

**ROLE OF EXERCISE AND GDNF IN AN ANIMAL MODEL  
OF PARKINSONS DISEASE:  
IMPLICATIONS FOR NEUROPROTECTION**

by

Ann D. Cohen

BA, University of Texas at Austin, 2000

Submitted to the Graduate Faculty of  
The School of Medicine in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy

University of Pittsburgh

2006

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Ann D. Cohen

It was defended on

August 16<sup>th</sup>, 2006

and approved by

Patrick Card, PhD, Department of Neuroscience

Brain Davis, PhD, Department of Medicine

Donald DeFranco, PhD, Department of Pharmacology

Teresa Hastings, PhD, Department of Neurology

Amanda D. Smith, PhD, Department of Neurology

Thesis Director/Dissertation Advisor: Michael J. Zigmond, PhD, Department of Neurology

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# **ROLE OF EXERCISE AND GDNF IN AN ANIMAL MODEL OF PARKINSON'S DISEASE: IMPLICATIONS FOR NEUROPROTECTION**

Ann D. Cohen

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Parkinson's disease (PD) is a progressive neurodegenerative disorder resulting in part from loss of nigrostriatal dopamine (DA) neurons. Treatments act only to relieve symptoms. It is therefore essential to develop treatments that slow or reverse the neurodegenerative process. Here, I explored exercise as a potential treatment against a 6-hydroxydopamine (6-OHDA) rat model.

6-OHDA causes selective loss of DA neurons, a PD model. Forced limb use after 6-OHDA ameliorates behavioral and striatal DA effects. Further, exercise increases trophic factors, such as GDNF, that have neuroprotective qualities in this model. I explored the effects of forced limb use prior to 6-OHDA on the effects of the toxin and GDNF levels in the striatum. I demonstrated that prior forced limb use attenuated the behavioral deficits and loss of DA typical of 6-OHDA and increased GDNF in the striatum of animals exposed to forced use.

The protective effect of exercise could reflect a decrease in the vulnerability of DA neurons, a regeneration of axons, or sprouting of axon terminals from undamaged neurons. Thus, I investigated the hypothesis that casting induced neuroprotection was due to the preservation of DA cells and terminals. Here, I demonstrated that forced limb use protected from 6-OHDA

induced loss of DA neurons and terminals. These findings suggest that exercise exerts its effects by decreasing the vulnerability of DA neurons and terminals to 6-OHDA.

Because casting increased GDNF, I next examined the effects of GDNF on 6-OHDA neurotoxicity during the 8 wk period after 6-OHDA. Using phenotypic markers of the nigrostriatal system, a non-DA cellular marker, and striatal DA content, I demonstrated these markers in the striatum and SN were not protected at 2 wks after 6-OHDA but recovered by 8 wks. No loss of DA cells in the SN or DA content in the striatum was observed in animals pretreated with GDNF. These data suggest that GDNF prevents 6-OHDA-induced DA cell death, but that weeks are required before these cells begin to normally express phenotypic markers. In conclusion, exercise may function to enhance the brain's ability to produce trophic factors, which may slow or halt the degenerative process in PD.

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## PREFACE

This thesis has been the culmination of many years of study and work through the course of my life. I have been incredibly fortunate to be guided by family, teachers, mentors, and friends who provided me the skills to accomplish this journey.

I would like to thank my co-mentors, Michael Zigmond and Amanda Smith for their guidance and mentorship over the past 5 years. They have provided excellent role models for me and without their knowledge and guidance this body of work would have not been possible.

I began my scientific career at the University of Texas at Austin, in Dr .Timothy Schallert's lab. Upon my move to gradate school, Dr. Schallert continued to serve as a collaborator with the Zigmond lab throughout my time here, and has provided invaluable insight and advice on detection of behavioral deficits and the forced limb use paradigm.

I would also like to thank the other member of my thesis committee, Drs. Brian Davis, Pat Card, Don DeFranco, and Terri Hastings, for their advice, time, and encouragement throughout this process. I thank the Zigmond lab for their assistance, support, and friendship. These individuals made my daily life in lab a pleasure. I would also like to acknowledge the Center for Neuroscience faculty, students, and staff for providing me with an amazing and supportive environment to attend graduate school.

Finally, I would like to thank my parents their love, support, and guidance has given me the tools to become the person I am today. Above all my parents have been the inspiration for

this work, their courage and strength in their struggle with Parkinson's disease, reminds me daily of the importance of our research.

This work was supported in part by USPHS grants NS19608, NS045698 and DAMD17-03-0479. ADC was supported as a predoctoral fellow by NS047831 and GM08424.

Chapter 2 has been previously published in the Journal of Neurochemistry.

Figure 2.4 represents a corrected version of the figure presented in the Journal of Neurochemistry. A calculation error occurred during the initial analysis and an erratum will be provided to the journal.

## **1.0 Introduction**

### **1.1 Parkinson's Disease is a progressive neurodegenerative disorder.**

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting 1% of individuals above age 55. The pathology of PD was first characterized by a loss of dopamine (DA)-containing neurons projecting from the substantia nigra (SN) to the dorsal striatum ( Hornykeiwicz, 1966) but it has been suggested that many other factors play a role in the symptoms and etiology of the disease. For example, damage to glutamatergic, GABAergic noradrenergic, cholinergic, and serotonergic neurons have all been reported in PD (Riederer and Lang, 1992; Braak et al., 1996; Hirsch, 2000; Braak et al., 2003). Indeed, some have suggested that several of these changes occur during the preclinical phase of PD before symptoms or loss of DA occur (Braak et al., 1996; 2003; Przuntek et al., 2004).

Despite the evidence of widespread pathology associated with PD, there is strong evidence to indicate that the loss of DA plays a crucial role in PD. The loss of DA in the putamen and DA cells in the SN is highly correlated with the motor symptoms associated with PD (see: Hornykeiwicz, 1966). Additionally, the primary treatment for the symptoms of PD is replacing DA levels through levodopa (L-DOPA) treatment, which is also consistent with the assumption that the loss of DA is a primary cause of the motor symptoms of PD. Further evidence for the critical role of DA in PD exists from animal models such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone, which have been shown to selectively destroy DA neurons while producing hallmark symptoms of PD.

### **1.1.1 PD produces motor deficits and non-motor symptoms**

PD produces hallmark clinical features including tremor; rigidity; bradykinesia, a slowness of movement; akinesia, an inability to move; and postural and gait abnormalities. Although PD is primarily associated with motor symptoms, several non-motor features commonly present in this disorder. Depression, masked face, cognitive deficits, and anosmia are all commonly reported in patients with PD.

### **1.1.2 PD produces a distinct neuropathology**

Late stages of PD produce losses of numerous cell types but typically the disease has been characterized by the loss of DA neurons. Postmortem examination of PD brains reveals a loss of DA neurons in the SN and a loss of DA terminals in the caudate and putamen of the dorsal striatum (for review see: Bernheimer et al., 1973). In addition to loss of nigrostriatal DA neurons and terminals, later stages of PD produce decreases in DA cell number in the ventral tegmental area and DA terminals in the nucleus accumbens and cortex. Additionally, loss of both norepinephrine and serotonin containing neurons has been reported in PD. It has been suggested these non-nigrostriatal losses are primarily associated with the non-motor deficits of the disease discussed above.

### **1.1.3 The causes of PD are unknown**

Little is known about the causes of PD, although a leading hypothesis is that PD results from the selective loss by apoptosis of DA neurons due to oxidative stress through the production of reactive oxygen species (ROS). In addition to oxidative stress, others have suggested dysfunction in mitochondrial respiration, proteasomal inhibition, as well as roles for

genetic mutations and environmental toxins (For reviews: Dawson & Dawson, 2003; Landrigan et al., 2005; Abou-Sleiman et al., 2006; Farrer, 2006). I have chose here to focus on oxidative stress and have selected an animal model that utilizes ROS as a primary means for producing DA cell death (see below).

#### **1.1.4 The role of oxidative stress in PD**

A leading hypothesis is that PD results from the selective loss by apoptosis of DA neurons due to oxidative stress through the production of ROS. ROS are highly reactive causing damage to cells through damage of proteins and DNA, impairing a cells ability to function (Cohen and d'Arcy Doherty, 1987). The brain is uniquely susceptible to ROS for several reasons including its high iron content and oxygen utilization and the presence of highly reactive neurotransmitters (for review see: Bharath et al., 2002). This seems to be particularly true in the DA system, where the highly reactive nature of DA has been suggested to produce damaging levels of ROS on its own and to enhance damage produced by other neurotoxins (for review see: Hastings and Zigmond, 1997). Indeed, increased indices of oxidative stress (Olanow and Tatton, 1999) and decreased levels of glutathione (GSH), an antioxidant, (Perry and Yong, 1986; Jenner, 1993) have been observed in postmortem brains from PD patients. Additionally, the catecholaminergic neurotoxin 6-OHDA, which is used to generate an animal model of PD, does so through oxidative stress (Sachs and Jonsson, 1975; Heikkila and Cohen, 1972; Zigmond and Keefe, 1997) and this toxicity can be enhanced by removing endogenous antioxidant defenses (Pileblad et al., 1989; Garcia et al., 2000).

#### **1.1.5 Role for genetics in the pathogenesis of PD**

Although no single gene is thought to cause PD, several genetic mutations have been found to play a role in the pathogenesis of the disease. Several genes including those encoding

alpha-synuclein, PINK-1, Parkin, and DJ-1 are associated with familial linked early onset PD, which is often accompanied by dementia (Polymeropoulos et al., 1997; Kitada et al., 1998; Bonifati et al., 2003; Valente et al., 2004; Abou-Sleiman et al., 2005). Later onset PD has been associated with several genetic mutations, including genes for the leucine-rich repeat kinase 2 (LRRK2) and ubiquitin carboxyl-terminal esterase L-1 (UCHL1). These genes vary in their function including disruption of intracellular signaling, impairment of cellular respiration, and impairment of cytoskeletal transport (for review see: Farrer, 2006). One prevailing hypothesis is that in idiopathic PD, these genetic disruptions paired with environmental stressors (see below) could combine to produce the pathology of the disease.

#### **1.1.6 Role for environment in the pathogenesis of PD**

The most well known environmental cause of PD can be attributed to MPTP, a synthetic chemical known to inhibit mitochondrial respiration, now utilized widely as an animal model of PD. MPTP was first described after use among a group of drug users in California who developed the cardinal symptoms of PD (Langston et al., 1983). In addition to MPTP, several pesticides have been implicated in the pathogenesis of PD. In fact, organochlorides have been found to be elevated in PD brains (Fleming et al., 1994). The insecticide rotenone, also a complex 1 inhibitor, is one of the most well known environmental toxins associated with PD, as it has recently become widely used in an animal model of PD (Betarbet et al., 2000). The herbicide paraquat and the fungicide maneb have both been associated in epidemiological studies with PD (eg. Brooks et al., 1999; McCormack et al., 2002) and have been shown to produce DA cell death in animal models (Thiruchelvam et al. 2000a; 2000b; Liu et al. 2003). In addition to pesticides, PD has been associated with environmental exposure to heavy metals, well water, and other agricultural chemicals (see Landrigan et al., 2005). It is likely that exposure to any of these



factors may pair with a genetic predisposition to develop PD.

## **1.2 There are various approaches that might be taken to slow or even reverse DA neuron degeneration.**

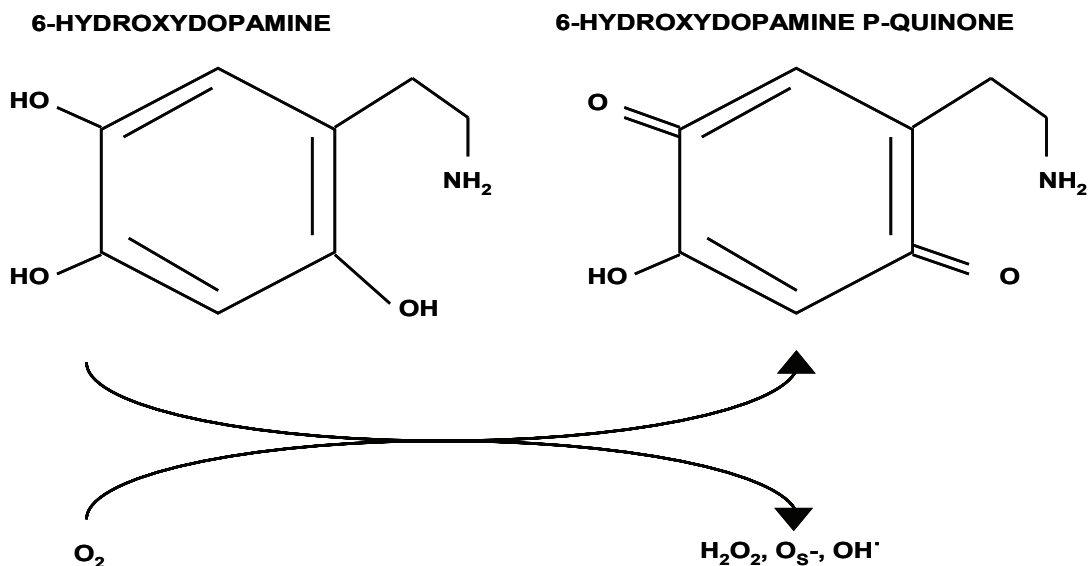
Most current forms of treatment are designed to provide a supplemental source of stimulation for DA receptors. Often these treatments seek to stimulate DA receptors, either directly or indirectly. L-DOPA is the most common such example. Other approaches involve neurosurgical techniques. However, none of the currently available treatments are able to completely eliminate the symptoms and none have been clearly shown to halt the progression of this disease. Thus, exploration of alternative approaches is warranted. Several longer-term approaches have been proposed. These include (a) transplantation of cells that can synthesize and release DA or its precursor, (b) administration of a trophic factor such as GDNF (see below), (c) introduction of a gene that would increase the synthesis of either DA or a neuroprotective factor, or (d) the administration of a drug (e.g., deprenyl or pramipexol) that has been shown in one or another model system to have neuroprotective effects.

## **1.3 6-OHDA rat model of PD**

### **1.3.1 6-OHDA-induced toxicity is a useful animal model of PD.**

Animal models of PD have been produced using the catecholaminergic neurotoxin 6-OHDA (Ungerstedt, 1971; Zigmond and Keefe, 1997). When delivered intracerebrally to rats in conjunction with a norepinephrine (NE) uptake inhibitor, 6-OHDA causes selective loss of DA neurons via oxidative stress, a proposed mechanism in the pathogenesis of PD (Halliwell,

2001). As 6-OHDA is a structural analog of catecholamines, it is able to enter DA neurons via the DA transporter (DAT) and is rapidly oxidized into 6-OHDA-quinone and hydrogen peroxide (Fig 1.1) (for review, see Zigmond and Keefe, 1997).



*Figure 1.1: 6-OHDA is rapidly oxidized to the toxic byproducts 6-OHDA quinone, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide radical (O<sub>2</sub><sup>-</sup>), and hydroxyl radical (OH<sup>·</sup>). (Modified from Zigmond and Keefe, 1997).*

As will be discussed below, the location of the infusion of 6-OHDA along the nigrostriatal bundle also plays a role in the magnitude and duration of DA neuronal degeneration.

### 1.3.2 Medial Forebrain Bundle Lesion

6-OHDA placed along the medial forebrain bundle (MFB) produces a rapid degeneration of DA neurons and terminals where a loss of DA levels in the striatum can be detected by 24 hours after 6-OHDA and a significant loss of DA neurons in the SN by 3 days post-6-OHDA (Willis et al., 1976; Perese et al., 1989; Smith et al., 2003). In addition to producing a large, reliable cell death to the nigrostriatal pathway, unilateral MFB infusions produce reliable, long-

lasting behavioral deficits that can be easily measured after 6-OHDA infusion (Schallert et al., 2000). A major issue regarding placement of 6-OHDA along the MFB is that of specificity. Because 6-OHDA is a catecholamine analog and not simply an analog of DA, when placed in the MFB 6-OHDA can produce damage to NE terminals. In order to create specific damage to only DA neurons, 6-OHDA can be used in conjunction with a NE uptake inhibitor, thereby blocking entry of 6-OHDA into NE terminals. Indeed, use of desipramine, an NE uptake inhibitor, has been shown to prevent loss of NE terminals but not DA terminals after 6-OHDA infusion (Breese and Traylor, 1971; Hernandez and Hoebel, 1982). One drawback to the MFB lesion is that it can produce (depending on dose) a rather large and rapid cell death that can sometimes overwhelm potential neuroprotective strategies that may take longer time periods to produce beneficial effects. Additionally, because of the speed with which MFB lesions produce death of DA neurons, it does not closely mimic the clinical condition.

### **1.3.3 Striatal Lesion**

6-OHDA delivered into the striatal DA terminals is another option for the destruction of the nigrostriatal pathway that has been widely used to examine neuroprotective strategies. Delivery of 6-OHDA into the striatum produces a slower, more progressive degeneration of DA neurons as death and retraction of the striatal terminals is required for subsequent neuronal death in the SN (Sauer and Oertel, 1994). One major advantage of this model is that it damages only a subset of DA neurons, allowing for examination of neuroprotective strategies. Because the cell death in the SN is protracted the striatal lesion model allows for an examination of neuroprotective strategies applied after the cell death process has already begun, which in the long term may be clinically relevant. Additionally, because in the striatum there are no NE terminals, this allows 6-OHDA to be specific to DA without drug pre-treatment. One drawback

to a striatal model is that behavioral deficits can be more difficult to detect because only a subset of DA neurons are affected, thus behavioral deficits are more subtle. Additionally, because loss of neurons in the SN is only partial it can be difficult to detect decreases in the number of neurons without use of a retrograde tracer, such as Fluoro-gold (eg Sauer and Oertel, 1994).

#### **1.3.4 DA depletions produce motor neglect**

One feature of PD is that movement is difficult and inactivity becomes more prominent as the disease progresses. Early in the disease, as the first fraction of the dopaminergic cells are in the process of degeneration, patients may learn to engage in alternative behavioral strategies that lead to even greater dependence on less compromised motor systems, an observation typical of animal models of the disease (Schallert & Hall, 1988; Schallert, 1988; Lees, 1992; Schallert *et al.*, 2000a; Whishaw, 2000). Additionally, like patients with PD, 6-OHDA-infused rats can show paradoxical kinesia. For example, they show transient improvements in motor performance when placed in an ice bath or exposed to a cat (Marshall, 1979), returned to their home cage after exposure to an unfamiliar environment (Schallert, 1989), or placed in a deep pool of water (Marshall, 1979; Keefe *et al.*, 1989; 1990). Thus, it is possible that a program of motor therapy that targeted motor processes normally affected by 6-OHDA might retard the neurodegenerative cascade.

### **1.4 Exercise is neuroprotective**

#### **1.4.1 Exercise is neuroprotective after brain damage**

Constraining the non-impaired upper extremity in stroke patients, thereby forcing use of

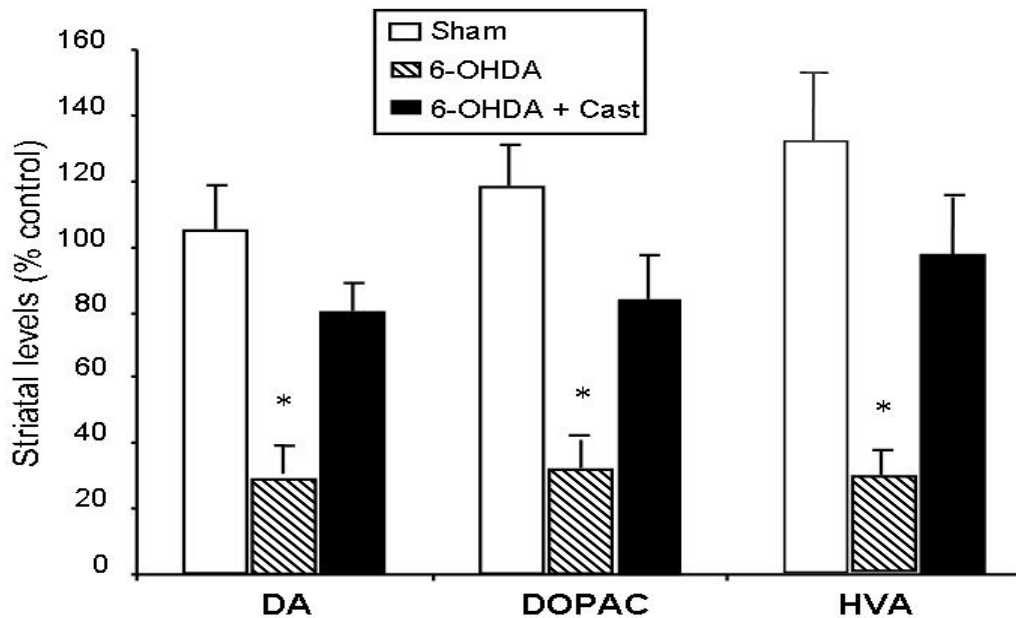
the affected limb, has been shown to improve motor function after stroke (Taub et al., 1999). Additionally, constraint induced therapy appears to induce reorganization in brain regions affected by ischemic brain damage (Taub and Morris, 2001; Sterr et al., 2002). Constraint therapy has also been used to improve functional outcome and expand neurophysiologically mapped limb representation areas in primate models and in humans after motor cortex injury (e.g., Liebert et al., 2000). Exercise and enriched environments have been shown to protect in several models of brain damage and have been shown to be neuroprotective in animals subjected to a variety of neurotoxic conditions, including ischemia, (Stummer et al., 1994, Li et al., 2004), 3-acetylpyrrole, domoic acid, (Carro et al. 2001), and genetic modifications associated with Alzheimer's disease (Allard et al., 2005; Jankowsky et al., 2005; Sisodia et al., 2005).

#### **1.4.2 Exercise is beneficial in PD and animal models of PD**

Increases in physical activity levels throughout life have been associated with a lower propensity to develop PD (Sasco et al., 1992; Tsai et al., 2002; Hirsch et al., 2003; Chen et al., 2005). There is also evidence for efficacy of physical therapy regimens in the treatment of PD (Toole et al., 1999, Bergen et al., 2002). A similar effect of exercise has been observed in animal models of PD. For example, treadmill and exercise in a running wheel, as well as an enriched environment, has been shown to reduce the effects of another DA-selective neurotoxin, MPTP in a mouse model (Bezard et al., 2003; Tillerson et al., 2003; Faherty et al., 2005). We and our colleagues have shown that placing a cast on one forelimb for 7 days immediately following the unilateral administration of 6-OHDA can attenuate both the behavioral and neurobiological effects of the toxin (Tillerson et al., 2001; 2002; Fig 1.2).

### 1.4.3 Exercise induces neuroprotective responses in the brain

Motor training and enriched experience have been associated with glial cell proliferation, changes in neurotransmitter levels (Bland et al., 1999; Humm et al., 1999), and the growth of neuronal processes (e.g., Jones & Schallert, 1994; Kleim et al., 1996; Schallert et al., 1997; Kempermann et al., 1998a; for reviews see: Kolb & Wishaw, 1998; Klintsova & Greenough, 1999, Ivanco & Greenough, 2000). Additionally, exercise produces a robust effect on trophic factor expression, including GDNF, BDNF, and FGF2. The mRNA for BDNF can be upregulated in the hippocampus and cerebral cortex in response to physical exercise (Neeper et al., 1995, 1996; Oliff et al., 1998; Russo-Neustadt et al., 1999; Widenfalk et al., 1999; Russo-



*Fig 1.2: Effect of forced use on DA, DOPAC, and HVA levels. Shown are means  $\pm$  SEM of sham (open bars, n=13), 6-OHDA lesioned (hatched bars; 12), and lesioned/casted*

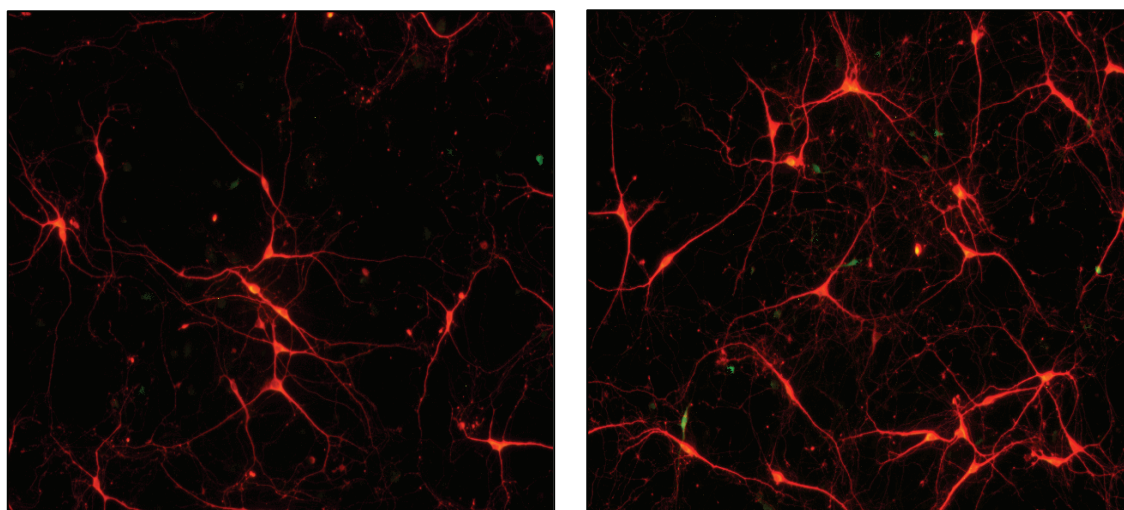
Neustadt et al., 2001). Additionally, exercise deprivation after a prolonged period of habitual running down-regulates hippocampal BDNF and TrkB (Widenfalk et al., 1999). NGF, another neurotrophin, is also increased in the hippocampus and cerebral cortex in response to physical exercise (Neeper et al., 1996). FGF2 is also increased during exercise (Gomez-Pinilla et al., 1997). Moreover, it has been observed that FGF2 is increased in astrocytes in the perinjury area of cortex in both the MCAo and focal ablation injury models, and that forced non-use of the impaired forelimb reduces both endogenous expression of FGF2 and the extent of dendritic arborization in layer V pyramidal neurons in the region surrounding the infarct (Schallert et al., 2000). Although not examined to the extent of BDNF, there is emerging evidence that GDNF may also play a role in the effects of exercise. For example, GDNF is upregulated in the hippocampus of rats raised in an enriched environment (Young et al., 1999).

Exercise may function to increase trophic factors in several ways. First, exercise could induce motor learning processes, which have been shown to increase trophic factors (Gomez-Pinilla et al., 1998). Second, exercise has been shown to increase glial cell proliferation and glia have been hypothesized to function to release endogenous trophic factors. Finally, another possibility is that exercise produces a drop in body weight, which has also been shown to increase endogenous levels of trophic factors (Lee et al., 2000).

## **1.5 Neuroprotective factors may be a useful treatment for PD.**

GDNF is a dimeric protein expressed in several cell populations and has been shown to be highly expressed in the developing rat striatum (Stromberg et al., 1993) making it an interesting factor to be exploited against degeneration of the nigrostriatal DA system. GDNF is a survival factor for DA neurons (Fig 1.3) that can protect against toxic insults in adult animals.

Early studies of DA neurons in culture indicated the presence of a survival factor (e.g., Engele and Bohn 1991), which was soon identified as GDNF (Lin et al., 1993). In the presence of this factor, DA neurons increase in size and have longer processes forming a denser network of dendrites and axons than DA cells lacking GDNF exposure (Lopez-Martin, 1999). GDNF is a member of the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily. In mouse and rat brains, GDNF expression rapidly falls off as development proceeds (Stromberg et al., 1993; Blum & Weickert, 1995). However, there are many instances in which aspects of recovery of function recapitulate ontogeny (see review by Cramer & Chopp, 2000), and there is evidence that GDNF expression can increase following injury (Naveilhan et al., 1997; Liberatore et al., 1997; Sakurai, et al., 1999; Wei et al., 2000).



**+ GDNF**

*Fig 1.3: The effects of GDNF on primary cultured TH<sup>+</sup> neurons (Adapted from Ding et. al., 2003)*



Increased GDNF expression, whether through the addition of exogenous GDNF or following the administration of a viral vector containing the GDNF gene, can protect DA neurons against the neurotoxic effects of 6-OHDA. This has been shown both in vivo and in vitro (e.g., Hoffer et al., 1994; Kearns & Gash, 1995; Choi-Lundberg et al., 1997; Akerud et al., 1999; Kramer et al., 1999; Gong et al., 1999; Schatz et al., 1999; Zigmond et al., 2000; Smith et al., 2003). The neuroprotective effects of GDNF extend to other insults and other cell types (e.g., McAlhany et al., 2000). Furthermore, decreased levels of GDNF are observed in patients with PD, which suggest loss of trophic support is a causal factor in the genesis of the disease (Siegel and Chauhan 2000). While animal studies have been quite promising, clinical data on the effects of GDNF on the progression of Parkinson's disease remains limited as some groups have shown improvements in symptoms and progression of PD (Gill et al., 2003; Patel et al., 2005; Slevin et al., 2006) while others have shown no clinical improvement (Nutt et al., 2003; Lang et al., 2006).

## **1.6 The dissertation**

Treatments for PD are generally limited and these treatments do not appear to reduce the progression of the disease. Moreover, side effects of these treatments gradually emerge and efficacy usually is reduced. Thus, developing strategies for slowing or preventing ongoing degeneration or even reversing the neurodegenerative process in PD is of paramount importance. I therefore set out in the broad aims of the dissertation to provide evidence for the following hypotheses:

*Exercise prior to oxidative stress directed toward DA neurons in the SN reduces the vulnerability of DA neurons to oxidative stress. The underlying mechanism of these*

*protective effects involves an increase in trophic factors, which protect DA neurons against such stress.*

To test these hypotheses I have used as my model adult male rats exposed to 6-OHDA. The exercise that I have chosen is 7 days of forced limb use caused by casting the contralateral limb. Moreover, the trophic factor on which I have focused is GDNF.

Our research group, working together with Dr. Tim Schallert and his associates at the University of Texas, Austin, had previously shown that forced reliance on the impaired forelimb is protective if performed for the 7 days immediately following 6-OHDA (Tillerson et. al., 2001; 2002). Chapter 2 examines the effect of forced reliance on a forelimb during the 7 days *prior to* ipsilateral infusion of 6-OHDA on the deficits characteristic of this lesion model. I additionally tested the hypothesis that forced-use might increase the expression of endogenous GDNF thereby attenuate the effects of 6-OHDA. Casted animals displayed no behavioral asymmetries and a marked attenuation in the loss of striatal DA and its metabolites. Animals receiving a unilateral cast alone showed an increase in GDNF protein in the striatum corresponding to the overused limb. These results suggest that pre-injury forced limb-use can prevent the behavioral and neurochemical deficits to the subsequent administration of 6-OHDA and that this may be due in part to neuroprotective effects of GDNF.

Chapter 2 demonstrates that prior forced limb use confers protection from the behavioral and neurochemical effects of 6-OHDA. However, it is not known whether this reflects (a) a decrease in the vulnerability of DA neurons to 6-OHDA, (b) a regeneration of damaged DA axons, or (c) the sprouting of DA axon terminals from undamaged neurons into areas initially affected by 6-OHDA. Chapter 3 investigated the hypothesis that the ability of casting to block the behavioral and neurochemical effects of 6-OHDA is due in part to the preservation of DA

cells and terminals, by using tyrosine hydroxylase (TH) to measure the loss of nigrostriatal terminals and neurons and Nissl to measure the presence of SN neurons. Animals casted prior to 6-OHDA showed a significant attenuation of normal loss of both TH immunoreactive and Nissl-stained cells in the SN. Precasted animals also showed a significant sparing of TH<sup>+</sup> terminals in the striatum. These data suggest that prior forced limb use produces behavioral and neurochemical protection by decreasing the vulnerability of DA neurons to oxidative stress.

The mechanism underlying the protective effect of forced limb use is unknown. However, in Chapter 2 I showed that glial cell-line derived neurotrophic factor (GDNF), a potent dopaminotrophic factor that protects against 6-OHDA toxicity (e.g., Kearns and Gash, 1995; Choi-Lundberg et al, 1998), was increased in the striatum with forced limb use, indicating that it may be involved in the protective effects of forced limb use against 6-OHDA.

In Chapter 4, I explored the effects of GDNF on 6-OHDA neurotoxicity during the 8 wk period following administration of the toxin, using several phenotypic markers of the nigrostriatal system, a non-DA cellular marker, and DA content in the striatum. At 2 wks post-infusion, TH, the vesicular monamine transporter 2 (VMAT2), and the dopamine transporter (DAT) displayed a loss in the striatum of GDNF-treated animals that was similar to the loss of these markers in 6-OHDA treated animals that did not receive GDNF. As expected, at 8 wks post-infusion, GDNF pre-treated animals had only a 20-30% loss depending upon the dopaminergic marker assessed. Similarly, when FG-positive cells were examined in the SN of GDNF pre-treated animals, no loss of cells was observed at 8 wks. Analysis of FG cells in SN and of DA levels in striatum indicated that GDNF-protection was complete by 2 wks. However, a marked loss of TH cells and of TH, VMAT2, and DAT in striatum was observed at that earlier time point. Additionally, no loss of DA content was observed in the striata of GDNF pre-treated

animals. Taken together, these data suggest that GDNF prevents 6-OHDA-induced DA cell death, but that weeks are required before these cells to begin to normally express many phenotypic markers.

In summary, the data presented in this dissertation suggest that exercise and GDNF are viable treatment options for Parkinson's disease. Further exploration of the neuroprotective effects of GDNF or exercise on a 6-OHDA model will provide insight into design and methodology of future clinical studies for treatment options in PD. Finally, as the mechanisms of the protective effects of GDNF and exercise are further explored more targets for drug creation may become available, particularly as early detection methods become more readily available.

## **2.0 Neuroprotective effects of prior limb use in 6-hydroxydopamine-treated rats: Possible role of GDNF**

### **2.1 Abstract**

Unilateral administration of 6-OHDA into the MFB causes a loss of DA in the ipsilateral striatum and contralateral motor deficits. However, if a cast is placed on the ipsilateral limb during the first 7 days following 6-OHDA infusion, forcing the animal to use its contralateral limb, both the behavioral and neurochemical deficits are reduced. Here, we examine the effect of forced reliance on a forelimb during the 7 days *prior to* ipsilateral infusion of 6-OHDA on the deficits characteristic of this lesion model. Casted animals displayed no behavioral asymmetries as measured 14-28 days post-infusion and a marked attenuation in the loss of striatal DA and its metabolites at 30 days. In addition, animals receiving a unilateral cast alone had an increase in GDNF protein in the striatum corresponding to the overused limb. GDNF increased within 1 day after the onset of casting, peaked at 3 days, and returned to baseline within 7 days. These results suggest that pre-injury forced limb-use can prevent the behavioral and neurochemical deficits to the subsequent administration of 6-OHDA and that this may be due in part to neuroprotective effects of GDNF.

### **2.2 Introduction**

PD is a neurological disorder characterized by the degeneration of DA cells in the substantia nigra. The loss of dopaminergic control over striatal output neurons leads to a variety

of neurological deficits, including akinesia. Most current treatments for PD are pharmacological, temporarily restoring dopaminergic tone in the striatum, and therefore focus on alleviating symptoms of the disorder. However, when the degree of DA cell loss becomes too extensive, the efficacy of drug treatment diminishes, and motor and psychiatric side effects become more problematic. Thus, it is essential to develop strategies for slowing or preventing ongoing cell death in this disorder.

Animal models of PD have been produced using the catecholaminergic neurotoxin 6-OHDA (Ungerstedt, 1971; Zigmond and Keefe, 1997). When delivered intracerebrally to rats, 6-OHDA causes selective loss of DA neurons via oxidative stress, a proposed mechanism in the pathogenesis of PD (Halliwell, 2001), and a marked impairment in limb use for movement initiation or skilled motor functions (e.g., Marshall et al., 1974; Spirduso et al., 1985; Schallert et al., 1992; Miklyeva and Whishaw, 1996). However, like patients with PD, 6-OHDA-infused rats can show paradoxical kinesia. For example, they show transient improvements in motor performance when placed in an ice bath or exposed to a cat (Marshall et al., 1976), returned to their home cage after exposure to an unfamiliar environment (Schallert, 1989), or placed in a deep pool of water (Marshall et al., 1976; Keefe et al., 1989; 1990).

Increases in physical activity levels throughout life have been associated with a lower propensity to develop PD (Sasco et al., 1992; Tsai et al., 2002). Additionally, constraining the non-impaired upper extremity in stroke patients, thereby forcing use of the affected limb, improves the motor function and increases the use of the affected limb (Taub et al., 1999; Taub and Morris, 2001; Sterr et al., 2002). There is also limited evidence for efficacy of physical therapy regimens in the treatment of PD (Toole et al., 1999).

We have recently shown that forcing animals to exercise their impaired limb for 7 days beginning 0-3 days after the unilateral administration of 6-OHDA can dramatically attenuate both the behavioral and neurobiological effects of the toxin (Tillerson et al., 2001; 2002). Although the mechanism by which forced use ameliorates behavioral and biochemical deficits is unknown, one hypothesis that can be drawn from the literature is that forced use of the impaired forelimb initiates a cascade of events that involves an increase in the availability of key neurotrophic factors in the brain. One such factor is GDNF.

GDNF has been shown to be a potent survival factor for DA neurons (Lin et al., 1993). Moreover, administration of exogenous GDNF or a viral vector containing the GDNF gene is known to protect DA neurons from the neurotoxic effects of 6-OHDA in vitro (Kramer et al., 1999; Gong et al., 1999; Schatz et al., 1999) and the behavioral and neurotoxic effects in vivo (Hoffer et al., 1994; Kearns & Gash, 1995; Choi-Lundberg et al., 1998; Akerud et al., 1999). Although levels of GDNF protein decrease markedly after development, there is evidence that GDNF can increase after experience, such as an enriched environment (Young et al., 1999). We, therefore, reasoned that forced-use might increase the expression of endogenous GDNF and attenuate the effects of 6-OHDA.

## **2.3 Materials and Methods**

### *Animals*

Male rats weighing 350-450 grams were used throughout these experiments. All animals were housed two per cage and maintained on 12 h light/dark cycle with food and water available ad libitum. All procedures were in strict accordance with the guidelines for the NIH Care and

Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees at the University of Texas and the University of Pittsburgh. The studies reported here of the effects of casting on the behavioral and neurochemical effects of 6-OHDA were carried out in hooded Long-Evans rats (Charles River Laboratories, Wilmington, MA), whereas studies of GDNF were carried out with Sprague-Dawley rats (Hilltop Lab Animals, Scottsdale, PA). A small pilot study indicated that the neuroprotective effects of casting seen with the Long-Evans strain (see Figures 2.1-2.3) were comparable to those seen with Sprague-Dawley strain (see legend to Figure 2.4). In addition, previous investigators also have found that forced exercise has the same effect on 6-OHDA infused rats from the Long-Evans and Sprague-Dawley strains (Moroz et al., 2002; Tillerson et al., 2002).

#### *Preoperative and Surgical Procedures*

Seven days prior to surgery animals were randomly assigned to one of four groups. *Sham-lesioned/casted animals* (n=3) received a cast for the 7 days prior to the sham lesion; *sham-lesioned animals* (n=2) received a sham lesion alone; *6-OHDA animals* (n=9) received unilateral 6-OHDA alone, whereas *casted/6-OHDA animals* (n=7) received a cast on one limb for 7 days prior to being given an ipsilateral infusion of 6-OHDA into the MFB.

Animals in the casted groups were fitted with plaster of Paris casts to immobilize the forelimb in a naturally retracted position against the sternum (Jones and Schallert, 1994). At the end of the 7-day casting period, all animals were anesthetized with Equithesin (25 mg/kg pentobarbital and 150 mg/kg chloral hydrate, i.p.), the casts were removed (if present), and the animals were prepared for surgery. Animals began to use the previously casted forelimb immediately upon awakening from surgery. Thirty minutes prior to surgery, animals were given



desipramine (15 mg/kg, i.p.), an inhibitor of norepinephrine uptake that serves to block the entry of 6-OHDA into noradrenergic neurons. 6-OHDA (10 µg in 4 µl of 0.9% NaCl, 0.02% ascorbic acid) was infused (0.5 µl/min) into either the right or left MFB (the side ipsilateral to the cast; 3.3 mm posterior, ± 1.7 mm lateral of bregma, and 9 mm ventral to dura) (Paxinos and Watson, 1982). Sham-lesioned animals received all surgical procedures up to but not including lowering of infusion cannulae.

### ***Behavioral Assessment***

One week before and 2-4 wks after infusion, animals were assessed for forelimb asymmetry and akinesia. The extent of asymmetry in the forelimbs during exploratory movements was determined by videotaping rats in a clear Plexiglass cylinder (20 cm diameter; 30 cm high) for 3 min and later analyzing a slow motion version of the tape (Schallert & Tillerson, 2000; Tillerson et al., 2001, 2002; Cenci et al., 2002; Schallert, 2002). We assessed the amount of time animals made independent use of the impaired forelimb, the independent use of the non-impaired forelimb, and the simultaneous use of both limbs (including alternating steps) for support and weight shifting movements along the walls. For a single score, these values were converted to percentages and the percent independent use of the non-impaired forelimb was subtracted from the percent independent use of the impaired forelimb. To assess forelimb akinesia, animals were held with their hindquarters suspended and allowed to initiate stepping movements in a 10-sec period. Stepping movements were assessed for both limbs and an asymmetry score was computed  $[(\text{ipsilateral steps}/\text{ipsilateral plus contralateral steps}) - (\text{contralateral steps}/\text{ipsilateral plus contralateral steps})]$  (Schallert and Tillerson, 2000; Schallert et al., 1992; Cenci et al., 2002). Three weeks post-infusion animals received apomorphine (0.5

mg/kg, s.c.), were placed in a 14.5 in. diameter plastic bowl and rotations were counted over a 90-min period to estimate the extent of denervation induced upregulation of DA receptors (Ungerstedt, 1971).

### *Neurochemical Assessment*

*Dopamine assay:* Thirty days post-infusion, animals from *sham-lesioned*, *sham-lesioned/casted*, *6-OHDA*, and *casted/6-OHDA* groups were sacrificed, brains were removed, and a section of the striatum from 1 to 2 mm anterior of bregma was dissected from both the ipsilateral and contralateral hemispheres. Dissected striata were assayed using minor modifications of previous methods (Smith et al., 2002). Striatal tissue was suspended in 0.1 M HClO<sub>2</sub> containing 347 μM NaHSO<sub>3</sub> and 134 μM Na<sub>2</sub> EDTA in a volume (μl) 20X weight in mg, homogenized and centrifuged at 16,000 x g for 20 min at 4°C, and the supernatant was removed. Tissue samples were assayed for DA and 3,4-dihydroxyphenylacetic acid (DOPAC) by injecting a 10 μl aliquot of the sample onto a Symmetry C18 column (3.9 x 140 mm, Waters Corporation, Milford, MA). The mobile phase consisted of 50 mM H<sub>2</sub>NaPO<sub>4</sub>, 0.72 mM sodium octyl sulfate, 0.075 mM Na<sub>2</sub>EDTA and 16% methanol (v/v), pH 2.7. The mobile phase was pumped through the system at 0.7 ml/min using a Shimadzu LC-10AD pump (Shimadzu Corp., Columbia, MD). Analyses were detected coulometrically using an ESA Coulochem Model 4100A detector, an ESA Model 5010 conditioning cell, and an ESA Model 5014B microdialysis cell (ESA, Inc., Chelmsford, MA). The settings for detection were E<sub>1</sub>=+0.26V, E<sub>2</sub>=+0.28V, and guard cell=+0.4 V. The limits of detection for DA and DOPAC were in the femtomole range.

*GDNF assay:* Thirty-three male Sprague Dawley rats were casted and decapitated 1-10 days after placement of the cast, and the striata were removed, homogenized in lysis buffer

containing 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride (10 µg/ml), and 0.5 mM sodium vanadate. Homogenate was then centrifuged at 12,000 x g for 20 min at 4°C, supernatant was removed, acid-treated with 1 N HCl (1 µl/10 µl of sample), and then neutralized with 1 N NaOH (1 µl/10 µl of sample), respectively, to increase the sensitivity of the assay (Okragly and Haak-Frendscho, 1997). Samples were assayed for GDNF using an ELISA kit (Promega Corporation, Madison, WI) according to the protocol provided. GDNF values were compared to those observed in uncasted animals.

### *Statistical Analysis*

Neurochemical, trophic factor, and rotational behavior data were analyzed using a one-way ANOVA, and post hoc analyses were carried out using Bonferonni-corrected multiple comparison tests. A two-way repeated measures ANOVA was used to analyze all other behavioral data. Post hoc analyses were performed using Bonferonni-corrected multiple comparison tests.

## **2.4 Results**

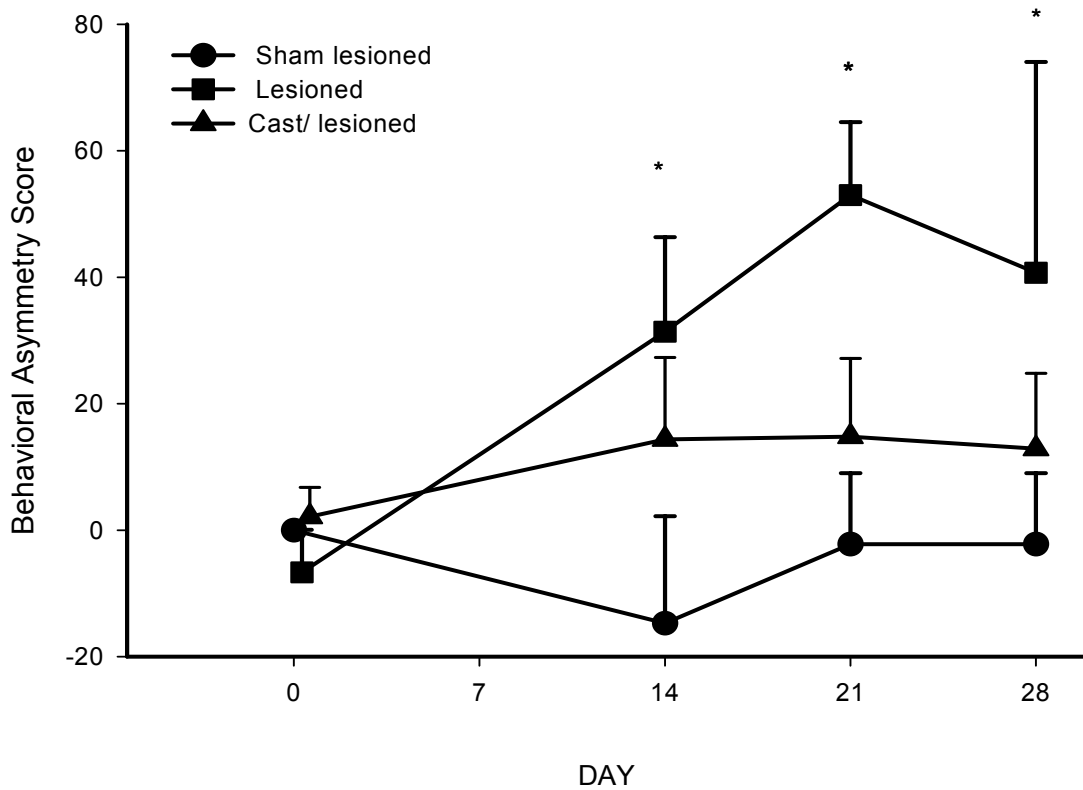
### *Effect of casting prior to injury on effects of unilateral 6-OHDA on forelimb rearing and akinesia*

Animals casted prior to 6-OHDA infusion showed reductions in forelimb asymmetry (Fig 2.1a) and akinesia (Fig 2.1b) normally associated with unilateral 6-OHDA toxicity. The casted sham group and non-casted sham group showed no significant difference in asymmetric behaviors, therefore these groups were pooled for further analysis ( $F_{(1,11)} = 2.336$ ;  $p > 0.05$ ). Analysis of variance indicated an overall group effect for forelimb asymmetry ( $F_{(2, 47)} =$

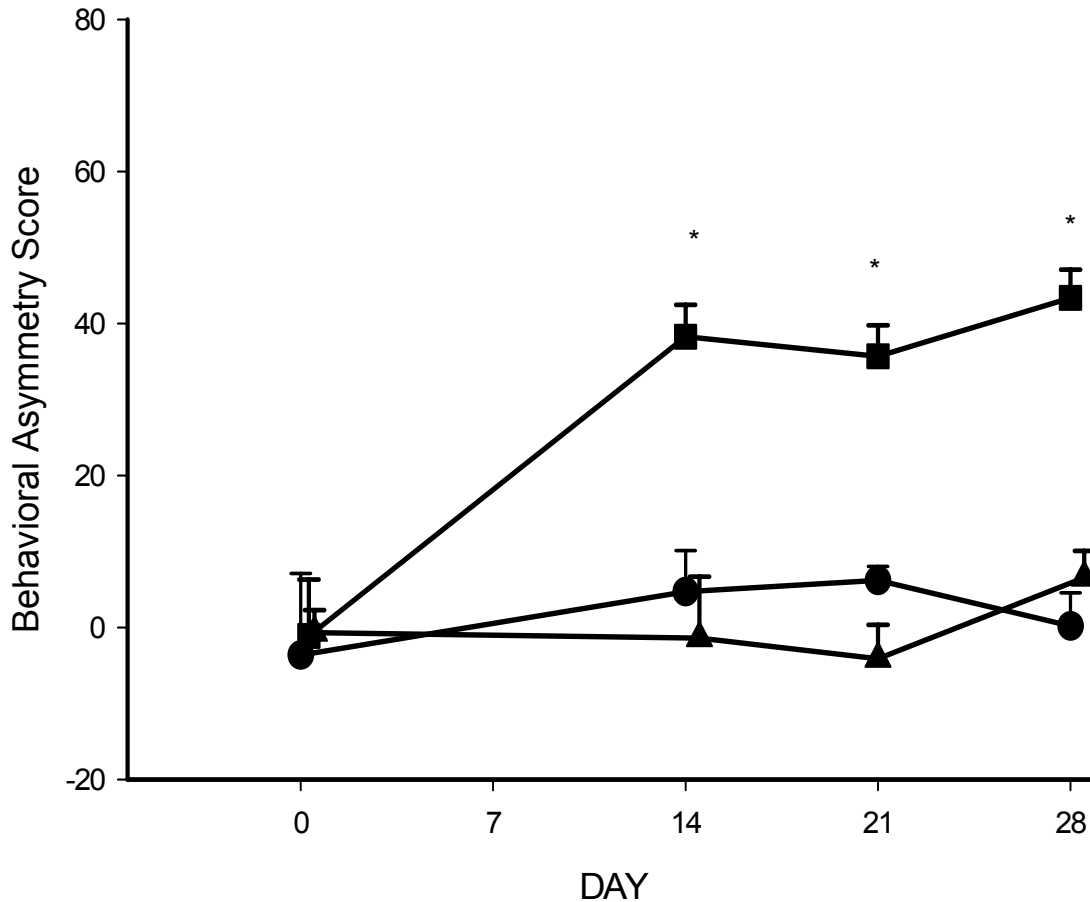
8.74;  $p < 0.01$ ) and forelimb akinesia ( $F_{(2, 39)} = 48.33$ ;  $p < 0.01$ ). Post hoc analysis of these behavioral measures indicated significant attenuation of asymmetric behavior in animals casted prior to infusion of 6-OHDA when compared with animals that received 6-OHDA alone ( $p < 0.01$ ).

*Effect of casting on apomorphine-induced turning normally associated with unilateral 6-OHDA.*

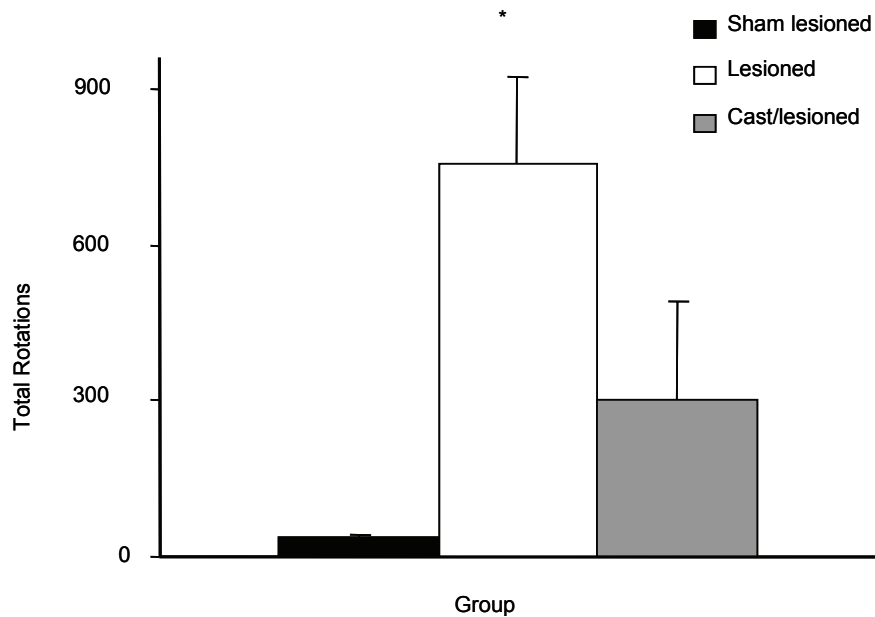
Casting-induced attenuation of the effects of 6-OHDA-induced forelimb rearing and akinesia might conceivably be caused by compensatory changes in pathways that parallel the nigrostriatal DA projection. Thus, we also used a third behavioral test, apomorphine-induced turning. This phenomenon depends on a large (>90%) reduction in the availability of DA at DA receptors (Ungerstedt, 1971). We observed that contralateral turning in response to apomorphine was markedly attenuated by casting prior to injury when compared to animals receiving a 6-OHDA alone ( $F_{(2, 18)} = 19.32$ ;  $p < 0.01$ ; Fig 2.2).



*Figure 2.1a: Effects of prior forced limb use on forelimb use asymmetry after 6-OHDA. Unilateral casting of a forelimb prior to ipsilateral infusion of 6-OHDA into the MFB prevented the limb use asymmetry associated with unilateral 6-OHDA lesion. Sham-lesioned animals showed no asymmetry of limb use while animals receiving 6-OHDA into the MFB without a cast showed a significant asymmetry of limb use (\*  $p < 0.01$  vs sham-lesioned animals). Animals that received a cast prior to 6-OHDA infusion displayed a significant decrease in limb use asymmetry (\*  $p < 0.01$  vs lesioned animals). All values are expressed as mean asymmetry score  $\pm$  S.E.M.*



*Figure 2.1b: Effects of prior forced limb use on forelimb akinesia after 6-OHDA. Forelimb akinesia was prevented by unilateral casting prior to ipsilateral 6-OHDA infusion into the MFB. Sham-lesioned animals showed no significant akinetic behavior. 6-OHDA-lesioned animals that were not fitted with a cast displayed significant akinetic behavior when compared to sham-lesioned animals (\*  $p < 0.01$ ). This akinetic behavior was prevented in casted/lesioned animals (\*  $p < 0.01$ , lesioned vs casted/lesioned) and comparable to behavior observed in sham-lesioned animals. All values are expressed as mean asymmetry score  $\pm$  S.E.M.*



*Figure 2.2: Effects of prior forced limb use on apomorphine-induced rotational behavior 28 d after 6-OHDA. Pre-6-OHDA forced limb use attenuated apomorphine-induced rotations. Sham-lesioned animals did not exhibit contralateral rotational behavior in response to apomorphine. 6-OHDA infusion increased the number of contralateral rotations in lesioned-animals (\* $p < 0.05$ , lesioned vs sham-lesioned). Forcing reliance on a forelimb prior to contralateral infusion of 6-OHDA greatly attenuated apomorphine-induced rotational behavior (\*  $p < 0.05$ , lesioned vs casted/lesioned animals). All values are expressed as mean rotations  $\pm$  S.E.M.*

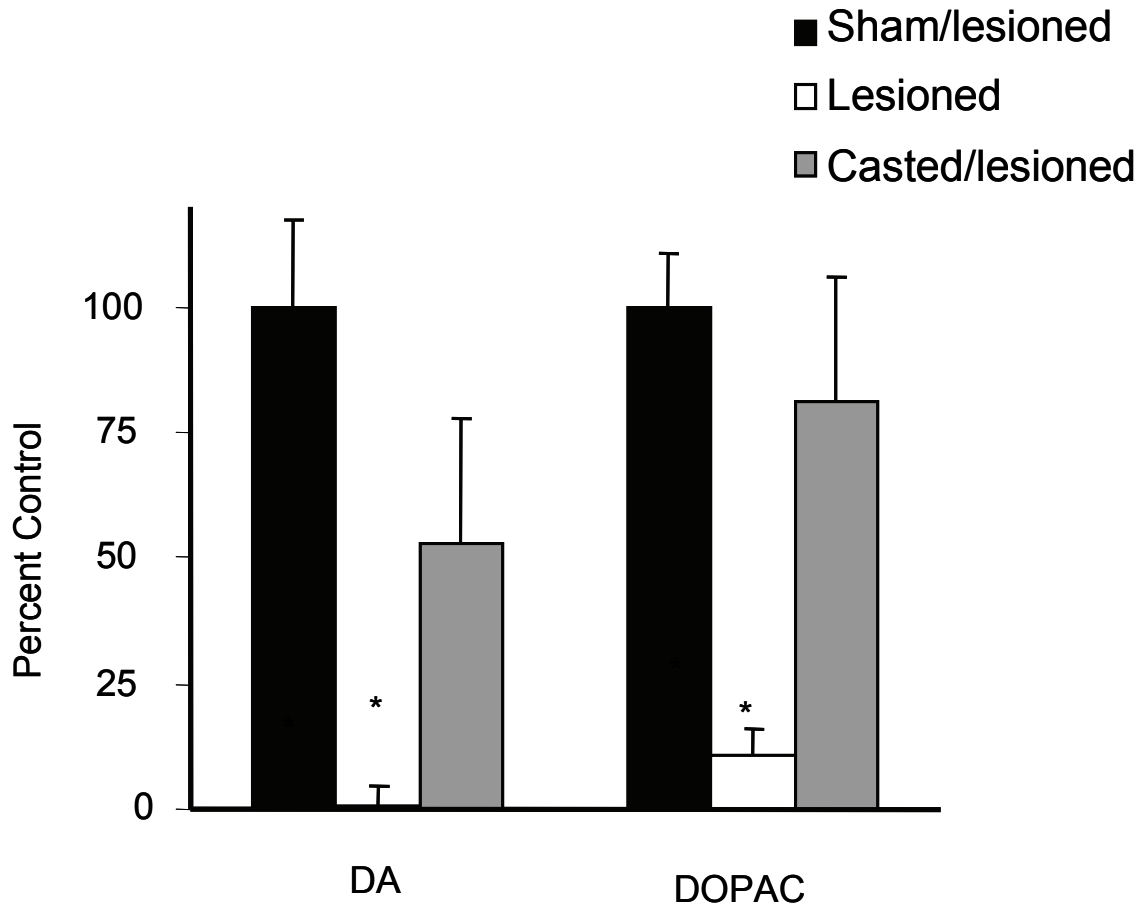
*Effect of casting prior to injury on 6-OHDA-induced changes in DA and DOPAC in the striatum*

There was no significant difference in striatal DA and DOPAC content between sham-lesioned animals and casted-sham-lesioned animals therefore data from these groups were pooled for further analysis ( $F_{(2, 2)} = 1.276$ ;  $p > 0.05$ ). A one-way ANOVA revealed a significant group effect for both DA and DOPAC content [DA: ( $F_{(2, 18)} = 10.03$ ;  $p < 0.01$ ); DOPAC: ( $F_{(2, 18)} = 11.16$ ;  $p < 0.01$ )]. 6-OHDA alone resulted in an almost complete loss of both striatal DA and DOPAC content compared to levels observed in sham-lesioned animals (Fig. 2.3;  $p < 0.01$ , shams vs. 6-OHDA alone). Post hoc analysis revealed a significant attenuation of the loss of striatal DA and DOPAC content in animals casted prior to 6-OHDA when compared to animals receiving 6-OHDA alone ( $p < 0.05$ ). No significant changes in striatal DA or DOPAC content were observed between groups in the non-infused hemisphere relative.

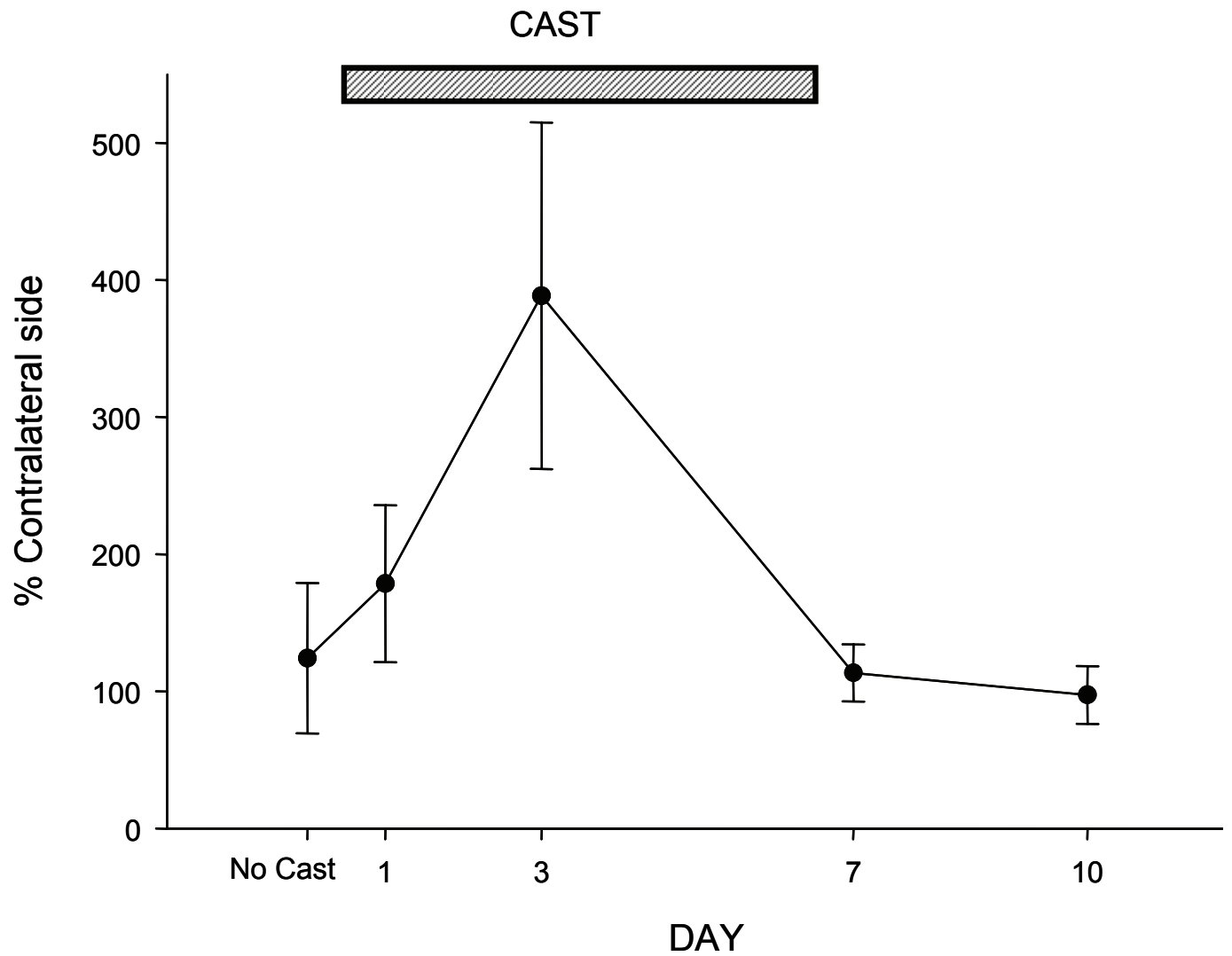
*Effect of casting on striatal GDNF*

Finally, we examined the profile of striatal GDNF protein levels during and after forced limb-use in Sprague Dawley animals that were not subjected to 6-OHDA. A one-way ANOVA revealed GDNF levels significantly increased in the striatum corresponding to the overused limb compared to non-casted control animals ( $F_{(2, 28)} = 5.934$ ;  $p < 0.01$ ), whereas GDNF levels in the opposite hemisphere were unchanged. Post hoc analysis indicated this increase was time dependent, increasing significantly above control 24 hr and 3 days after placement of the cast ( $p < 0.05$ ) and returned to near baseline levels on day 7 (Fig 2.4).





*Figure 2.3: Effects of prior forced limb use on striatal DA and its metabolites 30 d after 6-OHDA. Animals in the lesioned group showed a significant loss of DA and DOPAC when compared with sham-lesioned (DA:  $0.84 \pm 3.6\%$ , DOPAC:  $11 \pm 5\%$ , lesioned values;  $p < 0.01$ ) and casted/lesioned (DA:  $47 \pm 25\%$ ; DOPAC:  $81 \pm 21\%$ ;  $p < 0.05$ ) animals. This loss was attenuated in casted/lesioned animals such that no significant difference was detected between sham-lesioned and casted/lesioned animals. All results are expressed as mean percent of control  $\pm$  S.E.M., which were  $2.7 \pm 0.34$  ng/ 20  $\mu$ l for DA,  $1.2 \pm 0.16$  ng/ 20  $\mu$ l for DOPAC.*



*Figure 2.4: Effects of forced limb use on striatal GDNF levels. GDNF protein levels increased in a unilateral and time-dependent manner during unilateral casting. This increase was significant (\*  $p < 0.05$ ) at 72 hours after the cast was placed on the animal. The increase in GDNF was only observed in the striatum corresponding to the non-casted, overused limb. All results are expressed as a mean percent of contralateral side  $\pm$  S.E.M., which was  $0.31 \pm 0.04$  ng/mg wet tissue weight for GDNF.*

## 2.5 Discussion

Unilateral infusion of 6-OHDA along the MFB causes degeneration of DA neurons in the ipsilateral substantia nigra and loss of striatal DA and DOPAC content. This loss of striatal DA produces a number of motor deficits that include a tendency to limit the use of the contralateral limb during rearing, contralateral akinesia, and contraversive turning in response to the systemic administration of apomorphine (Ungerstedt, 1971; Marshall and Ungerstedt, 1977; Schallert and Tillerson, 2000). In this study, we report three observations. First, placing a cast on an animal for 7 days prior to the ipsilateral infusion of 6-OHDA prevented the development of behavioral deficits characteristic of this lesion model. Second, casting prior to injury greatly attenuated the loss of striatal DA. Third, casting prior to injury caused a significant but transient increase in striatal GDNF. These data extend our previous findings (Tillerson et al., 2001, 2002), indicating that casting the unimpaired forelimb for 7 days *prior* to and following 6-OHDA infusion protects against 6-OHDA toxicity.

It is our assumption that the ability of casting to protect against the behavioral effects of 6-OHDA results from the casting-induced attenuation of the loss of DA. The strongest support from this assumption comes from the ability of casting to greatly reduce the apomorphine-induced rotation normally associated with unilateral 6-OHDA infusions into the nigrostriatal DA projections. Our working hypothesis is that the casting-induced attenuation of the loss of striatal DA was, in turn, a result of an exercise-induced increase in GDNF in the region that subsequently received 6-OHDA. The neuroprotective effects of GDNF are well known (see Introduction). Although GDNF had declined to baseline prior to 6-OHDA infusion, this does not preclude GDNF as a possible participant in the neuroprotective effects of forced limb-use. Indeed, previous investigators have shown that exogenous GDNF can be neuroprotective if

administered as much as 7 days prior to 6-OHDA (Kearns & Gash, 1995; Choi-Lundberg et al., 1998), and in those experiments it is unlikely that levels of this trophic factor were still elevated at the time of toxin administration.

To be effective, GDNF would need to act on its receptor complex, thereby initiating a signaling cascade that produces cytoplasmic and/or translational changes in DA neurons. It is possible that these downstream effects of GDNF are activated after the protein has returned to baseline levels. Moreover, extracellular signal regulated kinase (ERK), a downstream effector of GDNF, has been shown to be activated after exercise, and to remain elevated for up to one month (Shen et al, 2001)

Although we have focused our initial attention on GDNF, there are many other factors that may play a role in the casting-induced protection that we observed. Exercise, primarily running, has been shown to increase brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Neeper et al., 1996), fibroblast growth factor-2 (FGF-2) (Gomez-Pinilla et al., 1995), and insulin-like growth factor 1 (IGF-1) (Carro et al., 2001) in certain regions of the CNS, particularly the hippocampus. Although there are no previous reports on the effects of exercise on trophic factors in the striatum, each of these factors has potent trophic activity towards DA neurons (Hyman et al, 1991; Altar et al., 1992; Lin et al., 1993; Winkler et al., 1996) and, where examined, has been shown to protect against the neurotoxic effects of 6-OHDA (Hoffer et al., 1994; Kearns & Gash, 1995; Levivier et al., 1995; Choi-Lundberg et al., 1998; Akerud et al., 1999; Gong et al., 1999; Kramer et al., 1999; Schatz et al., 1999; Shults et al., 2000; Wang et al., 2002). For example, rats exposed to enriched environments, exercise regimens, dietary restriction, and learning tasks all show an increase in protein and mRNA levels of trophic factors in the brain (Neeper et al., 1995; Gomez-Pinilla et al., 1998; Torasdotter et al., 1998; Pham et al.,

1999; Duan et al., 2001; Young et al., 1999). Many of these environmental changes are protective against hippocampal damage incurred from insults, such as ischemia, and it has been proposed that this neuroprotection is driven by increases in trophic factors (Duan et al, 2001; Young et al, 1999). Moreover, it seems likely that more than one factor will ultimately be found to be responsible for exercise-induced protection. In this regard, we note that FGF-2, one of the trophic factors shown to be upregulated by exercise (Gomez-Pinilla et al, 1995), has also been shown in vitro to induce expression of both GDNF and BDNF (Suter-Crazzolara and Unsicker, 1996; Kwon, 1997).

In conclusion, these data suggest that forced limb-use is protective against subsequent injury to the nigrostriatal DA system and this protection is accompanied by increases in striatal GDNF. These findings provide further evidence that physical therapy may be beneficial to patients with PD, as well as the possibility that exercise throughout life may protect against development of PD. In fact, our group has recently shown that forced use is not only protective but that lack of use may exacerbate damage associated with 6-OHDA, indicating that motor weakness early in the disorder may be an exacerbating factor (Tillerson et al, 2002). As early detection methods are developed, physical activity may be an important factor in slowing or even halting the neurodegenerative cascade associated with PD.

### **3.0 Effects of forced limb use on the vulnerability of dopamine neurons to 6-hydroxydopamine**

#### **3.1 Abstract**

Unilateral infusion of 6-OHDA into the MFB of the adult rat causes contralateral motor deficits and decreases in the apparent number of DA cell bodies and terminals in the SN and striatum, respectively. However, we have previously shown that casting of the ipsilateral forelimb for 7 days prior to 6-OHDA, causing forced use of the contralateral forelimb, significantly reduces this apparent neurodegeneration. It remains unknown, however, if the protection from 6-OHDA-induced depletion of striatal DA reflects a decrease in the vulnerability of SN neurons to 6-OHDA, a regeneration of processes after partial injury, or sprouting from undamaged axons into a region of DA terminal loss. In this report we show that animals casted prior to 6-OHDA infusion showed significant attenuation of normal toxin-induced loss of both tyrosine hydroxylase immunoreactive and Nissl-stained cells in the SN. Precasted animals also showed a significant sparing of tyrosine hydroxylase positive terminals in the striatum. These data suggest that prior forced limb use produces behavioral and neurochemical protection by decreasing the vulnerability of DA neurons to oxidative stress.

## 3.2 Introduction

PD is a progressive neurodegenerative disorder of unknown etiology that results in the loss of DA neurons projecting from SN to striatum. The most common treatments for PD focus on alleviating symptoms of the disorder by temporarily restoring dopaminergic tone in the striatum. However, these treatments do not appear to reduce the progression of the disease. Moreover, side effects of these treatments gradually emerge and efficacy usually is reduced (Pahwa and Lyons, 2004). Thus, developing strategies for slowing or preventing ongoing degeneration or even reversing the neurodegenerative process in PD is of paramount importance.

It is well established that exercise regimens after ischemic insult can produce functional recovery following brain injury. Constraining the non-impaired upper extremity in stroke patients, thereby forcing use of the impaired limb, improves motor function (Taub et al., 1999; Liepert et al., 2000; Taub and Morris, 2001; Sterr et al., 2002). Additionally, exercise and enriched environments prior to injury have been shown to be neuroprotective in animals subjected to a variety of neurotoxic conditions, including ischemia, (Stummer et al., 1994, Li et al., 2004), 3-acetylpyrine, domoic acid, (Carro et al. 2001), and genetic modifications associated with Alzheimer's disease (Adlard et al., 2005; Jankowsky et al., 2005; Lazarov et al., 2005).

Recent evidence suggests that exercise can be neuroprotective in PD as well. Increases in physical activity levels throughout life have been associated with a lower propensity to develop PD (Sasco et al., 1992; Tsai et al., 2002; Hirsch et al., 2003; Chen et al., 2005). There is also evidence for efficacy of physical therapy regimens in the treatment of PD (Toole et al., 1999, Bergen et al., 2002). Animal models provide further support for the hypothesis that exercise can be neuroprotective against PD. For example, treadmill and exercise in a running wheel, as well

as an enriched environment, has been shown to reduce the effects of another DA-selective neurotoxin, MPTP in a mouse model (Bezard et al., 2003; Tillerson et al., 2003; Faherty et al., 2005). Our own research has focused on the use of 6-OHDA to produce models of DA deficiency. Using a unilateral 6-OHDA rat model, we and our colleagues have recently shown that placing a cast on one forelimb for 7 days immediately prior to or following the unilateral administration of 6-OHDA can attenuate both the behavioral and neurobiological effects of the toxin (Tillerson et al., 2001; 2002; Cohen et al., 2003). However, it is not known in any of these cases whether the apparent neuroprotection reflects a decrease in the vulnerability of DA neurons to the toxin, a regeneration of damaged DA axons, or the sprouting of DA axon terminals from non-damaged neurons into areas initially affected by the toxin. Therefore, in the present study we have examined the profile of cell loss and degeneration of DA neurons and terminals in the nigrostriatal pathway over time in response to forced limb use followed by infusion of 6-OHDA into the MFB in adult rats. Our results suggest that exercise acts to reduce the vulnerability of DA neurons to the toxin.

### **3.3 Materials and Methods**

#### *Animals*

Male Sprague Dawley rats (Hilltop Lab Animals, Scottdale, PA) weighing 300-400 grams were used throughout these experiments. All animals were housed two per cage and maintained on 12 h light/dark cycle with food (Purina lab chow) and water available ad libitum. All procedures were in strict accordance with the guidelines for the NIH Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.



### *Preoperative and Surgical Procedures*

Seven days prior to surgery animals were randomly assigned to one of four groups: unilateral, intracerebral vehicle injection (n = 6), vehicle injection preceded by a forelimb cast (n = 6), unilateral infusion of 6-OHDA alone (n = 14), and 6-OHDA preceded by a forelimb cast (n = 13). Animals in the two casted groups were fitted with plaster of Paris casts to immobilize the right forelimb in a naturally retracted position against the sternum (Jones and Schallert, 1994; Cohen et al., 2003). After the seven days, all animals were anesthetized with Equithesin (25 mg/kg pentobarbital and 150 mg/kg chloral hydrate, i.p.), the casts were removed (if present), and the animals were prepared for surgery. Thirty minutes prior to surgery, animals were given desipramine (25 mg/kg, i.p.), an inhibitor of norepinephrine uptake that serves to block the entry of 6-OHDA into noradrenergic neurons. 6-OHDA (Sigma, St. Louis, MO; 3 µg in 2 µl of 0.9% NaCl, 0.02% ascorbic acid) or vehicle was infused (0.5 µl/min) into the right MFB (- 4 mm posterior, - 2.0 mm lateral of bregma, and 8 mm ventral to dura, according to the atlas of Paxinos and Watson (1982).

### *Behavioral Analysis*

A separate group of animals was examined to determine if forced limb use produces an increase in use of the non-casted forelimb. Animals were videotaped in their home cage during their awake (dark) cycle for a five minute period, the videotapes were the analyzed, and right, left and both forelimb movements were counted (Schallert and Tillerson, 2000). After the first night of testing, animals were anesthetized with Equithesin and randomly divided into two groups, one group (n = 6) was fitted with a plaster of Paris cast and the second group (n = 5) was

allowed to use both forelimbs freely. Animals were then videotaped 2, 3, 5, and 6 days after placement of the cast.

### *Histological Analysis*

At 2, 7, or 28 d post-infusion, animals were deeply anesthetized with Equithesin and sacrificed via transcardial perfusion using ice cold 0.9% saline followed by 4% paraformaldehyde. Brains were then sectioned at 60 microns using a cryostat. Every fifth section cut from the SN and striatum was collected for labeling with the antiserum for tyrosine hydroxylase (TH) or Nissl substance and sections from each group were processed together.

**TH<sup>+</sup> immunoreactivity** All sections were rinsed 3 times in 10 mM phosphate buffered saline (PBS) prior to and between each incubation. The sections were pretreated for 15 min with 3% H<sub>2</sub>O<sub>2</sub> in 10 mM PBS followed by blocking for 1 hr with 5% normal goat serum and then incubated overnight at 4°C in a 1:1000 dilution of mouse anti-TH antibody (Chemicon Inc, Temecula, CA; Cat # MAB318) in normal goat serum. This was followed by incubation for 1 hr at room temperature in a 1:200 dilution of biotinylated goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA). Tissue was then treated with avidin biotin peroxidase complex (ABC-Elite, Vector Laboratories, Burlingame, CA) with 3, 3'-diaminobenzadine as the chromagen to visualize the reaction. Sections were then mounted on gelatin coated slides, dehydrated in ascending concentrations of ethanol and coverslipped with Permount mounting medium (Fisher Scientific, Pittsburgh, PA).

**Nissl substance:** Nissl-positive cells were stained using a solution of 10% thionin (Sigma, St. Louis, MO). Sections were dehydrated through increasing concentrations of ethanol, defatted in chloroform and rehydrated through decreasing concentrations of ethanol. Sections

were then placed in thionin for 45 sec and rinsed in increasing concentrations of ethanol and coverslipped with Permount mounting medium (Fisher Scientific, Pittsburgh, PA).

### *Image Analysis*

**Densitometry in striatum:** Densitometric analysis was performed in the striatum using a M5 MCID system (Imaging Research Inc., Ontario, Canada). For quantification of TH<sup>+</sup> staining, the entire striatum was visualized under the camera and then analyzed for density of pixels present in each striatum.

**Stereology in SN:** For stereological analysis of cell counts in the SN, every third section from each animal was randomly selected for staining using a random number generated from a computer program. We then used systematic random sampling (using the fractionator approach) to determine cell number estimates using Stereoinvestigator software coupled to Microvid systems (Microbrightfield Inc.) (e.g., Pierri et al., 2001; Volk et al., 2001).

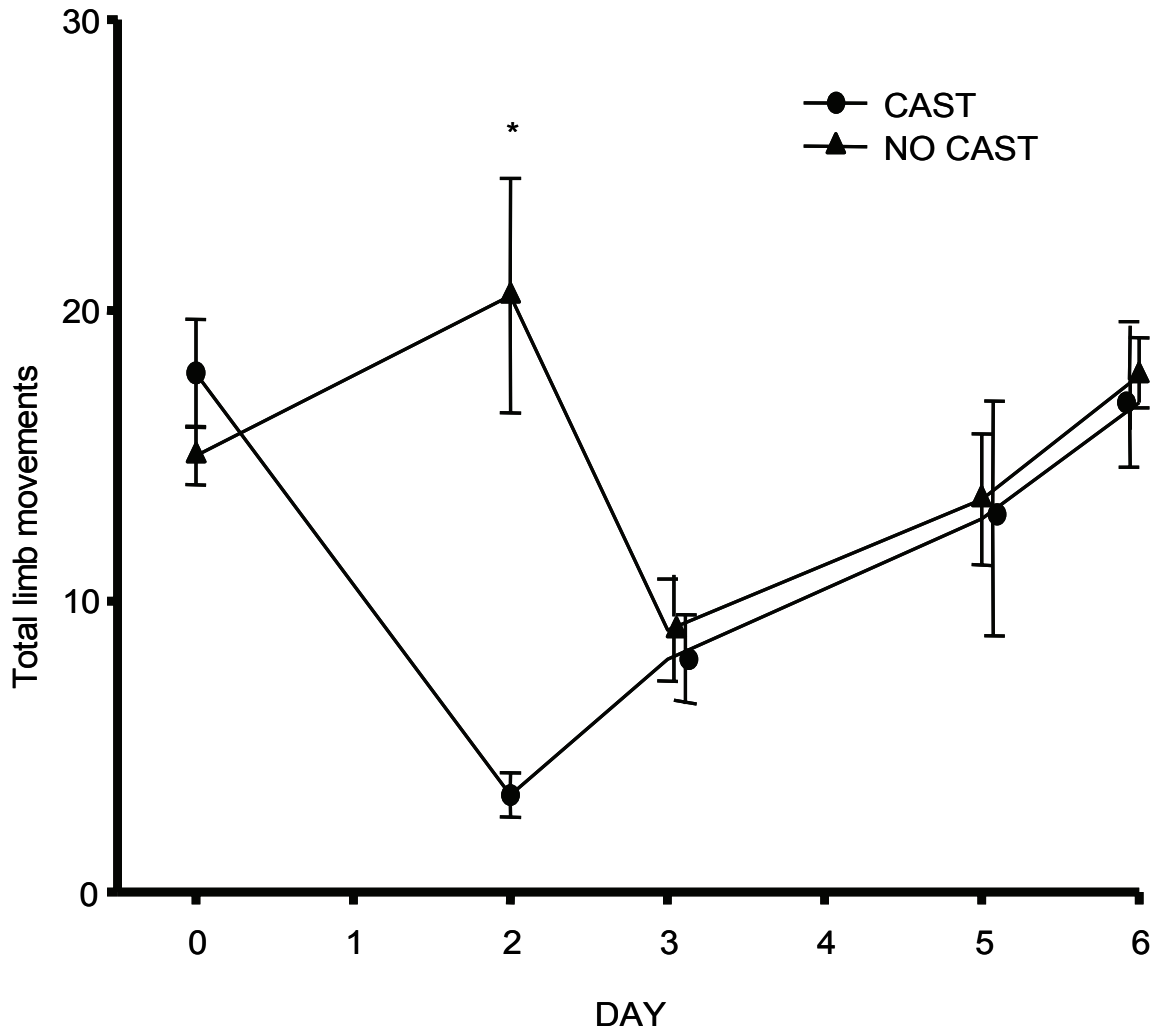
### *Statistical Analysis*

Data were analyzed with a one-way ANOVA, using Bonferonni-corrected multiple comparison tests for post hoc analysis. There was no significant difference between striatal or nigral measures in the two vehicle treated groups (vehicle-injected with or without a cast). Therefore these groups were pooled for further analysis (striatum:  $F_{(3, 2)} = 4.395$ ;  $p > 0.05$ ; SN:  $F_{(3, 2)} = 3.639$ ;  $p > 0.05$ ). No significant changes in cell number or terminal density were observed between groups in the non-infused hemisphere in any measures. All comparisons were made to vehicle treated animals.

### 3.4 Results

#### *Effect of forced limb use on limb use in the home cage*

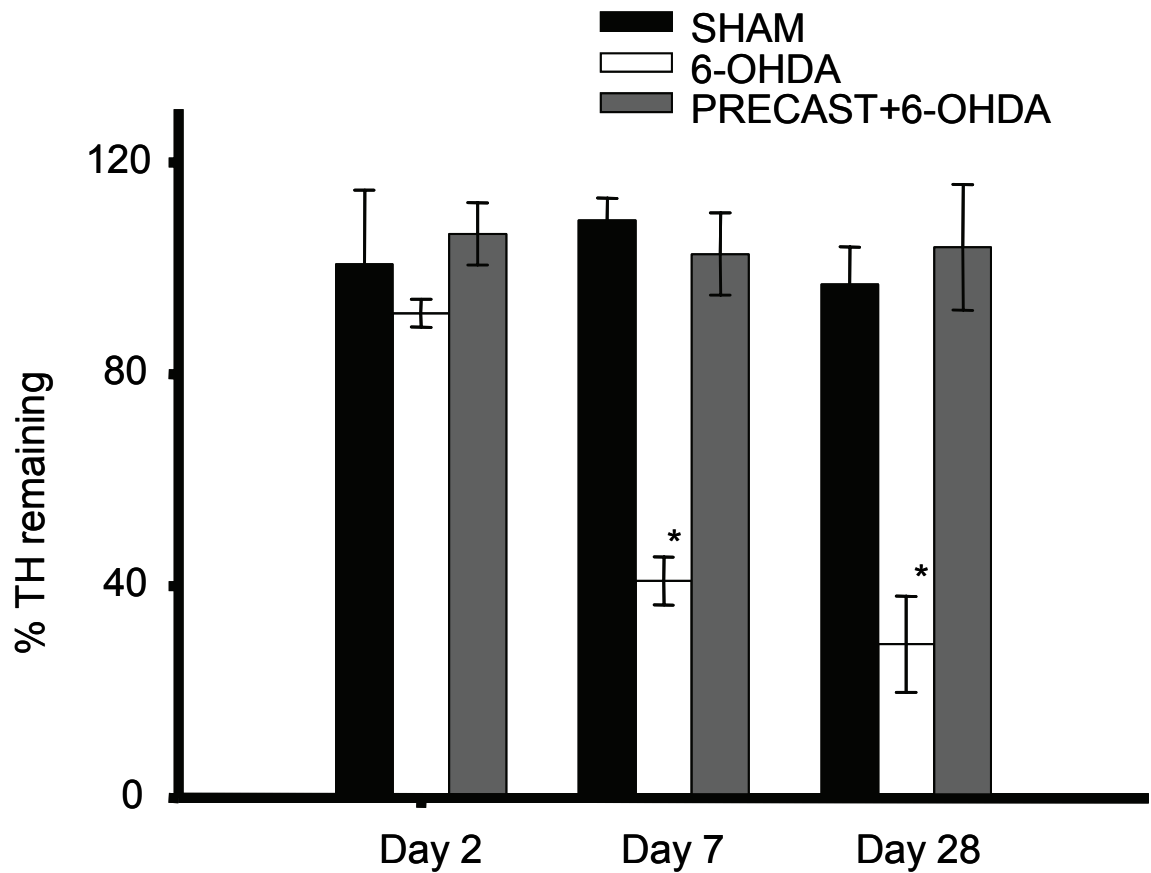
In order to determine if forced limb use produced an increase in limb use, we monitored behavior in the home cage in casted and uncasted animals. Over the 7 d observation period, normal animals placed their right and left forelimbs on the wall of the home cage approximately 15 times in 5 min. When casted animals were observed 2d after placement of the cast there was a significant decrease in the number of left, as the right limb was constrained, forelimb placements ( $3.3 \pm 0.7$  placements,  $p < 0.001$ , Fig 3.1). By 3 d after placement of the cast, animals began to use their single forelimb as much as non-casted animals used both forelimbs. As a result, there was no significant difference in limb use. This increased use of the uncasted limb persisted for the 6-day period examined.



*Figure 3.1: Effects of unilateral casting on limb placements in the homecage. Animals in the casted group showed a significant decrease ( $p < 0.001$ ) in placements of the non-casted forelimb at 2d after placement of the cast that was attenuated by 3d post-cast. By 3d post –cast casted animals were using their non-casted forelimb to the same degree that non-casted animals used both forelimbs.*

*Effect of forced limb use on the 6-OHDA-induced loss of TH<sup>+</sup> terminals in the striatum*

Neither the sham nor the precast groups exhibited any significant difference in striatal TH<sup>+</sup> density at 2, 7 and 28 d ( $p > 0.05$ ). In contrast, a significant difference in striatal TH<sup>+</sup> density was revealed between animals treated with 6-OHDA alone and sacrificed at 2 d and those sacrificed at 7 and 28 d ( $p < 0.001$ ). Although TH<sup>+</sup> was unaffected by 6-OHDA at 2 d postoperative ( $n = 4$ ), animals treated with 6-OHDA alone ( $n = 5$ ) had a  $65.3 \pm 3.9\%$  loss of TH immunoreactivity (TH<sup>+</sup>) in the striatum at 7 d postoperatively when compared to vehicle treated animals ( $n = 8$ ) who showed no loss of TH. This loss of TH was sustained at 28 d with animals receiving 6-OHDA alone ( $n=5$ ) showing a  $71.1 \pm 9.1\%$  ( $p < 0.001$ ; Fig 3.2). Forced limb use prior to infusion of 6-OHDA ( $n = 9$ ) significantly reduced the loss of TH observed as a result of 6-OHDA alone ( $p < 0.001$ ) such that there was no difference between these animals and vehicle treated control animals at either 7 or 28d ( $0.0 \pm 7.9$  vs.  $0.0 \pm 11.0\%$ , ns). [Overall group effect: ( $F_{(8, 25)} = 14.3$ ;  $p < 0.0001$ ; Fig 3.2)]



*Figure 3.2: Effects of unilateral casting on TH<sup>+</sup> terminals in the striatum after 6-OHDA. Loss of TH was prevented by casting of a forelimb prior to subsequent ipsilateral 6-OHDA infusion into the MFB. Sham animals showed no significant loss of TH. 6-OHDA animals that were not fitted with a cast displayed significant loss of TH when compared to sham animals (\*  $p < 0.01$ ). This loss was prevented in casted/6-OHDA treated animals (\*  $p < 0.01$ ), 6-OHDA vs casted/6-OHDA). All values are expressed as percent loss of TH<sup>+</sup> density ( $\pm$  SEM) compared to control animals.*

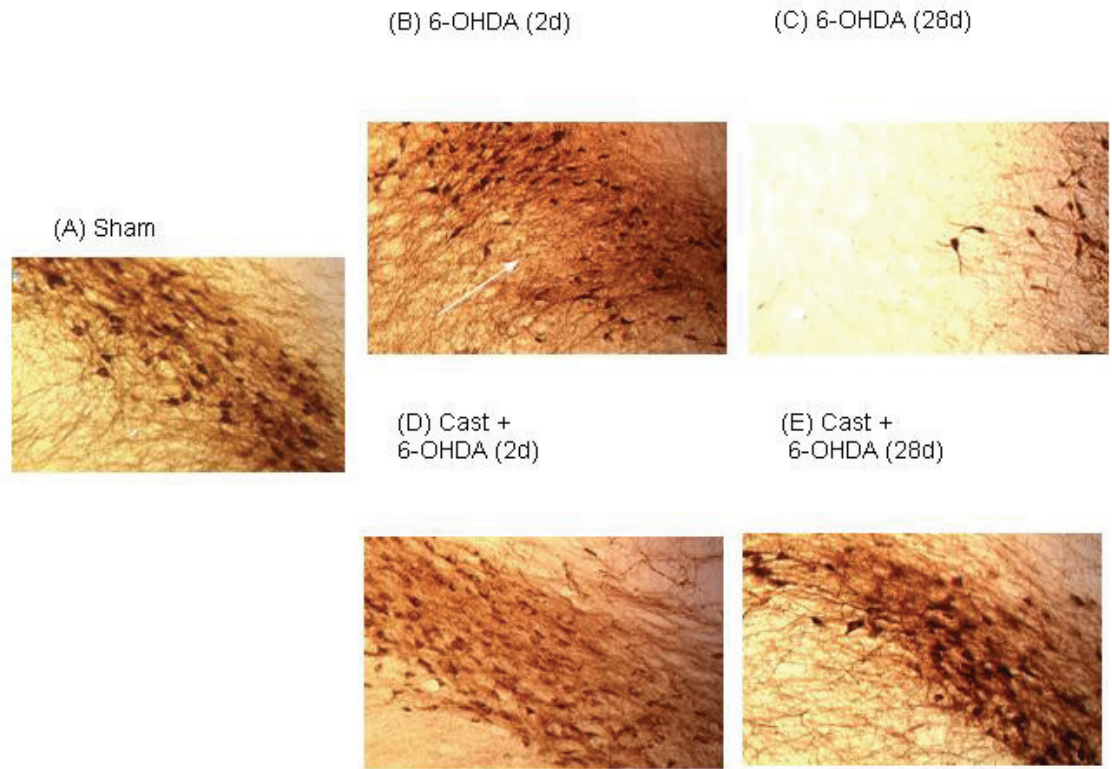
*Effect of forced limb use on the 6-OHDA-induced loss of TH<sup>+</sup> neurons in the SN*

Animals treated with 6-OHDA alone (n=10) showed an  $86.6 \pm 4.4\%$  loss of TH<sup>+</sup> cells in the SN at 7 and 28 d after 6-OHDA alone when compared to a  $0.0 \pm 3.26\%$  loss in vehicle treated animals (n = 8). Animals exposed to forced limb use prior to 6-OHDA (n=9) showed a significant reduction in loss of TH<sup>+</sup> cells in the SN ( $5.6 \pm 10.5\%$ ;  $p < 0.001$ ) when compared to 6-OHDA alone. Additionally, both the sham and precast groups showed no significant difference in the number of TH<sup>+</sup> neurons across 2, 7 and 28 d ( $p > 0.05$ ) while a significant difference in the number of TH<sup>+</sup> neurons was revealed between animals treated with 6-OHDA alone and sacrificed at 2d ( $62.2 \pm 14.5$ ) and those sacrificed at 7 ( $12.9 \pm 2.4$ ) and 28 d ( $13.4 \pm 10.5$ ) ( $p < 0.001$ ). [Overall group effect: 7 d ( $F_{(2, 13)} = 49.6$ ;  $p < 0.001$ ); 28 d ( $F_{(2, 12)} = 137.8$ ;  $p < 0.001$ ) Fig 3.3a&b]

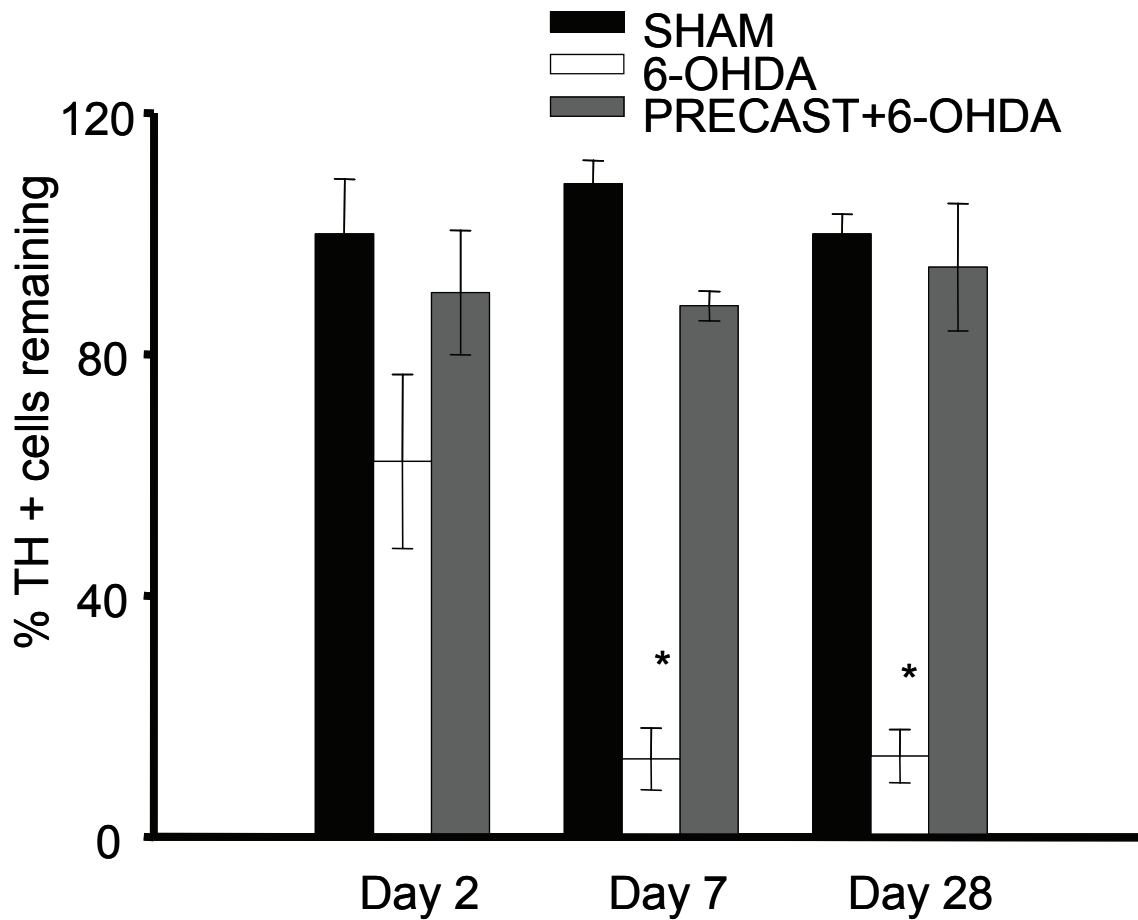
*Effect of forced limb use on the 6-OHDA-induced loss of Nissl positive neurons*

6-OHDA induced loss of TH<sup>+</sup> cells in the SN could be a result of a loss of the TH phenotype rather than the degeneration of the terminal itself. Therefore, we examined the number of Nissl<sup>+</sup> cells in the SN. A significant loss of Nissl<sup>+</sup> cells was observed at 7 and 28 d after 6-OHDA alone (n=10) when compared to vehicle treated animals ( $42.8 \pm 6.2\%$ ;  $p < 0.001$ ). Casting prior to 6-OHDA (n=9) completely blocked cell loss ( $0.0 \pm 4.1\%$ ) showing a significant difference from animals treated with 6-OHDA alone ( $p < 0.001$ ). A comparison of animals treated with vehicle alone (n=8) and those casted prior to 6-OHDA revealed no significant change in the number of Nissl<sup>+</sup> positive cells ( $p > 0.05$ ). Finally, there were no differences in any groups across 2, 7, and 28 d. [Overall group effect: 7 d ( $F_{(2, 13)} = 80.4$ ;  $p < 0.001$ ); 28 d ( $F_{(2, 13)} = 12.5$ ;  $p < 0.01$ ) Fig 3.4a&b]





***Figure 3.3a: Photomicrographs of TH<sup>+</sup> cells in the SN. Sham animals (a) showed no loss of TH<sup>+</sup> cells, while 6-OHDA treated animals showed little loss of TH<sup>+</sup> cells at 2d post-6-OHDA (b and arrow) and a significant loss at 28d post-6-OHDA (c). (D) Animals casted prior to 6-OHDA showed no loss of TH<sup>+</sup> cells at 2d post-6-OHDA and a significant attenuation at 28d post 6-OHDA (e).***



*Figure 3.3b: Effects of unilateral casting on loss of TH<sup>+</sup> cells in the SN after 6-OHDA. Pre-6-OHDA forced limb use attenuated loss of TH<sup>+</sup> cells in the SN. Sham animals had no detectable loss of TH<sup>+</sup> cells in the SN. 6-OHDA infusion significantly decreased the number of cells in the SN at 7 and 28 days post infusion in 6-OHDA treated animals (\* $p < 0.001$ , 6-OHDA vs sham). Forcing reliance on a forelimb prior to contralateral infusion of 6-OHDA greatly attenuated loss of TH<sup>+</sup> cells in the SN (\* $p < 0.001$ , 6-OHDA vs casted/6-OHDA animals). All values are expressed as percent cell loss (compared to controls)  $\pm$  S.E.M.*

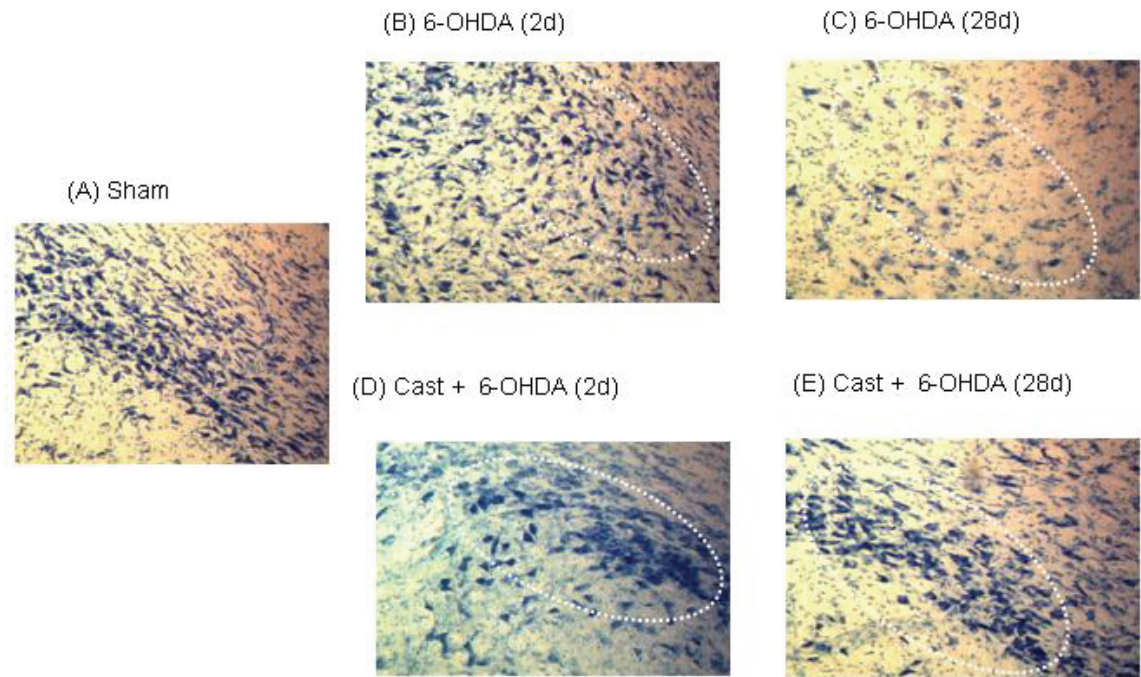
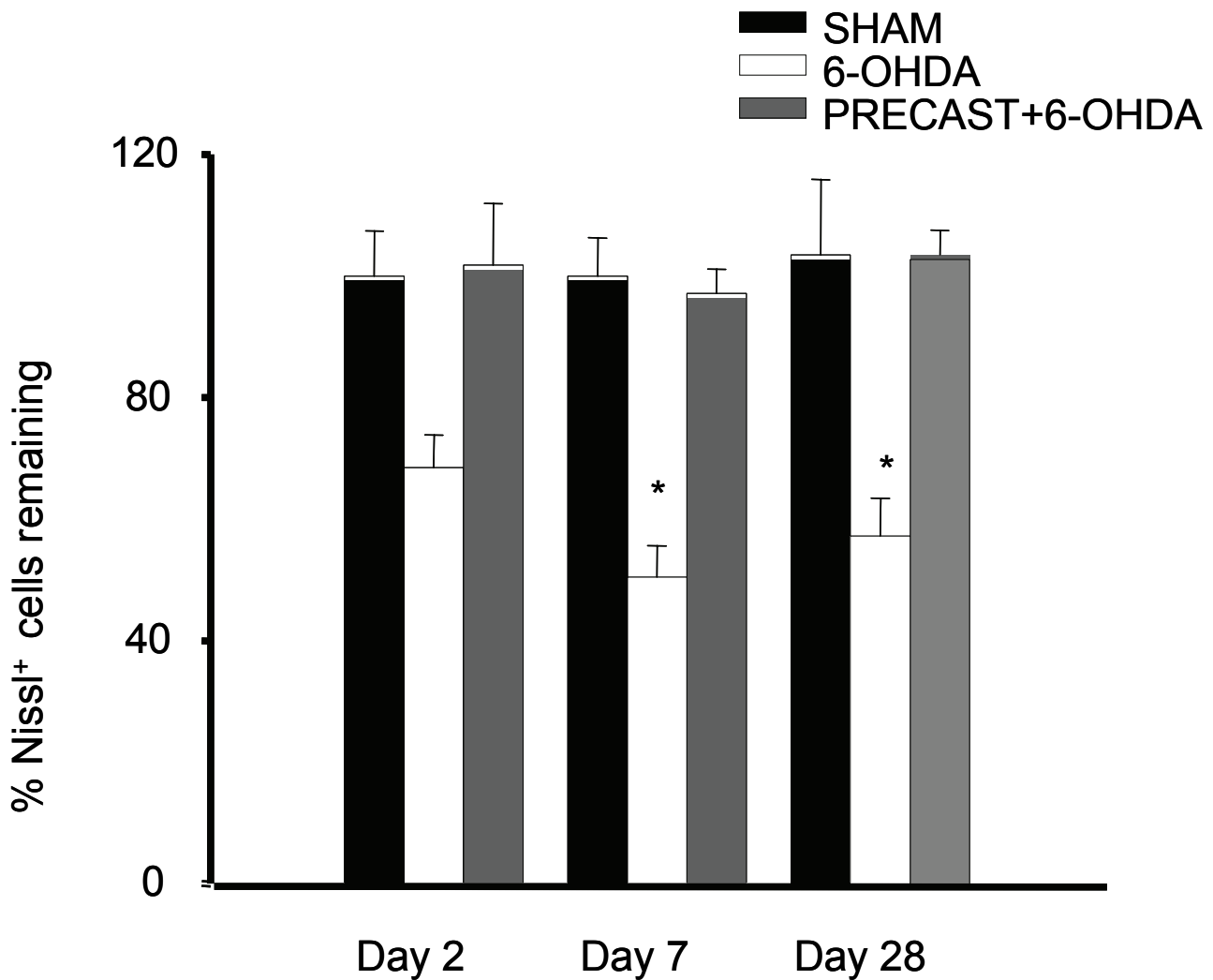


FIGURE 3.4a

*Figure 3.4a: Photomicrographs of Nissl<sup>+</sup> cells in the SN. Sham animals (a) showed no loss of nissl<sup>+</sup> cells, while 6-OHDA treated animals showed non-significant loss of Nissl<sup>+</sup> cells at 2d post-6-OHDA (b) and a significant loss at 28d post-6-OHDA (c). (D) Animals casted prior to 6-OHDA showed an attenuation loss of TH<sup>+</sup> cells at 2d post-6-OHDA and a significant attenuation at 28d post 6-OHDA (e).*



*Figure 3.4b: Effects of unilateral casting on loss of Nissl<sup>+</sup> cells in the SN. Animals in the 6-OHDA group showed a significant loss of Nissl<sup>+</sup> cells in the SN when compared with sham ( $p < 0.01$ ) and casted/6-OHDA animals ( $p < 0.01$ ) animals. This loss was attenuated in casted/6-OHDA animals such that no significant difference was detected between sham and casted/6-OHDA animals. All results are expressed as mean percent of control  $\pm$  S.E.M.*

### 3.5 Discussion

Current treatments for PD, such as L-dopa, only alleviate the symptoms of the disease and can lose their efficacy over time (Pahwa and Lyons, 2004). Thus, it is important that new therapeutic approaches that slow or block the neurodegenerative process be explored. One such approach is exercise. Exercise has been known for some time to have beneficial effects on the brain (for review see Mattson, 2000) but until recently was not widely explored in the treatment of PD. We have previously shown that forced exercise prior to 6-OHDA protects from both the behavioral and neurochemical effects of the toxin (Cohen et al., 2003). However, it remained unknown if this apparent neuroprotection was a result of sparing of nigrostriatal DA neurons and terminals or of sprouting of remaining neurons into areas primarily affected after administration of 6-OHDA. The present study was undertaken to more directly determine if forced limb use prior to 6-OHDA protects DA neurons from the toxic effects of 6-OHDA or if it stimulates sprouting in unaffected neurons following 6-OHDA.

We used immunohistological analysis to determine the effect of prior forced limb use on TH immunoreactive terminals and cells in the striatum and SN respectively after 6-OHDA toxicity. Previous studies have shown that a toxic insult such as MPTP or 6-OHDA can result in a loss of the TH phenotype without loss of actual DA neurons (Jackson-Lewis et al., 1995; Ara et al., 1998; Rosenblad et al., 2003). Thus, to distinguish between actual cell death and loss of the TH phenotype in the SN, we stained serial sections in the SN with TH or Nissl. After 6-OHDA treatment, a significant loss of TH<sup>+</sup> cells was observed by 7 days post-infusion, and persisted at least to 28 days. Additionally, 6-OHDA caused a similar loss of cells positive for Nissl substance indicating that 6-OHDA produces cell death in the SN rather than only a loss of the TH phenotype in these neurons. However, forced limb use blocked both the loss of TH<sup>+</sup> cells and

cells positive for Nissl substance at 7 and 28 d after infusion of 6-OHDA. These data support the hypothesis that forced limb use for the 7 d prior to 6-OHDA infusion prevents degeneration in the nigrostriatal DA system

We also observed that prior forced limb use prevents the loss of TH<sup>+</sup> terminals in the striatum. 6-OHDA caused a significant loss of TH<sup>+</sup> terminals in the striatum that was not detectable 2 d after 6-OHDA infusion but was complete by 7 d post-infusion. In contrast, animals exposed to prior forced limb use and 6-OHDA showed no sign of loss of TH<sup>+</sup> terminals in the striatum over a 28 d period. These data are in agreement with our previous finding in which we observed a protection of striatal DA content at 4 wks in 6-OHDA treated animals that underwent prior forced limb use. The sparing of DA terminals in the striatum further suggests that forced limb use confers a resistance of the nigrostriatal DA system to the effects of 6-OHDA.

In order to determine if casting a forelimb increases the use of the non-casted forelimb, we observed casted and non-casted animals in their home cage during the casting period. We determined that 2 d after placement of the cast, casted animals used their forelimb significantly less than non-casted animals. However, this effect was attenuated by 3 d post-cast. Indeed, not only did casted animals begin to use their non-casted limb as much as the corresponding limb of non-casted animals but also they began to use the non-casted limb as much as non-casted animals used both forelimbs. These data indicate that the protective effects of forced limb use could function through an increase in use of the non-casted forelimb.

The neuroprotective effects of forced limb use on TH in the nigrostriatal system and cell survival in the SN are consistent with our previous observations demonstrating the neuroprotective effects of prior forced limb use on DA levels in the striatum and behavior

(Cohen et al., 2003). Protection of behavior and DA content also has been observed with prior exposure to enriched environment and/or a running wheel in mice (Bezard et al., 2003; Faherty et al., 2004), casting following 6-OHDA in rats (Tillerson et al., 2001; 2002), and treadmill running following MPTP in mice (Tillerson et al., 2003). Collectively they demonstrate that under our conditions behavioral protection from 6-OHDA can be associated with protection of both DA neurons in the SN and DA content within striatal terminals.

Several other groups have demonstrated exercise-induced protection from the behavioral effects of MPTP and 6-OHDA. However, in some instances DA neurons have not been directly examined (Fisher et al., 2004; Mabandla et al., 2004; Howells et al., 2005). Moreover, in one case in which DA neurons have been examined, no protection was observed (Poulton & Muir, 2005). These data raise the possibility under some conditions preservation of DA terminals is not crucial for the protection of behavior after a toxic insult to the DA system. Although alternative explanations have not been carefully explored, compensatory changes in residual DA neurons have been proposed to underlie the ability of animals to sustain partial DA loss without severe behavioral consequences (Zigmond, 2000); perhaps exercise can promote such compensations under some circumstances. It is possible that exercise may stimulate remaining DA neurons to function at a higher than normal level in order to prevent behavioral consequences that would normally be associated with the level of loss of DA neurons observed.

Our working hypothesis is that the casting-induced attenuation of the loss of DA neurons and terminals was, in turn, a result of an exercise-induced increase in trophic factors in the nigrostriatal bundle. Indeed, we have shown that forced limb use increases GDNF in the striatum associated with the overused forelimb (Cohen et al., 2003). Additionally, exercise, primarily running, has been shown to increase trophic factors in certain regions of the CNS, particularly

the hippocampus (Gomez-Pinilla et al., 1995, 1998; Neeper et al., 1996; Carro et al., 2001; Faherty et al., 2005). Whereas multiple forms of exercise have been shown to increase trophic factors in various parts of the brain our paradigm is unique in several ways. First, we have shown that forced limb use not only increases GDNF in the striatum corresponding to the “exercised” forelimb but also confers protection from the behavioral, neurochemical, and neuroanatomical effects of a 6-OHDA infusion. Second, forced limb use differs from other types of exercise, such as treadmill running, in its duration and intensity possibly explaining the difference in neuroprotection conferred. These factors have potent trophic activity towards DA neurons (Hyman et al, 1991; Altar et al., 1992; Lin et al., 1993; Winkler et al., 1996) and, where examined, has been shown to protect against the neurotoxic effects of 6-OHDA (Hoffer et al., 1994; Kearns & Gash, 1995; Choi-Lundberg et al., 1998; Akerud et al., 1999; Gong et al., 1999; Schatz et al., 1999; Shults et al., 2000; Wang et al., 2002). Although it has been widely shown that exercise increases trophic factor production, it is unknown how exercise functions to stimulate trophic factor production. Moreover, although, trophic factors provide a promising target for the neuroprotective effects of exercise, the relationship is correlative. Further experiments examining the casual relationship between increases in trophic factors and the neuroprotective effects of exercise need to be performed before further conclusions can be drawn.

Although we have chosen to mainly focus on increases in trophic factors as the mechanism for forced-limb use induced neuroprotection, there are other factors that may play a role. First, although forced limb use has positive effects on the brain, it also can be regarded as a form of restraint stress and thus, would be expected to stimulate the hypothalamic-pituitary-adrenal (HPA) axis. Normally conceived of as neurotoxic, stress can also have neuroprotective



effects (Sloviter et al., 1989; Chadi et al., 1993; Follesa and Mocchetti, 1993; Mocchetti et al., 1996; Sousa et al., 1999). It is possible, therefore, that the HPA axis plays a pivotal role in forced limb use protection through stimulated neurotrophic factor expression or through the production of melanocortins, both of which have been shown to have neuroprotective effects in various peripheral and central nervous system injury models (Sloviter et al., 1989; Chadi et al., 1993; Follesa and Mocchetti, 1993; Mocchetti et al., 1996; Sousa et al., 1999).

There are many other factors that may play a role in the casting-induced protection that we observed. Exercise, primarily running, has been shown to increase brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Neeper et al., 1996), fibroblast growth factor-2 (FGF-2) (Gomez-Pinilla et al., 1995), and insulin-like growth factor 1 (IGF-1) (Carro et al., 2001) in the CNS, particularly the hippocampus. Although we have focused on GDNF, it seems likely that more than one factor will ultimately be found to be responsible for exercise-induced protection. It is important to note here that FGF-2, one of the trophic factors shown to be upregulated by exercise (Gomez-Pinilla et al., 1995), has also been shown in vitro to induce expression of both GDNF and BDNF (Suter-Crazzolara and Unsicker, 1996; Kwon, 1997).

Although forced-limb use has been shown to be clinically therapeutic in other conditions (Taub et al., 1999; Liepert et al., 2000; Taub and Morris, 2001), it is not clear if forced-limb use would be ideal for PD patients. Although the disease often begins with unilateral manifestations, it typically progresses to a bilateral disorder. On the other hand, the positive effects that others have had with bilateral forms of exercise in experimental animals and in patients support the hypothesis that exercise regimens can be used as a therapy.

In conclusion, these data suggest that forced limb-use is protective against subsequent injury to the DA neurons and terminals in the nigrostriatal system. These data are the first to

show that the protective effects of forced limb use are likely mediated by a protection of DA cells in the SN from the neurotoxic insult of 6-OHDA. Further exploration of the neuroprotective effects of forced limb use on a 6-OHDA model can provide targets for develop of neuroprotective drugs to treat PD, as well as lend support to the use of physical therapy as part of the treatment regimen for PD.

## **4.0 Effects of GDNF on the response of dopamine neurons to 6-hydroxydopamine: Time course of changes in phenotypic markers**

### **4.1 Abstract**

6-OHDA causes motor deficits and loss of DA cells of the SN and of their terminals in the rat striatum. GDNF can protect these neurons from the neurotoxic effects of 6-OHDA. However, there has been little exploration of the effects of GDNF on the temporal profile of phenotypic markers of the nigrostriatal pathway. Because the expression of phenotypic markers can be altered in response to injury, it is important to determine the profile of these markers in order to avoid incorrectly interpreting loss of phenotype. Thus in the present study, we explore the effects of GDNF on 6-OHDA neurotoxicity during the 8 wk period following administration of the toxin. Fluoro-gold (FG) was administered into a region located roughly in the middle of the striatum in order to label the cells projecting to this area. One week later, GDNF (9  $\mu$ g) or vehicle was injected into this same site, followed 6 hrs later by 6-OHDA (4  $\mu$ g) or its vehicle. When SN was examined 2-8 wks after 6-OHDA treatment, the toxin had caused a 83% loss of FG cells and a comparable loss of dopaminergic markers. The loss of FG cells was completely blocked by GDNF. In contrast, GDNF produced no significant protection of TH immunoreactivity in these FG-labeled cells at 2 wks, by 4 wks TH loss had reduced to 28%, ( $p > 0.01$ ) and by 8 wks no significant loss was observed ( $p > 0.01$ ). Recovery of tyrosine hydroxylase terminals and the high affinity DA transporter in the striatum followed a similar time course to the SN, the vesicular monoamine transporter was largely recovered within 4 wks (30% loss). Striatal DA showed the greatest and fastest GDNF-induced recovery, reaching normal levels within 2 wks. Thus, whereas GDNF blocks the degeneration of DA neurons, full restoration of the DA phenotype occurs gradually over several weeks.

## 4.2 Introduction

PD is a progressive neurodegenerative disorder of unknown etiology that results in the loss of DA neurons projecting from the SN to the striatum. Loss of dopaminergic tone in the striatum leads to a variety of neurological deficits, including resting tremor and akinesia. Current treatments for PD alleviate the symptoms of this disorder by temporarily restoring this dopaminergic tone and do nothing to slow or halt the degenerative process. Approximately 60,000 individuals a year are diagnosed with PD in the U.S. and 85% of this group is over the age of 50. Thus, as the population ages, developing strategies for slowing or preventing ongoing degeneration or even reversing the neurodegenerative process in PD are of paramount importance.

One promising therapy is the administration of trophic factors, such as glial cell line-derived neurotrophic factor (GDNF). GDNF is a dimeric protein that is highly expressed in the developing rat striatum (Stromberg et al., 1993). It has been shown to be a potent survival factor for DA neurons (Lin et al., 1993), and exogenous administration of GDNF or a viral vector containing the GDNF gene can protect DA neurons from the neurotoxic effects of 6-OHDA in vitro (Kramer et al., 1999; Gong et al., 1999; Schatz et al., 1999) and the behavioral and neurotoxic effects in vivo (e.g. Hoffer et al., 1994; Kearns & Gash, 1995; Choi-Lundberg et al., 1998; Smith et al., 2005). Although levels of GDNF are decreased in the brain during adulthood, injury to the brain can cause increases in the levels of GDNF (Naveilhan et al., 1997; Liberatore et al., 1997; Sakurai, et al., 1999; Wei et al., 2000; Smith et al., 2003) which may serve as a compensatory survival mechanism. However, even lower levels of GDNF are observed in patients with PD as compared to age-matched controls, which suggest that loss of trophic support may be a causal factor in the genesis of the disease (Siegel and Chauhan 2000).

Therefore, GDNF is a prime candidate as a therapeutic treatment against degeneration of the nigrostriatal DA system in PD.

Here, we explore the effects of GDNF on 6-OHDA neurotoxicity at 2 wk intervals during the 8 wk period following administration of the toxin, using several phenotypic markers of the nigrostriatal system, and a non-DA cellular marker for two principle reasons. First, we wanted to characterize the time course for the neuroprotective effects of GDNF because most studies to date have examined the effects of GDNF on 6-OHDA after an extended period, thus we know little about how quickly this protection develops. Second, we wanted to follow the time course of TH, dopamine transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2), as there has been little comparison of the differential protective effects of GDNF on these phenotypic markers of the nigrostriatal pathway. This aspect of this study could become quite important in a clinical setting where ligands for phenotypic markers are used in imaging studies to assess disease progression.

### **4.3 Materials and Methods**

#### *Animals*

Male Sprague Dawley rats (Hilltop Lab Animals, Scottdale, PA) weighing 250-350 grams were used in these experiments. All animals were housed two per cage and maintained on 12 h light/dark cycle with food (Purina lab diet) and water available ad libitum. All procedures were in strict accordance with the guidelines for the NIH Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

### *Surgical Procedures*

Seven days prior to GDNF and 6-OHDA infusion animals were anesthetized with isofluorane (1-2% in 100% O<sub>2</sub>, Inhaled; Halocarbon, River Edge, NJ), and Fluoro-gold (FG) (Fluorochrome, Denver, CO; 2% in sterile saline; 0.2 µl; 0.05 µl/min) was infused into the right striatum (+0.7 mm anterior, - 3.1 mm lateral of bregma, and 6.0 mm ventral to dura), according to the atlas of Paxinos and Watson (1982). One week after FG infusion animals were deeply anesthetized with isofluorane and 9 µg GDNF (Amgen, Thousand Oaks, CA) in 3 µl or vehicle (citrate buffer, pH 7.2) was infused into the right striatum at 0.5 µl/min at the same coordinates as FG. Six hours following GDNF administration animals were again deeply anesthetized with isofluorane and 6-OHDA (4 µg/0.75 µl; Regis, Morton Grove, IL) or vehicle (0.02% ascorbate in sterile saline; Sigma, St. Louis, MO) was infused into the right striatum at the same coordinates as above.

### *Histological Analysis*

At 2, 4, or 8 wks post-infusion, animals were deeply anesthetized with Equithesin and sacrificed via transcardial perfusion using ice cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.6. Following 48 hr cryoprotection in 30% sucrose, brains were sectioned at 60 µm on a cryostat at -20°C. Coronal slices were collected in a one-in-six series. Every sixth section cut from the SN was labeled and every sixth section from the striatum was labeled for TH, the vesicular monoamine transporter 2 (VMAT2), dopamine transporter (DAT), and GDNF.

**Immunohistochemistry:** All sections were rinsed 3 times in 10 mM phosphate buffered saline, pH 7.6 (PBS) prior to and between each incubation. PBS with 0.3% triton-X 100 was used as the diluent for all treatments unless specified otherwise. Sections were pretreated for 15 min with 1% H<sub>2</sub>O<sub>2</sub> in PBS followed by blocking for 1 hr with 10% normal donkey serum. Primary antibody incubations occurred on a rotator overnight at 4°C using a 1:1000 dilution of mouse anti-TH antibody (Chemicon Inc, Temecula, CA, Cat# MAB318); a 1:250 dilution of goat anti-VMAT2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, Cat# SC7721); a 1:500 dilution of rat anti-DAT antibody (Chemicon Inc, Temecula, CA, Cat# MAB369); or a 1:1000 dilution of a goat anti-GDNF antibody (R&D systems, Minneapolis, MN, Cat# AF-212-NA) with 1% of the appropriate normal serum. This was followed by incubation for 1 hr at room temperature in a 1:200 dilution of the appropriate biotinylated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Tissue was then treated with avidin biotin peroxidase complex (ABC-Elite, Vector Laboratories, Burlingame, CA) and subsequently with 0.02% 3, 3'-diaminobenzadine as the chromogen. Sections were then mounted on gelatin coated slides and dehydrated in ascending concentrations of ethanol before being rinsed in xylenes and coverslipped with Permount mounting medium (Fisher Scientific, Pittsburgh, PA).

**Double immunofluorescent labeling in SN:** To visualize FG and TH in the same neurons, immunofluorescent labeling was utilized. Sections were treated with 10% normal goat serum for 1 hr and then incubated overnight at 4°C in a 1:1000 dilution of mouse anti-TH antibody. Sections were subsequently labeled with Alexafluor 486 (goat anti-mouse; Molecular Probes, Invitrogen, CA) at a concentration of 1:500 in PBS for 2 hrs at room temperature, washed, mounted, coverslipped in DPX mounting medium (Fisher Scientific, Pittsburgh, PA), and viewed under epifluorescent illumination on a Nikon Inverted Eclipse TE microscope.

### *Image Analysis*

**Non-immunoreactive area in striatum:** Non-immunoreactive area analysis was performed in the striatum using MetaMorph software (Molecular Devices Corp., Downingtown, PA). For quantification of area devoid of immunoreactivity, the entire coronal section was visualized using a Nikon supercool scanner (Nikon Inc., Melville, NY). Images were then pseudo colored in MetaMorph and the region of low staining intensity on the three sections exhibiting the largest loss was circumscribed in blind fashion and the area within this region was calculated as mm<sup>2</sup> by MetaMorph. The average of these three sections was used as a representation of non-immunoreactive area for a given animal.

**Cell counts in SN:** For cell counts in the SN, one of every six sections from each animal was randomly selected for staining determined by randomly selecting number generated from a computer program. We then counted all FG<sup>+</sup>, TH<sup>+</sup>, and co-labeled cells in three sections of the SN.

### *HPLC Analysis*

Animals were sacrificed via decapitation and a 2 mm section of striatum was dissected, centered around the track made by prior injections. Dissected striata were assayed using minor modifications of previous methods (Smith et al., 2002). Striatal tissue was suspended in 0.1 M HClO<sub>2</sub> containing 347 μM NaHSO<sub>3</sub> and 134 μM Na<sub>2</sub> EDTA, homogenized in a volume 20 times (μl) the tissue wet weight (mg) and centrifuged at 16,000 x g for 20 min at 4°C, and the supernatant was removed. Tissue samples were assayed for DA by injecting a 20 μl aliquot of the sample onto a Reverse phase column (2.0 x 150 mm, ESA Inc., Chelmsford, MA). The



mobile phase consisted of 50 mM  $\text{H}_2\text{NaPO}_4$ , 0.72 mM sodium octyl sulfate, 0.075 mM  $\text{Na}_2\text{EDTA}$  and 16% methanol (v/v), pH 2.7. The mobile phase was pumped through the system at 0.3 ml/min using a ESA 580 pump (ESA Inc., Chelmsford, MA). Analyses were performed using an ESA Coulochem Model 4100A detector, an ESA Model 5010 conditioning cell, and an ESA Model 5014B microdialysis cell (ESA, Inc., Chelmsford, MA). The settings for detection were  $E_1 = -75\text{mV}$ ,  $E_2 = +220\text{mV}$ , and guard cell  $= +350\text{mV}$ . The limits of detection for DA were in the femtomole range.

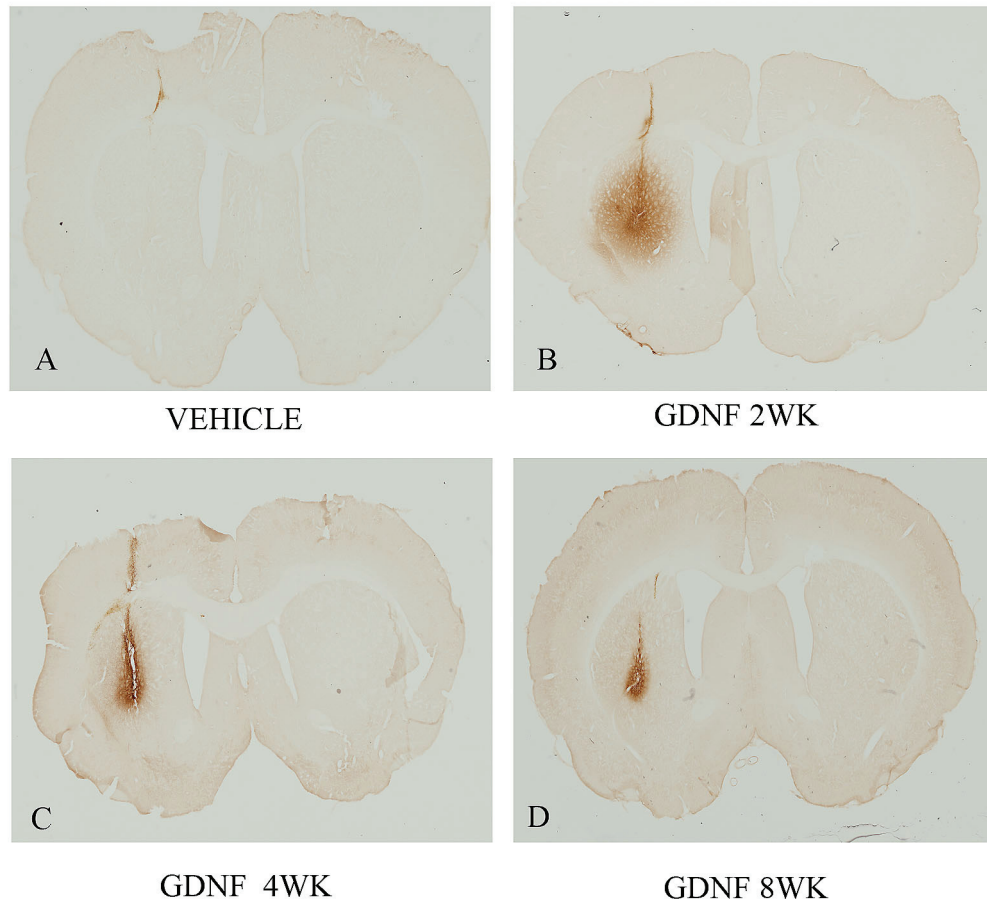
### *Statistical Analysis*

Data were analyzed with a two-way ANOVA, using Bonferonni-corrected multiple comparisons for post hoc analysis. There was no significant difference between the non-immunoreactive area in striatum of animals treated with 6-OHDA at the 2 (n = 9), 4 (n = 8) and 8 wk time points (n = 6), therefore these groups were pooled for further analysis.

## **4.4 Results**

### *Distribution of exogenous GDNF after intrastriatal administration*

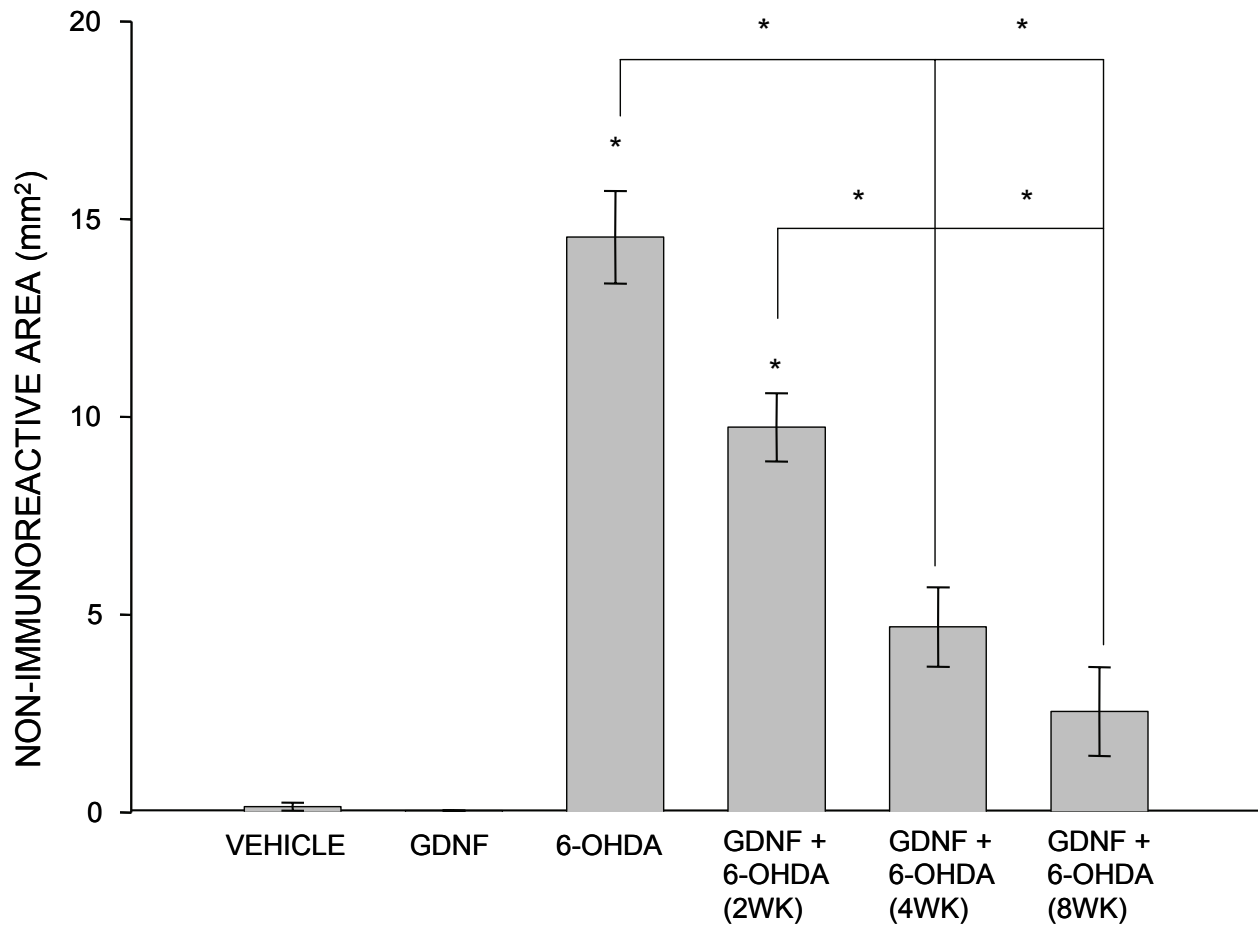
No GDNF immunoreactivity was observed in animals treated with 6-OHDA alone or with the 6-OHDA vehicle (Fig 4.1) at any time point examined. Two weeks after infusion of GDNF a large spread of GDNF immunoreactivity beyond the cannula track was observed in the striatum of animals given GDNF, either alone or together with 6-OHDA. However, by 4 and 8 wks, GDNF was largely confined to the cannula track. No GDNF was observed in the SN of any animals at any time points.



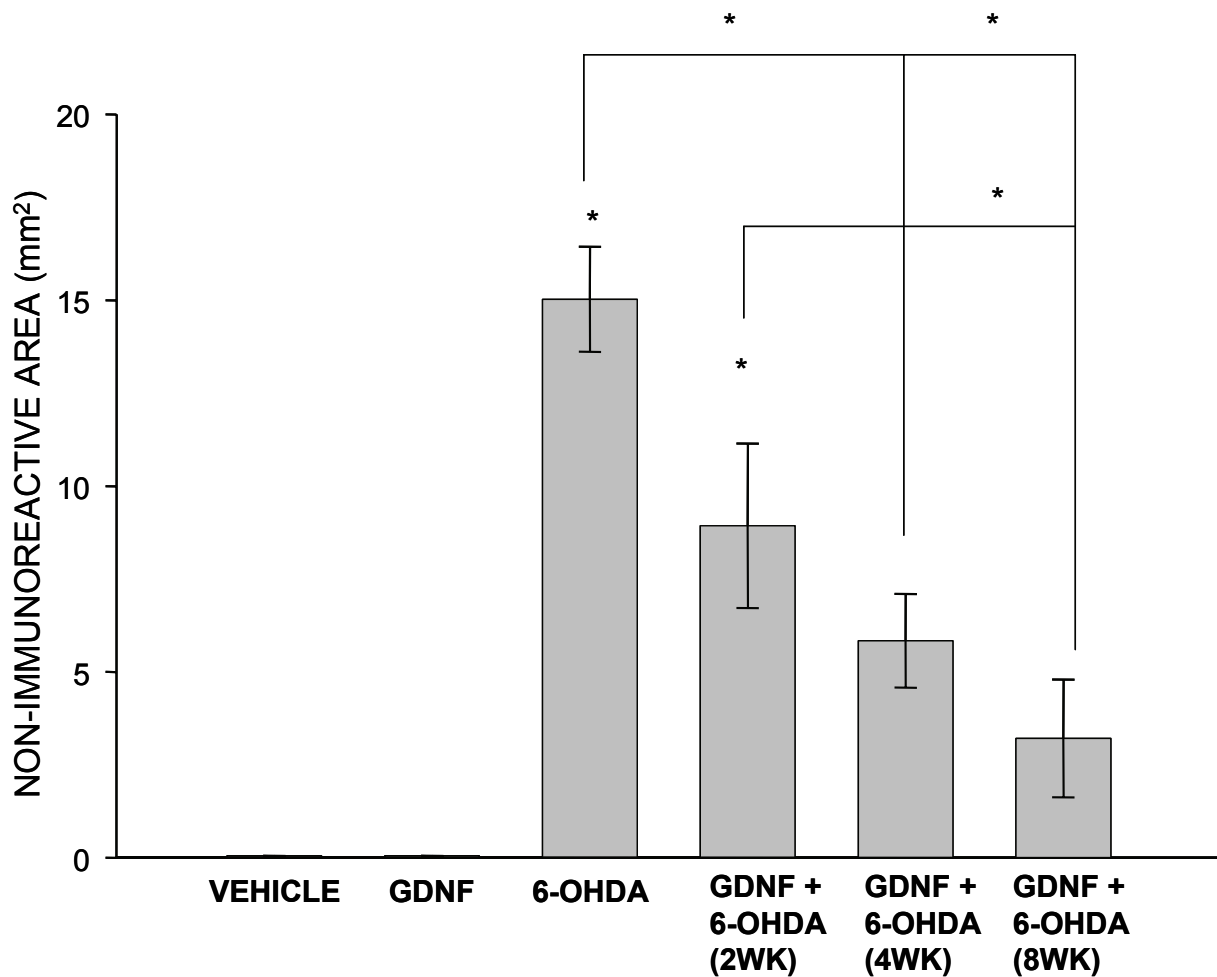
***Figure 4.1: Photomicrographs of GDNF-IR in the striatum. Sham animals (a) showed no GDNF-IR in the striatum, while GDNF-IR was present in the striatum of GDNF treated animals (2wks) (b), GDNF (4wks) (c), and GDNF (8wks) (d).***

*Effect of GDNF on the 6-OHDA-induced loss of TH, DAT and VMAT2 immunoreactivity in the striatum*

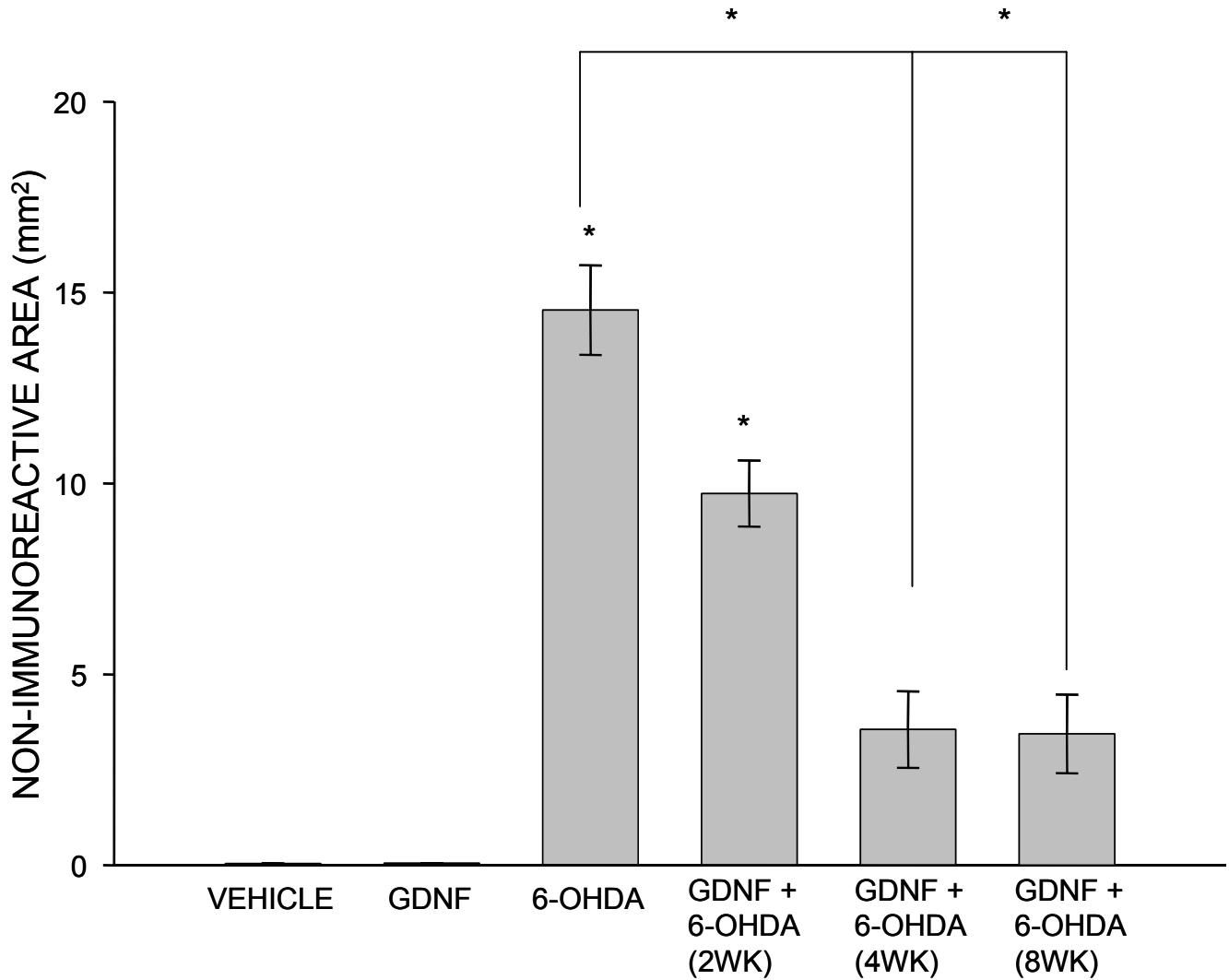
There was no significant difference in any of the three markers between animals treated with vehicle alone and GDNF alone. Animals treated with 6-OHDA alone displayed a significant increase in lesion area, measured by areas devoid of TH, DAT and VMAT2, as compared to vehicle treated (n = 7) and animals treated with GDNF alone (n = 10), who showed no loss of these markers at these same timepoints ( $p < 0.001$ ; Fig 4.2,4.3,4.4) [Overall group effect: TH: ( $F_{(6,55)} = 27.96$ ;  $p < 0.0001$ ; Fig 4.2); DAT: ( $F_{(6,55)} = 22.29$ ;  $p < 0.0001$ ; Fig 4.3); VMAT2: ( $F_{(6,55)} = 38.65$ ;  $p < 0.0001$ ; Fig 4.4) ]. At 2 wks, GDNF pre-treated animals (n = 10) did not show any appreciable protection against 6-OHDA toxicity when the TH, DAT, and VMAT2 markers were examined (Fig 4.2,4.3,4.4). At 4 wks, animals receiving GDNF prior to infusion of 6-OHDA (n = 9) showed a 70% reduction in lesion size with the TH and VMAT2 markers (Fig 4.2, 4.4). Conversely, no significant reduction in lesion size was observed at 4 wks using the DAT marker. A significant reduction in lesion size was observed with TH, DAT and VMAT2 at 8 wks (n = 6) post-infusion showing an 84, 80, and 76% reduction, respectively, in the loss immunoreactivity ( $p < 0.001$ ).



*Figure 4.2: Effects of GDNF on TH<sup>+</sup> terminals in the striatum after 6-OHDA. Loss of TH was prevented by GDNF prior to 6-OHDA infusion into the striatum at 4 and 8 but not 2wk post-6-OHDA. Vehicle and GDNF animals showed no significant loss of TH. 6-OHDA and GDNF+6-OHDA (2wk) animals displayed significant loss of TH when compared to vehicle treated animals (\* p<0.01). This loss was attenuated in GDNF+6-OHDA (4wk) and GDNF+6-OHDA (8wk) animals. All values are expressed as average lesion area in mm<sup>2</sup> ± SEM.*



*Figure 4.3: Effects of GDNF on DAT<sup>+</sup> terminals in the striatum after 6-OHDA. Loss of DAT was prevented by GDNF prior to 6-OHDA infusion into the striatum post-6-OHDA. Vehicle and GDNF animals showed no significant loss of TH. 6-OHDA animals displayed significant loss of TH when compared to vehicle treated animals (\* p<0.01). This loss was attenuated in GDNF+6-OHDA (2wk), GDNF+6-OHDA (4wk) and GDNF+6-OHDA (8wk) animals. All values are expressed as average lesion area in mm<sup>2</sup> ± SEM.*



*Figure 4.4: Effects of GDNF on VMAT2<sup>+</sup> terminals in the striatum after 6-OHDA. Loss of VMAT2 was prevented by GDNF prior to 6-OHDA infusion into the striatum at 4 and 8 but not 2wk post-6-OHDA. Vehicle and GDNF animals showed no significant loss of VMAT2. 6-OHDA and GDNF+6-OHDA (2wk) animals displayed significant loss of VMAT2 when compared to vehicle animals (\*  $p < 0.01$ ). This loss was attenuated in GDNF+6-OHDA (4wk) and GDNF+6-OHDA (8wk) animals. All values are expressed as average lesion area in  $\text{mm}^2 \pm \text{SEM}$ .*

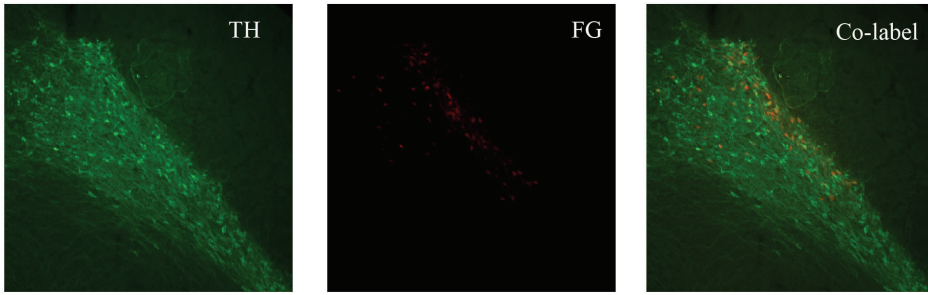
### *Effect of GDNF on the 6-OHDA-induced loss of TH positive neurons*

A significant loss of TH<sup>+</sup> cells was observed at 2, 4, and 8 wks after 6-OHDA alone when compared to vehicle and GDNF treated animals ( $p < 0.001$ ) [Overall group effect:  $F_{(7, 60)} = 15.14$ ;  $p < 0.001$ ; Fig 4.5]. GDNF infusion prior to 6-OHDA did not prevent the loss of the TH phenotype at 2 wks. However, by 4 and 8 wks GDNF prevented the loss of the TH<sup>+</sup> phenotype showing a significant difference from animals treated with 6-OHDA alone ( $p < 0.001$ ). A comparison of animals treated with vehicle alone and those treated with GDNF alone revealed no significant change in the number of TH<sup>+</sup> cells ( $p > 0.05$ ).

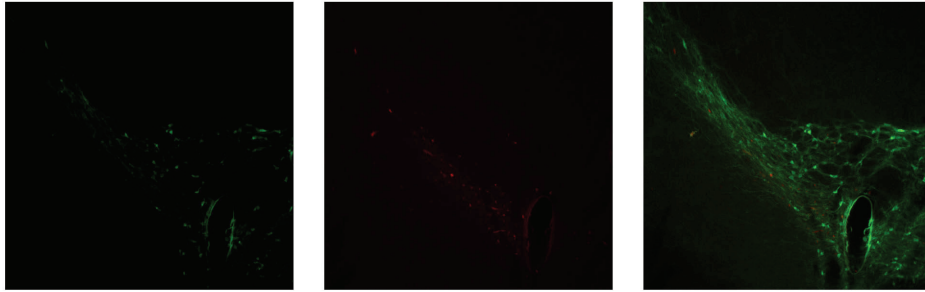
### *Effect of GDNF on the 6-OHDA-induced loss of Fluoro-gold positive neurons*

6-OHDA-induced loss of TH<sup>+</sup> cells in the SN could be a result of a loss of the TH phenotype rather than actual cell death or degeneration. Therefore, we examined the number of FG<sup>+</sup> cells in the SN, as well as cells co-labeled with TH and FG. A significant 57% loss of FG<sup>+</sup> cells was observed at 2 wks in animals given 6-OHDA alone ( $p < 0.001$ ). This loss of FG<sup>+</sup> cells was even greater at 4 wks (78%;  $p < 0.05$ ) and 8 wks (83%;  $p < 0.001$ ). In contrast, GDNF prior to 6-OHDA completely blocked loss of FG<sup>+</sup> cells at each time point examined ( $p < 0.001$ ).

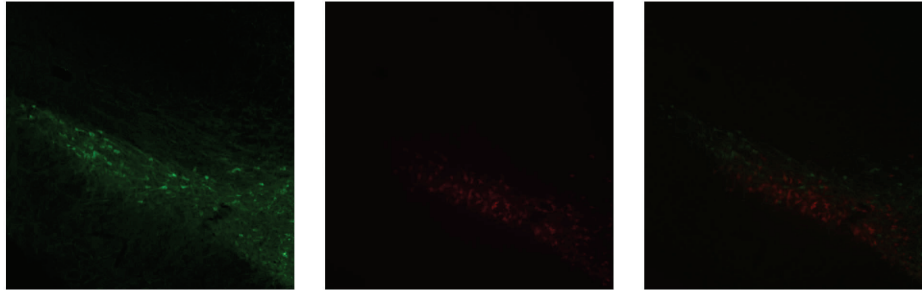
Co-labeling of cells with TH and FG was also examined. 6-OHDA produced a significant decrease of co-labeling of TH and FG at 2, 4, and 8 wks when compared to vehicle treated animals ( $p < 0.001$ ) [Overall group effect: Fluoro-gold: ( $F_{(7, 60)} = 18.74$ ;  $p < 0.001$ ) Co-label: ( $F_{(7, 60)} = 41.81$ ;  $p < 0.001$ ) Fig 4.5]. Similarly, animals treated with GDNF prior to 6-OHDA showed a significant decrease in co-labeling when examined at 2 wks and 4 wks post-6-OHDA when compared to controls ( $p < 0.001$ ). Animals treated with GDNF prior to 6-OHDA observed at 8 wks post-6-OHDA showed no significant decrease in co-labeling and were significantly different from the groups receiving 6-OHDA only.



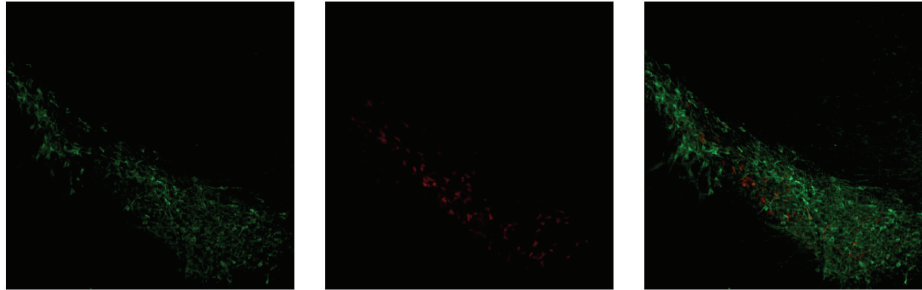
VEHICLE



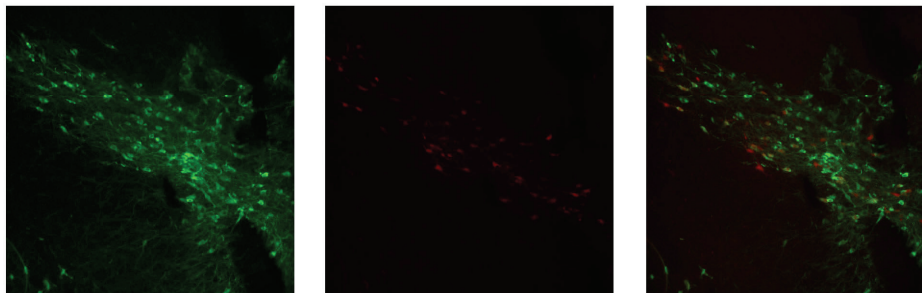
6-OHDA (8WK)



GDNF + 6-OHDA (2WK)



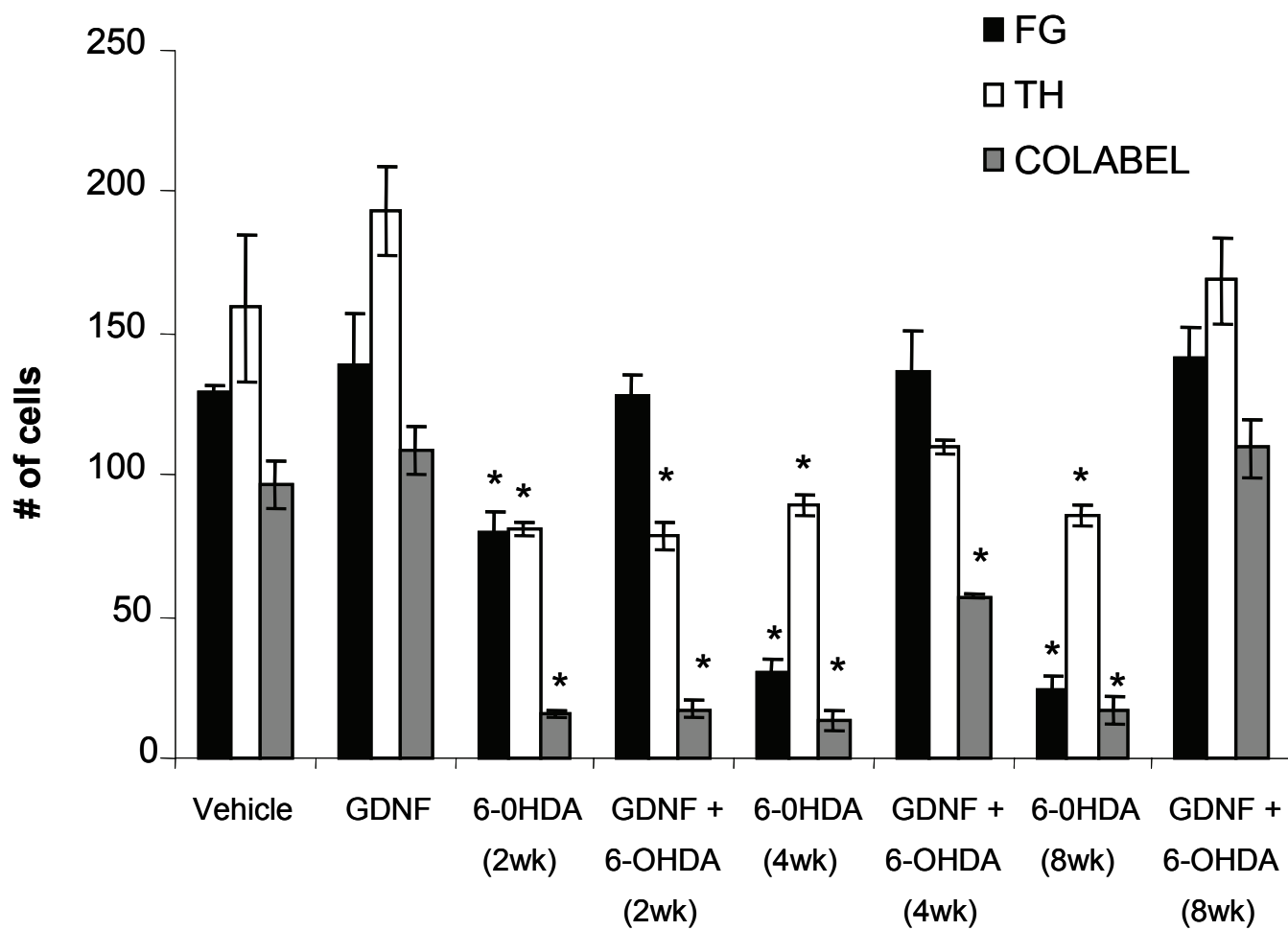
GDNF + 6-OHDA (4WK)



GDNF + 6-OHDA (8WK)



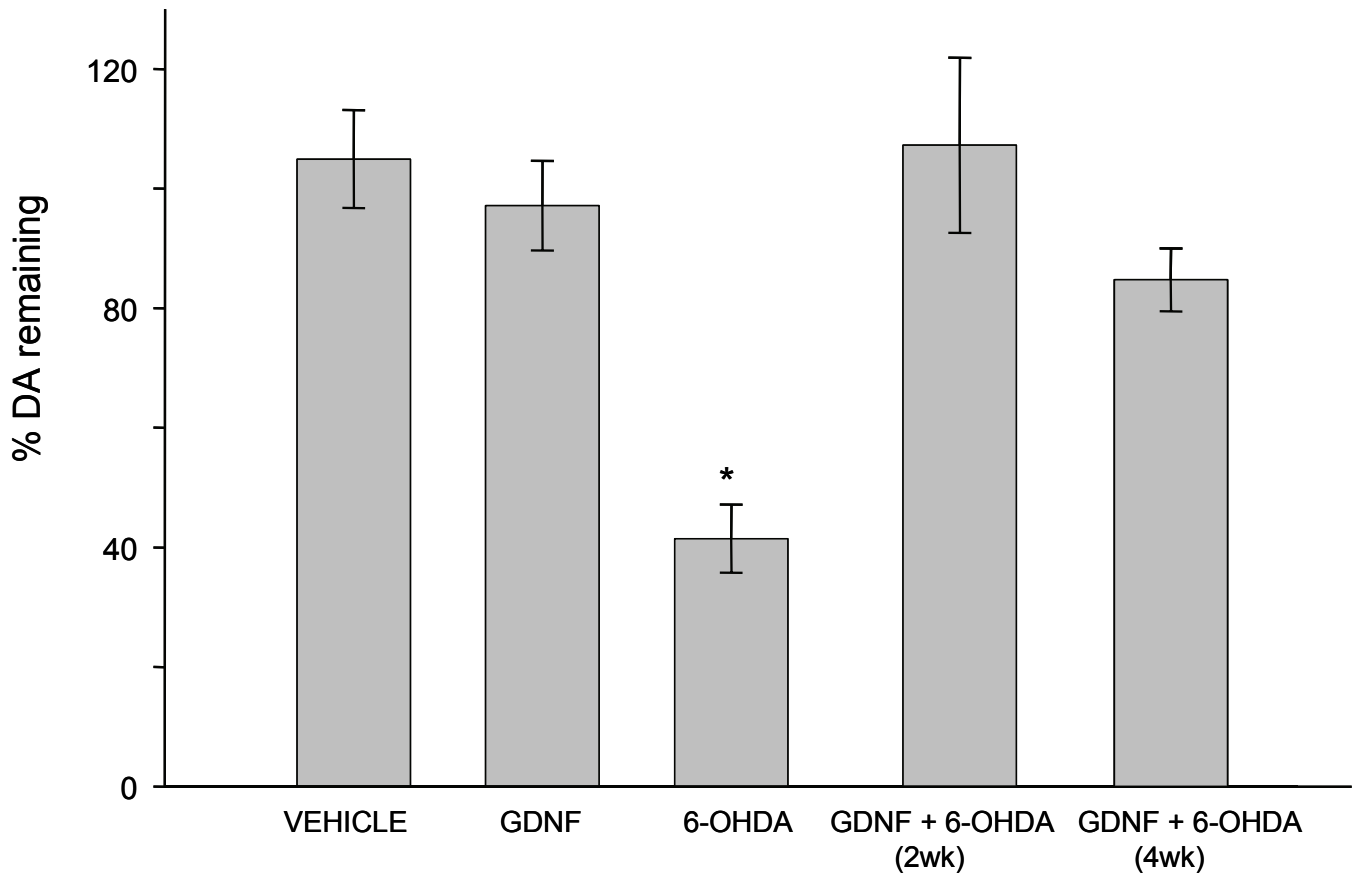
Figure 4.5a: Photomicrographs of TH<sup>+</sup> cells in the SN. Sham animals (a), (b) and (c) showed no loss of TH<sup>+</sup>, FG<sup>+</sup> or co-labeled cells, while 6-OHDA treated animals showed significant loss of TH<sup>+</sup>, FG<sup>+</sup> and co-labeled cells at 8wks post-6-OHDA (e), (f) and (g) and arrow). (H) Animals administered GDNF prior to 6-OHDA showed a loss of TH<sup>+</sup> cells at 2 wks post-6-OHDA but no significant loss at 4 and 8 wks post-6-OHDA (k) and (n). (I), (l) and (o) Animals administered GDNF prior to 6-OHDA showed no detectable loss of FG<sup>+</sup> cells at 2, 4 and 8 wks post-6-OHDA. (J) and (m) Animals administered GDNF prior to 6-OHDA showed a loss of co-labeled cells at 2 and 4 wks post-6-OHDA but no significant loss at 8 wks post-6-OHDA (p).



*Figure 4.5b: Effects of GDNF on loss of TH<sup>+</sup> and FG<sup>+</sup> cells in the SN after 6-OHDA. GDNF attenuated loss of TH<sup>+</sup> cells in the SN at 4 and 8 but not 2wks post-6-OHDA. Vehicle and GDNF animals had no detectable loss of TH<sup>+</sup> cells in the SN. 6-OHDA infusion significantly decreased the number of cells in the SN at 2, 4 and 8 wks post infusion in 6-OHDA animals (\*p < 0.001). Infusion of GDNF did not prevent a loss of TH<sup>+</sup> cells in GDNF+6-OHDA (2wks) animals (\*p<0.001) while no significant loss of TH<sup>+</sup> cells was observed in GDNF+6-OHDA (4wks) and GDNF+6-OHDA (8wks). GDNF attenuated loss of FG<sup>+</sup> cells in the SN at all timepoints post-6-OHDA. Vehicle and GDNF animals had no detectable loss of FG<sup>+</sup> cells in the SN. 6-OHDA infusion significantly decreased the number of cells in the SN at 2, 4 and 8 wks post infusion in 6-OHDA animals (\*p < 0.001). GDNF attenuated loss of cells co-labeled for TH and FG in the SN at 8 but not 2 or 4 wks post-6-OHDA. Vehicle and GDNF animals had no detectable loss of co-labeled cells in the SN. 6-OHDA infusion significantly decreased the number of cells in the SN at 2, 4 and 8 wks post infusion in 6-OHDA animals (\*p < 0.001). Infusion of GDNF did not prevent a loss of co-labeled cells in GDNF+6-OHDA (2wks) and GDNF+6-OHDA (4wks) animals (\*p < 0.001) while no significant loss of colabeled cells was observed in GDNF+6-OHDA (8wks). All values are expressed average number of cells ± S.E.M.*

***Effect of GDNF on the 6-OHDA-induced loss of DA content in the striatum***

We assessed the loss of DA content at 2 and 4 wks after 6-OHDA  $\pm$  GDNF pretreatment. No loss of DA was observed in animals treated with either vehicle alone or GDNF alone. 6-OHDA infused into the striatum produced a significant 60% loss of DA content in the striatum at both 2 and 4 wks post-6-OHDA when compared to animals treated with vehicle and GDNF alone ( $p < 0.01$ ) [Overall group effect: ( $F_{(4,24)} = 13.89$ ;  $p < 0.0001$ ; Fig 4.5)]. However, GDNF completely blocked the loss of DA content in the striatum when examined at 2 and 4 wks post-6-OHDA (Fig 4.6).



***Figure 4.6: Effects of GDNF on DA content in the striatum after 6-OHDA. Loss of DA content was prevented by GDNF prior to 6-OHDA infusion into the striatum at 2 and 4 wks post-6-OHDA. Vehicle and GDNF animals showed no significant loss of DA content in the striatum. 6-OHDA animals displayed a significant decrease in DA content when compared to sham animals (\*  $p < 0.01$ ). This loss was prevented in GDNF+6-OHDA (2wks) and GDNF+6-OHDA (4wks) animals. All values are expressed as % loss of DA content compared to the contralateral side  $\pm$  SEM.***

## 4.5 Discussion

GDNF is a promising neuroprotective factor in the treatment of PD. Animal studies have generally shown it to be protective against 6-OHDA and MPTP both in vitro and in vivo in animal studies (see Introduction). Clinical studies have been more equivocal with some groups reporting improvements in symptoms and pathology (Gill et al., 2003; Patel et al., 2005; Slevin et al., 2006), whereas others have shown no clinical improvement (Nutt et al., 2003; Lang et al., 2006) (see Sherer et al., 2006 for review of the issues). Despite this controversy, it seems likely that a full understanding of the neuroprotective effects of GDNF will be useful in the development of much needed therapies for PD. Indeed, a better understanding of the changes produced by GDNF on the DA system may shed light on the best paradigms and markers to use in quantifying the benefits treatment.

Our study confirms previous studies indicating that GDNF can protect DA neurons from 6-OHDA in the adult rat. It further extends that finding to examine the impact of GDNF on the phenotypic expression of DA neurons. This is critical since it has been reported that both MPTP and 6-OHDA can result in a loss of the TH phenotype without loss of DA neurons (Jackson-Lewis et al., 1995; Ara et al., 1998; Rosenblad et al., 2003), and that infusion of GDNF into the SN can block the loss of SN cells produced by intra-nigral 6-OHDA without affecting the loss of TH itself (Lu and Hagg, 1997). In order to distinguish actual cell death from loss of the TH phenotype in the SN, we infused FG into the striatum 7 d prior to infusion of 6-OHDA to retrogradely label SN neurons affected by 6-OHDA. After 6-OHDA, a significant loss of FG<sup>+</sup> cells was observed within 2 wks, which progressed over the time period examined. This is consistent with previous evidence of the progressive nature of the cell loss after intra-striatal 6-

OHDA (Sauer and Oertel, 1994). 6-OHDA also caused a loss of TH<sup>+</sup> cells. However, unlike the case for FG, this was maximal at the earliest time studied. This may reflect the fact that the initial loss of TH immunoreactivity demonstrates the downregulation of TH gene expression rather than actual cell loss. When neurons co-labeled for TH and FG were examined, 6-OHDA produced a sharp drop in the number of neurons at 2 wks, indicating that at this time point a large number of neurons labeled by FG do not contain TH at this time point (or the levels are below levels detectable by the assay). These data suggest that after a neurotoxic insult, loss of TH in SN neurons precedes actual cell death. This is in agreement with previous studies showing that TH is downregulated prior to actual cell death in response to injury to the nigrostriatal system (Jackson-Lewis et al., 1995; Ara et al., 1998; Rosenblad et al., 2003). GDNF did not block the loss of TH<sup>+</sup> phenotype or cells co-labeled for TH and FG 2 wks after infusion of 6-OHDA but the system was able to normalize by 8 wks post-infusion, whereas no decrease in the number of FG<sup>+</sup> neurons in animals treated with GDNF prior to 6-OHDA was observed between over 8 wks. These data suggest that whereas GDNF does not protect from an initial decrease in TH<sup>+</sup> expression within SN after 6-OHDA, the system is able to normalize by 8 wks post-infusion and that no loss of overall cell number occurs when 6-OHDA is preceded by GDNF.

A similar effect was observed in the striatum using immunohistochemistry to examine three phenotypic markers of DA terminal integrity, TH, DAT, and VMAT2. In animals exposed to GDNF and 6-OHDA there was a significant loss of all three markers 2 wks post-6-OHDA that had begun to recover at 4 wks and had further increased by 8 wks. The profiles and magnitude of the three markers differed to some extent. For example, 2 wks after animals received the combination of GDNF and 6-OHDA, the largest loss was with TH (20% of control); the loss of VMAT2 was smaller (35%), and the loss of DAT was smaller still (40%). This may suggest that

VMAT2 and DAT are less susceptible to stress-induced changes in the DA system. Indeed, others have suggested that VMAT2 is an optimal marker for DA terminal integrity because it is less susceptible to stress induced changes than other DA markers (Miller et al., 1999). Additionally, it is possible that because these proteins are rather different in location and function that the rates of turnover and synthesis of TH, DAT, and VMAT2 differ enough to account for the differences observed across this time course. These data are in agreement with the data presented above from the SN, suggesting that like TH in the SN, phenotypic markers of DA terminal integrity are affected by 6-OHDA even in the presence of GDNF but that these phenotypes are able to normalize at later time points following 6-OHDA.

GDNF itself has been shown to downregulate TH in the striatum and SN when overexpressed for an extended period of time in the striatum (Rosenblad et al., 2003). However, we feel that the possibility that GDNF alone is decreasing TH in these experiments is highly unlikely. First, in our study animals injected with GDNF alone showed no alterations in total TH protein at any of the 3 time points examined. Second, in the Rosenblad et al. (2003) study the GDNF gene was overexpressed using a viral vector producing levels of GDNF for much longer periods in the striatum than in the experiments discussed here.

The inability of GDNF to protect from the initial 6-OHDA-induced decreases in markers of the DA system, raises the possibility that that the downregulation of these markers reflects a shift in the DA system from normal maintenance to a survival mode where the system has prioritized repair or regenerative responses over the synthesis and packaging of DA. Indeed, others have shown changes in both the activity and amounts of TH following damage to the nigrostriatal system (Reis et al., 1975). These data would support the hypothesis that GDNF does not prevent 6-OHDA from affecting the DA system but it allows the system to recover from the



initial insult in a manner that would not occur without GDNF pretreatment. Future experiments will explore functional changes in the DA system, measure DA release and neuronal activity after GDNF pretreatment and 6-OHDA.

In order to determine the functional effects of GDNF following 6-OHDA, we examined DA content in a separate group of animals in the striatum at 2 and 4 wks post-infusion. 6-OHDA produced a significant loss of the neurotransmitter by 2 wks that persisted out to 4 wks following infusion of the toxin, while GDNF attenuated this loss at both time points. These data would suggest that although TH is decreased at 2 wks post-6-OHDA infusion in GDNF pretreated animals, GDNF allows the DA system to compensate and still function in a relatively normal manner. A likely explanation for this phenomenon is that because GDNF functions to enhance TH enzyme activity, normal levels of DA could be produced in the absence of normal levels of TH protein (Kirik et al., 2000; Lindgren et al., in preparation). Another possibility is that because GDNF has been shown to transiently increase tissue DA for 1-3 weeks in the striatum this may account for normal DA levels in the absence of the TH phenotype (Hudson et al., 1995; Beck et al., 1996; Martin et al., 1996, Rosenblad et al., 2003).

It had not previously been established whether GDNF remains present after infusion and if so for how long. We therefore used immunohistochemistry for the GDNF protein to determine how long GDNF remains present after our single infusion paradigm. In both animals treated with GDNF and 6-OHDA and animals treated with GDNF alone, GDNF was detectable at 2, 4, and 8 wks following infusion. The largest increase in GDNF staining was observed at 2 wks post-infusion, with staining at 4 and 8 wks largely confined to the cannula track. Although it is possible that the GDNF we detected includes endogenous GDNF increased in response to injury due to damage caused by 6-OHDA (Smith et al., 2003), this is unlikely

because animals treated with vehicle alone and 6-OHDA alone showed no immunoreactivity even along the cannula track.

It is unknown if the GDNF detected by immunohistochemistry at 2-8 weeks was biologically active. However, it is possible that the protein does continue to exert a physiological impact and thus plays a role in the dynamic changes in TH, DAT, and VMAT2 occurring over this time period. Another possibility is that the levels of GDNF detected between 2 and 8 wks are not functional, but that the GDNF quickly stimulates pro-survival intracellular signaling cascades whose end result is to protect DA neurons from cell death. These data would suggest that GDNF might provide long lasting effects on the nigrostriatal system long after the toxin is no longer present.

Lu and Hagg (1997) have demonstrated the downregulation of TH after GDNF and 6-OHDA and a neuroprotection of DA cell bodies measured by a retrograde tracer. Additionally, they reported that the recovery they observed disappeared if GDNF infusion was terminated. Although these data do not agree with the findings reported here there were several key differences in the experimental protocol in the two studies. First, in the previous report 6-OHDA was infused into the medial forebrain bundle, producing a much larger, more rapid loss of nigrostriatal DA neurons than in our striatal infusion model. Finally, while Lu and Hagg (1997) infused chronic GDNF into the SN, in our studies a single bolus of GDNF was infused in the striatum. This may be a crucial differences as it has been suggested that GDNF infused in the SN is ineffective at protecting axons and axon terminals from 6-OHDA (Kirik et al., 2004).

Our current hypothesis is that GDNF protects from the loss of SN neurons and striatal terminals without preventing an early downregulation of TH, DAT, and VMAT2. However, it remains possible that whereas GDNF protects SN neurons, it does not protect terminals from

degeneration and that recovery of striatal terminals at later time points is a result of sprouting. GDNF has been shown to produce axonal sprouting in the striatum after a 6-OHDA infusion (Rosenblad et al., 1998; Rosenblad et al., 1999). Additionally, it has been hypothesized that axonal sprouting may be the one of the principal processes responsible for recovery of function when GDNF is administered following damage to the nigrostriatal system (Beck et al., 1995; Tomac et al., 1995; Rosenblad et al., 1998; 1999; Grondin et al., 2002). However, this possibility seems unlikely for several reasons. First, unlike other studies where GDNF induced sprouting was observed, in our studies GDNF was administered prior to 6-OHDA infusion. Second, we observed no change in tissue DA levels in animals treated with GDNF just prior to 6-OHDA, indicating some function of DA terminals. Finally, preliminary studies examining the role of GAP43, a marker of sprouting, indicate no increase in this protein following GDNF or GDNF and 6-OHDA administration.

In conclusion, these data indicate that GDNF protects against the 6-OHDA induced loss of SN cells and DA transmitter levels but does not protect from the early decrease in phenotypic markers of the DA system. This would also suggest that the protective effects of GDNF from 6-OHDA are time dependent and that the loss of phenotypic markers in this system does not inevitably lead to cell death. They also suggest that human studies of GDNF-induced protection must be carefully timed in both the administration of the protein and measurement of its efficacy, in order to avoid the pitfalls of falsely interpreting temporary loss of phenotype. Because GDNF regulates TH directly (Kobori et al. 2004; Salvatore et al. 2004) it is possible that interpretation of the protective effect of GDNF on DA neurons using only the TH marker could be confounded. Further exploration of the neuroprotective effects of GDNF on a 6-OHDA model will provide insight into design and methodology of future clinical studies on the effects of GDNF and PD.

Additionally as the mechanisms of the protective effects of GDNF are further explored more targets for drug creation may become available, particularly as early detection methods become more readily available.

## 5.0 Discussion

Current treatments for PD, such as L-dopa, only alleviate the symptoms of the disease and can lose their efficacy and produce side effects over time (Pahwa and Lyons, 2004). Thus, it is important that new therapeutic approaches that slow or block the neurodegenerative process be explored. One such approach is exercise. Exercise has been known for some time to have beneficial effects on the brain (for review see Mattson, 2000). Indeed, exercise has been shown to protect from brain damage, in animal models of ischemia, (e.g. Stummer et al., 1994, Li et al., 2004), 3-acetylpyrrole, domoic acid, (Carro et al. 2001), animal models of Alzheimer's disease (Allard et al., 2005; Jankowsky et al., 2005; Sisodia et al., 2005). Similarly, in animal models of PD, running, either forced or voluntary, as well as an enriched environment, has been shown to reduce the effects of PD models (Bezard et al., 2003; Tillerson et al., 2003; Faherty et al., 2005).

Whereas the mechanism by which exercise confers its protective effects is unknown, it is well established that exercise confers changes in the brain that produce an environment conducive to cell survival. For example, as I have discussed in the Introduction, exercise produces increases in synaptogenesis, neurogenesis, and growth of neuronal processes. Here I have chosen to focus on exercise-induced increases in trophic factors. Exercise increases several trophic factors, including BDNF and GDNF (e.g. Neeper et al., 1995; 1996; Young et al., 1999), both of which promotes the survival of DA neurons and that are promising neuroprotective factors in the treatment of PD.

Based on this body of literature, in this dissertation I chose to focus on neuroprotective roles of exercise and GDNF on the effects of 6-OHDA in an animal model of PD. In this Chapter, I will first briefly summarize my results (Section 5.1). This will be followed by a discussion of some of the key issues raised by these results (Sections 5.2-5). Next, I will discuss the clinical significance of my results. I will end with a brief discussion of what I consider the most important future directions (Section 5.7) to be taken and the overall conclusions that I feel can be drawn from this work (Section 5.8).

## **5.1 Summary of Results**

### **5.1.1 Forced limb use protects from 6-OHDA induced behavioral deficits, loss of striatal DA, and loss of TH<sup>+</sup> neurons and terminals.**

In the Chapter 2, my colleagues and I report that forced limb use for the 7 days prior to 6-OHDA infusion along the MFB produced a robust attenuation of the behavioral and neurochemical effects of the 6-OHDA lesion model. Behaviorally, animals infused with 6-OHDA showed profound deficits in the use of the forelimb corresponding to the infused hemisphere and displayed both forelimb akinesia and robust rotation in response to apomorphine. In contrast, animals casted for the 7 days prior to 6-OHDA displayed no behavioral deficits in response to the toxin, such that they were statistically indistinguishable from controls. The ability of casting to protect against the behavioral effects of 6-OHDA is a likely result of the casting-induced attenuation of the loss of DA. The neuroprotective effects conferred by prior forced limb use on behavior and DA levels after 6-OHDA were striking, but several factors remained unknown. First, as discussed in section 5.2, it was not clear whether the forced limb use produced an overuse of the non-casted forelimb and thus could be referred to as

a form of exercise or *over*-use. Second, there were several possible explanations for the ability of forced motor activity to reduce the behavioral and neurochemical effects of 6-OHDA. For example, animals could be learning a different strategy for executing the behavioral tasks being tested, DA release from remaining DA terminals could increase, damaged axons could regenerate, intact neurons could sprout, or neurogenesis might occur. These are each explored below.

In Chapter 3, we used immunohistological analysis to determine the effect of prior forced limb use on TH<sup>+</sup> terminals and cell bodies in the striatum and SN respectively after 6-OHDA toxicity. Additionally, to distinguish between actual cell death and loss of the TH phenotype in the SN, we examined the SN for Nissl-positive neurons. Given the high proportion of DA neurons to total neurons within the SN, this provided an approximation of the number of DA neurons present that was independent of their expression of the TH<sup>+</sup> phenotype. We observed that prior forced limb use prevents the loss of TH<sup>+</sup> terminals in the striatum. 6-OHDA caused a significant loss of TH<sup>+</sup> terminals in the striatum, whereas animals exposed to forced limb use followed by 6-OHDA showed no sign of loss of TH<sup>+</sup> terminals in the striatum over a 28 day period. 6-OHDA treatment produced a significant loss of TH<sup>+</sup> cells and of cells positive for Nissl substance indicating that 6-OHDA produces cell death in the SN rather than only a loss of the TH phenotype in these neurons. However, forced limb use blocked both the loss of TH<sup>+</sup> cells and cells positive for Nissl substance at 7 and 28 days after infusion of 6-OHDA, supporting the hypothesis that forced limb use for the 7 days prior to 6-OHDA infusion prevents degeneration in the nigrostriatal DA system. The neuroprotective effects of forced limb use on TH in the nigrostriatal system and cell survival in the SN are consistent with the observations made in

Chapter 2 demonstrating the neuroprotective effects of prior forced limb use on DA levels in the striatum and behavior.

### **5.1.2 Forced limb use produces an increase in GDNF in the striatum**

Our working hypothesis was that the casting-induced attenuation of the loss of striatal DA was, in turn, a result of an exercise-induced increase in GDNF in the region that subsequently received 6-OHDA. In order to examine the role GDNF plays in casting-induced neuroprotection we examined levels of GDNF during the casting period in Chapter 2. GDNF was increased significantly in the striatum at 1 and 3 day following placement of the cast but had returned to baseline levels by day 7. Although GDNF had declined to baseline prior to 6-OHDA infusion, this does not preclude GDNF as a possible participant in the neuroprotective effects of forced limb-use. Indeed, previous investigators have shown that exogenous GDNF can be neuroprotective if administered as much as 7 days prior to 6-OHDA (Kearns & Gash, 1995; Choi-Lundberg et al., 1998). This may mean that GDNF triggers longer lasting secondary intracellular responses such as the activation of specific intracellular cascades (see section 5.7.5).

### **5.1.3 GDNF protects SN neurons from 6-OHDA induced toxicity but does not immediately prevent from loss of the TH phenotype**

In the previous chapters of this dissertation, I have explored the neuroprotective effects of prior forced limb use on the effects of 6-OHDA in a rat model. I have shown that forced limb use for the 7 d prior to 6-OHDA infusion produces a robust neuroprotective effect on the behavioral deficits, loss of DA and loss of TH<sup>+</sup> terminals and cell bodies. Additionally, I have shown that a



rise in GDNF is correlated with the protective effects of forced limb use. However, it will be necessary to perform additional studies to more stringently test the hypothesis that changes in GDNF expression are actually causally related to the changes in behavior and/or DA levels.

One would like to examine the neuroprotective effects of forced limb use in animals in which endogenous GDNF is blocked. This could be achieved using several approaches including, blocking antibodies, antisense RNA to block production of the protein, or use transgenic animals. Whereas these approaches may be the most direct test of the hypothesis that GDNF is responsible for the neuroprotective effects of 6-OHDA, it faces several drawbacks. Although some investigators have succeeded in studying the role of GDNF with **blocking antibodies** (Oo et al., 2003), in vivo **antisense** technology generally has not been capable of completely blocking synthesis of the protein (see Batchelor et al., 2000). Additionally, both blocking antibodies and antisense probes for GDNF may cause an alternate pathway (see below) to increase. Thus, it may be necessary to block multiple trophic factors at one time in order to establish a role for trophic factors in the protective effects of forced limb use. Although **knockout animals** generally can avoid the pitfalls discussed above, mice homozygotic for  $GFR\alpha 1$ , the GDNF receptor, expire soon after birth (Rossi et al., 1999). In summary, in the experimental paradigms discussed above interpretation of negative data poses a major challenge, presenting several possibilities including that the trophic factor in question is truly not involved, the blocking technique utilized did not work, or that an alternate pathway is compensating for the decrease in trophic factor signaling.

Given these technical and interpretational problems, I chose an alternate approach to establish a neuroprotective role for GDNF, direct infusion of the exogenous protein. Although this approach does not directly mimic the GDNF induced increase in forced limb use, I believed

it would provide insight into the neuroprotective effects observed in Chapters 2 and 3 without the technical burden of the techniques discussed above.

To elucidate further the role GDNF may play in neuroprotection, in Chapter 4 we examined the time course of these effects. Most studies to date have examined the effects of GDNF on 6-OHDA after an extended period, but there is very little evidence on the effects of GDNF on multiple phenotypic markers during the progression of cell death that typically occurs after 6-OHDA infusion in the striatum. Thus, in Chapter 4, we examined the role of GDNF after 6-OHDA infusion over an 8-wk period, using three phenotypic marker of the DA system, TH, DAT, and VMAT; a non-phenotypic marker in FG; and tissue DA levels.

After administration of 6-OHDA, a significant loss of TH<sup>+</sup> cells was observed by 2 wks post-infusion and persisted at 4 and 8 wks. 6-OHDA caused a loss of cells positive for FG as well a progressive loss at each time point examined demonstrating the progressive nature of the striatal lesion model (Sauer and Oertel, 1994). Additionally, where neurons co-labeled for TH and FG were examined, 6-OHDA produced a sharp drop in the number of neurons at 2 wks, indicating that a large number of neurons sending projections to the striatum from the SN do not contain TH or the levels are below our limits of detection. These data indicate that after a neurotoxic insult, loss of TH in SN neurons precedes actual cell death.

GDNF did not block the loss of TH<sup>+</sup> phenotype or cells co-labeled for TH and FG 2 wks after infusion of 6-OHDA. However, the number of TH<sup>+</sup> and co-labeled neurons began to increase by 4 wks and had returned to baseline by 8 wks after infusion of GDNF and 6-OHDA. Conversely, when the number of FG<sup>+</sup> neurons was examined in animals treated with GDNF prior to 6-OHDA, no appreciable difference was observed in comparison to control animals at 2, 4, or 8 wks. These data suggest that whereas GDNF does not protect from an initial decrease in TH<sup>+</sup>

cells after 6-OHDA, the system is able to rebound its ability to produce TH by 8 wks post-infusion, and that no loss of overall cell number occurs as a result of 6-OHDA infusion. This could suggest that the downregulation of TH reflects a shift in the DA system from normal maintenance to a survival mode where the system has prioritized repair or regenerative responses over the synthesis and packaging of DA (Reis et al., 1975).

#### **5.1.4 GDNF-induced protection from the loss of TH, DAT, or VMAT immunoreactive terminals in the striatum is delayed by several weeks**

In Chapter 4, DA terminals in the striatum were examined for several phenotypic markers of DA neurons, TH, DAT and VMAT immunoreactivity following GDNF and 6-OHDA infusions. Similar to the effects observed in the SN, 6-OHDA produced a loss of all three markers at 2 wks that persisted to 8 wks. In animals exposed to both GDNF and 6-OHDA there was still a significant loss of all three phenotypes at 2 wks post-6-OHDA. However, this had begun to recover at 4 wks and had further increased by 8 wks. The profiles and magnitude of the three markers differed slightly. The largest loss at 2 wks after GDNF and 6-OHDA was with TH, followed by a smaller loss of VMAT2 and an even smaller loss of DAT. This may suggest that VMAT2 and DAT are less susceptible to stress-induced changes in the DA system. Indeed, others have suggested that VMAT2 is an optimal marker for DA terminal integrity due its stability (Miller et al., 1999).

These data provide further support the hypothesis that the phenotypic markers of DA terminal integrity are affected by 6-OHDA even in the presence of GDNF, but that these phenotypes are able to normalize by 8 wks following 6-OHDA. These data also highlight the

importance of using multiple markers of terminal and neuronal integrity when examining cell death.

### **5.1.5 GDNF prevents loss of tissue DA following 6-OHDA**

Whereas we have established that GDNF protects from loss of nigral neurons, it was not clear if the changes in TH, DAT, or VMAT2 had an impact on DA content in the striatum. 6-OHDA produced a significant loss of the neurotransmitter by 2 wks that persisted out to 4 wks following infusion of the toxin, while GDNF attenuated this loss at both time points. These data would suggest that although TH is decreased at 2 wks post-6-OHDA infusion in GDNF pretreated animals, GDNF allows the DA system to compensate and still function in a relatively normal manner. The ability of DA to remain normal in the absence of TH may be related to the ability of GDNF to regulate the activity of the TH enzyme, thereby increasing the activity of the remaining TH to a degree where DA synthesis appears normal (Kirik et al., 2000; Lindgren et al., in preparation).

### **5.2 Forced limb use produces “overuse” of the non-casted forelimb**

The protective effects of forced limb use were established in Chapters 2 and 3, but it remained unknown if the effects of forced-limb use can be attributed to “overuse” of the non-casted forelimb. Tillerson and colleagues (2002) have suggested that use of the impaired limb following the removal of the cast is imperative for maintenance of the behavioral effects observed in a model using forced limb use following 6-OHDA infusion. They demonstrate that if, after casting the unimpaired forelimb on days 1-7, and subsequently casted the impaired forelimb for days 7-14, the behavioral sparing that had been evident following forced use was lost and remained

compromised for at least 40 days post-6-OHDA. These data indicate the importance of continued use of the affected limb (Tillerson et al., 2002). In order to determine if casting a forelimb increases the use of the non-casted forelimb in the model of **prior** forced limb use, in Chapter 3 we observed casted and non-casted animals in their home cage during the casting period. Two days after placement of the cast, casted animals used their forelimb significantly less than non-casted animals. However, this effect was attenuated by 3 days post-cast. Indeed, not only did casted animals begin to use their non-casted limb as much as the corresponding limb of non-casted animals but they began to use the non-casted limb as much as non-casted animals used both forelimbs. These data indicate that the protective effects of forced limb use could function through an increase in use of the non-casted forelimb.

### **5.3 Mechanism of exercise induced GDNF**

It is not known how exercise functions to increase trophic factor expression although several possibilities exist. The first possibility is that forced limb use functions to increase motor learning, forced use requires the animals to engage in alternative strategies for eating, drinking, and ambulating in their cages. These adaptive responses would likely produce motor learning responses in the brain that are well known to induce trophic factor expression (Gomez-Pinnila et al., 1998; Kliem et al., 2003). Exercise has been shown to increase glial cell proliferation (Gomez-Pinnila et al., 1998; Holmes et al., 2004; Li et al., 2005) and the role of glial cells providing trophic support for neurons is well established (Darlington, 2005). Therefore, it is possible that a forced limb use-induced increase in glia could function to increase GDNF. Because the RET and GFR- $\alpha$  are located on DA neurons, another cell type could likely function to release GDNF (Treanor et al., 1996; Smith et al., 2003). Additionally, it has been shown that

GDNF mRNA is expressed in the adult brain in several regions receiving projections from the nigra, including striatum, nucleus accumbens, thalamic nuclei, olfactory tubercle, hippocampus, cerebellum, cingulate cortex and olfactory bulb (Trupp et al., 1997). It will be important in future experiments to localize the increase in GDNF to particular cell type to further examine this hypothesis. Finally, as discussed in detail below it is possible that stress may play a large role in the exercise induced increase of GDNF.

#### **5.4 Differences in exercise induced and GDNF induced neuroprotection**

Important differences in the types of neuroprotective effects of GDNF and exercise were observed in these studies. First, forced limb use prior to 6-OHDA confers a more complete neuroprotection with no downregulation of TH, whereas, exogenous GDNF pretreatment does not protect from the initial decrease in TH. Although this difference may indicate that GDNF is not involved in the protective effects of exercise, as discussed below, I believe that it is more likely that it reflects one or more of several differences between the use of exercise and exogenous GDNF as neuroprotective treatments. First, in our study the exercise occurred over a period of 7 days prior to the administration of 6-OHDA (Chapter 2 and 3), whereas exogenous GDNF was given in a single bolus 6 hours before 6-OHDA (Chapter 4). Second, the amount and location of the increased endogenous GDNF that developed after exercise was undoubtedly different from that for exogenous GDNF. Third, it is highly likely that exercise produced many changes in addition to the rise in GDNF that we reported. My conclusion is that that exercise is likely to be a more potent protective approach than administration of GDNF alone.

## **5.5 GDNF remains present in the striatum up to 8 weeks following infusion**

It is not well established how long GDNF remains present following infusion but as discussed above, it has been shown that GDNF can confer neuroprotection when administered long before infusion of a neurotoxin. Establishing the temporal kinetics of GDNF remained an important issue, as it was unknown how long exogenous GDNF could confer protective effects on a compromised nigrostriatal pathway. Other groups have shown that removal of the GDNF stimulus reverses the protective benefits of the protein (Lu and Hagg, 1997). We therefore examined the presence of GDNF in the striatum at 2, 4, and 8 wks following infusion. In both animals treated with GDNF, the protein was detectable at 2, 4, and 8 wks following infusion. The largest increase in GDNF staining was observed at 2 wks post-infusion, with staining at 4 and 8 wks largely confined to the cannula track. I assume that this GDNF staining is due to the actual infusion of GDNF rather than a generic reaction to injury as animals treated with vehicle alone and 6-OHDA alone showed no GDNF immunoreactivity even along the cannula track. These data suggest that the effects of a single bolus of GDNF can be long lasting conferring beneficial effects long after infusion of the protein.

Although these data are not in agreement with previous findings, it is likely that differences in location of the infusion of GDNF could result in differences in the kinetics of the protein. Lu and Hagg (1997) infused chronic GDNF into the SN, whereas in our studies a single bolus of GDNF was infused in the striatum. This may be a crucial differences as it has been suggested that GDNF infused in the SN is ineffective at protecting axons and axon terminals from 6-OHDA (Kirik et al., 2004).

## **5.6 Clinical applications of exercise and GDNF**

The data presented in this dissertation suggest the both exercise therapy and GDNF infusion are able to prevent the neurotoxic effects of oxidative stress and thus are treatment alternatives for PD. Whereas forced-limb use has been shown produce beneficial effects clinically in other conditions (Taub et al., 1999; Liepert et al., 2000; Taub and Morris, 2001), it may not be an ideal physical therapy regime for PD patients. Although PD commonly presents itself unilaterally, it typically progresses to a bilateral disorder, making forced use of a single forelimb not ideal. However, others have focused on bilateral forms of exercise in experimental animals and in patients, supporting the hypothesis that exercise regimens can be used as a therapy (see Introduction).

Despite the likely efficacy of bilateral forms of exercise in PD, additional issues remain. As the disease progresses, movement becomes more difficult and therefore exercise regimes for later-stage patients will have to be tailored to the motor progression of PD and compliance to an exercise program may prove to be more difficult. Third, most of the exercise regimes that have been used in experimental animals (e.g., treadmill, running wheels, balance beams) are not readily available to many individuals.

One plausible clinical alternative to the use of exercise in the treatment of PD would be to take advantage of the molecules that appear to be endogenously increased by exercise. One such example is GDNF. Infusion of GDNF into patients with PD has been explored by several groups but the data remain quite controversial, as some groups have reported improvements in symptoms and pathology (Gill et al., 2003; Patel et al., 2005; Slevin et al., 2006) while others have shown no clinical improvement (Nutt et al., 2003; Lang et al., 2006). For GDNF or GDNF agonists to become an effective treatment option for PD, it will be necessary to standardize methods for delivery and the markers used for reporting improvements, particularly in the



pathology of the disease. As indicated in Chapter 4, it may be crucial to develop markers not reliant on phenotypes in the DA system, as they seem to change in response to stress, as this may lead to falsely discarding treatment options that may in fact be effective over time. A challenge to the clinical use of GDNF has been the inability of the molecule to cross the blood brain barrier requiring intracranial delivery of the protein, if a small molecule that mimicked the actions of GDNF would be ideal for the treatment of PD (eg Lin et al., 2004). Another related approach might be to develop drugs that act intracellularly to trigger processes that normally are stimulated by GDNF, such as the activation of ERK or Akt (see below). However, by bypassing the receptor for GDNF, one runs the risk of increasing the non-specific effects of such treatments.

## **5.7 Future directions**

### **5.7.1 Analysis of other trophic factors**

In this dissertation, I have chosen to focus on GDNF but there are many other trophic factors that may play a role in casting-induced neuroprotection. Exercise has been shown to increase BDNF and NGF (Neeper et al., 1996), FGF-2 (Gomez-Pinilla et al., 1995), and IGF-1 (Carro et al., 2001) in the CNS. Each of these factors could be a likely candidate for protection of the DA system, as these factors have been shown to have potent trophic activity towards DA neurons (Hyman et al., 1991; Altar et al., 1992; Lin et al., 1993; Winkler et al., 1996) and, where examined, has been shown to protect against the neurotoxic effects of 6-OHDA (Hoffer et al., 1994; Kearns & Gash, 1995; Levivier et al., 1995; Choi-Lundberg et al., 1998; Akerud et al., 1999; Gong et al., 1999; Kramer et al., 1999; Schatz et al., 1999; Shults et al., 2000; Wang et al., 2002).. Examining each of these factors will be crucial in establishing the mechanism for exercise-induced neuroprotection as it seems likely that more than one factor will ultimately be

found to be responsible for exercise-induced protection. To this end, FGF-2, one of the trophic factors shown to be upregulated by exercise (Gomez-Pinilla et al, 1995), has also been shown in vitro to induce expression of both GDNF and BDNF (Suter-Crazzolara and Unsicker, 1996; Kwon, 1997).

### **5.7.2 Analysis of the role of stress in the neuroprotective effects of exercise**

Although our focus has been on trophic factors as the mechanism for forced-limb use induced neuroprotection, there are other factors that may play a role. One such factor is stress, although forced limb use has positive effects on the brain, it also can be regarded as a form of restraint stress and thus, would be expected to stimulate the hypothalamic-pituitary-adrenal (HPA) axis. Whereas stress is normally classified as a neurotoxic factor, stress has also been shown to have neuroprotective effects (Sloviter et al., 1989; Chadi et al., 1993; Follesa and Mocchetti, 1993; Mocchetti et al., 1996; Sousa et al., 1999). It is possible, therefore, that the HPA axis plays a pivotal role in forced limb use protection through stimulated neurotrophic factor expression or through the production of melanocortins, both of which have been shown to have neuroprotective effects in various peripheral and central nervous system injury models (Sloviter et al., 1989; Chadi et al., 1993; Follesa and Mocchetti, 1993; Mocchetti et al., 1996; Sousa et al., 1999). It is possible that the increases in GDNF observed following forced limb use are a result of HPA axis induced increases in trophic factors, as discussed above, rather than as a result of an increase in limb use.

### **5.7.3 Analysis of other exercise regimes**

As noted above, forced limb use may not be the ideal form of exercise to use in the clinical setting, it is therefore important to examine other forms of exercise in animal models of PD to determine if another form of exercise could be more efficacious and more clinically

applicable. Indeed, others have examined treadmill and exercise in a running wheel, as well as an enriched environment and have shown neuroprotective effects in an MPTP in a mouse model of PD (Bezard et al., 2003; Tillerson et al., 2003; Faherty et al., 2005). It is important to note that in some studies where the effects of exercise have been examined complete protection has not been observed, Poulton and Muir (2005) observed behavioral protection from 6-OHDA but failed to demonstrate protection of DA neurons in animals who ran prior to 6-OHDA. These data raise the possibility that different types and durations of exercise may produce different results, demonstrating the necessity of further exploration of these differences.

#### **5.7.4 Role of exercise in non-motor effects of PD**

As discussed in Chapter 1, PD is not only manifested as a motor disorder but also produces non-motor symptoms, including depression, cognitive deficits, autonomic dysfunction and anosmia. To date, the focus on the protective effects of exercise has been on the motor symptoms and systems associated with an animal model of PD. I believe this will be an important effect to explore as others have shown that exercise and neurotrophic factors both decrease depressive symptoms and cognitive deficits in animal models (for reviews see: Mattson et al., 2001; Duman et al., 2005). Indeed, our laboratory has preliminary results indicating that forced limb use protects from 6-OHDA induced damage to the nucleus accumbens and the ventral tegmental area.

#### **5.7.5 The mechanism of trophic factor induced neuroprotection**

I believe that trophic factors play a major role in the protective effects of forced limb use, but it remains unknown how specifically they confer these effects. GDNF appears to act via a plasma membrane receptor complex consisting of GFR $\alpha$ 1 and RET, which in turn triggers several signaling cascades (for review see: Airaksinen et al., 1999). Two possible signaling

cascades that have been shown to be activated by GDNF are the phosphatidylinositol 3-kinase (PI 3K)/Akt and the Ras/extracellular signal-regulated kinase (ERK) signaling pathways. It is known that GDNF activates ERK (Soler et al., 1999; Trupp et al., 1999) and that blockade of the ERK pathway attenuates GDNF-induced survival in cultures of hippocampal neurons and ciliary ganglion neurons (e.g., Lenhard et al., 2002; Peterziel et al., 2002; but see also Soler et al., 1999). In addition, to the ERK pathway, some of the effects of GDNF may be mediated by Akt. GDNF has been shown to activate Akt (van Weering & Bos, 1998; Soler et al., 1999; Besset et al., 2000; Jin et al., 2002; Neff et al., 2002; Perez-Garcia et al., 2004) and that blockade of this pathway blocks GDNF induced survival (Soler et al., 1999). Data from our lab also indicate that the protective effects of GDNF against 6-OHDA may be mediated in part by activation of Erk1/2 and Akt in a DA-like cell line (Ugarte et al., 2003). Additionally, preliminary data from our lab indicate that in vivo exercise and GDNF increase Erk 1/2 and that GDNF increases Akt in the striatum and SN. These data suggest that further exploration of the time course of activation of these factors is merited, particularly in light of the fact that a causal relationship can be easily established because pharmacological inhibitors for both the ERK and Akt pathways exist. In addition, further analysis of these pathways may provide more targets for drug creation, particularly as early detection methods become more readily available.

## **5.8 Conclusions**

The data presented in this dissertation provide a basis for the neuroprotective effect of exercise in an animal model of PD, with that effect involving an increase in GDNF. We demonstrated here that forced limb use substantially attenuates the behavioral, neurochemical, and neuroanatomical effects of 6-OHDA into the nigrostriatal pathway. Additionally, we showed

that forced limb use produces a robust increase of GDNF protein in the striatum, indicating a role for trophic factor signaling in the neuroprotective effects of forced limb use. Finally, to further explore the protective effects of GDNF, we observed that GDNF protects DA neurons from cell death after 6-OHDA but does not protect from the early loss of phenotypic markers of the DA system, but is able to normalize after 6-OHDA infusion. Taken together these data suggest that exercise and GDNF are viable potential treatment options for PD, but that care must be taken in the analysis of markers of the DA system when evaluating neuroprotection.

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