TUMOR NECROSIS FACTOR DEPENDENT MECHANISMS AND

NEUROPROTECTIVE STRATEGIES IN MODELS

OF PARKINSON'S DISEASE

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To my family and friends for their love and support

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TUMOR NECROSIS FACTOR DEPENDENT MECHANISMS AND

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Parkinson's disease is a chronic, progressive, neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra that innervate the striatum.

Although the nigral cell loss that causes motor dysfunction in Parkinson's disease has been identified for some time, the mechanisms that lead to this dopaminergic neuron loss are unclear. Elevated levels of the cytokine tumor necrosis factor in cerebrospinal fluid and postmortem brains of Parkinson's patients and in animal models of the disease implicate tumor necrosis factor in contributing to disease pathology; but a specific role for this cytokine in mediating loss of dopaminergic neurons in Parkinson's disease has not been clearly established. Here I demonstrate that neutralization of soluble tumor necrosis factor *in vivo* with a recombinant dominant-negative tumor necrosis factor inhibitor reduced 6-hydroxydopamine-induced nigral degeneration by 50% and attenuated amphetamine-induced rotational behavior, indicative of striatal dopamine preservation in a rodent model of Parkinson's disease. Similar protective effects were observed with *in vivo* chronic co-infusion of dominant-negative tumor necrosis factor inhibitor with a proinflammatory initiator, low-dose lipopolysaccharide, into the substantia nigra of rodents, confirming a role for soluble tumor necrosis factor inhibitor - dependent neuroinflammation in contributing to nigral degeneration. In rat embryonic midbrain neuron/glia mixed cell cultures exposed to lipopolysaccharide, delayed administration of dominant-negative tumor necrosis factor inhibitor prevented degeneration of dopaminergic neurons despite sustained microglia activation. Addition of a dominant-negative tumor necrosis factor inhibitor also attenuated 6-hydroxydopamine-induced dopaminergic neuron toxicity *in vitro*. In this dissertation the ability of lentiviral-encoded dominant-negative tumor necrosis factor inhibitor to provide neuroprotection was also investigated. Intranigral delivery of lentiviral dominant-negative tumor necrosis factor inhibitor in a hemiparkinsonian rat 6-hydroxydopamine model attenuated nigral

dopaminergic neuron loss and reduced behavior deficits when compared to control lentiviral-infected animals. Collectively, these data identify tumor necrosis factor signaling in contributing to dopaminergic neuron loss *in vitro* and *in vivo* in two chronic rat models of Parkinson's disease, and provide evidence that delaying the progressive degeneration of the nigrostriatal pathway in the early stages of Parkinson's disease in humans may be therapeutically feasible with agents that block soluble tumor necrosis factor signaling.

TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AADC – Aromatic L-amino Acid Decarboxylase

AAV – Adeno-Associated Virus

ADAS – Adipose-Derive Adult Stromal Cells

ANOVA – One-way Analysis of Variance

ASK1 – Apoptosis Signal-regulating Kinase 1

BDNF – Brain-Derived Neurotrophic Factor

bFGF – basic Fibroblast Growth Factor

BMSCs – Bone Marrow Stromal Cells

C3R – Complement 3 Receptor

cIAPs – cellular Inhibitor of Apoptosis proteins

COR – C-terminal of Ras of complex proteins

COX – Cyclooxygenase

DA – Dopamine

DBS – Deep Brain Stimulation

DD – Death Domain

DN-TNF – Dominant Negative Tumor Necrosis Factor

ESCs – Embryonic Stem Cells

EVM – Embryonic Ventral Mesencephalon

FADD – Fas-Associated Death Domain

FBS – Fetal Bovine Serum

GABA – Gamma-Aminobutyric Acid

GAD – Glutamatic Acid Decarboxylase

GDNF – Glial-Derived Neurotrophic Factor

GFAP– Glial Fibrillary Acidic Protein

GSH – Glutathione

GTPases – Guanosine Triphosphate Phosphohydrolases

HTRA2 – high temperature requirement A2

IFNγ – Interferon-γ

IL-1 – Interleukin-1

IL-6 – Interleukin-6

iNOS – inducible Nitric Oxidase Synthase

JNK – c-Jun N-terminal Kinase

KO – Knockout

LPS – Lipopolysaccharide

LRRK2 – Leucine-Rich-Repeat Kinase 2

LTα3 – Lymphotoxin alpha

MAO-B – Monoamine Oxidase B

MEA – Multi-Electorode Array

MnSOD – Manganese Superoxide Dismutase

MPDP+ – 1 -Methyl-4-phenyl- 2,3 –dihydropyridinium,

MPP+ – 1-methyl-4-phenylpyridinium

MPPP – 1-methyl-4-phenyl-4-propionoxypiperidine

MPTP – 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NDM – Neural Differentiation Medium

NeuN – Neuron-specific Nuclear protein

NF-κB – Nuclear Factor kappa-B

nNOS – neuronal Nitric Oxide Synthase

NOD2 – Nucleotide-binding Oligomerization Domain containing 2 protein

NOS – Nitric Oxide Synthase

Nrf2 – Nuclear factor erythroid 2-related factor

NSAIDS – Non-Steroidal Anti-Inflammatory Drugs

NSCs – Neural Stem Cells

PD – Parkinson's disease

PINK1 – Tensin homologue (PTEN)-induced kinase 1

RIP – Receptor Interacting Protein

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

SD – Standard Deviation

SEM – Standard Error of the Mean

SKPs – Skin-derived Precusors

SNpc – Substantia Nigra pars compacta

SODD – Silencer Of Death Domains

solTNF – soluble Tumor Necrosis Factor

TACE – Tumor necrosis factor Alpha Converting Enzyme

TGF-β – Transforming Growth Factor-β

TBI – Traumatic Brain Injury

TH – Tyrosine Hydroxylase

tmTNF – transmembrane Tumor Necrosis Factor

TNF – Tumor Necrosis Factor

TNF-ICD – Tumor Necrosis Factor Intracellular Domain

TNFR1 – Tumor Necrosis Factor Receptor 1

TNFR2 – Tumor Necrosis Factor Receptor 2

TRADD – Tumor necrosis factor Receptor Associated Death Domain

TRAF2 – Tumor necrosis factor Receptor Associated Factor 2

UCH-L1 – Ubiquitin Carboxy-terminal Hydrolase L1

UPS – Ubiquitin Proteosome System

WT – Wild Type

CHAPTER ONE
Introduction

PARKINSON'S DISEASE OVERVIEW

Identification, Pathology, Clinical Symptoms

Identification

Parkinson's disease (PD) is a chronic, progressive, neurodegenerative disorder with age-associated onset. The prevalence of PD is approximately 1% in individuals between 65–70 years of age, increasing to 4–5% of the population in 85-year-olds (Fahn, 2003). Sporadic PD has a mean age of onset of 70 (Farrer, 2006; Tanner, 2003); but inherited familial forms of the disease, which are currently estimated at 4% of total cases, are typically early-onset with diagnosis occurring before the age of 50 (Mizuno et al., 2001; Van Den Eeden et al., 2003). PD has been recognized by physicians for centuries, but was first described by James Parkinson in "An Essay on the Shaking Palsy" initially published in 1817 in which he describes the hallmark clinical manifestations of PD including tremor, rigidity, bradykinesia, akinesia, and postural instability (Parkinson, 1817; Parkinson, 2002).

Pathology

Parkinson's disease is characterized by the loss of melanized dopamine (DA)-producing neurons in the substantia nigra pars compacta (SNpc) region of the midbrain. Loss of these nigral DA neurons leads to decreased innervation of the dorsal striatum, and it is this loss of striatal DA that results in the motor symptoms of PD and clinical diagnosis of

the disease. Histologically, PD is identified by the presence of Lewy bodies, intraneuronal cytoplasmic inclusions whose main components are ubiquitin and α-synuclein, first identified and linked to PD pathology in 1912 by Frederic Lewy. PD pathology, although diagnosed by DA neuron loss in the SNpc, is known to affect additional neuronal cell types in both the central and peripheral nervous systems. Braak developed criteria to define stages of the progressive pathology in PD by assessing the accumulation of Lewy neurites and overt Lewy body formation throughout the nervous system including in the dorsal motor nuclei of glossopharyngeal and vagal nerves, the olfactory bulb, nuclei of the lower brainstem including the locus coeruleus, and eventually subsets of thalamic nuclei (Braak et al., 2003; Braak et al., 2004; Braak et al., 2000). In the periphery the enteric nervous system, particularly the ganglion cells of Auerbach's and Meissner's plexi, is affected by Lewy body pathology (Wakabayashi et al., 1993; Wakabayashi et al., 1988).

Clinical symptoms

The motor symptoms of PD include postural instability, tremor, rigidity, bradykinesia, akinesia, and difficulty speaking and swallowing. These motor symptoms occur due to the degeneration of SNpc DA neurons that project to the striatum; their loss results in alteration of the basal ganglia neural circuit activity that regulates movement by inhibition of the direct pathway and excitation of the indirect pathway. The direct pathway promotes motor activity and the indirect pathway inhibits movement, therefore reduction of DA innervation results in a hypokinetic movement disorder by increasing inhibition of the ventral lateral nucleus of the thalamus, which sends excitatory

projections to the motor cortex. In addition to symptoms stemming from the loss of SNpc DA neurons, degeneration of non-nigral neurons in PD leads to non-motor aspects of the disease including depression, anxiety, fatigue, sleep disorders, and autonomic and olfaction dysfunction (reviewed in (Ziemssen and Reichmann, 2007)). Many of these non-motor symptoms occur before the significant loss (60-80%) of striatal DA innervation that results in the more recognized motor symptoms of disease has occurred (Ponsen et al., 2004; Sommer et al., 2004). Epidemiological evidence suggests that autonomic and olfaction deficits precede motor symptoms and may be useful in the development of criteria to diagnosis PD before a substantial deficit in DA production has occurred (reviewed in (Becker et al., 2002)).

CONTRIBUTION OF GENETIC AND ENVIRONMENTAL FACTORS TO PARKINSON'S DISEASE INITIATION AND PROGRESSION

Involvement of the Ubiquitin Proteosome System, Mitochondrial Dysfunction, and Oxidative Stress in PD

A prevalent current hypothesis is that a combination of genetic and environmental factors may both be important components of disease initiation and progression. Many current research programs aim to understand the common mechanisms between environmental disease-causing agents and genetic mutations. Three main themes of cellular dysregulation have been implicated in disease progression resulting from both

environmental and genetic causes; these include ubiquitin proteosome dysfunction, increased oxidative stress, and mitochondrial dysfunction.

The Ubiquitin Proteosome System (UPS) is the primary cellular mechanism by which misfolded and aggregated proteins are removed from the cytosol. This system is also responsible for the majority of homeostatic intracellular protein degradation. Involvement of UPS dysfunction in initiating or contributing to PD pathology is indicated by the presence of Lewy bodies which are intracellular proteinacious aggregates, and by mutations in UPS-associated proteins that have been linked to familial PD. Lewy body formation is thought to be a protective mechanism by which proteins that can not be degraded by the proteosome due to aggregation or decreased proteosomal function are sequestered. In addition, triplication of or mutations in α-synuclein, a major component of Lewy bodies, results in familial PD (Singleton et al., 2003). Another line of evidence for proteosome involvement in PD is that several of the mutations identified to cause familial PD function in the UPS including Parkin, Ubiquitin carboxy-terminal hydrolase L1 (UCHL-1), and α-synuclein (reviewed in (Le and Appel, 2004)).

Mitochondrial dysfunction is a second cellular mechanism that is believed to influence DA neuron loss. Normal mitochondrial function is necessary for cellular metabolism and energy homeostasis through ATP generation. Mitochondrial dysfunction as a contributor to PD was first recognized when drug addicts presented with severe parkinsonism after injecting MPPP (1-methyl-4-phenyl-4-propionoxypiperidine), a synthetic opiod, which was contaminated with MPTP (1-methyl 4-phenyl 1,2,3,6-

tetrahydropyridine) that resulted in irreverable parkinsonism (Langston et al., 1983). After conversion of MPTP to MPP+ (1-methyl-4-phenylpyridinium) by glial cells, MPP+ is taken up by the dopamine transporter where once inside the cell it inhibits mitochondrial complex 1 eventually resulting in DA neuron death. In addition to DA neuron-specific toxin-induced inhibition of complex 1 as a cause of PD, systemic deficits in mitochondrial respiration in PD patients have been recognized for some time based on work in which platelet-derived mitochondria from PD patients were used to repopulate neuroblastoma cells in which mitochondria were absent. These cybrid cells were found to have decreased basal mitochondrial complex 1 activity, increased oxidative stress, and enhanced susceptibility to mitochondrial complex 1 inhibitors (Swerdlow et al., 1996). In addition, an intrinsic sensitivity to mitochondrial complex I defects or inhibitors of complex I activity specifically in DA neurons is evidenced by the selective toxicity of the pesticide rotenone for DA neurons despite rotenone-mediated inhibition of mitochondrial complex I throughout the brain (reviewed in (Sherer et al., 2002a)). Moreover, PD-linked genes implicate mitochondria either directly or indirectly in disease pathogenesis, these mutations include DJ-1, tensin homologue (PTEN)-induced kinase 1 (PINK1), leucine-rich-repeat kinase 2 (LRRK2), and high temperature requirement A2 (HTRA2) (Lin and Beal, 2006).

Increased oxidative stress is a third mechanism of cell death in PD and is common to both mitochondrial dysfunction and protein aggregation. Oxidative stress occurs when there is an intracellular accumulation of reactive oxygen and nitrogen species (ROS/RNS) due to lower endogenous anti-oxidant capacity and overproduction

of ROS within the cell. One source of intracellular ROS accumulation is through mitochondrial production of oxidative by-products during oxygen consumption in the electron transport chain, which would render neurons especially sensitive as the brain consumes 20% of the total oxygen in the body (reviewed in (Floyd, 1999)). Oxidative stress-driven mitochondrial dysfunction is thought to be a particular problem in DA neurons due to a high metabolic rate, low content of antioxidants, and elevated levels of oxidizable species including neuromelanin, polyunsaturated fatty acids, iron, and dopamine (Peter, 2003). The synthesis, degradation, and auto-oxidation of dopamine into melanin all generate hydrogen peroxide which decomposes to hydroxide, a highly oxidative oxygen free radical. The generation of hydroxide from hydrogen peroxide is accelerated by the presence of free ferrous ion, which is also abundant in substantia nigra (Spatz, 1922). Increased oxidative stress leads to the oxidation of proteins, lipids, and DNA, impairs cellular functions including mitochondrial respiration, and can increase the formation of toxic species all of which can induce proapoptotic signaling (reviewed in (Barnham et al., 2004)). ROS accumulation also contributes to abberant calcium signaling and the eventual induction of apoptosis (Ermak and Davies, 2002). In further support for oxidative stress as a contributor to PD pathology are the findings that protein oxidation, nucleoside oxidation, and lipid peroxidation are all increased in the SNpc of PD brains compared to healthy controls (Floor and Wetzel, 1998; Yoritaka et al., 1996; Zhang et al., 1999).

Contribution of genetics to Parkinson's disease

Although the majority of PD cases are sporadic, meaning there is no known disease mutation, PD can be inherited and specific mutations have been demonstrated to be causal. Both autosomal dominant and autosomal recessive genes have been linked to disease (Table 1.1; reviewed in (Hardy et al., 2006).

Dominantly inherited mutations

The first mutation associated with PD was an A53T point mutation in the α-Synuclein gene (Polymeropoulos et al., 1997). α-Synuclein is a soluble and natively unstructured protein with unclear function, but it is the main component of Lewy bodies. Three dominant point mutations (A53T, A30P, and E46K) and gene triplication have been associated with inherited disease, indicative of a gain of function disease mechanism (Kruger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003; Zarranz et al., 2004). The A53T and E46K mutations lead to early onset PD with rapid progression wheras the A30P mutation results in disease onset and progression that is similar to sporadic disease (Kruger et al., 2001; Spira et al., 2001; Zarranz et al., 2004). In addition to severe, early onset disease as a result of point mutations and gene triplication, α-synuclein duplication and promoter and 3' region polymorphisms have been associated with increased idiopathic disease risk (Chartier-Harlin et al., 2004; Ibanez et al., 2004; Maraganore et al., 2006; Pals et al., 2004; Tan et al., 2004), with disease severity correlating to α-synuclein gene dosage (Farrer et al., 2004). Due to the presence of wild-type α-synuclein in Lewy bodies found in sporadic PD, and the higher propensity of the

mutated protein to aggregate in comparison to wild-type α-synuclein (Conway et al., 1998; Giasson et al., 1999; Hashimoto et al., 1998; Pandey et al., 2006), UPS dysfunction leading to oxidative stress is thought to be the cellular mechanism of α-synuclein toxicity.

A second protein which has been found to have autosomal dominant inheritance in PD is leucine-rich repeat kinase 2 (LRRK2), mutations in which lead to dominant inheritance with variable penetrance. Screening has shown LRRK2 to be the most prevalent cause of familial PD, particularly within specific ethnic groups, and mutations have been found in sporadic disease as well (Bonifati, 2007; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Patients with LRRK2 mutations have variable disease onset and although many have pathology indistinguishable from idiopathic disease with the presence of Lewy bodies, pathology can differ even among carriers of the same mutation (Bonifati, 2006). LRRK2, a member of the ROCO family of Guanosine Triphosphate Phosphohydrolases (GTPases), is a large protein containing many discrete domains including a Roc GTPase, an associated C-terminal of Roc (COR) domain, a WD40 repeat, an armadillo and anykrin repeat region, leucine-rich repeats, and a kinase domain (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). This protein is known to have both kinase and weak GTPase activity, although the PD-relevant *in vivo* substrates are unknown (Lewis et al., 2007; West et al., 2005). More than 20 point mutations have been reported in LRRK2, and many of those which are known to confer pathogenicity are located throughout and between the kinase and Roc GTPase domains (Mata et al., 2005). The many mutations and variable onset and pathology associated with these mutations suggest LRRK2 may have multiple functions. Several disease-causing mutations located

within the kinase region and particularly the G2019S mutation have been consistently demonstrated to increase the *in vitro* kinase activity of LRRK2 on phosphorylation of nonspecific substrates and autophosphorylation consistent with a dominant phenotype, however the effect of many mutations on kinase activity is unclear (Gloeckner et al., 2006; Greggio et al., 2006; Jaleel et al., 2007; Luzon-Toro et al., 2007; MacLeod et al., 2006; West et al., 2005). In addition to mutations affecting kinase activity, the R1441C/G mutation within the GTPase domain decreases GTP hydrolysis (Lewis et al., 2007; Li et al., 2007). Although the pathogenic mechanism(s) of LRKK2 mutations are not known, the kinase activity of LRRK2 is required for increased toxicity resulting from mutations even in cases where the mutation does not enhance kinase activity (Greggio et al., 2006; Smith et al., 2006). Due to the presence of many protein interaction domains in LRRK2, pathogenic mutations located outside of the kinase domain are thought to alter LRRK2 association with other proteins to influence pathology.

UCH-L1 is a deubiquitinating enzyme in which a missense mutation, I93M, has been linked to autosomal dominant PD in one kindred (Leroy et al., 1998). However the relevance of mutation in UCH-L1 to PD is unclear as additional families with UCH-L1 mutations have not been identified. UCH-L1 has several described functions including ubiquitination (Liu et al., 2002), deubiquitination (Larsen et al., 1998; Wilkinson et al., 1989), and mono-ubiquitin stabilization (Osaka et al., 2003). In an effort to validate UCH-L1 mutations as a cause of DA neuron loss, transgenic mice overexpressing the human UCH-L1 I93M mutation were generated and a 30% reduction of SNpc DA neurons was observed in these mice at 20 weeks of age (Setsuie et al., 2007). Further

evidence linking UHC-L1 function to PD is that a gene polymorphism that results in the substitution of serine 18 to tyrosine has been associated with decreased susceptibility to PD (Maraganore et al., 1999; Maraganore et al., 2004) due to decreased ubiquitin ligase activity of the protein towards α-synuclein leading to stabilization of the protein (Liu et al., 2002).

Recessively inherited mutations

Parkin is a RING finger-containing protein that functions as an E3 ubiquitin ligase and has been identified as causing autosomal recessive juvenile-onset Parkinson's disease (Shimura et al., 2000). Parkin mutations are highly prevalent in juvenile onset PD; 50% of familial and 70% of sporadic PD patients bear Parkin mutations when disease is diagnosed before the age of 20 ((Lucking et al., 2000; Periquet et al., 2003); reviewed in (Mata et al., 2004)). Although Parkin has typical E3 ligase activity and can ubiquitinate through lysine 48 linkages to direct proteins to the proteosome, it has also been demonstrated to catalyze lysine 63 linkages which may be involved in intracellular signaling pathways and in the enhancement of Lewy body formation (Lim et al., 2005). Supporting a role for Parkin in influencing Lewy body deposition, it has recently been demonstrated that Parkin overexpression protects DA neurons from mutant α-synuclein overexpression and this protection correlates with increased accumulation of hyperphosphorylated α-synuclein inclusion bodies (Lo Bianco et al., 2004). Although this evidence indicates Parkin may contribute to PD pathology through changing the activity of the UPS, Parkin-deficient mice also display reductions in proteins involved in oxidative stress and mitochondrial dysfunction which correlate with reduced

mitochondrial respiration in the striatum, decreased serum antioxidants, and increased markers of oxidative stress including protein and lipid modification, leaving open many pathways by which loss of Parkin activity may impinge upon DA neuron survival (Palacino et al., 2004).

Mutations in PTEN-induced kinase 1 (PINK1) have been linked to autosomal recessive parkinsonism with early onset and slow progression (Valente et al., 2004). PINK1 is a serine/threonine kinase with high homology to calcium/calmodulin-dependent serine/threonine kinases, that contains a mitochondrial targeting sequence; both mutant and wildtype proteins are targeted to the mitochondria (Gandhi et al., 2006; Silvestri et al., 2005). Mutations in PINK1 are found throughout the coding region with missense mutations commonly found in the kinase region. PINK1 is protective against mitochondrial dysfunction, oxidative stress, and proteosome-activated apoptosis and this PINK1-mediated protection is dependent upon intact kinase activity (Petit et al., 2005; Sim et al., 2006; Valente et al., 2004). PINK1 has been linked to both Parkin and DJ-1: in drosophila, Parkin overexpression can rescue from PINK1 knockout-induced toxicity; and physical interaction between PINK1 and DJ-1 has been demonstrated to have a synergistic protective effect under conditions of oxidative stress in cell culture systems (Clark et al., 2006; Tang et al., 2006). Elucidation of the protective mechanism of PINK1 is hampered by the lack of identified *in vivo* substrates resulting in the dependence on studies of autophosphorylation or *in vitro* phosphorylation of generic substrates to determine the function of wildtype PINK1 and alterations in activity due to Parkinson's disease linked mutations. However, recently the mitochondrial chaperone tumor necrosis

factor receptor-associated protein 1 was shown to be a PINK1 substrate (Pridgeon et al., 2007) and identification of additional substrates should help to determine protective PINK1 activities.

Loss of function mutations in DJ-1 lead to early-onset autosomal recessive PD (Bonifati et al., 2003). Several lines of evidence suggest that DJ-1 acts as an oxidant sensor or antioxidant protein. DJ-1 undergoes an isoelectric shift under conditions of oxidative stress as a result of the oxidation of cysteine 106 to a cysteine sulfinic acid resulting in increased mitochondrial localization (Canet-Aviles et al., 2004). DJ-1 overexpression also protects cells from a variety of oxidative stress-inducing toxins (Inden et al., 2006; Paterna et al., 2007; Taira et al., 2004). Many DJ-1 mutations have been shown to destabilize the protein, some through disruption of DJ-1 homodimers, leading to degradation of DJ-1 (Gorner et al., 2007; Hulleman et al., 2007; Ooe et al., 2006). Possible mechanisms of DJ-1-provided increased antioxidant capacity include stabilization of Nrf2 (nuclear factor erythroid 2-related factor) which is antioxidant transcriptional regulator (Clements et al., 2006), and DJ-1-induced increases in the level of the intracellular antioxidant glutathione (Zhou and Freed, 2005). DJ-1 also appears to have chaperone activity as DJ-1 overexpression can attenuate α-synuclein aggregation resulting from oxidative stress if DJ-1 itself is in an oxidized state which permits its association with α-synuclein (Shendelman et al., 2004; Zhou et al., 2006). Mice lacking DJ-1 protein expression have been generated and although they do not develop neuronal cell loss they do exhibit age-dependent motor deficits, hypokinesia, dopaminergic system

dysfunction, and increased sensitivity to the neurotoxin MPTP (Chen et al., 2005b; Goldberg et al., 2005; Kim et al., 2005).

Contribution of environmental factors to Parkinson's disease

Until the recent identification of PD-linked gene mutations, environmental agents were thought to be the main cause of DA neuron loss in PD. These environmental factors associated with an increased risk of non-familial, idiopathic PD include traumatic head injury, viral infection, rural living, well-water consumption, occupations including welding and mining, and exposure to heavy metals, organophosphate compounds, neurotoxins including MPTP, and certain pesticides such as paraquat and rotenone (Casals et al., 1998; Firestone et al., 2005; Goldman et al., 2006; Jankovic, 2005; Kamel et al., 2007; Liu et al., 2003; Priyadarshi et al., 2001; Stern et al., 1991; Thiruchelvam et al., 2002). However, causative environmental chemical agents have not been identified definitively in the etiology of Parkinson's disease despite intensive research, suggesting that multiple environment and genetic triggers may be necessary for the development of idiopathic disease.

Viral encephalitis

One environmental risk that has been well established in PD susceptibility, and promoted the belief that PD could be caused by environmental stimuli, is viral encephalitis. The influenza pandemic of 1918 was associated with a dramatic increase in post-encephalitic parkinsonism in the 1920s and 30s, comprising approximately half of all parkinsonism

cases (Dale et al., 2004; Josephs et al., 2002). Furthermore, it is well known that individuals infected with Japanese encephalitis virus for longer than one year are at risk for developing post-encephalitis parkinsonism with many of the same pathological and clinical symptoms as those found in sporadic PD (Shoji et al., 1993). Japanese encephalitis virus has been used to model post-encephalitic parkinsonism in rats as infection in rodents results in catecholamine reduction and severe hypokinesia redolent of parkinsonism (Hamaue et al., 2006; Ogata et al., 1997).

Gastrointestinal infections and autoimmunity

Autonomic disturbances are an early feature of sporadic PD and it has been suggested that diseases of the gastrointestinal tract could contribute to enhanced vulnerability for the disease (Przuntek et al., 2004; Weller et al., 2005a; Weller et al., 2005b). Supporting a role for chronic systemic inflammation in leading to increased risk of sporadic PD, polymorphisms in the nucleotide-binding oligomerization domain containing 2 protein (NOD2) have been shown to be associated with Crohn's disease, a chronic inflammatory disease of the intestinal tract (Hugot et al., 2001; Ogura et al., 2001), and more recently have been shown to be over-represented in sporadic PD patients (Bialecka et al., 2007). Moreover, supporting an association between infectious diseases of the gastrointestinal tract and increased risk of PD is the evidence that parkinsonism has been loosely associated with peptic ulceration and patients diagnosed with sporadic PD are more likely than control individuals to be seropositive for *H. pylori,* a causative agent for peptic ulcers (Dobbs et al., 2000). The mechanism underlying the correlation between chronic gastrointestinal tract infection and inflammation and PD susceptibility is not well

understood, but could be related to autonomic dysfunction which might then sensitize an individual to a subsequent insult that contributes to PD risk.

Traumatic brain injury (TB1)

Head injury is an inconsistently reported risk factor for PD (Factor and Weiner, 1991; Goldman et al., 2006; Nayernouri, 1985; Stern et al., 1991; Stern, 1991), but recent twin studies provide data that head injury resulting in amnesia or loss of consciousness increases PD risk with the association being stronger in monozygotic twins than in dizygotic twins (Goldman et al., 2006). If both twins in the study had PD, the twin with younger onset was more likely to have sustained a previous head injury. The mechanisms by which TBI contributes to increased PD risk are unknown, but a murine experimental model of TBI suggests that in aged mice one possibility is injury-induced nitration of α-synuclein resulting from neuroinflammation (Uryu et al., 2003). Although it is unclear how nitrative stress contributes to the development of α-synuclein pathology in neurodegenerative diseases or traumatic brain injury, studies suggest that oxidative and nitrative stress may play a role in promoting the aggregation of α -synuclein (Norris et al., 2003; Paxinou et al., 2001) or in stabilizing aggregated forms of α-synuclein (Souza et al., 2000), resulting in further oxidative and proteolytic stress.

1 -Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)

MPTP was discovered as a DA neurotoxin when it was intravenously injected as an impurity in a meperidine analog, 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP), which was being produced as a synthetic opioid. Several days after repeated intravenous

injection four individuals exhibited severe parkinsonism that responded to dopamine replacement therapy (Langston et al., 1983). After identification of the neurotoxin animal models were developed, initially through repeated injections of MPTP in rhesus monkeys, which results in selective destruction of the SNpc dopamine neurons with concomitant behavioral deficits and responsiveness to L-dopa (Burns et al., 1983). Shortly after the MPTP model was developed in monkeys, a protocol to induce MPTP-dependent parkinsonism in mice was developed (Heikkila et al., 1984a). Development of these animal models of dopaminergic neuron loss allowed for the investigation of DA neuron sensitivity, PD therapies, and neuroprotective agents. After systemic injection, MPTP readily crosses the blood brain barrier where it is converted to 1 -methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) within glial cells by monoamine oxidase B, and then to the active neurotoxin MPP+ (1-methyl-4-phenylpyridinium). MPP+ is next released to the extracellular space where it enters cells through selective uptake by the dopamine transporter as well as noreprinephrine and serotonin transporters (reviewed in (Przedborski and Vila, 2003)). Once inside the neuron, MPP+ is concentrated in mitochondria where it binds to complex 1 and disrupts the flow of electrons along the electron transport chain resulting in inhibition of ATP formation, as well as increases in the generation of reactive oxygen species (Chan et al., 1991; Rossetti et al., 1988).

Pesticides

Pesticide exposure has been linked to PD through the epidemiologic evidence that rural living and increased pesticide exposure results in a higher incidence of PD (Ascherio et al., 2006; Liou et al., 1997; Priyadarshi et al., 2000). Further associating pesticide

exposure with increased disease susceptibility is the ability of many pesticides and herbicides to inhibit mitochondrial complex I respiration, and the structural similarity of the pesticide rotenone to MPTP. The inhibition of mitochondrial complex I activity by pesticides enhances hydrogen peroxide and superoxide generation which cause further mitochondrial dysfunction as well as promote the formation of hydroxide and peroxinitrite; all of which have an additive effect to increase complex I damage and mitochondrial dysfunction (reviewed in (Gomez et al., 2007)). This hypothesis of pesticide/herbicide-induced mitochondrial dysfunction and oxidative stress leading to DA neuron loss has been supported by the evidence that treatment of rodents with the pesticide rotenone (Betarbet et al., 2000; Sherer et al., 2003b), the herbicide paraquat (Brooks et al., 1999), and the fungicide maneb with paraquat (Thiruchelvam et al., 2002) results in parkinsonian behavior and DA neuron loss. Treatment with rotenone, a potent and selective inhibitor of complex 1, results in increased oxidative stress including glutathione depletion and increased oxidative modification of DNA and proteins and is toxic to DA neurons at concentrations below those which result in ATP depletion (Sherer et al., 2002b). Paraquat has been reported to impair mitochondrial function in rat liver through uncoupling of oxidative phosphorylation and inhibiting electron transfer (Palmeira et al., 1995), and in the brains of rodents administered paraquat decreased mitochondrial complex I activity and increased lipid peroxidation have been observed (Tawara et al., 1996).

Heavy metals

Implicating chronic heavy metal exposure to increased PD susceptibility, copper and iron levels are elevated in cerebrospinal fluid and postmortem brains of PD patients (Dexter et al., 1987; Pall et al., 1987; Sofic et al., 1988), and exposure to manganese or copper for more than twenty years has been associated with increased PD risk (Gorell et al., 1999). Chronic exposure to high levels of manganese has been linked to the neurological disorder manganism with similarities to idiopathic PD characterized by slowing motor function, tremor, rigidity, and psychosis (Mergler and Baldwin, 1997). Manganese toxicity, although causing symptoms mirroring idiopathic PD, is frequently associated with atypical L-Dopa responses which are not maintained and are not accompanied by the development of dyskinesia, indicative of a different disease mechanism and pathology (Huang et al., 1993). In fact, manganese toxicity has been associated with degeneration in the globus pallidus, substantia nigra pars reticularis, and striatum as opposed to the SNpc degeneration present in PD (reviewed in (Olanow, 2004)). Heavy metal exposure is likely to cause selective nigrostriatal dysfunction and death through increased oxidative stress as iron, copper, and manganese can increase auto-oxidation of dopamine to dopamine quinone, and iron can promote hydroxyl free-radical generation by catalyzing the Fenton reaction (reviewed in (Montgomery, 1995)).

Interplay between genetics and environment in disease initiation and progression

As many genetic mutations linked to Parkinson's disease have a variable age of onset, and in some instances variable penetrance, and as exposure to environmental toxins appears to contribute to sporadic PD, but exposure can differ greatly in affected individuals, it is thought that susceptibility to disease initiation and progression may

result from a combination of genetic susceptibility and environmental exposure. For example, sporadic PD in patients bearing single nucleotide polymorphisms in Parkin exhibits earlier onset in individuals exposed to environmental risk factors including pesticides and organic solvents compared to individuals who have the polymorphism but no environmental exposures (Ghione et al., 2007). Furthermore individuals with identical LRRK2 mutations have variable age of onset even within members of the same family (Civitelli et al., 2007; Kachergus et al., 2005; Roberta Marongiu, 2006). Also in support of a "multiple hit hypothesis" is that both environmental agents and genetic determinants of PD all appear to act through the common pathways of UPS dysfunction, oxidative stress, and mitochondrial inhibition (reviewed in (Sulzer, 2007)).

INFLAMMATION AND PARKINSON'S DISEASE

We recently published an in-depth review of inflammatory processes which regulate cellular mechanisms that may contribute to PD including mitochondrial dysfunction, oxidative stress, and proteolytic stress, as well as PD-linked environmental risk factors which may trigger neuroinflammation and affect disease onset or progression (Tansey, 2007).

Characteristics of Neuroinflammation

Although the immune system in the brain differs from that in the peripheral body, the brain is capable of mounting an inflammatory response in reaction to many stimuli

including pathogens, traumatic injury, and stroke primarily through the actions of macrophage-derived resident microglia. When stimulated, these immune surveillance cells produce cytokines, chemokines, prostaglandins, reactive oxygen and nitrogen species, and growth and trophic factors and are capable of phagocytosis. Peripheral circulating immune cells can also invade the central nervous system particularly when the blood brain barrier has been compromised by disease or injury. Neuroinflammation has been described aptly as a two-edged sword (McGeer and McGeer, 2004; Wyss-Coray and Mucke, 2002). Inflammation which occurs immediately after injury or infection and is short-lived limits injury and promotes healing; however when an inflammatory stimulus is not resolved or is mis-directed, neuroinflammation can be neurotoxic. Under normal and disease conditions microglia serve an essential role in brain health by mediating innate immune responses to invading pathogens (reviewed in (Wersinger and Sidhu, 2002; Wyss-Coray and Mucke, 2002)). In a healthy, uninjured brain, microglial cells are present with ramified morphology and low expression of surface receptors that mediate pro-inflammatory and chemoattractant properties (reviewed in (Kreutzberg, 1996)). In nonactivated and mildly activated states, microglia have a homeostatic role in the brain in which they function to scavenge neurotoxins, dying cells, and cellular debris and provide trophic factors that promote neuronal survival and sprouting (reviewed in (Aloisi, 1999; Batchelor et al., 1999; Nakamura, 2002; Orr et al., 2002)). Upon injury or infection microglia can mount a response and become rapidly activated when pathological events occur (reviewed in (Wojtera et al., 2005)). In the continued presence of the activating stimulus additional microglia are attracted to the site of injury by secretion of chemokines (Aloisi, 2001; Aloisi et al., 2000). Microglia, although they are important mediators of

neuroinflammation, are not the only cell type that participates in neuroinflammatory responses, astrocytes and oligodendrocytes can also participate in the process. Astrocytes, like microglia, have a homeostatic role in the brain and are important in the regulation of extracellular glutamate concentration and permeability of the blood brain barrier, but after injury reactive gliosis can occur in which astrocytes become activated to upregulate glial fibrillary acidic protein (GFAP) and the gap-junction protein Connexin 43 which can result in glial scarring and permanent tissue damage (Haupt et al., 2007; Tilleux and Hermans, 2007).

Cytokines, prostaglandins, and reactive oxygen and nitrogen species

Upon activation of the immune response in the brain, secretion of many substances by glial cells occurs including that of growth factors, reactive oxygen and nitrogen species, prostaglandins, chemokines, and cytokines. Some of these products are neurotrophic and serve to limit injury whilst others induce DA neuron loss through increases in local oxidative stress and increased pro-apoptotic signaling.

Cytokines

Microglia and, to a lesser degree, astrocytes secrete immunoregulatory proteins called cytokines including Tumor Necrosis Factor (TNF), the Interleukin-1 (IL-1) and Interleukin-6 (IL-6) families, Interferon-γ (IFNγ), and Transforming Growth Factor-β (TGF-β), all of which modulate inflammatory processes and can influence the permeability of the blood brain barrier (reviewed in (Benveniste, 1992; Sedgwick et al.,

2000; Whitton, 2007)). Cytokines can exert neuroprotective effects; however, particularly under conditions of sustained activation, cytokines and chemokines promote pro-apoptotic signaling in neurons, oligodendrocytes, and astrocytes. Whether cytokine signaling has beneficial or detrimental effects in the brain depends on the dynamics, cellular source, localization, and context of cytokine release as well as the presence of co-expressed factors. Chronic inflammation and elevated cytokine levels have been noted and implicated in contributing to the pathology of a wide range of neurodegenerative disorders including Multiple Sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, tauopathies, and Age-related Macular Degeneration (reviewed in (Block and Hong, 2005; McGeer and McGeer, 2004; Mrak and Griffin, 2005; Nagatsu and Sawada, 2006)). While there is no evidence to support a role for any cytokine in the direct triggering of any of these neurodegenerative conditions, cytokine-driven neuroinflammation and neurotoxicity may modify disease progression in a number of these disorders.

Prostaglandins

Prostaglandins are fatty acids derived from arachidonic acid in which the rate limiting step is catalyzed by cyclooxygenase (COX) enzymes. One of the most important functions of prostaglandins is their ability to modulate inflammatory processes. Drugs which inhibit the COX enzymes (COX-1 and COX-2) are Non-Steroidal Anti-Inflammatory Drugs (NSAIDS), whose chronic use has been shown in a prospective study to lower the incidence of PD by 46% (Chen et al., 2005a; Chen et al., 2003). COX-1 is a constitutively expressed enzyme whose level remains relatively constant, whereas

COX-2 is inducibly expressed and more spatially restricted. In the brain, microglia express primarily COX-1 whereas dopaminergic neurons, especially under inflammatory conditions, express COX-2 (Hoozemans et al., 2001). The nonselective COX inhibitors aspirin and salicylic acid have been found to provide partial neuroprotection in the MPTP mouse model of PD (Aubin et al., 1998; Ferger et al., 1999). The protective effects of salicylic acid in the MPTP model have been ascribed to scavenging of hydroxyl radicals and preventing depletion of the antioxidant glutathione as opposed to COX inhibition of prostaglandin production (Ferger et al., 1999; Mohanakumar et al., 2000). In addition to attenuation of DA neuron loss by nonspecific COX inhibitors, inhibition of COX-2 specifically also reduced MPTP-induced DA neuron damage, implicating COX-2 inhibition as a neuroprotective strategy (Teismann and Ferger, 2001). In the MPTP model, treatment with rofecoxib, a specific COX-2 inhibitor, prevents the increase of prostaglandin PGE_2 in the midbrain as well as the accumulation of DA quinones, and increases DA neuron survival without altering neuroinflammation (Teismann et al., 2003). Inhibition of COX-2 activity has also been shown to be protective in the rat 6-hydroxydopamine (6-OHDA) model of PD during the progressive phase of DA neuron loss correlating with a decrease in activated microglia and no change in astrocyte numbers (Sanchez-Pernaute et al., 2004).

Reactive oxygen and nitrogen species (ROS/RNS)

Elevated inflammation results in the overproduction of ROS and RNS both via production by activated microglia and astrocytes and through the activation of COX and lipoxygenase enzymes in all cell types. COX enzyme activity leads to increased oxidative

stress through decreased glutathione activity and reduced mitochondrial membrane potential, as well as increased production of peroxidated lipids and proteins (reviewed in (Phillis et al., 2006)). Lipoxygenase enzymes also increase cellular oxidative stress by the synthesis of eicosanoids which are known to activate programmed cell death (reviewed in (Maccarrone et al., 2001)). Microglial cells express nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is responsible for inflammation-induced production of ROS (Klegeris and McGeer, 1994; Lavigne et al., 2001). NADPH oxidase has been shown to be more highly expressed in microglia of the SNpc from sporadic PD patients compared to unaffected individuals as well as in microglia following MPTP intoxication of mice; furthermore, genetic deletion of an essential subunit of NAPDH oxidase attenuates DA neuron loss in MPTP lesioned mice (Wu et al., 2003).

Superoxide, generated by NADH oxidase, reacts with nitric oxide (NO) to produce peroxynitrite which can cross neuronal cell membranes and increase intracellular oxidative stress (reviewed in (Calabrese et al., 2004)). NO is generated by nitric oxide synthases (NOS) including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS. nNOS and iNOS expression are both upregulated in sporadic PD and in some animal models of disease, and genetic deletion or inhibition of either of these NOS isoforms is neuroprotective in the MPTP model (Liberatore et al., 1999; Przedborski et al., 1996). The evidence above supports neuroinflammation-driven generation of ROS and RNS in inducing and exacerbating DA neuron dysfunction.

Evidence of chronic inflammation in Parkinson's disease

In the last decade, the contribution of inflammation-driven oxidative stress and cytokine-dependent toxicity in inducing DA neuron dysfunction and death in PD has been extensively studied (reviewed in (Hald and Lotharius, 2005; Hirsch et al., 2005; Nagatsu and Sawada, 2005; Nagatsu and Sawada, 2006; Tansey et al., 2007; Wersinger and Sidhu, 2006; Whitton, 2007)). The participation of persistent inflammation to PD progression is supported by evidence of increased microglial activation, cytokine accumulation, and oxidative damage in the CSF and post-mortem brains of PD patients (Banati et al., 1998; Gerhard et al., 2006; Hunot et al., 1999; McGeer et al., 1988; Vawter et al., 1996). Furthermore, a common feature of experimental models of PD is a neuroinflammatory response characterized by microglial activation and oxidative and cytokine-driven stress (Cassarino et al., 1997; Castano et al., 1998; Czlonkowska et al., 1996; Dehmer et al., 2000; Gao et al., 2002a; Gayle et al., 2002; Herrera et al., 2000; Kohutnicka et al., 1998; Liberatore et al., 1999; Mandel et al., 2000; Mogi et al., 2000b; Nakagawa and Schwartz, 2004). This neuroinflammation associated with neurotoxin-induced DA neuron death persists as demonstrated by the evidence of activated microglia in the substantia nigra after MPTP intoxication for at least six months in mice and for years in monkeys and humans (Langston et al., 1999; McGeer et al., 2003; Yasuda et al., 2007). Moreover, *in vivo* positron emission tomography scans of PD patients with the peripheral benzodiazepine receptor binding ligand [^{11}C]-(*R*) PK11195 demonstrate markedly elevated neuroinflammation in the pons, basal ganglia, striatum, and frontal and temporal cortical regions compared to age-matched healthy controls (Gerhard et al., 2006). The

persistence of neuroinflammation during and following DA neuron loss is evident, but perhaps the most compelling evidence supporting inflammatory processes in DA neuron loss comes from epidemiological studies in which chronic use of NSAIDS reduces PD risk (Chen et al., 2003). This epidemiological evidence in conjunction with experimental data in which aspirin and other NSAIDS provide DA neuron protection in animal models of PD point to neuroinflammation in contributing to neurotoxic processes (reviewed in (Chen et al., 2005a; Esposito et al., 2007)), and suggest that early intervention with NSAIDS may delay or prevent the onset of PD. The evidence linking inflammation-driven cytokine accumulation to PD includes the association of genetic polymorphisms connected with the overproduction of cytokines, chemokines, and acute phase proteins to sporadic PD susceptibility (Hakansson et al., 2005a; Hakansson et al., 2005b; Kruger et al., 2000; Nishimura et al., 2005; Nishimura et al., 2001), and the sensitivity of DA neurons to cytokine-driven oxidative stress and pro-apoptotic signaling which is evident from a number of studies (Aloe and Fiore, 1997; Carvey et al., 2005; Ferrari et al., 2006; Gayle et al., 2002; McGuire et al., 2001; Mount et al., 2007).

TUMOR NECROSIS FACTOR AND PARKINSON'S DISEASE

Tumor Necrosis Factor (TNF) signaling

Introduction to TNF and TNF receptors

TNF is a potent pro-inflammatory cytokine whose presence in serum as the factor responsible for bacterial endotoxin-induced tumor necrosis was identified in 1975 and

cloned in 1984 (Carswell et al., 1975; Pennica et al., 1984). The TNF receptors were cloned the following year (Aggarwal et al., 1985). Since the identification of TNF and its receptors, TNF has been shown to activate many downstream signaling pathways implicated in a variety of normal cellular and disease processes. TNF is synthesized as a monomeric type-2 transmembrane protein (tmTNF) that is inserted into the membrane as a homotrimer and cleaved by the matrix metalloprotease TNF alpha converting enzyme (TACE) to a soluble 51 kDa circulating form (solTNF); both the transmembrane and soluble forms of TNF are biologically active (reviewed in (Aggarwal, 2000b; Idriss and Naismith, 2000; MacEwan, 2002)). TNF receptor 1 (TNFR1, p55, CD120a) and TNF receptor 2 (TNFR2, p75, CD120b) are membrane glycoprotein receptors that specifically bind TNF and homotrimers of lymphotoxin α (LTα3), but the two receptors differ in their expression profiles, ligand affinity, cytoplasmic tail structures, and downstream pathway activation (reviewed in (Aggarwal, 2000a)). Signaling of TNF through TNF receptors requires that receptors preassemble into trimers on the cell membrane prior to ligand binding, this trimerization occurs through the intracellular cytoplasmic tail of the receptors and trimers are composed of like receptors (Chan et al., 2000; Engelmann et al., 1990).

TNFR1 is expressed in all cell types, whereas TNFR2 is expressed primarily by cells of the immune system (including microglia) and by endothelial cells (Aggarwal, 2000a). TNFR1 is activated by both soluble and transmembrane TNF, but TNFR2 is preferentially activated by tmTNF (Grell, 1995; Grell et al., 1998). The balance between tmTNF and solTNF production is determined by the cell type, the activation status of the

cell, the stimulus eliciting TNF production, the amount of active TACE, and the expression of TACE inhibitors (Gearing et al., 1994; Smookler et al., 2006). Interestingly, in addition to TNF signaling through TNFR1 and TNFR2, tmTNF has also been shown to induce reverse signaling such that the tmTNF expressing cell is activated via interaction between tmTNF and its receptors. Forward signaling through TNFR1 and TNFR2 and reverse signaling through tmTNF can elicit a variety of cellular responses depending on the metabolic state of the cell and the adaptor proteins present in the cell, thus influencing the activation of a number of intracellular signaling pathways including nuclear factor kappa-B (NF-κB), p38, c-jun N-terminal kinase (JNK), and the sphingomyelinase signaling pathway; the activation of these pathways can elicit a number of responses including inflammation, proliferation, cell migration, apoptosis, and necrosis (Eissner et al., 2000; Eissner et al., 2004; Harashima et al., 2001; Ware, 2005).

TNFR-mediated signaling

It is believed that most of the biologic effects of solTNF signaling are mediated through TNFR1 activation. TNFR1 contains a prototypical death domain (DD) in its cytoplasmic tail that characterizes many members of the TNF superfamily (Tartaglia et al., 1993; Ware et al., 1996). TNFR1 mediated signaling is initiated by TNF homotrimer binding which leads to the dissociation of silencer of death domains (SODD) allowing the binding of TNF receptor associated death domain (TRADD) and the subsequent recruitment of other adaptor proteins including receptor interacting protein (RIP) and TNR receptor associated factor 2 (TRAF2) (Hsu et al., 1996a; Hsu et al., 1996b; Hsu et al., 1995; Jiang et al., 1999). This membrane assembled signaling complex then leads to

RIP-dependent activation of NFκB signaling resulting in NFκB translocation to the nucleus and increased transcription of NFκB-dependent target genes leading to prosurvival signaling, cellular proliferation, and cytokine production. The membrane associated complex of ligand-engaged TNFR1 with TRADD, TRAF2, and RIP recruits inhibitor of apoptosis proteins 1 and 2 resulting in activation of ERK, JNK, and p38 MAP kinase pathways via RIP- and MAP kinase-activating DD-dependent phosphorylation of several upstream MAP kinase kinase kinases (Lee et al., 2003; Schievella et al., 1997; Shu et al., 1996; Winston et al., 1995). Particularly important in determining the outcome of a TNF signal are the kinetics of JNK activation which are dependent on the activating kinase. Acute and transient JNK activation by TNF, which is cytoprotective, involves the activation of TAK1 (Sato et al., 2005), whereas sustained JNK activation leading to caspase-dependent apoptosis depends upon phosphorylation by apoptosis signal-regulating kinase 1 (ASK1) (Tobiume et al., 2001). ROS signaling is critical for activation of ASK1 leading to sustained JNK activation; ASK1 is usually sequestered by the regulatory redox protein thioredoxin, but under oxidative stress conditions thioredoxin is inactivated by oxidation permitting ASK1 activation (Saitoh et al., 1998; Sakon et al., 2003).

Activated TNFR1 can also be internalized and this leads to dissociation of the TRADD/TRAF2/RIP complex and association of Fas-associated DD (FADD) which recruits procaspase 8 resulting in formation of the death-inducing signaling complex, and eventual triggering of the executioner caspases through the extrinsic apoptosis pathway (Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004). Caspase-8, in addition to

triggering the extrinsic pathway, also triggers the intrinsic apoptosis pathway by cleaving the pro-apoptotic Bcl-2 family members Bax and Bid to initiate mitochondrial-induced apoptosis (Gross et al., 1999; Wang et al., 2006; Zhao et al., 2001). If activation of the NFκB signaling pathway is impaired or absent the pro-apoptotic signaling arm of TNF-mediated signaling predominates leading to cellular death upon TNFR1 activation (reviewed in (Hehlgans and Pfeffer, 2005; Ware, 2005)). In order for a TNF stimulus not to activate apoptosis, NFκB signaling activation must not only initiate TNF-dependent anti-apoptotic signaling, but also antagonize JNK signaling. This NFκB-dependent interference of JNK activation occurs by several mechanisms. First, GADD45b, whose expression is regulated by NFκB, inhibits MAP kinase kinase 7, which is an upstream JNK activator (De Smaele et al., 2001; Papa et al., 2004). Second, another crucial cell-signaling pathway that can act as a switch between pro- and anti- apoptotic TNF signaling and is regulated by complex cross-talk between JNK and NFκB is ROS signaling. JNK is activated by elevated intracellular ROS levels and through induction of anti-oxidant genes including manganese superoxide dismutase (MnSOD) and glutathione, can dampen oxidative stress and prevent a feed-forward cycle of JNK-driven expression of pro-inflammatory cytokines which further enhance ROS levels (Pang et al., 1992; Sakon et al., 2003).

TNFR2-mediated signaling

TNFR2-dependent signaling is less well understood and is likely to have fewer biologic outcomes based on its relatively restricted expression profile that is limited primarily to endothelial and hematopoietic cells and on its preferential binding to tmTNF (Grell,

1995). Signaling through TNFR2 activates primarily inflammatory and pro-survival signaling pathways through recruitment of TRAF1 and 2 adaptor proteins and subsequent activation of cellular Inhibitor of Apoptosis Proteins (cIAPs) and the NF-κB pathway (Rao et al., 1995; Rothe et al., 1995; Rothe et al., 1994). TNFR2 has also been shown to activate phosphatidylinositol 3-kinase-dependent signaling to promote retinal and hippocampal neuron survival (Fontaine et al., 2002; Marchetti et al., 2004). Although the direct signaling through TNFR2 is protective, TNF receptors compete for binding of adaptor proteins including TRAF2 which could tip the balance of TNFR1 signaling towards receptor internalization and assembly of the death inducing signaling complex if the adaptor TRAF2 is primarily bound to TNFR2 (Wajant et al., 2003).

Reverse signaling through transmembrane TNF

Upon receptor binding, tmTNF has been demonstrated to initiate intracellular signaling in the tmTNF-expressing cell (Harashima et al., 2001; Higuchi et al., 1997). This signaling is mediated, at least in part, through casein kinase 1 phosphorylation of the cytoplamsic tail of TNFR2 resulting in increased intracellular calcium levels and activation of p38 and MAP kinase pathways (Kirchner et al., 2004; Pocsik et al., 1995; Watts et al., 1999). Another type of reverse signaling though tmTNF is possible; after release of solTNF by TACE, the TNF intracellular domain (TNF-ICD) can be released into the cell through regulated intramembrane proteolysis by signal peptide peptidase-like proteases where it is trafficked to the nucleus by virtue of a nuclear localization signal resulting in increased production of pro-inflammatory cytokines (Domonkos et al., 2001; Fluhrer et al., 2006; Friedmann et al., 2006). Understanding of the signaling that occurs through tmTNF is

still under investigation and the *in vivo* significance of these reverse signaling pathways remains unclear.

TNF inhibitors

Currently TNF inhibitors are approved for use in the treatment of peripheral autoimmune disorders including rheumatoid arthritis, ankylosing spondylitis, and Crohn's disease. These inhibitors are infliximab, etanercept, and adalimumab, which all bind to and sequester TNF from its receptors. Infliximab is a chimeric bivalent IgG1 monoclonal antibody composed of a human constant region and murine variable regions. Adalimumab is a humanized bivalent mouse IgG1 monoclonal antibody. Etanercept is a fusion protein comprised of human IgG fused to a dimer of the extracellular regions of TNFR2. There are some differences in the properties of these TNF inhibitors. Infliximab and Adalimumab, by virtue of being IgGI antibodies, can activate complement and bind FcReceptor and they also can bind both monomeric and trimeric solTNF whereas Etanercept, like TNFR2, only binds TNF trimers, however all three inhibitors bind tmTNF (Mitoma et al., 2005; Mitoma et al., 2004; Scallon et al., 2002; Tracey et al., 2007). Another difference between Etanercept and Infliximab and Adalimumab is the ability of Etanercept to neutralize lymphotoxin, a property that is shared by its parent receptor TNFR2 (Gudbrandsdottir et al., 2004; Scallon et al., 2002). In addition to these approved inhibitors several similar antibody and receptor based TNF inhibitors are under clinical investigation.

A class of TNF inhibitor with a different mechanism of action, and the one I used in my studies is dominant negative TNF. These are dominant negative TNF variants engineered with one or two point mutations that are designed to interrupt the binding of TNF to receptor, without interfering with the ability of TNF to form trimers (Steed et al., 2003). Due to the need for these trimers to exchange with endogenous TNF monomers to form heterotrimers incapable of binding to and initiating signaling through TNF receptors, they are specific for solTNF and do not inhibit tmTNF signaling (Steed et al., 2003).

Additionally, less specific inhibitors of TNF including minocycline and thalidomide have been used in the treatment of inflammatory conditions. Minocycline, a broad-spectrum tetracycline antibiotic, has been shown to have both bacteriostatic and anti-inflammatory actions (Sapadin and Fleischmajer, 2006). Minocycline does not only inhibit TNF synthesis, but also inhibits matrix metalloproteases, reduces COX-2 activity and prostaglandin E2 production, and attenuates apoptosis (Gabler and Creamer, 1991; Golub et al., 1998; Matsuki et al., 2003; Wang et al., 2004; Wang et al., 2003; Whiteman and Halliwell, 1997; Yrjanheikki et al., 1999; Zhu et al., 2002). Thalidomide is an immunomodulatory drug which means it can attenuate a variety of cytokines and immune cell-mediated responses. Thalidomide inhibits TNF through enhanced degradation of TNF mRNA (Melchert and List, 2007; Moreira et al., 1993), however is can also alter the expression of COX-2, IL-1β, TGF-β, IL-12 and IL-6 affecting immune cell regulation and migration separate from its TNF-dependent effects (reviewed in (Melchert and List, 2007)).

TNF signaling in PD

The presence of elevated TNF levels in postmortem PD brains and in tissues of PD patients has been known for some time; however its importance in contributing to DA neuron dysfunction and death has only recently been appreciated. TNF was first implicated in PD as a result of microglial activation that was presumed to occur after DA neuron loss had begun when elevated levels of TNF and soluble TNFR1 (cleaved in response to local elevations of TNF protein) were detected in the cerebrospinal fluid of PD patients and in the SN of postmortem PD brains (Boka et al., 1994; Mogi et al., 1994; Mogi et al., 2000a). Further implicating TNF signaling in PD, is the evidence that in experimental models of PD increased TNF mRNA and protein are detectable in rodent midbrain SN within hours of *in vivo* administration of 6-OHDA (Mogi et al., 1999), MPTP (Ferger et al., 2004; Rousselet et al., 2002; Sriram et al., 2002), or lipopolysaccharide (LPS) (Gao et al., 2002b). Furthermore, TNF levels were shown to remain elevated in MPTP-treated non-human primates one year after treatment (Barcia et al., 2005). Although this evidence places TNF at the site of DA neuron loss it does not address a causal or contributing role for TNF in DA neurodegeneration. However DA neurons have been demonstrated to be exquisitely sensitive to elevated TNF levels in both *in vitro* primary cultures (Clarke and Branton, 2002; Gayle et al., 2002; McGuire et al., 2001), and *in vivo* (Aloe and Fiore, 1997; Carvey et al., 2005). Lastly, and perhaps the most compelling reason to investigate TNF in the initiation and progression of DA neuron loss, is the epidemiologic evidence demonstrating that a polymorphism (−1031 C)

in the TNF promoter that drives transcriptional activity and results in higher than normal TNF production is more prevalent in a cohort of Japanese early-onset PD patients compared to late-onset PD patients and unaffected controls (Nishimura et al., 2001). Another polymorphism in the TNF gene promoter (−308 G/A) that is associated with elevated serum TNF levels has also been found to be overrepresented in sporadic PD (Kruger et al., 2000; Wahner et al., 2007). These epidemiologic data support a causal role for TNF in DA neuron loss. Initially, the importance of TNF in mediating DA neuron loss was investigated using mice deficient in either TNF or its receptors with conflicting results. TNFR1 or TNFR2 single-knockout mice were not protected from MPTP-induced fiber damage in the striatum or cell body loss in nigra (Leng et al., 2005; Rousselet et al., 2002). However receptor double-knockout mice had less striatal damage (Sriram et al., 2002; Sriram et al., 2006a), but in severe MPTP regimens (4 or 8 injections of 15mg/kg) protection of nigral cell bodies was not seen (Leng et al., 2005; Rousselet et al., 2002). Mice lacking TNF were also shown to be protected against MPTP-induced striatal dysfunction, but not DA cell body loss (Ferger et al., 2004). These studies are hard to interpret due to the severe nigrostriatal lesion resulting from many dosing paradigms, making neuroprotection a difficult target to reach. Additionally, TNF deficient animals have been demonstrated to have peripheral immune cell maturation deficiencies, alterations in expression of brain proteins including DJ-1, altered dopamine metabolism, and reduced microglial responses to MPTP making it difficult to attribute differences in pathology specifically to TNF signaling (Pasparakis et al., 1996; Pejovic et al., 2004; Ritter et al., 2003; Rousselet et al., 2002; Sriram et al., 2006a). The effect of TNF inhibition with non-specific TNF inhibitors including thalidomide, a potent anti-

inflammatory and sedative, and minocycline, a tetracycline antibiotic that inhibits TNF synthesis, have also been investigated in the MPTP mouse and intranigral LPS rat models of PD with some (Ferger et al., 2004; Tomas-Camardiel et al., 2004) but not all (Sriram et al., 2006b) studies showing nigrostriatal protection. However, due to the TNF-independent anti-inflammatory effects of these compounds, the specific role of TNF in DA neuron death was not evident. Owing to limitations in interpreting the role of TNF signaling in DA neuron loss obtained by studies using TNF- and TNF receptor- knockout mice or inhibitors with general anti-inflammatory properties that were being investigated at the time I was beginning my studies, I tested and confirmed an important role for TNF signaling in the *in vivo* degeneration of DA neurons using specific pharmacologic inhibition of TNF in rat models of PD. In both the 6-OHDA oxidative neurotoxin and chronic LPS models of PD dominant-negative TNF administration attenuated nigral DA neuron loss by approximately half (McCoy et al., 2006). Together, combined evidence from histopathologic, epidemiologic, and pharmacologic studies support a role for TNF-mediated nigral DA neuron degeneration and death and implicate elevated midbrain TNF levels in Parkinson's disease susceptibility (reviewed in (Tansey and Wyss-Coray, 2008)).

RODENT MODELS OF PARKINSON'S DISEASE

The generation of animal models in the study of PD has been crucial in understanding both the cellular and molecular mechanisms that contribute to DA neuron loss, as well as in providing a setting in which to investigate palliative, neuroprotective, and

neurorestorative strategies in the treatment of PD. A number of non-genetic rodent models of PD have been studied and although each model exhibits some aspects of PD, no single model recapitulates all aspects of the disease pathology seen in human sporadic PD and new models are continually being developed in the search for more faithful representations of sporadic PD pathology.

1 -Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)

After the recognition of MPTP as the causal neurotoxic agent in individuals who injected synthetic opioids, animal models were quickly developed initially through repeated injections of MPTP in rhesus monkeys (Burns et al., 1983; Langston et al., 1983). Shortly after the MPTP model was developed in monkeys, a protocol to induce MPTP-dependent parkinsonism in mice was developed (Heikkila et al., 1984a). Following systemic injection, MPTP readily crosses the blood brain barrier where it is converted to MPDP+ within glial cells by monoamine oxidase B, then to the active neurotoxin MPP+ (1-methyl-4-phenylpyridinium). MPP+ is then released to the extracellular space where it enters cells through selective uptake by the dopamine transporter as well as noreprinephrine and serotonin receptors (reviewed in (Przedborski and Vila, 2003)). MPTP intoxication in mice causes both acute DA neuron dysfunction and death within days, and a prolonged sequelae of events that continue to result in DA neurodegeneration for months to years (Jackson-Lewis et al., 1995). In the acute phase of MPTP-induced death, once inside the neuron MPP+ is concentrated in mitochondria where it binds to complex 1 and disrupts the flow of electrons along the electron transport chain resulting

in decreased ATP formation and increased generation of ROS (Chan et al., 1991; Rossetti et al., 1988). It is believed that MPTP-induced toxicity resulting from mitochondrial impairment may be more dependent on ROS signaling then on ATP depletion as MPTP-related ATP depletion causes only an acute 20% reduction in the striatum and midbrain which is not expected to be a severe enough depletion for this to be the primary cause of cell death (Chan et al., 1991; Davey and Clark, 1996). Support for the hypothesis that MPTP toxicity is exerted through increased oxidative stress is the evidence that mice overexpressing or deficient in anti-oxidant genes have attenuated MPTP-induced DA neuron loss (Klivenyi et al., 2000; Przedborski et al., 1992; Wu et al., 2003; Zhang et al., 2000). In addition, increased NO signaling has been specifically implicated in MPTP toxicity both through the use of transgenic animals and through pharmalogic inhibition of NOS enzymes (Dehmer et al., 2000; Liberatore et al., 1999; Przedborski et al., 1996; Schulz et al., 1995; Watanabe et al., 2007).

There is a second, prolonged phase of nigrostriatal degeneration which occurs after the acute phase of cell death in first 24-48 hrs following MPTP intoxication. A wealth of evidence supports that this delayed phase of DA neuron death is associated with persistent increased neuroinflammation (Czlonkowska et al., 1996; Kohutnicka et al., 1998; Kurkowska-Jastrzebska et al., 1999; Sugama et al., 2003; Yasuda et al., 2007), and can be attenuated with anti-inflammatory agents and in animals that are deficient in pro-inflammatory cytokines and COX-2 (Czlonkowska et al., 1996; Kohutnicka et al., 1998; Kurkowska-Jastrzebska et al., 2004; Sugama et al., 2004; Teismann and Ferger, 2001; Teismann et al., 2003; Vijitruth et al., 2006; Wu et al., 2002; Wu et al., 2003). The

contribution of this second phase of neuron death to MPTP-induced pathology suggests that the initial mitochondrial dysfunction and increased oxidative stress can initiate a cascade of neuroinflammatory processes that persist to impact DA neuron survival.

The extent as well as the timecourse of DA neuron loss in the MPTP mouse model can vary depending on the cumulative MPTP dose, the number of doses, and the frequency of administration. Four distinct dosing paradigms have been recognized and reviewed (Schmidt and Ferger, 2001). The first model results in presymptomatic changes which include nigrostriatal dyfunction measured by decreased stiatal DA and its metabolites, but does not induce significant nigral neuron loss and is obtained by a single peripheral injection of MPTP (10-20mg/kg). Acute, necrotic DA neuron loss can be obtained with four peripheral injections of 20-30mg/kg separated by two hours each. A slightly more progressive model associated with apoptotic DA neuron loss requires one or two daily injections of 20mg/kg for a minimum of five days (Tatton and Kish, 1997), with the majority of cell death occurring in the first five days. The last and most progressive MPTP model is attained with a single daily low dose injection of four mg/kg for twenty days (Bezard et al., 1997a; Bezard et al., 1997b).

These routes of MPTP intoxication result in many of the same neuro-histopathologic changes as are seen in sporadic PD; these include loss of SNpc DA neurons with relative sparing of ventral tegmental area DA neurons, and behavioral deficits. MPTP models are dissimilar from idiopathic disease in that DA neuron death is very acute, degeneration of certain neuron populations such as those in the olfactory bulb

and locus coeruleus that degenerate in PD are unaffected in MPTP intoxication, and accumulation of Lewy bodies does not occur in mice (Przedborski and Vila, 2003; Shimoji et al., 2005). However α-synuclein postitive inclusions have been identified in primate models which could be interpreted as pre-Lewy body pathology (Forno et al., 1993). Despite these limitations, the MPTP model has been valuable in the investigation of mitochondrial-dependent DA neurotoxicity and primates with MPTP-induced parkinsonism are the standard model for investigations into the mechanisms behind and therapies for L-Dopa-induced dyskinesias.

6-hydroxydopamine (6-OHDA)

The catecholamine analog 6-OHDA has been used as a noradrenergic and catecholaminergic neurotoxin in the study of both the peripheral and central nervous systems and was first used in rats to cause nigrostriatal toxicity bilaterally in 1968 and unilaterally in 1970 (Ungerstedt, 1968; Ungerstedt and Arbuthnott, 1970). When injected, 6-OHDA is concentrated in neurons via the dopamine and norepinephrine reuptake transporters. As 6-OHDA does not readily cross the blood brain barrier it must be injected directly into the striatum, the substantia nigra, or the medial forebrain bundle to selectively produce nigrostriatal degeneration. With nigral or medial forebrain bundle administration of 6-OHDA, DA neurons die rapidly within the first 24 hours and striatal dopamine reaches maximum depletion within three to four days (Faull and Laverty, 1969). In addition, nigral delivery of 6-OHDA is associated with damage to the ventral tegmental area and substantia nigra pars reticularis, resulting in nearly complete DA deinnervation. Striatal injection of 6-OHDA using either a single or multiple dose

regimen results in a more spatially defined, selective, and gradual loss of DA neurons in the SNpc via retrograde degeneration beginning at the striatal DA neuron terminals and progressing to DA neuron cell body loss in the SNpc (Bjorklund et al., 1997; Kirik et al., 1998; Przedborski et al., 1995). Once inside DA neurons, 6-OHDA leads to cellular dysfunction and death through the generation of ROS and quinones (Cohen, 1984), both because 6-OHDA is easily oxidized and because 6-OHDA treatment causes mitochondrial impairment and therefore contributes to the formation of superoxide free radicals (Cohen, 1984; Glinka et al., 1996; Lotharius et al., 1999). This oxidative stress hypothesis of 6-OHDA-induced toxicity is supported by the observation that 6-OHDA treatment reduces striatal glutathione and superoxide dismutase enzyme activity and increases levels of malondialdehyde (Kumar et al., 1995; Perumal et al., 1992). Furthermore, treatment with anti-oxidants or overexpression of anti-oxidant genes protects against 6-OHDA induced toxicity both *in vitro* and *in vivo* (Asanuma et al., 1998; Bensadoun et al., 1998; Cadet et al., 1989; Callio et al., 2005; Davison et al., 1986; Mayo et al., 1998; Ridet et al., 2006; Tiffany-Castiglioni et al., 1982). Although the 6-OHDA model has been used extensively to induce DA neuron loss and is more progressive than the MPTP model, it does not reproduce all of the cardinal PD features as no Lewy body pathology has been detected, and due to the site-specific injection of 6-OHDA to target the nigrostriatal pathway, 6-OHDA does not affect other brain areas involved in PD such as the olfactory bulb or the locus coeruleus (reviewed in (Dauer and Przedborski, 2003)).

Lipopolysaccharide (LPS)

As DA neurons are extremely sensitive to inflammation-induced toxicity (De Pablos et al., 2005), and as microglia have been implicated in contributing to PD progression, intranigral administration of LPS in rats has been used to provoke DA neuron degeneration. Initially, a single injection of 2uL of 1mg/mL (2ug total) LPS was delivered in the SN of rats to achieve acute DA neuron loss which was evident by four days after the injection and reached its maximal level at fifteen days. This DA neuron loss was accompanied by rapid (within two days) microglial activation which returned to near baseline activation within nine days (Castano et al., 1998; Herrera et al., 2000). A second more progressive model in which DA neuron loss is selective and delayed is achieved by chronic supranigral infusion of 5ng/hr LPS for two weeks (1.68ug total). In this chronic neuroinflammatory model microglial activation occurs rapidly, plateaus at 2 weeks, and remains elevated for at least eight weeks after the start of infusion. DA neuron loss begins four to six weeks after the start of infusion and loss reaches 70% by ten weeks (Gao et al., 2002b). LPS-induced DA neuron loss also occurs in animals which have been exposed to LPS *in utero*. The offspring of gravid rats systemically injected with 1mg/kg LPS at gestational day 10.5 have irreversible, significant, and progressive DA neuron loss three weeks after birth (Carvey et al., 2003; Ling et al., 2002). Prenatal exposure to LPS also results in increased susceptibility of nigral DA neurons to a second adult exposure to the toxin (Ling et al., 2006). Lastly, a single intraperitoneal LPS injection of 5mg/kg in adult mice was shown to induce persistent microglial activation and DA neuron loss which reached significance at seven months post injection and had progressed at ten months (Qin et al., 2007). A benefit of some of these more chronic,

delayed, and progressive models of DA neuron loss is that they more closely follow the protracted loss of DA neurons seen in idiopathic PD. In addition, selective DA neuron loss achieved through LPS administration supports an important role for inflammatory processes in contributing to parkinsonian pathology.

Pesticides

Rotenone

Rotenone is a highly lipophilic pesticide that crosses both the blood brain barrier and cellular membranes quickly. Rotenone can accumulate in and inhibit the function of mitochondria through binding to and inhibiting complex 1 oxidative phosphorylation (Chance et al., 1963), leading to mitochondrial oxidative-stress driven DA toxicity. Rotenone is most often systemically administered to rodents to cause DA neuron loss as oral delivery does not induce degeneration unless given at extremely high doses (Bove et al., 2005). In male rats intravenous or subcutaneous infusion of 2-3 mg rotenone/kg daily for 28-56 days or daily intraperitoneal injections of 2.5 mg/kg for 60 days have been demonstrated to induce DA neuron loss with severity being moderately variable within the same strain and highly variable between rat strains (Alam and Schmidt, 2002; Betarbet et al., 2000; Hoglinger et al., 2003; Sherer et al., 2003b). These rotenone exposures result in complex 1 inhibition throughout the rat brain, yet cause selective degeneration of the dopaminergic neurons in the SN via retrograde degeneration of the striatal terminals (Betarbet et al., 2000). In addition to progressive and selective cell loss, many of the remaining dystrophic neurons have intracytoplasmic inclusions containing α-

synuclein and ubiquitin as well as increased microglial activation and oxidative stress; all features of sporadic PD (Betarbet et al., 2000; Hoglinger et al., 2003; Sherer et al., 2003a; Sherer et al., 2003b). Negative aspects of the rodent rotenone PD model include variable pathology and high lethality during treatment particularly at high doses (Betarbet et al., 2000; Hoglinger et al., 2003; Lapointe et al., 2004; Sherer et al., 2003b; Zhu et al., 2004).

Paraquat and maneb

Paraquat and maneb are two other pesticides which have also been used to model PD both singly and in combination. Paraquat was first considered as a possible DA neurotoxin based on its structural similarity with MPP+. Although paraquat crosses the blood brain barrier poorly it can be measured in the brain of rodents after systemic injection (Corasaniti et al., 1998). Initially systemic paraquat exposure was not found to cause striatal DA depletion (Perry et al., 1986), however in subsequent studies chronic systemic paraquat administration resulted in nigral DA neuron loss, decreased dopamine nerve terminal density at the striatum, and Lewy body-like pathology (Brooks et al., 1999; Li et al., 2005; Manning-Bog et al., 2002; Ossowska et al., 2005). The mechanism of paraquat-induced toxicity is likely to result from increased oxidative stress as paraquat is readily converted to a free radical leading to superoxide generation and lipid peroxidation (Hara et al., 1991a; Hara et al., 1991b; Hara et al., 1991c; Hara et al., 1991d; Yumino et al., 2002). Paraquat also triggers caspase-mediated DA neuron death via the activation of JNK signaling both *in vitro* and *in vivo* (Peng et al., 2004). As ROS generation has been demonstrated to lead to JNK activation and subsequent apoptotic cell death (Sakon et al., 2003), JNK-induced apoptosis would be consistent with the

hypothesis that paraquat toxicity is oxidative-stress dependent. Paraquat has also been demonstrated to induce excitotoxic cell death through depolarization of N-methyl-D-aspartate receptor channels and augmented calcium influx (Shimizu et al., 2003).

Maneb, an additional pesticide which has not been extensively studied, interacts with paraquat to cause greater nigrostriatal dysfunction than treatment with either agent alone when mice are intraperitoneally injected with 5-10 mg/kg paraquat and 15-30 mg/kg maneb weekly for four weeks (Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b). Paraquat and maneb models have not been extensively characterized and the selectivity of agents to induce exclusively, or primarily, DA neuron loss is unclear.

Proteosome inhibition

Treatment of rodents with proteosome inhibitors has been used to model PD based on the ubiquitin proteosome dysfunction hypothesis of PD. However, results obtained from systemic proteosome inhibition have been varied and have dampened enthusiasm about the utility of this model (Beal and Lang, 2006; Bove et al., 2006; Kordower et al., 2006b; Manning-Bog et al., 2006; McNaught and Olanow, 2006; McNaught et al., 2004; Schapira et al., 2006b; Zeng et al., 2006). In 2004 McNaught et al. administered proteosome inhibitors for two weeks, three times per week by subcutaneously injection and animals developed L- dopa responsive parkinsonian behavior within one to two weeks after the injections and DA neuron degeneration that progressed between two weeks and six weeks following the end of injections (McNaught et al., 2004). In addition,

analogous to sporadic PD, degeneration occurred in other brain areas including the locus coeruleus, dorsal motor nucleus of the vagus, and the nucleus basalis of Meynert and lewy-body like pathology was seen (McNaught et al., 2004). Although the pathology seen in this initial study has been partially recapitulated by other investigators there are some discrepancies (Schapira et al., 2006b; Zeng et al., 2006), and additional carefully controlled studies have not been able to replicate these findings (Bove et al., 2006; Kordower et al., 2006b; Manning-Bog et al., 2006). In one study the motor deficits obtained after proteosome inhibition were neither progressive nor L-dopa responsive, and, as in the initial study, DA neuron loss was evaluated without the use of stereology (Zeng et al., 2006). In another study proteosome inhibitors were subcutaneously administered as done previously but no significant motor impairment or inclusion bodies were observed. DA neuron loss was reported although the stereologic counting methods that were employed were not standard (Schapira et al., 2006b). Three additional published studies in which proteome inhibitors were delivered to mice, rats, and monkeys have failed to observe DA neuron loss, inclusion body formation, or behavior deficits despite conducting the studies with exactly the same protocol as originally published with carefully managed blinded analysis (Bove et al., 2006; Kordower et al., 2006b; Manning-Bog et al., 2006).

CURRENT AND PROSPECTIVE TREATMENTS FOR PD

Pharmacologic and surgical treatments for PD

Pharmacologic treatment of motor symptoms has been the standard of care for Parkinson's disease. Historically dopaminergic drugs have been primarily used to alleviate motor aspects, but novel non-dopaminergic therapies are being developed both to treat non-motor aspects of the disease as well as motor complications that arise with continued use of dopamine replacement therapy (reviewed in (Schapira et al., 2006a)).

Dopamine replacement (levodopa-carbadopa)

Initially the treatment of PD was limited to the use of anticholinergic drugs, pallidotomy, and thalamic ablation. However in the 1960's treatment was drastically changed by the demonstration of dopamine depletion in the striatum of postmortem PD brains which lead to the investigation and use of the blood brain permeant dopamine precursor L-dopa in PD afflicted individuals (Birkmayer and Hornykiewicz, 1961; Ehringer and Hornykiewicz, 1960). Later that decade, addition of a dopa decarboxylase inhibitor to L-dopa therapy increased the effectiveness of the treatment by preventing peripheral breakdown of dopamine, and has resulted in dopamine replacement as the gold standard of PD therapy. Although the most effective treatment for PD symptoms, L-dopa responsiveness declines after years of use and patients often exhibit dyskinesia. In addition dopamine replacement therapy is palliative and not disease modifying.

Deep brain stimulation (DBS)

Although surgeries to lesion the globus pallidus and thalamus were early treatments for PD, their use dramatically decreased with the advent of L-dopa therapy. However, due to L-dopa-induced dyskinesia and loss of effectiveness in many patients, interest in surgical

treatments has become popular again. Current surgeries pioneered by Dr. A Benabid in the 1990s do not lesion brain areas, but instead implant electrodes to stimulate deep brain regions resulting in reduced tremor and rigidity; unilateral DBS was FDA approved in 1997 to treat tremor and bilateral DBS in 2002 to treat additional motor symptoms including rigidity and akinesia (Benabid et al., 2000). DBS is currently applied to the thalamus, globus pallidus and subthalamic nucleus. DBS of the ventral intermediate nucleus of the thalamus is used to reduce tremors; DBS of the subthalamic nucleus can improve tremor, rigidity, and dyskinesia through influencing the input to the globus pallidus and is the most effective surgical treatment for the reduction of parkinsonian symptoms. DBS of the globus pallidus in the basal ganglia can also be performed with results similar to stimulation of the subthalamic nucleus.

Dopamine agonists

D_2-like DA receptor agonists of two classes, ergoline and non-ergoline, are used to treat PD through the direct activation of striatal DA receptors. Apomorphine which is both a D_1 and D_2 DA receptor agonist is also used (reviewed in (Brooks, 2000)). These agonists are prescribed as an adjunct therapy to L-dopa treatment to decrease dyskinesia by allowing lower doses of L-dopa to be administered, and alone in early stages of disease before initiation of L-dopa therapy (Adler et al., 1997; Rinne, 1985). In addition to having symptomatic benefit, dopamine agonists may also be disease modifying through their reported abilities to scavenge oxidant species, upregulate antioxidant and growth factor genes, and promote anti-apoptotic pathways in culture and animal models (Gassen et al., 1996; Grunblatt et al., 2001; Gu et al., 2004; Iida et al., 1999; Kakimura et al.,

2001; Ohta et al., 2004; Tanaka and Ogawa, 2005; Zou et al., 1999). Clinically the ability of dopamine agonists to modify disease progression has been investigated and these agents have been demonstrated to delay the rate of decline in imaging studies measuring either loss of dopamine transporters or fluorodopa uptake (Parkinson's Study Group, 2002; Whone et al., 2003).

Monoamine oxidase B (MAO-B) inhibitors

Monoamine oxidase B (MAO-B) inhibitors block one of the enzymes that degrade dopamine in the brain. MAO-B inhibitors have been demonstrated to protect against MPTP-intoxication due to their ability to prevent the conversion of MPTP into the active neurotoxic metabolite MPP+ in animal models of Parkinson's disease (Heikkila et al., 1984b). In addition, a study demonstrated increased lifespan in PD patients using MAO-B inhibitors (Birkmayer et al., 1985). The possibility that MAO-B inhibitors may be neuroprotective through modulation of oxidative stress and production of neuronal growth factors has not been convincingly demonstrated in PD patients, and it is unclear if this therapy is disease modifying, or merely lowers the requirement for L-dopa (Am et al., 2004; Calne, 1995; Kontkanen and Castren, 1999; Magyar and Szende, 2004; Mizuta et al., 2000; Wadia et al., 1998). Clinical trials have demonstrated that treatment with the MAO-B inhibitor rasagiline reduces motor deterioration compared to patients in which the therapy was delayed, and that selegegiline, another MAO-B inhibitor, could delay initiation of L-dopa therapy in early PD (reviewed in (Rascol, 2003; Schapira et al., 2006a)).

Gene therapy for PD

The use of gene therapy in PD treatment is very attractive for several reasons including: the spatially defined and cell type specific pathology, the need for consistent drug administration to avoid dose fluctuations, and the difficulty in chronic administration of drugs which can not cross the blood brain barrier. These characteristics of PD have lead to the advancement of gene therapy clinical trials to assess the safety and feasibility of several agents (reviewed in (Cress, 2008; Fiandaca et al., 2008; Porras and Bezard, 2008)).

Neurotrophic factor replacement

Ceregene has recently completed a Phase I clinical trial delivering neurturin, a potent neurotrophic factor, into the putamen of advanced Parkinson's patients using an adeno-associated virus (AAV). This trial with AAV-neurturin (designated CERE-120) was successful with the conclusions that CERE-120 is safe, well-tolerated, and has promising indications of efficacy with persistent improvement in clinical rating scores for motor symptoms prompting the initiation of a Phase II trial (reviewed in (Fiandaca et al., 2008)). CERE-120 delivery in MPTP lesioned non-human primates has been shown to increase striatal dopamine and provide histologic and behavioral protection (Kordower et al., 2006a).

An additional, closely related neurotrophic factor glial-derived neurotrophic factor (GDNF) has been well studied in animal models of PD and has been demonstrated

to protect against 6-OHDA and MPTP-induced DA neuron loss and behavioral deficits (reviewed in (Kirik et al., 2004)). However expression of GDNF has been demonstrated to have different effects on DA neuron function depending upon the species, animal model, and procedure used (reviewed in (Eslamboli, 2005)). Amgen has recently completed a Phase II clinical trial in which recombinant GDNF was infused into the putamen of patients via catheterization, but unfortunately a clear clinical benefit was not demonstrated and several adverse reactions occurred (Lang et al., 2006).

GAD gene therapy

The first human PD gene therapy trial delivered glutamic acid decarboxylase (GAD) to the subthalamic nucleus. GAD is the rate-limiting enzyme in the synthesis of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). This strategy for symptomatic PD treatment is based on previous lesioning and DBS interventions targeted to the subthalamic nucleus to decrease its activity, and by the observation that GABA receptor agonists could decrease activity of the subthalamic nucleus in PD patients (Levy et al., 2001). Initially AAV-GAD was tested in 6-OHDA lesioned rats where it attenuated apomorphine-induced rotational behavior (Luo et al., 2002). Neurologix Inc. has sponsored a Phase I clinical trial to deliver GAD to the subthalamic nucleus using an AVV vector (Kaplitt et al., 2007). Although the administration of AAV-GAD has been demonstrated to be safe, its efficacy has not been rigorously tested in PD patients due to limited participants and unilateral delivery; AAV-GAD has provided modest but sustained behavioral improvement in MPTP lesioned monkeys (Emborg et al., 2007;

Kaplitt et al., 2007). GAD gene therapy, like subthalamic DBS, may provide symptomatic benefit but is not likely to be disease modifying.

Aromatic L-amino acid decarboxylase (AADC)

Aromatic L-amino acid decarboxylase (AADC) is the enzyme responsible for converting L-dopa to dopamine and its expression declines in late PD. Therefore, enhancing AADC expression in the striatum would increase the conversion of L-dopa to dopamine in PD patients resulting in a lower needed dose, but not removing the need for dopmaine replacement therapy. AAV-AADC delivery to the striatum is effective in MPTP lesioned primates resulting in significantly lower L-dopa requirement, improvement in clinical rating scores, and reduced dykinesia. Transgene expression and the associated behavioral impairments have been maintained in primates for up to six years (Bankiewicz et al., 2006; Forsayeth et al., 2006). Based upon this preclinical development, AAV-AADC entered clinical trials by Avigen, Inc. and bilateral expression has been shown to increase AAV expression in the striatum and improve L-dopa responsiveness (Fiandaca et al., 2008).

Cell replacement in PD

Cell replacement for PD treatment is one of the most intensely investigated areas of stem and progenitor cell research in neurologic disorders owing to the spatially and cell-type limited pathology that needs correction in order to provide substantial clinical benefit. Clinically, human fetal cells have been used in Parkinson's patients, but the prospects in

cell replacement for PD have diversified to include neural stem cells (NSCs) and non-neural adult progenitor cells (reviewed in (Correia et al., 2005; Galvin and Jones, 2006; Lindvall and Bjorklund, 2004)).

Fetal cell transplant

Fetal cell grafts for PD are generally harvested from the embryonic ventral midbrain at a developmental stage associated with terminal neuronal differentiation. These cells are then transplanted into the striatum of Parkinson's patients where they can form synapses with medium spiny neurons (their usual target cell). In animal PD models and in human patients, grafted fetal cells have been demonstrated to provide functional benefits and these improvements are dependent upon cell survival, engraftment, and production of DA at the striatum (Bjorklund, 1992; Brundin et al., 1986; Elsworth et al., 1996; Hauser et al., 1999; Herman and Abrous, 1994; Kordower et al., 1998; Polgar et al., 2003; Studer et al., 1998). The effects of fetal cell transplants in PD patients are somewhat variable. In some patients there is stable, significant improvement in symptoms making dopamine replacement therapy unnecessary (Piccini et al., 1999). However, in many patients the improvement in symptoms is modest and gradual, but efficacy is usually limited to relatively young patients and is dependent upon the number of surviving transplant cells and their limited ability to engraft (reviewed in (Bjorklund and Lindvall, 2000; Freed et al., 2001; Olanow et al., 2003)). A significant negative side effect of fetal transplants is their likelihood to cause medication-independent dyskinesias presumably due to ectopic or focal expression of dopamine (Freed et al., 2001; Hagell et al., 2002). Technical and ethical difficulties with fetal cell transplants include the difficulty in obtaining and

preparing a sufficient number of DA progenitors that are capable of engraftment which has led to the investigation of alternative cell sources.

Embryonic stem cells (ESCs)

As a result of the limited availability of fetal tissue, the possibility of embryonic stem cells (ESCs) as a potential source of DA neurons has been considered. ESCs are derived from the inner cell mass of the pre-implantation embryo (blastocyte) and are pluripotent (Thomson et al., 1998). A major hurdle in the use of ESCs is the need for these cells to differentiate into functional DA neurons to prevent the formation of tumors and irrelevant cell types. Necessary signaling cues for DA neuron fate determination in mice including the growth factors FGF-8 and sonic hedgehog have been identified (Ye et al., 1998), and were subsequently used to coax ES cells into a DA neuron fate (Kawasaki et al., 2000; Kim et al., 2006; Lee et al., 2000; Studer et al., 2000). Lineage commitment can also be induced by overexpression of the transcription factor neuron receptor related-1, and selection of a more homogenous DA neuron population can be obtained through genetic modification with a construct containing a resistance or reporter gene under the control of a lineage-restricted promoter (Aubert et al., 2003; Li et al., 1998; O'Shea, 2001). Implantation of DA neuron-induced and enriched populations into 6-OHDA lesioned rats and MPTP intoxicated monkeys restores striatal DA deficiency (Barberi et al., 2003; Kim et al., 2002; Takagi et al., 2005). Despite these promising results there are substantial hurdles to overcome before advancement to clinical trials can be considered including the presence of feeder cell layers and serum in many of the preparations, the evidence that human-derived and DA-differentiated ESCs survive poorly in PD models, and the

concern of teratoma formation (reviewed in (Bjorklund and Isacson, 2002; Correia et al., 2005)).

Adult stem and progenitor cells

In addition to ESCs and fetal neural stem cells, multipotent stem and progenitor cells are present in several peripheral tissues including liver, intestine, bone marrow, skin, heart, and adipose. Bone marrow stromal cells (BMSCs) (Bonilla et al., 2005; Bossolasco et al., 2005; Dezawa et al., 2004; Hermann et al., 2004; Hermann et al., 2006; Jiang et al., 2002a; Jiang et al., 2002b; Wislet-Gendebien et al., 2005; Woodbury et al., 2002; Woodbury et al., 2000), Skin-derived precursors (SKPs) (Amoh et al., 2005; Fernandes et al., 2004), and Adipose-derived adult stromal (ADAS) cells have all been demonstrated to express markers of neural progenitors (Ashjian et al., 2003; Fujimura et al., 2005; Kang et al., 2004; McCoy et al., 2008; Ning et al., 2006; Safford et al., 2002; Safford et al., 2004).

Adult mouse BMSCs have been grafted into MPTP intoxicated mice resulting in significant behavioral improvements despite these grafts not having appreciable numbers of tyrosine hydroxylase expressing, differentiated donor cells (Li et al., 2001). Furthemore, transplantion of naïve BMSCs and BMSCs engineered to express trophic factors including GDNF and neurturin have been injected into the striatum of 6-OHDA lesioned rats where they result in improvements in behavioral deficts despite poor survival in many cases (Dezawa et al., 2004; Offen et al., 2007; Suon et al., 2006; Ye et al., 2007). In addition to BMSC-provided benefit in animal models of PD, I have shown

that ADAS cells, when transplated in the nigra of 6-OHDA lesioned rats, result in attenuation of DA neuron loss and improvement in rotational behavior (McCoy et al., 2008). As has been demonstrated with BMSCs, I show that ADAS cell grafts do not appear to terminally differentiate into mature DA neurons despite their ability to reduce 6-OHDA-induced damage (Appendix A). Exposure of cells to conditioned media from BMSCs has been demonstrated to protect DA neuron cultures from 6-OHDA induced cell death, as well as to increase the survival of fetal DA neuron grafts presumuably through the abiltity of BMSCs to secrete a number of neurotrophic factors (Shintani et al., 2007). ADAS cells are also able to produce many potent neurotrophic factors, and it is likely that the mechanism of protection afforded by transplantation of cells from alternative adult progenitor populations will be similar (McCoy et al., 2008).

Ethical and technical hurdles to successful cell replacement

Fetal midbrain cells, although able to survive, make synaptic contacts,and adopt DA phenotypic traits in animal models and in some recipient PD patients, have serious technical and ethical hurdles. The technical hurdles include poor grafting in some patients and graft-induced dyskinesias. Differences in the ability of transplanted cells to engraft may be attributable to graft-induced inflammation as clinical trials which employ immunosuppressant therapy have reported greater therapeutic benefit (reviewed in (Lindvall and Bjorklund, 2004)). Ethical difficulties arise in some nations from the need to use brain tissue from multiple, aborted embryos (Boer, 1999). Use of ESCs has been investigated for PD cell replacement therapy, but has even more substantial technical difficulties to overcome. Perhaps most troubling is the propensity of poorly differentiated

transplanted grafts to generate undifferentiated teratomas (Bjorklund et al., 2002). Protocols to enrich for more lineage committed DA neurons and precursors to lessen the risk have yielded positive results (Barberi et al., 2003; Kawasaki et al., 2000; Kim et al., 2002; Takagi et al., 2005), but the propensity of ES cells to generate tumors is still a concern and limits the clinical advancement of ESC based therapy.

Although an attractive cell source, peripheral adult stem cells have not been demonstrated to efficiently and stably differentiate into DA producing neurons even though they express a number of neuronal markers (reviewed in (Correia et al., 2005)). Despite the lack of evidence that these cells are capable of replacing DA neurons, their value may lie in their capability to produce and secrete trophic factors as well as their amenability to genetic modification by which they may be induced to overexpress anti-inflammatory or trophic factors that limit progressive DA neuron loss (McCoy et al., 2008; Shintani et al., 2007).

Table 1.1 Genetic mutations linked to PD

Locus	Chromosome position	Gene	Protein	Inheritance	Average age of onset (yrs)	Mutations	Pathology	Proposed function
Park1/4	*4q21*	*SNCA*	α-synuclein	Autosomal dominant	30-60	A30P, E46K, A53T, duplication, and triplication	Nigral degeneration with Lewy bodies	Unclear; possibly vesicle trafficking
Park2	*6q25.2-q27*	*PRKN*	Parkin	Autosomal recessive	20-40	Recessive homozygous and compound heterozygous mutations, exon deletions, duplication, and triplication	Nigral degeneration without Lewy bodies	E3 Ubiquitin ligase, scaffolding protein
Park5	*4p14*	*UHCL1*	UCHL1	Autosomal dominant	50s	I93M	Not reported	E3 Ubiquitin ligase
Park6	*1p35-p36*	*PINK1*	PINK1	Autosomal recessive	20-40	G309D, missense mutations, exon deletions	Not reported	Mitochondrial kinase
Park7	*1p36*	*DJ-1*	DJ-1	Autosomal recessive	20-40	exon deletion, L166P, deletions, and compound missense.	Not reported	Oxidative stress signaling
Park8	*12p12*	*LRRK2*	Dardarin/ LRRK2	Autosomal dominant	50-70	R1441C/G/H, Y1699C, G2019S, I2020T, and G2385R	Variable α-synuclein and tau pathology	Unclear; possses GTPase and kinase activities, may also act as a scaffold protein.

Modified from: Belin and Westerlund, 2008; Farrer, 2006; Gasser, 2005; Hardy et al., 2006

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CHAPTER TWO

Blocking soluble tumor necrosis factor signaling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease

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ABSTRACT

The mechanisms that trigger or contribute to loss of dopaminergic (DA) neurons in Parkinson's Disease (PD) remain unclear and controversial. Elevated levels of Tumor Necrosis Factor (TNF) in cerebrospinal fluid and postmortem brains of PD patients and animal models of PD implicate this pro-inflammatory cytokine in the pathophysiology of the disease; but a role for TNF in mediating loss of DA neurons in PD has not been clearly demonstrated. Here we report that neutralization of soluble Tumor Necrosis Factor (solTNF) *in vivo* with the engineered dominant-negative TNF (DN-TNF) compound XENP345 reduced by 50% the retrograde nigral degeneration induced by a striatal injection of the oxidative neurotoxin 6-hydroxydopamine (6-OHDA). XENP345 was neuroprotective only when infused into the nigra, not the striatum. XENP345/6-OHDA rats displayed attenuated amphetamine-induced rotational behavior indicating preservation of striatal dopamine levels. Similar protective effects were observed with

chronic *in vivo* co-infusion of XENP345 with bacterial lipopolysaccharide (LPS) into the substantia nigra, confirming a role for solTNF-dependent neuroinflammation in nigral degeneration. In embryonic rat midbrain neuron/glia cell cultures exposed to LPS even delayed administration of XENP345 prevented selective degeneration of DA neurons despite sustained microglia activation and secretion of solTNF. XENP345 also attenuated 6-OHDA-induced DA neuron toxicity *in vitro*. Collectively, our data demonstrate a role for TNF *in vitro* and *in vivo* in two models of PD, and raise the possibility that delaying the progressive degeneration of the nigrostriatal pathway in humans is therapeutically feasible with agents capable of blocking solTNF in early stages of PD.

INTRODUCTION

Parkinson's Disease (PD) is the second most prevalent neurodegenerative disease in the U.S. with a 5% incidence in individuals over 65 (Moore, 2005). Its clinical manifestations result from selective loss of dopaminergic (DA) neurons in the ventral mesencephalon substantia nigra pars compacta (SNpc), with a resulting decrease in striatal dopamine. The critical molecular mediators and mechanisms that elicit death of nigral DA neurons have yet to be identified; but a wealth of studies implicate microglia and inflammatory processes in the pathophysiology of PD (Hald and Lotharius, 2005; Hirsch et al., 2005; McGeer et al., 1988; Vawter et al., 1996), and chronic use of non-steroidal anti-inflammatory drugs can lower risks for development of PD in humans by 46% (Chen et al., 2003).

Cerebrospinal fluid and post-mortem brains of PD patients display elevated levels of the pro-inflammatory cytokine Tumor Necrosis Factor (TNF) as do animals treated with the dopaminergic neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) used to model nigral degeneration in non-human primates and rodents (Barcia et al., 2005; Boka et al., 1994; Hunot et al., 1999; Mogi et al., 2000; Nagatsu and Sawada, 2005; Sriram et al., 2002). TNF is synthesized as a type II transmembrane trimeric protein cleaved by the TACE metalloprotease to a soluble form (Aggarwal, 2000; MacEwan, 2002); both forms are biologically active, but their relative roles in mediating DA neuron survival are unknown. Soluble TNF (solTNF) transduces inflammatory stimuli through the canonical death receptor TNFR1 (Tartaglia et al., 1993) which is highly expressed in nigrostriatal DA neurons rendering them vulnerable to TNF-induced toxicity (Aloe and Fiore, 1997; Carvey et al., 2005; Gayle et al., 2002; McGuire et al., 2001). The role of transmembrane (tm) TNF is less well understood, but it can mediate pro-survival effects through TNFR2 in cortical (Marchetti et al., 2004) and hippocampal (Heldmann et al., 2005) neurons.

We hypothesized that solTNF is a major mediator of neurotoxic mechanisms contributing to degeneration of nigral DA neurons *in vivo*; therefore blocking its activity should yield neuroprotection in animal models of PD. To test our hypothesis, we employed TNF variants engineered to disrupt binding of the solTNF trimer to its receptors by forming dominant-negative TNF (DN-TNF) heterotrimers that eliminate solTNF homotrimers, and thus inhibit its signaling (Steed et al., 2003). To elucidate the cellular mechanisms by which TNF promotes DA neuron death, we utilized rat

embryonic ventral mesencephalon (EVM) neuron/glia cultures. Our findings that solTNF, but not tmTNF, contributes significantly to the progressive loss of DA neurons induced by bacterial and oxidative neurotoxins in cellular and animal models of PD are relevant for the design and testing of novel therapeutic strategies for PD.

MATERIALS AND METHODS

Materials

Rabbit anti-tyrosine hydroxylase (TH), guinea pig anti-GABA, mouse anti-MAP2b, and mouse anti-NeuN (neuron specific nuclear protein) antibodies were obtained from Chemicon (Temecula, CA). Mouse anti-rat complement 3 receptor (C3R) antibody Ox-42 was obtained from Santa Cruz and mouse anti-CD45 antibody was obtained from Serotec. FITC conjugated isolectin-B4, LPS (from *E. coli* 0111:B4, Lot # 114K4133 1.5 x 10^6 EU/mg), 6-OHDA, poly D-lysine, and D-amphetamine were obtained from Sigma-Aldrich Corporation (St. Louis, MO) and a single stock of each was used for all experiments. Cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA). Laminin was obtained from BD Biosciences (San Jose, CA). The recombinant dominant-negative TNF XENP345, a PEGylated version of the TNF variant A145R/I97T (Steed et al., 2003), was bacterially produced and formulated by Xencor Inc. to contain less than 0.1 EU/mL. Recombinant mouse TNF was obtained from R&D systems (Minneapolis, MN). Antibodies for quantitative TNF ELISA were obtained from Biosource/Invitrogen Corporation (Carlsbad, CA). Osmotic pumps were purchased from

Alzet Corporation (Cupertino, CA), cannulae and tubing from Plastics One Corp. (Roanoke, VA). All other reagents were obtained from Sigma-Aldrich.

Animal studies

Young adult and timed-pregnant Sprague Dawley SASCO and CDF/Fischer 344 rats were purchased from Charles River Laboratories (Wilmington, MA) and housed in pathogen-free climate-controlled facilities at the Animal Resources Center at The University of Texas Southwestern Medical Center. All animal studies were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center at Dallas.

Methods

Intrastriatal 6-OHDA injection and XENP345 infusions

Young adult female Sprague Dawley SASCO rats (200-225 g) (n= 6 per group, total of 30) were anesthetized with halothane (2%) and placed in a stereotaxic frame. Their eyes were protected with ophthalmic ointment and body temperature was monitored with a rectal probe and maintained with radiant heat under feedback control. The scalp was prepped under sterile conditions and the skull exposed and incised. We chose a previously published regimen of 6-OHDA to induce a mild-to-moderate retrograde lesion in the nigrostriatal pathway (Kirik et al., 1998). Burr holes were drilled to permit unilateral injection of 20 μg 6-OHDA (4 μL of 5 μg/μL) at a rate of 1 μL/min into the striatum on the right hemisphere (stereotaxic coordinates: anteroposterior (AP): -1.2 mm

from bregma, mediolateral (ML): -3.9 mm; and dorsoventral (DV): -5 mm below surface of dura) (Paxinos et al., 1985). Cannulae (gauge 28, Plastics One) connected via polyethylene tubing to a subcutaneously implanted osmotic minipump (Alzet 2002) preloaded with vehicle (sterile saline) or the treatment agent XENP345 (0.08 mg/kg/day) were then stereotaxically inserted through the burr holes into the same site as the 6-OHDA lesion or into an area just above the substantia nigra pars compacta (stereotaxic coordinates from bregma: AP: -4.8mm from bregma, ML: -1.7 mm, and DV: -8 mm below surface of dura) through another burr hole and were left in position for 3 weeks. Cannulae were secured to the skull with surgical glue (Plastics One).

Intranigral LPS or LPS/XENP345 infusions.

The low-dose chronic LPS infusion model published previously was used to induce selective, delayed and progressive death of DA neurons *in vivo* (Gao et al., 2002). LPS (5 ng/hr) was unilaterally infused for 2 wk via a 28 gauge cannula into the SNpc (coordinates from bregma AP: -4.8 mm, ML: -1.7 mm, and DV: -8 mm) (Paxinos et al., 1985) of young adult male CDF rats (200-240g) (n=6 per group, 3 sets of experiments) under the same surgical procedures described above. Cannulae were connected via polyethylene tubing (Plastics One) to a subcutaneously implanted osmotic minipump (Alzet 2002) preloaded with the treatment agent. Vehicle (sterile saline) or XENP345 (0.03 mg/kg/day, representing a 5:1 ratio XENP345:LPS) was preloaded along with LPS into the pump and infused for 2 wk (n= 6 per group).

Rotational behavior analyses.

At 1, 2 and 3 wk post 6-OHDA lesion, amphetamine-induced rotational behavior was monitored in a glass cylinder (diameter 24.5 cm). Animals received 2.5 mg/kg D-amphetamine prepared in sterile saline (Sigma, St. Louis) i.p. and 60 min after the injection, rotational asymmetry was monitored for 20 min. Rotation towards the lesion (ipsilateral) was scored as positive and net rotational asymmetry score was expressed as full body turns/min.

Perfusion and tissue processing for histology.

At 3 wk post 6-OHDA lesion or 8 wk post start of LPS infusion, animals were deeply anesthetized with pentobarbital and intracardially perfused with 300 ml of heparinized PBS pH 7.4, followed by 500 mL of 4% paraformaldehyde in PBS, pH 7.4. Brains were postfixed for 24 h in the same solution and cryoprotected in 20% sucrose in PBS for 18-24 hr. Coronal sections (40 μm-thickness) were cut through the striatum and substantia nigra pars compacta on a Leica cryostat and mounted on glass slides (SuperFrost Plus, Fisher) for immunohistological analyses and stereological estimate of DA neuron number in a fixed 200 μm area in SNpc.

Immunohistochemistry of brain sections.

Sections on glass slides were fixed for an additional 15 min in 4% paraformaldehyde, followed by a PBS rinse (pH 7.4). Mounted sections (rather than the standard free-floating sections) were chosen because of the critical importance of maintaining right/left hemisphere orientation at all times for comparison of ipsilateral (lesioned) to contralateral (unlesioned) side in both the unilateral 6-OHDA and LPS lesion models. Even with the

use of standard brain notching techniques, re-establishing correct R/L orientation prior to mounting free-floating sections would have been technically laborious and prone to error. Prior to immunohistochemistry, sections were incubated in 0.2 M glycine (pH 7.4) for 30 min to minimize tissue autofluorescence due to the aldehyde fixative. Pilot experiments were conducted using 40µm brain sections from control (unlesioned rats) to establish the optimum blocking and antibody incubation times to completely penetrate the section all the way to the 2µm bottom guard zone used in the stereological analyses of neuron number. Sections were permeabilized for 35 min in TBS containing 0.3% Triton-X-100 and 1% normal goat serum (NGS), followed by blocking for 60 min in TBS containing 1% NGS. Primary antibody incubations were done for 48 hr at 4˚C in TBS containing 0.1% Triton-X-100 and 1% NGS. Secondary antibody incubations were performed for 4 hr at room temperature in the same dilution buffer. Nuclei were counterstained with 0.5 µg/mL Hoechst 33258. Sections were coverslipped with aqueous-based mounting media with anti-fade reagent (Biomeda).

Nigral DA neuron counts

StereoInvestigator analyses software (Micro Bright Field Inc., Williston, VT) was used to perform unbiased stereological counts of NeuN/TH-immunoreactive (NeuN/TH-IR) cell bodies in the SNpc using the optical fractionator method (West et al., 1991) for both LPS and 6-OHDA studies. The boundary of SNpc was defined according to previous anatomical demarcation in the rat (German and Manaye, 1993). For analysis, the treatment of the various brain sections was blinded to the observer. Cells were counted with a 100X oil immersion objective (1.3 NA) using an Olympus BX61 microscope.

Random and systematic counting frames (each 50 x 50 x 5 μm with 2 μm upper and lower guard zones) on cryosections (40μm serial sections placed 4 per slide) obtained from over 40 rats through the extent of SNpc (from AP:-3.3mm to -5.3mm behind bregma) were sampled using a 20 μ optical dissector. We systematically chose to stain every other slide for TH/NeuN and the intervening slide for complement 3 receptor (OX-42 antibody). A dopaminergic neuron was defined as a NeuN/TH immunoreactive cell body with a clearly visible unstained nucleus. For estimating total neuron number (number of NeuN-positive cell), a cell was defined as a soma with a nucleolus in focus within the counting frame. A microglial cell was defined as an OX-42-immunoreactive cell with processes (ramified) or without processes (ameboid-shape).

Striatal TH fiber density

Fluorescence intensity of TH-immunoreactive fibers above a fixed threshold using entorhinal cortex for background subtraction was used to estimate striatal TH-fiber density. For this analysis, cryosections were taken from a region 1mm anterior to olfactory bulb, through the CPu complex, an ending 1mm posterior to SNpc. Areas at the striatal lesion site (AP: -1.2 mm from bregma, ML: -3.9 mm from midline in right hemisphere; and DV: -5 mm below surface of dura (Paxinos et al., 1985) devoid of cellular material were excluded from the analysis and normalized for comparison to the contralateral (unlesioned side) according to standard practice.

Mesencephalic mixed neuron/glia cultures.

Primary rat ventral mesencephalic neuron/glia cultures were prepared by modification of a published protocol (Liu et al., 2000). Briefly, ventral mesencephalic tissues were dissected from embryonic day 14 Fischer 344 rats into Hank's balanced salt solution and dissociated with mild, mechanical trituration in cold media containing 10% FBS. Following trituration, cells were centrifuged at 1200g for 5 minutes, resuspended in 3-4mL complete media for counting, and plated into 4-well chamber slides (two 25 μL-microislands per well at a density of 7.5×10^5 cells/mL) according to the method of (Takeshima et al., 1994) or in 96 well culture plates (100 μL/well at a density of 2×10^5 cells/mL) precoated with poly-D-lysine (0.1 mg/mL) and laminin (20 μg/mL) in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10 ng/mL basic Fibroblast Growth Factor (bFGF). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cultures were replenished two days later with 0.5 mL/well (chamber slides) or 0.1 mL/well (96-well plate) fresh media lacking bFGF and were used for treatment five days later. For treatment (in duplicate or triplicate) with LPS or 6-OHDA the cultures were maintained in 0.5 mL/well (chamber slides) or 0.1 mL/well (96-well plate) of media supplemented with 2.5% FBS and lacking bFGF.

Quantitative TNF ELISA

The culture medium was collected after treatment of cells with LPS or 6-OHDA as indicated. The quantities of TNF-α were measured with a mouse TNF-α ELISA kit from Biosource/Invitrogen Corporation (Carlsbad, CA) as per manufacturer's instructions.

Immunocytochemistry in EVM cultures

Neurons were stained with anti-MAP2b antibody to detect both somata and neurites or anti-Neuronal antigen N (NeuN) antibody to detect somata only. Dopaminergic neurons were detected with anti-TH antibody. GABAergic neurons were identified with anti-GABA antibody. Microglia were detected with an anti-complement type 3 receptor antibody (OX-42), CD-45 antibody, or FITC-BS-1 isolectin B4. Cells were fixed with 4% paraformaldehyde, permeabilized with TBS containing 3% gelatin from cold water fish skin (Sigma), 1% BSA, and 0.3% Triton-X-100, blocked with TBS containing 3% gelatin from cold water fish skin and 1% BSA. Primary antibody incubations were done for 2 hr at room temperature with primary antibodies diluted in TBS containing antibody diluent (TBS containing 3% gelatin from cold water fish skin, 1% BSA, and 0.1% Triton-X-100) anti-MAP2b, 1:400; anti-Neu-N, 1:400; anti-TH, 1:250; OX-42 1:60; anti-CD-45 1:600; anti-GABA 1:1500; FITC-BS-1 isolectin B4, 0.1 mg/mL; or anti-GFAP, 1:1000). Except for FITC-isolectin B4, the bound primary antibody was visualized on an Olympus BX61 fluorescence microscope after incubation with an appropriate Molecular Probes/Invitrogen Alexa-conjugated secondary antibody (1:1000). Images were captured with either a CoolSnap CCD ES monochromatic or CF color camera and analyzed with MetaMorph software (Universal Imaging Systems, West Chester, PA). For analysis, the treatment of the various cultures was blinded to the observer. Counts were performed in a minimum of 6 fields per well per treatment condition. To obtain average cell counts per well, 20X images were taken throughout the extent of each microisland. The number of

cells per 20X field were averaged and multiplied by the number of fields to obtain the averages per well. Each experimental set was repeated two to three times.

Uptake assays for tritiated DA or GABA.

Neurotransmitter uptake was measured using published protocols (Gao et al., 2002). Mixed neuron/glia cultures grown in 96 well plates were washed two times with 100 μL Krebs-Ringer buffer [containing 16 mM sodium phosphate, 119 mM sodium chloride, 4.7 mM potassium chloride, 1.8 mM calcium chloride, 1.2 mM magnesium sulfate, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4]. For DA and GABA uptake the cultures were incubated for 15 min at 37°C with 50 μL of 10 μM [^{3}H]DA (30 Ci/mmol) and 50 μM [^{3}H]GABA (90 Ci/mmol) in Krebs-Ringer buffer, respectively. After being washed three times with 100 μL ice-cold Krebs-Ringer buffer, the cells were collected in 50 μL 1N sodium hydroxide, combined with 1 mL scintillation fluid, and radioactivity was counted with a liquid scintillation counter. Nonspecific uptake was determined in parallel wells that received both the tritiated tracer and 10 μM mazindol (to block specific DA uptake) or 2 mM β-alanine (to block glial GABA uptake) (Mabjeesh et al., 1992). Each treatment condition was done in triplicate. Each experimental set was repeated at least two times.

Statistical Analyses

Differences among means were analyzed using one-way analysis of variance (ANOVA). When ANOVA showed significant differences, comparisons between means were tested by the Tukey-Kramer Multiple Comparisons post-hoc test. Left versus right differences from the same animals were analyzed using two-tailed paired Student's t test. Values

expressed are the group mean ± SEM. For culture experiments, differences in TH cell number among the different groups were analyzed by ANOVA followed by the Newman-Keuls post-hoc test for *p* values significance. Values expressed are the mean ± SD; **p* values < 0.05, ***p* values < 0.01, *** *p* values < 0.001.

RESULTS

Blocking soluble TNF signaling *in vivo* provides protection to dopamine neurons from 6-OHDA-induced death and attenuates rotational behavior.

TNF levels have been reported to increase in striatum and substantia nigra of 6-OHDA injected rats (Mogi et al., 1999). To determine if TNF signaling has a critical role in nigral DA neuron loss *in vivo*, we tested the ability of a DN-TNF inhibitor (XENP345), to provide neuroprotection from 6-OHDA-induced lesions in rats. 6-OHDA neurotoxin rodent models of PD are characterized by increased oxidative stress and striatal terminal die-back followed by loss of DA cell bodies within the SN (Przedborski, 2005; Przedborski and Ischiropoulos, 2005). In these experiments a lesion was induced by a unilateral intrastriatal pre-terminal injection of 6-OHDA into rats (Kirik et al., 1998). Immediately following administration of 6-OHDA (or vehicle) into the striatum, a cannula connected to an osmotic minipump preloaded with XENP345 or saline was stereotaxically positioned into the ipsilateral striatum or ipsilateral SNpc. Using unbiased design-based stereology, the number of TH/MAP2b-positive soma was estimated within the substantia nigra 3 weeks after administration of the 6-OHDA striatal injection. We

found that administration of the XENP345 into the nigra rescued about 50% of the nigral DA neurons from 6-OHDA-induced death and prevented the decrease in TH fiber density (Figure 2.1 a-e). In contrast, no statistically significant difference was detected between groups when rats were implanted with striatal pumps pre-loaded with vehicle or XENP345. These findings suggest that the key TNF signaling events mediating neurodegeneration of DA neurons occur at the cell somata in the region of the substantia nigra pars compacta.

As a physiological measure of 6-OHDA-induced striatal DA depletion, we tested amphetamine-induced rotational behavior weekly for 3 weeks following the 6-OHDA lesion. We found that the protection of DA neurons in SNpc achieved with nigral delivery of XENP345 correlated with attenuated ipsiversive circling behavior induced by D-amphetamine (2.5 mg/kg i.p.) (Figure 2.1 f). These data suggest that TNF may be crucial for the progressive phase of 6-OHDA induced dopaminergic cell loss, and support a role for TNF signaling in DA neuron loss induced by oxidative neurotoxins.

Blocking soluble TNF signaling *in vivo* during a chronic neuroinflammatory stimulus protects DA neurons from death.

Since inhibition of solTNF signaling was efficacious in protecting DA neurons from death induced by the oxidative neurotoxin 6-OHDA, we hypothesized that a TNF-dependent neuroinflammatory or neurotoxic component is required for robust killing of nigral DA neurons. To test this, we used a purely neuroinflammatory *in vivo* model of PD

and compared survival of TH+ neurons in SNpc after infusion of LPS alone or LPS and XENP345. Previous studies by (Gao et al., 2002) reported that a 2-week supra-nigral infusion of LPS (5 ng/hr) increased the number of complement 3 receptor (Ox-42 antibody) immunoreactive microglia with rod-like morphology as early as three days after the start of LPS infusion which subsequently peaked at 2 weeks and persisted for 8 weeks. This microglial activation profile is consistent with this being a chronic neuroinflammatory model. We have confirmed the persistent presence of Ox-42-positive ameboid-shaped microglia at week 8 after a 2-week chronic LPS infusion into SNpc (Figure 2.2). We reasoned that since TNF is a potent activator of microglia (Aggarwal, 2000; Hinkerohe et al., 2005) and a known mediator of LPS action in peripheral tissues (Beutler, 2005), chronic infusion of DN-TNFs to block solTNF signaling during an inflammatory stimulus might prevent TH-positive cell loss in the SNpc of LPS-infused rats. Unbiased stereological measurements of TH-IR/NeuN neuron soma and fluorescence densitometry of TH-positive fiber density revealed that co-infusion of XENP345 (70 ng/hr) with LPS (5 ng/hr) for 14 days into rat substantia nigra rescued about 50% of the LPS-induced nigrostriatal degeneration measured 8 weeks after the start of infusion (Figure 2.2c). In addition, rescue of DA neurons was accompanied by a decreased number of C3R-immunoreactive (IR) microglia in SNpc (Figure 2.2a). Infusion of XENP345 alone, chronically or in a single bolus (20 μg), did not induce microgliosis and was not associated with any tissue damage (Figure 2.2a) consistent with a lack of TNF-like agonistic activity of DN-TNFs published in previous work (Steed et al., 2003). Together with our findings in the 6-OHDA model, these data indicate that solTNF signaling is a primary mediator of DA neuron loss *in vivo* induced by either

neuroinflammatory agents or oxidative neurotoxins, and that inhibition of solTNF can reduce neuroinflammation and subsequent neurodegeneration.

Soluble TNF is a critical mediator of LPS- and 6-OHDA-induced dopaminergic neuron loss *in vitro*.

Given the fact that *in vivo* administration of TNF inhibitors selective for solTNF signaling rescued only 50% of DA neurons from LPS- or 6-OHDA-induced degeneration, it was important to determine whether additional rescue of DA neurons could be achieved by also blocking signaling by tmTNF. To determine the contribution from each form of the ligand to the death of DA neurons in LPS-treated embryonic (E14) rat ventral mesencephalon (EVM) cultures, we compared sparing of TH neurons by two inhibitors with different modes of action. We first measured LPS-induced solTNF production in the media by quantitative ELISA to determine the doses of TNF inhibitors needed to block TNF signaling during an LPS stimulus. We found that treatment of EVM cultures with 10 ng/mL LPS led to rapid release of solTNF which peaked at around 500pg/mL (Figure 2.3 a). In pilot experiments, we next confirmed the lack of toxic effects of DN-TNFs alone (XENP345 and others) or etanercept alone and determined the optimal dosing of TNF inhibitors necessary to completely block DA neuron death induced by exogenous solTNF (data not shown). Next we treated EVM neuron/glia cultures with 10 ng/mL LPS (Figure 2.3 b) in the presence of either XENP345 (3 or 200 ng/mL) to block solTNF exclusively (Steed et al., 2003) or with the soluble decoy receptor etanercept (Fc-TNFR2, 100 ng/mL) to block both solTNF and tmTNF signaling (Agnholt et al., 2003; Mitoma et al.,

2005; Scallon et al., 2002). Four days after LPS stimulation, DA neuron survival was measured by counting tyrosine hydroxylase and MAP-2b co-labeled neurons (Figure 2.3 b). We found that blocking TNF signaling with either inhibitor during an LPS stimulus attenuated LPS-induced loss of DA neuron number (Figure 2.3 c) and their neurites (Figure 2.3 b) equally well and by approximately half, indicating that the gradual loss of TH positive neurons induced by LPS treatment and dependent on TNF signaling is mediated exclusively by solTNF, since blocking of both tmTNF and solTNF with etanercept yielded no further rescue compared to XENP345. The slight differences between XENP345 and etanercept in ability to rescue DA neurons *in vitro* may be related to pharmacokinetic differences since the doses of both TNF inhibitors used *in vitro* were in excess of that needed to neutralize the amount of secreted solTNF measured by ELISA in response to the LPS challenge (Steed et al., 2003). Given that solTNF and tmTNF bind TNFR1 and TNFR2 with different affinities, and the normal biological role of TNF and its receptors in the nigrostriatal pathway is not at all clear, our findings that XENP345 significantly attenuated the death of DA neurons induced by 6-OHDA or LPS *in vivo* support a direct and neurotoxic role for solTNF as the relevant microglial-derived neuroinflammatory mediator of nigral degeneration. To determine if the rescued neurons retained function, we measured tritiated dopamine uptake in these cultures. Rescue of TH positive neurons correlated with increased DA uptake (Figure 2.3 d). Nonspecific dopamine uptake was measured (and subtracted as background) in these cultures using the specific dopamine transport inhibitor mazindol. In agreement with published observations (Gao et al., 2002), GABAergic neurons were unaffected by LPS treatment (Figure 2.3 e).

Based on our findings that *in vivo* inhibition of TNF signaling with XENP345 attenuated 6-OHDA-induced loss of DA neurons and decreased amphetamine-induced ipsiversive circling behavior, we predicted that 6-OHDA-induced neurotoxicity of DA neurons and rescue by XENP345 could also be reproduced *in vitro,* thus allowing identification of the TNF-dependent mechanisms mediating 6-OHDA-induced neurodegeneration. Therefore, we treated EVM cultures with a concentration range of 6-OHDA (5-100 μM) for 24 hr and assayed solTNF release as well as DA uptake in the surviving DA neurons at 4 days after initial exposure to 6-OHDA in the presence or absence of TNF inhibitors. We found that 20 μM 6-OHDA induced neurotoxicity and death in DA neurons comparable to 10 ng/mL LPS (Figure 2.4) under the same media conditions and time-course of the experiment. Lower concentrations of 6-OHDA did not induce significant DA neuron death (due to the protective effects of 2.5% FBS) and higher concentrations induced excessive death of both DA and GABA neurons (data not shown). As expected, the level of solTNF release (~ 50pg/mL) evoked by this amount of 6-OHDA (Figure 2.4 a) was lower than that evoked by the inflammogen LPS; however, the robust rescue with XENP345 and etanercept indicated this level of solTNF was sufficient to account for a significant fraction (>50%) of the 6-OHDA-induced neurotoxicity (Figure 2.4 c). Together, these studies are the first to demonstrate a critical role for solTNF signaling in mediating death of DA neurons *in vitro* and *in vivo* induced by two different neurotoxins used in rodent models of PD.

TNF-dependent potentiation of microglia activation is not the primary mechanism mediating DA neuron degeneration.

Given that microglial-derived oxidative stress has been proposed to be involved in the loss of DA neurons in the LPS model (Gao et al., 2002), we investigated the extent to which rescue of DA neurons by inhibition of TNF signaling correlated with attenuated microglia activation (measured using antibodies against the microglial markers complement 3 receptor, isolectin B4, and CD45). EVM cultures were treated with LPS (10 ng/mL) or with LPS plus XENP345 (200 ng/mL co-added, 100 ng/mL re-added every 24 hr) to measure microglia activation at 12, 24, 48 and 96 hr after addition of LPS. Blocking solTNF signaling with XENP345 did not completely abolish LPS-induced microglia activation, but had its greatest attenuating effects in the early time points (<24 hr) after LPS stimulation (Figure 2.5 a,b). In a control experiment, we found that robust induction of microglia activation by 4 ng/mL solTNF can be inhibited completely by a ratio of XENP345 to solTNF of 50:1 (Figure 2.5 c), suggesting that partial inhibition of LPS-induced microglia activation with XENP345 was not due to incomplete neutralization of solTNF. From these data we conclude that solTNF is required for potentiation of early LPS-induced microglia activation, yet only partially mediates the neuroinflammatory response elicited by LPS. Therefore, we conclude that the critical microglial-derived mediator responsible for DA neuron loss is solTNF, and attenuation of TNF-mediated effects is sufficient to provide significant rescue of DA neurons, since neuroprotection can be achieved with XENP345 despite persistent microglia activation. Experiments to identify the cellular and signaling mechanisms mediated by TNF and

necessary for 6-OHDA-induced neurodegeneration are in progress. It is well known that the action of the oxidative neurotoxin 6-OHDA involves formation of 6-OHDA-paraquinones and leads to glutathione (GSH)-depletion of mitochondria (Przedborski, 2005; Przedborski and Ischiropoulos, 2005). Such depletion sensitizes many cells to the toxic actions of TNF via mitochondrial targeting of glycosphingolipids and potentiation of intracellular ROS accumulation (Garcia-Ruiz et al., 2003; Garcia-Ruiz et al., 1997). Therefore, these TNF-dependent signaling cascades may have important roles in triggering the death of DA neurons and are therefore under investigation.

Delayed inhibition of TNF signaling also rescues DA neurons from LPS-induced death.

We reasoned that since low-dose LPS exposure results in delayed, progressive and selective death of DA neurons, delayed administration of XENP345 after an LPS insult may still be capable of preventing a substantial portion of DA neuron loss. In these experiments, we treated EVM cultures with LPS and then added XENP345 after delays of 12-72 hr (and re-adding inhibitor every 24 hr). Microglial activation and DA neuron survival were measured 4 days after the initial LPS stimulus. XENP345 was still efficacious in attenuating DA neuron loss (Figure 2.6 a) even when added several days after the LPS insult. Despite the ability of delayed addition of XENP345 to block DA neuron loss, we found that delayed inhibition of TNF was unable to block sustained LPS-induced microglia activation, as measured by three immunocytochemical markers of microglial activation (Figure 2.6 b). These data suggest that direct neurotoxicity of

microglial-derived TNF on DA neurons, presumably through TNFR1 receptors present on these neurons (McGuire et al., 2001), is likely to be the primary mechanism eliciting delayed death of DA neurons in LPS-treated neuron/glia cultures. However, a second mechanism by which TNF may mediate dopaminergic cell loss is by perpetuating microglial-derived extracellular ROS and RNS production, thus increasing oxidative stress on DA neurons and leading to degeneration**.**

DISCUSSION

This is the first comprehensive study to employ TNF-selective pharmacological inhibitors in two different models of PD to investigate the direct contribution of the two biologically active forms of TNF in mediating degeneration and death of DA neurons in wild-type rodents. Although it has been known that PD brains possess increased gliosis, cytokine levels, and reactive oxygen and nitrogen species, the mechanisms underlying neuroinflammation-mediated DA neuron death have remained unclear. We hypothesized that, independent of the trigger that elicits its production or its cellular source, solTNF might act at two different cellular sites: directly on DA neurons to exert neurotoxic effects and indirectly on microglia to potentiate microglial-derived oxidative stress, and that activation of both routes results in progressive degeneration and loss of DA neurons. Our results that nigral delivery of the soluble TNF inhibitor XENP345 attenuated loss of rat DA neurons, independent of whether this loss is induced by the bacteriotoxin LPS or by the oxidative neurotoxin 6-OHDA, confirm that solTNF is a critical neuroinflammatory mediator involved in the neurodegenerative actions of both LPS and 6-OHDA *in vivo*. The neuroprotective effects of the immunosuppressant FK-506 in 6-OHDA lesioned rats has been ascribed to inhibition of microglial-derived TNF (Mogi et al., 1999) since FK-506 has been shown to inhibit secretion of pro-inflammatory cytokines by macrophages (Keicho et al., 1991). Mechanistically, our results further indicate that TNF drives late signaling events in eliciting neurotoxicity and triggering DA neuron death, since delayed (up to 72 hr) addition of XENP345 in a 4-day low-dose LPS treatment regimen can markedly attenuate DA neuron death independent of persistent

microglial activation as assessed by several microglial markers. Given that clinical diagnosis of PD in humans occurs only after a significant loss (>80%) of nigral DA neuron has occurred, most interventions are in essence 'delayed' so these findings have potential clinical relevance; there may be a therapeutic window during which inhibition of TNF signaling could slow disease progression. Consistent with a role of TNF in the pathophysiology of PD, a cohort of Early Onset PD patients in Japan have an increased frequency of a polymorphic allele (-1031C) in the TNF gene promoter that results in higher transcriptional activity (Nishimura et al., 2001) and causes them to be high TNF producers. A recent prospective study indicating that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) lowers the risk of developing PD by 46% (Chen et al., 2003) also supports a role for chronic inflammation in triggering or accelerating development of PD in humans.

There are several possible explanations as to why co-infusion of a solTNF inhibitor into the SNpc did not completely abrogate loss of nigral NeuN/TH-positive neurons. We can rule out a role for tmTNF since etanercept did not yield greater neuroprotection compared to XENP345 *in vitro*. We can also rule out technical problems with immunohistological detection as a reason for the apparent partial rescue. Specifically, the protocol used for staining and detection of NeuN/TH-positive cells throughout the extent of the 40µm sections required for performing stereology using a 20µm optical fractionator was optimized in pilot experiments to ensure complete penetration of primary and secondary antibodies to the bottom guard zone used in the stereology parameters (See Methods). One possible explanation for the partial rescue that

is currently being investigated relates to the potential for limited diffusion of XENP345 away from the delivery site during the chronic infusion. Second, in these experiments, TNF signaling was blocked only in a small region of SNpc for 2 weeks coincident with the chronic LPS infusion or immediately following induction of the intrastriatal 6-OHDA lesion; yet the cascade of neurotoxic and neuroinflammatory events that occurs in these less acute animal models of PD is known to extend beyond the 2-week interval during which TNF signaling was pharmacologically inhibited (Sanchez-Pernaute et al., 2004). Third, partial neuroprotection may be indicative of a component of DA neuron loss that is TNF-independent and might involve the action of other pro-inflammatory cytokines, including IL-1β or IL-6 (Allan and Rothwell, 2001; Hald and Lotharius, 2005; Nagatsu and Sawada, 2005). To distinguish between these three possibilities, experiments are in progress to determine whether longer or continuous solTNF signaling inhibition across a larger volume of SNpc affords greater neuroprotection to nigral DA neurons, and whether continuous TNF signaling inhibition blocks the progressive phase of 6-OHDA-induced DA neuron death shown to be attenuated by selective COX-2 inhibition (Sanchez-Pernaute et al., 2004) Nonetheless, a reduction of nigral DA neuron death by 50% with delivery of DN-TNF biologics into the CNS would have a significant and positive impact on delaying progression of DA neuron loss in individuals with PD should these *in vivo* results in animal models be realized in clinical trials. Because of their size (~51 kD trimers), XENP345 would not be expected to cross the blood-brain barrier but this possibility is being investigated. Clinically, anti-TNF biologics presently used to treat patients with rheumatoid arthritis (etanercept and infliximab) have been linked to increases in demyelinating disease due to their ability to block tmTNF function (Arnett et

al., 2001; Sukal et al., 2006); therefore, a tmTNF-sparing TNF inhibitor for use in PD may be a safer therapy in humans.

To date, evaluation of the role of TNF in mediating DA neuron death in mature animals has only been investigated using acute MPTP intoxication in mice deficient in TNF pathway genes (Ferger et al., 2004; Leng et al., 2005; Rousselet et al., 2002; Sriram et al., 2002), and in wild-type mice treated with thalidomide (Ferger et al., 2004), which inhibits synthesis of TNF and many other genes. However, although these early studies implicated TNF signaling in DA neuron death, these null mice are not ideal models in which to critically address the direct role of TNF in nigral degeneration in adult animals. The brain proteome of TNFR double knockout mice indicates significant changes in expression of numerous genes (Pejovic et al., 2004), including the redox sensor DJ-1 which itself has been shown to be important in protecting DA neurons from oxidative stress (Goldberg et al., 2005; Kim et al., 2005). Moreover, mice that develop without any TNF signaling display arrested dendritic cell development and blunted systemic inflammatory responses (Pasparakis et al., 1996; Ritter et al., 2003; Sriram et al., 2006). Our own unpublished observations that treatment of neuron/glia cultures from TNF KO mice with TNF or LPS elicits blunted microglial activation and attenuated DA neuron death compared to treatment of cultures from WT mice. Therefore, it is impossible to discern whether resistance (or lack thereof) to MPTP injury in adult mice that developed without TNF signaling is a direct result of no TNF production (i.e., in TNF KO) or inability to bind TNF (i.e., in TNFR1/R2 double knockout) and/or from modifications in the function of downstream TNF-dependent targets (including microglia). In summary,

although studies with genetic models suggested that pharmacological manipulation of the TNF pathway may offer neuroprotection, our study is the first to directly demonstrate the feasibility and efficacy of this approach; plus the translational value of studies in adult rats with the soluble TNF-selective pharmacological inhibitor XENP345 makes our direct approach biologically and clinically relevant to humans.

Our data demonstrate that solTNF significantly contributes to toxicity and degeneration of DA neurons, independent of the trigger that elicits its production. This raises the exciting possibility that anti-TNF therapy specifically targeted against solTNF may be an effective treatment for prevention or attenuation of PD progression without interfering with important tmTNF functions such as maintenance of immune function and resistance to infection (Olleros et al., 2005). Lastly, we posit that our findings regarding a critical role of TNF signaling in death of DA neurons may be applicable to other neurodegenerative diseases in which the role of neuroinflammation is being intensely investigated both as a contributing factor, and as the basis for development of new vaccination therapies. For example, levels of TNF were found to be elevated in entorhinal cortex coincident with the earliest appearance of pathology (Janelsins et al., 2005) in a triple transgenic Alzheimer's Disease mouse model harboring mutations in presenilin 1, amyloid precursor protein, and tau (Oddo et al., 2003), and chronic exposure to systemic LPS accelerated development of amyloid and tau pathology in these mice (Kitazawa et al., 2005). Given its role as a major effector of LPS action, we hypothesize that TNF is a key mediator of LPS-enhanced neuropathology in these mice perhaps by promoting mitochondrial dysfunction and activation of apoptotic death cascades. Investigations of

TNF-dependent neuroinflammatory mechanisms that exacerbate neuropathology and hasten neuron loss may unveil opportunities for development of new anti-inflammatory therapeutics to treat human neurodegenerative diseases like PD and Alzheimer's disease.

FIGURES

Figure 2.1

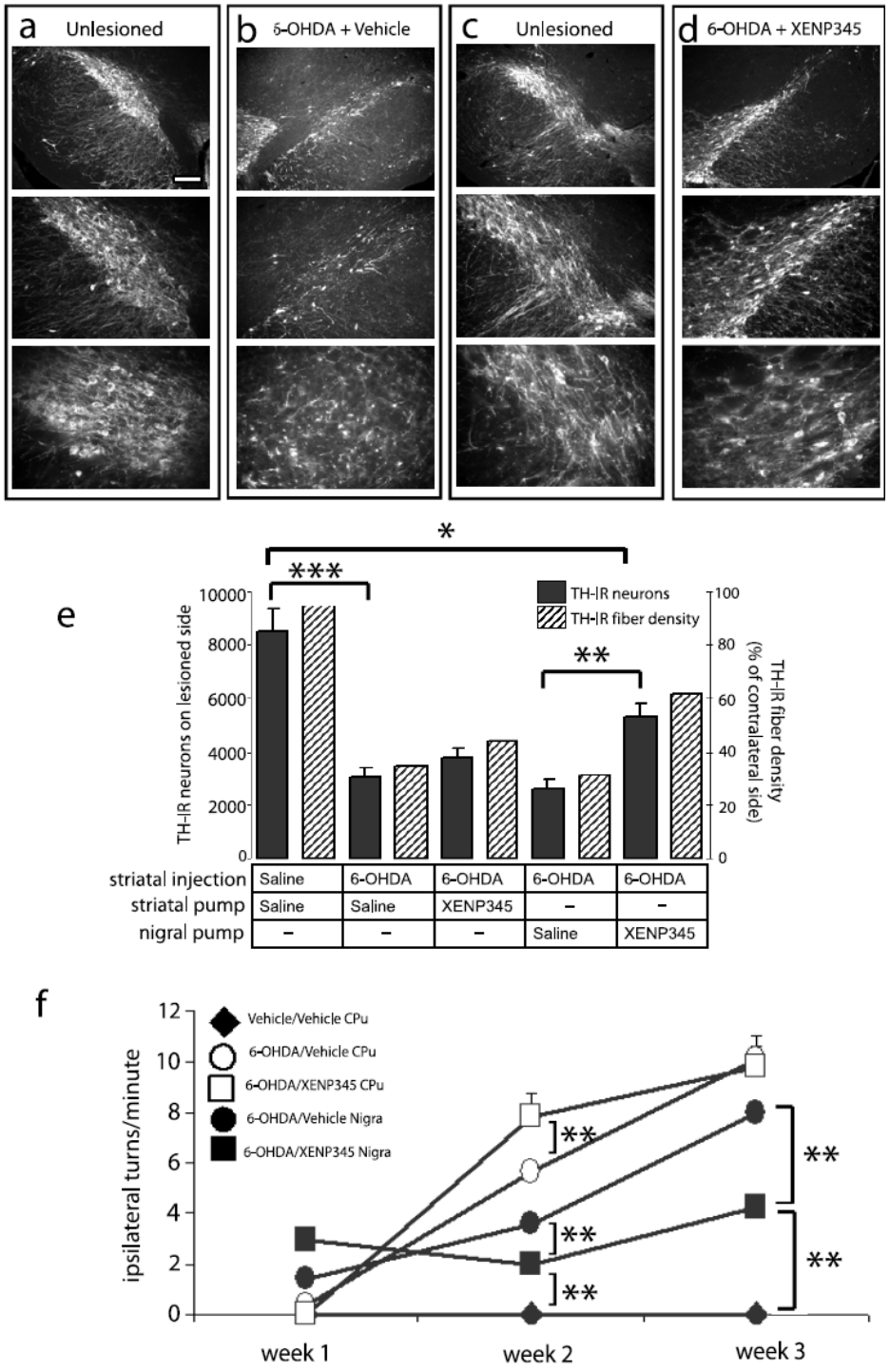

Figure 2.1. Blocking TNF signaling in the nigra attenuates striatal 6-OHDA-induced loss of nigral DA neurons and drug-induced rotational behavior.

A unilateral striatal lesion was induced by injecting 6-OHDA (20 μg) into the caudate-putamen complex of young adult rats; mock-lesioned animals received an injection of saline. Animals were stereotaxically implanted with an ipsilateral striatal or nigral indwelling cannula connected to an osmotic pump to deliver saline vehicle or XENP345 (0.08 mg/kg/day) over a two-week period. Animals were anesthetized and brains were fixed for IHC analyses of TH/NeuN-IR neurons by intracardiac perfusion three weeks after the lesion. Panels from top to bottom represent increasing magnification of representative brain sections used to obtain stereological estimates of nigral DA neuron number in 6-OHDA lesioned animals implanted with pump preloaded with saline (a, contralateral unlesioned side; b, lesioned/pump side) or implanted with nigral pump preloaded with XENP345 (c, contralateral unlesioned side; d, lesioned/pump side). Scale bar top panel = 100μm, middle panel = 50μm and bottom panel = 10μm. (e) Stereological estimate of DA neuron number (TH/NeuN-IR cells) in SNpc expressed as a percentage of the contralateral side (solid bars). Statistical significance was evaluated by ANOVA followed by post-hoc comparison test between groups and to unlesioned control group. Values expressed are group mean + SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Fluorescence intensity of TH-immunoreactive fibers was used to estimate striatal TH-fiber density (hatched bars) on the lesioned side, expressed as a percentage of the unlesioned contralateral side. (f) As a physiological measure of striatal DA depletion in mock- or 6-OHDA-lesioned animals, rotational behavior induced by an i.p. injection of 2.5 mg/kg amphetamine was measured weekly in all animals and expressed as the number of ipsilateral turns/min. Values expressed are group mean ± SEM; ** $p < 0.01$.

Figure 2.2

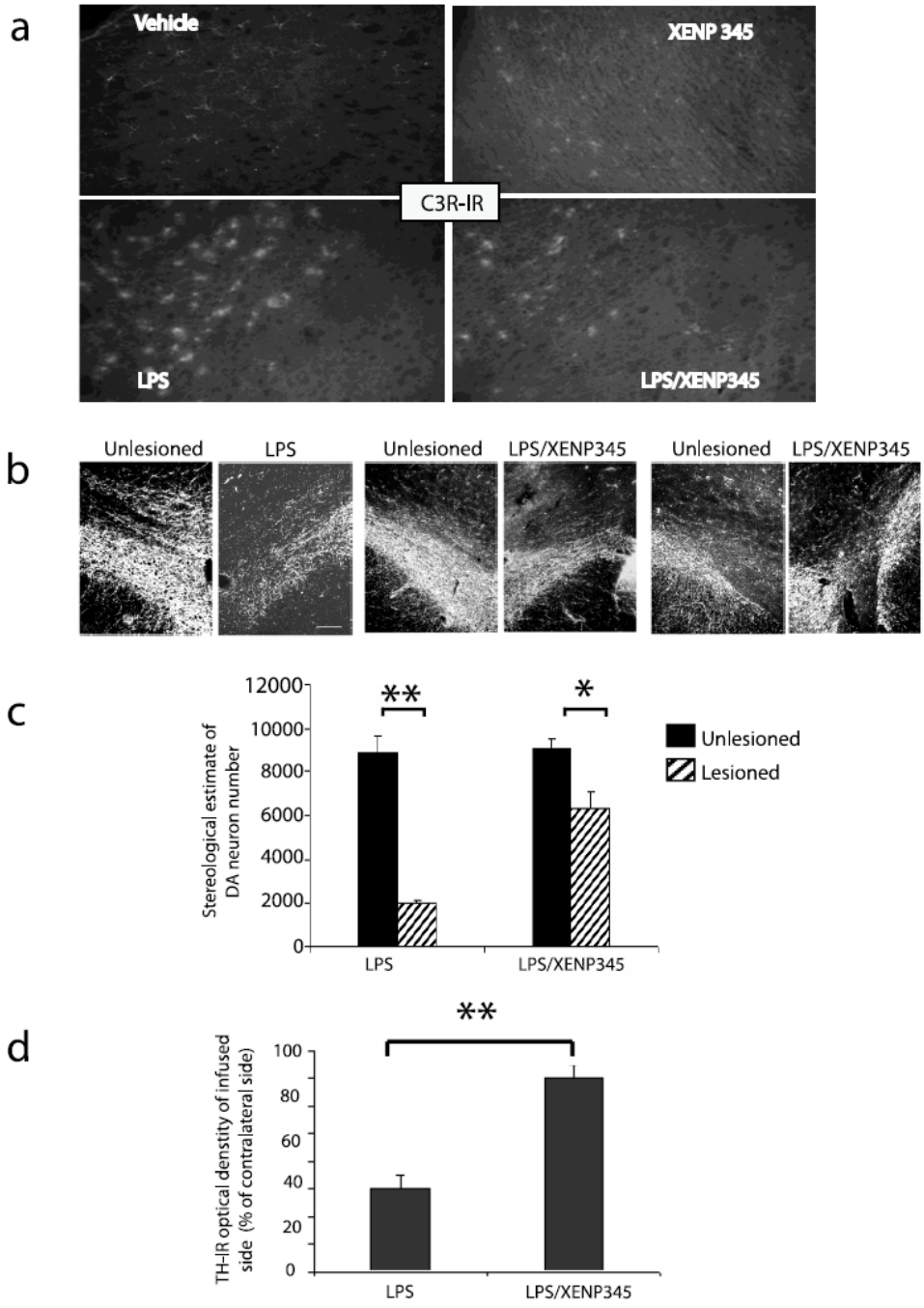
a
Vehicle
XENP 345
C3R-IR
LPS
LPS/XENP345
b
Unlesioned
LPS
Unlesioned
LPS/XENP345
Unlesioned
LPS/XENP345
c
Stereological estimate of DA neuron number
12000
10000
8000
6000
4000
2000
0
**
*
Unlesioned
Lesioned
LPS
LPS/XENP345
d
TH-IR optical denstity of infused side (% of contralateral side)
100
80
60
40
20
0
**
LPS
LPS/XENP345

Figure 2.2. Inhibition of soluble TNF signaling *in vivo* with XENP345 protects DA neurons from LPS-induced death.

Low-dose LPS (5 ng/hr in normal saline) was infused chronically for 2 weeks into the substantia nigra of CDF/Fischer 344 rats with or without XENP345 (70 ng/hr in normal saline). (a) Representative sections of microglia activation detected by complement 3 receptor (C3R)-positive cells in nigral sections from rat SNpc chronically infused with vehicle, LPS, LPS plus XENP345, or a single bolus of XENP345 (20 μg) scale bar = 10 μm. A ramified (resting) morphology is evident in vehicle and XENP345 alone brains. LPS/XENP345 co-infused SNpc displayed fewer ameboid (activated) microglia compared to LPS alone. (b) Representative sections of TH-IR from an animal infused with LPS and two different animals co-infused with LPS/XENP345 (B, scale bar = 100 μm). (c) Stereological estimates of nigral DA neuron number (TH/NeuN co-immunoreactive neurons) after LPS or LPS/XENP345 infusion in SNpc on the unlesioned (contralateral) side in solid bars and lesioned (ipsilateral) side shown in gray hatched bars. Left versus right differences from the same animals were analyzed using two-tailed paired Student's t test. Values were expressed as the group mean ± SEM; **p*< 0.05, ***p* < 0.01 significantly different from unlesioned side. (d) Striatal TH-IR optical density on infused side (expressed as a percentage of contralateral side). Values expressed are the mean ± SEM; **p< 0.01 significantly different from LPS only infused group.

Figure 2.3

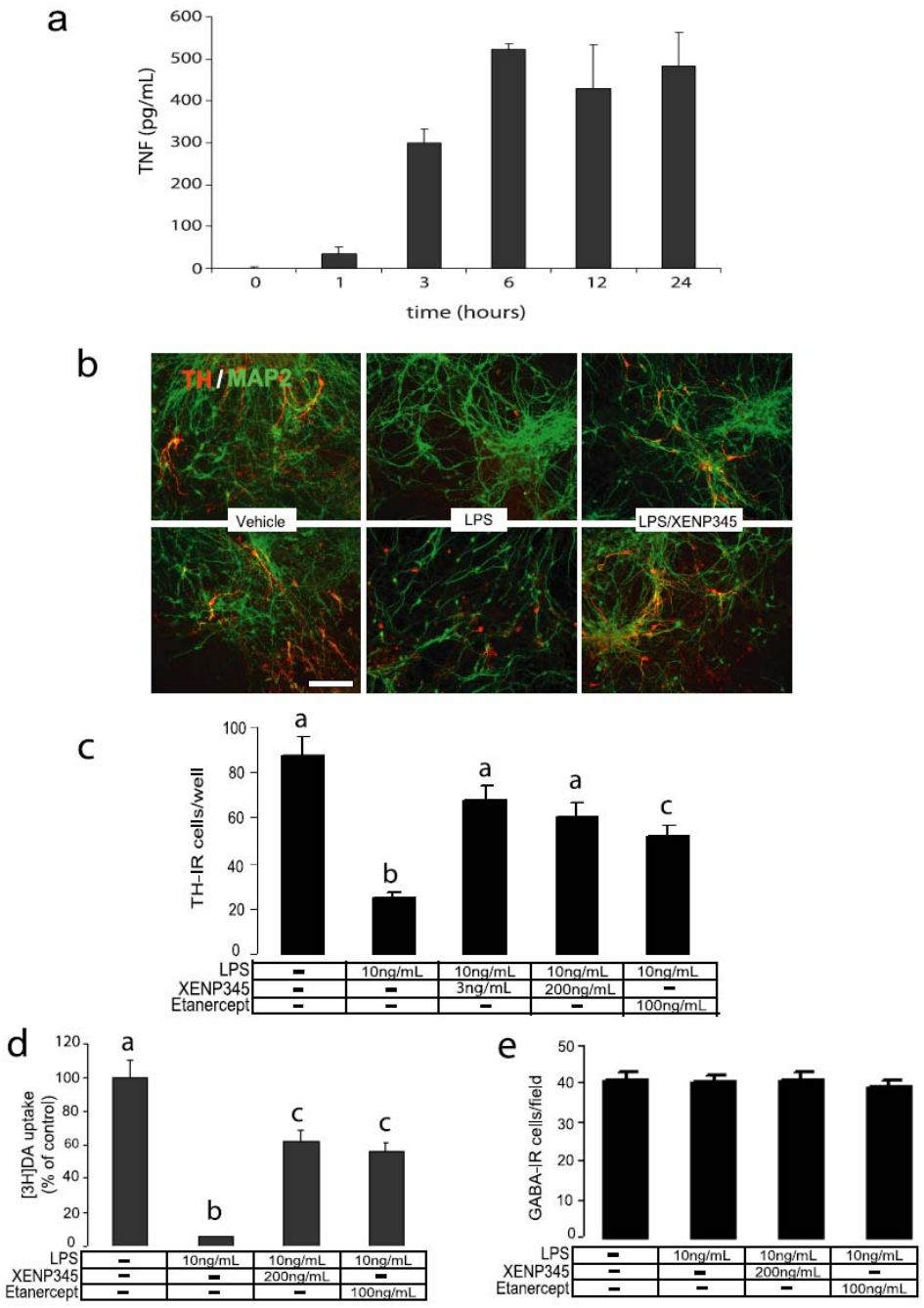
a
TNF (pg/mL)
600
500
400
300
200
100
0
0
1
3
6
12
24
time (hours)
b
TH / MAP2
Vehicle
LPS
LPS/XENP345
c
TH-IR cells/well
100
80
60
40
20
0
a
b
a
a
c
LPS
XENP345
Etanercept
10ng/mL
10ng/mL
3ng/mL
10ng/mL
200ng/mL
10ng/mL
100ng/mL
d
[3H]DA uptake (% of control)
120
100
80
60
40
20
0
a
b
c
c
LPS
XENP345
Etanercept
10ng/mL
10ng/mL
200ng/mL
10ng/mL
100ng/mL
e
GABA-IR cells/field
50
40
30
20
10
0
LPS
XENP345
Etanercept
10ng/mL
10ng/mL
200ng/mL
10ng/mL
100ng/mL

Figure 2.3. Inhibition of TNF signaling by anti-TNF biologics attenuates LPS-induced neurotoxicity and death of dopaminergic (DA) neurons in EVM cultures.

(a) Production of solTNF into culture media elicited by LPS (10 ng/mL) in rat embryonic day 14 (E14) ventral mesencephalon (EVM) neuron/glia cultures was measured by quantitative ELISA. (b) EVM cultures were treated with LPS (10 ng/mL) in media supplemented with 2.5% FBS (see Methods) for 4 d in the presence or absence of solTNF-selective XENP345 or etanercept, a soluble decoy human Fc-TNFR2 receptor that inhibits both tmTNF and solTNF. DA neurons were identified as double-labeled TH/MAP2b cells. Two representative panels are shown for vehicle-, LPS- or LPS plus XENP345-treated cultures. Similar results were obtained in a minimum of three independent experiments. (c) Quantification of DA neuron survival in EVM cultures treated with LPS (10 ng/mL) for 4 days in the presence or absence of TNF inhibitors. Similar results were obtained in a minimum of three independent experiments. Values shown are mean $\pm$ SEM; histogram bars with different letters are significantly different ($p < 0.05$). (d) Uptake of [^{3}H]-dopamine was measured in LPS-treated EVM cultures in the presence or absence of TNF inhibitors. Values shown are background-corrected for non-specific uptake measured by including mazindol during incubation with the tritiated neurotransmitter. Values shown are mean + SEM; histogram bars with different letters are significantly different ($p < 0.05$). Similar results were obtained in three independent experiments. (e) GABAergic neuron survival, assessed by counting number of GABA/NeuN double-labeled cells, was unaffected by LPS treatment. No statistical significant difference was found between groups. Similar results were obtained in two independent experiments.

Figure 2.4

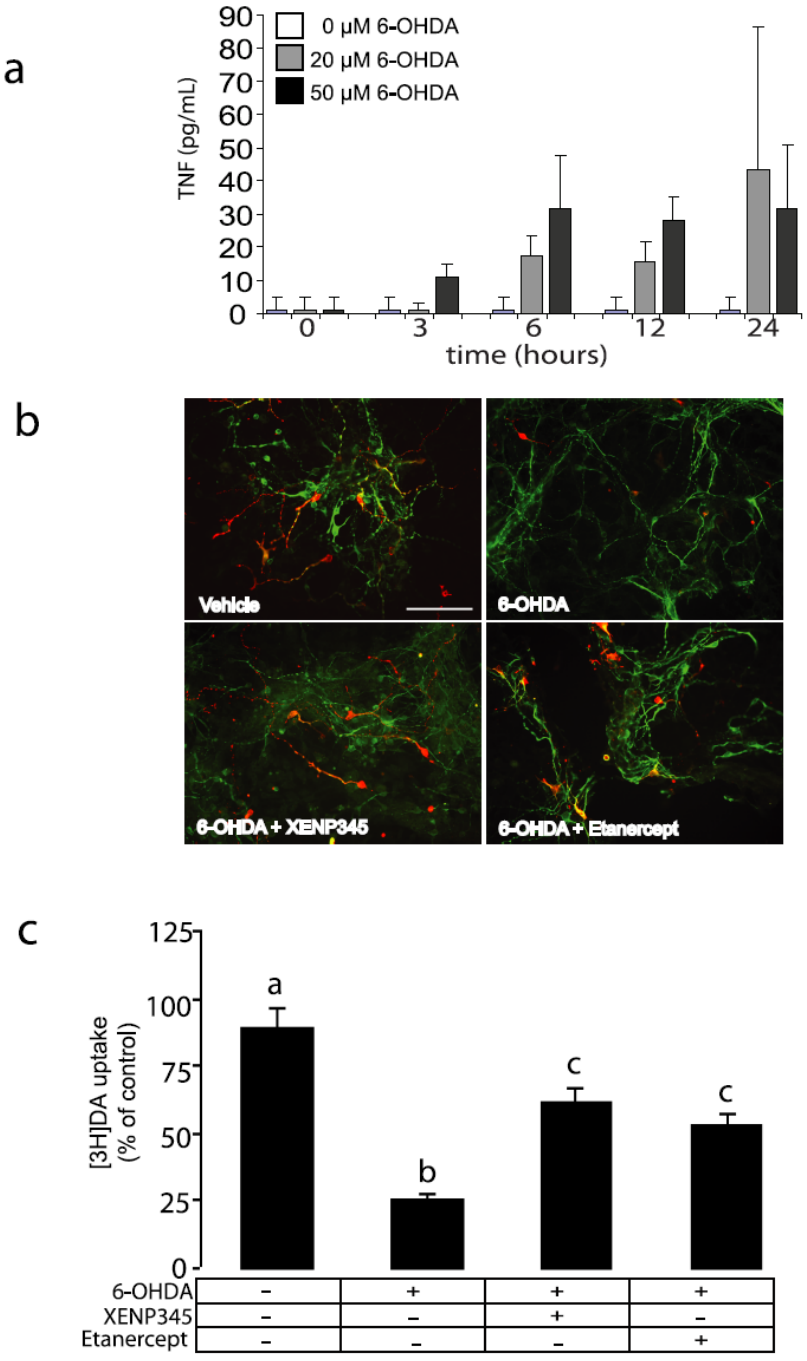
a
0 μM 6-OHDA
20 μM 6-OHDA
50 μM 6-OHDA
TNF (pg/mL)
90
80
70
60
50
40
30
20
10
0
0
3
6
12
24
time (hours)
b
Vehicle
6-OHDA
6-OHDA + XENP345
6-OHDA + Etanercept
c
[3H]DA uptake
(% of control)
125
100
75
50
25
0
a
b
c
c
6-OHDA
XENP345
Etanercept
–
+
+
+
–
–
+
–
–
–
–
+

Figure 2.4. Inhibition of soluble TNF signaling attenuates 6-OHDA-induced death of dopaminergic (DA) neurons in EVM cultures.

(a) Production of solTNF into culture media elicited by 6-OHDA (0μM (white), 20μM (gray), or 50μM (black)) in rat embryonic day 14 (E14) ventral mesencephalon (EVM) neuron/glia cultures was measured by quantitative ELISA. (b) EVM cultures were treated with 6-hydroxydopamine (20 μM) for 24 hr in media supplemented with 2.5% FBS (see Methods) in the presence or absence of the solTNF-selective XENP345 (200 ng/mL) or etanercept (200 ng/mL). DA neuron survival was assessed 96 hr after exposure to 6-OHDA. DA neurons were identified as double-labeled TH/MAP2b cells. Representative panels are shown for vehicle-, 6-OHDA-, 6-OHDA/XENP345-, or 6-OHDA/etanercept-treated cultures (scale bar = 100 μm). (c) Uptake of [^{3}H]-dopamine was measured in 6-OHDA-treated EVM cultures in the presence or absence of TNF inhibitors. Values shown are background-corrected for non-specific uptake measured by including mazindol during incubation with the tritiated neurotransmitter. Values shown are mean $\pm$ SEM; histogram bars with different letters are significantly different ($p < 0.05$).

Figure 2.5

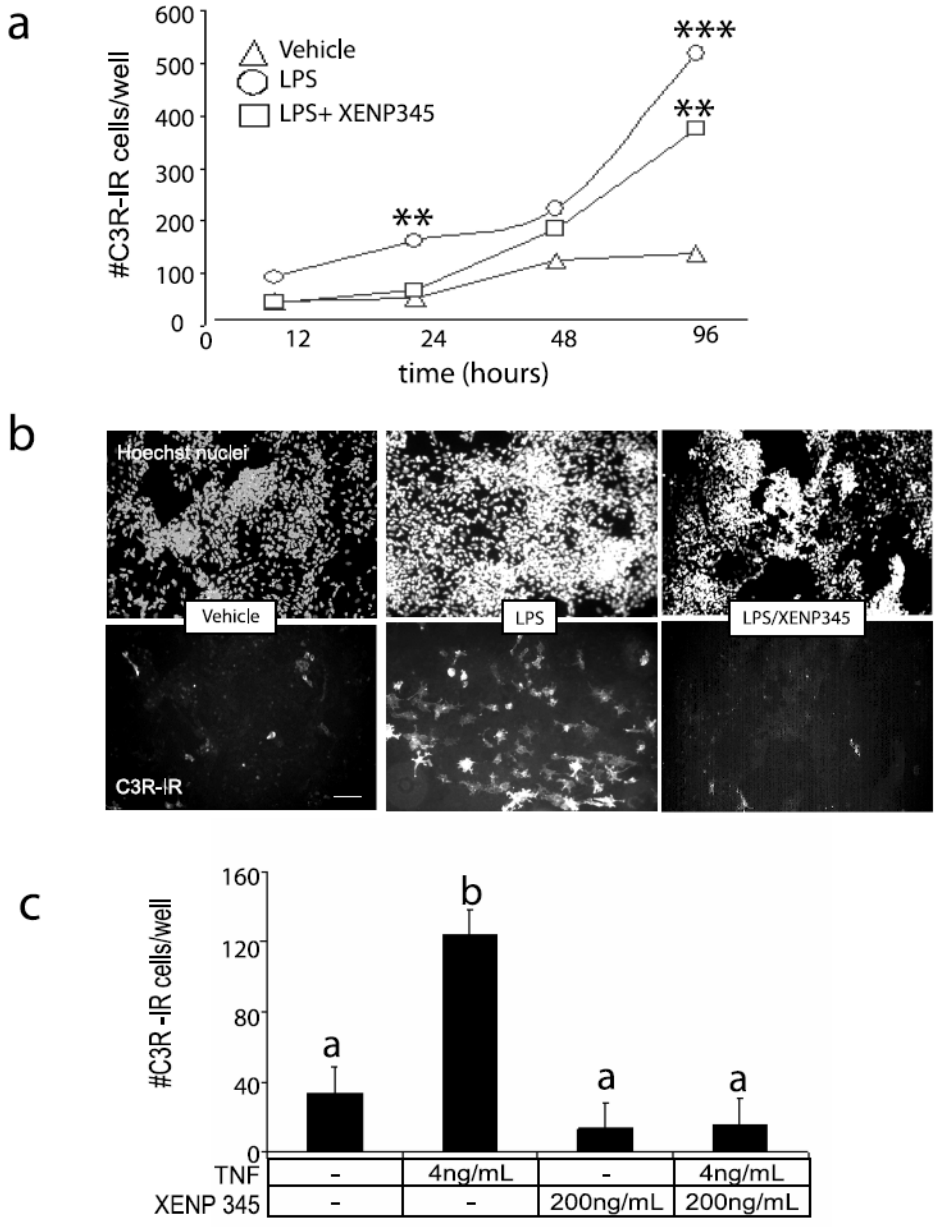
a
#C3R-IR cells/well
600
500
400
300
200
100
0
Vehicle
LPS
LPS+ XENP345
***
**
**
0
12
24
48
96
time (hours)
b
Hoechst nuclei
Vehicle
LPS
LPS/XENP345
C3R-IR
c
#C3R -IR cells/well
160
120
80
40
0
a
b
a
a
TNF
-
4ng/mL
-
4ng/mL
XENP 345
-
-
200ng/mL
200ng/mL

Figure 2.5. Soluble TNF is a primary mediator of LPS-induced microglia activation and death of DA neurons in ventral mesencephalon neuron/glia cultures.

(a) Quantification of microglia activation (measured by C3R immunoreactivity) induced by LPS treatment for 12, 24, 48, or 96 hr in the presence (squares) or absence (circles) of XENP345. Vehicle treated cultures are indicated by triangles. Values are expressed as the mean ± SEM; error bars are smaller than the symbols; $^{**}p < 0.01$, $^{**}p < 0.001$. Similar results were obtained in at least three independent experiments. (b) Representative images of microglia activation measured after 24 hr of LPS stimulation in the presence or absence of XENP345 (scale bar = 50 μm) is indicated by number of ameboid-shaped CR3-immunoreactive microglia in the neuron/glia cell cultures. Similar results were obtained in three independent experiments. (c) Control experiment showing neutralization of solTNF-induced microglia activation by XENP345 and absence of TNF-like activity when used alone. EVM cultures were treated with TNF (4 ng/mL) in the presence or absence of XENP345. After 24 hr stimulation, cells were fixed and stained with an antibody specific for complement 3 receptor (C3R) to quantify extent of microglia activation. Values shown are mean $\pm$ SEM; histogram bars with different letters are significantly different ($p < 0.05$).

Figure 2.6

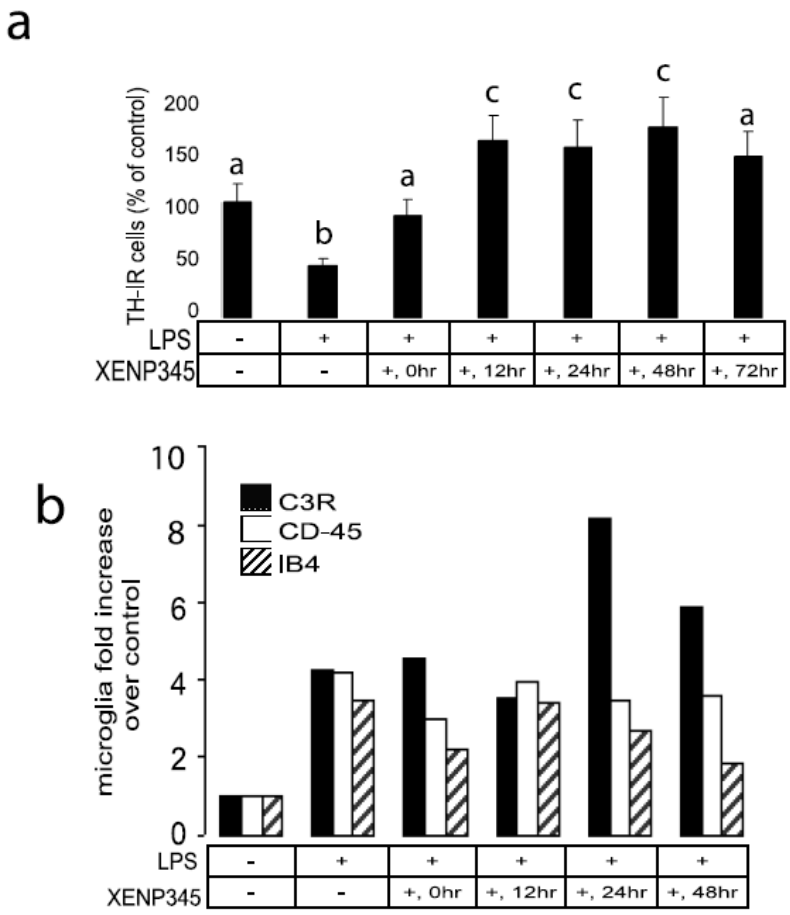

Figure 2.6. Delayed addition of XENP345 provides robust rescue of DA neurons despite sustained microglia activation.

(a) Quantification of DA neuron survival after co-addition or delayed addition of XENP345 (200 ng/mL) following LPS (10 ng/mL) treatment. Values shown are mean + SEM; histogram bars with different letters are significantly different ($p < 0.05$). Similar results were obtained in two independent experiments. (b) Quantification of microglia activation in response to LPS (10 ng/mL) in the presence or absence of XENP345 (200 ng/mL) using three different immunocytochemical microglial markers (C3R, CD45, IB4). Similar results were obtained in two independent experiments. Values shown are mean $\pm$ SEM; histogram bars with different letters are significantly different ($p < 0.05$).

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CHAPTER THREE

Intranigral lentiviral delivery of dominant negative tumor necrosis factor in hemiparkinsonian rats attenuates neurodegeneration and behavioral deficits

ABSTRACT

Neuroinflammatory processes have been implicated in the progressive loss of ventral midbrain dopaminergic neurons that give rise to Parkinson's disease (PD), a late-onset movement disorder that affects 2% of the population over age 70. Previously, we demonstrated that inhibition of the pro-inflammatory cytokine TNF through nigral infusion of dominant-negative TNF (DN-TNF) protein (XENP345) in two rat models of Parkinson's disease attenuates dopaminergic neuron loss. The objective of this study was to develop a constitutive lentiviral vector encoding DN-TNF and to determine if a gene therapy approach to deliver DN-TNF directly into the rodent substantia nigra prevents or attenuates neurotoxin-induced dopaminergic neuron loss and associated behavioral deficits. Here we demonstrate that a single injection of lentivirus expressing DN-TNF into rat substantia nigra administered concomitant with a striatal 6- hydroxydopamine lesion resulted in sufficiently high expression of inhibitor *in vivo* to attenuate both dopaminergic neuron loss and behavioral deficits resulting from striatal dopamine depletion. Our findings demonstrate the feasibility and efficacy of DN-TNF gene transfer as a novel neuroprotective strategy to prevent or delay nigrostriatal pathway degeneration with potential future therapeutic applications in the treatment of Parkinson's disease.

INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons (DA) in the substantia nigra pars compacta (SNpc) that project to the striatum. This selective loss of dopaminergic neurons results in striatal dopamine depletion which is responsible for the clinical manifestations of the disease. Although the critical mechanisms responsible for dopaminergic neuron loss are not fully understood, many studies implicate pro-inflammatory processes in contributing to disease progression (McGeer and McGeer, 2004; Tansey et al., 2007; Whitton, 2007), and prospective studies have shown that chronic use of non-steroidal anti-inflammatory drugs (NSAIDS) can lower the incidence of PD by 46% (Chen et al., 2003).

The pro-inflammatory cytokine Tumor Necrosis Factor (TNF) is elevated in postmortem brains and cerebrospinal fluid of PD patients (Mogi et al., 1994), and TNF mRNA and protein levels are increased in many experimental models of PD including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sriram et al., 2002) and 6-hydroxydopamine (6-OHDA) (Mogi et al., 1999). We have shown previously that chronic infusion of dominant-negative TNF (DN-TNF) protein, a selective inhibitor of soluble TNF, into the substantia nigra via an osmotic pump attenuates dopaminergic neuron loss resulting from a striatal 6-OHDA lesion or from chronic intranigral lipopolysaccharide (LPS) infusion (McCoy et al., 2006). DN-TNF-dependent nigrostriatal protection in the 6-OHDA model correlates with preservation of striatal

dopamine as measured by amelioration of amphetamine-induced rotational behavior (McCoy et al., 2006). To circumvent the inherent limitations associated with chronic infusion of such biologics through an invasive infusion device, in this report we investigate the feasibility and efficacy of lentiviral delivery of DN-TNF to achieve nigrostriatal protection in the rat 6-OHDA model of PD.

Our neurohistological and behavioral results demonstrate that a single intranigral injection of lentivirus expressing DN-TNF (lenti-DN-TNF) can significantly attenuate dopaminergic neuron loss and striatal dopamine depletion induced by the oxidative neurotoxin 6-OHDA. These findings provide further validation of soluble TNF as a therapeutic target in PD and suggest that anti-TNF gene transfer of a dominant negative TNF biologic into the substantia nigra may be an effective and novel therapeutic strategy to delay or prevent the progressive loss of dopaminergic neurons in humans with early stage PD.

MATERIALS AND METHODS

Materials

Cloning of DN-TNF and GFP sequences into lentiviral vectors. The human full length DN-TNF DNA sequence required to generate XENP345 protein (TNF variant A145R/I97T) in mammalian cells (Zalevsky et al., 2007), provided to us by David E. Szymkowski (Xencor, Inc., Monrovia, CA), included the signal peptide sequence required for membrane insertion and TACE recognition sequence required for natural

cleavage and extracellular secretion. The DN-TNF sequence was subcloned into a constitutive self-inactivating lentiviral vector based on the plasmid pLV (Pfeifer et al., 2002) 5' of an internal ribosome entry site (IRES) followed by the GFP coding sequence. The GFP-expressing lentiviral plasmid has been described previously (Pfeifer et al., 2002; Taylor et al., 2006). DN-TNF or GFP expression was driven by the CMV/ß-actin hybrid promoter (CAG).

Preparation and purification of lentivirus stocks. Lentivirus stocks were produced and purified according to a previously published protocol (Taylor et al., 2006). The final titer was 125µg/mL p24 and 1.6 x 10^9 infectious units (IU)/mL for the negative control lentivirus-GFP and 980µg/mL p24 and 8 x 10^8 IU/mL for lentivirus-DN-TNF. Vector stocks were diluted in Hanks balanced salt solution (HBSS) (Invitrogen, Carlsbad CA) as indicated.

Methods

Measurement of p65Rel A cytoplasmic-to-nuclear translocation. MN9D dopaminergic cells were plated onto 4-well dishes (Nunc, Rochester NY) in DMEM (Sigma, St Louis MO) supplemented with 10% Fetal Bovine Serum (FBS) (Gemini, West Sacramento CA) at 90,000 cells/well. Twenty-four hours later cells were infected at an MOI of 10 in a total of 250µL per well. At 24 hr post transduction, cells were switched to DMEM containing 0.5% FBS. At 48 hr post transduction, cells were stimulated for 15 min with 2ng/mL recombinant mouse TNF (R&D systems, Minneapolis MN), fixed in 4%

paraformaldehyde (Sigma), and assessed for p65Rel A nuclear translocation using an antibody specific for NFκBp65 (Santa Cruz Biotechnology, Santa Cruz CA; 1:200 dilution). Supernatants from the transduced cells were collected to measure DN-TNF production by hTNF ELISA (Invitrogen)**.**

Animal studies. Young adult Sprague Dawley SASCO and timed-pregnant young adult CDF/Fischer 344 rats were purchased from Charles River Laboratories (Wilmington MA) and housed in pathogen-free climate-controlled facilities at The University of Texas Southwestern Medical Center. All animal studies were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center at Dallas.

Expression of lenti-GFP and lenti-DN-TNF in E14 rat ventral mesencephalon (EVM) neuron/glia cultures. Primary rat EVM cultures were prepared by modification of a published protocol (Liu et al., 2000; McCoy et al., 2006). Briefly, ventral mesencephalic tissues were dissected from embryonic day 14 Fischer 344 rats and dissociated with mild mechanical trituration. Cells were pre-plated at a density of 1×10^6 cells/mL in 100μL DMEM/F12 (Sigma) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville GA), 2mM L-glutamine, 100μM nonessential amino acids, 50U/mL penicillin, 50μg/mL streptomycin (Sigma), and 10ng/mL Fibroblast Growth Factor (FGF-2) (R&D Systems) into a 48 well plate precoated with 0.1mg/mL poly-D-lysine (Sigma) and 20μg/mL laminin (BD Biosciences, Franklin Lakes NJ), and after 1hr, an additional 200μL media was added. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cultures were replenished two days later with 0.5mL/well fresh media lacking bFGF. To determine the

cell types that were infected by lentivirus, cells were transduced after 5 days *in vitro* (DIV) with lenti-GFP using 250,000 IU in 200μL DMEM/F12 containing 2.5% FBS and lacking bFGF. Twenty-four hours after infection, 150μL per well of DMEM/F12 with 2.5% FBS was added, 72 hours after infection cultures were fixed in 4% paraformaldehyde (Sigma) and immunocytochemical analysis was performed using rabbit anti-tyrosine hydroxylase (Chemicon, Temecula CA; 1:250 dilution), mouse anti-MAP2 (Chemicon; 1:400 dilution), mouse anti-complement type 3 receptor (Ox-42) (Chemicon; 1:60 dilution), or rabbit anti-GFAP (Dako, Carpinteria CA; 1:1000 dilution), and the appropriate Alexa 594-conjugated secondary antibodies (Invitrogen) diluted to 1:1000. To determine DN-TNF production of lentivirus infected cultures, at 5 DIV cells were infected with lenti-GFP or lenti-DN-TNF at 250,000 IU in 200μL DMEM/F12 with 2.5% FBS lacking bFGF. Twenty-four hours after infection, 150μL per well of DMEM/F12 with 2.5% FBS was added and hDN-TNF expression was measured by hTNF ELISA (Invitrogen) 24 and 48hr after viral transduction.

Measurement of TNF-induced microglial activation in EVM cultures. Microislands (2, 25-microliter volumes) of a neuron/glia single-cell suspension from E14 ventral mesencephalon resuspended at a density of 1×10^6 cells/mL were plated onto 4-well chamber slides (Fisher Scientific, Waltham MA) precoated with 0.1mg/mL poly-D-lysine and 20μg/mL laminin. At 5 DIV, the cells were infected with 250,000 IU of lenti-GFP or lenti-DN-TNF in a 300μL volume. Forty-eight hours post-transduction, the volume was brought up to 500μL with media containing the treatment (vehicle, TNF 5ng/mL, or TNF+ XENP345 at 200ng/mL as a positive control) cells were fixed 24 hours after the addition of TNF or TNF+ XENP345 in 4% paraformaldehyde and immunocytochemical

analysis was performed using goat anti-Iba-1 (Abcam, Cambridge MA; 1:150 dilution) and Donkey Anti Goat-Alexa 594 (Invitrogen; 1:1000 dilution). The total number of activated microglia per well was counted manually (an average of 13 fields per well, each condition in triplicate) in mock-infected, lenti-GFP-infected, or lenti-DN-TNF-infected cultures.

Measurement of 6-hydroxydopamine (6-OHDA) induced dopaminergic neuron loss in EVM cultures. Microislands (2, 25-microliter volumes) of a neuron/glia single cell suspension from E14 ventral mesencephalon resuspended at a density of 750,000cells/mL were plated onto 4-well chamber slides. At 5 DIV, the cells were infected with 250,000IU in a 300µL volume. Forty-eight hours post-transduction, the volume was brought up to 500µL with media containing the treatment (saline vehicle, 10µM 6-OHDA (Sigma), or 20µM 6-OHDA) cells were fixed 48 hours after 6-OHDA treatment in 4% paraformaldehyde (Sigma) and immunocytochemical analysis was performed using rabbit anti-tyrosine hydroxylase (Chemicon; 1:250 dilution) and DAG-Alexa 594 diluted to 1:1000. The total number of tyrosine hydroxylase positive dopaminergic neurons per well were counted in mock-infected, lenti-GFP-infected, or lenti-DN-TNF-infected cultures on an average of 8 fields per well. Two wells per treatment condition were counted.

Substantia nigra lentivirus infection to measure DN-TNF production. Briefly, 2 young adult female Sprague Dawley SASCO rats (200-250g) and 2 young adult male Sprague Dawley SASCO rats (340-400g) were anesthetized with halothane (2%) and placed in a

stereotaxic frame. Burr holes were drilled to permit unilateral stereotaxic injection of 2uL of lenti-DN-TNF (diluted in HBSS to a concentration of 100ug/mL p24) at a rate of 0.5uL/min into the substantia nigra pars compacta (stereotaxic coordinates from bregma: AP: -5.3mm from bregma, ML: -2.3mm, and DV: -7.3mm below surface of dura). Two weeks after lenti-DN-TNF injection, rats were deeply anesthetized, and brains were quickly harvested, midbrain was dissected, divided into left and right hemispheres, and flash-frozen in liquid nitrogen. hTNF ELISA was performed on brain homogenates and DN-TNF production was determined for each animal.

Intrastriatal 6-OHDA injection and substantia nigra lentivirus infection. 6-OHDA lesions were performed as previously described (Kirik et al., 1998; McCoy et al., 2006). Briefly, young adult female Sprague Dawley SASCO rats (200-250g) were anesthetized with halothane (2%) and placed in a stereotaxic frame. Burr holes were drilled to permit unilateral injection of 20μg 6-OHDA or sterile saline (4μL of 5μg/μL) at a rate of 1 μL/min into the striatum on the right hemisphere (stereotaxic coordinates: anteroposterior (AP): -1.2mm from bregma, mediolateral (ML): -3.9mm; and dorsoventral (DV): -5mm below surface of dura) (Paxinos et al., 1985). Vehicle (sterile HBSS), 2uL of Lenti-DN-TNF or –GFP (diluted 1:10 in sterile HBSS) was stereotaxically injected at a rate of 0.5uL/min through a burr hole into the substantia nigra pars compacta (stereotaxic coordinates from bregma: AP: -5.3mm from bregma, ML: -2.3mm, and DV: -7.3mm below surface of dura) immediately following intrastriatal 6-OHDA delivery.

Vibrissae-evoked forelimb placing. Measuring forelimb placement by both same-side and cross-midline vibrassae stimulation on each forelimb allows the differentiation between deficits in sensorimotor integration (as would occur in conditions such as stroke) and motor function, as occurs in parkinsonism (Woodlee et al., 2005). In unilateral 6-OHDA-lesioned animals, placement of the forelimb contralateral to the lesion is expected to be impaired upon stimulation of whiskers on either side of the head; while the forelimb ipsilateral to the lesion should be unaffected with either same-side or crosslimb placement. This pattern of same-side and cross-midline placing deficits is indicative of motor initiation deficits and not of decreased sensorimotor integration which affects only cross-midline placing. At 1, 2 and 3 weeks post 6-OHDA or sham lesions the vibrissae-evoked forelimb test was performed as previously described (Woodlee et al., 2005) for both same-side forelimb placing and cross-midline placing. Forelimb placing was measured as the successful number of paw reaches out of five trials for all four combinations of vibrissae stimulation and limb placement (i.e., the ipsilesional or contralesional vibrissae were stimulated for each forelimb) to measure deficits in motor initiation giving an indication of 6-OHDA-induced parkinsonian akinesia. During testing, animals were held by the torso and the hindlimbs and forelimb not being testing were gently restrained.

Rotational behavior analyses. At 1, 2 and 3 wk post 6-OHDA lesion, amphetamine-induced rotational behavior was monitored in a glass cylinder (diameter 24.5cm). Animals received 2.5mg/kg D-amphetamine (Sigma) i.p. and 60 min after the injection, rotational asymmetry was monitored for 20 min. Rotation towards the lesion (ipsilateral) was scored as positive and net rotational asymmetry score was expressed as full body turns/min.

Perfusion and tissue processing for histology. At 3 weeks post 6-OHDA lesion and lentivirus infection, animals were deeply anesthetized with Eusthasol (Butler Animal Health Supply, Dublin OH) and intracardially perfused with 300ml of heparinized (Butler Animal Health Supply, Dublin OH) PBS, pH 7.4, followed by 500mL of 4% paraformaldehyde in PBS, pH 7.4. Brains in the skulls were postfixed for 24hr in the same PFA solution. Following postfix, brains were dissected out of the skull and cryoprotected in 20% sucrose in PBS for 24-28hr. Coronal sections (30μm-thickness) were cut through the striatum and substantia nigra pars compacta on a Leica cryostat and mounted on glass slides (SuperFrost Plus, Fisher) for immunohistological analyses and stereological estimate of DA neuron number in a fixed 200μm area in SNpc.

Brightfield immunohistochemistry of brain sections. Nigral sections on glass slides were fixed for an additional 15 minutes in 4% paraformaldehyde, followed by 2 PBS rinses (pH 7.4). Sections were permeabilized for 45 minutes in PBS containing 0.3% Triton-X-100 and 1% normal goat serum (NGS), followed by a Tris Buffered Saline (TBS) rinse and blocking for 60 minutes in TBS containing 1% NGS. Primary antibody incubations were done for 24 hr at room temperature in TBS containing 0.1% Triton-X-100 and 1% NGS and rabbit anti-tyrosine hydroxylase (1:750). After 3 PBS washes, secondary antibody incubations were performed for 4hr at room temperature in PBS containing biotinylated goat anti-rabbit (1:400) (Vector Laboratories, Burlingame CA). After 3 PBS washes a 2 hr room temperature incubation of peroxidase labeled neutravidin at 1:5000 (Vector Laboratories) in PBS was performed followed by a PBS wash and 2 washes in Tris buffer. The signal was detected by using 0.6mg/mzl diaminobenzidine with 6.0mg/mL nickel ammonium sulfate and 0.006%

hydrogen peroxide as substrate in Tris buffer. Following immunohistochemistry for tyrosine hydroxylase the sections were blocked again in PBS containing 1% normal horse serum and the same protocol followed using mouse anti-NeuN (Chemicon; 1:300 dilution) as the primary antibody, and biotinylated horse anti-mouse secondary antibody. The NeuN signal was detected with diaminobenzide lacking nickel ammonium sulfate. Sections were dehydrated and mounted in a xylene base mounting media.

Nigral DA neuron counts. StereoInvestigator analyses software (Micro Bright Field Inc., Williston VT) was used to perform unbiased stereological counts of NeuN/TH-immunoreactive (NeuN/TH-IR) cell bodies in the SNpc using the optical fractionator method (West et al., 1991). The boundary of SNpc was defined according to previous anatomical demarcation in the rat (German and Manaye, 1993). For analysis, the treatment of the various brain sections was blinded to the observer. Cells were counted with a 40X oil immersion objective (1.3 NA) using a Nikon 80i microscope. Random and systematic counting frames (each 190 x 130μm with 2μm upper and lower guard zones) on cryosections (30μm serial sections with a mounted thickness of 25μM placed 6 per slide) obtained through the extent of SNpc (from AP:-3.3mm to -5.3mm behind bregma) were sampled using a 20μm optical dissector. A dopaminergic neuron was defined as a TH immunoreactive cell body with a NeuN stained nucleus in focus within the counting frame.

Fluorescence immunohistochemistry. Immunohistochemisty was performed as described previously (McCoy et al., 2006). Antibody dilutions were as follows: MAP2 (Chemicon) 1:400, TH (Chemicon) 1:250, Ox-42 (BD Pharmingen) 1:60, GFAP (Dako) 1:1000, hTNF (R

&D systems) 1:500, or GFP (Chemicon or Rockland, Gilbertsville PA) 1:1000. The appropriate Invitrogen Alexa-conjugated secondary antibodies were used at a dilution of 1:1000 in cell culture and 1:500 in tissue. Images were captured with a CoolSnap CCD ES monochromatic camera and analyzed with MetaMorph software (Universal Imaging Systems, West Chester, PA).

RESULTS

We previously demonstrated that the soluble TNF-selective dominant negative TNF inhibitor XENP345 (Steed et al., 2003; Zalevsky et al., 2007) was efficacious in attenuating 6-OHDA-induced DA neuron loss (McCoy et al., 2006) when delivered directly into the midbrain substantia nigra through a chronically implanted cannula connected to an osmotic pump. Given the neuroprotective effects of infusion of recombinant DN-TNF observed in these previous studies, we investigated the extent to which intranigral delivery of lenti-DN-TNF could protect against DA neuron loss and behavioral deficits *in vivo* in a unilateral 6-OHDA lesion model of PD. The sequence of green fluorescent protein (GFP) (Figure 3.1a) or the DN-TNF variant A145R/I97T (Figure 3.1b) was subcloned into a self-inactivating lentiviral vector downstream from the cytomegalovirus/β-actin hybrid (CAG) promoter. The DN-TNF vector included the TNF-alpha converting enzyme (TACE) cleavage site necessary for the conversion of the transmembrane form of the protein to soluble TNF (Aggarwal, 2000) as well as an internal ribosome entry site (IRES)-driven GFP sequence to monitor *in vivo* gene expression.

Efficient in vitro transduction of dopaminergic neuron-like cells with lenti-DN-TNF

To measure the steady-state levels of DN-TNF protein following transduction, we first evaluated transduction efficiency, protein expression, and inhibition of TNF signaling by lentivirus derived DN-TNF in the MN9D dopaminergic cell line (Choi et al., 1991). Infection of MN9D dopaminergic cells with lentivirus expressing GFP (lenti-GFP) or lenti-DN-TNF resulted in transduction efficiencies of greater than 50% (Figure 3.1c) and production of DN-TNF protein (> 30ng/mL) was detectable in the conditioned medium as early as 24 hours (Figure 3.2a) after infection and remained at this steady state level for up to 72 hours, as measured by a human TNF quantitative ELISA which recognizes the human DN-TNF (h-DN-TNF) sequence but does not detect rodent-derived TNF.

Lenti-DN-TNF blocks TNF-induced NFκB pathway activation

Next, we tested the ability of lentivirus-derived DN-TNF to prevent activation of the nuclear factor-kappa B (NFκB) signaling pathway by exogenously added soluble murine TNF. Transduction of MN9D cultures with lenti-DN-TNF, but not lenti-GFP or mock transduction, attenuated TNF-dependent nuclear enrichment of the p65 RelA subunit of NFκB that normally occurs within 15 minutes of stimulation with soluble TNF (Figure 3.2b)(Hohmann et al., 1990). These data demonstrate that transduction of dopaminergic cells with lenti-DN-TNF results in efficient production of functionally active DN-TNF protein that can inactivate signaling by exogenously added soluble TNF.

Inhibition of microglia activation and neuroprotection by lenti-DN-TNF in rat primary ventral midbrain cultures

To validate the transduction efficiency and DN-TNF production attained by lenti-DN-TNF transduction of primary cells, we used mixed neuron/glia cultures obtained from embryonic (E14) rat ventral mesencephalon (EVM). Based on GFP fluorescence driven by the IRES, we estimated that between 30 - 50% of cells were transduced in primary cultures (Figure 3.3a) depending on the plating density. Lenti-GFP infection of primary cultures resulted in transduction of microtubule-associated protein 2 (MAP2)-positive neurons, CD11c (OX-42)-positive microglia, and to a lesser degree glial fibrillary acidic protein (GFAP)-positive astrocytes (Figure 3.4). Also as seen in rat substantia nigra, tyrosine hydroxylase (TH)-immunoreactive dopaminergic neurons appeared to be relatively resistant to lentiviral infection compared to other neurons. DN-TNF protein expression in primary cultures was detectable in lenti-DN-TNF, but not lenti-GFP-infected cultures as early as 24 hours after transduction with secreted levels of DN-TNF peaking between 35 and 50ng/mL (data not shown). To determine the ability of lentivirus-derived DN-TNF to prevent TNF signaling, we performed microglial activation assays. After 5 days *in vitro*, rat EVM cultures were transduced with lenti-GFP, lenti-DN-TNF, or received no virus, and 48 hours after transduction they were stimulated with 5ng/mL recombinant murine TNF with or without co-addition of the recombinant DN-TNF biologic XENP345 (as a positive control for TNF inhibition). Cultures were fixed after a 24-hour stimulation and immunostained for the microglial activation marker ionized calcium-binding adaptor molecule 1 (Iba-1). Mock- and lenti-GFP infected

cultures exhibited TNF-induced microglial activation that could be blocked by the dominant negative protein XENP345. In contrast, lenti-DN-TNF cultures displayed a lower basal level of microglial activation and showed no increase in microglial activation upon addition of soluble TNF (Figure 3.3b). DN-TNF-mediated neuroprotection was further examined in lentivirus-transduced rat EVM cultures in the presence of 6-OHDA, a known neurotoxin which induces DA neuron death (Kirik et al., 1998). Cultures transduced with lenti-GFP and exposed to 6-OHDA displayed dose-dependent 50 - 75% loss of TH-immunoreactive neurons compared to vehicle-treated cultures (Figure 3.3c). In contrast, cultures transduced with lenti-DN-TNF were resistant to 6-OHDA-induced death consistent with our previous observations that soluble TNF is a critical mediator of 6-OHDA-induced dopaminergic death [8]. On the basis of these cell culture data we predicted that lentiviral transduction of neurons and/or glia in the midbrain would result in secretion of sufficient amounts of bioactive DN-TNF protein to antagonize midbrain TNF production elicited by 6-OHDA and its death-inducing effects on ventral midbrain dopaminergic neurons.

In vivo neuroprotection of the nigrostriatal pathway by nigral delivery of lenti-DN-TNF

Pilot studies established that a single intranigral stereotaxic injection of 2μL lenti-DN-TNF (100μg/mL p24) resulted in production of DN-TNF protein (0.6-2.0ng per midbrain hemisphere, n = 4), as detected by hTNF ELISA (which specifically detects the hDN-TNF sequence and does not cross react with endogenous rat TNF) in homogenates of

microdissected midbrain two weeks after injection. We investigated the transduction of specific cell types after lenti-DN-TNF infection of rat mibrain, and found that the majority of lentivirus-transduced cells were microglia as assesed by immunofluorescence detection of co-expressed GFP or hDN-TNF with cell type specific markers (Figure 3.4). Transduction of predominantly microglial cells *in vivo,* and neuronal cells to a lesser extent, is consistent with *in vitro* infection results as DA neurons do not appear to be transduced efficiently either *in vitro* or *in vivo*; GFAP- immunoreactive astrocytes were present at much lower density in the substantia nigra relative to that of microglia. Next, lenti-DN-TNF or lenti-GFP (as a negative control) was injected into the substantia nigra immediately following intrastriatal 6-OHDA- or mock- lesion to investigate the neuroprotective effects of DN-TNF gene transfer. Using unbiased stereologic methods to estimate the number of nigral DA neurons, we found that 6-OHDA-lesioned lenti-DN-TNF-injected animals displayed significantly less nigral degeneration compared to 6-OHDA-lesioned lenti-GFP injected animals. Mock-lesioned lenti-DN-TNF-injected animals displayed a slight increase in the number of nigral dopaminergic neurons that was not significantly different from that in mock-lesioned lenti-GFP-injected animals (Figure 3.5a). On average, 47% of TH positive dopaminergic neurons remained in the lesioned substantia nigra in lenti-DN-TNF injected animals, whereas in lenti-GFP animals only 24% of nigral DA neurons survived compared to the contralateral side, constituting a 50% reduction in DA neuron loss. Neither lenti-GFP nor lenti-DN-TNF transduction caused a significant change in DA neuron number in unlesioned control animals. These neurohistological findings indicate that delivery of DN-TNF into the

nigrostriatal pathway by gene transfer affords significant neuroprotection that is comparable to chronic infusion of DN-TNF protein [8].

To investigate whether neuroprotection by lenti-DN-TNF was accompanied by amelioration of 6-OHDA-induced behavioral deficits, we performed two routine behavioral tests to estimate the degree of striatal DA depletion: amphetamine-induced rotational behavior and vibrissae-evoked forelimb placing. Both behavioral tests were performed at weekly intervals after the induction of 6-OHDA lesion and lentivirus injection. To test vibrissae-evoked forelimb placement we measured forelimb placing (out of 5 trials) for both forelimbs of the animal upon stimulation of the whiskers on each side of the head. Measuring forelimb placement by both same-side and cross-midline vibrassae stimulation on each forelimb allows the differentiation between deficits in sensorimotor integration (as would occur in conditions such as stroke) and motor function, as occurs in parkinsonism (Woodlee et al., 2005). In unilateral 6-OHDA-lesioned animals, placement of the forelimb contralateral to the lesion is expected to be impaired upon stimulation of whiskers on either side of the head; while the forelimb ipsilateral to the lesion should be unaffected with either same-side or cross-midline limb placement. We found there was no difference between mock lesion and 6-OHDA-lesioned groups in placement of the forelimb ipsilateral to the 6-OHDA lesion when whiskers on either side of the head were stimulated (data not shown). However, 6-OHDA lesioned groups displayed a decrease in successful forelimb placement for the forelimb contralateral to the lesion upon stimulation of both ipsilateral and contralateral vibrissae, indicative of motor initiation deficits characteristic of parkinsonian akinesia (Figure 3.6b

and data not shown). The deficit in forelimb placement of lesioned animals which received lenti-DN-TNF was reduced but was not significantly different from unlesioned lenti-GFP infected rats at any time post lesion. In contrast, 6-OHDA-lesioned animals that received lenti-GFP had a deficit in forelimb placement that progressed over time and became statistically significant three weeks after the lesion. The deficits in forelimb placement were consistent when vibrissae on either side of the head were stimulated, indicating a lack of significant deficits in sensorimotor integration.

In addition to vibrissae-evoked forelimb placin*g*, we measured amphetamine-induced rotational behavior as an indirect measure of striatal DA depletion. Consistent with results of forelimb placing tests, 6-OHDA-lesioned/lenti-GFP-injected animals displayed progressive amphetamine-induced rotational behavior that was significantly greater than that in unlesioned animals at all time-points (One-way ANOVA, $p<0.05$). In contrast, 6-OHDA/lenti-DN-TNF injected animals displayed reduced rotational behavior which was not significantly different from unlesioned control animals at the two and three weeks post-lesion (Figure 3.6a). The improvements in both forelimb placement and amphetamine-induced rotational behavior suggest that lenti-DN-TNF injections may have prevented striatal DA depletion, thus resulting in functional neuroprotection of the nigrostriatal pathway in the 6-OHDA model.

DISCUSSION

Our results demonstrate the feasibility and efficacy of DN-TNF gene transfer as a potential therapeutic strategy to achieve nigrostriatal neuroprotection in PD. Specifically, here we show that lentiviral DN-TNF infection results in detectable secretion of DN-TNF both *in vitro* in a dopaminergic cell line and in primary mixed cultures, as well as *in vivo* in the SNpc. Lentivirus-derived DN-TNF was efficacious in blocking TNF-induced signaling and microglial activation *in vitro* and attenuated neurotoxin-induced DA neuron loss *in vitro* and *in vivo*. Results from this study in which nigral lenti-DN-TNF infection led to an approximate doubling of remaining nigral dopaminergic neurons (47% of nigral DA neurons remaining in lenti-DN-TNF infected animals compared to 24% remaining in lenti-GFP infected animals) are consistent with our previous report of nigral DA neuron protection mediated by the chronic infusion of the recombinant DN-TNF biologic XENP345 (31% of nigral neurons remained with vehicle infusion compared to 62% with DN-TNF infusion) (McCoy et al., 2006). There are a number of possible explanations for the greater variability in DA neuron survival obtained with lenti-DN-TNF delivery as opposed to infusion of the DN-TNF protein XENP345 [8]. One possibility is that the amount of DN-TNF production in the lentivirus-infected SNpc may have been more variable compared to the constant amount of XENP345 protein delivered by chronic infusion through an osmotic pump; alternatively, the spread of lentivirus-derived DN-TNF protein may have been more restricted. Another difference in experimental design that may account for the greater variability in DA neuron rescue with lenti-DN-TNF versus infusion of DN-TNF protein is the inherent lag time (typically 3-4 days) required

for gene expression following transduction with the lentivirus; this is not the case with infusion of DN-TNF inhibitor which is available immediately to block the potent neurotoxic effects of TNF. Thus, injection of the lentivirus days or weeks prior to 6-OHDA lesions (as done in several glial-derived neurotrophic factor (GDNF) studies (Bensadoun et al., 2000; Dowd et al., 2005; Georgievska et al., 2002)) might have led to an even greater rescue of DA neurons and larger behavioral effect. In addition to doubling the number of remaining nigral DA neurons after a striatal 6-OHDA lesion, lentiviral delivery of DN-TNF also attenuated behavioral deficits both in forelimb placement and drug-induced rotation resulting from 6-OHDA lesions. We did not investigate the neuroprotective effects of intrastriatal delivery of lenti-DN-TNF in these studies because previous experience with intrastriatal delivery of XENP345 afforded no neuroprotection (McCoy et al., 2006). Taken together, our studies provide proof of concept for the feasibility and efficacy of DN-TNF gene delivery as a means to administer TNF inhibitors into specific regions of the CNS without the need for an invasive chronic infusion device. In the future, it will be of interest to determine the extent to which significantly delayed TNF signaling inhibition still affords neuroprotective effects, a question of therapeutic relevance when one considers a clinical diagnosis of Parkinson's disease means significant dopamine neuron loss has already occurred.

Although anti-TNF drugs may be effective disease-modifying therapies in other conditions characterized by chronic inflammation (Scheinfeld, 2004), the currently FDA-approved systemic administration of anti-TNF biologics (i.e., large pegylated Fc-fused

TNF decoy receptors or TNF antibodies) to treat peripheral autoimmune diseases such as rheumatoid arthritis and Crohn's disease, is unlikely to provide adequate brain penetration to affect TNF signaling in the brain (Tweedie et al., 2007; van Oosten et al., 1996; William H. Robinson, 2001). Therefore, CNS applications may require use of chronic infusion devices or alternative delivery methods such as the one reported here. Gene therapy approaches are attractive for use in PD treatment for several reasons including the spatially-defined and cell type-specific pathology of the disease, the requirement for consistent drug administration to minimize or prevent dose fluctuations, and the difficulty in chronically administering drugs which can not cross the blood brain barrier.

Several gene therapy approaches to deliver proteins targeting a number of pathways including: enzymes involved in neurotransmitter metabolism, neurotrophic factors, and anti-apoptotic proteins are currently under preclinical and clinical investigation for the treatment of PD (Porras and Bezard, 2008). Initially adeno-associated virus (AAV)-glutamatic acid decarboxylase (GAD) delivery to the subthalamic nucleus was investigated in 6-OHDA-lesioned rats where its expression attenuated apomorphine-induced rotational behavior (Luo et al., 2002). A Phase I clinical trial using AAV-GAD in the subthalamic nucleus of Parkinson's patients has demonstrated the safety of this therapy, but efficacy of this treatment has not been rigorously tested in patients due to unilateral delivery of AAV-GAD (Kaplitt et al., 2007) (Fiandaca et al., 2007). It has been demonstrated that AVV-GAD administration in

MPTP-intoxicated monkeys yields modest behavioral improvement (Emborg et al., 2007).

AAV-Aromatic amino acid decarboxylase (AADC) delivery to the striatum is effective in limiting L-dopa requirements, improving clinical rating scores, and reducing dyskinesia in MPTP-lesioned primates; AADC expression and the associated behavioral improvement has been shown to last up to six years (Bankiewicz et al., 2006; Forsayeth et al., 2006). These preclinical studies have lead to clinical trials in which bilateral delivery of AAV-AADC was demonstrated to increase striatal AADC expression and improve L-dopa responsiveness (Fiandaca et al., 2008). Most notably, promising early results have been obtained from clinical trials in which AAV expressing the potent dopaminergic survival and trophic factor neurturin was delivered into the putamen of PD patients (Bartus, 2007). In Phase I clinical trials AAV-delivered neurturin was well tolerated and resulted in persistent improvements in PD symptoms as measured by the Unified Parkinson's Disease Rating Scale motor "off" score; this therapy is currently in Phase II trials. In addition to these promising early results from clinical trials, development of inducible viral vectors is also underway and is expected to advance gene therapy efforts that can progress to clinical trials in humans (reviewed in (Cress, 2007)). In addition to AAV-based vectors, lentiviral vectors have been shown to be efficient tools for gene transfer into the CNS (Jakobsson and Lundberg, 2006). Transduction of astrocytes with a lentiviral vector encoding glial-derived neurotrophic factor (GDNF) afforded neuroprotection in an *ex vivo* intranigral gene delivery approach (Ericson et al., 2005). Moreover, development of cell-type specific disease-regulated lentiviral vectors

has now been demonstrated in glial cells by using the glial-fibrillary acidic protein (GFAP) promoter or the proenkephalin promoter in neurons (Jakobsson et al., 2006). In short, great progress in generation of second and third generation viral vectors for therapeutic gene transfer has been made in the last decade, but the safety and efficacy of regulatable vectors has not been extensively investigated in preclinical models and may require further validation prior to advancement for use in PD treatment (Kordower and Olanow, 2008).

In summary, although the exact mechanisms responsible for degeneration of nigral DA neurons in PD have not been fully delineated, the wealth of data implicating inflammatory processes in the progressive loss of these neurons coupled with the protective effects of NSAIDs against idiopathic PD, provide compelling reasons to accelerate research approaches to selectively target inflammatory factors with demonstrated neurotoxic effects on DA neurons. Our studies demonstrate the feasibility and efficacy of dominant negative Tumor Necrosis Factor gene transfer as a novel neuroprotective strategy to prevent or delay nigrostriatal pathway degeneration in a pre-clinical model of PD.

Figure 3.1

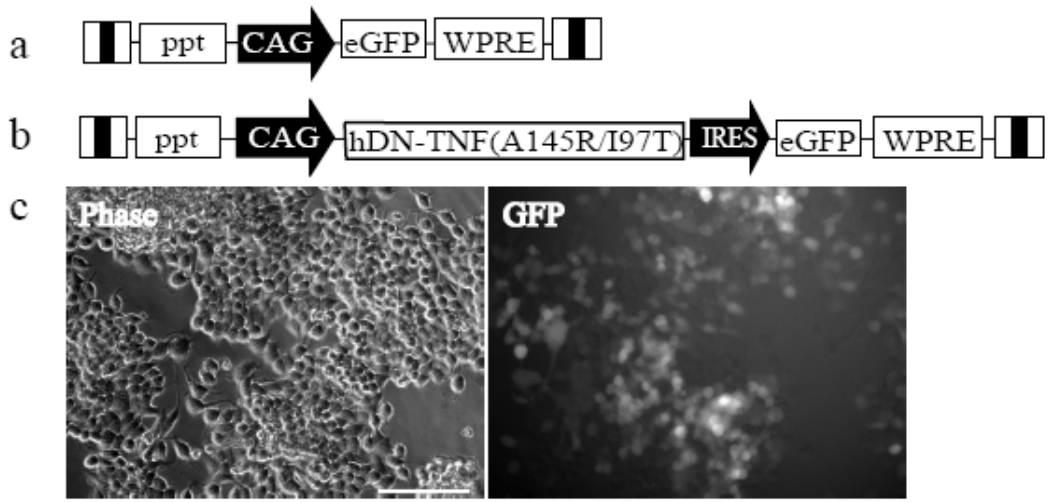


Figure 3.1. Lentiviral vector design and *in vitro* transduction of MN9D cultures with lenti-DN-TNF-IRES-GFP results in high transduction efficiency. (a,b) Schematic of lentiviral vectors. Both the lenti-GFP and lenti-DN-TNF viral vectors contain a chicken β-actin cytomegalovirus enhancer/promoter (CAG), ß-globin intron, a central polypurine tract of HIV-1 (ppt), a woodchuck hepatitis virus posttranscriptional response element (WPRE), and a self-inactivating deletion in the 3' LTR. The lenti-DN-TNF vector contains an internal ribosome entry site (IRES) for GFP expression following the sequence for pro-human DN-TNF (A145R/I97T). (c) Phase contrast and fluorescence images of the dopaminergic MN9D cell line transduced with lenti-GFP and imaged under an inverted microscope with a FITC fluorescence filter 48 hours after transduction. Scale bar in **c** = 50μm

Figure 3.2

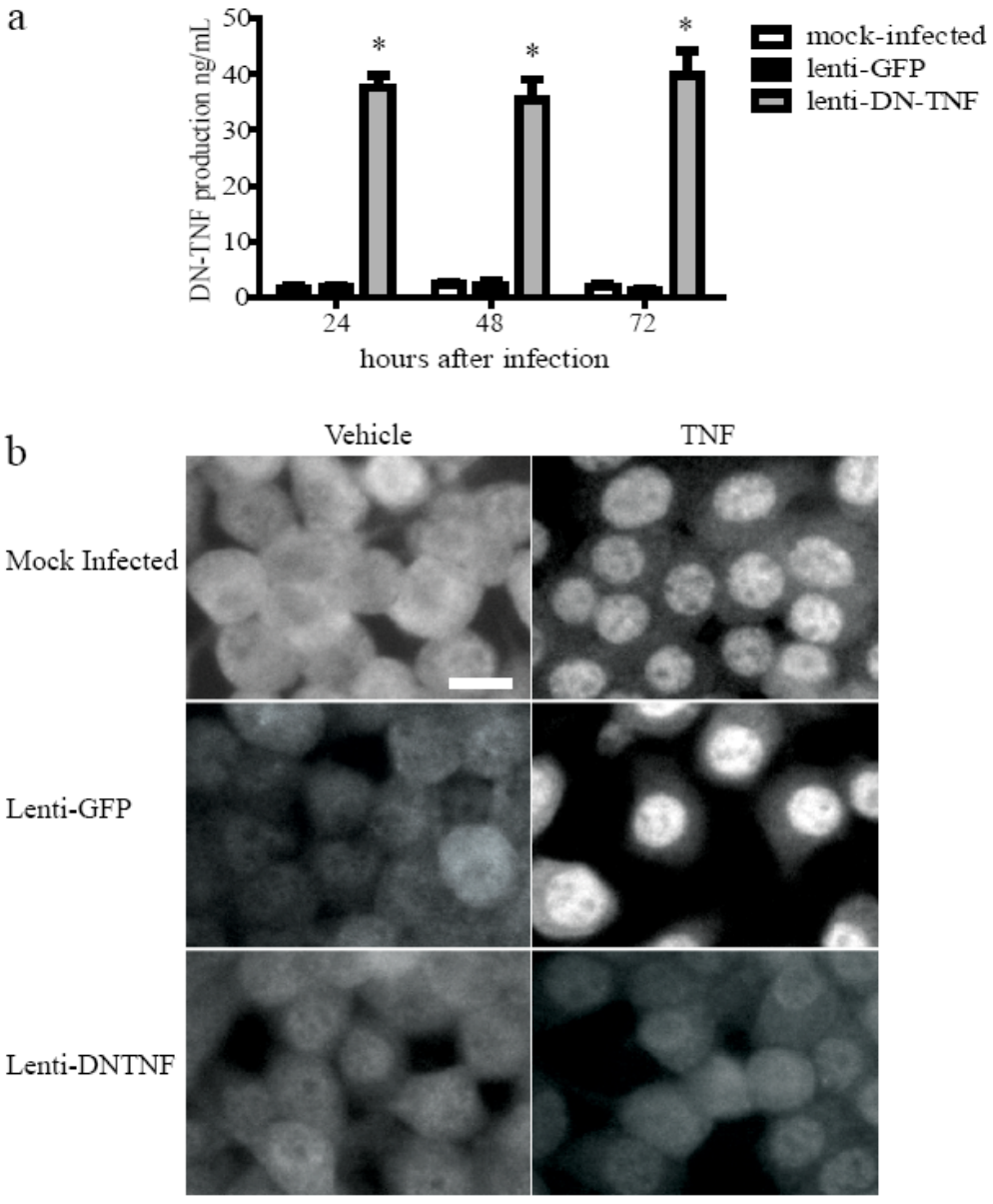

a
DN-TNF production ng/mL
50
40
30
20
10
0
24
48
72
hours after infection
*
*
*
mock-infected
lenti-GFP
lenti-DN-TNF
b
Vehicle
TNF
Mock Infected
Lenti-GFP
Lenti-DNTNF

Figure 3.2. *In vitro* transduction of MN9D dopaminergic cell cultures with lenti-DN-TNF results in the production of DN-TNF and prevents TNF-induced signaling. (a) MN9D cultures were mock-, lenti-GFP-, or lenti-DN-TNF- transduced and DN-TNF production was measured by hTNF ELISA analysis of the culture supernatants 24, 48, and 72hr after infection. Values were analyzed by one way ANOVA followed by Tukey's post hoc test, n = 3 per condition, values shown as mean + SEM, * significantly different from mock-infected cultures at $p < 0.0001$. (b) Transduction with lenti-DN-TNF but not lenti-GFP prevents nuclear translocation and enrichment of the p65RelA subunit required for activation of NFκB-dependent gene transcription in response to stimulation with soluble TNF. MN9Ds were mock-, lenti-GFP-, or lenti-DN-TNF-infected 24hr after plating. At 24hr after infection cultures were switched to 0.5% FBS containing media; 24hr later cultures were stimulated with 2ng/mL TNF for 15 minutes. After fixation cells were immunolabeled for the localization of NFκB p65RelA to assess cytoplasmic to nuclear translocation. Scale bar = 10μM.

Figure 3.3

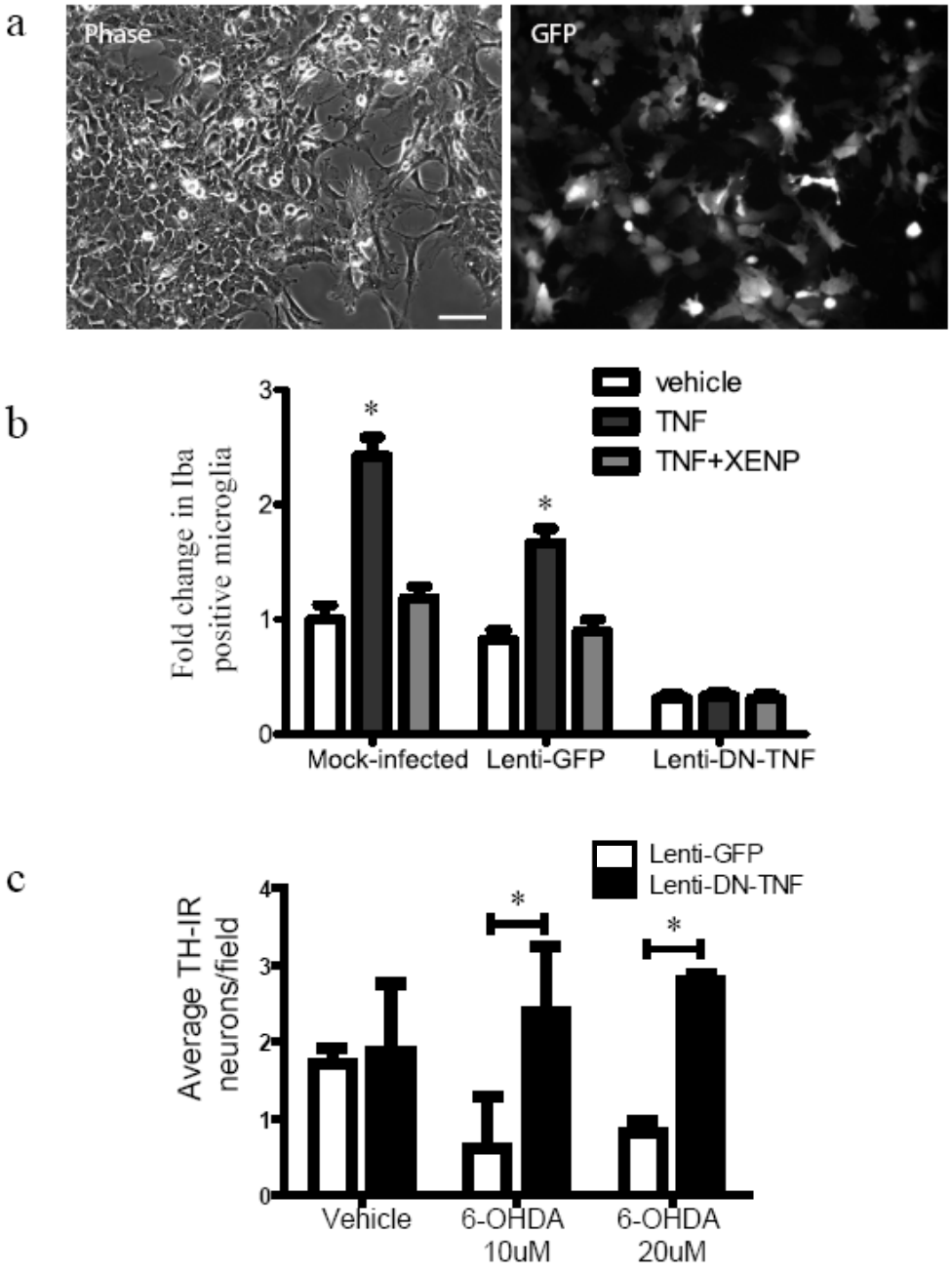

a
Phase
GFP
b
vehicle
TNF
TNF+XENP
Fold change in Iba positive microglia
0
1
2
3
*
*
Mock-infected
Lenti-GFP
Lenti-DN-TNF
c
Lenti-GFP
Lenti-DN-TNF
Average TH-IR neurons/field
0
1
2
3
4
*
*
Vehicle
6-OHDA 10uM
6-OHDA 20uM

Figure 3.3. Lentiviral-DN-TNF transduction of rat embryonic ventral midbrain results in production of bioactive DN-TNF as measured by reduced microglial activation resulting from TNF treatment and increased DA neuron survival after 6-OHDA exposure. (a) Phase and GFP fluorescence images of rat EVM cultures 72hr after lenti-GFP infection. Scale bar = 50μm. (b) EVM cultures were infected with lenti-GFP or lenti-DN-TNF. 48 hours after infection 5ng/mL TNF was added +/- 200ng/mL XENP345. 24 hours after TNF stimulation the cultures were fixed and labeled with anti-Iba1. Iba1-immunoreactive microglia were counted manually in mock-infected, lenti-GFP-infected, or lenti-DN-TNF-infected cultures. Basal microglia activation in mock-infected cultures treated with vehicle was set to 1 and other values were expressed as fold change relative to this condition. Values shown are mean Iba-immunoreactive microglia per field + SEM. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test; * significantly different from vehicle treatment a $p<0.001$. (c) The number of tyrosine hydroxylase positive dopaminergic neurons was counted to obtain an average number per well in mock-infected, lenti-GFP-infected, or lenti-DN-TNF-infected cultures treated with 6-OHDA on an average of 8 fields per well. Two wells per treatment condition were counted. Values shown are average TH immunoreactive neurons per well + SEM. Data was analyzed by one way ANOVA $p=0.05$ followed by a one-tailed Tukey's post hoc test; * significantly different from lenti-GFP infection at $\alpha < 0.05$.

Figure 3.4

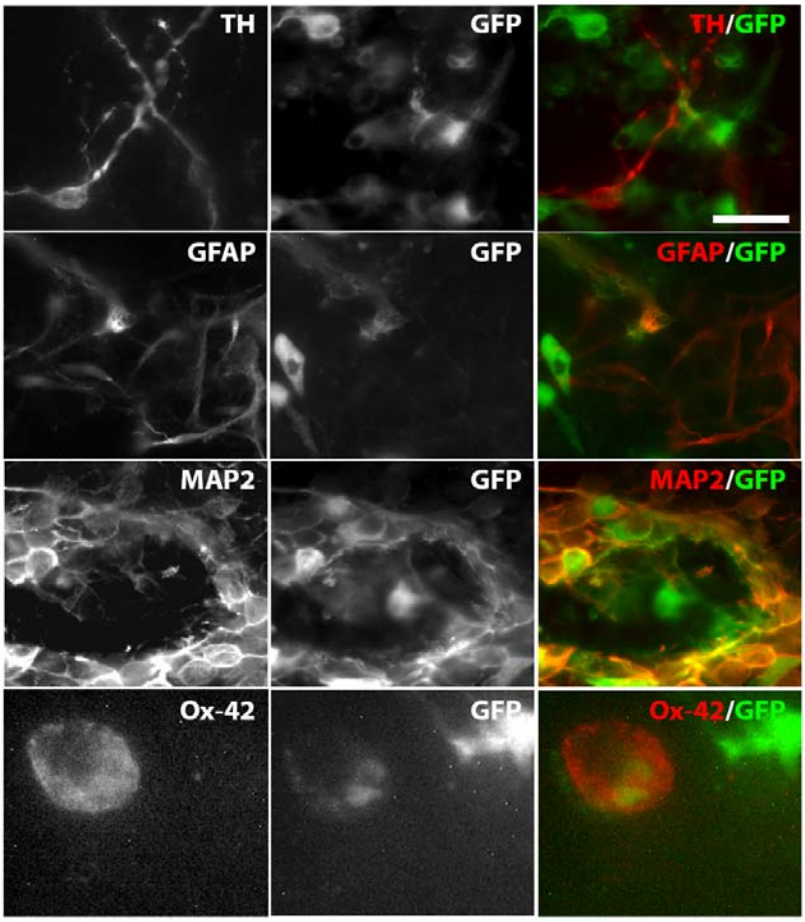


Figure 3.4. Lentiviral-GFP transduction of multiple cell types in rat embryonic ventral midbrain cultures. EVM cultures were infected with lenti-GFP and 72 hours after infection cultures were fixed and labeled with antibodies against tyrosine hydroxylase to detect dopaminergic neurons, GFAP to detect astrocytes, MAP2 to detect neurons, and OX-42 to detect microglia. Scale bar = 20μm

Figure 3.5

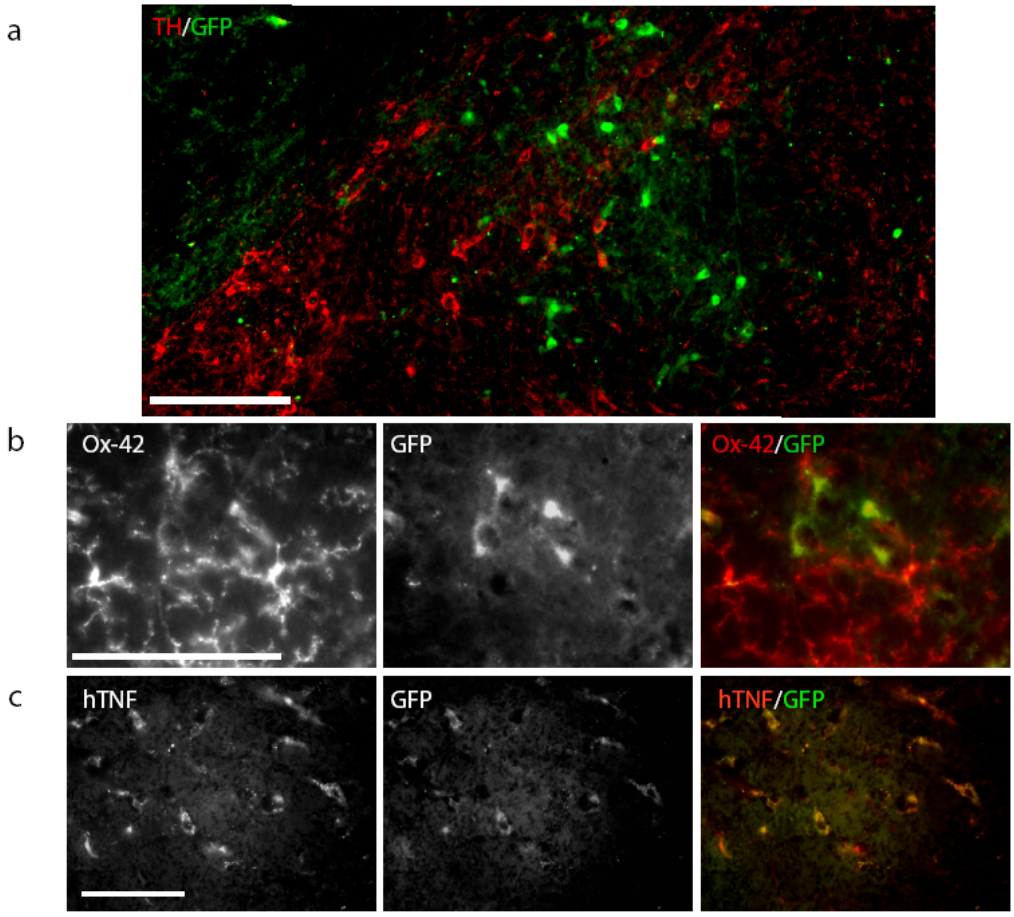
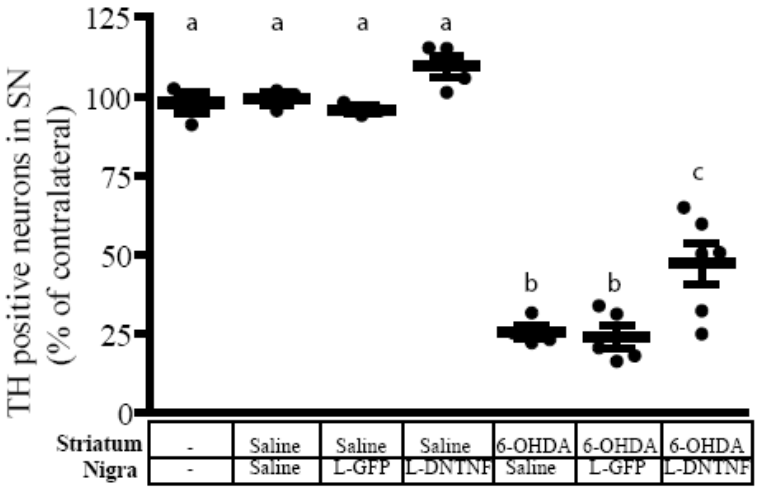


Figure 3.5. *In vivo* lentiviral transduction. Animals were stereotaxically injected with lentivirus expressing GFP (a) or DN-TNF (b,c) into the nigra. Animals were perfused three weeks after the lesion and brains were harvested for immunohistology and labeled with anti-GFP to detect lenti-infected cells, tyrosine hydroxylase (TH) to detect dopaminergic neurons, hTNF to detect DN-TNF, and OX-42 to detect microglia. Scale bars=100uM.

Figure 3.6

a


TH positive neurons in SN
(% of contralateral)
125
100
75
50
25
0
a
a
a
a
b
b
c
Striatum
Nigra
- / -
Saline / Saline
Saline / L-GFP
Saline / L-DNTNF
6-OHDA / Saline
6-OHDA / L-GFP
6-OHDA / L-DNTNF

b

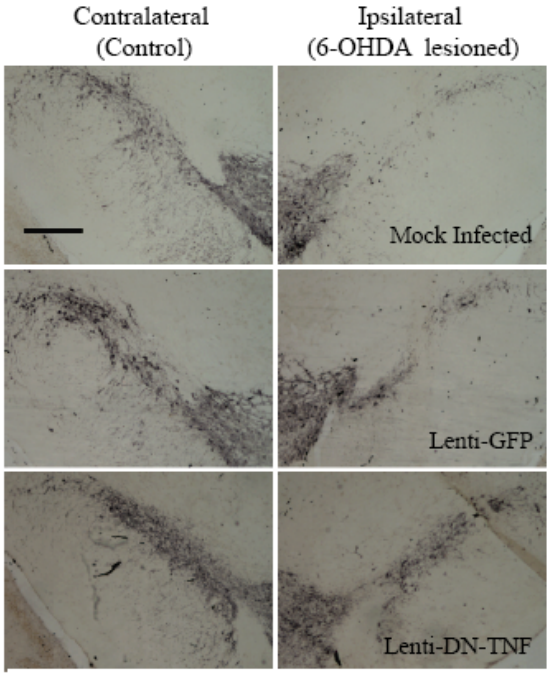

Contralateral
(Control)
Ipsilateral
(6-OHDA lesioned)
Mock Infected
Lenti-GFP
Lenti-DN-TNF

Figure 3.6. Transduction of rat midbrain with lenti-DN-TNF attenuates 6-OHDA-induced DA neuron loss. A unilateral striatal lesion was induced by injecting 6-OHDA into the caudate-putamen (CPu) of rats. Animals were stereotaxically injected with lentivirus expressing GFP or DN-TNF into the substantia nigra. Animals were perfused three weeks after the lesion and brains were harvested for immunohistology. (a) Stereological estimate of DA neuron number (TH/NeuN-IR cells) in SNpc after a striatal 6-OHDA lesion and transduction of the substantia nigra with lentiviral GFP or DN-TNF. 6-OHDA induced significant ($p < 0.05$) loss of DA neurons. Inhibition of TNF with lentivirus-derived DN-TNF significantly ($p < 0.05$) rescued DA neurons. For comparison of groups, data were analyzed by one-way ANOVA followed by Tukey's post hoc test for significance. Values are expressed as means ± SEM; Groups with different letters are statistically different from each other at $p < 0.05$. (b) TH-IR in nigral sections from 6-OHDA lesioned rats. Scale bar = 400μM.

Figure 3.7

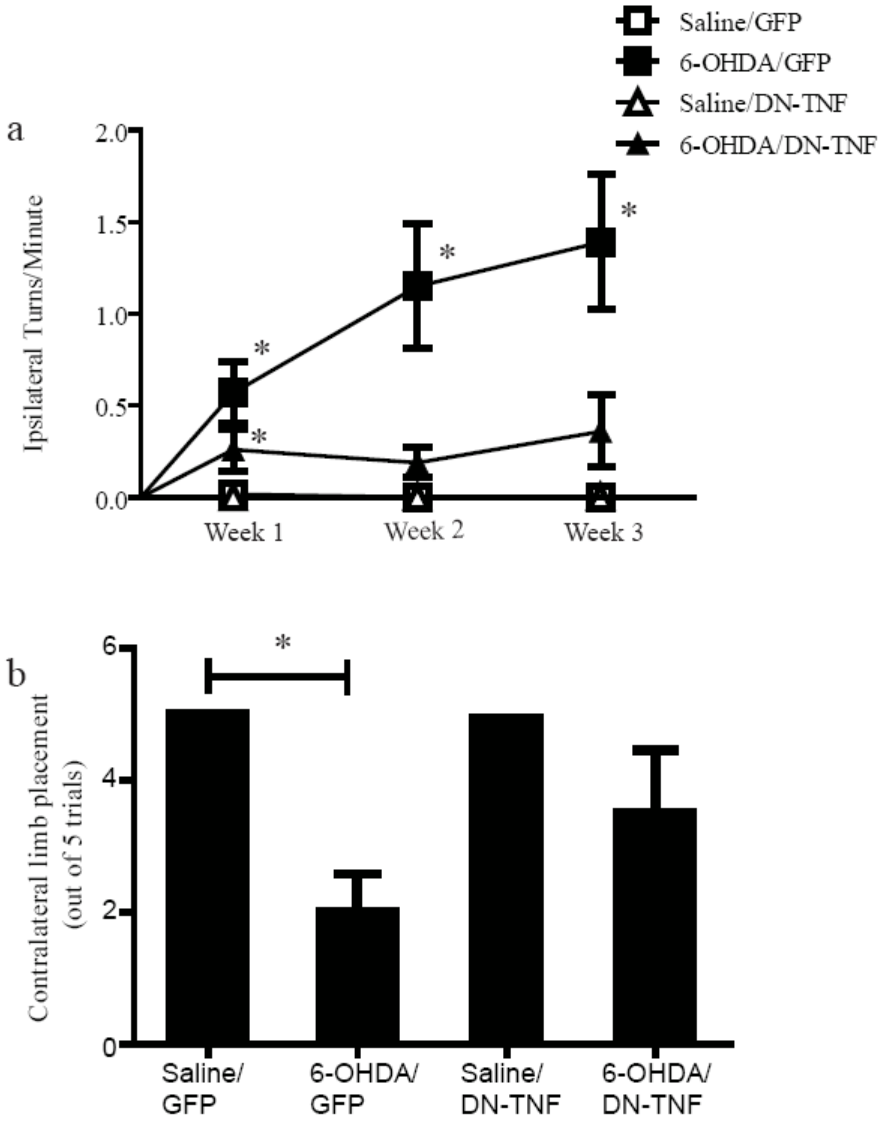

Saline/GFP
6-OHDA/GFP
Saline/DN-TNF
6-OHDA/DN-TNF
a
Ipsilateral Turns/Minute
2.0
1.5
1.0
0.5
0.0
Week 1
Week 2
Week 3
*
b
Contralateral limb placement (out of 5 trials)
6
4
2
0
Saline/ GFP
6-OHDA/ GFP
Saline/ DN-TNF
6-OHDA/ DN-TNF

Figure 3.7 Transduction of rat midbrain with lenti-DN-TNF attenuates 6-OHDA-induced behavioral deficits. (a) Measurement of amphetamine-induced rotations indicated significant attenuation of ipsiversive behavior. Values shown are mean (n = 3 for striatal saline with lenti-GFP, n = 4 for saline/DN-TNF, and n = 6 for both 6-OHDA lesioned groups), +/- SEM. One way ANOVA followed by Tukey's post hoc test, * significantly different from vehicle lesioned, lenti-GFP infected controls at $p < 0.05$. (b) The vibrissae-evoked forelimb placing test was performed weekly after rats were lesioned with 6-OHDA and virus injection. 6-OHDA lesioned animals that received lenti-DN-TNF never develop any significant deficits in forelimb placement whereas lenti-GFP injected animals display significant deficits 3 weeks after lesions. Values are plotted as contralateral limb placing out of 5 trials at three weeks after 6-OHDA lesioning; with whiskers on the right (ipsilateral) of the head being stimulated. Values plotted are means + SEM (n = 3 for saline/GFP, n = 4 for saline/DN-TNF, and n = 6 for both 6-OHDA lesioned groups) One way ANOVA followed by Tukey's post hoc test. * significantly different at $p < 0.05$.

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CHAPTER FOUR

Conclusions and Future Directions

CONCLUSIONS

The presence of inflammatory mediators at the end stages of Parkinson's disease and other neurodegenerative disorders has been recognized for some time. However it has only been appreciated recently that the occurrence of activated microglia and increased cytokine levels might indicate inflammation as an active mechanism of disease, and not simply a result of the pathologic changes that occur over the course of a chronic disease. At the time this thesis research began, the investigation into inflammation and TNF as contributors to pathology was just beginning. This thesis has contributed to the understanding of inflammation and, specifically, TNF signaling in the degeneration of dopaminergic neurons in models of PD by demonstrating that specific inhibition of soluble TNF signaling attenuates DA neuron loss resulting from chronic inflammation or treatment with an oxidative neurotoxin.

The possibility that inflammation and TNF signaling might contribute to PD pathology was first articulated in 1994, when elevated TNF levels were measured in the cerebrospinal fluid of PD patients and in the postmortem brains of PD affected individuals (Mogi et al., 1994). Shortly after the identification of increased TNF levels in PD patients, increased expression of TNF receptors by DA neurons in the SNpc of postmortem PD brains was detected; suggesting that midbrain DA neurons might be susceptible to TNF-induced signaling and inhibition of its signaling may perhaps be

neuroprotective (Boka et al., 1994). Despite the evidence that elevated levels of TNF and TNF receptors were present in the parkinsonian brain, the sensitivity of DA neurons to TNF was not investigated for some time. In 2001 it was demonstrated that dopaminergic neurons present in *in vitro* cultures of embryonic rat brain were sensitive to TNF dependent toxicity in a dose dependent manner (McGuire et al., 2001), providing experimental evidence for a role of TNF in mediating neuronal cell death. The same year it was also recognized that a cohort of patients who developed early onset Parkinson's disease were also more likely to share a polymorphism in the TNF gene promoter that resulted in elevated TNF production (Nishimura et al., 2001). Over the next several years conflicting results were published regarding the susceptibility of TNF and TNF receptor deficient mice to DA neurotoxins (Ferger et al., 2004; Leng et al., 2005; Rousselet et al., 2002; Sriram et al., 2002; Sriram et al., 2006). However the specific role of TNF was not clear from these studies due to both the conflicting results that had been obtained and to the very likely possibility of development compensation in inflammatory reactions in these mice (Pasparakis et al., 1996; Pejovic et al., 2004; Ritter et al., 2003). Further experiments which implicated, but did not authoritatively demonstrate TNF toxicity in contributing to toxin-induced DA neuron loss were performed using the non-selective TNF inhibitors minocycline and thalidomide (Ferger et al., 2004; Tomas-Camardiel et al., 2004) both of which have profound effects on neuroinflammatory processes.

Based upon the evidence of inflammation in PD and the studies that TNF is toxic to midbrain DA neurons, I hypothesized that TNF would be a critical mediator of DA neuron loss in Parkinson's disease models and that specific inhibition of soluble TNF

signaling would provide neuroprotection. I demonstrated that infusion of the recombinant dominant negative peptide XENP345 in the 6-OHDA neurotoxin model of PD reduced DA neuron loss by approximately 50% with a corollary preservation of striatal tyrosine hydroxylase immunoreactive fibers (Chapter 2; (McCoy et al., 2006)). This DA neuroprotection resulted in behavioral improvements as measured by amphetamine-induced rotation indicative of striatal DA preservation. TNF inhibition was also sufficient to attenuate DA neuron loss by approximately 50% in a second animal model of PD where DA neuron degeneration is induced by chronic infusion of LPS. In this model XENP345 infusion also resulted in reduced LPS-induced microglial activation within the midbrain. To extend these findings I used primary cultures of embryonic rat midbrain and demonstrated that delayed addition of XENP345 up to 72 hours after an LPS stimulus could still provide robust neuroprotection, but that this neuroprotection did not correlate with decreased microglial activation. The disparity between DA neuron loss and microglial activation as well as the ability of XENP345 to block LPS-induced toxicity after the neuroinflammatory response is well established and is consistent with a mechanism of direct TNF toxicity resulting from activation of TNFR1 on DA neurons, rather than a general anti-inflammatory mechanism of action. Following these results obtained by treatment with recombinant DN-TNF, I tested the ability of lentivirus derived DN-TNF to provide neuroprotection, as development of these vectors would allow flexibility in the time of DN-TNF delivery as well as allow TNF inhibition lasting longer than the two weeks obtained by chronic infusion. I demonstrated that lentiviral DN-TNF could block downstream TNF signaling in a DA cell line and that it could prevent TNF-induced microglial activation and DA neuron loss in primary embryonic rat midbrain

cultures (Chapter 3). Based on these *in vitro* results, I investigated the ability of lenti-DN-TNF transduction of rat SNpc to attenuate DA neuron loss resulting from a striatal 6-OHDA lesion. I found that lenti-DN-TNF infection of the SNpc in 6-OHDA lesioned animals could reduce DA neuron loss and could improve behavioral deficits. This validation of lentiviral DN-TNF both *in vitro* and *in vivo* provides evidence of both the feasibility and efficacy of DN-TNF gene transfer as a potential therapeutic strategy in Parkinson's disease.

FUTURE DIRECTIONS

Due to the therapeutic implications of DN-TNF mediated DA neuron rescue in models of PD, it would be of great interest to determine if delayed delivery of DN-TNF could attenuate DA neuron loss resulting from neurotoxin injection. It has been demonstrated previously that selective inhibition of COX-2 in the delayed phase of DA neuron loss in a progressive 6-OHDA model is neuroprotective (Sanchez-Pernaute et al., 2004). Since COX-2 inhibition would dampen the immune response of microglia, this latter phase of neuron loss is likely to be inflammation driven, and as TNF is a potent pro-inflammatory cytokine, I hypothesize that this delayed neuron loss is TNF-dependent. Through the use of inducible lentiviral vectors DN-TNF production could be initiated at the time of 6-OHDA lesion, at time points after DA neuron loss is underway, or DN-TNF production could be limited to the initial phase of cell death and production stopped during the progressive phase. Investigating when in the pathologic process TNF signaling is

important and how late DN-TNF therapy could be initiated after DA neuron loss has begun and still provide histologic and functional benefit would have clinical relevance as in PD patients DA neuron loss is well underway at the time of diagnosis. Currently, clinical trials are underway in which neurotrophic factors, as well as GAD and AADC, are being delivered to the striatum patients by viral transduction, and this mode of treatment has thus far been demonstrated to be safe and well tolerated.

A second area of investigation stemming from these results would be the identification of the downstream mechanisms that mediate TNF-dependent DA neuron death resulting from the oxidative neurotoxin 6-OHDA. 6-OHDA-induced toxicity is believed to result from pro-oxidative conditions (Cohen, 1984), and DN-TNF administration would not be expected to modulate the initial 6-OHDA driven oxidative stress. Although the initial phase of cellular dysfunction might not be affected by DN-TNF administration there are several downstream pathways by which TNF signaling inhibition may afford neuroprotection.

One such mechanism of TNF-induced toxicity after a 6-OHDA treatment may result from increased sensitivity of DA neurons to TNF such that a TNF stimulus which would usually not cause DA neuron dysfunction can cause cellular death in the presence of 6-OHDA. If increased TNF sensitivity of DA neurons after 6-OHDA treatment occurs it could result from 6-OHDA-induced glutathione depletion. 6-OHDA treatment results in decreased cellular glutathione (Kumar et al., 1995; Perumal et al., 1992), and it has been demonstrated that depletion of mitochondrial glutathione in hepatocytes sensitizes

these cells to a subsequent TNF stimulus (Fernandez-Checa et al., 1997). Therefore it is possible that a similar mechanism of increased susceptibility to TNF toxicity is operating in 6-OHDA treated DA neurons.

A second mechanism of TNF-dependent toxicity may result from the activation of stress-induced kinases, particularly ASK1. ASK1 can be activated by increases in oxidative stress resulting from hydrogen peroxide, 6-OHDA, or TNF treatment (Gotoh and Cooper, 1998; Ouyang and Shen, 2006). Activation of ASK1 leads to phosphorylation of p38 MAPK and sustained activation of JNK. The duration of JNK activation is crucial in determining whether downstream signaling pathways lead to cellular proliferation or pro-apoptotic signaling; persistent JNK signaling has been associated with caspase activation (Chen et al., 1996). Therefore if JNK signaling is activated through 6-OHDA-driven increases in oxidative stress any subsequent TNF signaling might be expected to further activate ASK1-mediated p38 and JNK dependent apoptosis, and thus overpower NFκB dependent pro-survival signaling.

Because oxidative stress and mitochondrial dysfunction are implicated in both 6-OHDA- and TNF-induced DA neuron loss, it is likely that these two stimuli would overwhelm the anti-oxidant capacity of the cell and act synergistically to induce mitochondrial dysfunction and activation of downstream pathways that are driven by increased oxidative stress. Given that there are many potential intersection points between 6-OHDA and TNF induced signaling, the possibility that several downstream pathways converge to enhance cellular dysfunction and death is very possible.

Considering that the mechanisms of both toxin-induced cell loss and sensitization by genetic mutation have consistently been linked to mitochondrial dysfunction and oxidative stress, TNF signaling is likely to be an important mediator in DA neuron loss resulting from many factors. Therefore targeting TNF signaling may be beneficial to protect DA neurons from a variety of pathologic insults.

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APPENDIX A

Autologous transplants of Adipose-Derived Adult Stromal (ADAS) cells afford dopaminergic neuroprotection in a model of Parkinson's disease

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ABSTRACT

Adult adipose contains stromal progenitor cells with neurogenic potential. However, the stability of neuronal phenotypes adopted by Adipose-Derived Adult Stromal (ADAS) cells and whether terminal neuronal differentiation is required for their consideration as alternatives in cell replacement strategies to treat neurological disorders are largely unknown. We investigated whether *in vitro* neural induction of ADAS cells determined their ability to neuroprotect or restore function in a lesioned dopaminergic pathway. *In vitro*-expanded naïve or differentiated ADAS cells were autologously transplanted into substantia nigra 1-week after an intrastriatal 6-hydroxydopamine injection. Neurochemical and behavioral measures demonstrated neuroprotective effects of both ADAS grafts against 6-hydroxydopamine-induced dopaminergic neuron death, suggesting that pre-transplantation differentiation of the cells does not determine their ability to survive or neuroprotect *in vivo*. Therefore, we investigated whether equivalent

protection by naïve and neurally-induced ADAS grafts resulted from robust *in situ* differentiation of both graft types into dopaminergic fates. Immunohistological analyses revealed that ADAS cells did not adopt dopaminergic cell fates *in situ*, consistent with the limited ability of these cells to undergo terminal differentiation into electrically active neurons *in vitro.* Moreover, re-exposure of neurally-differentiated ADAS cells to serum-containing medium *in vitro* confirmed ADAS cell phenotypic instability (plasticity). Lastly, given that gene expression analyses of *in vitro*-expanded ADAS cells revealed that both naïve and differentiated ADAS cells express potent dopaminergic survival factors, ADAS transplants may have exerted neuroprotective effects by production of trophic factors at the lesion site. ADAS cells may be ideal for *ex vivo* gene transfer therapies in Parkinson's disease treatment.

INTRODUCTION

Because central nervous system tissue has limited capacity for intrinsic repair after injury, cell replacement strategies represent an attractive approach for neurorestorative medicine (Weissman, 2000). Endogenous neural stem cells within certain regions of the adult brain can be proliferated *in vitro* (Gage, 2000; Gage et al., 1995). However, regulation of their growth and proliferation potential *in vivo* remains a major problem (Belmadani et al., 2006; Conti et al., 2006; Hermann et al., 2004b; Miller, 2006). Similarly, widespread use of fetal tissue or embryonic stem cells in cell replacement therapies has been limited by histocompatibility issues, availability, and ethical concerns prompting the search for alternative cell sources (Frankel, 2000; Sonntag and Sanchez-

Pernaute, 2006). Of particular interest are other adult tissues with progenitor populations that could be easily harvested, expanded, and used in autologous transplantation strategies to protect vulnerable neuronal populations and/or to accelerate repair after neural injury or neurodegeneration without the need for immunosuppression. Subpopulations of cells residing within adult liver (Alison and Sarraf, 1998), intestine (Potten, 1998), skin/hair-follicles (Amoh et al., 2005; Fernandes et al., 2004), and bone marrow (Jiang et al., 2002a; Jiang et al., 2002b) express neuroectodermal or neural crest cell markers *in vitro* and/or *in vivo*. Similarly, adult bone marrow-derived mesodermal stromal cells (MSC) display neurogenic properties (Bonilla et al., 2005; Bossolasco et al., 2005; Dezawa et al., 2004; Hermann et al., 2004a; Hermann et al., 2006; Jiang et al., 2002a; Jiang et al., 2002b; Woodbury et al., 2002; Woodbury et al., 2000) including the ability to fire action potentials and respond to neurotransmitters, including GABA, glycine, and glutamate (Wislet-Gendebien et al., 2005).

In recent years, adult adipose was shown by several laboratories (reviewed in (Schaffler and Buchler, 2007) to be a source of multipotent cells from which to derive progenitors such as chondrocytes, adipocytes, osteoblasts, and myocytes for tissue engineering and repair of mesodermal or mesenchymal-derived tissues (Tholpady et al., 2003; Zuk et al., 2002; Zuk et al., 2001) while others reported adipose may also have sub-populations of cells with neurogenic potential *in vitro* (Ashjian et al., 2003; Fujimura et al., 2005; Kang et al., 2004; Ning et al., 2006; Safford et al., 2002; Safford et al., 2004). The term Adipose-Derived Adult Stem (ADAS) cells was the original term used to refer to these cells on the basis of their potential for multi-lineage specification (Safford et al.,

2002). However, strict criteria for 'stemness' has not been met conclusively (Easterday et al., 2003; Lakshmipathy and Verfaillie, 2005; Weissman et al., 2001). Therefore, the preferred term for these cells, and the one which we use in our study, is Adipose-Derived Adult Stromal (ADAS) cells (Safford et al., 2004). Despite significant progress in characterization of cell surface markers for ADAS cells (Guilak et al., 2006; Izadpanah et al., 2006), therapeutic benefit derived from transplantation of ADAS cells has yet to be demonstrated in animal models of neurodegeneration.

The primary goal of these studies was to investigate the extent to which autologous grafts of naïve or neurally-induced rat ADAS cells protect, repair, or restore function of the nigrostriatal pathway in a neurotoxin model of PD. Neurochemical and behavioral measures confirmed the neuroprotective effects of both kinds of autologous ADAS cell grafts against 6-OHDA-induced dopaminergic neuron death. On the basis of neurohistological, cell biological, electrophysiological, and gene expression studies, we concluded that the mechanism by which ADAS cell grafts contributed to improved nigrostriatal function does not involve stable differentiation of ADAS cells into functional dopaminergic neurons. Our findings suggest that modulation of the oxidative stress-induced neuroinflammatory environment in the lesioned substantia nigra and/or ADAS-derived production of growth factors known to promote dopaminergic survival and neuroprotection at the lesion site may have contributed to the therapeutic effects of ADAS cell transplants.

MATERIALS AND METHODS

Materials

Animals

All animal procedures were approved by the Institutional Care and Use Committee at UT Southwestern. Animals were housed in a climate controlled facility staffed with certified veterinarians.

Real-time Quantitative PCR .and RT-PCR Primers:

Rat primers sequences are as follows:

Cyclophilin forward: 5'-CCC TGA AGG ATG TGA TCA TTG-3'

Cyclophilin reverse: 5'-GGA AAA GGG TTT CTC CAC TT-3'

Nestin forward: 5'-CAA GTG CCC CCG GTA CTG-3'

Nestin reverse: 5'-TCA GCA AAC CCA TCA GAT TCC-3'

GFAP forward: 5'-TGG CCA CCA GTA ACA TGC A-3'

GFAP reverse: 5'-CAA ACT TGG ACC GAT ACC ACT CT-3'

NeuroD forward: 5'-CCC AGA GGC AGC CAA GTC-3'

NeuroD reverse: 5'-AGC CTT TAG TAA AAC AAT TGA ATG TCT AG-3'

S100beta forward: 5'-ATC AAC AAC GAG CTC TCT CAC TTC-3'

S100beta reverse: 5'-CAC TTC CTG CTC TTT GAT TTC CT-3'

Tuj-1/β-tubulin III forward: 5'-GAG GCC TCC TCT CAC AAG TAT GT-3'

Tuj-1/β-tubulin III reverse: 5'-ACG CTG TCC ATG GTT CCA-3'

VE cadherin forward: 5'-CAC GAC AAT ACC GCC AAC A-3'

VE cadherin reverse: 5’-AAC TTG GTA TGC TCC CGA TTA AA-3’

Persephin forward: 5’-GAC CTG GAA GCC CCA TCA- 3’

Persephin reverse: 5’-GCC GGC ACA AAC CAG GTA- 3’

Artemin forward: 5’-TTG GAG AC CTA CTG CAT TGT C- 3’

Artemin reverse: 5’-CAG CTA GGG TTG GCC ACA AG- 3’

Neurturin forward: 5’-CAG CGG AGG CGC GTG CGC AGA GA -3’

Neurturin reverse: 5’- CGG CTG TGC ACG TCC AGG AAG GA-3’

α-actin forward: 5’-TGT AAG GCG GGC TTT GCT-3’

α-actin reverse: 5’-CCC ACG ATG GAT GGG AAA -3’

Tyrosine hydroxylase forward: 5’-TGT TGG CTG ACC GCA CAT T- 3’

Tyrosine hydroxylase reverse: 5’-GCC CCC AGA GAT GCA AGT C- 3’

Nerve growth factor forward: 5’-CTC TGA GGT GCA TAG CGT AAT GTC- 3’

Nerve growth factor reverse: 5’-AAA ACG CTG TGA GAG TGT AGA AC- 3’

Neuron Specific Enolase forward: 5’-GCT TTG CCC CCA ATA TCC T-3’

Neuron Specific Enolase reverse 5’-CCT TGT CAA TGG CTT CCT TCA-3’

Choline acetyltransferase forward: 5’-AGC CAA TCG CTG GTA TGA CA-3’

Choline acetyltransferase reverse: 5’-CAC CGC AGG TGC CAT CT-3’

Smooth muscle alpha actin forward: 5’-TGT AAG GCG GGC TTT GCT- 3’

Smooth muscle alpha actin reverse: 5’-CCC ACG ATG GAT GGG AAA-3’

Platelet derived growth factor forward: 5’-AAT GAC CAC GGC GAT GAG A- 3’

Platelet derived growth factor reverse: 5’-TCT TCC AGT GTT TCC AGC AGC- 3’

Ciliary neurotrophic factor forward: 5’ -CTG GCT AGC AAG GAA GAT TCG- 3’

Ciliary neurotrophic factor reverse: 5’-CAG GCC CTG ATG TTT TAC ATA AGA -3’

Brain-derived neurotrophic factor forward: 5'-CGC ACC TCT TTA GGC ATC CT -3'

Brain-derived neurotrophic factor reverse: 5'-TCC CGG ATG AAA GTC ACT -3'

Vascular endothelial growth factor forward: 5'-AAC GAA AGC CGA AGA AAT CC- 3'

Vascular endothelial growth factor reverse: 5'-CGC TCT GAA CAA GGC TCA CA- 3'

Glial-derived neurotrophic factor forward: 5'-CTC CAA TAT GCC CGA AGA TTA TC- 3'

Glial-derived neurotrophic factor reverse: 5'-AGT CTT TTG ATG GTG GCT TGA A -3'

METHODS

Survival surgeries to harvest adipose tissue.

For survival surgeries, adult female Sprague-Dawley rats (200-225g) were anesthetized with 2% halothane and a 0.75cm^2 portion of the interscapular fat pad was excised during a survival surgery when a unilateral striatal 6-hydroxydopamine injection was performed. The adipose tissue was rinsed in cold sterile PBS (Ca/Mg Free, Invitrogen Inc., Carlsbad, CA) and incubated for 1 hr in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and antibiotic/antimycotic (Invitrogen) prior to dissociation. Fat pads were mechanically triturated then enzymatically digested in collagenase (Invitrogen) for 1 hr at 37 degrees C followed by mechanical dissociation to yield a stromal cell suspension which was collected by centrifugation (x1000g). The cell pellet was resuspended in DMEM

supplemented with 10% FBS (D10 medium) and plated (Day 0, P0) onto 35mm tissue culture-treated dishes.

Expansion and Neural induction of ADAS cells for Immunocytochemical Analyses. Non-adherent cells in P0 cultures were removed 24h post-plating in order to expand the small number of adherent stromal cells (< 10% of total cells plated) by serial passage. Media was replenished every 3 days and cultures were serially passaged at 75% confluence at a 1:4 ratio. Differentiation of cultures (P2 to P4) consisted of pre-induction of cultures plated in D10 with 10ng/ml EGF (R&D Systems, Minneapolis, MN) and 20ng/ml FGF-2 (R&D) for 2 to 3 days prior to a 4-day exposure to either of the following: Neural Differentiation Medium [NDM: D10, 120uM indomethacin (Sigma-Aldrich, St. Louis, MO), 3ug/mL insulin (Sigma-Aldrich), 300uM isobutyl-methylxanthine (Sigma-Aldrich)] or N2-supplemented/VPA [N2/VPA: DMEM, N2 supplement (Invitrogen), 0.5mM valproic acid (Sigma-Aldrich)] to induce gradual neuronal differentiation and outgrowth of processes. Alternatively, differentiation was achieved by pre-induction of cultures grown in D10 by exposure to 10ng/ml EGF + 20ng/ml FGF-2 (E/F) in NS-A Proliferation medium (Stem Cell Technologies, Vancouver, BC) for 3 days followed by 4-day differentiation in NS-A Differentiation supplement (Stem Cell Technologies). Where indicated, specific differentiation factors [1uM all-trans retinoic acid, 1-10uM forskolin, 10-50ng/ml Glial cell-derived neurotrophic factor (GDNF) or 10ng/ml Brain-Derived Neurotrophic Factor (BDNF)] were used to investigate their effects on process outgrowth of ADAS cells.

Immunocytochemical detection of neural and glial markers.

Cells in culture were fixed in 4% paraformaldehyde (PFA) for 20 minutes prior to blocking/permeabilization for immunocytochemistry with antibodies to Nestin (BD Pharmingen, San Diego, CA), vimentin (Chemicon, Temecula, CA), Fibronectin (Sigma-Aldrich), Neuron Specific Enolase (Polysciences Inc., Warrington, PA), VE cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), Neuro D (Santa Cruz), Tubulin beta III/Tuj1 (Covance, Princeton, NJ), MAP2b (Chemicon), NeuN (Chemicon), Gamma-Amino Butyric Acid or GABA (Chemicon), TrkB (Chemicon), Choline Acetyl Transferase or ChAT (Chemicon), tyrosine hydroxylase or TH (Chemicon), smooth muscle actin or SMA (Sigma-Aldrich), CD11b or OX-42 (Santa Cruz), Glial Fibrillary Acidic Protein or GFAP (Chemicon), oligodendrocyte marker O4 (R&D), the Schwann cell marker S100β (Sigma-Aldrich), low-affinity neurotrophic factor receptor p75NTR (Chemicon), and myelin basic protein or MBP (Chemicon). Detection of desired antigens was achieved through use of the appropriate secondary antibody conjugated to Alexa-488 or Alexa-594 diluted 1:1000 (Invitrogen). IgG or IgM control sera were used at the same concentration as the corresponding primary antibodies to establish the specificity of staining. Total cell nuclei were visualized with the nuclear dye Hoechst 33258 (bis-benzimide). A CoolSnap ES (monochrome) camera mounted on an upright Olympus BX61 or an inverted Olympus CK40 fluorescence scope and MetaMorph software were used for image capture and analyses.

Survival surgeries for fat pad harvest and intrastriatal 6-OHDA injection.

6-OHDA lesions were performed as described previously (McCoy et al., 2006). Briefly, young adult female Harlan Sprague Dawley SASCO rats (200-225g) (n= 4 or 5 per group) were anesthetized with halothane (2%) and placed in a stereotaxic head-holder frame with the incisor bar set at -2.5 mm as per the rat stereotaxic atlas (Paxinos, 1998). Burr holes were drilled at stereotaxic coordinates (AP): -1.2mm from bregma, mediolateral in right hemisphere (ML): -3.9mm; and dorsoventral (DV): -5mm below surface of dura) (Paxinos et al., 1985) to perform unilateral injection of 20μg 6-OHDA (4μL of 5μg/μL in ascorbic acid, Sigma-Aldrich) at a rate of 1μL/min into the striatum with a 5uL Hamilton micro syringe and a 30 gauge needle. Fat pads from the interscapular region were also harvested from all experimental animals at this time (including rats that were to receive a sham-transplant) for *in vitro* expansion of ADAS to passage 2 (P2) as described above. This unilateral intrastriatal 6-OHDA injection was chosen to induce a moderate retrograde lesion in the nigrostriatal pathway resulting in the loss of approximately 65-70% of TH-positive somata in the SNpc three weeks after the lesion (Kirik et al., 1998; McCoy et al., 2006). Seven days after 6-OHDA lesion, drug-induced rotational behavior testing was conducted to establish a baseline for locomotor behavior just prior to cell transplantation.

Rotational behavior analyses.

The extent of the retrograde nigrostriatal lesion was physiologically characterized using amphetamine-induced rotational behavior testing (Ungerstedt and Arbuthnott, 1970). Animals received 2.5mg/kg D-amphetamine (Sigma, St. Louis) i.p. and were subsequently placed in a glass cylinder (diameter 24.5cm) to monitor rotational

asymmetry for 20 min. Drug-induced rotational behavior was measured prior to the transplant (one week post 6-OHDA injection) as well as at 1, 2, and 3 weeks post-transplantation to assess effects of the ADAS grafts. Rotation towards the lesion (ipsilateral) was scored as positive and net rotational asymmetry score was expressed as full body turns.

Stereotaxic transplantation of MTR-labeled ADAS grafts.

One week after intrastriatal 6-OHDA or saline injection and fat pad harvesting, cell transplants were performed. On the day of transplantation, passage 2 ADAS were labeled with 95-100% efficiency with the fixable mitochondrial dye CMH_2Xros MitoTracker Red (MTR) which fluoresces in a membrane potential-dependent manner (Poot et al., 1996). After MTR loading, the cells were rinsed and resuspended at a density of 4×10^6 per mL in order to deliver approximately 40,000 cells (4ul of 10,000/ul) per animal into the substantia nigra in the hemisphere ipsilateral to the 6-OHDA- or saline-injected striatum via stereotaxic injection using a 10ul Hamilton microsyringe with a 30 gauge needle at rate of 1uL/min via automated infusion pump using the following stereotaxic coordinates: AP -5.3mm, ML -2.4mm, DV -6.0mm below surface of dura; incisor bar at -2.5mm. Lesioned rats received E/F treated naïve ADAS (n = 5), NDM differentiated ADAS (n = 5), or saline (n = 4). A control (unlesioned) group of rats (n = 4) was also included.

Perfusion and tissue processing for histology.

Animals were deeply anesthetized with Euthasol and intracardially perfused as described previously (Kirik et al., 1998; McCoy et al., 2006) four weeks after autologous

transplantation (i.e. five weeks post lesion). Brains were then removed from the skull and post-fixed for 24 h in the same PFA solution, cryoprotected in 20% sucrose in PBS for 18-24 hr, and frozen by embedding in Tissue-tek cooled by a dry-ice/isopentane solution. Coronal sections (30µm-thickness) were cut through the striatum and substantia nigra pars compacta (SNpc) on a Leica CM1850 cryostat and mounted on glass slides (SuperFrost Plus, Fisher) for immunohistological analyses and stereological estimate of DA neuron number in SNpc.

Immunohistochemistry of brain sections.

Processing of brain sections was done as described previously (Kirik et al., 1998; McCoy et al., 2006). IgG or IgM sera were used at the same concentration as the corresponding primary antibodies to confirm the specificity of staining.

Stereological Nigral DA neuron counts

StereoInvestigator analyses software (Micro Bright Field Inc., Williston, VT) was used to perform unbiased stereological counts of TH-immunoreactive (TH-IR) cell bodies in the SNpc using the optical fractionator method (West et al., 1991) as described previously (Kirik et al., 1998; McCoy et al., 2006). The boundary of SNpc was outlined under magnification of the 4X objective as defined according to previous anatomical demarcation in the rat (German and Manaye, 1993). For analysis, the treatment of the various brain sections was blinded to the observer. Cells were counted with a 60X oil immersion objective (1.3 NA) using an Olympus BX61 microscope. Serial sections through the extent of SNpc (from AP:-3.3mm to -5.3mm behind bregma) were cut on a

Leica cryostat and placed 4 per slide (cut thickness of 30μm and mounted thickness of 22 μm) for systematic analysis of randomly placed counting frames (size 50 x 50μm) on a counting grid (size of 160μm x 120μm) and sampled using an 18μm optical dissector with 2μm upper and lower guard zones. Every other slide was stained for TH/NeuN and the intervening slide was analyzed for MitoTracker Red fluorescence. A dopaminergic neuron was defined as a TH immunoreactive cell body with a clearly visible TH-negative nucleus. An ADAS cell was defined as a MTR-positive cell. The reason for assessing *in vivo* differentiation of ADAS post transplantation was to determine if MitoTracker Red-positive cells also co-expressed a particular cell fate marker (i.e. tyrosine hydroxylase for differentiation into dopaminergic cell fate).

Quantification of striatal TH-fiber density.

Striatal optical density (OD) of TH immunostaining, determined by digital image analysis on Image Pro Plus 5.1, was used as an index of striatal density of TH innervation; densitometric measurements have been shown to provide valid relative indices of extent of fiber innervation on the same brain section of an animal receiving a unilateral 6-OHDA lesion (Burke et al., 1990). ODs were measured to estimate dopaminergic fiber innervation into the striatum. The density readings of TH-immunoreactivity in the striatum on both hemispheres of each animal were corrected for nonspecific background density, as measured on sections stained with non-immune IgG serum. Striatal images taken under a 4x objective converted to gray scale were then delineated and the intensity of staining was thus assessed for the entire striatal region (boundaries according to the rat stereotaxic atlas) of the four sections sampled for the ipsilateral and for the contralateral

striatum, subsequently averaged for each animal to obtain average optical density (AOD). The data are expressed as percent fiber density of the ipsilateral (experimental) side relative to the contralateral (control) side. Values expressed are group means $\pm$ S.D. Values were compared by one-way ANOVA followed by Tukey-Kramer HSD post-hoc test. Groups with different letters are significantly different at $p<0.05$.

Quantification of microglial burden.

To estimate the microglial burden in the SNpc, optical density of the OX-42 immunoreactivity in the SNpc was obtained by digital image analysis using the Image-Pro Plus 5.1. The density readings were corrected for nonspecific background density as measured on sections stained with non-immune IgG serum. SNpc images taken under a 4x objective converted to gray scale were then delineated and the intensity of staining was thus assessed for the entire SNpc region (boundaries according to the rat stereotaxic atlas) of the six sections sampled throughout the extent of SNpc, subsequently averaged for each animal. The data was expressed as integrated optical density (IOD). All data are expressed as mean ± S.E.M. Inter-group differences between the various dependent variables were assessed using one-way ANOVA, followed by the Tukey's post hoc multiple comparisons test. Data obtained were analyzed by GraphPad statistic software; $p < 0.05$ were considered significant.

Assessment of Phenotypic Stability of ADAS cells in vitro.

Cells were proliferated in base media (D10) to passage 2 or 3 followed by supplementation with 10ng/mL EGF and 20ng/mL FGF-2 (E/F) for 2-3 days.

Differentiation was induced by mitogen withdrawal and exposure to the indicated medium formulations (NDM or N2/VPA). Following a period of 7 days of differentiation, the cells were returned to D10 medium for 24 or 48 hrs prior to harvesting for marker analyses by immunocytochemistry. A qualitative survey of immunoreactivity for each protein marker was performed at high (40X) and low (10X) magnification and scores between 1 and 4 were given based on fluorescence intensity and fraction of total cells expressing that particular marker according to the following scale: 4 = intense immunoreactivity in many (> 50 %) of the cells, 3 = strong staining in ~ 50% of the cells, 2 = detectable staining in less than 50% of the cells, 1 = detectable staining in a small fraction (< 10%) of the cells, 0 = no detectable immunoreactivity.

Real-time Quantitative PCR.

Quantitative real-time PCR was performed as previously described (Kurrasch et al., 2004). Briefly, total RNA was isolated with RNAStat60 (Tel-Test Inc., Friendswood, TX) from cultured cells or rat tissues, treated with DNase I, and reverse transcribed using Superscript II RNase H- reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using an ABI Prism 7900HT Detection System (Applied Biosystems, Foster City, CA). Each reaction was performed in 384-well plate format in a volume of 10ul that contained 3.5ng cDNA, 7ul SYBR green PCR Master Mix, and 200nM of each PCR primer. All reactions were performed in duplicate. Relative mRNA abundance for each sample were normalized to those of cyclophilin.

RT-PCR:

Total RNA was isolated with RNA STAT60 from cultured cells or rat brain, treated with DNase I, and reverse transcribed using Superscript II RNase H- reverse transcriptase. Each PCR reaction was performed in a reaction volume of 10uL that contained 5uL GoTaq green (Sigma-Aldrich), 180nM of each PCR primer, and cDNA was added at 20, 4.0, or 0.8pg per reaction. The reaction conditions were as follows: initial denaturation at 95 ºC for 5 min and 3 cycles of amplification (95 ºC for 30 s, 60 ºC for 30 s and72 ºC for 60 s), followed by 35 cycles of amplification (94 ºC for 30 s, 57 ºC for 60s and 72 ºC for 60 s), followed by a final extension step for 10 min at 72 ºC. The PCR reaction products were separated by electrophoresis in a 2.0% agarose gel and stained with ethidium bromide. Relative mRNA abundance for each sample was determined by normalizing the optical density of NTN to that of alpha actin. Total optical densities were quantified using ChemiImager software (Alpha Innotech , San Leandro, CA).

Multi-Electrode Arrays (MEAs).

ADAS cells were passaged to P2 plated into D10 on poly-D-lysine coated multielectrode arrays to record any spontaneous or evoked neural network activity as described previously (Keefer et al., 2001; Mistry et al., 2002). Cells were proliferated in D10 supplemented with 10ng/mL EGF and 20ng/mL FGF-2 for 2 days then differentiated by mitogen withdrawal and exposure to the indicated differentiation conditions (NDM or N2/VPA) for 5 days. Application of the excitatory neurotransmitter glutamate (5μM), the GABA A receptor antagonist bicuculline (20μM), or trains of electrical stimulation (0.75 V biphasic pulses, trains of 10 pulses repeated 5 times with 30 seconds between pulse trains) were performed to evoke neural network activity.

Statistical Analyses.

Intergroup differences among the means between the various dependent variables were analyzed using one-way ANOVA. When ANOVA showed significant differences, comparisons between means were tested by Tukey's multiple-comparison post hoc test (Graph Pad Prism, San Diego, CA). Values expressed are the group means $\pm$ S.E.M or group means $\pm$ S.D. as indicated. Groups represented by histogram bars labeled with different letters are significantly different at $p < 0.05$. Groups with asterisks (*) are significantly different from sham-transplanted group at $p < 0.05$.

RESULTS

Morphological differentiation and expression of neuro-glial markers in naïve and differentiated ADAS cells *in vitro.*

The initial selection of the adipose stromal cell population at P0 was accomplished by retaining a relatively small fraction of adherent cells (<10%) and discarding non-adherent cells 24 hrs after plating. In agreement with previous reports, we were able to grow and expand flat and fibroblast-like adipose-derived adult stromal (ADAS) *in vitro* with high efficiency for a number of passages (~P15). Pre-exposure to a mitogenic cocktail consisting of EGF/FGF-2 promoted expansion of a spindle-shaped putative progenitor pool within the heterogenous stromal cell population. To induce differentiation, the mitogenic cocktail was withdrawn and cells were exposed to serum-containing Neural Differentiation Medium (NDM) or to the histone deacetylase inhibitor valproic acid

(VPA) in N2-supplemented serum free medium. The latter was chosen because exposure to VPA/N2 *in vitro* has been shown to induce neuronal differentiation of adult hippocampal neural progenitors via expression of the neurogenic basic helix-loop-helix transcription factor NeuroD (Hsieh et al., 2004). Under these treatment conditions, a large fraction (>50% of total cells) of the cells in ADAS cultures pre-induced with EGF/FGF-2 for 24 hrs displayed bipolar and multipolar morphologies with extensive process outgrowth (Figures A.1 and A.7) appearing by 12 hrs compared to cells not pre-induced with the mitogenic cocktail (5% of total cells) in agreement with morphological differentiation described by others (Ashjian et al., 2003; Safford et al., 2004).

Prior to embarking on transplantation studies, we first established the extent to which the morphological differentiation of ADAS cells into neuron-like cells was coincident with expression of cellular markers of differentiation. Two-day treatment with EGF/FGF-2 induced expression of markers characteristic of early neural progenitors (including nestin, vimentin, and glial fibrillary acidic protein) in a significant fraction of the cells (Table A.1). Molecular markers denoting early commitment to neuronal fates such as β-tubulin III/Tuj1 and neuron specific enolase (NSE) were detectable in a significant fraction of cells only after exposure to NDM or N2/VPA (Figure 1, Table A.1, Figure A.2). Molecular markers of mature neurons, including neuronal N antigen (NeuN), microtubule-associated protein-2 (MAP2), TrkB, tyrosine hydroxylase (TH), choline acetyltransferase (ChAT), and gamma amino butyric acid (GABA) were detected with low frequency in a small population of the cells treated with either differentiation medium and supplemented with factors such as brain-derived neurotrophic factor

(BDNF) or glial-derived neurotrophic factor (GDNF) and did not increase further with additional time (7-10 days) in culture (Figure A.2). Lastly, expression of Smooth Muscle Actin (SMA) and the endothelial cell marker VE cadherin was detectable in a subpopulation of the cells after proliferation in EGF/FGF-2 and after NDM treatment (Table A.1, Figure A.2). As indicated in Table A.1, mRNA expression of several of these protein markers was confirmed by real-time quantitative PCR analysis (data not shown). These findings are in agreement with reports made for adipose-derived stromal cells from humans, rodents, and non-human primates (Ashjian et al., 2003; Fujimura et al., 2005; Ning et al., 2006; Romanov et al., 2005; Safford et al., 2002; Safford et al., 2004; Zuk et al., 2002).

Autologous ADAS cell grafts attenuate 6-OHDA-induced nigrostriatal pathway degeneration and behavioral deficits.

Given that ADAS cells can be coaxed to display morphological, molecular, and cellular markers characteristic of neuroblasts in response to two different neuritizing cocktails, the critical question is whether neural induction of ADAS *in vitro* prior to transplantation is required to derive therapeutic benefit from autologous ADAS grafts in a rat model of Parkinson's disease. To ensure that ADAS cells could be tracked after autologous transplantation to assess ADAS engraftment and neuronal differentiation in brain sections upon completion of the study, we conducted pilot experiments to select a stable cell labeling method that did not compromise cell viability. ADAS cells were harvested from adult rats, expanded *in vitro* to passage 2 with EGF/FGF-2, and pre-labeled with the

fixable mitochondrial-specific probe CMX-H2 MitoTracker Red (MTR) before prior to transplantation. We found that ADAS transplants were able to survive, engraft, and retain MTR fluorescence in unlesioned animals for at least three weeks (Figure A.3). Importantly, because MTR fluorescence in cells requires an intact mitochondrial membrane potential, this cell labeling method enabled us to track and identify *in situ* only those cells which were viable and healthy. Labeling of ADAS cells with a lentivirus encoding Green Fluorescent Protein (GFP) confirmed these findings but was not chosen for these studies to avoid the potential toxicity of GFP overexpression. In addition, GFP fluorescence does not depend on the viability of the cells and could have confounded interpretation of survival and engraftment studies.

Next, we investigated the ability of autologous ADAS cell transplants to protect, repair or restore function in a model of PD. We chose a model in which there is progressive degeneration of the nigrostriatal pathway for at least three weeks following a neurotoxic injury induced by striatal injection of 6-OHDA (Kirik et al., 1998). Autologous transplants of naïve or differentiated (NDM-treated) ADAS cells were injected into the rostral midbrain one week after striatal 6-OHDA admistration while the lesion was still progressing. The extent of the nigrostriatal lesion was evaluated prior to transplantation and weekly thereafter using a standard amphetamine-induced rotational behavior test. Quantitative stereological analyses of TH-positive neuron number in SNpc indicated that the group of 6-OHDA-lesioned rats that received nigral ADAS grafts displayed significantly reduced loss of TH-immunoreactive neurons on the lesioned side 4 weeks post-transplantation compared to sham-transplanted 6-OHDA lesioned rats

(Figure A.4 a and b, Table A.2)**.** In addition, quantification of TH-positive fiber density in the striatum indicated that ADAS cell transplants spared striatal terminals; specifically, 6-OHDA-lesioned/sham transplanted animals displayed 35% of control versus 65-72% of control in 6-OHDA-lesioned/ADAS-transplanted animals (Figure A.4 c). It is difficult to ascertain with certainty whether the increased TH-immunoreactivity in the ADAS cell-transplanted animals reflects true sparing of terminals, re-growth, and/or attenuated down-regulation of TH expression induced by 6-OHDA neurotoxicity, but we speculate it may be a combination of all three processes. Importantly, neurohistological protection by ADAS cell transplants was accompanied by improvement in locomotor deficits. Behavioral analyses indicated that 6-OHDA-lesioned rats that received naïve or differentiated ADAS cell transplants displayed significantly attenuated amphetamine-induced rotational behavior compared to sham-transplanted rats that received no ADAS cells (Figure A.5), suggesting that ADAS grafts prevented the progressive retrograde degeneration of the nigrostriatal pathway and contributed to striatal dopamine preservation. To our knowledge, this is the first demonstration that autologous transplantation of ADAS cells provides robust protection to rostral midbrain dopaminergic neurons against oxidative neurotoxins independent of the pre-differentiation status of the cells. Similar neuroprotective effects of ADAS cells have been reported in stroke and spinal cord injury models using rat, non-human primate, and human adipose-derived adult stromal cell populations (Kang et al., 2003b; Kang et al., 2006; Tansey, 2005).

ADAS cells survive after transplantation but do not differentiate into dopaminergic neurons.

The equivalent degree of nigral dopaminergic neuron protection achieved by transplantation of either naïve or NDM differentiated ADAS into hemi-parkinsonian rats suggested that the pre-transplantation differentiation status of the cells did not determine their ability to survive or neuroprotect *in vivo* after transplantation. This observation led us to hypothesize that either both types of grafts had undergone robust differentiation into dopaminergic fates *in vivo* and partially replaced the lost nigral dopaminergic neurons; or alternatively, that the neurally induced ADAS cells failed to maintain the neural phenotypes *in vivo* but afforded neuroprotection to the same extent as the naïve ADAS cells, implying that a mechanism other than cell replacement had contributed to functional recovery in the hemi-parkinsonian rats. To distinguish between these two mutually exclusive possibilities, we performed immunohistological analyses of midbrain sections. Four weeks after transplantation, examination of brain cryosections revealed the presence of MTR-labeled ADAS cells in close proximity to TH-positive neurons in substantia nigra; however, co-expression of TH (or any other neural markers) and MTR was not detected (Figure A.6 a-c). On the basis of these findings we concluded that neuroprotection by ADAS cells was achieved without them adopting dopaminergic neuron fates *in vivo.*

ADAS cell transplants attenuate microglial activation in SNpc of 6-OHDA-lesioned animals.

Because neuroinflammation has been implicated in the progressive degeneration of the nigrostriatal pathway in humans and in experimental models of PD (reviewed in (Hald and Lotharius, 2005; Hunot and Hirsch, 2003; Whitton, 2007) and previous work from our laboratory implicated microglial-derived soluble TNF as a critical mediator of nigral dopaminergic neuron loss induced by 6-OHDA (McCoy et al., 2006), we aimed to determine whether ADAS cell transplants had a modulatory effect on the neuroinflammatory reaction in the nigrostriatal pathway following a 6-OHDA lesion. Immunohistological analyses of the microglial activation markers OX-42 and F4/80 in SNpc of unlesioned, 6-OHDA-lesioned/sham-transplanted, and 6-OHDA-lesioned ADAS cell-transplanted animals indicated attenuation of the neuroinflammatory response 4 weeks post-transplant in animals that received the ADAS cell transplants compared to lesioned animals that received a sham-transplant (Figure A.6 d). Specifically, quantification of microglial burden in SNpc indicated a reduction in microglial burden of 45% (Figure A.6 e). Although not direct proof that ADAS cells produced anti-inflammatory mediators in the nigral environment, these data raise the interesting possibility that ADAS cell-derived molecular mediators may have the capacity to influence microglial activity at a site of injury.

Phenotypic stability, cell cycle exit, and terminal differentiation of ADAS cells *in vitro*.

The lack of detectable differentiation of ADAS into DA neurons after transplantation left us to consider the alternative hypothesis that ADAS cell differentiation induced *in vitro* was short-lived *in vivo* and both naïve and NDM-treated cell types afforded neuroprotection through a mechanism unrelated to their ability to adopt neuronal fates. We sought empirical support for this hypothesis by investigating the relative stability of the *in vitro* differentiated ADAS phenotypes after re-exposure to serum-containing medium. We monitored P2 and P3 cultures for changes in expression of the early neural progenitor marker nestin and the nuclear proliferation marker Ki67 in ADAS cell cultures grown in serum-containing medium D10, in response to mitogens (EGF and FGF-2), after 4- or 7-day neural induction in NDM, and following re-exposure to D10 for 1 or 2 days. Expression of the nuclear proliferation antigen Ki67 (Figure A.7 a and b) and nestin (Figure A.7 c) increased upon stimulation with mitogens, decreased after 4 days in differentiation media, and continued to decline by 7 days in the same; while expression of the neuronal commitment marker Tuj-1 (Figure A.7 d) was increased in a small fraction of the cells in response to mitogens, peaked in differentiation media after 4 days (see also Table A.1) and remained highly expressed at 7 days**.** However, re-exposure of differentiated cultures to serum-containing medium induced a second wave of proliferation evidenced by Ki67 (Figure A.7 a and b) and nestin (Figure A.7 c) re-emergence in the cultures; concomitantly, Tuj-1 expression (Figure A.7 d) began to decline immediately to levels below those observed after 7 days in NDM. Taken together,

these findings indicate that the *in vitro* differentiated ADAS cell phenotypes induced by the neuritizing cocktails NDM are relatively unstable. Mechanistically, this plasticity is consistent with the possibility that NDM-treated ADAS cells lost their neurally-induced phenotypes *in situ* after transplantation. To extend and confirm the observations that neurally induced ADAS cells do not display characteristics of terminally differentiated neurons, we exposed ADAS cell cultures to a 4-day treatment of VPA-supplemented N2 serum-free medium, conditions previously reported to induce terminal differentiation of neural stem cells (Hsieh et al., 2004). Consistent with results obtained after exposure to NDM, ADAS did not become terminally differentiated into mature neurons after exposure to N2/VPA as evidenced by persistent expression of the transcription factor NeuroD (Figure A.8, top panel) which is present in neuroblasts during the early stages of neural lineage commitment (Amrein et al., 2004; Katayama et al., 1997). Moreover, exposure to NDM induced outgrowth of processes in a subpopulation of the cells in the culture; yet these cells continued to express Ki67 (Figure A.8, white ovals) much like cells treated with the mitogenic cocktail EGF/FGF-2 in D10**.** In fact, proliferation arrest in NDM-treated cultures was not evident in morphologically differentiated cells until supplementation with agents that induce rapid and robust differentiation of neuroblasts into mature neurons (i.e., all-trans retinoic acid and forskolin); under these conditions, only flat fibroblast-like undifferentiated cells without processes continued to cycle (Figure A.8, white rectangles). Together with results from experiments addressing phenotypic stability, these findings suggest that neural induction of ADAS cells *in vitro* prior to transplantation does not induce terminal differentiation of ADAS into mature neurons. Given that morphological and molecular differentiation status of ADAS cells is

determined by the strength and duration of the differentiation signal, supraphysiological stimuli may be required for ADAS to undergo terminal differentiation and cell cycle exit.

ADAS cell cultures do not display spontaneous or evoked electrical activity *in vitro.*

Next, we investigated the extent to which morphological differentiation of ADAS cells correlated with functional differentiation of ADAS cells into mature neurons. Upon elevation of extracellular potassium to 20mM, a subset of morphologically differentiated ADAS cells responded to depolarizing stimuli by increasing intracellular free calcium concentrations as measured by increasing fluorescence intensity of the calcium indicator fluo-4 (data not shown), consistent with published reports that ADAS obtained from human processed lipo-aspirates express a delayed-rectifier type K+ current expressed during early neuronal development (Ashjian et al., 2003). However, attempts to measure spontaneous or evoked action potentials via intracellular recordings were unsuccessful. Likewise, attempts to measure spontaneous neural network activity from ADAS cells grown on multi-electrode arrays (MEAs) or evoked activity in response to glutamate (5 μM), the GABA A receptor antagonist bicuculline (20 μM), or trains of electrical stimulation (0.75 V biphasic pulses, trains of 10 pulses repeated 5 times with 30 seconds between pulse trains) in NDM or N2/VPA-treated cells were also unsuccessful (data not shown) despite their differentiated morphology (i.e., extended processes) (Figure A.9). Taken together, our findings indicate that terminal differentiation of ADAS cells into electrically mature neurons does not occur *in vitro* under the conditions tested. In this respect, our results are similar to those reported for multipotent skin-derived progenitor

(SKP) cells which express neuro-glial markers but fail to progress from neuroblast to neuron-like stages *in vitro* (Fernandes et al., 2006), possibly due to lack of electrical stimulation (Waragai et al., 2006) or other critical signaling cues which may be possible *in vivo* through interactions with endogenous cell populations.

ADAS cells express neurotrophic factors that protect dopaminergic neurons and promote their survival.

We hypothesized that one mechanism by which transplanted ADAS grafts could contribute to attenuated DA neuron loss after toxin-induced death without undergoing terminal differentiation into DA neurons might be through production of trophic factors at the site of injury that can protect DA neurons and promote their survival and/or differentiation of endogenous progenitor populations into dopaminergic neurons. On the basis of previous work (Milbrandt et al., 1998), we determined that the most likely candidates to examine would be the glial-derived neurotrophic factor (GDNF) family ligands (GFLs), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) because multiple *in vitro* and *in vivo* studies have demonstrated their ability to protect dopaminergic neurons and/or help repair a damaged nigrostriatal pathway. To test this idea, we performed real-time quantitative PCR analysis (or RT-PCR in the case on NTN) of ADAS cells proliferated *in vitro* in the presence of EGF/FGF-2 for the exact amount of time as cells used in transplantation studies. We found that ADAS cells express high levels of mRNA for BDNF, GNDF, and NGF relative to the levels normally detected in rat brain (Figure A.10). All of these factors exert potent trophic and

neuroprotective effects on nigral dopaminergic neurons (Hyman et al., 1991; Lin et al., 1993; Stromberg et al., 1985)**.** Levels of these neuroprotective growth factors were generally highest in EGF/FGF-2 expanded cultures. These *in vitro* findings suggest one possible mechanism by which autologous ADAS cell transplants might have mediated neuroprotection.

DISCUSSION

Several studies to date have reported that Adipose-Derived Adult Stromal (ADAS) cells from rats (Ning et al., 2006; Tholpady et al., 2003; Yang et al., 2004), mice (Fujimura et al., 2005; Safford et al., 2002), rhesus monkeys (Kang et al., 2004), and humans (Ashjian et al., 2003; Fujimura et al., 2005; Kang et al., 2003a; Safford et al., 2002; Zuk et al., 2002) can be coaxed to differentiate into neuron-like morphologies and to express neuro-glial markers *in vitro*. Importantly, our study is the first to demonstrate that *in vitro* neural induction and stable terminal differentiation of ADAS cells into functionally mature neurons *in vivo* are not necessary for ADAS cells to exert neuroprotective effects in models of neurological injury and thus be considered viable tissue sources to treat neurodegenerative diseases. It was somewhat surprising that the neuroprotective effects achieved in our studies were derived from transplantation of a small number of naïve or neurally-induced ADAS cells (~40,000). However, neuroprotective effects in hemi-parkinsonian rats have also been reported with small numbers of bone marrow-derived stromal (BMSC) (Aggarwal et al., 2006; Dezawa et al., 2004; Hellmann et al., 2006). We have not transplanted 6-OHDA-lesioned animals with ADAS cells at any other time-

point during the lesion; but we predict that if ADAS cells can survive in the hostile oxidative and neuroinflammatory environment of the SNpc during the first week after a striatal 6-OHDA injection, they are likely to survive at a later date. Future experiments will investigate whether delayed transplantation of ADAS cells affords similar neuroprotective effects.

Ideally, viable sources for cell replacement therapies in neurological disease would limit the magnitude of injury or degeneration or their sequelae, replace lost neurotransmitters, secrete neurotrophic factors or neuromodulatory substances to promote proliferation and survival of endogenous precursors, or promote restoration of function in neural circuits. Successful outcomes of neural stem cell grafts in restoration of function after 6-OHDA-induced nigrostriatal degeneration has been attributed to a combination of neural differentiation and trophic factor production (Yasuhara et al., 2006) in large part based on the observation that a large fraction of adult neural stem cells within transplanted grafts retain detectable nestin expression and never fully mature post-transplantation. Likewise, the *in vivo* neuroprotective effects of other cell types with demonstrated *in vitro* multi-lineage potential, including mesenchymal stem cells (MSC) (Scuteri et al., 2006), umbilical cord matrix stem cells (Weiss et al., 2006), and bone marrow derived stromal cells (BMSCs) (Carvey et al., 2005; Garcia et al., 2004; Ye et al., 2005), has been shown to be mediated in part through a mechanism of trophic support.

ADAS cells may have afforded neurohistological protection and ameliorated functional deficits induced by the striatal 6-OHDA lesion through multiple mechanisms.

Our findings from *in vitro* studies on neurotrophic factor gene expression suggest that one likely mechanism by which both kinds of ADAS cell grafts may have contributed to neuroprotection or attenuated dopaminergic dysfunction and neuronal loss in the 6-OHDA rat model is through production of neurotrophic factors at the lesion site. Consistent with this idea, NGF has been shown to promote survival of fetal ventral mesencephalic cells and rescue dopaminergic neurons (Chaturvedi et al., 2006; Kavanagh et al., 2006) and its mRNA expression in neurally-induced and naïve ADAS cells was approximately 2- and 30-fold, respectively that of total brain. Secretion of BDNF by engineered fibroblasts transplanted into the striatum has been shown to attenuate loss of dopaminergic neuron cell bodies within the SN pars compacta induced by striatal 6-OHDA injection (Levivier et al., 1995). Notably, ADAS cells expressed this and other potent dopaminergic survival factor *in vitro*. Specifically, BDNF and GDNF expression in ADAS cells was 2- to 4-fold that of total brain; we posit that secretion of these potent trophic factors *in situ* may have contributed to protection of vulnerable DA neurons. Similarly, BMSCs engineered with Neurturin were reported to reduce striatal dopamine deficiency and rotational behavior without significantly affecting 6-OHDA-induced TH-neuron loss (Ye et al., 2007). In the future, it may in fact be possible to augment the neuroprotective effects of ADAS grafts in the nigrostriatal pathway even further by engineering them to secrete more of these factors. In support of this idea, human ADAS grafts transduced with an adenovirus encoding BDNF improved functional deficits in a model of stroke (Kang et al., 2003b). Other mechanisms that have been proposed to explain the neurorestorative properties of ADAS cells in other studies include stimulation of migration and proliferation of endogenous neural precursor populations to the lesion

site and production of pro-angiogenic factors including VEGF, hepatocyte growth factor, or TGFβ (Rehman et al., 2004).

In summary, non-engineered ADAS cells may never replace neural stem cells or embryonic cells as a source of multipotent neural progeny for cell replacement therapies to treat CNS diseases. Nevertheless, the discovery that a stromal cell population residing in adult adipose, a tissue of mesodermal origin that is readily accessible and easy to harvest, displays molecular properties of neural progenitor cells *in vitro* and neuroprotective properties *in vivo* has important basic science and clinical implications. Critical advances in the field of neurorestorative medicine may soon be possible due to the identification of adult adipose as a novel non-immunogenic and easy-to-harvest tissue source that contains large numbers of cells with neuroprotective properties towards dopaminergic neurons that can be expanded *in vitro* for autologous transplantation. In addition to the inherent non-immunogenic properties of autologous ADAS grafts and their demonstrated neuroprotective capacity, we predict that the latter could be enhanced further by genetic engineering with GFLs. Alternatively, engineering of ADAS cells with neuroimmune modulatory peptides may also warrant investigation in pre-clinical models of PD in light of the overwhelming amount of recent evidence implicating neuroinflammatory processes in the progressive degeneration of the nigrostriatal pathway and development of PD in humans (reviewed in (Tansey et al., 2007; Whitton, 2007).

FIGURES AND TABLES

Figure A.1

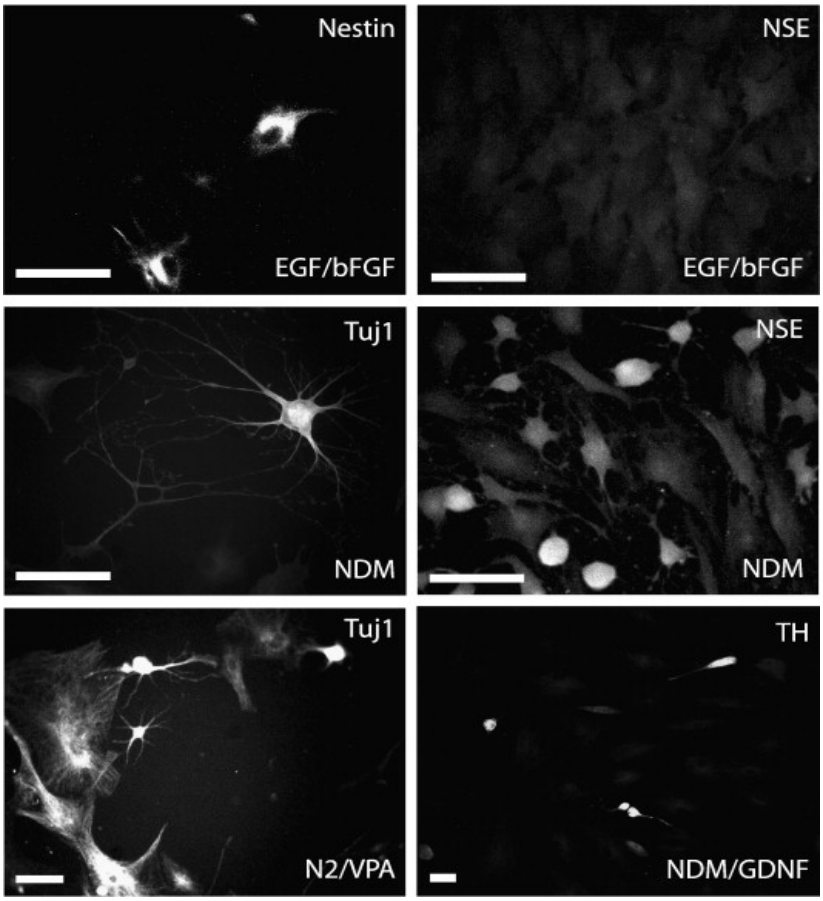

Figure A.1. Immunocytochemical analyses of cellular marker expression in naïve and differentiated rat ADAS cells.

Representative examples of passage 3 naïve and differentiated ADAS cells immunostained for specific neural cell markers (See Materials and Methods). Treatments were as follows: EGF/FGF-2 for 2 days; NDM: 2 days in EGF/FGF-2 plus 4 days in NDM; N2/VPA: 2 days in EGF/FGF-2 plus 4 days in N2 supplement + 0.5mM VPA; 10ng/mL GDNF. Data shown are representative of six independent experiments. Scale bar = 50 um.

Table A.1

Markers	D10 Medium	D10→ EGF/FGF-2 2d	D10→ EGF/FGF-2 → NDM 4d	D10→ EGF/FGF-2 → N2/VPA 4d	NSA Prolif→ NSA Differentiation 4d
Fibronectin	+++	+++	++	++	++
Vimentin	+	++	++	+	++
Nestin*	+	++	+	+	+
VE Cadherin*	-	+	++	++	+
Neuro D*	-	+/-	++	+++	+
Tuj1*	-	+/-	++	++	++
NSE*	-	-	++	++	++
NeuN	-	-	+/-	+/-	++
MAP2b	-	-	+/-	+/-	++
GABA	-	-	+	+/-	+/-
ChAT*	-	-	+/-	+/-	+/-
TH*	-	-	+/-	+/-	+/-
GFAP*	-	+	+/-	+/-	+
S100β*	-	+/-	+/-	+/-	+/-
O4	-	+/-	+/-	+/-	+/-
SMA*	++	++	+/-	+/-	++
TrkB	-	-	+	+	+
OX-42	-	+/-	-	-	-
p75NTR	-	+/-	-	-	+/-
MBP	-	-	-	-	-

Summary of Immunocytochemical analyses of cell marker expression in naive (undifferentiated) and differentiated ADAS cells

* denotes that mRNA confirmed by real time QPCR. Studies were performed on ADAS cells at passages 2 through 4.

+++ = robust expression in >50% of the cells
++ = strong expression in ~ 50% of the cells
+ = detectable expression in < 50% of the cells
+/- = detectable expression in small percentage (<10%) of the cells
- = no detectable expression

Figure A.2

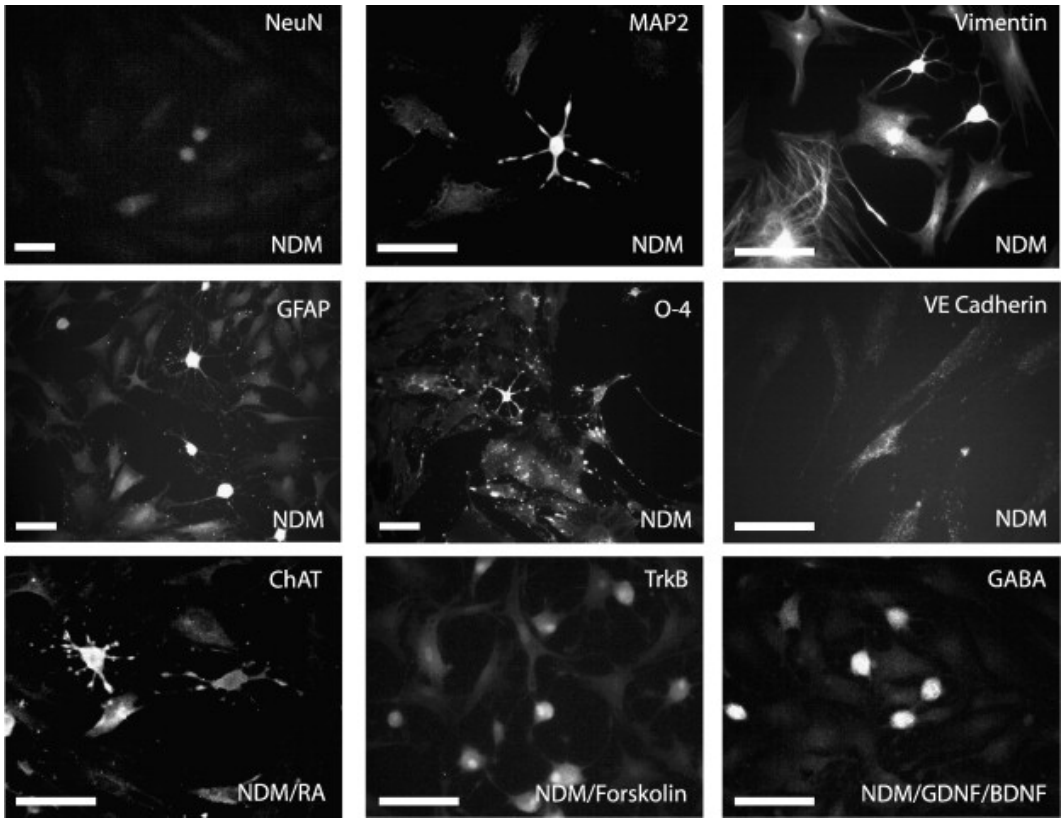

Figure A.2. Immunocytochemical analyses of cellular marker expression in naïve and differentiated rat ADAS cells at passage 4.

Representative examples of naïve and differentiated ADAS cells immunostained for specific neural, glial, and endothelial cell markers (See Materials and Methods). Treatments were as follows: EGF/FGF-2 for 2 days; NDM: 2 days in EGF/FGF-2 plus 4 days in NDM; N2/VPA: 2 days in EGF/FGF-2 plus 4 days in N2 supplement + 0.5mM VPA; 1uM RA (retinoid acid); 10um Forskolin; 10ng/mL GDNF; 10ng/mL BDNF. Data shown are from one of six independent experiments. Scale bars = 50 um.

Figure A.3

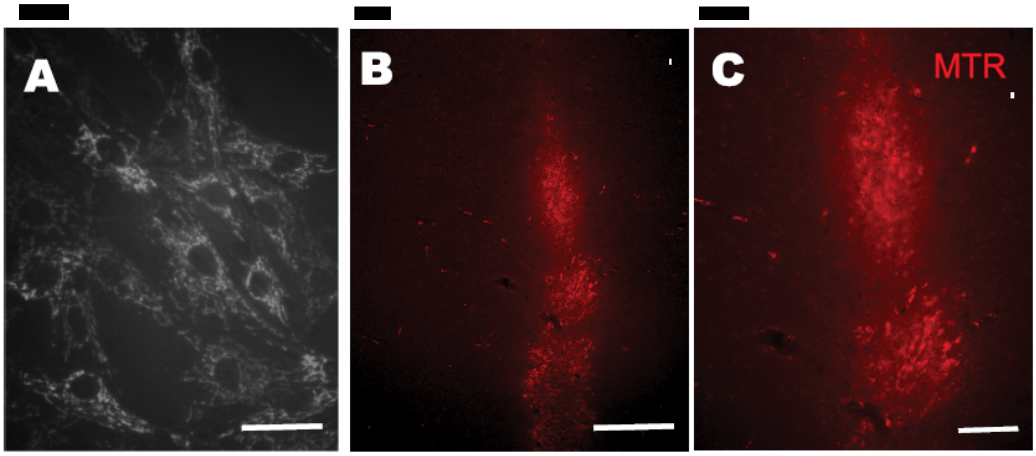

Figure A.3. Mitotracker labeled ADAS cells are detectable in unlesioned rat brain a. MitoTracker Red-labeled ADAS cells *in vitro*, scale bar =50uM. b and c, MitoTracker-Red-labeled ADAS cells (MTR) transplanted into unlesioned rat midbrain are detectable three weeks after transplantation (b, scale bar = 400um, c, scale bar = 200uM).

Figure A.4

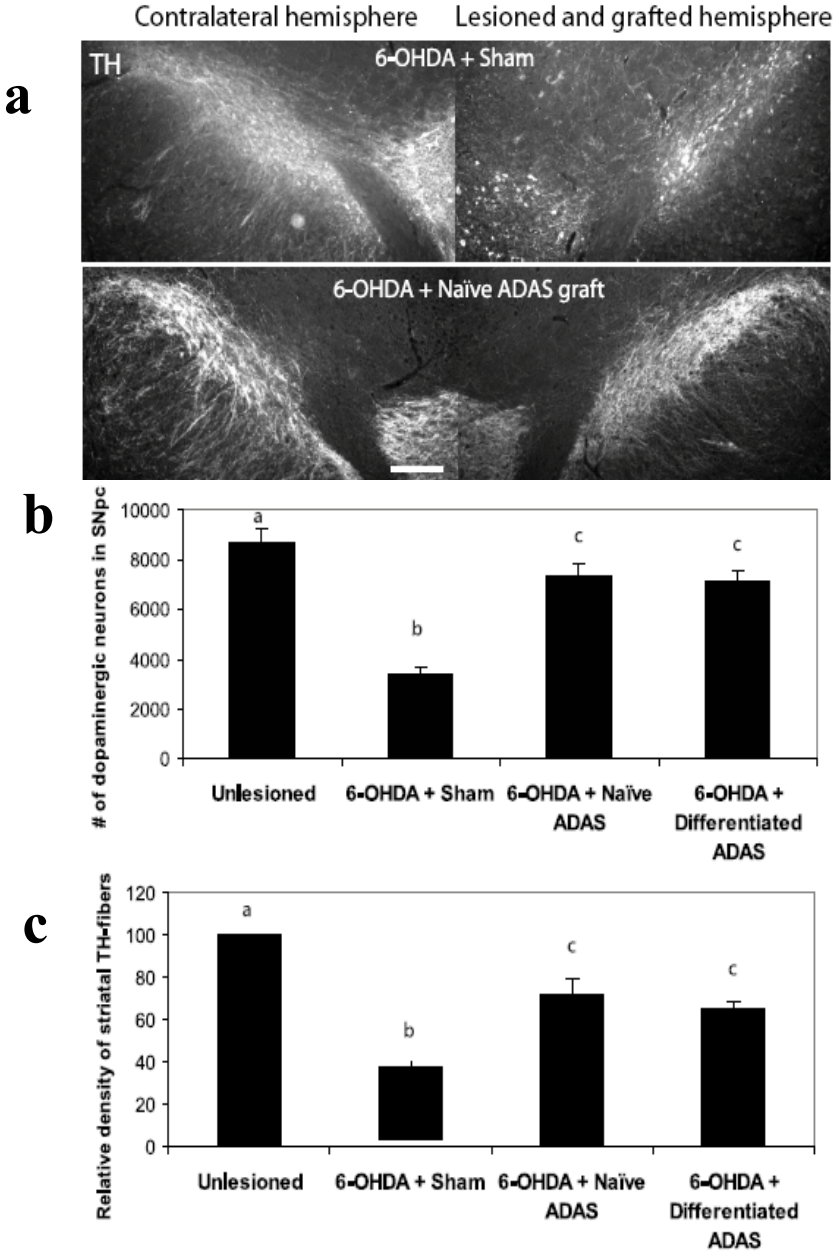
a
Contralateral hemisphere
Lesioned and grafted hemisphere
TH
6-OHDA + Sham
6-OHDA + Naïve ADAS graft
b
# of dopaminergic neurons in SNpc
10000
8000
6000
4000
2000
0
a
b
c
c
Unlesioned
6-OHDA + Sham
6-OHDA + Naïve ADAS
6-OHDA + Differentiated ADAS
c
Relative density of striatal TH-fibers
120
100
80
60
40
20
0
a
b
c
c
Unlesioned
6-OHDA + Sham
6-OHDA + Naïve ADAS
6-OHDA + Differentiated ADAS

Figure A.4. ADAS cell transplants attenuated loss of nigral and striatal tyrosine hydroxylase immunoreactivity in 6-OHDA lesioned rats.

Comparison of midbrain dopaminergic (tyrosine hydroxylase-positive) cell bodies and fiber immunoreactivity at 4 weeks post-lesion in control (unlesioned, n = 4), 6-OHDA-lesioned (n = 4), 6-OHDA-lesioned with sham (n = 4) transplant, 6-OHDA-lesioned with passage 2 naïve ADAS cells transplant (n = 5), or passage 2 differentiated ADAS cells transplant (n = 5). a, Representative rostral midbrain sections from 6-OHDA/Sham and 6-OHDA/naïve ADAS transplanted rats were stained with an antibody against tyrosine hydroxylase (TH). The 6-OHDA-lesioned hemisphere in the sham-transplanted rat displayed significant loss of TH-immunoreactivity in SNpc relative to the contralateral (unlesioned) side whereas 6-OHDA-lesioned hemisphere of rat that also received a naïve ADAS cell transplant displayed significant sparing of TH-positive cell bodies and fibers. Scale bar = 400 um. b, Stereological estimates of nigral DA neuron number, and c, Relative density of striatal TH-positive fibers confirm the neuroprotective effects of the autologous grafts of naïve and differentiated ADAS cells against 6-OHDA-induced loss of nigral TH-positive dopaminergic neurons (see Materials and Methods). Values expressed for neuron number and striatal density are group means $\pm$ S.D. Values were compared by one-way ANOVA followed by Tukey-Kramer HSD post-hoc test. Groups with different letters are significantly different at $p<0.05$.

Figure A.5

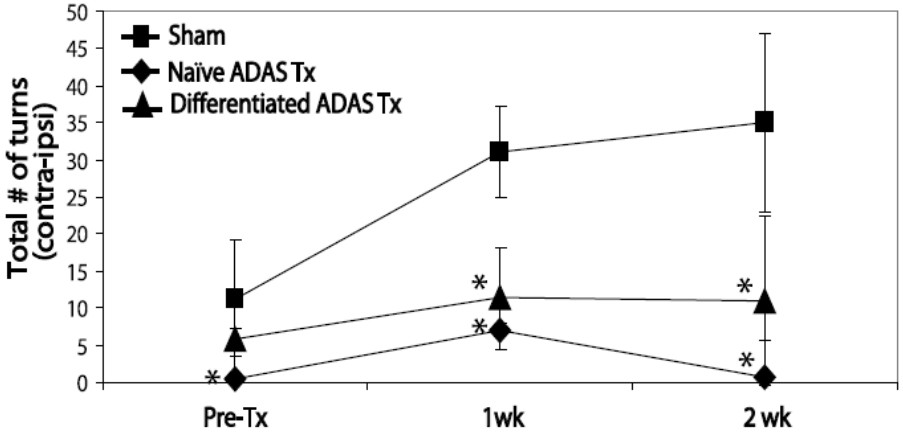

Figure A.5. ADAS cell transplants attenuated rotational behavior in 6-OHDA lesioned rats.

Rotational behavior was monitored for 20 minutes after amphetamine administration at 2.5 mg/kg i.p. (see Materials and Methods) as an indirect measure of striatal dopamine depletion induced by 6-OHDA. 6-OHDA-lesioned rats that received passage 2 naïve ADAS cells (black diamonds, n = 5) or NDM-treated ADAS cells (black triangles, n = 5) displayed attenuated amphetamine-induced rotational behavior compared to 6-OHDA-lesioned sham-transplanted (black squares, n = 4) rats. Values expressed are the group means $\pm$ S.D. Values were compared by one-way ANOVA followed by Tukey-Kramer HSD post-hoc test. Groups marked with an asterisk (*) are significantly different from sham at $p < 0.05$.

Table A.2

Stereological estimate of TH-positive neurons in SNpc one month post-transplantation

Group	Right SNpc (ipsilateral)	Left SNpc (contralateral)
Unlesioned (n = 4)	**8664 ± 587**	**8829 ± 1073**
6-OHDA + Sham (n = 4)	**3361 ± 300**	**9002 ± 660**
6-OHDA + Naïve ADAS (n = 5)	**7335 ± 539**	**8488 ± 520**
6-OHDA + Differentiated ADAS (n = 5)	**7109 ± 449**	**9115 ± 547**

Figure A. 6

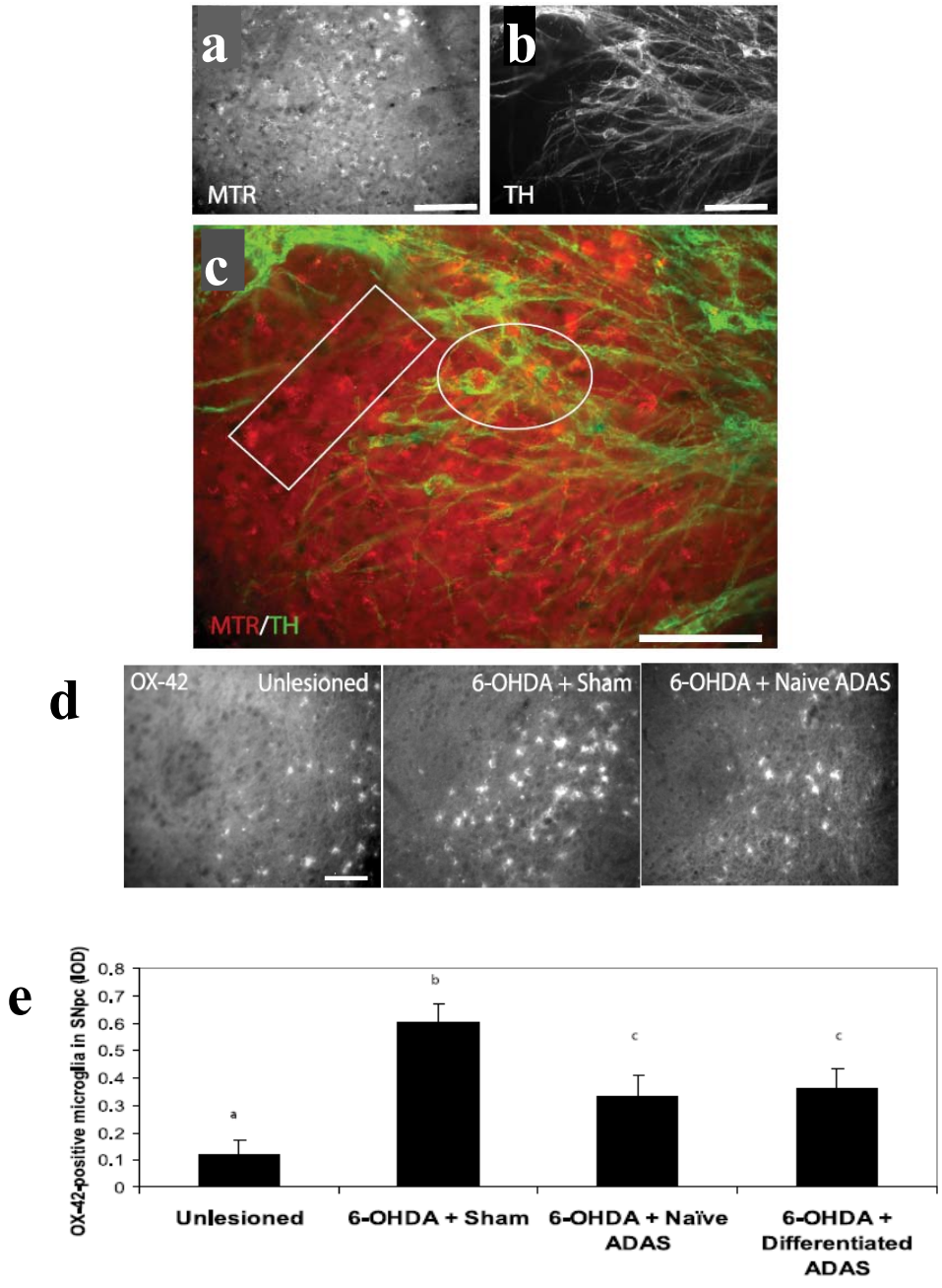
a
MTR
b
TH
c
MTR/TH
d
OX-42
Unlesioned
6-OHDA + Sham
6-OHDA + Naive ADAS
e
OX-42-positive microglia in SNpc (IOD)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
c
c
Unlesioned
6-OHDA + Sham
6-OHDA + Naïve ADAS
6-OHDA + Differentiated ADAS

Figure A.6. Transplanted ADAS cells engraft in 6-OHDA lesioned brains and attenuate microglial activation.

a, Mitotracker labeled ADAS cells in midbrain sections of rats lesioned with the oxidative neurotoxin 6-hyroxydopamine (see Materials and Methods). b, Expression of the dopaminergic marker TH is not detectable in ADAS cells after transplantation. c, In merged images, the white box indicates a region with singly labeled (MTR-positive) ADAS cells and the white oval indicates a region where MTR-labeled ADAS cells (red) were in close proximity to TH-positive (green) neuron cell bodies. d, Representative images of OX-42-positive microglial staining in SNpc of unlesioned, 6-OHDA-lesioned/sham-transplanted, and 6-OHDA-lesioned naïve ADAS cell-transplanted animals reveal attenuation of neuroinflammatory response in animals that received the ADAS cell transplants compared to lesioned animals that received a sham-transplant. e, Quantification of microglial burden in SNpc, IOD = integrated optical density (see Materials and Methods). Values are means $\pm$ S.E.M . Values were compared by one-way ANOVA followed by Tukey-Kramer HSD post-hoc test. Groups with different letters are significantly different at $p<0.05$. Scale bars=200 um.

Figure A.7

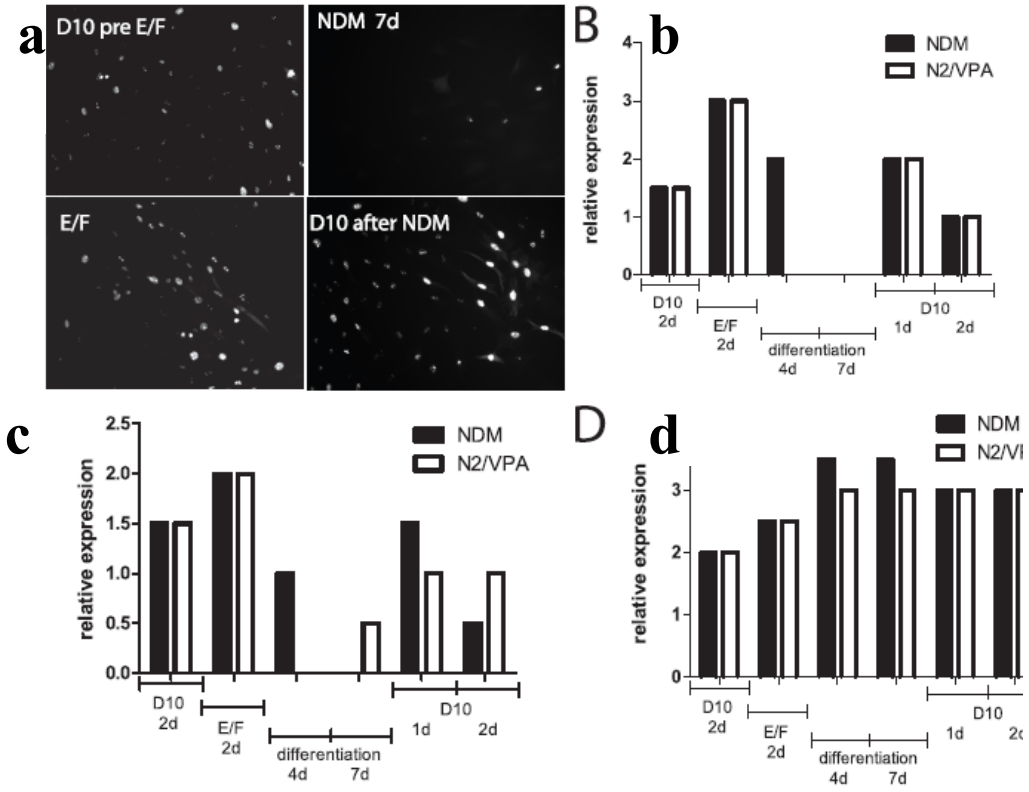

Figure A.7. Stability of differentiated phenotypes of ADAS cells *in vitro*.
a. survey of immunoreactivity for each protein marker was performed after the indicated treatment conditions leading up to, during, and following differentiation with NDM (black bars) or N2/VPA exposure (white bars) at high (40x objective) and low (10x objective) magnification. Scores between 1 and 4 were assigned by investigators blinded to the treatment based on fluorescence intensity and fraction of total cells expressing that particular marker according to the following scale: 4 = intense immunoreactivity in many (> 50 %) of the cells, 3 = strong staining in approximately 50% of the cells, 2 = detectable staining in less than 50% of the cells, 1 = detectable staining in a small fraction (< 10%) of the cells, 0 = no detectable immunoreactivity (Panels b, c, d). Independent of which differentiation medium was used (NDM (black bars) or N2/VPA (white bars), See Materials and Methods), results were similar for each of the different markers. a, Nuclear proliferation antigen Ki-67 expression (immunofluorescence) in ADAS cells under the conditions indicated. b, Relative expression of Ki-67 in ADAS cell culture under the conditions indicated, c, Relative expression of nestin in ADAS cell culture under the conditions indicated. d, Relative expression of Tuj-1 in ADAS cell culture under the conditions indicated. Data shown are representative of two independent experiments.

Figure A.8

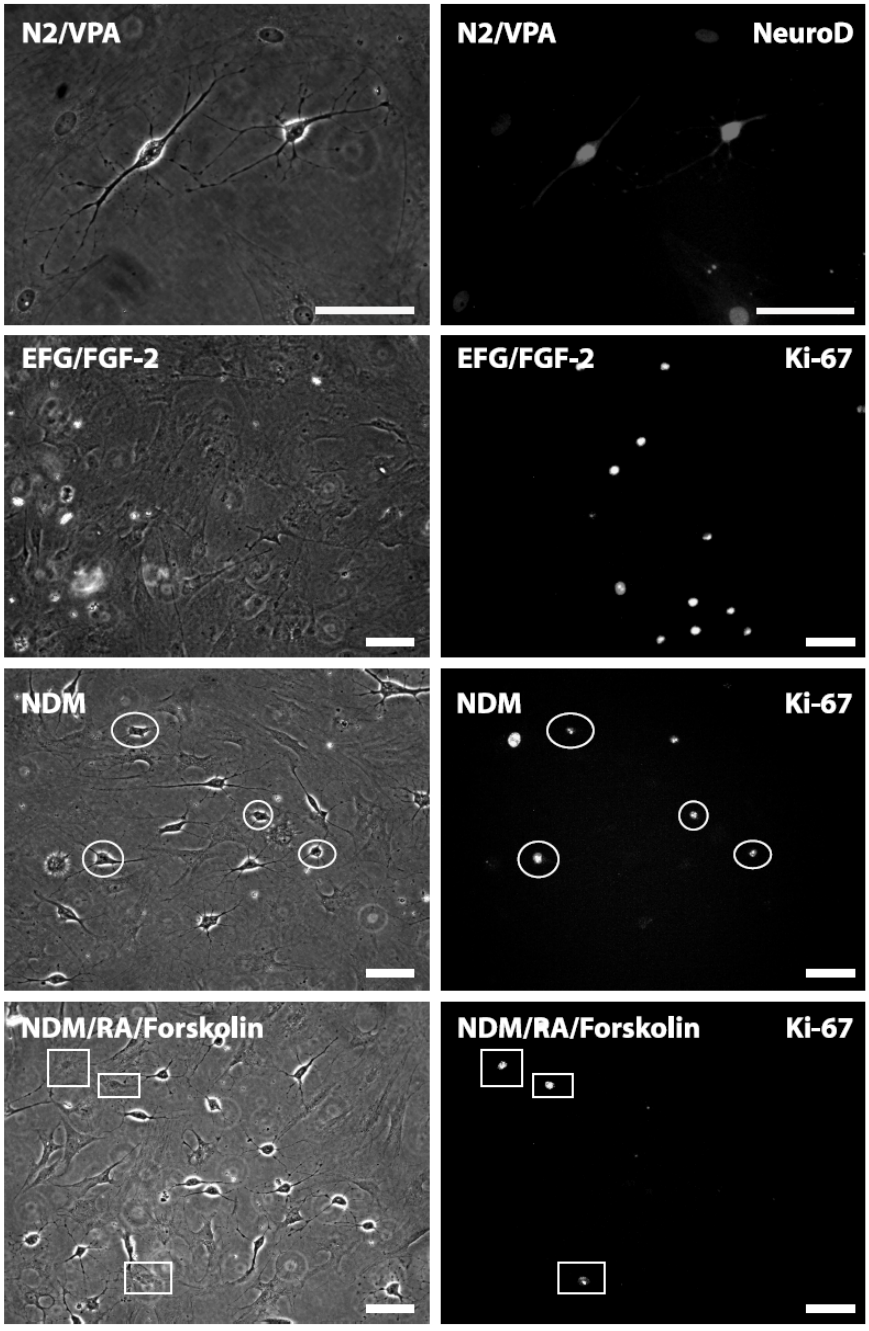
N2/VPA
N2/VPA
NeuroD
EFG/FGF-2
EFG/FGF-2
Ki-67
NDM
NDM
Ki-67
NDM/RA/Forskolin
NDM/RA/Forskolin
Ki-67

Figure A.8. Cell cycle arrest and terminal differentiation of morphologically differentiated ADAS cells requires exposure to retinoid acid and forskolin.

N2/VPA-treated ADAS cell cultures (passage 3) were immunostained with an antibody against NeuroD. Persistent expression of Neuro D was detectable in both undifferentiated (flatter) and in morphologically differentiated ADAS cells. Expression of nuclear proliferation antigen Ki-67 was detectable in subpopulations of ADAS cells growing in EGF/FGF-2 and in some morphologically differentiated ADAS cells exposed to NDM (white ovals). Cell cycle arrest was achieved in morphologically differentiated cells only after supplementation of NDM with RA and forskolin, as evidenced by presence of Ki67 only in non-differentiated cells (white rectangles). Treatments were as follows: EGF/FGF-2 2 days, NDM: 2 days EGF/FGF-2 plus 4 days NDM; N2/VPA: 2 days EGF/FGF-2 plus 2 days N2/VPA; NDM/RA/Forskolin: 2 days EGF/FGF-2 plus 4 days NDM + 1uM RA/10uM Forskolin. Data shown are representative of three independent experiments. Scale bar = 50 um.

Figure A.9

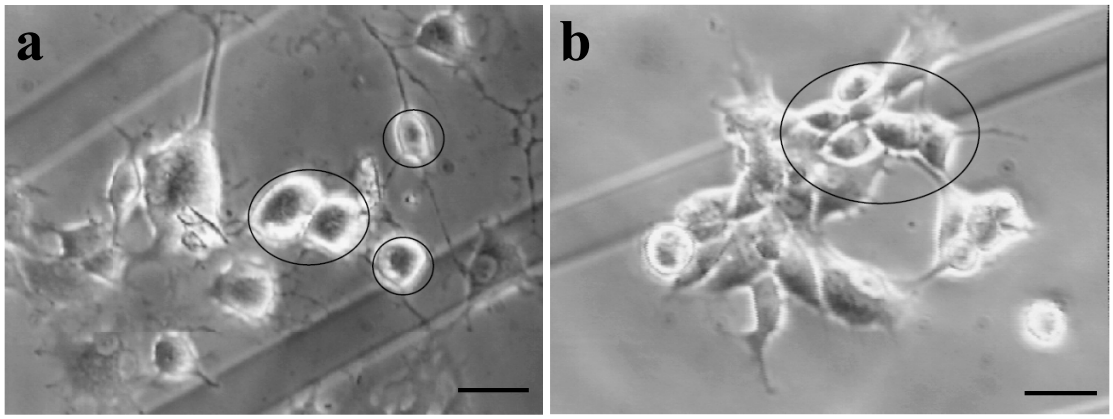

Figure A.9. ADAS cells did not display spontaneous or evoked electrical activity on multi-electrode arrays (MEAs).

ADAS cells (passages 2 through 4) were plated into D10 on poly-D-lysine coated MEAs to record spontaneous and evoked neural network activity as described previously (MEA recordings were performed by Edward Keefer). Cells were proliferated in D10 supplemented with 10ng/mL EGF and 20ng/mL FGF-2. a, 2 days in EGF/FGF-2 plus 4 days in N2 supplement + 0.5mM VPA; b, 2 days in EGF/FGF-2 plus 4 days in NDM supplemented with 1uM retinoid acid, and 1uM Forskolin 5uM. No basal or evoked activity was measured despite the presence of cells with extensive process outgrowth.

Figure A.10

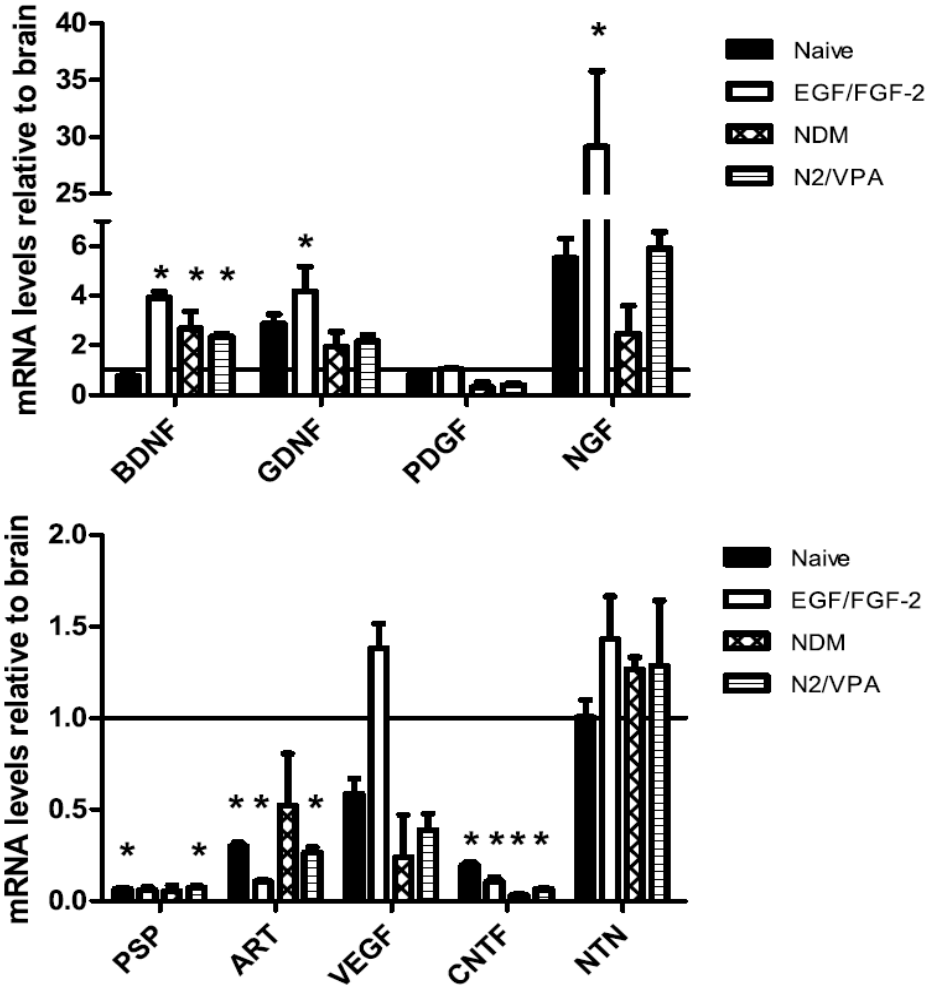
mRNA levels relative to brain
40
35
30
25
6
4
2
0
BDNF
GDNF
PDGF
NGF
Naive
EGF/FGF-2
NDM
N2/VPA
mRNA levels relative to brain
2.0
1.5
1.0
0.5
0.0
PSP
ART
VEGF
CNTF
NTN
Naive
EGF/FGF-2
NDM
N2/VPA

Figure A.10. ADAS cells express potent dopaminergic survival factors *in vitro*.

To investigate the growth factor expression profile of the cells relative to total brain levels just prior to transplantation, ADAS cells were plated and treated as follows at passage 2 or 3 prior to harvesting for real-time quantitative PCR analysis or in the case of neurturin, for semi-quantitave RT-PCR analysis (see Materials and Methods). a, Expression of BDNF, GDNF, and NGF by ADAS was found to be higher than brain levels. b, Expression of NTN and VEGF by ADAS was found to be equivalent to that in total brain; while expression of ART and CNTF was below that of total brain and PSP was undetectable. Cells were grown in D10, grown in D10 then proliferated for 2 days in D10 supplemented with 10ng/mL EGF and 20ng/mL FGF-2, or grown in D10, proliferated in EGF/FGF-2, and exposed to NDM or N2/VPA for 2 days. Differences in expression of mRNAs between rat brain and ADAS cultures were analyzed by single factor ANOVA followed by Tukey's post hoc test (GraphPad). Values expressed are means +/- SEM normalized to rat brain (indicated by a solid horizontal line). Genes with asterisks (*) are significantly different from brain at the level of $p<0.05$). Each condition was plated in triplicate. Differences between each group and whole brain were analyzed by single factor ANOVA followed by Tukey's HSD post-hoc test (GraphPad). Values expressed are mean mRNA levels relative to brain levels +/- SEM. Bars marked with an asterisk (*) significant at the level $p < 0.05$).

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VITAE

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