZ that binds to the vesicular monoamine transporter, a second section is used with tritiated WIN 35,428 [N-methyl-3H] that binds to the plasma membrane dopamine transporter. The slide-mounted sections were first washed for 10 min in 4% paraformaldehyde. There was a pre-wash for 5 min in potassium buffer, pH 8, containing 137 mM KCl, 3mM NaCl, Na₂H₂PO₄ 1.5 mM, K₂HPO₄ 8 mM and EDTA 1 mM. In the same solution the slides were subsequently incubated for 180 min at pH 8 with 10 nM MTBZ. Control sections were incubated with 10 μM unlabelled DTBZ to determine the
non-specific binding. At the end of the incubation, the slide-mounted sections were washed 3 x 3 min in the potassium buffer.

**In situ hybridization histochemistry (ISHH):** The probes were oligodeoxyribonucleotides (NAPS, Vancouver) complementary to nucleotides 322-360 of the cloned preproenkephalin cDNA (Howells 1984), and nucleotides 934-982 of the cloned prodynorphin gene (Young 1986). Oligonucleotides (0.5mM) were labelled at the 3' end with 25uL [a-\(^{35}\)S]dATP (9.26M bq/25uL; Amersham) using 80-100 U of terminal deoxynucleotidyltransferase (TdT; Amersham) for 60-90 min at 37°C. The labelled probes were purified on Nick™ column nucleic acid purification column (Amersham) to > 10\(^5\) cpm/\(\mu\)L of probe.

All solutions and buffers were prepared with distilled water treated with diethyl-pyrocarbonate (DEPC) of Sigma to inhibit RNAse activity and then autoclaved. The slide-mounted sections were air-dried and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min, then rinsed three times in 1 x PBS (phosphate buffered saline), 5 min each at RT. Equilibrate sections with 10mM DTT in 1 x PBS for 10min at hybridization temperature (42°C) and followed by incubating sections in 350 mL of blocking solution (10mM DTT, 18mM iodoacetamide, 0.01mM N-ethylmaleimide and 1 x PBS) at 42°C. Slides were washed in 2 x 5 min of 1 x PBS and followed by the immersion in 400mL of 0.1M TEA (triethanolamine) buffered at pH 8.0 for 5 min at room temperature. Subsequently, 0.25% acetic anhydride is add to 0.1M TEA buffer and slides are incubated for 10 min at room temperature. The sections were washed in 2 x SSC (300mM NaCl, 30mM sodium citrate and DEPC H\(2\)O) for 2min at room temperature and dehydrated in a series of ascending concentrations of ethanol and air-dried. The slides were then incubated with the hybridization mixture, which comprised 50% formamide (deionized), 4x SSC (1x SSC = 0.15 M NaCl and 0.015 M sodium citrate), 1x Denhardt's solution (0.02 % Ficoll, 0.02 % polyvinyl pyrrolidone, 10 mg/ml of RNAse free bovine serum albumine), 1 % sarcosyl, 10 % dextran sulphate, 500 \(\mu\)g/ml sheared and denatured salmon sperm DNA, 200 mM dithiothreitol, and 10\(^7\) cpm/ml of \(^{35}\)S-labeled oligonucleotide probe. Approximately 50 \(\mu\)l of hybridization cocktail were
added to each section. Slides were cover slipped with parafilm and incubated overnight (16-18 hours) at 42 °C in a humid chamber. After hybridization, the parafilm cover slips were floated off in 1x SSC at 55 °C, and the sections were given three washes (15 min each) in 1x SSC at 55 °C, plus a final wash beginning at 55 °C and cooling to room temperature in 0.1 x SSC. The slides were then rinsed twice in distilled water, dehydrated in a series of ascending concentrations of ethanol and air-dried. They sections are exposed to phosphor plates at room temperature (Fuji Films) for 2 days. A phospho-imager was used to generate the image.

Quantitative image analysis: Autoradiographic images were digitized and analysed using Optiquant. The optical density was converted to the intensity of radioactivity using $^{14}$C standards (Amersham) for ISHH or $[^3]H$ standards (Amersham) for $[^3]H$DTBZ and $[^3]H$WIN35,428 binding. Striatal expression of binding or neuropeptide mRNA was analyzed in 4-5 sections of the neostriatum, rostrocaudally at the levels of 10 to 18 according to the atlas of Paxinos and Watson (Paxinos 1997).

4.3.1 Results (pilot)

Treatment-induced changes in L-dopa response

With daily L-dopa treatment (8 mg/kg, i.p.), in conjunction with dose ranging treatments, the majority of rats with unilateral 6-OHDA lesions rapidly developed AIM on the side contralateral to the lesions. Among the group of 26 rats treated, 4 severely dyskinetic and 4 non-dyskinetic rats were selected for microdialysis investigation. The mean total AIM score was 73 (SE=4.6) in the severe dyskinesia group with a maximum magnitude (Emax) of 13.75 (SE=0.63). The severe dyskinesia group had complete lesions whereas the non-dyskinetic group showed moderate lesions based on DTBZ binding (the groups will be referred to by their lesion levels).

We compared the time concentration curve of striatal DA, DOPAC and HVA after L-dopa injection between the severe and moderate lesion groups. The results for these parameters have been corrected for probe recovery. The rats
with severe lesions had a mean basal DA level of 1.25 nM (SD=0.42). This group had a monophasic DA time concentration curve with a magnitude from baseline of 8.04 nM (SD=3.98, 643% change) and a time until maximum magnitude (Tmax) of 66 minutes (SD=9.47 min) (Figure 6). The rats with moderate lesions had an average basal DA level of 9.44 (SD=1.981), and a modest increase of 5.94 nM (SD=2.32, 63% change); there was no definitive peak response. Both the DA baseline and the percent change from baseline were significant based on ANOVA group comparisons (p=0.0004 and p=0.0090 respectively).

DOPAC and HVA showed similar patterns of the time concentration curve over the testing period (Figure 7.). The basal levels of DOPAC and HVA were 14.2 uM (SD=4.47) and 9.1 uM (SD=2.92) for the moderate lesion group. The complete lesion group had significantly lower basal levels of DOPAC, 0.625 uM (p=0.041) and HVA at 0.61 uM (p=0.034). The magnitude of DOPAC increase was 10.1 uM (71%) for the moderate and 4.53 uM (726%) for the complete lesion group. HVA showed similar findings with an increase of 9.55 uM (105%) and 5.16 uM (847%), for the moderate and complete lesions respectively.

**Treatment-induced changes in the pre-synaptic and post-synaptic markers**

The two groups had significant differences in the severity of DA terminal loss based on DTBZ bindings (p=0.0475) and DAT binding by WIN35,428 (p=0.0230). In the rats with severe DA terminal loss, mean DTBZ binding was 12% of the unlesioned side, and 29% of the unlesioned side in the partial group. The levels of the baseline dopamine corresponded strongly with WIN and DTBZ as did the metabolites. In regard to the postsynaptic factors there was a negative correlation between the baseline levels of DA and prodynorphin mRNA (Part. Corr.=-0.844, p=0.017). Table 5 summarizes the results of autoradiographic ligand binding and in situ hybridization histochemistry studies for pre-synaptic and postsynaptic markers (Figure 8). Both groups of rats had an upregulation of striatal preproenkephalin mRNA. The prodynorphin mRNA expression was significant upregulated in severely lesioned rats (p=0.0089). In contrast, the rats with moderate DA terminal loss showed no significant regulatory changes in prodynorphin mRNA in the striatum.
Figure 6. The time action response curve of AIM and DA concentration for dyskinetic (severely lesioned) rats. AIM (red diamonds) is graphed on the left y-axis. DA (black squares) is graphed on the right y-axis in nM concentration. The L-dopa line indicates the injection point. The DA baseline has been subtracted to graph alongside the AIM response (N=4)(error bars = SEM).
Figure 7. DA (black squares) and DOPAC (green diamonds) concentration time curves. Magnitude of L-dopa-induced DA efflux was greater in the striatum with severe DA denervation but had a smaller increase in DOPAC (Fig A)(N=4). The striatum with moderate DA denervation had no peak response to L-dopa but a large uM change in DOPAC (Fig .B)(N=3). Magnitude of DA efflux is defined as a difference between the peak level and baseline before injection of L-dopa. The black line indicates the injection of L-dopa. (error bars=SEM)
Table 5. Mean microdialysis data for metabolites as well as pre- and post-synaptic marker averages.

<table>
<thead>
<tr>
<th>Mean values</th>
<th>Partial Lesions</th>
<th>Severe Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>L-dopa Max</td>
</tr>
<tr>
<td>[DA] (nM)</td>
<td>9.44</td>
<td>15.383</td>
</tr>
<tr>
<td>[DOPAC] (uM)</td>
<td>14.23</td>
<td>24.3</td>
</tr>
<tr>
<td>[HVA] (uM)</td>
<td>9.085</td>
<td>18.633</td>
</tr>
<tr>
<td>(DOPAC+[HVA])/[DA]</td>
<td>2.47</td>
<td>2.791</td>
</tr>
<tr>
<td>AIM (Emax)</td>
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</tr>
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<td>WIN</td>
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</tr>
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</tr>
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<td>PPE</td>
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</tr>
<tr>
<td>Pdyn</td>
<td></td>
<td>0.984</td>
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</table>
Figure 8. Autoradiographic images of [3H]DTBZ binding (A1, A2) WIN 35,428 binding (B1, B2) and in situ hybridization histochemistry for prodynorphin (PDyn) mRNA (C1, C2) in striatal sections. Left column (A1, B1, C1): representative striatal sections of rats with complete lesions (<80%) and L-dopa-induced abnormal involuntary movements (AIM); Right column (A2, B2, C2): representative striatal sections of rats with partial lesions (80-50%) and no AIM. The right hemisphere of the brain has a 6-OHDA lesions.
4.2.2 Methods and Materials (microdialysis)

Female Sprague-Dawley rats (Vancouver, Canada), weighing 250-275 g at the time of lesion surgery, were given unilateral stereotaxic injections of 6-OHDA (3 µg/µl in 0.02 % ascorbate-saline). The lesions were formed in two locations under isoflurane anesthesia, the right ascending (1) mesostriatal DA bundle (7.5ug) and the (2) ventral tegmental area (9ug). The stereotaxic coordinates relative to bregma and the dural surface are: (1) A= −4.4, L= 1.2, V= 7.8, tooth bar= −2.4: (2) A= −4.0, L= 0.75, V= 8.0, tooth bar= −3.4. The toxin was infused at a rate of 1 µl/min and the cannula was left in place for one-minute post infusion. Following lesions nitric acid-passivated stainless steel guide cannula (19 gage, 15 mm) were implanted bilaterally 1 mm below dura, directly above the striatum (+1.0 mm AP, +/-3.1 mm ML from bregma and -3.0 to -7.0 DV from dura). Dental acrylic and jeweler's screws were used to form a head cap that held the cannula in place. Following surgery, rats were housed individually in plastic cages.

**Pulsatile L-dopa treatment:** Four weeks after surgery, 33 lesioned rats, and 8 sham lesioned rats received L-DOPA methyl ester (Sigma, USA) at a rate of 10mg/kg mixed with 15mg/kg of benserazide. Another 10 lesioned rats received saline injections. The drugs were dissolved in saline and injected I.P. 6 days a week, (Sigma, USA) for 3.5 weeks to establish stable dyskenisia levels. During the treatment period rats were tested by the AIM rating system. After 3.5 weeks microdialysis testing was conducted at a dose of 10mg/kg, with simultaneous AIM testing.

**Measurements of L-dopa-induced dyskinesia:** L-dopa-induced motor fluctuations were assessed using the abnormal involuntary movements (AIM) scale (Lee 2000, Cenci 1998, Lundblad 2002). This includes three subtypes of movements and stereotypy that are based on topographic distribution: limb dyskinesia, axial dystonia, and locomotor activity (Table 1). The scoring is based on a scale from 0 to 4 for each subtype of behaviour with 0 illustrates no dyskinesia and 4 being severe (Lee 2000). These categories were quantified
during the test period, at injection of L-dopa and every 10 minutes thereafter for
160 minutes. The AIM score is a sum of all measurements from sequential
assessments after injection of L-dopa.

**Microdialysis and HPLC:** Microdialysis probes were concentric in design
with silica inlet/outlet lines. The active surface consisted of a semi-permeable
membrane 4 mm in length (340 um o.d., 65 000 MW cut-off, Filtral 12, Hospal).
Probes were flushed continuously at 1 ul/min with a modified Ringer's solution
(perfusate); 10 mM sodium phosphate, 1.2 mM CaCl₂, 3.0 mM KCl, 1.0 mM
MgCl₂, 147.0 mM NaCl, pH 7.4) using a 2.5 ml gas tight syringe (Hamilton, Reno,
NV, USA) and a syringe pump (model 22, Harvard Apparatus, South Natick, MA,
USA). Typical *in vitro* probe recoveries of DA conducted at room temperature
were 18±1% of a standard DA solution (Ahn 2002) DA in microdialysates were
separated from other chemical species by high-pressure liquid chromatography
(HPLC) and quantified by electrochemical detection (Antek system). The details
of the HPLC-ED methods have been described previously (Ahn 2002).

Microdialysis sessions were conducted from 08:00–13:00 h in a Plexiglas
chamber (42 cm x 38 cm x 38 cm) fitted with a two-channel liquid swivel (Instech
Laboratories Inc., Plymouth Meeting, PA, USA). Each session involved bilateral
dialysis of the neostriatum. Probes were implanted 14–16 h prior to the collection
of the first microdialysis sample. Implanted animals remained overnight in the
test chamber with their daily ration of food and access to water. Dialysis samples
were collected at 10 min intervals and immediately assayed for DA using HPLC-
ED. Baseline samples were collected until DA levels were stable (less than 5%variation). The HPLC system used Antek systems.

**Data analyses:** For the purposes of statistical analyses and graphical
representation, neurochemical data were normalised to a baseline value
(calculated by averaging the concentration of DA in the three samples preceding
the final baseline sample). All data were analysed using repeated measures
analysis of variance (ANOVA) tests and also stepwise regression was used to
correlate relationships between the data. The statistics were conducted using
Statview. Statistical significance was set at \( p \leq 0.05 \). Magnitude of L-dopa
response (Emax) was defined as a mean of three highest consecutive AIM scores in the time response curve. The peak response time was the latency from the administration of L-dopa to the time at Emax. Rising time was the interval from the onset of L-dopa response to the peak response whereas decay time was the interval from the peak response to the offset of L-dopa response.

**Autoradiographic study**

**Tissue preparation:** Three days after the last dose of L-dopa, the animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and killed by decapitation. The brains were removed, frozen immediately in cold iso-pentane (Sigma, USA), and stored at -80 °C. Serial sections (16 μm thick) through the basal ganglia were cut coronally on a cryostat and mounted onto superfrost® microscope glass slides (Fisher, USA). The location of the probes implanted into the brain were calculated during slicing. The microdialysis probes descended into the striatum within the regions of 12-14 (2.5 lateral and -7 from bregma) based on Paxinos and Watson atlas (Paxinos 1997).

**Ligand binding autoradiography for pre-synaptic marker:** To label pre-synaptic markers in the dopaminergic nerve terminals in the striatum, one series of sections was incubated with DTBZ that binds to the vesicular monoamine transporter, a second section was used with tritiated **WIN 35,428** [N-methyl-³H] that binds to the plasma membrane dopamine transporter. The slide-mounted sections were first washed for 10 min in 4% paraformaldehyde. There was a prewash for 5 min in potassium buffer, pH 8, containing 137 mM KCl, 3mM NaCl, Na₂H₂PO₄ 1.5 mM, K₂HPO₄ 8 mM and EDTA 1 mM. In the same solution the slides were subsequently incubated for 180 min at pH 8 with 10 nM MTBZ. Control sections were incubated with 10 μM unlabelled DTBZ to determine the non-specific binding. At the end of the incubation, the slide-mounted sections were washed 3 x 3 min in the potassium buffer. Then slides were dried under a cold air stream, and exposed to phosphor plates at room temperature (Fuji Films) for 5 days. A phospho-imager was used to generate the image.

**Quantitative image analysis:** Autoradiographic images were digitized and analysed by the image analysis program **Optiquant.** The optical density was
converted to the intensity of radioactivity using $^{3}$H standards (Amersham) for $^{3}$H-WIN 35,428 binding. Striatal expression of $^{3}$H-WIN 35,428 and DTBZ binding were analysed in 4 sections per brain in the neostriatum, rostrocaudally at the levels of 11 to 18 according to the atlas of Paxinos and Watson (Paxinos 1997).

4.3.2 Results (Microdialysis)

AIM behavioural response

Daily L-dopa treatment (10mg/kg) resulted in gradual development of AIM on the side contralateral to the unilateral 6-OHDA lesions. The study included 51 rats that were in three testing groups, 43 were lesioned of which 33 were treated with L-dopa, while the other 10 were given saline injections. The remaining 8 animals served as sham lesion controls. Within the L-dopa group a variety of lesion severities were evident based on autoradiographic binding results. Thirteen of these rats developed dyskinesia. The saline group also had 3 rats that presented with dyskinesia on their first day of treatment during microdialysis testing. The L-dopa induced dyskinesia group had a mean maximum average magnitude AIM of 8.2 (SE=2.77). The saline group had a magnitude of 4.8 (SD=1.49). All other groups showed no dyskinesia. The time until maximum AIM amplitude was 72.27 min (SD=16.5) and 76.7 min (SD=5.8) for the treated and saline groups.

Autoradiography of pre-synaptic markers

The loss of DA terminals by 6-OHDA lesions were estimated by $[^{3}\text{H}]$DTBZ and $[^{3}\text{H}]$WIN 34,582 autoradiography. The groups were matched for lesion severity based on the VMAT2 binding. The $[^{3}\text{H}]$WIN 34,582 values were higher in the non dyskinetic groups, indicating that more reuptake sites were available in these animals. All rats that developed AIM with L-dopa treatment showed severe DA terminal loss. Complete lesion group showed $>80\%$ loss of DA terminals. The moderately lesioned animals ($80\% - 60\%$) and mildly lesioned animals ($>60\%$), were also examined (figure 9). The last group was the sham lesioned rats that served as a control.
DA Baselines levels

The basal extracellular DA levels in the striatum were measured before treatment. The complete lesioned rats had 4.3nM (SD=3.77) remaining DA in the denervated side (Table 6). The moderate lesion group had similar levels at 4.96nM (SD=3.66) for the denervated side of the brain. The mild lesion group (10.5nM) and sham lesion group (23.4nM) had higher values. Comparing the data of intact and DA-depleted animals revealed that destruction of the nigrostriatal pathway by 6-OHDA, led to a partial correlation (std coeff. 0.645) between the lesioned baseline DA levels and the VMAT totals.

DA 'Surge' Response

The administration of a therapeutic dose of L-DOPA increased extracellular DA levels in the DA-denervated striatum. In the dyskinetic group, extracellular DA levels increased by 12.61nM (SD=7.01), amounting to 673% of basal level. In the non-dyskinetic group, DA levels increased by 4.5nM (SD=4.35), amounting to 74.9% of basal levels without a distinct peak response. This trend continued in the other non-dyskinetic groups as the moderate lesioned animals elevated by 2.243 (SD=3.7) a response of 36.8%. In the saline group, those that developed dyskinesia response at the first dose of L-dopa showed a distinct phasic response to L-dopa with the peak level of 32.6 nM (SD=21.5; 775.1% of basal level). Those that did not develop dyskinesia in the saline group showed a similar response to their treated counterparts. Although the basal DA levels were lower in the complete lesion group, the maximal concentration of extracellular DA converted from L-DOPA was not statistically different compared to intact rats. The percentage increases were significant for both dyskinetic groups compared to the treated non-dyskinetic group and the other lesion severities. It is evident that a DA surge on a magnitude greater than 100% increase is necessary to induce dyskinetic behaviour (Figure 10).

Time changes in DA response

The DA peak had time related changes in the curve in response to L-dopa treatment. There was a significant difference demonstrated between the dyskinetic groups and the other groups in relation to the time to maximum
amplitude of the DA curve. The treated dyskinetic group reached its max at 68.6 minutes (SD=19.6) while the drug naive rats had their max at 118.3 minutes (SD=32.15)(Figure 11). The p-value for the change based on the ANOVA was 0.0071. The value for the max for the non-dyskinetic group was also delayed at a point of 95.0 and was significant to the dyskinetic group (p=0.0169). This information indicates that treatment in conjunction with a dyskinetic response is capable of moving the DA curve forward.

AIM and DA level comparisons

There is dissociation between the DA and the dyskinetic response especially within the saline dyskinetic group. The peak latencies are 76 min for AIM and 118min for DA (Figure 12). This is less evident in the treated group where the AIM and DA are at 72min and 68min. The onset and ascending time of the responses increase in a similar fashion although the DA response is often delayed approximately 5 min compared to the behaviour. The descending half of the DA response once the peak is reached was characteristic in that the treated dyskinetic rats had a much greater duration of time at 52min (se=7.5). While the other fully lesioned groups only remained above 50% for approximately 17min.
Figure 9. Autoradiographic images of [3H]DTBZ binding (A1, A2, A3) and WIN 35,428 (B1, B2, B3) in striatal sections. Row 1 (A1, B1): representative striatal sections of rats with complete lesions (<80%) and L-dopa-induced abnormal involuntary movements (AIM); Row 2 (A2, B2): representative striatal sections of rats with partial lesions (80-50%) with no AIM. Row 3 (A3, B3) Mild lesion rats (50-20%) dopamine terminal depletion. The right hemisphere of the brain has a 6-OHDA lesions.
Table 6. Autoradiography binding results for the pre-synaptic markers and baseline DA values for the microdialysis groupings. The baseline of the lesioned sides and control hemisphere have been reported as well as the percent change to Emax. The DA levels are in nM (Mean values and standard deviation).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>DTBZ</th>
<th>WIN</th>
<th>DA lesion baseline</th>
<th>DA control baseline</th>
<th>% to Emax (lesion)</th>
<th>AIM max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyskinetic L-dopa</td>
<td>0.077 (.018)</td>
<td>0.077 (0.008)</td>
<td>3.47 (.55)</td>
<td>16.4 (2.6)</td>
<td>673.4 (234.3)</td>
<td>8.20 (.83)</td>
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</tr>
<tr>
<td>Non dysk L-dopa</td>
<td>0.156 (.018)</td>
<td>0.132 (0.028)</td>
<td>7.35 (3.9)</td>
<td>23.6 (4.8)</td>
<td>75.0 (10.4)</td>
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<td></td>
</tr>
<tr>
<td>partial L-dopa</td>
<td>0.344 (.058)</td>
<td>0.280 (0.019)</td>
<td>6.86 (2.25)</td>
<td>17.0 (4.6)</td>
<td>49.2 (17.3)</td>
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<td></td>
</tr>
<tr>
<td>poor L-dopa</td>
<td>0.834 (.044)</td>
<td>0.792 (.054)</td>
<td>10.54 (2.4)</td>
<td>21.79 (2.6)</td>
<td>74.7 (14.9)</td>
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<td></td>
</tr>
<tr>
<td>sham L-dopa</td>
<td>0.966 (.030)</td>
<td>1.011 (0.026)</td>
<td>27.9 (3.0)</td>
<td>33.3 (2.6)</td>
<td>44.0 (18.8)</td>
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<td></td>
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<tr>
<td>Dyskinetic Saline</td>
<td>0.106 (.048)</td>
<td>0.086 (.009)</td>
<td>4.21 (1.1)</td>
<td>43.13 (4.75)</td>
<td>775.1 (268.7)</td>
<td>4.78 (.86)</td>
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<tr>
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<td>0.297 (.034)</td>
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<td>48.61 (12.9)</td>
<td>97.9 (14.7)</td>
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Figure 10. The DA concentration time course in response to L-dopa treatment. The dotted line indicates the point at which L-dopa is injected. The DA level is in nM. The N-values are mild 13 rats, moderate 7 rats, severe dyskinetic 11 rats and severe no dyskinesia 5 rats.
Figure 11. DA time response changes resulting from pulsatile L-dopa treatment. DA levels are calculated as the nM change from baseline. The L-dopa treated group is in red (N=11). The rats treated with saline and then given L-dopa on the day of microdialysis are in black (N=3). The black line indicates the point at which L-dopa was injected.
Figure 12. The DA time course compared to the AIM response. L-dopa treated severe dyskinesia (Emax >8)(N=6)(top figure), L-dopa treated mild dyskinesia (Emax <8)(N=5)(bottom figure). DA scale is calculated as the ratio to baseline. AIM score is in red and the scale is on the right. The black line indicates the time of L-dopa injection.
4.4 Discussion

Metabolites

We compared the time concentration curve of striatal DA and DOPAC after L-dopa injection between rats with moderate and severe DA denervation. The rats with complete lesions showed a monophasic DA time concentration curve with low peak DOPAC and HVA levels. In contrast, the rats with partial DA denervation did not show a monophasic DA time concentration curve, but showed high metabolite peaks. These findings suggest that the absence of DA surge with moderate lesions is likely caused by high-capacity DA reuptake since DOPAC reflects metabolism of cytosolic DA. Based on these preliminary observations, we propose that these results are consistent with the view that the function of DA transporter is in the uptake mode when the DA denervation is moderate, but it is reversed when the DA denervation is severe.

It is evident that exogenous L-dopa is converted to DA in both dopaminergic neurons and non-dopaminergic cells in the striatum (Hefti 1981, Mizoguchi 1993, Sarre 1994). This in conjunction with the DAT change may be enough to overcome homeostatic regulatory mechanisms. Diffusion may play a key role in removing extracellular DA from the synapse in a dopamine-depleted striatum because there is a decrease in metabolite formation. The elevated metabolite values in the moderate lesion group supports the view that peripheral breakdown of the L-dopa is not responsible for the absence of a DA peak in these animals. Also, an increased ratio between HVA and DA has been observed in patients with Parkinson's disease and in rats with nigrostriatal denervation (Herrero 1996), suggesting hyperactive DA neurons that could support rapid release mechanisms.

DA Surge Response

These experiments demonstrated that the administration of L-dopa resulted in a very significant increase in extracellular dopamine levels in the dopamine-depleted striatum. We have defined this as a surge response because of its pulsatile nature and specificity to dyskinetic animals. The surge response
exceeded a 100% increase when behaviour was induced. This response was very small in intact and moderately denervated striatum but increased in full lesions especially in dyskinetic animals where it exceeded 700% in some cases. Not only is a dopamine surge a key factor in the initiation of dyskinesia, it is a trigger for the cascade of post synaptic and pre-synaptic changes that can lead to a worsening of the L-dopa side effects. Dyskinetic problems can be activated with the initial dose of L-dopa. The factors that appear to contribute to this response are the extent of the lesion and the treatment dose level.

The DA surge effect is an essential characteristic in the differences in response that individuals have to the dopamine influx. The different groups in this study had characteristic shapes to the DA response. Within minutes of administering the L-dopa treatment most dyskinetic rats showed an increase in the DA levels in the striatum as well as increased behavioural activity in the form of dyskinetic behaviour. The surges evoked by repeated L-Dopa treatment up-regulate neurotransmitter-related gene expression in post-synaptic striatal neurons. The implications of this are that DAT along with glutamate and post-synaptic interactions predominantly with dynorphin are key factors in this response.

The minor increases in striatal dopamine after L-dopa administration in non-dyskinetic and moderately lesioned animals are not associated with enhanced motor behaviour. Although these groups lacked a DA surge after L-dopa, they did show a higher rise of DOPAC levels than those with severe lesions. This suggests that the absence of DA surge with moderate lesions is likely caused by high capacity DA uptake since DOPAC reflects metabolism of cytosolic DA. This is evidence for an increase ability to cope with the DA influx. In moderate lesions it can be assumed that there is more dopamine uptake and distribution of the incoming dopamine. However in the fully lesioned animals it is more difficult to define, reasons for the reduced response because lesion severity should be comparable. This indicates a compensatory mechanism may be more active in these animals possibly from DAT or post-synaptic changes.
Time Course Changes

The DA surge in the L-dopa treated group had an earlier onset than in the saline group. This observation suggests that repeated L-dopa treatment may enhance L-dopa-induced DA efflux. It also indicates that not only are the behavioural manifestations capable of shifting to shorter latency to the peak as the fluctuations and dyskinesia increases, but that the DA response is capable of similar changes. An example being that the saline treated dyskinetic group had a strong surge of dopamine levels that did not occur until 40 minutes after the AIM maximum. Although there is disconnect between the behaviour and the DA surge, they may in fact have a strong underlying correlation. Based on these observations, we propose that changes in the time action curve of dopamine may play a critical role in fluctuating responses by patients.

The postsynaptic changes are not evident after the first treatment because time is required to develop the immediate early gene (IEG) response. Therefore, the initial delayed surge in saline treated animals is not controlled by postsynaptic factors, but after treatment the accelerated occurrence of the response coupled with the broadening of the peak may be strongly related to the post synaptic levels. In this experiment, the DA curve and amplitude responses do not correlate inversely with the level of denervation. This lack of correlation between increases in DA efflux in striatum and motor behaviour has been discussed in other labs (Jonker 2000, Fisher 2000). One hypothesis for this discrepancy is that dopamine release in the substantia nigra more closely reflects behaviour than axonal release in the striatum (Fisher 2000).

The human data sheds some light on what is happening with the delayed onset of the dopamine peak in the saline group. The time course of antiparkinsonian effects of L-Dopa after a single dose does not correspond to the time course of plasma L-Dopa concentrations ("pharmacokinetic-dynamic dissociation") (Contin 1993) and neither does the density of DA synaptic vesicles (Lee 2000). This lends credence to our findings that the DA response is capable of an accelerated occurrence with treatment. The length of response was also a factor as the dopamine and behaviour levels had different durations in animals.
with different responses. This can be paralleled in human studies in the DA levels of fluctuating patients do not remain elevated as long as stable responders (de la Fuente-Fernandez 2001). This is indicative of the wearing off phenomena, and may indicate an increased DA release.

**Basal Levels and Synaptic Factors**

The baseline levels of DA were significantly different between partial and complete lesions within the metabolite experiment. This was also the case in the larger study although there was not as strong a trend as it appeared that factors other than merely the level of denervation were influencing DA levels. Although one cannot rule out the possibility that minor changes in the location of the lesion could result in fluctuations of DA levels depending on probe location, it appears more likely that the DA levels may be modulated in part by the involvement of dynorphin. This is evident from the metabolite experiment as a key factor in controlling the release of dopamine in the direct pathway.

Homeostatic equilibrium should not be compromised by DA terminal loss in the PD striatum providing that extracellular DA levels remain within a specified range (4-20 nM). This range does relate to our rats especially at the lower levels of detection. The amount of fluctuation evident in the baseline DA levels may indicate individualized levels of compensation in each rat, but more likely the efficiency of DA recovery between microdialysis probes is a contributing factor. The existence of effective regulatory mechanisms for extracellular DA is evidenced by microdialysis studies showing that extracellular DA levels remain within a normal range until striatal tissue DA is less than 20% of control values (Abercrombie 1990, Robinson 1988). This is partly attributed to an increase in DA synthesis and release, and potentially dynorphin as well as changes in DAT in the remaining DA terminals (Zigmond 1990).

The percent increase in the DA surge is considerably greater in dyskinetic animals than non-dyskinetic rats. However, the overall concentration of DA obtained even at the height of response rarely exceeds normal baseline levels. This is direct evidence of sensitization in the system as sub normal responses are able to result in detrimental behaviours.
Regulatory changes serve to maintain extracellular DA during the process of progressive nigrostriatal degeneration and treatment. One of the main factors in this process appears to be DAT. As the extent of DA terminal loss increases, the DAT undergoes compensatory regulation in two stages geared towards optimizing/maximizing extracellular levels of DA. This is done by an initial down regulation or internalization of DA transporter in the pre-symptomatic period, followed by reversal of DA transporter commencing when the extracellular DA homeostasis is compromised in the striatum.

Dopamine biosynthesis decreases when reuptake is inhibited, which indicates that the transporter is necessary for maintaining neurochemical homeostasis at the synaptic level (Raevskii 2002). This balance can be undermined by a single dose of L-dopa that must be developed by pre-synaptic changes primarily DAT modulation. Whereas down-regulation of DAT is related to extracellular DA levels, the reversal of DAT is affected by several factors including denervation, a significant increase in glutamatergic activity, and a rapid increase in cytosolic DA levels.

Conclusions

We observed that the DA time action curve is capable of an accelerated occurrence and a surge of DA is responsible for the onset of dyskinetic behaviour even when there is disconnect between observable responses. There is a higher formation of DA metabolites, primarily DOPAC in the partially denervated striatum than in fully lesioned rats. Many of these changes can be attributed to changes in the function of the DA transporter and potentially dynorphin.
Chapter V
Discussion and Conclusion

5.1 Discussion

The selective damage to DA neurons in the striatum decreases extracellular levels of striatal DA significantly (Jonkers 2000). This denervation creates a condition in which changes in the baseline levels of DA and also the pharmacokinetic response to the repeated administration of L-dopa are formed. Whether it is the time course, response amplitude, or synaptic factors there is a considerable difference seen between dyskinetic animals and non-dyskinetic animals and further differences between fully and moderately denervated rats. Through these experiments it was our intention to explore and document some of these factors to determine their cause.

The results of the reproducibility study indicate that the AIM model is not only reproducible across test days and within its individual components, but offers a testing accuracy that allows for an expanded analysis of the time action curve of dyskinetic movements induced by the administration of L-dopa. The AIM scoring system offers a solid foundation based upon clinical observation and how these observations can be translated into the 6-OHDA behavioural rat model. The reliability and possible implications for modelling key factors such as amplitude, duration and response times of the pharmacodynamic response to L-dopa treatment makes this model a useful tool in the attempts to understand the processes involved in parkinsonian dyskinesia.

The pre- and post-synaptic study investigated the correlations between the pharmacodynamic interpretations and post mortem information. This allowed exploration of several key characteristics that are evident within the time action curve of dyskinesia. The Emax correlated negatively with latency to onset, positively with the rising time, and negatively with the decay time. When the curve data were compared to the in situ hybridization histochemistry results a negative correlation was evident between decay time and dynorphin. Furthermore, a positive correlation was seen between Emax and dynorphin.
These findings suggest that there are common pharmacodynamic characteristics between dyskinesia and antiparkinsonian responses, and that dynorphin may play a role in both L-dopa-induced response fluctuations and dyskinesia in PD.

The microdialysis experiments allowed our research to connect the behavioural parameters with the central pharmacokinetics. We found that only rats with severe lesions showed the DA surge. Furthermore, only dyskinetic rats had a DA surge response. The partially lesioned animals had a higher rise of DOPAC levels than those with severe lesions. These findings suggest that the absence of DA surge with moderate lesions is likely caused by high-capacity DA reuptake. This also indicates that DA release may be more predominant than DA uptake in severe DA terminal loss conditions. Although severely lesioned animals often had reduced baseline extracellular DA levels the correlation was not significant. This may indicate individualized compensatory mechanisms, such as the negative correlation evident between baseline levels of DA and the dynorphin levels.

Pharmacodynamic time action curves in response to L-dopa treatment are capable of shifting to earlier onset and response severity. This appears to also be the case for the DA curve as L-dopa naive rats had a dopamine peak that was delayed. This clearly indicates that treatment causes the DA concentration curve to peak with a shorter latency. A potential factor is that the reversal of DAT removes buffering function of DA terminals in the striatum, causing the rapid increase ("surge") of extracellular DA efflux even with a therapeutic dose of L-Dopa. This surge of extracellular DA efflux could account for the transition of L-Dopa response from "stable" to "wearing-off" by increasing the peak DA level and shortening the duration of effective extracellular DA levels. In addition, recurrent DA surges evoked by repeated L-Dopa treatment up-regulate neurotransmitter-related gene expression in postsynaptic striatal neurons, which account for further transition of L-Dopa response into unpredictable "on-off fluctuations, and the development of L-Dopa-induced dyskinesia (Lee 2000, Cenci 1998).
5.2 Future Directions

The research presented here has opened up an exciting new level of collaboration. The potential studies into PD through this behavioural model and the use of microdialysis could lead to many important findings. In conjunction with the microdialysis study a pilot was conducted to examine the glutamate and GABA levels in both the GPe and the striatum. Due to problems pertaining to the amino acid HPLC machine, only a limited amount of data could be generated. It was evident however that the glutamate levels in the striatum could play a key role in the activity of DA, as it is capable of pre-synaptic facilitation of the release of DA in the striatum by glutamate (Shimizu 1990, Foster 2003). This information indicates that glutamate in conjunction with other factors could cause the reversal of DAT and “on-off” fluctuations of the basal ganglia.

The rats with a dopamine surge exhibited dyskinesia, and the saline treated rats had a much greater latency to the peak DA response. These observations suggest that repeated L-dopa treatment might enhance L-dopa-induced DA efflux. Based on this repeated L-dopa treatment may enhance reversal of DAT. Therefore experiments that incorporate testing at different points in the treatment paradigm or a pre- and post- treatment strategy may allow insight into the rate and cause of changes to the DA response curve. This research may benefit further from exploration into the down regulation (internalization) and the reversal mechanism of the DA transporter.

The use of proteomics would be a distinct advantage. We encountered great difficulty with in situ hybridization histochemistry techniques during the course of these studies. Therefore using this method would allow us to accurately calculate the levels for Dynorphin and GABA in the brain post mortem without having to speculate the expression from mRNA levels. Further investigation into the role of Dynorphin is required as it may be critical not only for the expression of dyskinesia but it may have a key role in maintaining the baseline DA levels in the striatum.
5.3 Conclusion

Several factors pertaining to L-dopa induced dyskinesia were investigated. The AIM reliability was examined to illustrate our ability to calculate changes in the time course response to treatment. The results of this study indicate that the AIM model is reproducible across consecutive test days. This allows for an expanded analysis of the peak dose AIM response to L-dopa. Next we explored how the pharmacodynamic changes are related to post mortem analysis of the pre- (DAT and VMAT) and post-synaptic (dynorphin, GABA and enkephalin) markers. This showed that prodynorphin mRNA expression was correlated positively with magnitude and negatively with the AIM response. Following this we incorporate real-time AIM analysis with DA levels in the striatum to compare the time action responses to L-dopa. This microdialysis experiment presented clear evidence that the AIM response is associated with L-dopa-induced phasic rise in DA levels in the striatum. Also, severe terminal loss and pulsatile L-dopa treatment influence the central pharmacokinetics of L-dopa. This three-sided approach brought about angles to allow insight into the dyskinesia phenomenon.

When this information is put together it is evident that there is a breakdown in the natural homeostatic level of the basal ganglia. The motor fluctuations are primed largely by DA denervation. But it is the DA surge even at therapeutic doses that gradually develops that creates the conditions necessary for changes to DAT and post-synaptic opioid peptides. This is eventually translated into the characteristics seen in the dose response curve and how there is a dynorphin involvement in the shape of the dyskinetic manifestation. This indicates and strengthens the view that pre and post-synaptic factors are instrumental components in the mechanism of response fluctuations and L-dopa-induced dyskinesia.
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Chromatogram of DA microdialysis results for an individual rat. Dopamine is evident at the 6.5 minute mark post injection to the HPLC machine.