A novel neuroprotective role for the Fas molecule in models of Parkinson's Disease

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Dedication

A thesis dedicated to my grandmother, Czernia Kegel,
who through her illness, has moved me to study neuroprotection.

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Abstract

Fas (CD95), a member of the tumor necrosis factor receptor (TNF-R) superfamily, has been extensively studied as a death-inducing receptor in immune cells. However Fas is also widely expressed in a number of other cell-types, including in neurons. We have found that defects in the Fas/Fas Ligand system render mice highly susceptible to neural degeneration in models of Parkinson's disease (PD). Fas-deficient *lpr* mice develop a dramatic phenotype resembling clinical PD (i.e., characterized by extensive nigrostriatal degeneration accompanied by tremor, hypokinesia, and loss of motor coordination) upon treatment with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) at a dose which causes no neural degeneration nor behavioural impairment in wild-type mice. Moreover, Fas engagement directly protects neuronal cells from MPTP/1-methyl-4-phenylpyridinium ion (MPP^+) toxicity *in vitro*. Our data show that decreased Fas expression renders dopaminergic neurons more prone to degeneration in response to a neurotoxin and imply a neuroprotective role for Fas.

Defects in the ubiquitin-proteasome system have been implicated in PD; therefore we investigated the role of the proteasome in Fas-induced neuroprotection. Wild-type and Fas-deficient mice have similar baseline neuronal proteasomal activity. However, *lpr* mice treated with MPTP demonstrate decreases in proteasomal activity compared with MPTP-treated wild-type mice. To examine these findings in a second model *in vivo*, we stereotaxically injected adeno-associated viral vectors containing α-synuclein or control green fluorescent protein (GFP) into the substantia nigra of *lpr*

and wild-type mice. As seen with the MPTP model, α-synuclein-injected *lpr* mice demonstrate behavioural deficits and nigrostriatal neuropathology compared with *lpr* mice receiving a control GFP injection or wild-type mice receiving either α-synuclein or GFP. This indicates that Fas may be exerting its neuroprotective effect, at least in part, through regulation of proteasomal activity.

Consistent with Fas mediated-neuroprotection in Parkinsonian models, we have demonstrated that patients with sporadic PD manifest a defect in inducible Fas expression, suggesting that individuals with an impaired ability to upregulate Fas may be at increased risk of developing PD. Studies of Fas as a neuroprotective factor could lead to treatments which promote survival of dopamine neurons and reduce protein aggregation *in vivo*.

Résumé

Fas a été principalement étudié dans le système immunitaire pour son rôle de récepteur induisant la mort cellulaire. Pourtant, Fas est également exprimé dans plusieurs autres tissus, dont les neurones. Nous montrons qu'un défaut dans le système Fas/Fas Ligand rend les souris hautement susceptibles à une dégénérescence neuronale dans un modèle de la maladie de Parkinson (MP). Les souris de souche *lpr*, déficientes en Fas, développent un phénotype similaire à la MP clinique quand elles sont traitées avec des doses de la neurotoxine MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) qui ne causent pas de dégénérescence neuronale ni de déficits comportementaux chez les souris de type sauvage. Nos données démontrent qu'une diminution de l'expression de Fas rend les neurones dopaminergiques plus susceptibles de subir une dégénérescence suite à l'exposition à une neurotoxine, ce qui suggère un rôle neuroprotecteur pour Fas.

Un défaut dans le système ubiquitine-protéasome ayant été impliqué dans la MP, nous examinons le rôle du protéasome dans la neuroprotection induite par Fas. Dans les neurones de souris déficientes en Fas et de type sauvage, le niveau d'activité protéasomale de base est similaire. Par contre, les souris *lpr* traitées avec du MPTP ont un taux d'activité protéasomale plus bas que les souris de type sauvage traitées de la même façon. Afin d'examiner ces résultats dans un deuxième modèle *in vivo*, nous avons injecté stéréotactiquement, dans la subtantia nigra (substance noire) de souris de type sauvage ou *lpr*, un vecteur adenoviral-associé contenant le gène de l'α-synucléine ou d'une protéine de control fluorescente verte (GFP). Conformément aux

résultats du modèle MPTP, les souris *lpr*, mais non les souris de type sauvage, injectées avec le gène de l'α-synucléine accusaient un déficit comportemental et une neuropathologie nigrostriatale. Ces résultats indiquent que Fas exercerait son influence neuroprotectrice à travers la régulation de l'activité protéasomale, du moins en partie.

Nous avons montré, en accord avec la neuroprotection que Fas procure dans les modèles parkinsoniens, que les patients ayant une MP idiopathique ont un défaut dans l'induction de l'expression de Fas. Ces résultats suggèrent que les individus ayant une régulation déficiente de Fas ont plus de risque de développer la MP. L'étude des propriétés neuroprotectrices de Fas pourrait conduire à des traitements favorisant la survie des neurones dopaminergiques et la réduction des agrégats de protéines *in vivo*.

Contributions of Authors

For the three manuscripts included in this thesis, Dr. Julie Desbarats was instrumental in supervising my research.

Manuscript 1: Defective Fas expression exacerbates neurotoxicity in a model of Parkinson's disease

A. M. Landau, K.C. Luk, M.-L. Jones, R. Siegrist-Johnstone, Y.K. Young, E. Kouassi, V.V. Rymar, A. Dagher, A.F. Sadikot, and J. Desbarats. Journal of Experimental Medicine, 202(5):575-81,2005.

I set up the cellular and mouse models of Parkinson's disease and performed most of the experiments for this manuscript with the valuable collaboration from the following people:

- Dr. Kelvin Luk, under the supervision of Dr. Abbas Sadikot, did a stereological assessment of our stained sections in order to produce an unbiased neuron count.
- Michelle-Lee Jones, a medical student, recruited PD patients and controls with the help of Dr. Alain Dagher at the Montreal Neurological Institute Movement Disorders Clinic, and did flow cytometry experiments.
- Rosmarie Siegrist-Johnstone and Yoon Kow Young both provided technical assistance for the injections, perfusions and dissections of mice.
- Dr. Vladimir Rymar taught us how to perform striatal dissections in mice.

- Dr. Edouard Kouassi arranged for our samples to be run by HPLC.

Manuscript 2: The PSI model of Parkinson's Disease in mice is confounded by neurotoxicity of the ethanol vehicle

A.M. Landau, E. Kouassi, R. Siegrist-Johnstone, and J. Desbarats.

Movement Disorders, accepted, October 2006

I set up and evaluated a new model of Parkinson's disease. I did all of the experiments for this paper.

- Dr. Edouard Kouassi arranged for our samples to be run by HPLC.
- Rosmarie Siegrist-Johnstone provided technical assistance for the injections and dissections of the mice.

Manuscript 3: Fas enhances proteasomal activity and is neuroprotective in α-synuclein-induced Parkinsonism in mice

A.M. Landau, W. Ruan, T. Yasuda, R. Siegrist-Johnstone, H. Mochizuki, and J. Desbarats. To be submitted, 2007

I wrote the manuscript and performed the experiments for figures 2.2, 2.5 and 2.6. The data for Figure 2.2 were generated under the guidance of Dr. Julie Desbarats

and the data for Figures 2.4 and 2.5 were generated during a summer fellowship in Japan under the guidance of Dr. Hideki Mochizuki

- Dr. Wenjing Ruan designed and performed the *in vitro* experiments for Figures 2.3 and 2.4.
- Dr. Toru Yasuda made the adenoviral vectors and helped me with the intranigral injections.
- Dr. Hideki Mochizuki provided support and guidance.

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Abbreviations

5-HIAA	5-Hydroxyindoleacetic Acid
6-OHDA	6-Hydroxydopamine
AAV	Adeno-Associated Virus
ALS	Amyotrophic Lateral Sclerosis
ANOVA	Analysis of Variance
ATP	Adenosine Tri-Phosphate
B6	C57BL/6
CHIP	C-Terminus of hsc 70-Interacting Protein
CMV	Cytomegalovirus
CNS	Central Nervous System
CRD	Cysteine-Rich Domains
D1	Dopamine Receptor Subtype 1
D2	Dopamine Receptor Subtype 2
DAT	Dopamine Transporter
DD	Death Domain
DED	Death Effector Domain
DISC	Death-Inducing Signalling Complex
DNA	Deoxyribonucleic Acid
DOPAC	3,4-dihydroxyphenylacetic Acid
DUB	Deubiquitinating Enzyme
E1	Ubiquitin-Activating Enzyme
E2	Ubiquitin-Conjugating Enzyme

E3	Ubiquitin Protein-Ligase
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal growth factor
ERK	Extracellular-Signal-Regulated Kinase
FADD	Fas Associated Death Domain Protein
FAIM	Fas Apoptosis Inhibitory Protein
FAP-1	Fas Associated Phosphatase
FasL	Fas Ligand
FITC	Fluorescein Isothiocyanate
FLICE	FADD-Like Interleukin-1 Converting Enzyme
FLIP	FLICE Inhibitory Protein
GDNF	Glial-Derived Neurotrophic Factor
GFP	Green Fluorescent Protein
Gld	Generalized Lymphoproliferative Disease
GPe	*Globus Pallidus Pars Externa*
GPi	*Globus Pallidus Pars Externa*
hFAF1	Fas-Associated Factor 1
HPLC	High Performance Liquid Chromatography
HVA	Homovanillic Acid
IL	Interleukin
JNK	c-Jun N-Terminal Kinase
KO	Knockout
LB	Lewy Bodies

LBD	Ligand Binding Domain
L-DOPA	Levodopa
LHON	Leber's Hereditary Optic Neuropathy
Lpr	Lymphoproliferative
MAO-B	Monoamine Oxidase B
MEK	MAP/ERK kinase
MHC	Major Hisotcompatibility Complex
MG-132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
MPP+	1-methyl-4-phenylpyridinium ion
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAcc	Nucleus Accumbens
NF	Nuclear Factor
NGF	Nerve Growth Factor
NIK	NFκB-Inducing Kinase
NSAID	Non-Steroidal Anti-Inflammatory Drugs
NT	Neurotrophin
OT	Olfactory Tubercle
$p75^{NTR}$	Low Affinity Neurotrophin Receptor
PBL	Peripheral Blood Lymphocytes
PBS	Phosphate Buffered Saline
PBT	Peripheral Blood T Cells
PD	Parkinson's Disease

PE	Phycoerythrin
PEA-15/PED	Phosphoprotein enriched astrocytes-15 kDa/phosphoprotein enriched in diabetes
PI3K	Phosphatidylinositol 3-kinase
PKB	Akt/Protein Kinase B
PLAD	Pre-Ligand Assembly Domain
PINK1	Phosphatase and Tensin Homologue (PTEN)-Induced Kinase 1
PSI	Z-Ile-Glu(O*t*Bu)-Ala-Leu-al
PTEN	Phosphatase and Tensin Homologue
RIP	Receptor Interacting Protein
RAIDD	RIP-Associated ICH-1/Ced3-Homologous Protein with a Death Domain
ROS	Reactive Oxygen Species
SE	Standard Error
SN	*Substantia Nigra*
SNc	*Substantia Nigra Pars Compacta*
SNP	Single Nucleotide Polymorphism
SNr	*Substantia Nigra Pars Reticulata*
SOD	Superoxide Dismutase
STN	Subthalamic Nucleus
TH	Tyrosine Hydroxylase
TM	Transmembrane Domain
TNF-R	Tumour Necrosis Factor Receptor

TRK	Tyrosine Kinase Neurotrophin Receptor
TUNEL	Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
UPS	Ubiquitin-Proteasome System
UCHL1	Ubiquitin C-Terminal Hydrolase 1
VTA	Ventral Tegmental Area
Wt	Wild-type

Chapter 1

Introduction and Background

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1. Parkinson's disease

1.1 Introduction

Parkinson's disease (PD) was first described over 3000 years ago in the *Ayurvedic* Indian medical texts, as *kampavata*, a clinical disorder with symptoms of tremor and akinesia (Manyam, 1990). Treatment for this syndrome was derived from *Mucuna pruriens*, a plant which contains levodopa (L-DOPA) (Katzenschlager et al., 2004), a drug still important today in the treatment of PD.

PD was then described in "An Essay on the Shaking Palsy", published in 1817 by James Parkinson (Parkinson, 1817, 2002). Based on Dr. Parkinson's original observations of individuals suffering from this movement disorder, PD was described as a slowly progressive affliction comprising, sequentially (i) weakness and trembling in one affected area, usually in the hands, (ii) development of stooped posture and finally (iii) sleep disturbances and difficulties in everyday functions due to violent tremulous agitation (Parkinson, 2002). Following Dr. Parkinson's death, Shaking Palsy was renamed as PD in his honour.

PD is the second most common neurodegenerative disease worldwide (after Alzheimer's disease), affecting more than 1% of North Americans over the age of 60 (Nussbaum and Ellis, 2003). It is a relentlessly progressive movement disorder resulting from selective degeneration of dopaminergic neurons in the *substantia nigra* (SN) of the brain. The four cardinal symptoms of PD are tremor, rigidity, bradykinesia, or slowness of movement, and postural instability. Secondary indicators

of PD may include an inability to plan and carry out goal-directed behaviour, difficulty in initiating movements, a shuffling gait, stooped posture, reduced facial expressions, speech impairments, sleep disturbances, and sexual difficulties. Depression is common in PD patients with a frequency of 40%, which may be due to a serotonergic deficit occurring in PD. For nearly one quarter of the immediately aforementioned subset of PD patients, depression precedes the onset of motor dysfunction (Cooper et al., 1991; Giladi et al., 2000). Depressed patients with PD have a more rapid cognitive decline (Starkstein et al., 1990) than non-depressed PD patients. Up to 25-40% of PD patients eventually develop dementia (Emre, 2003), representing a 6-fold increase relative to the general population (Aarsland et al., 2001).

1.2 Diagnosis

Diagnosis of PD is based on clinical symptoms since no assay for biochemical appraisal is yet available. Existing diagnostic criteria are characterized by the presence of at least two of the major symptoms including tremor, bradykinesia, rigidity and postural imbalance. Asymmetric onset and positive response to L-Dopa are also factors which are important in the diagnosis of PD. Potential causes of secondary parkinsonism must be excluded, eg., response to medications for another disorder such as to dopaminergic receptor-blocking drugs used for treating schizophrenia. The aforementioned diagnostic criteria can distinguish PD from other neurodegenerative diseases and movement disorders including progressive

supranuclear palsy, multiple-system atrophy and dementia with Lewy bodies (LB) as well as other causes including head injury, exposure to toxins and hydrocephalus. Definitive diagnosis can only be confirmed at autopsy (Litvan et al., 2003).

1.3 Epidemiology

The incidence of PD is 8-18 per 100,000 individuals (de Lau and Breteler, 2006). PD onset rarely occurs before the age of 50 years and the incidence greatly increases after age 60. In industrialized countries, the prevalence of PD is estimated as 0.3% of the general population and 1% in people over the age of 60 (Nussbaum and Ellis, 2003). PD may be less common in African and Asian individuals than in Caucasians; however results from different studies are conflicting. It has been reported that men exhibit higher prevalence than women and the neuroprotection offered by estrogen is thought to represent a possible explanation (Saunders-Pullman, 2003).

1.4 Environmental Factors -Lifestyle

Environmental agents have been revealed as significant risk factors for PD. A positive association has been found between pesticide exposure and PD risk in several studies (reviewed in (Lai et al., 2002)). Men who worked on a plantation for more than ten years had a significantly higher risk of PD ostensibly due to pesticide

exposure (Petrovitch et al., 2002). Exposure to some specific metals like manganese, mercury, iron, copper and zinc have also been shown to increase the risk of PD via their accumulation in the SN and increased oxidative stress (reviewed in (Lai et al., 2002)).

Intriguingly, cigarette smoking has been consistently shown to be inversely correlated with PD risk (Grandinetti et al., 1994; Gorell et al., 1999). Current smokers and even former smokers have lower risks of developing PD. This may be attributable to a neuroprotective effect of nicotine. Indeed, nicotine has been shown to increase dopamine release, act as an antioxidant, and alter monoamine oxidase B (MAO-B) activity (Quik, 2004). It should also be mentioned that PD patients may be less likely to develop addictive behaviours due to possible dopamine deficit.

There is also a reduced risk of PD in coffee drinkers (Hernan et al., 2002). Men who do not drink coffee have a 5-fold risk of developing PD compared with men who drink 5 cups of coffee per day (Ross et al., 2000). There is a strong inverse association between PD and caffeine consumption in men and a "U" shaped relationship in women, where the lowest risk of PD was in moderate coffee drinkers (1-3 cups per day) (Ascherio et al., 2001). Caffeine is an inhibitor of the adenosine A receptor, which results in an increase in dopamine and improvement of motor deficits in a mouse model of PD (Ross et al., 2000). Other sources of caffeine, including tea, cola drinks and chocolate may also have neuroprotective properties. Green tea contains both caffeine and antioxidants (Yang and Wang, 1993), and therefore it may

have a preventative effect even greater than that of coffee with respect to PD. Antioxidants such as Vitamin C and E may protect against oxidative damage to neurons by neutralising free radicals, however conflicting evidence has been presented in animal models (Ren et al., 2006).

The use of anti-inflammatory drugs has been shown to be beneficial for some PD patients. Non-steroidal anti-inflammatory drug (NSAID) use is associated with a lower risk of PD. NSAID users have a relative PD risk of only 0.55 compared with non-users (Chen et al., 2005). More recently, a study by Hernan and colleagues demonstrated that the use of nonaspirin NSAIDS was associated with a 20% decrease in the incidence of PD in men and a 20% increase in women (Hernan et al., 2006). Therefore, as in the case of the relationship between caffeine consumption and the incidence of PD, there are sex differences which warrant further investigation.

2. Neuropathology

2.1 Neuropathology associated with PD

Even though PD pathology can be present in several areas of the brain, including the *locus coeruleus*, dorsal motor nucleus of the vagus, *nucleus basalis* of Meynert and peripheral catecholaminergic neurons (Braak et al., 2003; Zarow et al., 2003), the main PD-associated area is the SN. In PD, the pigmented dopaminergic neurons of the SN degenerate. The SN projects through the median forebrain bundle to the striatum where it controls catecholamine content (Poirier and Sourkes, 1965). In PD, these projections to the striatum are reduced, resulting in a decline of dopamine in this portion of the brain, and ultimately, a decrease in smooth and purposeful motor activity. Often, symptoms do not appear until 80% and 60% of putamenal dopamine and of SN dopaminergic neurons, respectively, have been depleted (Marsden, 1990; Fearnley and Lees, 1991).

Until recently, the traditional view was that PD is initiated via degeneration of dopaminergic neurons in the SN. However, others have more recently asserted that the initial event is degeneration of the olfactory bulb and the anterior olfactory nucleus, leading to olfactory dysfunction, followed by pathology in the lower brainstem (Braak et al., 2003). It is only in later stages that deterioration of the SN and eventually of the neocortex may occur (Braak et al., 2003).

2.2 Lewy Bodies

Lewy Bodies (LB) are pathological hallmarks of PD. These are comprised of lipids at the core and are surrounded by filamentous proteins including ubiquitin, neurofilament, α-synuclein and various proteasomal elements (McNaught, 2001). Mutated α-synuclein can be a major component of LBs in rare familial cases (Polymeropoulos et al., 1997), whereas sporadic cases are often characterized by accumulation of wild-type (wt) α-synuclein. LBs are found in the remaining dopaminergic neurons in the SN in PD patients. They are also present in extranigral neurons of the cerebral cortex and the basal forebrain nuclei (Braak et al., 1995). The fact that LBs appear in several areas of the brain may explain the presence of non-dopaminergic symptoms of PD such as sleep disorders, neuropsychiatric disorders, dysautonomia and olfactory disturbances.

There is a controversy in the literature as to whether LBs are harmful or cytoprotective. The accumulation of protein aggregates may cause stress to the cell and affect the ubiquitin-proteasome system (UPS, discussed below), which may be toxic to neurons. On the other hand, the presence of LBs may be beneficial to cells via sequestration of abnormally folded proteins from important cellular compartments. Interestingly, patients with PD caused by a mutation in *parkin* do not often develop LBs, indicating that formation of the latter is not absolutely required for disease progression. In fact, patients with the *parkin*-mutated form of PD tend to

acquire a more aggressive form of the disease at a younger age which may imply that the lack of LBs exacerbates the disease and therefore that LB formation is protective against cell death.

2.3 Circuitry

Due to the neuropathology described above, the circuitry of the nigrostriatal system becomes disrupted. The motor circuit originates in the cortex and projects to subcortical areas via projections to the putamen. Movement is controlled by putamenal output through direct and indirect pathways to the internal portion of the *globus pallidus (Gpi)* and *SN pars reticulata (SNr)*, with resulting projections to the thalamus and cortex (Figure 1.1). The direct excitatory pathway projects from putamen to the *GPi/SNr* and to the thalamus before returning to the motor cortex. The indirect inhibitory circuit projects from the putamen to the subthalamic nucleus (STN) and *GPi/SNr* via the external portion of the *globus pallidus (GPe)*, then to the thalamus and back to the cortex (Hazrati and Parent, 1991).

PD patients lose the projections from the *SN pars compacta* (*SNc*) to the striatum as a consequence of nigral degeneration. The resulting loss of dopamine at striatal dopamine subtype 1 (D1) receptors leads to a decreased inhibition from the putamen to the *GPi*. Furthermore, the loss of dopamine at inhibitory dopamine subtype 2 (D2) receptors increases the activity of inhibitory putamenal neurons reducing *GPe* activity and overexciting the STN and the *GPi/SNr*. Therefore there is

an increase in inhibitory activity from the *GPi* to the thalamus, which occurs via both the direct and indirect pathways. Excessive thalamic inhibition results in a decreased activation of the motor cortex and has been proposed as the primary cause of the development of the cardinal motor symptoms of PD (DeLong, 1990) (Figure 1.1).

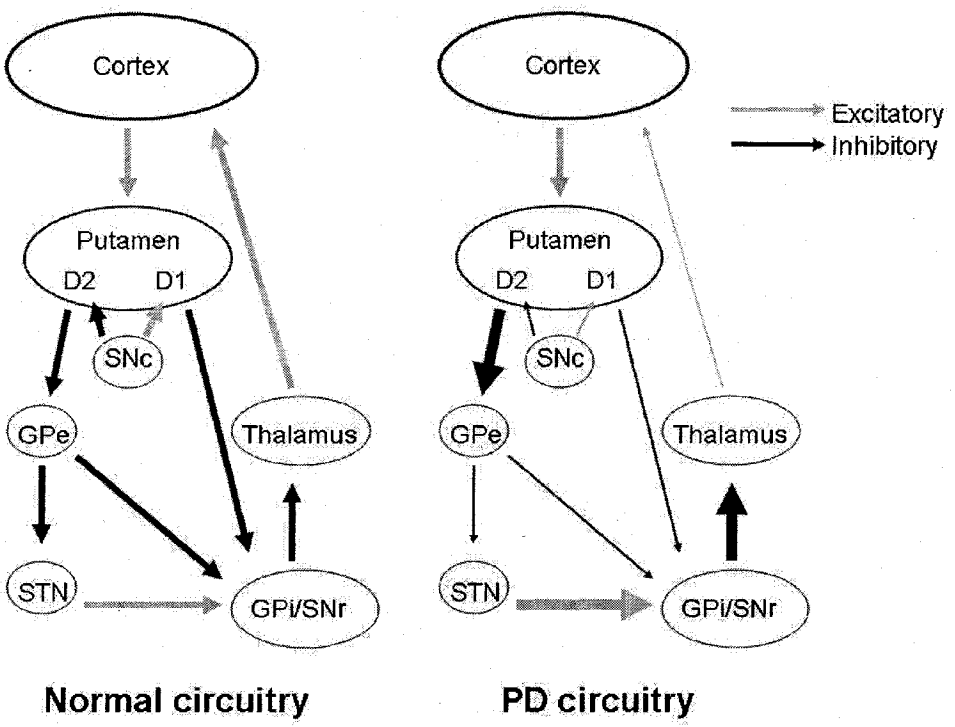

Figure 1.1: Proposed circuitry accounting for normal movement and Parkinsonian symptoms. Excitatory projections are represented by grey arrows and inhibitory projections, by black arrows. In PD circuitry, the wider arrows than in normal circuitry represent an increased activity and the narrower arrows represent a decrease. Abbreviations: D1, dopamine receptor subtype 1; D2, dopamine receptor subtype 2; *GPe, globus pallidus pars externa; GPi, globus pallidus pars interna; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata*; STN, subthalamic nucleus.

2.4 Cell death in PD

Certain cell death-related molecular pathways like c-Jun N-terminal kinase (JNK) (Hunot et al., 2004), cyclin-dependent kinases (Smith et al., 2003) and apoptosis have been implicated in the cell death in PD (Vila and Przedborski, 2003). Studies using the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining method have shown morphological changes representative of apoptosis including nuclear condensation, chromatin fragmentation and formation of apoptotic-like bodies in autopsied PD brains (Mochizuki et al., 1996; Tompkins et al., 1997). However, other groups did not find TUNEL-positive neurons in the PD SN (Kosel et al., 1997; Banati et al., 1998; Wullner et al., 1999). Ablation of Bax, a pro-apoptotic protein which is expressed at high levels in dopaminergic neurons, has been found to render mice more resistant to neurotoxic agents (Vila et al., 2001) further implicating apoptosis as an important mechanism in dopaminergic cell death.

3. Mechanisms of neurotoxicity in PD

3.1 Etiology and Pathogenesis

Despite the compendium of research on PD, the precise etiology remains elusive. PD is idiopathic in approximately 90% of patients. The recent discovery of genes which contribute to rare familial forms of PD and the use of well established toxin- and genetic-based animal models have provided insight into the molecular pathways involved in disease pathogenesis. There is an abundance of evidence which indicates that oxidative stress, mitochondrial impairment, and proteasomal dysfunction may represent principal molecular hallmarks of disease pathogenesis. The timing and selectivity of dopaminergic neuron killing probably arises from the convergence of all of these factors.

3.2 Genetics

Genetic factors have been shown to be important in PD, eg., first-degree relatives of PD patients have a 2-3 fold higher chance of developing PD compared with the general population (Payami et al., 1995; Marder et al., 1996; Elbaz et al., 1999; Rybicki et al., 1999). Furthermore, twin studies demonstrate 100% concordance in monozygotic twins with onset under the age of 50 and only a 10% concordance rate when disease onset was after the age of 50. In dizygotic twins, the

rates were 17% and 10% with onset before and after the age of 50 respectively (Tanner et al., 1999).

Single gene mutations result in a small portion of PD cases which account for 1- 3% of common late-onset PD, and 20% of younger-onset PD (Farrer, 2006). There are five clearly defined genetic causes of PD: *α-synuclein, parkin, DJ-1, PINK1*, and *LRRK2*, and one potential genetic cause *UCH-L1*, to date only implicated in one family. Understanding the mechanisms by which mutations in specific genes result in PD can shed enlightenment on the more common idiopathic form.

The specific genes and molecular pathways involved in PD and the animal models used to probe these pathways will be discussed as they pertain to the pathological mechanism under investigation.

3.3 Mitochondrial Dysfunction

The first evidence implicating mitochondrial dysfunction in PD emerged following the accidental exposure of drug addicts to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) in the ealy 1980's. Addicts developed parkinsonian symptoms following intravenous injection of the meperidine analog (MPPP) (Langston et al., 1983). MPTP, formed during the synthesis of MPPP, turned out to be a neurotoxin that produces a rapid and irreversible parkinsonian syndrome (Langston et al., 1983). MPTP crosses the blood brain barrier where it is converted in

glial cells to its toxic metabolite, the 1-methyl-4-phenylpyridinium ion (MPP^+). MPP^+ resembles dopamine and is taken up by the dopamine transporter (DAT). It accumulates in dopaminergic cells where it inhibits complex 1 of the mitochondrial electron transport chain (Nicklas et al., 1985) resulting in increased free radical production and oxidative stress and decreased ATP production ultimately leading to cell death. The decreased ATP production impairs all ATP-dependent processes (including the UPS described below). Furthermore, brief exposure to MPTP can engage an ongoing inflammatory response (McGeer et al., 2003) which can perpetuate neuronal death long after the incipient exposure to MPTP. Sporadic PD patients demonstrate deficiencies in complex 1 in the SN, skeletal muscle, and platelets (Mizuno et al., 1989; Parker et al., 1989; Schapira et al., 1989) which demonstrates a link between mitochondrial dysfunction and idiopathic PD.

3.3.1 MPTP model of PD – Complex 1 inhibitor

In light of the above, MPTP has been used to induce PD in several different animal models including primates, rodents, cats, pigs and fish. MPTP produces an irreversible and severe parkinsonian syndrome in humans and primates characterized by all of the cardinal features of PD, including tremor, rigidity, slowness of movement and postural instability.

For many years, the main shortcoming of the MPTP model was the lack of development of α-synuclein containing LB, however, it has been reported that MPTP

in fact does stimulate α-synuclein aggregation and upregulation in the SN (Kowall et al., 2000; Vila et al., 2000). Recently, a chronic infusion of MPTP in mice produced a model which recapitulated the pathological features of PD with α-synuclein positive aggregates (Fornai et al., 2005).

3.3.2 Rotenone model of PD - Complex 1 inhibitor

Pesticides and herbicides have been implicated as causative factors in PD. They may also reveal genetic vulnerability to dopaminergic toxins. Rotenone, an insecticide and pesticide, is an example of both a complex 1 inhibitor and an environmental agent which could influence the pathogenesis of PD. Rotenone is lipophilic, and like MPTP, readily crosses the blood brain barrier. It also crosses all cellular membranes and can therefore accumulate in the mitochondria, where it is suspected to exert its pathogenic role by (like MPTP) inhibiting complex 1 activity. In a study by Betarbet and colleagues, rotenone was intravenously administered to rats over 3 weeks and this produced nigrostriatal dopaminergic degeneration (Betarbet et al., 2000). In contrast to the MPTP model, rotenone-treated rats develop proteinaceous inclusions which are reminiscent of the LBs seen in PD patients (Betarbet et al., 2000; Hoglinger et al., 2003; Sherer et al., 2003). Behaviourally, rotenone-treated rats display a 70% reduction in spontaneous motor activity as well as flexed posture and rigidity (Hoglinger et al., 2003; Sherer et al., 2003).

The rotenone model is still relatively new and has not been applied as often as the MPTP one. There are important limitations to the former model. Rotenone-induced degeneration depends highly on the route of administration and may not be specific for the dopaminergic neurons in the SN (Hoglinger et al., 2003). The lesion can be variable in rats (Betarbet et al., 2000) and rotenone has not been shown to induce PD in mice, which is an important shortcoming if one wishes to assess the role of a gene using genetically modified mice.

3.4 Oxidative Stress

Oxidative stress results from an increased production of reactive oxygen species (ROS) which in turn react avidly with DNA, lipids and proteins. ROS are highly unstable molecules produced during normal cellular metabolism. In fact, approximately 3-10% of oxygen utilized by tissues is converted to reactive intermediates which impair the functioning of cells and tissues. If not neutralized, ROS accumulate and cause random damage within cells. For example, the presence of nitric oxide and superoxide anion can result in peroxynitrite formation (Guzik et al., 2002). Peroxynitrite toxicity can occur *via* lipid peroxidation, sulfhydryl oxidation, generation of ROS, and nitration of phenolic residues (Beckman, 1991). Paradoxically, ROS can also play a role as second messenger signalling molecules in cellular functions including angiogenesis, growth and differentiation (Irani, 2000; Ushio-Fukai and Alexander, 2004). The source, redox status and amount of ROS can

be determining factors in their potential as signalling molecules versus toxic agents (Irani, 2000).

As mentioned above, complex 1 inhibition due to administration of MPTP increases ROS production that perpetuates neurotoxicity. As well, oxidative damage to α-synuclein can enhance the latter's ability to misfold and aggregate (Hashimoto et al., 1999b; Hashimoto et al., 1999a). Oxidative stress also occurs due to the normal metabolism of dopamine which is vulnerable to hydroxyl radicals (Slivka and Cohen, 1985), auto oxidation (Hirrlinger et al., 2002) and intracellular nitration (LaVoie and Hastings, 1999), potentially accounting in part for selective damage to dopamine neurons in PD.

Through studies on *postmortem* brains, it was found that PD patients have elevated levels of lipid peroxidation- and protein nitration-markers in the SN and LBs (Andersen, 2004). Decreased levels of reduced glutathione and the presence of oxidized glutathione have also been observed in these areas (Sian et al., 1994). These data link oxidative stress to human PD pathogenesis.

3.4.1 Mutations implicated in Oxidative Stress and Mitochondrial Impairment

Strong evidence for mitochondrial dysfunction and oxidative stress in the pathogenesis of PD was recently procured *via* the identification of mutations in a possible redox sensor, *DJ-1*, and in a mitochondrial kinase (phosphatase and tensin

homologue (PTEN)-induced kinase 1 (PINK)) (Bonifati et al., 2003; Valente et al., 2004). However, the mechanisms of neuronal degeneration corresponding to these mutations are still under investigation.

DJ-1

Endogenous DJ-1 is present in the mitochondria (Zhang et al., 2005). The exact function of DJ-1 is unknown, however its overexpression appears to protect M17 human neuroblastoma cell lines against mitochondrial complex 1 inhibitors and hydrogen peroxide-induced oxidative stress (Canet-Aviles et al., 2004). These protective effects are abolished by mutating a cysteine residue (C106A) in DJ-1 (Canet-Aviles et al., 2004). Additionally, knocking down DJ-1 in SH-SY5Y cells with short interfering RNA renders cells highly susceptible to MPP^+, 6-OHDA and hydrogen peroxide (Taira et al., 2004). The inactivation of DJ-1 in zebrafish results in loss of dopaminergic neurons after exposure to oxidative stress and proteasomal inhibition, and this was found to be mediated by p53 (Bretaud et al., 2006). Recombinant wt DJ-1 protein stereotaxically injected into the SN, but not mutated DJ-1, protects against neurotoxicity due to simultaneous injection of 6-hydroxydopamine (6-OHDA) into the SN (Inden et al., 2006). Embryonic stem cells deficient in DJ-1 display increased sensitivity to oxidative stress leading to apoptotic death (Martinat et al., 2004). A proteomic study of the SN of mice treated with MPTP revealed a significant increase in DJ-1 in the mitochondrial fraction of the SN after MPTP exposure (Jin et al., 2005). This same study demonstrated the presence of DJ-1 in the cytoplasmic inclusions in mice, and in the LBs of PD patients (Jin et al.,

2005). DJ-1 mutant mice are more sensitive to MPTP than wt mice (Kim et al., 2005). The protection conferred by DJ-1 may be due to its direct oxidation at the cysteine residue C106 and DJ-1 is therefore a potential antioxidant (Kinumi et al., 2004). This is supported by a study in which the protective effect of DJ-1 is abolished by mutating this residue (Canet-Aviles et al., 2004). Together, this evidence suggests that DJ-1 may play an important role in neuroprotection against oxidative stress caused by mitochondrial toxins.

PINK1

Both missense and nonsense mutations within the highly conserved kinase domain of the *PINK1* gene were found to cause autosomal recessive PD (Valente et al., 2004). Patients with *PINK1* gene mutations are indistinguishable from idiopathic PD patients, apart from the earlier age of onset and sustained positive response to L-Dopa (Albanese et al., 2005). PINK1 contains a mitochondrial targeting motif which explains its localization to the mitochondria (Beilina et al., 2005). Like several genes which have been shown to underlie autosomal recessive parkinsonism, *PINK1* has been shown to encode neuroprotective properties against mitochondrial dysfunction induced by the proteasome inhibitor MG-132, a function abrogated by the mutation G309D identified in certain Parkinson's families (Valente et al., 2004).

3.5 Ubiquitin-proteasome system

There are two types of ubiquitin-dependent protein degradation, the endosomal/lysosomal system and the ubiquitin-proteasome system (UPS). The endosomal/lysosomal system uses mono-ubiquitination or oligo-ubiquitination in order to target many cell surface proteins for degradation (Ciechanover, 2006). The UPS system, which was the subject of the Nobel prize awarded in Chemistry in 2004, is the major pathway for regulated, ATP-dependent, non-lysosomal proteolysis and involves poly-ubiquitination of the target protein (Goldberg, 2005).

The UPS degrades most cellular proteins and targets damaged proteins for degradation. A series of enzyme-mediated reactions occur in which several ubiquitin molecules are ligated to the substrate to be degraded as a targeting signal to the proteasome. The process is carried out in three sequential steps by three types of enzymes which act together: E1 (ATP-dependent ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzymes) and E3 (ubiquitin-protein ligases), in order to tag the substrate with a poly-ubiquitin chain. The E1 activating enzyme forms a linkage between a cysteine residue and a carboxy-terminal glycine of ubiquitin via a thiol ester linkage (Hochstrasser, 1996). Activated ubiquitin is then transferred to an E2-conjugating enzyme. E3 ligase enzymes are responsible for binding to E2-conjugating enzymes (Imai et al., 2000) and ensuring the addition of the poly-ubiquitin chain to substrates to be degraded (von Coelln et al., 2004a). It is the E3 ligases of which there

are approximately 1000 (eg., parkin) which are responsible for substrate specificity (Ciechanover, 1998) (Figure 1.2 A).

Mono-ubiquitination can alter the function of a protein, activate transcription and route proteins to the lysosome/vacuole (Ciechanover, 2006). The attachment of multiple ubiquitin molecules (poly-ubiquitination) of the substrate allows proteins to be recognized and degraded by the 26S proteasome depending on the ubiquitin-ubiquitin linkages (Hicke et al., 2005). Substrates tagged with at least four ubiquitin molecules (with K48 linkages) are recognized by the 26S proteasome complex. The proteasome degrades the protein into small peptides (Figure 1.2 B) and ensures the release of ubiquitin molecules with the help of deubiquitinating enzymes (DUB), an example of which is ubiquitin C-terminal hydrolase 1 (UCH-L1). Genes encoding parkin and UCH-L1 are implicated in familial PD.

The proteasome is a large, structurally-complex, dynamic and multi-catalytic protein complex. It has protease activity and can degrade through both ubiquitin-dependent and -independent pathways. Proteasomes are present in the cytoplasm, perinuclear region, and the nucleus.

The 20S proteasome is a hollow cylinder-shaped complex consisting of 4 stacked rings formed by 7 distinct subunits each (Voges et al., 1999). There are two identical outer α rings and two inner β rings. The β rings contain catalytic sites with chymotrypsin-like, trypsin-like and caspase-like proteolytic activities. The

chymotrypsin-like catalytic sites are the ones most often targetted by proteasome inhibitors (Adams, 2003).

The 26S proteasome complex consists of the 20S catalytic proteasome core with 19S (also known as PA700) regulatory complexes on each end (Figure 1.2 B). The non-catalytic outer α rings serve as an anchor for the 19S complex. The 19S complexes include ubiquitin-recognition and -binding subunits. It is therefore the 19S complex which determines substrate specificity. The 19S regulatory complex selectively opens the channel through the 20S proteasome and unfolds ubiquitinated proteins in an ATP-dependent manner, allowing entry of substrates into the catalytic core, and cleaves off the poly-ubiquitinated chain from the substrate. At this point, the ubiquitin molecules are recycled by a DUB like UCH-L1.

Three of the β subunits (β1, β2, β5) of the 20S proteasome possess constitutive protease activity. The exposure of cells to pro-inflammatory molecules such as IFN-γ, TNF-α and LPS can induce the synthesis of other catalytic subunits, known as β1*i*, β2*i* and β5*i*, which are incorporated into an alternate form of the proteasome, the immunoproteasome (Kloetzel, 2001). Immunoproteasomes degrade proteins into peptides which associate with major hisotcompatibility complex (MHC) class I molecules (Fruh et al., 1994). Another regulator/activator of the 20S proteasome induced by inflammatory stimuli is PA28. PA28 α and β subunit expression is induced by IFNγ (Tanahashi et al., 1997). PA28 associates with the 20S proteasome (Cascio et al., 2002), and can promote the generation of MHC class 1

binding peptides. Some proteins which are degraded by the 20S proteasome can be further digested by cytosolic peptidases; others are transported into the endoplasmic reticulum for binding to MHC class 1 molecules and cell surface presentation (Rock et al., 1994). Therefore, there are at least three levels of regulation of proteasomal activity: 19S/PA700, the β1*i*, β2*i* and β5*i* subunits, and PA28.

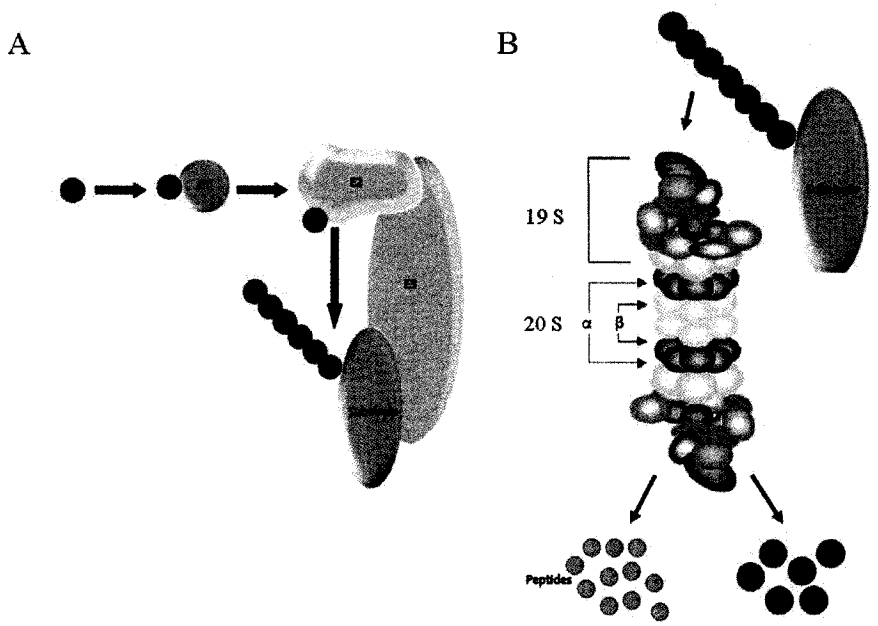

Figure 1.2: Ubiquitination and degradation by the Ubiquitin Proteasome System. A) The ubiquitination process whereby ubiquitin molecules are conjugated to a substrate as a degradation signal to the proteasome. B) The 26S proteasomal complex which is formed by the 20S proteasome and 19S regulatory complexes on each end. The proteasome recognizes the substrate bound to a poly-ubiquitin chain and degrades the substrate into small peptides and free, reusable ubiquitin. Abbreviations: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-ligase; Ub, ubiquitin. Image reproduced and modified from (Reinstein and Ciechanover, 2006) and (Wang and Maldonado, 2006).

3.5.1 UPS in PD patients

In cases where the UPS is impaired, toxic proteins can accumulate which may lead to neuronal dysfunction and death. A common pathology in several neurodegenerative diseases is the accumulation and aggregation of modified proteins such as β-amyloid fibrils in Alzheimer's disease, polyglutamine repeats in Huntington's disease and α-synuclein in PD, which implicates defects in the UPS as an important pathway in neurodegeneration (Ciechanover and Brundin, 2003). The aggregates in PD are ubiquitinated, which represents failed attempts to eliminate abnormal proteins, further suggesting an impairment of the UPS in this disease.

Defects in the UPS have been implicated in the pathophysiology of PD. Selective impairment of proteasome activity and reduced expression of proteasomal subunits have been reported in *postmortem* tissue from the SN of patients with sporadic PD (McNaught et al., 2002a; McNaught et al., 2003). These patients showed significant loss of proteasomal α-subunits in the dopaminergic neurons, which can prevent assembly of the 26/20S proteasome and impair its activity (McNaught et al., 2002a), and in fact, proteasome activities are significantly decreased in the SN of PD patients (McNaught et al., 2001). In addition, the proteasome activator 19S/PA700 is decreased in the SN of sporadic PD patients (McNaught et al., 2003). Proteasome 20S activity was found to be reduced in the peripheral blood lymphocytes in PD patients compared with healthy controls and patients with Alzheimer's disease. The duration and severity of PD was found to be inversely correlated with proteasome 20S activity

(Blandini et al., 2006). There are low baseline levels of proteasome activators in the SN of PD patients which may render nigral dopaminergic cells more susceptible to oxidative stress (McNaught et al., 2002a). Neuromelanin, which is contained in dopaminergic neurons, has been shown to inhibit the activity of the 26S proteasome. It reduces the 19S/PA700 regulatory subunit of the 26S proteasome, which may influence the selective vulnerability of dopamine neurons in PD (Shamoto-Nagai et al., 2004). A dysfunctional UPS may underlie abnormal protein accumulation, thereby facilitating the formation of toxic protein aggregates and increasing the vulnerability of nigral dopaminergic neurons to degeneration in sporadic PD. Since a decrease in proteasome activity can be detected in the blood of PD patients, this may lead to the development a non-invasive biomarker for PD (Blandini et al., 2006).

3.5.2 Clues into UPS dysfunction in PD

In PD, UPS dysfunction may lead to α-synuclein aggregation and LB formation. Parkin is an E3 ligase and UCH-L1 is a DUB. Mutations in these three genes have been linked to human PD which further supports a role for proteasomal dysfunction in the pathophysiology of PD (Polymeropoulos et al., 1997; Leroy et al., 1998; Shimura et al., 2001).

α-synuclein

An alanine-to-threonine missense mutation (A53T) in α-synuclein was uncovered as the cause of PD in an Italian-American family (the Contursi kindred)

(Polymeropoulos et al., 1996; Polymeropoulos et al., 1997). It was later discovered that duplications (Chartier-Harlin et al., 2004) and triplications (Singleton et al., 2003) of *α-synuclein* can lead to familial PD as well. α-synuclein can cause a very rare, autosomal-dominant form of PD. PD patients with α-synuclein mutations are characterized by an early age of disease onset. They are responsive to treatment with L-Dopa and often afflicted by cognitive impairment.

α-synuclein is an 140-amino acid presynaptic protein which has been proposed to be involved in vesicle handling and neurotransmitter release (Cabin et al., 2002; Yavich et al., 2004). α-synuclein is localized mainly to the pre-synaptic terminals and the cytosol (Iwai et al., 1995; Goedert, 2001), and in its native state is a soluble and unfolded protein; however, due to its central hydrophobic region, it has a high propensity to aggregate and form insoluble fibrils (Giasson et al., 2001). α-synuclein is one of the major components of LBs in both familial and sporadic forms of the disease (Spillantini et al., 1997), suggesting that abnormal variants of this protein might be crucial for the pathogenesis in several forms of PD. Indeed, the formation of aggregates is the leading hypothesis to explain the pathogenicity of α-synuclein. Additionally, this protein can bind to synaptic vesicles forming protofibrils and pores that cause permeabilization of the vesicle membranes and subsequent release of excess dopamine into the cytosol (Lashuel et al., 2002). The formation of protofibrils is enhanced by the oxidation of dopamine, which could contribute to the vulnerability of the SN dopaminergic neurons to α-synuclein-induced toxicity. Furthermore, it has been found that proteasomal activity is decreased in cells

overexpressing α-synuclein (Fujita et al., 2006) and therefore impairment to the UPS may be a mechanism of α-synuclein-induced toxicity.

α-synuclein as a genetic mouse model of PD

Missense mutations (A30P and A57T) (Polymeropoulos et al., 1997; Kruger et al., 1998), duplications (Chartier-Harlin et al., 2004) and triplications (Singleton et al., 2003) of α-synuclein were all found to lead to PD in humans, and therefore alterations in α-synuclein are now being used to model PD in mice. Overexpression of α-synuclein results in behavioural, neurochemical and neuropathological defects in mouse models (Masliah et al., 2000; Richfield et al., 2002; Rockenstein et al., 2002). Several lines of α-synuclein-null mice have been found to be partially resistant to MPTP (Dauer et al., 2002; Schluter et al., 2003; Drolet et al., 2004), suggesting that α-synuclein is involved in the pathophysiology of MPTP-induced parkinsonism. Unfortunately, not all of the transgenic α-synuclein mouse models can recapitulate all the features of PD including dopaminergic cell loss. Expression of wt or A53T α-synuclein in mice results in neurological dysfunction and early death associated with the formation of extensive α-synuclein-positive inclusions in the neocortex, hippocampus, SN, spinal cord and cerebellum (Masliah et al., 2000; Giasson et al., 2002), whereas expression of the A30P mutated α-synuclein resulted in no phenotype in mice (Lee et al., 2002).

Parkin

The first description of an autosomal-recessive early-onset form of PD was in a Japanese family (Yamamura et al., 1973). The clinical characteristics included an early onset, usually under the age of 40, and the presence of dystonia (sustained muscle contractions which cause twisting and repetitive movements or abnormal postures), in addition to other hallmark PD symptoms. These patients respond well to L-Dopa, but present an increased rate of L-DOPA-induced dyskinesia.

Mutations in the *parkin* gene were found to be linked to autosomal-recessive early-onset PD (Kitada et al., 1998). Several different *parkin* mutations have been identified including deletions, insertions, multiplications and point mutations and these are responsible for approximately half of the cases of familial PD (von Coelln et al., 2004a).

Pathologically, *parkin* mutations are associated with significant dopaminergic neuronal loss in the SN and the *locus coeruleus* (Mizuno et al., 2001). Interestingly, there is an absence of LBs in patients with the homozygous deletions of *parkin*, although LBs have been found to be present in a patient with compound heterozygous *parkin* mutations (Farrer et al., 2001). These findings suggest a role for parkin in LB formation; however, nigral cell loss and clinical parkinsonism can occur in the absence of inclusion body pathology.

As mentioned earlier, parkin is an E3 ubiquitin ligase and has an important role in the UPS, binding to E2-conjugating enzymes (Imai et al., 2000) and ensuring

the addition of the poly-ubiquitin chain to substrates to be degraded (von Coelln et al., 2004a). Moreover mutations in *parkin* may decrease or abolish its E3 ligase activity. Loss of parkin function may be detrimental due to the accumulation of substrates such as synphilin-1, O-glycosylated α-synuclein, Pael receptor, C-terminus of hsc70-interacting protein (CHIP), cdc-Re11A, cyclin E, p38 and synaptotagmin X1 (Cookson, 2005), which may be toxic to dopaminergic neurons. For example, overexpression of a parkin substrate, the Pael receptor, produces dopaminergic cell death *in vitro*, which can be rescued by parkin overexpression (Yang et al., 2003). Overexpression of the aminoacyl-tRNA synthetase cofactor p38 induces cell death which can also be rescued by wt, but not mutated parkin (Ko et al., 2005). α-synuclein is not a substrate of parkin, however, a form of O-glycosylated α-synuclein and synphilin-1, an α-synuclein associated protein, are parkin substrates (Chung et al., 2001; Shimura et al., 2001). Overexpression of parkin is protective against hydrogen peroxide and heat shock (Pasquali et al., 1996) as well as α-synuclein induced proteasomal dysfunction and toxicity (Petrucelli et al., 2002).

A novel mechanism of parkin-mediated neuroprotection has been recently identified in a proteasome-independent ubiquitination pathway (Fallon et al., 2006). Parkin can ubiquitinate an endocytic scaffold protein, Eps15, and the ubiquitination of this protein interferes with internalization and degradation of the epidermal growth factor (EGF) receptor, thus promoting phosphoinositide 3-kinase (PI3K)-Akt signalling (Fallon et al., 2006). The PI3K-Akt signalling pathway represents a neuronal survival pathway, involved in GDNF-mediated neuroprotection in the 6-

OHDA model of PD (Ugarte et al., 2003). Expression of EGF receptors and EGF are decreased in the prefrontal cortex and striatum of PD patients (Iwakura et al., 2005). Furthermore, similar effects are observed in the ipsilateral side in the 6-OHDA rat model of PD, indicating that EGF receptor signalling may be protective for dopaminergic neurons (Iwakura et al., 2005).

Unlike with α-synuclein, attempts to model parkin-associated PD *in vivo* have not been very successful (Perez and Palmiter, 2005). Mice with genetically inactivated parkin do not show nigral neuronal loss but do have nigrostriatal deficits (Goldberg et al., 2003), alterations in behaviour, dopamine transmission (Itier et al., 2003), and loss of neurons in the *locus coeruleus* (Von Coelln et al., 2004b). The *parkin* knockout model in Drosophila clinically shows locomotor dysfunction due to peripheral muscle degeneration rather than dopaminergic neuronal loss (Greene et al., 2003). Although this study and one other (Pesah et al., 2004) demonstrate no loss of dopaminergic neurons, shrinkage and degeneration has been reported in dopaminergic neurons in *parkin*-mutated flies (Cha et al., 2005; Whitworth et al., 2005).

UCH-LI

UCH-L1, a neuron specific DUB which represents 2% of total neuronal protein, has been found to be mutated in two German family members with autosomal dominant PD (Leroy et al., 1998). Neuropathology has not yet been confirmed since *postmortem* tissue is not available. The PD related missense mutation I93M decreases catalytic activity of UCH-L1 by 50% and may be the cause of α-synuclein

accumulation (Leroy et al., 1998). UCH-L1 is implicated in PD by virtue of its presence in LBs and its involvement in the UPS. Loss of UCH-L1 activity in PD ostensibly results in decreased ubiquitination due to a decrease in free ubiquitin molecules, and therefore impaired clearance of proteins.

A mouse model with an in frame deletion of exons 7 and 8 of UCH-L1 does not demonstrate cell death in the SN; rather mice develop gracile axonal dystrophy, sensory and motor ataxia with accumulation of β-amyloid and ubiquitin deposits (Saigoh et al., 1999). It has thus been suggested that mice carrying *UCH-L1* mutations do not model PD and therefore that mutated *UCH-L1* may not be sufficient for the initiation of PD. However in light of a recent study in which transgenic mice expressing the I93M *UCH-L1* mutation exhibited a loss of dopaminergic neurons (Setsuie et al., 2007), this theory is now up for reevaluation.

3.5.3 PSI mouse model: a new, controversial model of PD

Proteasomal inhibition has also been used to induce experimental PD in rodents. Recently, an *in vivo* rat model of PD was developed using a reversible lipophilic synthetic small peptide proteasome inhibitor, PSI (Z-Ile-Glu(O*t*Bu)-Ala-Leu-al) (McNaught et al., 2004). PSI-treated rats displayed a progressive parkinsonian syndrome including hypoactivity, tremor, and neuropathology characteristic of PD (McNaught et al., 2004). This was very exciting to researchers in the PD field; rats receiving systemic injections over two weeks developed a slowly

progressive form of the disease. This could allow investigation during the pre-symptomatic phase, and therefore initiation, of the disease. In contrast to some of the toxin-induced PD models, accumulation of protein aggregates was observed and neurodegeneration was also found to occur in other areas affected in human PD including the *locus coeruleus*, dorsal motor nucleus of the vagus, and the *nucleus basalis* of Meynert (McNaught et al., 2004).

The apparent success of this new model was consistent with other work by McNaught's group which demonstrates PD-like neuropathology and behavioural symptoms in rats by direct injection of lactacystin, a proteasome inhibitor, into the SN (McNaught et al., 2002b). As well, other groups have reported neurotoxicity of the nigrostriatal system due to proteasome inhibition. Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), another proteasome inhibitor, was found to be cytotoxic to dopaminergic cells in culture and leads to a 70% decrease in proteasomal function and an accumulation of aggregates in the cells (Sun et al., 2006). In the same paper, it was shown that stereotaxic injection of MG-132 into the SN of C57Bl/6 (B6) mice resulted in a loss of tyrosine hydroxylase-positive (TH^+) neurons and a decrease in dopamine and metabolite content on the ipsilateral side 12 days after injection (Sun et al., 2006). Fornai and colleagues also demonstrated that impairment of the UPS through the administration of lactacystin or epoxomycin produces cell death and neuronal inclusions *in vitro* and *in vivo* (Fornai et al., 2003). Conversely, it has also been found that injection of proteasome inhibitors lactacystin or MG-132 directly into the rat SN was not toxic to dopaminergic neurons, and in fact, this injection protected

cells from 6-OHDA-induced neurotoxicity and behavioural deficits (Inden et al., 2005). In this case, proteasome inhibition caused the formation of intracellular protein inclusions, which may have been responsible for the neuroprotection, supporting a potential neuroprotective role of LBs against 6-OHDA-mediated neurotoxicity.

Many of the most experienced laboratories in PD research set out to reproduce the above-described, potentially exciting PSI model of PD originally put forth by Kevin McNaught's group. Although two studies managed to confirm at least some of the original findings in rats (Schapira et al., 2006; Zeng et al., 2006), most of these groups have been unable to replicate the model. Negative findings have been demonstrated in mice (Bove et al., 2006), rats (Bove et al., 2006; Kordower et al., 2006; Manning-Bog et al., 2006) and monkeys (Kordower et al., 2006). It has been concluded that PSI, at this point in time, does not induce a reproducible model of PD (Beal and Lang, 2006).

4. Search for Neuroprotective strategies for PD

PD is a chronic neurodegenerative disease for which at present there is no cure. There is a great need for neuroprotective strategies to prevent dopaminergic neurons from undergoing cell death. Based on the pathological mechanisms of PD some neuroprotective agents have been designed to reduce oxidative stress, boost mitochondrial function, provide neurotrophic factor support and inhibit apoptosis. In light of the new evidence of proteasomal involvement in PD, future research should focus on neuroprotective strategies which may promote functionality of the proteasome.

In this thesis, I demonstrate that the Fas (CD95) molecule has neuroprotective properties in both cellular and mouse PD models; furthermore, this neuroprotection may be mediated in part through regulation of proteasomal activity.

5. Fas

5.1 History of the molecule's discovery

Fas (also known as APO-1 or CD95) first appeared in the literature in 1989, when it was described by two independent groups (Trauth et al., 1989; Yonehara et al., 1989) as a cell surface molecule expressed on human lymphocytes. Fas was found to trigger cell death when cross-linked with anti-Fas antibodies. The relationship between Fas and lymphocyte cell death was reinforced in 1992, when Nagata's group discovered that the lymphoproliferative (*lpr*) phenotype in mice, characterised by defective lymphocyte apoptosis, was caused by a mutation in *Fas* (Watanabe-Fukunaga et al., 1992). Then, in 1995, three papers in the same issue of Nature reported that Fas and Fas Ligand (FasL) expressed on the same cell could interact, resulting in cell "suicide" or "cell automomous death" (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). These findings all strengthened the notion that the main physiological role of Fas is the induction of apoptosis in lymphocytes. Throughout the last decade, reviews about Fas with titles such as "The Fas death factor" (Nagata and Golstein, 1995), "A license to kill" (Fraser and Evan, 1996), "Apoptosis by death factor" (Nagata, 1997), and "Death the Fas way" (Sharma et al., 2000) further entrenched this paradigm. It is not surprising that Fas has become reflexively and inextricably linked to cell death in the collective mind of the scientific community. However, as early as 1993, reports emerged documenting non-apoptotic roles for Fas (Alderson et al., 1993; Owen-Schaub et al., 1993). Although these

received relatively little attention compared with the well established apoptotic pathway, there is now a solid body of work demonstrating that Fas can transduce activation, proliferation and differentiation signals as well as apoptotic signals.

5.2 The Molecule

Fas is a member of the tumour necrosis factor receptor (TNF-R) superfamily of cytokine receptors (reviewed in ref. (Baker and Reddy, 1998)). Membership in this family is defined by characteristic extracellular cysteine-rich domains (CRDs), which control ligand binding and spontaneous self-assembly into trimers (Chan et al., 2000) (Figure 1.3). A subset of TNF-R superfamily members, including Fas, TNF-R1, death receptor 3, 4 (TRAIL-R1) and 5 (TRAIL-R2) and the low affinity neurotrophin receptor ($p75^{NTR}$), are often termed "death receptors" due to their intracellular "death domains" (DD) (Peter et al., 1999). A DD consists of a series of six α-helices, which can bind other DD-containing proteins through homotypic interactions (Huang et al., 1996). Thus, the DDs couple cell surface Fas with intracellular signal transduction cascades via adaptor molecules which also contain DDs. The DDs were originally named to reflect their ability to couple Fas to apoptotic pathways. However, DDs can initiate other pathways, some resulting in proliferation or survival instead of death. Thus, the appellation "death domain" may be as misleading as the term "death receptor", since both the receptor itself and its intracellular DD may transduce multiple types of signals.

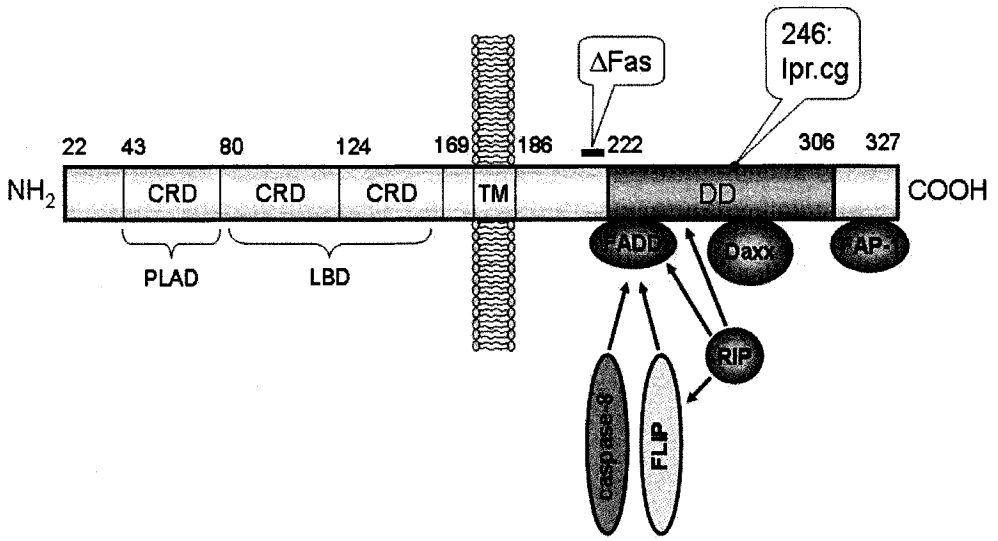

Figure 1.3: Linear representation of the mouse Fas receptor with known Fas-interacting proteins. The amino acid numbering is based on the translated Fas protein (beginning at position 22) as found on the NCBI sequence viewer webpage (ref. P25446). The exact binding sites of Daxx and FADD are currently unknown. The *lpr.cg* point mutation locally unfolds the death domain, prevents FADD binding and decreases Daxx binding. The human ΔFas mutation generated by Chang et al. prevents FADD but not Daxx from binding the receptor (Chang et al., 1999). Abbreviations: CRD, Cysteine-Rich Domain; PLAD, Pre-Ligand Assembly Domain; LBD, Ligand Binding Domain; TM, Transmembrane Domain; FADD, Fas-Associated Death Domain; DD, Death Domain; RIP, Receptor Interacting Protein; FLIP, FLICE (caspase 8) Inhibitory Protein; FAP-1, Fas Associated Phosphatase-1.

6. Fas signalling

Fas monomers spontaneously self-assemble into trimers in the cell membrane (Chan et al., 2000). When bound by FasL or by agonistic anti-Fas antibodies, these trimers presumably undergo a conformational change resulting in activation of downstream pathways. Ligand binding may allow the recruitment of new adaptor proteins to the DD, or may bring previously recruited proteins into proximity, allowing their autocatalytic activation. Three Fas DD-binding molecules have been well characterised: the Fas Associated Death Domain protein (FADD / MORT-1) (Strasser and Newton, 1999), the death associated protein Daxx (Michaelson, 2000), and the Receptor Interacting Protein (RIP) (Stanger et al., 1995). The Fas Associated Phosphatase (FAP-1) is a fourth protein that has been well characterised as a Fas-binding protein, although it does not appear to couple Fas to downstream signalling cascades. Instead, FAP-1 associates with the 15 C-terminal amino acids of the Fas intracellular domain, and negatively regulates apoptotic signalling (Sato et al., 1995) (Figure 1.3). It is unknown whether FAP-1 also regulates Fas non-apoptotic signalling, either negatively or positively.

6.1 FADD

FADD is an adaptor protein that couples Fas to caspase 8 (also known as FADD-like interleukin-1 converting enzyme, FLICE) and possibly to other signalling pathways as mentioned above. FADD binds to Fas via homotypic DD interactions

and to caspases 8 and / or 10 via a homotypic death effector domain (DED) interaction (Figure 1.4 A). The complex formed by Fas, FADD, and caspase 8 / 10 is known as the Death-Inducing Signalling Complex (DISC). Caspases 8 and 10 are activated by autocatalytic processing in the DISC when FasL binds Fas. Subsequently, processed caspase 8 / 10 can initiate two death pathways: a cytoplasmic caspase cascade, and a mitochondrial destabilization via Bid resulting in the release of cytochrome c. These two pathways converge on caspase 3 activation, leading to apoptosis.

The Fas/FADD/caspase 8/10 initiated pathways appear to be the main mediators of Fas-induced apoptosis (extensively reviewed in (Nagata, 1998; Wajant, 2002)). In fact, the absence of functional caspase 8 or FADD abolishes Fas-induced apoptosis (Varfolomeev et al., 1998; Wajant et al., 1998; Yeh et al., 1998; Zhang et al., 1998; Chang et al., 1999). Caspase 8 or FADD targeted deletions (knockouts) in mice led to early embryonic death, around day E11.5 - E12.5 (Varfolomeev et al., 1998; Yeh et al., 1998). As expected for the deletion of an apoptotic gene, the salient pathology in the caspase 8 knockout embryos was an abnormal accumulation of cells, in this case a prominent hyperaemia (accumulation of red blood cells) which led to embryonic death (Varfolomeev et al., 1998). In contrast, $FADD^{-/-}$ embryos did not demonstrate decreased apoptosis. Instead, $FADD^{-/-}$ mice experienced a developmental delay, suggesting that FADD normally may be involved in embryonic growth (Yeh et al., 1998). Furthermore, in chimeric $FADD^{-/-}$ mice and mice selectively lacking FADD function in T cells, thymocyte survival was drastically reduced, and peripheral

T cell activation and proliferation were defective (Newton et al., 1998; Zhang et al., 1998; Zornig et al., 1998; Hueber et al., 2000; Mack and Hacker, 2002). Fas-mediated death was also abolished in these cells (Wajant et al., 1998; Yeh et al., 1998; Zhang et al., 1998). Thus, FADD couples to a proliferative or survival pathway as well as to apoptosis. A mutation in FADD that abrogates its role in proliferation without impairing its apoptotic function has been described (Hua et al., 2003). FADD, therefore, may control proliferation and apoptosis via separate domains. Several mechanisms for FADD-dependent proliferation have been proposed. Phosphorylation of FADD at Ser194 has been shown to be critical for FADD-dependent cell proliferation (Park et al., 2005). Most of the mechanisms of FADD-dependent proliferation involve the FLICE Inhibitory Protein (FLIP) (Thome and Tschopp, 2001). FLIP is a caspase 8 homologue with at least two splice isoforms: the short form, $FLIP_S$, lacks the catalytic domain, and the long form, $FLIP_L$, has a caspase domain that lacks catalytic function. FLIP effectively inhibits apoptosis through the Fas / FADD / caspase 8 pathway by binding to FADD and thus preventing the binding and activation of caspase 8 (Irmler et al., 1997). FLIP, like its homologue caspase 8, is essential for embryonic survival, and FLIP knockout mice die by day E10.5 of development (Yeh et al., 2000). In contrast to the caspase 8 knockouts and consistent with an anti-apoptotic role for FLIP, FLIP-null fibroblasts are hypersensitive to Fas-induced death (Yeh et al., 2000). Furthermore, FLIP appears to have a distinct role as a mediator of Fas-induced proliferation. FLIP can activate the extracellular-signal-regulated kinase (ERK) pathway, an evolutionarily highly conserved MAP kinase cascade involved in proliferation / differentiation (Kataoka et

al., 2000). In many cells, growth factor receptors are coupled to the ERK pathway by Ras, a small GTPase that activates Raf-1, which in turn phosphorylates the MAP / ERK kinase (MEK), which specifically activates ERK (Derkinderen et al., 1999). FLIP may short circuit this process by recruiting Raf-1 directly into the DISC, thus bypassing Ras (Kataoka et al., 2000) (Figure 1.4 A). Interestingly, ERK activation upregulates FLIP expression in some cells (Aoudjit and Vuori, 2001). Together, FLIP-induced ERK activation and ERK-induced FLIP upregulation may establish a positive feedback cycle which simultaneously promotes Fas-induced proliferation while inhibiting Fas-induced apoptosis.

6.2 Daxx

Daxx is predominantly localised to the nucleus where it is thought to act as a transcriptional regulator (Salomoni and Khelifi, 2006). It can also be translocated to the cytoplasm by the apoptosis signalling kinase 1 (ASK1) (Figure 1.4 B) (Ko et al., 2001). ASK1 is required both for the cytoplasmic localization of Daxx, and for the Daxx / Fas interaction (Ko et al., 2001). Daxx can bind to the Fas DD; this interaction is independent of FADD binding, and FADD and Daxx binding occur at different locations on the DD (Chang et al., 1999). Daxx can enhance Fas-induced apoptosis by at least two independent mechanisms, namely by activation of c-Jun N-terminal kinase (JNK) (Yang et al., 1997), and by transcriptional regulation of genes involved in caspase activation (Torii et al., 1999). However, in some systems Daxx appears to have no effect on apoptosis, and Daxx alone has not been documented to

induce apoptosis in the absence of a functional FADD / caspase pathway (Michaelson, 2000). Interestingly, like FADD, Daxx may also transduce survival and / or proliferative signals from Fas (Michaelson et al., 1999; Michaelson, 2000). Daxx deletion mutations in mice result in early embryonic lethality (day E8.5 – E9.5), accompanied by global apoptosis (Michaelson et al., 1999). Pronounced, widespread apoptosis in the Daxx knockouts suggests that Daxx is predominantly a survival factor, at least during development (Michaelson et al., 1999; Michaelson, 2000).

6.3 RIP

The receptor interacting proteins (RIP, RIP2, RIP3, RIP4) are death domain-containing proteins which possess serine / threonine kinase activity (Stanger et al., 1995; Kelliher et al., 1998; Thome et al., 1998; Yu et al., 1999; Holler et al., 2000; Kasof et al., 2000; Meylan et al., 2002). RIP associates with the Fas death domain in yeast-two-hybrid screens (Stanger et al., 1995), but can also interact directly with FADD (Varfolomeev et al., 1996; Holler et al., 2000; Hu et al., 2000) and FLIP (Kataoka et al., 2000). *In vitro*, RIP overexpression results in NFκB translocation, JNK activation, and apoptosis (Stanger et al., 1995; Kelliher et al., 1998; Thome et al., 1998; Yu et al., 1999; Holler et al., 2000; Kasof et al., 2000; Kim et al., 2000a; Meylan et al., 2002). RIP deletion mutant mice are runted at birth and die at postnatal day 1-3 (Kelliher et al., 1998). RIP knockout mice displayed marked apoptosis in lymphoid and adipose tissues, and their thymocyte (immature T cell) survival was dramatically reduced (Kelliher et al., 1998; Cusson et al., 2002). However, unlike

FADD knockout T cells, $RIP^{-/-}$ mature T cells demonstrate normal proliferation and Fas-mediated apoptosis (Kelliher et al., 1998; Cusson et al., 2002). Thus, *in vivo* RIP appears to function as a survival factor, rather than as an inducer of proliferation or apoptosis. RIP family proteins may transduce non-apoptotic signal through several pathways, including NFκB (Kelliher et al., 1998; Thome et al., 1998; Yu et al., 1999; Kasof et al., 2000; Meylan et al., 2002) and ERK (Navas et al., 1999). NFκB activation by RIP is dependent on functional NFκB-inducing kinase (NIK), and may involve NIK recruitment into the DISC (Figure 1.4 C) (Malinin et al., 1997; Pazdernik et al., 1999; Hu et al., 2000). RIP-mediated activation of NFκB, while RIP is bound to FLIP, may account at least in part for the observation that FLIP can regulate NFκB activation. Activation of NFκB results in its translocation to the nucleus, where it acts as a transcription factor. NFκB activation usually induces proliferation, differentiation, or inflammation, but may also promote apoptosis, perhaps accounting for the discrepancies in reported RIP functions in different cellular contexts (Li and Stark, 2002). As mentioned above, RIP possesses serine / threonine kinase activity. Activated RIP2 can phosphorylate ERK, thus activating the ERK pathway independently of MEK (Figure 1.4 C). Finally, RIP can also activate caspase 1 directly (Thome et al., 1998), and caspase 2 via the adaptor protein RAIDD (RIP-associated ICH-1/Ced3-homologous protein with a death domain) (Duan and Dixit, 1997). *In vivo*, activation of caspase 1 allows the processing of pro-interleukin 1β (IL-1β) to active IL-1β, a potent inducer of inflammation (Cerretti et al., 1992). Thus, RIP can mediate Fas-induced differentiation and inflammation through a number of different pathways.

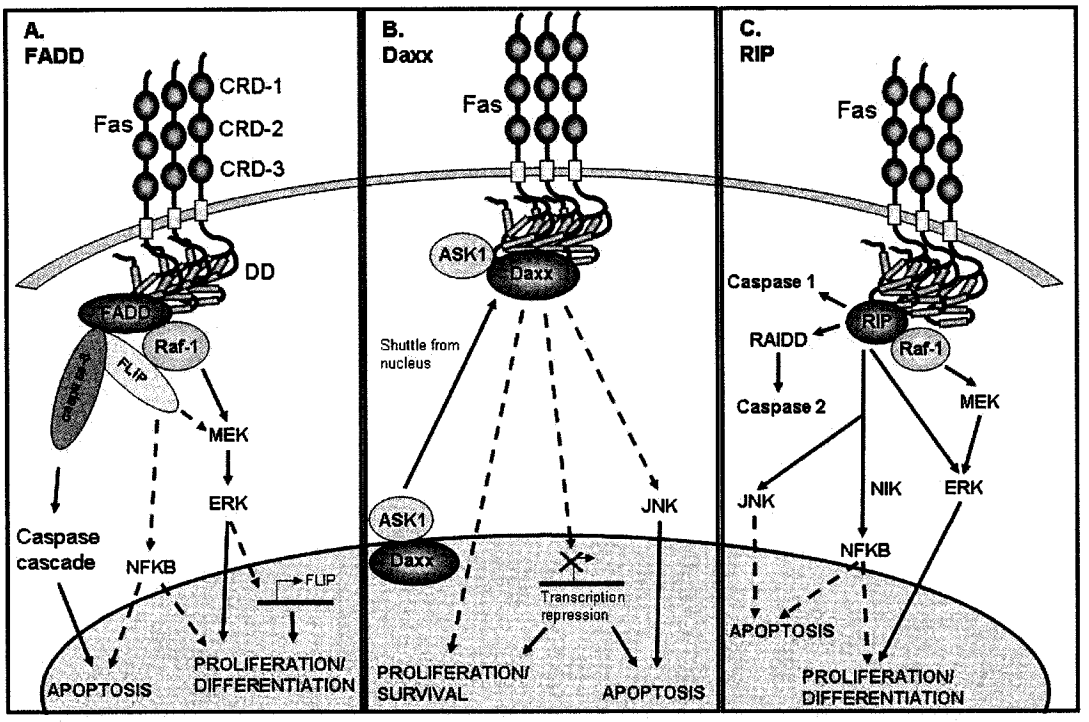

Figure 1.4: Pathways initiated by Fas. Interaction with FADD (panel A), Daxx (panel B) and RIP (panel C) are shown. Many of these interactions likely take place simultaneously, but the pathways are shown separately for the sake of clarity. Molecules which have been shown to associate with the DISC are circled. The Fas death domain is drawn based on the NMR structure (Huang et al., 1996). Abbreviations: MEK, MAP/ERK Kinase; ERK, Extracellular-signal Regulated Kinase; ASK 1, Apoptosis Signal-Regulating Kinase-1; JNK, c-Jun N-Terminal Kinase; RAIDD, RIP Associated ICH-1/Ced-3-homologous protein with a Death Domain; NIK, NFκB Inducing Kinase.

6.4 Fas signalling: all paths lead to multiple outcomes

FADD, Daxx, and RIP can couple Fas to proliferative / survival pathways as well as to apoptosis. In addition, most of the known Fas-associated signal transducing molecules are essential for survival: knockouts of FADD (Yeh et al., 1998), FLIP (Yeh et al., 2000), caspase 8 (Varfolomeev et al., 1998), Daxx (Michaelson et al., 1999), and RIP (Kelliher et al., 1998) are all lethal during the embryonic or early postnatal period. Furthermore, only the caspase 8 knockout has a phenotype clearly related to defective apoptosis (Varfolomeev et al., 1998). All other Fas interacting proteins, when deleted, result in phenotypes suggestive of predominantly proliferative or survival roles. Clearly, these molecules have functions far beyond the induction of lymphocyte apoptosis. Each of these proteins associate with multiple receptors, implying that Fas itself need not be essential for survival.

7. Mutations in the Fas gene

7.1 Mouse models of Fas deficiency

In mice, three Fas mutations exist: *lpr* and *lpr*.cg, which are naturally occurring mutations of *Fas*; and the Fas "null" mice, representing a deletion of part of the Fas molecule, created by Dr. Nagata's group. *Lpr* mice have a transposable element in intron 2 of the *Fas* gene resulting in decreased cell surface Fas expression (around 15% of wt levels, depending on the tissue) (Adachi et al., 1993). *Lpr*.cg mice bear a point mutation at amino-acid 246, which is predicted to locally unfold the death domain (Kimura and Matsuzawa, 1994; Eberstadt et al., 1997). The *lpr*.cg mutation prevents Fas-induced apoptosis, but is permissive for at least some Fas-induced proliferation / differentiation pathways (Desbarats and Newell, 2000; Desbarats et al., 2003). Finally, the Fas null mice produced by Nagata's group have a targeted deletion of most of exon 9. Fas in these mice lacks part of the DD, but retains a sequence subsequently implicated in FADD binding (Adachi et al., 1995). Furthermore, the Fas null mice express exon-9-deleted Fas protein at the cell surface, albeit at lower levels than wt mice (Adachi et al., 1995). A FasL mutant also exists, the generalized lymphoproliferative *(gld)* mouse. *Gld* mice have a point-mutation in the extracellular domain of FasL which decreases the affinity of the Fas/FasL interaction resulting in reduced signalling through Fas (Takahashi et al., 1994; Karray et al., 2004). FasL is expressed at normal or elevated levels in *gld* mice. All of these mutations may retain some proliferative Fas function despite their impaired Fas-

induced apoptosis. Thus, the true *in vivo* impact of Fas proliferative signalling remains to be determined.

7.2 Fas mutations in human

Autoimmune Lymphoproliferative Syndrome (ALPS) is a human condition which afflicts patients without a functional Fas/FasL system. It involves chronic lymphoproliferation similar to what is found in *lpr* mice (Rieux-Laucat, 2006). ALPS patients have defects in apoptosis but may have normal proliferative signalling, similar to what occurs in *lpr.cg* mice. Type Ia patients bear dominant negative mutations in Fas and type Ib patients have a mutated FasL. There are also patients with ALPS type II with mutations in caspase 10 (Rieux-Laucat, 2006).

8. Non-apoptotic roles for Fas

Although historically Fas is known mainly as a "death receptor", Fas can also induce proliferation, differentiation, cytokine secretion and tissue regeneration. Unlike the well-defined apoptotic pathway, the molecular mechanisms underlying other Fas functions have not yet been established in detail, although the ERK, NFκB, and JNK pathways have all been implicated (Lambert et al., 2003).

8.1 Proliferation

The earliest indication that Fas could induce outcomes other than apoptosis appeared in 1993, when Alderson *et al.* and Owen-Schaub *et al.* independently reported that Fas could enhance proliferation in activated T cells, B cells, and some tumour cells (Alderson et al., 1993; Owen-Schaub et al., 1993). Other reports soon followed demonstrating that Fas stimulation promoted proliferation in lymphocytes, fibroblasts, hepatocytes, and in tumour cells of many different tissue origins (Alderson et al., 1994; Aggarwal et al., 1995; Freiberg et al., 1997; Husain et al., 1998a; Jelaska and Korn, 1998; Newton et al., 1998; Zhang et al., 1998; Borset et al., 1999; Desbarats et al., 1999; Desbarats and Newell, 2000; Shinohara et al., 2000; Mitsiades et al., 2006). In terms of molecular mechanism, different studies implicated FADD, FLIP and caspase 8 in Fas-induced proliferation in T cells (Zhang et al., 1998; Zornig et al., 1998; Kennedy et al., 1999; Hueber et al., 2000; Kataoka et al., 2000; Thome and Tschopp, 2001; Lens et al., 2002; Mack and Hacker, 2002; Hua

et al., 2003), and the ERK pathway in fibroblasts, glioma and neural stem cells (Shinohara et al., 2000; Ahn et al., 2001; Tamm et al., 2004). No one specific, well-defined pathway has yet provided a unified explanation for Fas-induced proliferation.

8.2 Inflammation

Apart from promoting proliferation, Fas engagement can also trigger a number of inflammatory changes. IL-1 is a central activator of the inflammatory response, and its secretion can be triggered by Fas engagement (Miwa et al., 1998). Fas can also activate caspase 1 via RIP, and caspase 1 cleaves pro-IL-1 to its mature secreted form (Thome et al., 1998). Thus, Fas may be involved both in the processing and release of IL-1, a primary mediator of immunity (Rosenwasser, 1998) and a modulator of neurological function (O'Connor and Coogan, 1999). Other inflammatory cytokines released in response to Fas engagement include IL-6, an inducer of inflammation that may also have neurotrophic properties (Juttler et al., 2002), and IL-8, a chemokine which recruits neutrophils to inflammatory sites (Harada et al., 1994). Fas engagement induces secretion of IL-6 from glioma cells *in vitro* (Choi et al., 2002), and triggers elevated plasma IL-6 levels when Fas is engaged in the brain *in vivo*, by the intracerebroventricular injection of agonistic Fas antibodies (Benigni et al., 1998). Fas engagement induces secretion of IL-8 from several cell types including colon epithelial cells and synoviocytes (Abreu-Martin et al., 1995; Sekine et al., 1996; Schaub et al., 2000; O'Brien et al., 2002). Further inflammatory changes induced by Fas include upregulation of cell surface integrins

accompanied by increased cell migration (Jarad et al., 2002), and induction of inflammatory angiogenesis (Biancone et al., 1997). Inflammatory angiogenesis involves new capillary growth after injury, during inflammation, or during tumour growth. Fas engagement has been shown to induce the growth of new blood vessels into a matrigel™ implant *in vivo* (Biancone et al., 1997).

8.3 Tumour progression

Fas has been implicated in tumour progression (Reichmann, 2002). Tumour cells may concomitantly become resistant to Fas-induced apoptosis, and sensitive to accelerated proliferation mediated by Fas engagement (Ungfroren et al., 1998; Borset et al., 1999; Gerharz et al., 1999; Yakirevich et al., 2000; Bodey et al., 2001; Osaki et al., 2001; Mitsiades et al., 2006). In fact, many tumours co-express Fas and FasL, which could act in an autocrine manner to promote proliferation (Xerri et al., 1997; Husain et al., 1998b; Ungfroren et al., 1998; Gerharz et al., 1999; Yakirevich et al., 2000; Bodey et al., 2001; Osaki et al., 2001). FasL expression on the tumour could also promote tumour vascularization by stimulating angiogenesis as described above; tumour vascularization is essential for tumour survival and is a rate limiting step for tumour growth and malignancy (Biancone et al., 1997). FasL stimulation of apoptosis-resistant tumour cells increases tumour motility and invasiveness through activation of NKκB, ERK and caspase 8 (Barnhart et al., 2004). Given these findings, it is not surprising that a correlation has been noted between increased Fas expression and increased tumour malignancy (Donin et al., 2000).

9. Fas in the nervous system

Fas is widely expressed in the nervous system, both in neurons and in glial cells (Nat et al., 2001). Fas expression has been documented in cortical (Park et al., 1998), hippocampal (Park et al., 1998), sensory (Desbarats et al., 2003), and motoneurons (Raoul et al., 1999; Raoul et al., 2002), and in all types of glia: oligondendrocytes (Casha et al., 2001), astrocytes (Choi et al., 1999; Bechmann et al., 2000), microglia (Spanaus et al., 1998), and Schwann cells (Wohlleben et al., 2000). Fas-induced neural cell death has been extensively reported, although it was recently reported that FasL does not induce apoptosis in embryonic hippocampal and cortical neurons (Zuliani et al., 2006) or in astrocytes (Song et al., 2006). There is also mounting evidence that Fas plays other, non-apoptotic roles in the nervous system, as it does in other tissues described above.

During embryonic development and in the early postnatal period, neurons co-express Fas and FasL. In this context, Fas / FasL probably promote branching in axons and dendrites rather than control cell death (Zuliani et al., 2006). In adults, neurons generally express very low or undetectable levels of Fas constitutively, but readily upregulate Fas in response to stressors, including oxidative stress, traumatic injury, ischemia, pharmacological toxicity, excitotoxicity, and during some neurodegenerative diseases (Lambert et al., 2003).

9.1 Fas-induced death in the nervous system

Fas-induced death in the nervous system occurs under circumstances of oxidative stress. Oxidative stress induces expression of Fas and FasL in neuronal cells *in vitro* (Vogt et al., 1998; de la Monte et al., 2000; Facchinetti et al., 2002) and *in vivo*, experimental ischemia / reperfusion (a model for stroke and neonatal hypoxia) generates a large burden of ROS, and results in increased Fas expression, activation of caspase 8, and neuronal death (Felderhoff-Mueser et al., 2000; Matsushita et al., 2000; Harrison et al., 2001; Jin et al., 2001; Morita-Fujimura et al., 2001; Northington et al., 2001b; Northington et al., 2001a; van Landeghem et al., 2002). Importantly, Fas-deficient *lpr* mice have reduced infarct volume following ischemic insult (Martin-Villalba et al., 1999). Similarly, infarct volume is reduced in normal mice by treating them with FasL antibodies and neutralizing TNF antibodies, 30 minutes after inducing the stroke (Martin-Villalba et al., 1999; Martin-Villalba et al., 2001). Additionally, functional recovery in stroke and spinal cord injury was improved in Fas-deficient mice, and was promoted in wt mice by neutralizing antibodies to Fas (Martin-Villalba et al., 2001; Demjen et al., 2004). These findings demonstrate a causal link between Fas function and neuronal death.

Furthermore, mutations and chemicals that promote the accumulation of ROS sensitise cells to Fas-induced death (Giardina et al., 1999; Danielson et al., 2002; Raoul et al., 2002; Xiong and McNamara, 2002; Nitobe et al., 2003). Specifically, mutations in superoxide dismutase, which occur in some cases of human

Amyotrophic Lateral Sclerosis (ALS) and in ALS mouse models, cause abnormal ROS accumulation and progressive motoneuron degeneration (Raoul et al., 2002; Xiong and McNamara, 2002).

Also, mutations in mitochondrial DNA which affect Complex I of the electron transport chain also result in damaging ROS production and neurodegenerative disease, in this case Leber's Hereditary Optic Neuropathy (LHON), characterised by retinal ganglion cell death and optic neuropathy (Danielson et al., 2002). Both these mutations render neurons unusually sensitive to Fas-induced apoptosis (Danielson et al., 2002; Raoul et al., 2002; Xiong and McNamara, 2002). In fact, motoneurons are unusually sensitive to oxidative stress even in the absence of SOD mutations, and motoneurons are the only neuronal cell type tested that is spontaneously sensitive to Fas-induced apoptosis, when motoneurons, sensory neurons, cerebellar granule neurons, cortical neurons, and astrocytes were tested in parallel (Raoul et al., 2002). Indeed, the acute sensitivity of motoneurons to Fas-induced death was blocked by ROS scavengers (Raoul et al., 2002). Fas mediates cell death in motoneurons both *in vitro*, when the neurons are deprived of neurotrophic factors, and *in vivo*, after facial nerve axotomy (Ugolini et al., 2003). In ALS, Fas-induced death is thought to involve crosstalk between two signalling pathways; the classical apoptosis cascade as outlined above, and a newly identified cascade involving the upregulation of neuronal nitric oxide (Raoul et al., 2002; Raoul et al., 2006). Taken together, these findings provide strong evidence suggesting that Fas triggers neuronal apoptosis predominantly in an oxidative environment.

9.2 Fas-induced neural survival and regeneration

Fas clearly induces neuronal apoptosis under certain circumstances. However, there is compelling evidence that Fas engagement can also stimulate axon regrowth after injury, instead of leading inevitably to apoptosis. Work from Dr. Desbarats' lab has shown that Fas engagement with FasL or with agonistic Fas antibodies induces neurite outgrowth *in vitro*, and accelerates peripheral nerve regeneration *in vivo* (Desbarats et al., 2003; Pettmann and Henderson, 2003). Additionally, Fas / FasL promote branching in axons and dendrites in cultured immature neurons and this occurs in a caspase-independent and DD-dependent manner (Zuliani et al., 2006).

Further evidence that Fas can contribute to neuron survival and regeneration in the nervous system comes from studies done in *lpr* mice. Fas-deficient *lpr* mice display cognitive and sensorimotor deficits, *stria vascularis* cell degeneration, progressive atrophy of pyramidal neuron dendrites, and delayed neurite regeneration (Hess et al., 1993; Hikawa et al., 1997; Sakic et al., 1998; Ruckenstein et al., 1999a; Ruckenstein et al., 1999b). *Lpr* mice are primarily used as an immunological, not a neurological, model, and therefore it is likely that other neurological systems are also affected, but have not yet been studied in *lpr* mice. Most neurological findings in the *lpr* mouse have been attributed to the lupus-like autoimmune disease that they eventually develop (Sakic et al., 1998). However, potent immunosuppressive drugs, which completely prevent autoimmunity, do not prevent the neurological

manifestations in the *lpr* mouse (Ruckenstein et al., 1999a; Ruckenstein et al., 1999b). Furthermore, the neurological findings precede the autoimmune changes (Hess et al., 1993). Together, these observations suggest that neurodegeneration in the *lpr* mouse has a genetic etiology and may result directly from low Fas expression.

9.3 Mechanisms of Fas-induced neural regeneration

Work from Dr. Desbarats' lab has found that Fas-induced neurite growth in primary sensory neurons was blocked by inhibitors of the ERK pathway (Desbarats et al., 2003). Furthermore, Fas engagement induced ERK activation in neuroblastoma cells, which in turn resulted in expression of p35, a neuron-specific activator of cyclin-dependent kinase 5 essential for neurite outgrowth (Desbarats et al., 2003). The ERK / p35 pathway triggered by Fas in neurons had already been described as the molecular mechanism underlying nerve growth factor (NGF)-induced axon regeneration in sensory neurons (Tsai et al., 1994; Pang et al., 1995; Nikolic et al., 1996; Harada et al., 2001). Fas engagement can similarly activate ERK in neural stem cells (Ceccatelli et al., 2004). Thus, Fas and neurotrophin receptor signalling can converge on the ERK pathway, possibly at the level of Raf-1 (Figure 1.5). As described above, Raf-1 can be recruited into the DISC surrounding the activated Fas receptor (Kataoka et al., 2000). Raf-1 then activates MEK, the kinase which specifically activates ERK. The high affinity, tyrosine kinase neurotrophin receptors (TRKs) also activate Raf-1, but via the Grb / sos / Ras pathway (reviewed in (Patapoutian and Reichardt, 2001)). Although Fas and neurotrophin receptor

signalling can converge on one pathway, they can also both activate other, independent, pathways. Thus, the overall physiological outcomes induced by the two receptors are likely different, although they result in similar morphological effects in terms of axon regrowth in sensory neurons *in vitro* (Desbarats et al., 2003).

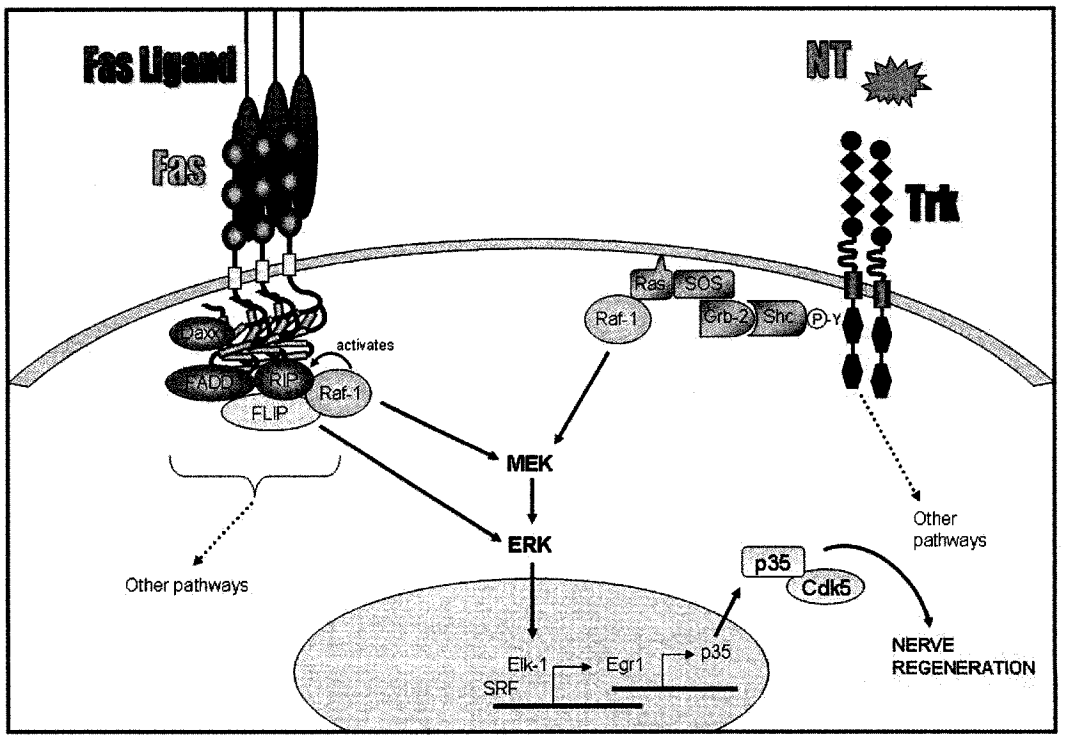

Figure 1.5: Convergence in signalling pathways activated by engagement of neurotrophin receptors and Fas. Both the neurotrophin receptors (TRK, Tyrosine Kinase Receptor) and Fas signalling pathways converge on the ERK pathway leading to axon regrowth, but each receptor also triggers other pathways. These pathways may be non-overlapping / non-convergent, and therefore result in different biological outcomes when a neuron is stimulated with Fas Ligand versus with neurotrophin (NT).

9.4 Fas-induced upregulation in the injured nervous system

Fas has been implicated in neuronal apoptosis during trauma, stroke, neurodegenerative diseases, infectious disease, drug use, and epilepsy. However, many of these studies have been correlational rather than causal: because Fas is best known as a death receptor, Fas upregulation alone is often interpreted as a sign of imminent Fas-induced apoptosis. Therefore, increased Fas expression in response to trauma or in the presence of disease has been cited extensively as evidence for Fas-induced death in the nervous system. An alternative explanation may be that Fas expression or upregulation on neurons following traumatic injury may be a protective mechanism designed to increase pro-regenerative signalling to the cell, in the same way that neurotrophin receptors are upregulated following injury (Cui et al., 2002; Qiao and Vizzard, 2002). In support of this possibility, excitotoxic central nervous system (CNS) injury induces Fas and FasL expression in conjunction with new fiber sprouting (Shin et al., 1998). Changes in Fas expression were observed for up to 40 days after the initial injury - during which time these cells co-expressing Fas and FasL failed to die - suggesting that long term upregulation of Fas may underlie an ongoing synaptic reorganization in response to injury (Shin et al., 1998). Furthermore, Fas expression is virtually absent in areas manifesting severe degeneration in Alzheimer's disease brains, while it is upregulated in adjacent, relatively spared areas (de la Monte et al., 1997). Similarly, in Creutzfeldt-Jacob disease, a human spongiform encephalopathy, subsets of neurons which are resistant to the disease express increased Fas and FasL, in conjunction with increased ERK and

MEK (Puig and Ferrer, 2001). Together, these findings further reinforce the notion that Fas expression may increase during some neuropathologies to subserve a neuroprotective or neuroregenerative function.

9.5 What regulates the outcome of Fas signalling in the nervous system?

Fas engagement can result in cell death, but also in cell proliferation, differentiation, or cytokine secretion. Which factors regulate the pathway(s) that will be engaged, and hence, the physiological outcome(s) of Fas signalling?

As discussed above, oxidative stress sensitises neurons to Fas-induced death. In motoneurons, Fas-induced death could be blocked by caspase 8 inhibitors, or by dominant-negative Daxx / ASK1, or by ROS scavengers (Raoul et al., 2002). Thus, neither the classical, caspase 8-dependent Fas death pathway, nor ROS production, was by itself sufficient to kill the motoneurons. These findings suggest that the abnormal accumulation of ROS in neurons can act as a molecular switch, turning Fas function to "death".

A second molecular switch regulating Fas function may be the ratio of caspase 8 to FLIP present in the cell, where increasing caspase 8 promotes death and increasing FLIP favours growth. Neural tissues tend to express very low levels of caspase 8, especially compared with cells that are highly Fas sensitive, such as thymocytes and T cell lines. Neurons, glial cells, and neural derived tumours all

express exceptionally low levels of caspase 8 (Teitz et al., 2001; Wosik et al., 2001; Desbarats et al., 2003). In contrast, FLIP is constitutively expressed and readily upregulated in many neural tissues (Raoul et al., 1999; Wosik et al., 2001; Desbarats et al., 2003) and can inhibit Fas-mediated apoptosis (reviewed in (Park et al., 2005). Motoneurons which have undergone *in vitro* maturation and differentiation are resistant to Fas-induced apoptosis, possibly due to the upregulation of FLIP (Raoul et al., 1999). FLIP can trigger signalling through the NFκB and ERK pathways (Kataoka et al., 2002). Thus, in the nervous system Fas may be a predominantly neuroprotective factor, rather than a death factor, due to a tissue-specific favourable FLIP to caspase 8 ratio.

Other proteins have been identified that also have the ability to block Fas-mediated apoptosis, allowing Fas to mediate other roles. Fas apoptosis inhibitory molecule (FAIM) blocks apoptosis and allows neurite outgrowth through activation of ERK and NFκB signalling pathways (Sole et al., 2004). Phosphoprotein enriched astrocytes-15 kDa/phosphoprotein enriched in diabetes (PEA-15/PED) is a protein with a DED which can also block Fas and TNF-R1-mediated apoptosis (Condorelli et al., 1999). PEA-15/PED localizes to the DISC and prevents caspase 8 activation (Ricci-Vitiani et al., 2004). Inhibition of either PEA-15/PED or FLIP can sensitize human astrocytes to Fas-mediated cell death (Song et al., 2006). Another intracellular molecule which can regulate the outcome of Fas signalling is lifeguard, a post-synaptic neural membrane protein, which is upregulated by the PI3K / Akt pathway

and mediates the resistance of cerebellar granule neurons to FasL-induced death (Beier et al., 2005).

The presence of growth factors, cytokines, and neurotrophins influences the outcome of Fas signalling through numerous mechanisms. Exposure to growth factors before Fas engagement can confer resistance to Fas-induced apoptosis (Kosai et al., 1998; Gibson et al., 1999; Haeffner et al., 1999; Boehme et al., 2000; Chodon et al., 2000; Kazama and Yonehara, 2000; Suzuki et al., 2000; Chen et al., 2001; Mitsunaka et al., 2001; Kanda et al., 2002; Steinbach et al., 2002). In fact, growth factors not only impart resistance to Fas-induced death, but often allow the cell to respond to Fas engagement with accelerated proliferation – that is, they selectively block Fas apoptotic pathways and / or promote Fas-induced proliferative signalling. Growth factors can function to protect cells against apoptosis by upregulating survival factors, particularly the antiapoptotic protein bcl-2 (and other bcl-2 family members), and the serine/threonine kinase Akt (also known as Protein Kinase B). Bcl-2 and its homologues function by stabilizing the mitochondrial membrane, which selectively blocks the mitochondrial branch of Fas-induced apoptotic signalling, while allowing parallel Fas growth signals to be transduced unimpaired (Haeffner et al., 1999; Boehme et al., 2000; Mitsunaka et al., 2001). Akt functions via several pathways, notably by interfering with DISC assembly, thus blocking apoptotic signalling at its most upstream point (Gibson et al., 1999; Suzuki et al., 2000). On the other hand, growth factors that stimulate the ERK pathway may impact on Fas growth signalling directly by upregulating FLIP. FLIP simultaneously prevents caspase 8-

triggered apoptosis, and promotes further ERK phosphorylation via FLIP-mediated recruitment of Raf-1 into the DISC (Wilson et al., 1999; Holmstrom et al., 2000; Tran et al., 2001).

The internalization of Fas can play an important role in controlling signalling pathways. Blocking Fas internalization impairs DISC formation and apoptosis, whereas when internalization is impaired and Fas is stimulated with FasL, the ERK and NFκB signalling pathways are activated (Lee et al., 2006). The Tyr 291 posttranslational modification site in the intracellular domain of Fas has been found to be important in Fas receptor internalization (Lee et al., 2006). Palmitoylation of Cys 199 is required for the formation of stable aggregates (Feig et al., 2007) and the localization of Fas to lipid rafts (Chakrabandhu et al., 2007), which are both necessary for Fas internalization.

Finally, in the physiological context it is important to consider the availability of FasL. Interestingly, following nerve injury, FasL is upregulated on glial cells (Wohlleben et al., 2000). The glial cells' response to injury is complex, and involves the production of multiple growth factors. Thus, glial cells may be expressing FasL as a means to promote regeneration, just as they are secreting neurotrophic factors. Furthermore, activated immune cells express FasL (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995) and infiltrate the injured site (Moalem et al., 1999; Schwartz et al., 1999). T cell FasL may contribute to regeneration by ligating neuron Fas, as T cell infiltration after traumatic neural injury is known to be neuroprotective and to

decrease the extent of secondary degeneration (Moalem et al., 1999; Schwartz et al., 1999). Conversely, chronic inflammation can result in the production of ROS by immune cells and activated microglia (Hensley et al., 2000). In this case, the presence of FasL on glia and immunocytes may contribute to Fas-induced apoptosis. To further complicate the situation, Fas and FasL can both be produced in soluble form. *In vitro*, soluble FasL constructs are highly effective at promoting axon regeneration (Desbarats et al., 2003). *In vivo*, soluble FasL is detected in the cerebrospinal fluid (but not in the serum) of patients after severe brain injury, and may be acting as a soluble growth factor to promote regeneration (Ertel et al., 1997). In contrast, chronic neurodegeneration seems to promote elevated levels of soluble Fas, detected in the brains of patients suffering from Alzheimer's and Parkinson's diseases (Mogi et al., 1996; Martinez et al., 2000). Soluble Fas can act as a decoy for FasL, preventing signalling through cell surface Fas, and reducing beneficial regenerative Fas signals as well as pro-apoptotic signals.

Thus, cellular context, as defined by the presence of growth factors and their receptors, expression of pro-apoptotic (e.g. caspase 8) and pro-survival (e.g. Akt) proteins, and by oxidative stress, determine whether Fas engagement will lead to death or regeneration in the nervous system. An acute injury, accompanied by growth factor secretion and in the absence of significant ischemia, may favour regenerative Fas signalling, while ROS accumulation due to mutation or chronic inflammation may promote Fas-induced apoptosis.

10. Fas and Parkinson's disease

In PD patients, Fas and FasL expression is reduced in the neurons of the SN (Ferrer et al., 2000). Concomitantly, the soluble form of Fas is increased in tissue from the nigrostriatal region, but not the cerebral cortex, of PD brains (Mogi et al., 1996). Soluble Fas can act as a decoy receptor, and can block FasL binding to cell surface Fas. Thus, Fas signalling in PD patients may be diminished both by decreased cell surface Fas expression, and by the presence of soluble Fas.

A study by Hayley and colleagues recently reported that Fas immunoreactivity in the SN of mice was significantly increased one day after MPTP exposure and suggested that this increase in Fas expression was responsible for the death of the dopaminergic neurons (Hayley et al., 2004). However, it is possible that Fas may be upregulated as a protective mechanism of dopaminergic cells. Furthermore, this study found that "Fas-deficient" mice were resistant to MPTP-induced dopaminergic neuron death, suggesting that disruption of the Fas-FasL system may be protective in PD (Hayley et al., 2004). However, the mutant mice used for these studies were not in fact Fas-deficient mice, but were mice bearing a mutation in Exon 9 of the DD of Fas. These Fas mutants are unable to signal death, but are still able to trigger other signalling pathways, and therefore are not true knockouts or nulls.

Data presented in my thesis will demonstrate that Fas is exerting a neuroprotective role in the MPTP mouse model of PD using Fas-deficient *lpr* mice

instead of the Fas "null" mice used in the Hayley study. The *lpr* mouse provides a more appropriate model to determine the function of the intact Fas molecule as these mice bear a mutation resulting in low Fas expression instead of a partial deletion to only the DD. Furthermore, I will demonstrate that Fas can regulate proteasomal activity which may account in part for its neuroprotective role in mouse PD models.

Objectives

PD is the second most common neurodegenerative disorder, affecting more than 1% of North Americans over the age of 60 (Nussbaum and Ellis, 2003). It is a relentlessly progressive degenerative disease of the nigrostriatal system and results from the selective degeneration of dopamine neurons in the SN of the brain. There are currently no proven neuroprotective or regenerative therapies available to halt disease progression.

Fas, a member of the TNF-R superfamily, has been extensively studied as a death-inducing receptor in the immune system. However, Fas is also widely expressed in a number of other cells, including in neurons, and can induce proliferation and differentiation as well as cell death. In this thesis, I present evidence supporting a neuroprotective role for Fas in PD.

The objectives of my thesis are as follows: 1) To identify the role of Fas in the well established MPTP mouse model of PD, 2) To assess the expression of Fas in peripheral blood lymphocytes of PD patients, 3) To evaluate a model of PD induced by proteasomal-inhibition in mice with the goal of studying proteasomal regulation as a potential mechanism of Fas-induced neuroprotection and 4) To determine whether Fas potentiates proteasome activity and α-synuclein degradation in an *in vivo* mouse model of PD induced by α-synuclein overexpression in the SN.

Chapter 2:

Defective Fas expression exacerbates neurotoxicity in a model of Parkinson's disease

Defective Fas expression exacerbates neurotoxicity in a model of Parkinson's disease

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Abstract

Fas (CD95), a member of the tumor necrosis factor receptor (TNF-R) superfamily, has been extensively studied as a death-inducing receptor in the immune system. However, Fas is also widely expressed in a number of other tissues, including in neurons. Here we report that defects in the Fas/Fas Ligand system unexpectedly render mice highly susceptible to neural degeneration in a model of Parkinson's Disease. We found that Fas-deficient *lpr* mice develop a dramatic phenotype resembling clinical Parkinson's Disease, characterized by extensive nigrostriatal degeneration accompanied by tremor, hypokinesia, and loss of motor coordination, when treated with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) at a dose which causes no neural degeneration nor behavioural impairment in wild-type (wt) mice. *Gld* mice, which express a mutated Fas Ligand, display an intermediate phenotype between that of *lpr* and wt mice. Moreover, Fas engagement directly protects neuronal cells from MPTP/1-methyl-4-phenylpyridinium ion (MPP^+) toxicity *in vitro*. Our data show that decreased Fas expression renders dopaminergic neurons highly susceptible to degeneration in response to a Parkinson-causing neurotoxin. These findings constitute the first evidence for a neuroprotective role for Fas *in vivo*.

Keywords: Fas, CD95, MPTP, Parkinson's Disease, neuroprotection, midbrain, *lpr*

Introduction

Fas is commonly categorized as a death receptor due to its well-defined role in apoptosis (Nagata, 1997). It is expressed throughout the central nervous system, including in glia and neurons (Choi and Benveniste, 2004) and induces neuronal apoptosis under certain conditions, such as in models of stroke and amyotrophic lateral sclerosis (Martin-Villalba et al., 1999; Raoul et al., 1999; Raoul et al., 2002). In this report, we investigated the role of Fas signalling in a mouse model of Parkinson's Disease (PD). PD is a chronic and debilitating neurodegenerative disorder, characterized by degeneration of the midbrain dopaminergic neurons of the *substantia nigra pars compacta* (SN), resulting in the hallmark symptoms of the disease, namely tremor, bradykinesia, rigidity and postural instability. The etiology of PD is unknown and in up to 95% of cases there is no identified genetic linkage (Dauer and Przedborski, 2003). Environmental factors, such as neurotoxic pesticides, have been implicated in disease pathogenesis. Exposure to the dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides a well established model of PD in rodents and primates, and has been shown to cause PD in humans (Dauer and Przedborski, 2003). MPTP crosses the blood-brain barrier where it is metabolized to its active form, the 1-methyl-4-phenylpyridinium ion (MPP^+), which is selectively concentrated in dopaminergic neurons by the dopamine transporter, resulting in dopaminergic neuronal death.

In PD patients, membrane-bound Fas and Fas Ligand (FasL) expression are reduced in the SN (Ferrer et al., 2000). Concomitantly, soluble Fas, which acts as a decoy receptor and blocks the binding of FasL to Fas, is elevated in PD (Mogi et al., 1996; Hartmann et al., 1998). Thus, Fas signalling in PD patients may be diminished both by reduced cell surface Fas expression, and by the presence of soluble Fas. Apoptosis is believed to be a factor in the neurodegeneration of PD (Vila and Przedborski, 2003), but *in vitro* models indicate that caspase-8, the upstream caspase activated during Fas-mediated apoptosis, is not involved, suggesting that Fas may not be the principal death effector in PD neurodegeneration (Gomez et al., 2001).

We used mice bearing mutations in the Fas/FasL system to directly determine the role of Fas signalling in the MPTP model of PD. We demonstrate here that reduced Fas expression dramatically increases neuronal susceptibility to MPTP toxicity *in vivo*, strongly supporting a neuroprotective role for Fas.

Materials and Methods

Mice and MPTP treatment

We used 8 to 10 week old female C57BL/6 (B6, Charles River), *lpr*, *gld*, Fas exon 9 knockout ("Fas null"), CBA and *lpr.cg* mice (Jackson Laboratories). We administered five subcutaneous injections of 25 mg/kg MPTP (Sigma) or saline, once per day for five consecutive days. Mice were used according to the McGill University Animal Care Committee.

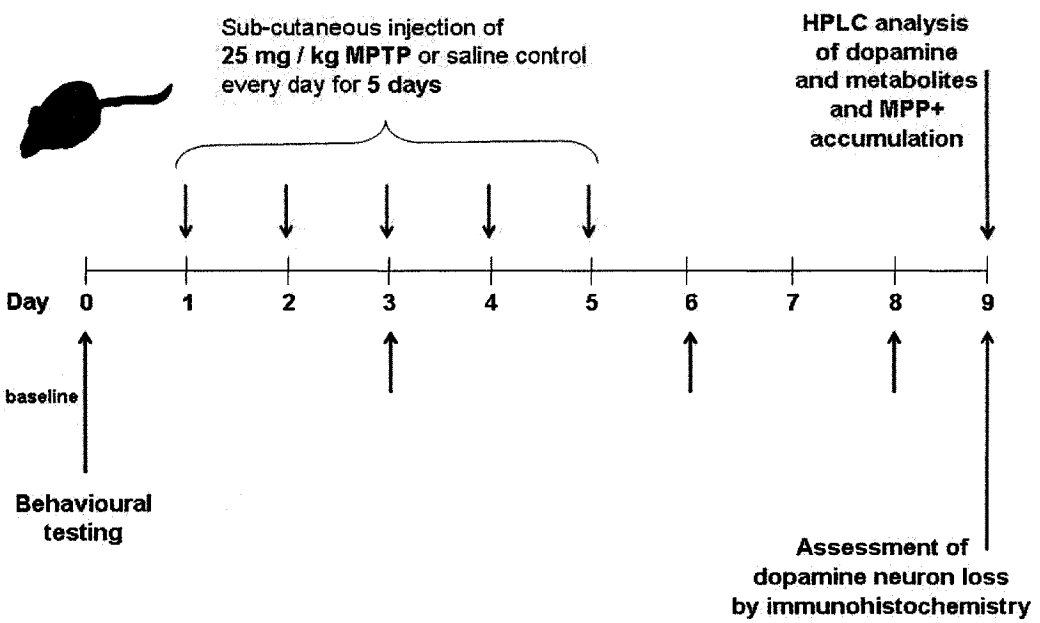

Figure 2.1 Dosing scheme for MPTP treatment and analysis

Behavioural Analysis

We carried out automated activity monitoring (AM1053 system, Cambridge Neurotechnology Ltd.) over 5 minute periods. We assessed Rotarod (IITC/Life Science) performance as the average of 3 trials of one minute each at 20 rpm. Statistical analysis was by ANOVA and Tukey test.

Brain preparation, immunohistochemistry and stereology

We perfused mice and cut 50 μm coronal brain sections through the striatum and midbrain using a freezing sledge microtome (Leica), and performed immunohistochemistry with anti-TH antibody (1:2000, Pel-Freez) on every third serial sections. A second series was processed using 0.1% cresyl violet as a Nissl stain. We obtained unbiased stereology estimates of midbrain dopaminergic neurons using StereoInvestigator software (Microbrightfield) as described (van den Munckhof et al., 2003).

Primary cultures

We prepared and treated P2-P5 midbrain cells from B6 mice with MPTP as described (Smeyne and Smeyne, 2002). FasL construct (400ng/ml, Sigma) was administered where indicated 30 min prior to MPTP. After 7 days, TH+ cells were

stained with IgG-PE Zenon kit (Molecular Probes) bound to monoclonal mouse anti-TH antibody (Sigma).

Neuroprotection assay

We plated 2 x 10^4 Fas-positive SH-SY5Y neuroblastoma cells per well in phenol-red-free DMEM in 96 well plates. We pretreated the cells with FasL construct (100ng/ml) and added MPP^+ at the indicated concentrations 30 min later, then incubated the cells for 3 days at 37° C / 5%CO_2. We assayed cell viability using WST-1 reagent (Roche Diagnostics) according to the manufacturer's instructions. As a control, the same was done on Fas-negative SH-SY5Y cells. IETD-fmk (BD Biosciences) was used where indicated and was tested by incubating 10^4 Jurkat cells/well at 37°C for 1 hour with IETD-fmk to which FasL (100ng/ml) was added for 2.5 hours. WST-1 was used to assay viability.

HPLC

HPLC was performed as described (Przedborski et al., 1996) with modifications (see supplemental methods). Monoamines were detected with an ESA system with 5011 analytical cell, and a Higgins Analytical HAISIL 100 C18 column (5µm, 150 x 4.6 mm). MPP^+ levels were measured using a Beckman 32 Karat System with a diode array detector (295 nm) on a Gemini C18 RP 150 x 4.6 mm analytical column (Phenomenex).

Patients

We recruited patients with idiopathic PD from five consecutive weekly Montreal Neurological Institute Movement Disorders Clinics. Diagnosis of idiopathic PD was established according to the following criteria: tremor, bradykinesia and rigidity (two of three), asymmetric onset, response to dopaminergic medication (in patients taking medication), and absence of the following signs suggesting another diagnosis: early dementia, early falls or balance problems, severe autonomic dysfunction and use of neuroleptic medications (Calne et al., 1992; Hughes et al., 1992). Patients with concurrent inflammatory diseases were excluded. Only men were included in this study, as we were unable to recruit enough women for statistical significance. Aged-matched men with no history of PD or inflammatory disease were recruited as controls (average ages: PD patients, 65.78 ± 6.94 years; control subjects, 68.43 ± 15.41). We obtained informed consent from all participants and all experiments involving humans were approved by the Institutional Review Board of McGill University.

Flow cytometry

We stimulated mouse lymph node cells overnight with 2.5μg/ml Concanavalin A (Sigma) in RPMI/10% FCS, then labelled the cells with phycoerythrin (PE)-conjugated anti-mouse CD95 or PE-conjugated isotype-matched

control antibodies (BD Biosciences). Human PBLs were prepared by ficoll density centrifugation from 10 ml blood. PBLs were divided into unstimulated cells, and stimulated cells using Concanavalin A as described above. Cells were labelled with fluorescein isothiocyanate (FITC) conjugated anti-human CD3, and PE conjugated anti-human CD95 or PE-conjugated isotype-matched control antibodies (BD Biosciences). Fas upregulation was defined as the change in mean PE fluorescence between the unstimulated and stimulated populations, gated on live $CD3^+$ cells.

Supplemental methods:

Measurement of striatal monoamines and metabolite levels

Striatal dopamine and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), as well as serotonin were measured by HPLC with electrochemical detection on an ESA system (Bedford, MA) equipped with a model 5011 analytical cell. Briefly, the striata were dissected, immediately frozen on dry ice and stored at –80° C. On the day of the analysis, the striata were weighed and sonicated in 500 µl of 0.12M perchloric acid containing 3.3 ng/ml dihydroxybenzylamine (DHBA) as an internal standard. After centrifugation at 13,000 g for 5 min at 4°C, 10 µl of the supernatant were injected on a Higgins Analytical (Mountain View, CA) HAISIL 100 C18, 5µm, 150 x 4.6 mm column maintained at 25° C. The mobile phase consisted of 90 mM sodium acetate, 38 mM citric acid, 0.16 mM EDTA and 0.51 mM sodium heptane sulfonate in 12% methanol

(pH adjusted at 4.25 with HCl 12N). Flow rate was 0.7 ml/min and the potentials applied across the electrodes were –175 mV for E1 and + 300 mV for E2.

MPP^+ accumulation assay

Mice were treated with 3 doses of 25 mg/kg MPTP over three days and striata were dissected 4 hours after the third dose. MPP^+ levels were measured by HPLC using a Beckman 32 Karat System (Beckman Coulter, Fullerton, CA) equipped with a diode array detector (295 nm). Briefly, the striata were treated as above and sonicated in 250 µl of 0.12 M perchloric acid containing 0.5 µg/ml of 4-phenylpyridine as internal standard. After centrifugation (as above), 40 µl of the supernatant were injected on a Gemini C18 RP 150 x 4.6 mm analytical column (Phenomenex, Torrance, CA). The mobile phase consisted of 90% 0.1 M acetic acid, 75 mM triethylamine·HCl solution (pH 2.35 adjusted with formic acid) and 10% acetonitrile at a flow rate of 1.5 ml/min at room temperature.

Results and Discussion

Fas deficiency results in markedly increased susceptibility to MPTP-induced dopaminergic neuron degeneration

We compared the MPTP susceptibility of wt C57BL/6 (B6), Fas-deficient lymphoproliferative (*lpr*), and FasL-mutated generalized lymphoproliferative (*gld)* mice, which all share the B6 genetic background. *Lpr* mice express low to absent cell surface Fas due to the insertion of a transposable element within a Fas intron (Adachi et al., 1993). *Gld* mice have a point-mutation in the extracellular domain of FasL which decreases the affinity of the Fas/FasL interaction resulting in reduced signalling through Fas (Takahashi et al., 1994; Karray et al., 2004). We treated mice with a subacute toxicity regimen of MPTP consisting of an injection of 25 mg/kg/day for five consecutive days, and quantified tyrosine hydroxylase positive (TH+) neurons as a marker for dopaminergic neurons in the midbrain (SN and ventral tegmental area, VTA) four days after the final injection of MPTP. Consistent with previous findings (Petroske et al., 2001), wt mice treated with this subacute regimen of MPTP exhibited only a marginal non-significant loss of midbrain dopaminergic neurons at this time point (Fig. 2.2 A-C). In contrast, we observed a dramatic loss of dopaminergic neurons in Fas-deficient mice. We found that the SN and VTA of MPTP-treated *lpr* mice showed a 58% ($P<0.001$) and 40% ($P<0.05$) loss of dopaminergic neurons, respectively, compared with saline-treated *lpr* mice. MPTP-treated *gld* mice lost 26% ($P<0.05$) of the dopaminergic neurons in the SN compared

with saline-treated *gld* mice (Fig. 2.2 A-C). The pre-treatment number of TH+ neurons was not significantly different between *lpr*, *gld* and wt midbrains (Fig. 2.2 B-C). The depletion of neuron cell bodies in the SN of MPTP-treated *lpr* mice was confirmed in cresyl-violet stained sections (data not shown). We also observed a striking decrease in dopaminergic innervation to the striatum in MPTP-treated *lpr* mice and to a lesser extent in *gld* mice (Fig. 2.2 D). In fact, the loss of terminals in the caudate putamen of the striatum and the sparing of the olfactory tubercle (Ot) and nucleus accumbens (NAcc) in MPTP-treated *lpr* mice resemble the pattern of terminal loss seen in the striatum of PD patients. The increased susceptibility of *lpr* mice to MPTP occurs despite lower levels of MPP+ accumulation in the striatum than wt B6 mice, as determined by HPLC four hours after the third dose of MPTP (B6: 3.29 ± 0.15 μg/g tissue, *lpr*: 2.29 ± 0.12 μg/g, *gld* 2.13 ± 0.37 μg/g). Increased neurodegeneration in the presence of decreased bioavailable MPP^+ further emphasizes the enhanced sensitivity to neurodegeneration in the absence of Fas. Thus, Fas-deficient *lpr* mice treated with MPTP display a dramatic degeneration of the nigrostriatal system. *Gld* mice, which have a partial Fas-signalling deficit (Karray et al., 2004), demonstrate an intermediate degree of neuronal loss.

Fas deficiency results in tremor, hypokinesia and decreased motor coordination following MPTP treatment

Wt mice did not exhibit significant spontaneous behavioural changes during or following the MPTP treatment course. In sharp contrast, we found that Fas-

deficient *lpr* mice displayed marked hypokinesia (Fig. 2.3 A) and developed a tremor after the second to third dose of MPTP (Video S1 A-C). Over 4 independent experiments, 78% of the *lpr* mice (n=32) became immobile during the first 4 days following the initiation of MPTP treatment, although they gradually recovered spontaneous mobility (Fig. 2.3 B). Wt mice maintained normal activity levels throughout the treatment course (Fig. 2.3 A-B), and *gld* mice displayed a behavioural phenotype more closely resembling wt mice, which correlates with their less severe neuronal loss (Fig. 2.3 A).

Over the five days of MPTP treatments, the *lpr* mice were too impaired for assessment of coordination. Three days following the final MPTP injection we found no impairment in wt mice, consistent with previous work (Petroske et al., 2001). However, we observed that *lpr* mice had impaired coordination by Rotarod testing, achieving only 39% (P <0.05) of the score of their wt counterparts (Fig. 2.3 C). *Gld* mice appeared slightly impaired, but this decrease did not reach statistical significance. Thus, Fas-deficient mice became severely hypokinetic, developed tremor, and displayed decreased coordination after exposure to MPTP at a dose that causes no spontaneous behavioural deficits in wt mice.

These results suggest that Fas is providing a neuroprotective signal which is missing in *lpr* mice and reduced in *gld* mice. This putative Fas-induced neuroprotective signal is likely ligand-dependent, since *gld* mice, like *lpr* mice, displayed increased susceptibility to neuronal loss after MPTP administration,

although degeneration was less extensive in *gld* than in *lpr* mice. The residual signal transmitted through Fas in *gld* mice (Karray et al., 2004) might explain the less severe degenerative and behavioural phenotype seen in *gld* compared with *lpr* mice.

Fas engagement is protective against MPTP/MPP$^+$ neurotoxicity *in vitro* and this neuroprotection is independent of caspase 8 activation

To determine whether Fas engagement is directly protective against MPTP toxicity in dopaminergic neurons, we treated cultured midbrain neurons with a FasL construct to induce Fas signalling, followed by exposure to MPTP. Treatment with FasL protected primary TH+ neurons from MPTP toxicity (Fig. 2.4 A). We repeated this experiment using the Fas-positive neuroblastoma cell line SH-SY5Y to determine whether Fas-induced neuroprotection operated independently of glia. We found that administration of FasL significantly reduced MPP$^+$ toxicity (Fig. 2.4 B). Fas-mediated protection did not occur in a Fas-negative subline of SH-SY5Y cells (Fig. 2.4 B). To determine whether the neuroprotective effect of Fas required caspase 8 activation, we used a specific cell permeant caspase 8 inhibitor, IETD-fmk, which effectively protects Jurkat T cells from FasL-induced apoptosis (Fig. 2.4 C). Unlike Fas-mediated apoptosis, Fas-mediated neuroprotection was not blocked by IETD-fmk (Fig. 2.4 D). Thus, Fas engagement protected dopaminergic neurons from MPTP / MPP$^+$ toxicity even in the absence of glia, and independently of caspase 8 activation.

Fas-mediated neuroprotection *in vivo* is independent of the Fas death domain

Our *in vitro* data suggested that Fas-mediated neuroprotection may be independent of the Fas death domain (DD), as the DD serves to anchor and activate caspase-8 via the adaptor protein Fas-Associated Death Domain (FADD). We tested this hypothesis in two strains of mice bearing disruptions in their Fas DD. Mice bearing an engineered disruption of the Fas death domain (Adachi et al., 1995) are commonly referred to as "Fas null" because Fas-induced death is abolished in these mice. However, the mice express a truncated Fas molecule lacking exon 9, which encodes most of the death domain, but retain the remainder of the intracellular Fas domains as well as Fas expression at the cell surface, unlike *lpr* mice which express no detectable Fas by flow cytometry (Fig. 2.5 A). These Fas exon 9 knockout (KO) mice are resistant to MPTP ((Hayley et al., 2004) and Fig. 2.5 A), in contrast to the dramatic susceptibility of *lpr* mice, as shown by striatal dopamine and dopamine metabolite levels. These mice show no significant differences in MPP^+ levels (Exon 9 KO: 3.48 ± 0.34 μg/g, B6: 3.29 ± 0.15 μg/g). We propose that this difference in susceptibility between *lpr* and exon 9 KO mice is based on Fas expression levels (Fig. 2.5 A), with Fas expression being neuroprotective.

An analogous, naturally occurring point mutation, *lpr.cg*, causes local unfolding of the death domain and likewise abolishes Fas-induced apoptosis, although *lpr.cg* mice express normal levels of cell surface Fas, and can mediate non-apoptotic signalling (Desbarats et al., 2003). The *lpr.cg* mutation is only available on

the CBA background, and therefore cannot be directly compared with the *lpr*, Fas exon 9 KO, and *gld* mice, which are all on the B6 background. Therefore, we compared MPTP susceptibility of *lpr.cg* mice to their wt CBA counterparts. We found no significant difference in TH+ neuron loss in the SN of *lpr.cg* versus wt CBA mice (Fig. 2.5 B) and in fact we observed a statistically significant protection in the VTA of *lpr.cg* compared with CBA mice (Fig. 2.5 B). Behaviourally, both Fas exon 9 KO and *lpr.cg* mice were resistant to MPTP administration ((Hayley et al., 2004) and Fig. 2.5 B, respectively). Taken together, these data indicate that a functional DD is not required for Fas-mediated neuroprotection. The observation that Fas exon 9 KO and *lpr.cg* mice are slightly more resistant to MPTP than wt mice suggests that Fas neuroprotective signalling in the absence of Fas apoptotic signalling may confer additional protection from MPTP toxicity. The abnormal immune system which is characteristic of mice lacking Fas-induced death is present in Fas exon 9 KO, *gld*, *lpr.cg*, and *lpr* mice, demonstrating that the immune abnormality in itself does not alter neuronal susceptibility to MPTP toxicity, since *lpr.cg* and exon 9 KO mice are protected while *lpr* mice become dramatically more susceptible.

Fas is upregulated in the midbrain in response to MPTP (Hayley et al., 2004), and indeed, is upregulated by multiple types of injuries or stress to the CNS (Choi and Benveniste, 2004). We propose that Fas upregulation may represent an adaptive response to stress, resulting in neuroprotection when the cell is salvageable, and neuronal apoptosis when the damage is too severe for the cell to be rescued. We have recently shown that Fas engagement can mediate neurite outgrowth via activation of

the Extracellular-signal Regulated Kinase (ERK) pathway, independent of caspase-8 (Desbarats et al., 2003). Fas engagement can similarly activate ERK in neural stem cells (Ceccatelli et al., 2004). Furthermore, it has previously been demonstrated that ERK phosphorylation can mediate a protective stress response in neurons (Anderson and Tolkovsky, 1999), and there is growing evidence that neuroprotective kinase pathways, when disrupted, may lead to genetically determined PD in humans (Shen, 2004). Thus, Fas expressed by stressed neurons may provide protective signals via protein kinase pathways, which, when disrupted, lead to increased susceptibility to PD.

Peripheral blood lymphocytes (PBL) from PD patients show decreased Fas upregulation upon activation

Due to the extreme susceptibility to toxin-induced PD demonstrated by mice with reduced Fas expression, we investigated Fas expression in patients diagnosed with idiopathic PD. We obtained blood samples from PD patients and controls without PD as a simple and minimally invasive means to measure Fas expression. We found that baseline Fas expression in PBLs from patients with PD did not differ from those of control subjects without PD. However, when we stimulated the peripheral blood T cells (PBT) with mitogen to induce Fas upregulation, we found a highly significant deficit ($P<0.001$) in the ability of PBTs from PD patients to upregulate Fas (Fig. 2.6). Thus, PD patients show an impairment of Fas upregulation, at least in PBTs.

Although it is possible that the medication prescribed for PD may influence Fas regulation, two of the patients included in our study were not on any medication, and both of these individuals displayed very low levels of Fas upregulation, similar to those of treated PD patients (Fig. 2.6). Furthermore, there was no correlation between level of Fas upregulation and disease duration in our patients. Thus, it is possible that decreased ability to upregulate Fas was a pre-existing condition in these patients. If this impairment in Fas upregulation in T cells correlates with defective upregulation of Fas in other tissues upon cellular stress, this may confer predisposition to PD. Although the clinical data are in accordance with the results from our mouse study, it will be important to validate the clinical findings in a larger patient population.

Our studies demonstrate for the first time that a reduction in Fas expression or signalling dramatically increases neuronal susceptibility to neurodegeneration *in vivo*, strongly supporting a neuroprotective role for Fas, at least in some contexts. We propose that the lack of Fas in *lpr* mice, and the decreased Fas signalling in *gld* mice, is responsible for the increased neuronal death due to the lack of a Fas-dependent neuroprotective signal. The striking level of dopaminergic neuron degeneration in the midbrain of MPTP-treated *lpr* mice, together with their profound behavioural phenotype, may provide a new model for PD research in which to test potential therapeutics and neuroprotective strategies. Moreover, individuals with an impaired ability to upregulate Fas expression may be at increased risk of developing PD following exposure to environmental neurotoxins.

Figures

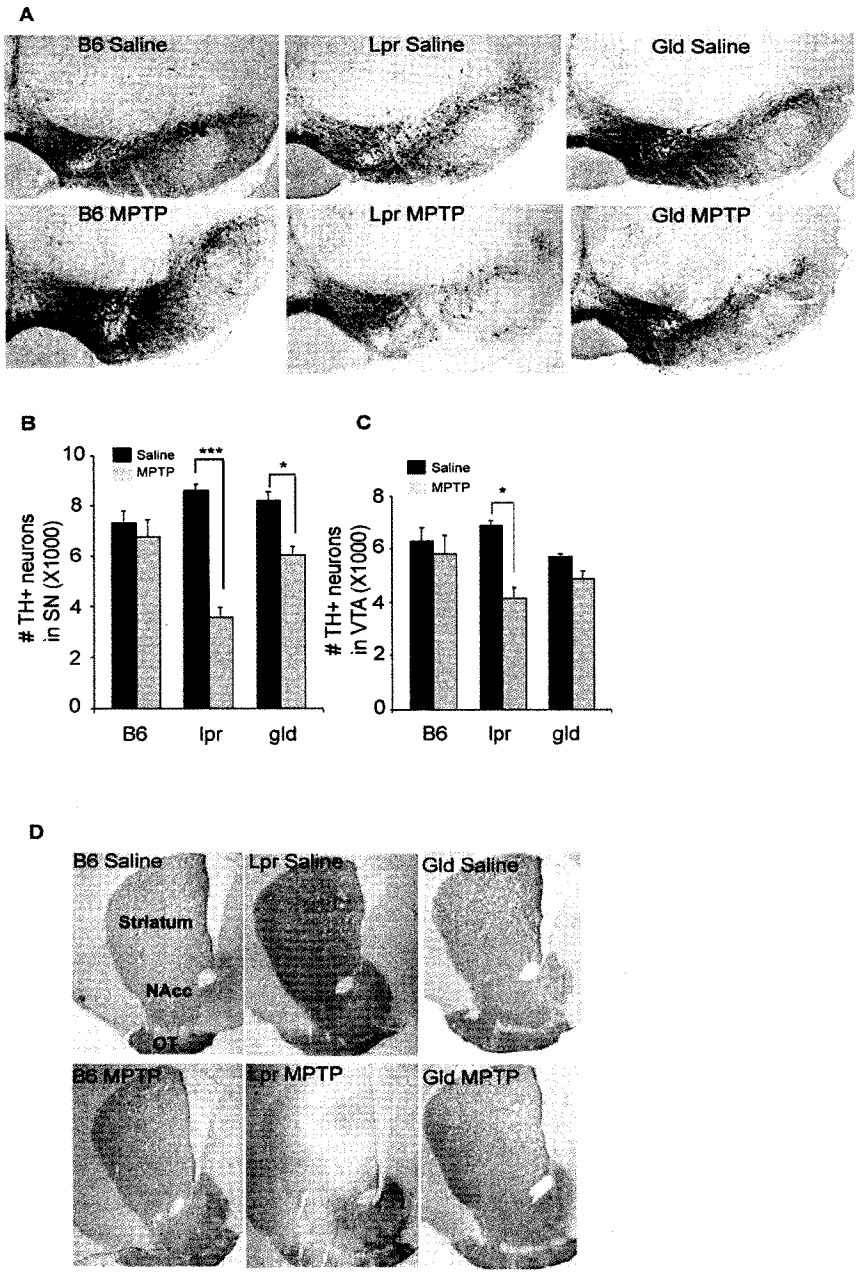

Figure 2.2: Massive loss of dopaminergic neurons and striatal projections in MPTP-treated Fas-deficient mice. (A) TH stained sections through the midbrain (SN and VTA) in MPTP or saline treated wt, *lpr* and *gld* mice. (B, C) The number of

TH+ neurons in the SN (B) and VTA (C) were determined by unbiased stereology. (n=4, error bars represent standard error (SE), *$P<0.05$, **$P<0.01$, ***$P<0.001$.) (D) TH stained sections through the striatum in saline or MPTP-treated wt, *lpr* and *gld* mice. No loss of staining is observed in wt striata, but extensive depletion of SN projections to the caudate putamen with relative sparing of the nucleus accumbens (Nacc) and olfactory tubercle (Ot), is seen in *lpr* mice.

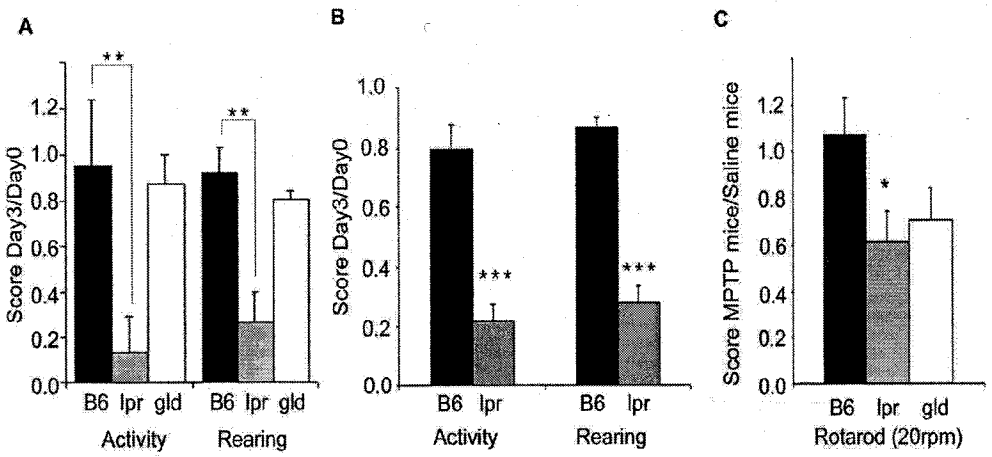

Figure 2.3: *Lpr* mice become profoundly hypokinetic after exposure to MPTP.

(A) Total activity and rearing are shown for wt, *lpr* and *gld* mice (same mice as in Fig. 2). Scores were generated as the ratio of pre-treatment to post treatment activity for each individual mouse. Activity was measured 20 hours after the second injection of MPTP, in order to avoid the acute effects of the toxin. (B) Pooled behavioural analysis over 4 experiments is shown. (B6 n=18, *lpr* n= 32) (C) Rotarod scores were calculated as the ratio of performance of MPTP-treated to saline-treated mice, 3 days after the final injection of MPTP.

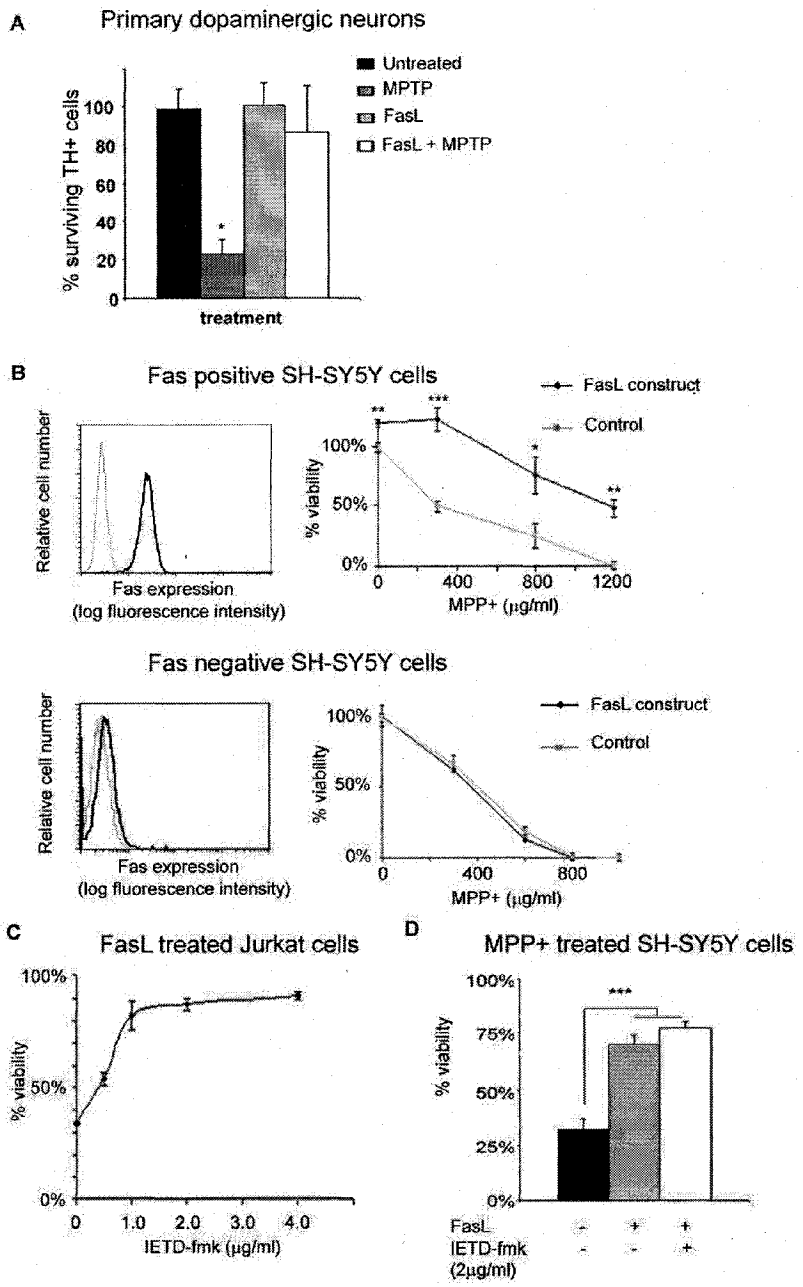

Figure 2.4: Fas-induced neuroprotection is independent of caspase 8 activation. (A) Primary dopaminergic neurons were treated as shown with FasL and/or 50µM MPTP. Untreated cells are defined as having 100% viability. (B) Fas positive and

negative SH-SY5Y cells (as assessed by flow cytometry) were treated with FasL (black line) or PBS (grey line) 30 min prior to MPP^+ exposure. 100% viability represents untreated SH-SY5Y cells. (C) Jurkat cells were killed by FasL and this cell death was blocked by the caspase 8 inhibitor IETD-fmk. (D) SH-SY5Y cells were treated with IETD-fmk one hour before addition of FasL and/or 800μg/ml MPP^+ to the culture. Error bars show SE.

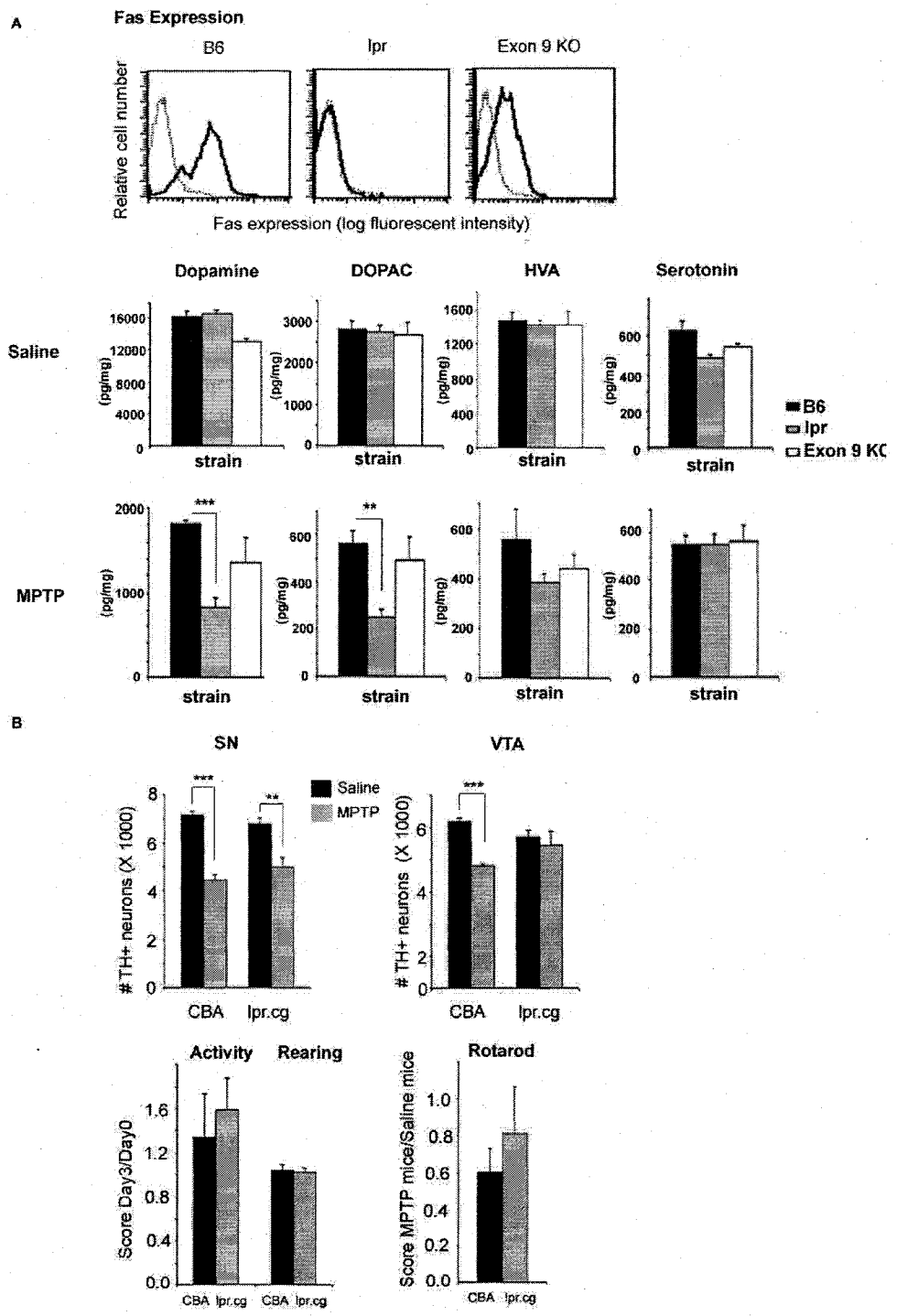

Figure 2.5: Fas-mediated neuroprotection *in vivo* is independent of the Fas death domain. (A) Exon 9 KO mice have a deleted DD and are no more susceptible to

MPTP than wt mice. *Lpr* mice (MPTP-susceptible) do not express detectable Fas, whereas wt and exon 9 KO mice express Fas as determined by flow cytometry. (Fas expression, solid line; background staining, dotted lines). MPTP-treated *lpr* mice show reduced striatal dopamine, DOPAC and HVA, as determined by HPLC, compared with B6 and exon 9 KO mice. Serotonin levels were included as a control and were not affected by MPTP. (B) *Lpr.cg* mice have a disrupted DD and are no more susceptible to MPTP than wt CBA mice, as shown by TH+ neuron counts (saline-treated, black bars; MPTP, grey bars; n=3) and behaviour (CBA, black bars; *lpr.cg*, grey bars; n= 4-6). Methodology was as described in Figs. 2 and 3.

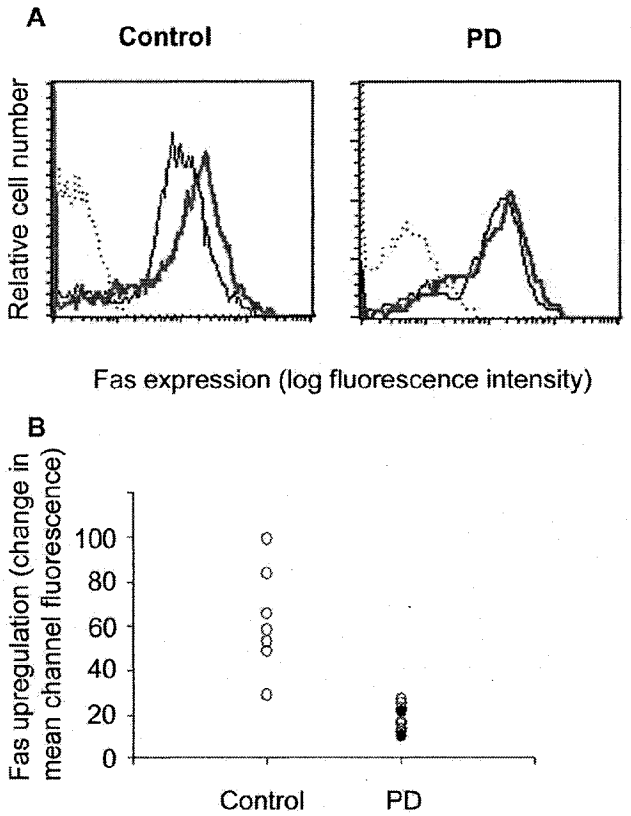

Figure 2.6: Fas upregulation is decreased in PD patients. (A) Representative flow cytometry histograms show that cell surface Fas expression is upregulated by activation on PBTs from control subjects, but not from PD patients. Histograms show background staining (dotted lines), Fas expression on unstimulated T cells (black lines), and Fas expression on activated T cells (thick grey lines). (B) Fas upregulation on T cells, calculated as the difference in mean Fas fluorescence between unstimulated and activated T cells (as shown in A), is shown for control subjects and PD patients ($P<0.001$). Filled circles represent the two PD patients not taking medication. Proliferation (% blast, 12.0 ± 2.5 for controls; 13.7 ± 4.9 for PD patients) and viability (97 ± 1 live cells for controls, 95 ± 2 for PD patients) were equivalent in the T cells from control and PD patients.

Acknowledgements

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Transition to Chapter 3

In Chapter 2 we demonstrated that despite the fact that Fas is often considered to be an inducer of apoptosis, Fas can also be a neuroprotective agent in the nervous system. Fas provided protection for dopaminergic neurons in cellular and mouse models of PD. Furthermore, we demonstrated that PD patients have a deficit in Fas upregulation which may, in part, explain their susceptibility to this debilitating neurodegenerative disease.

Next, we aimed to evaluate the role of the proteasome in Fas-deficient mice, as defects in the UPS are implicated in PD. This was to be done with the goal of elucidating the mechanism of Fas-mediated neuroprotection. However, before looking at the role of Fas, it was first necessary to evaluate proteasomal inhibition as a model of PD in wt mice. At the time we began these studies, there was only one published paper using the PSI model in rats, and none in mice. The PD research community had very high expectations based on the promising data from this one study done in rat, however, these data had not yet been replicated and mouse studies had not been performed with the proteasomal inhibitor PSI. Chapter 3 represents the work in which we evaluated the PSI-induced proteasome inhibition as as model of PD in wt mice.

Chapter 3

The PSI model of Parkinson's disease in mice is confounded by neurotoxicity of the ethanol vehicle

The PSI model of Parkinson's disease in mice is confounded by neurotoxicity of the ethanol vehicle

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Running Title: Ethanol-Induced Nigrostriatal Damage

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Abstract

Defects in the ubiquitin-proteasome system have been implicated in Parkinson's Disease (PD). Recently, a rat model of PD was developed using a synthetic proteasome inhibitor, PSI (Z-Ile-Glu(O*t*Bu)-Ala-Leu-al). We attempted to transfer this model to mouse studies, where genetics can be more readily investigated due to the availability of genetically modified mice. We treated C57BL/6 (B6) mice with six intraperitoneal injections of 6mg/kg PSI in 50 µl of 70% ethanol over a two week period. We found significant decreases in nigrostriatal dopamine in PSI-treated compared to saline-treated mice. However, we observed similar decreases in the ethanol-treated vehicle control group. Administration of ethanol alone led to significant long-term alterations in dopamine levels. Ethanol significantly eclipses the effects of PSI in the dopamine system and therefore is a confounding vehicle for this model.

Keywords: Parkinson's Disease, proteasome, PSI, mouse model, ethanol, neurotoxicity

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, after Alzheimer's disease. It is a relentlessly progressive degenerative disease of the nigrostriatal system and results from the selective degeneration of dopamine neurons in the SN of the brain. The consequent deficiency in striatal dopamine gives rise to the characteristic symptoms of the disease, including tremor, bradykinesia, rigidity and postural instability.

The proteasome is the main intracellular mechanism responsible for proteolytic degradation, and is critical in the clearance of misfolded and oxidized proteins (Sherman and Goldberg, 2001). Defects in protein degradation by the ubiquitin proteasome system (UPS) have been reported in clinical PD. In familial PD, rare mutations in parkin and ubiquitin C-terminal hydrolase 1 are implicated in dysfunction of the UPS (Leroy et al., 1998; Shimura et al., 2001). In sporadic PD, patients exhibit structural and functional defects in the 26/20S proteasome in the SN of the brain (McNaught et al., 2003). Proteasome inhibition has also been used to induce experimental PD. Recently, an *in vivo* rat model of PD was developed using a synthetic small peptide proteasome inhibitor, PSI (Z-Ile-Glu(O*t*Bu)-Ala-Leu-al) (McNaught et al., 2004). PSI-treated rats displayed a progressive Parkinsonian syndrome including hypoactivity and tremor and exhibited neuropathology characteristic of PD (McNaught et al., 2004). However, recently numerous groups have been unable to replicate this model (Beal and Lang, 2006). In our study, we

attempted to transfer the rat PSI model of PD to mouse studies, where genetic factors can be more readily investigated using genetically modified mice.

Materials and Methods

Mice and behaviour

Eight to ten week old C57BL/6 (B6) mice (Charles River) were used in this study according to the McGill University Animal Care Committee Guidelines. PSI (Calbiochem) was dissolved in 100% ethanol and diluted in water to 70%. Mice were injected with six subcutaneous injections of 6 mg/kg PSI in 70% ethanol, or ethanol alone as a vehicle, over a two week period (on Mondays, Wednesdays and Fridays). A separate group of mice was treated with saline as an additional control. Weight gain and behavioural deficits were monitored weekly over the next 8 weeks using automated activity monitoring (AM1053 system, Cambridge Neurotechnology Ltd.) for 5 minute periods to assess total activity and rearing.

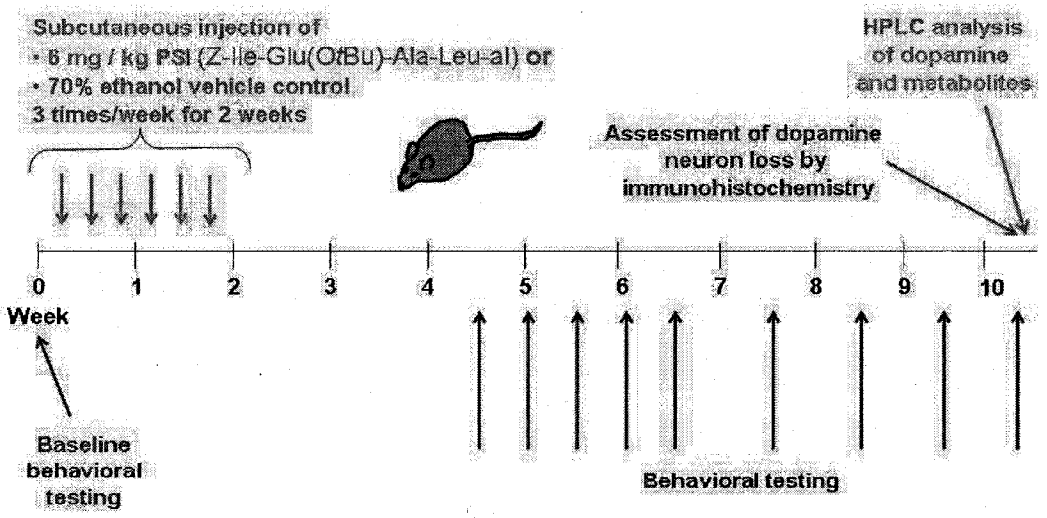

Figure 3.1: Dosing scheme for PSI treatment and analysis

Pathology and HPLC

At the 8th week following the final injection, mice were killed by cervical dislocation and their brains were quickly removed. The right side of the brain was fixed in paraformaldehyde, cryoprotected in 30% sucrose and cut into 20 μm coronal brain sections through the striatum and midbrain using a freezing sledge microtome (Leica). Immunohistochemistry was performed with anti-tyrosine hydroxylase (TH) antibody (1:2000, Pel-Freez) on every fourth serial section and TH positive neurons in the SN were quantified using Metamorph software. This method correlated well with results from unbiased stereology in our previous study (Landau et al., 2005). Striata were dissected from the left half of the brain and analyzed for dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by High Performance Liquid Chromatography (HPLC) as previously described (Landau et al., 2005).

Results

Mice treated with PSI gained significantly less weight than untreated or ethanol-treated mice over the ten week period studied (Fig 3.2 A). Two weeks after the final injection, PSI-treated mice had significantly lower total activity scores than ethanol-treated mice (Fig 3.2 B). However, scores did not get progressively worse during the course of the study. Rearing activity was significantly decreased in PSI-treated mice two weeks after the final injection compared to ethanol treated mice, but recovered to normal by the end of the study (Fig 3.2 C). Together, these data show that PSI induces a significant but transient decrease in activity.

PSI-treated mice displayed a 34% loss of TH+ neurons in the SN compared to saline-treated control mice. Surprisingly, we observed a similar (29%) loss of dopaminergic neurons in the ethanol-treated mice (Fig 3.3 A). Consistent with this, we found a 2-3 fold decrease in dopamine, DOPAC and HVA by HPLC in the striata of PSI-treated mice compared to saline-treated mice. Again, we observed a similar decrease in dopamine and its metabolites in the ethanol-treated vehicle control group (Fig 3.3 B). Together, these data indicate that ethanol alone, in the absence of PSI, was sufficient to cause depletion of striatal dopamine and loss of dopaminergic neurons.

Discussion

There has been some conflicting data in the literature as to the effects of proteasomal inhibition in rats. Two reports by McNaught and colleagues demonstrate PD-like neuropathology and behavioural symptoms in rats using proteasomal inhibition by systemic administration of epoxomicin and PSI (McNaught et al., 2004) and by direct injection of lactacystin into the brain (McNaught et al., 2002b). As well, other groups have reported that the stereotaxic injection of proteasome inhibitors into the brain can induce nigrostriatal degeneration (Fornai et al., 2003; Sun et al., 2006). However, in a study by Inden et al., the injection of proteasome inhibitor directly into the rat SN was not toxic to dopaminergic neurons, and in fact, this injection protected cells from 6-OHDA neurotoxicity (Inden et al., 2005). Recently, several studies have attempted to validate the proteasome inhibition rat model put forth by McNaught et al. (McNaught et al., 2004), and it was concluded that PSI, at this point in time, does not induce a reproducible model of PD (Beal and Lang, 2006).

In our study, we did not intend to reproduce the findings of McNaught's group; instead, we attempted to extend the model to mice. Mice treated with PSI in ethanol displayed small but significant, transient behavioural deficits compared to ethanol-treated mice. McNaught et al. (McNaught et al., 2004) reported progressive behavioural impairment and tremor in PSI-treated rats, which we did not see here. The behavioural deficits observed in our study, although not progressive, do suggest that there is a small but significant effect of the proteasome inhibitor PSI in mice.

The ethanol vehicle alone led to a significant decrease in striatal dopamine and metabolite levels, and induced loss of dopaminergic neurons in the SN, similar to those induced by PSI dissolved in ethanol. This introduces a serious confounding factor in this protocol of PSI-induced PD. In agreement with our data, Przedborski and colleagues find that there is no significant difference in dopaminergic neuron numbers in the SN of ethanol compared with PSI in ethanol-treated mice (Bove et al., 2006). However, in addition, we demonstrate here that both treatment groups experience significant losses of nigrostriatal TH^+ neurons, dopamine, and dopamine metabolites compared with saline-treated mice. The finding that the ethanol vehicle can act as significant confounding factor in the model of McNaught et al.(McNaught et al., 2004) may help to explain the poor reproducibility of the PSI model in the hands of different investigators.

Ethanol has been previously shown to cause pathological changes in different brain regions in experimental animals. Chronic ethanol administration suppressed axon sprouting in the dentate gyrus of the hippocampus (Lind et al., 1988). Postnatal cerebellar rat purkinjie cells and occipital neurons are vulnerable to ethanol exposure (Mooney and Napper, 2005; Pierce et al., 2006). Chronic alcohol exposure decreases levels of dopamine and DOPAC in the ventral striatum and results in a twenty percent decrease in tyrosine hydroxylase protein levels (Rothblat et al., 2001). Another group found that dopamine and its metabolites were depleted 23 weeks after an ethanol diet,

and attributed this to nigrostriatal neuronal loss (Collins et al., 1990). These alterations in the dopamine system due to ethanol are consistent with our findings.

Mechanistically, Collins proposed that alcohol abuse can favor the formation of endogenous alkaloids analogous to 6-hydroxydopamine (6-OHDA) that might have the potential to cross-link proteins, generate reactive oxygen species and damage neurons (Collins, 1982, 2002). In addition, ethanol can induce fibrillization of α-synuclein *in vitro* (Munishkina et al., 2003), which might contribute to ethanol-induced toxicity.

Ethanol, as well as its metabolite acetaldehyde, have previously been shown to potentiate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice by enhancing the depletion of dopamine and dopamine metabolites in the striatum and increasing MPTP-induced death of dopaminergic neurons in the SN (Zuddas et al., 1989). Another study found the same to be true in the 6-OHDA model (Ming et al., 2004). One potential explanation for this effect could be the decrease in proteasome function due to ethanol and its metabolite acetaldehyde (Rouach et al., 2005). In a microarray analysis in which cortical neurons were treated with ethanol for five consecutive days, the expression of several genes involved in the UPS, including the α and β subunits of the 20S proteasome, were decreased (Gutala et al., 2004).

Furthermore, our data show that ethanol administration may result in long-lasting effects on striatal dopamine metabolism up to two months after exposure. This may be due to ethanol-induced loss of dopaminergic neurons in the SN of the brain. The long-term effects on dopamine metabolism seen in this study are consistent with work done using the 6-OHDA model of PD. Casolini and colleagues found a depletion of dopamine in the ventral and dorsal striatum three weeks after destruction of dopamine neurons in the SN by injection of 6-OHDA (Casolini et al., 1993).

Despite our findings which could implicate alcoholism as a risk factor in PD, there has been no proven positive or negative clinical link between alcohol and PD (Hernan et al., 2004) and no differences in allele frequencies of aldehyde dehydrogenase 2 between people with PD and controls (Fujii et al., 1998). However, people with PD consume less alcohol than controls (Hellenbrand et al., 1996; Fujii et al., 1998). Interestingly, chronic alcoholics demonstrated transient signs of parkinsonism during alcohol withdrawal or severe intoxication (Carlen et al., 1981).

In summary, we propose that ethanol, independent of PSI, can provoke a loss of dopaminergic neurons in the SN and a long-lasting depletion of nigrostriatal dopamine. While PSI may induce certain behavioural elements of a PD syndrome, PSI administered in 70% ethanol does not provide an adequate model of PD in B6 mice. Finally, the interesting long-term effects of ethanol on the dopaminergic system described here deserve further investigation.

Figures

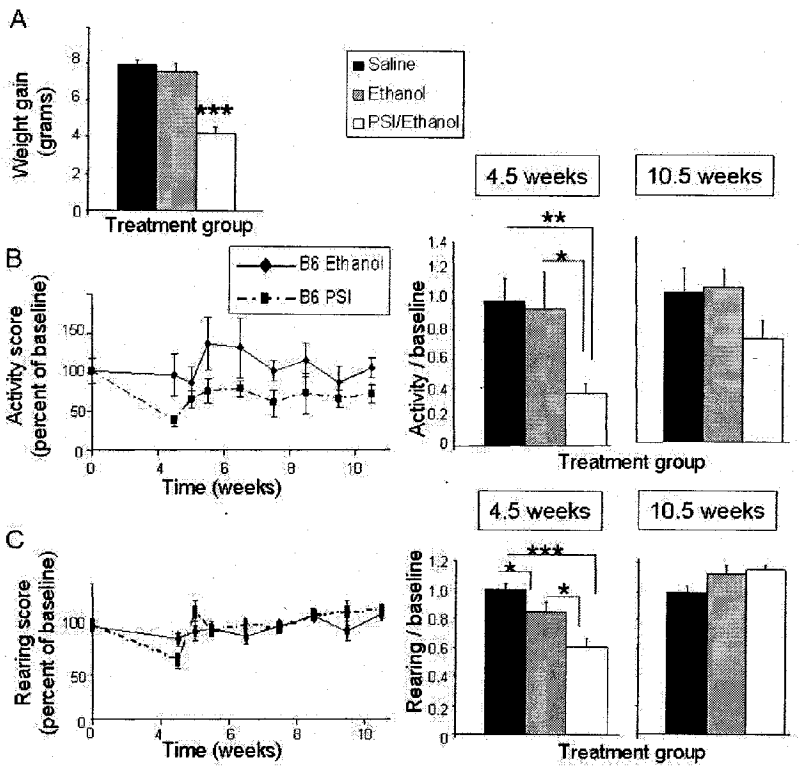

Figure 3.2: Delayed weight gain and hypokinesia in PSI-treated mice. (A) Weight gain over the 10-week study period is shown for saline (black bars), ethanol (grey bars) and PSI/ethanol-treated mice (white bars). (B) Total activity is shown for ethanol (solid line) and PSI-treated mice (dotted line) over time as percent of baseline, and at 4.5 and 10.5 weeks after initiation of the study. (C) Rearing is shown for ethanol and PSI-treated mice over time as percent of baseline, and at 4.5 and 10.5 weeks. (n=5 mice per group, error bars represent standard error (SE), $*P<0.05$, $**P<0.01$, $***P<0.001$.)

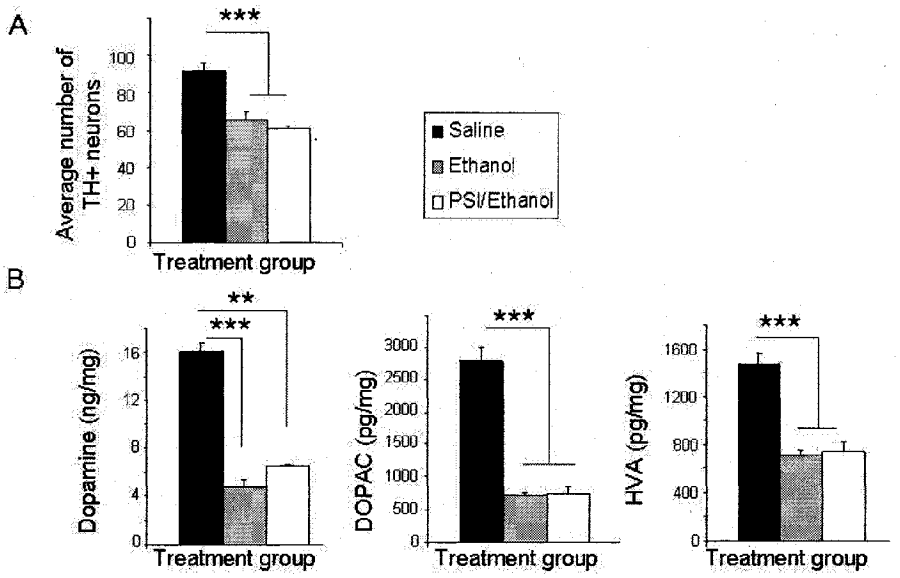

Figure 3.3: PSI and ethanol alone induce dopamine cell loss and alterations in dopamine metabolism. (A) Number of dopaminergic (tyrosine hydroxylase-stained) neurons in the SN pars compacta is shown for saline, ethanol and PSI/ethanol-treated B6 mice. (B) Striatal dopamine, DOPAC and HVA levels are compared in saline, ethanol and PSI/ethanol-treated B6 mice. (n=4-6 mice per group, error bars represent SE, $^{**}P<0.01$, $^{***}P<0.001$.)

Acknowledgements

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Supplemental data

PSI and Ethanol induce long term increases in striatal serotonin levels

We evaluated the PSI proteasome inhibition model of PD in wt mice and found that the use of ethanol as a control was a major confounding factor. Ethanol on its own caused a decrease in dopamine and its metabolites and a reduction in TH+ dopaminergic neurons in the SN. Furthermore, we found increased levels of serotonin and its metabolite 5-hydroxyindoleacetic acid (5HIAA) in both ethanol and PSI/ethanol treated mice (Figure 3.4). This long-term upregulation of serotonin is consistent with work done using the 6-OHDA model of PD. Previous reports indicate that dopamine cell loss induced by 6-OHDA is followed by serotonergic hyperinnervation throughout the striatum (Guerra et al., 1997), and this is thought to correspond closely to the loss of tyrosine hydroxylase terminals in the striatum (Towle et al., 1989).

Fas is not protective against ethanol-induced neurotoxicity

As we have provided evidence of Fas-mediated neuroprotection in the MPTP model of PD in Chapter 2, we decided to evaluate a potential neuroprotective role for Fas in the ethanol-induced neurotoxicity model. We found that *lpr* mice are not more susceptible to ethanol-induced toxicity than wt mice. There were no significant differences in levels of striatal dopamine and metabolite content or in the percentage of TH cell loss in wt versus Fas-deficient *lpr* mice when exposed to ethanol (Figure 3.5). These data indicate that Fas does not provide protection against ethanol-induced

neurotoxicity, highlighting the fact that the roles of Fas may depend on the mechanisms of toxicity.

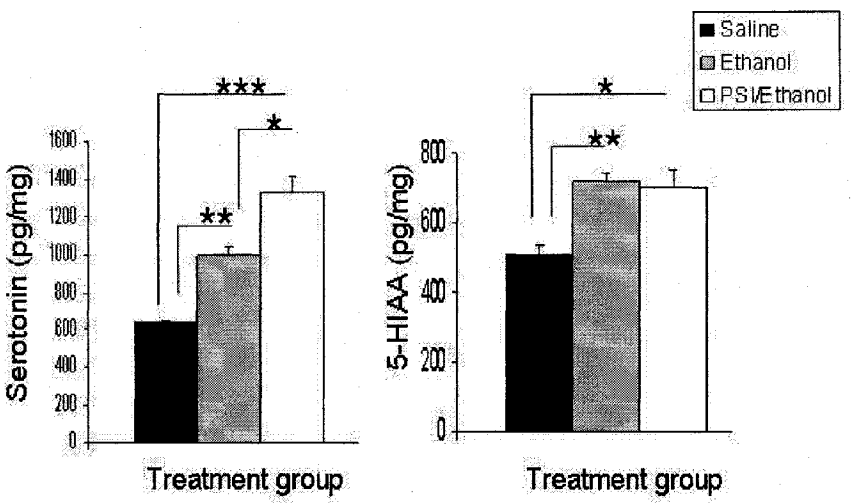

Figure 3.4: PSI and ethanol both induce long term changes in striatal serotonin and its metabolite 5HIAA. PSI and ethanol-treated B6 mice show increased striatal serotonin and 5HIAA as determined by HPLC, compared with saline-treated mice. (n=4-6 mice per group, error bars represent SE, *$P<0.05$, **$P<0.01$, ***$P<0.001$.)

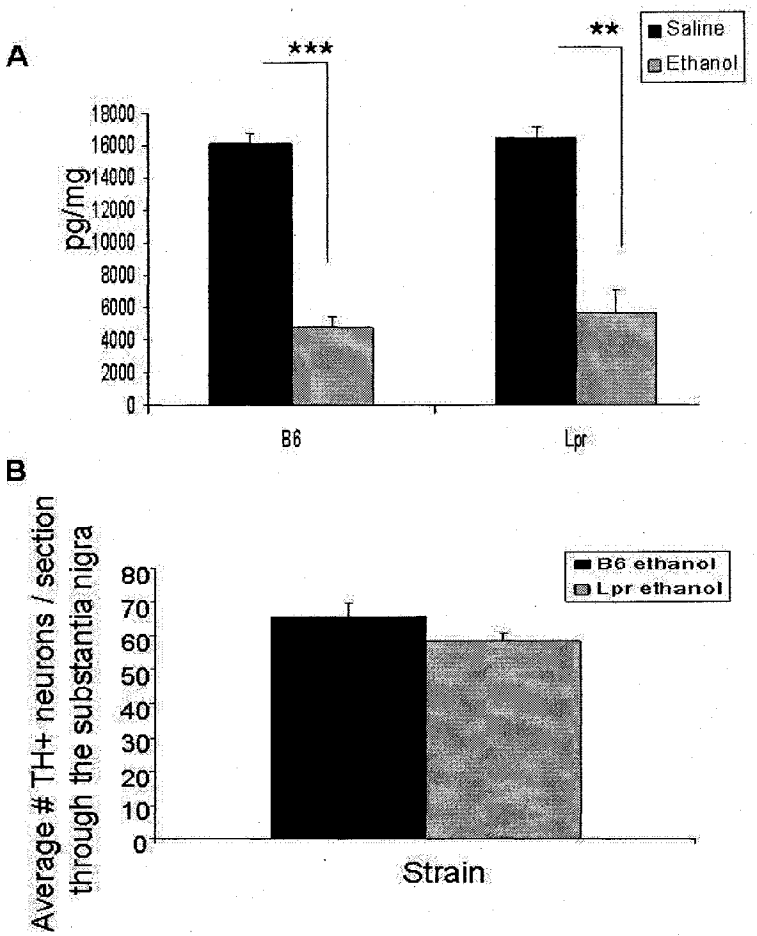

Figure 3.5: Fas is not protective against ethanol-induced neurotoxicity A) No differences are found between wt and Fas-deficient *lpr* mice strains in striatal dopamine content (or metabolite levels, data not shown) by HPLC in control saline-treated or ethanol-treated mice. B) No difference in the number of dopamine neurons in the SN are observed between the two strains after ethanol treatment. (n=4-6 mice per group, error bars represent SE, $^{**}P<0.01$, $^{***}P<0.001$.)

Transition to Chapter 4

In Chapter 2 we demonstrated a neuroprotective role for Fas in an MPTP-induced mouse model of PD. In Chapter 3 we evaluated a new, proteasomal-inhibition mouse model of PD with the intention of studying the relationship between Fas and the UPS. We determined that the PSI model of PD in mice had a major shortcoming, the neurotoxicity induced by the ethanol vehicle, and we were therefore unable to use this model to evaluate Fas-mediated neuroprotection.

In Chapter 4 we attempted to evaluate the role of Fas in modulating UPS function in PD models. In this chapter we measure proteasomal activities in wt and *lpr* mice treated with MPTP to see if there are any deficits in proteasome activity in *lpr* mice due to their lack of Fas expression. We also study the relationship of Fas and the UPS in the α-synuclein model of PD.

Chapter 4

Fas induces proteasome activity and is neuroprotective in α-synuclein-induced Parkinsonism

Fas induces proteasome activity and is neuroprotective in α-synuclein-induced Parkinsonism

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To be submitted

Abstract

Fas is a prototypical death receptor that can paradoxically exert regenerative and neuroprotective effects through largely unknown mechanisms. We have recently demonstrated that Fas is neuroprotective in a mouse model of Parkinson's disease. Decreased activity of the ubiquitin-proteasome system and accumulation of α-synuclein aggregates have been implicated in Parkinson's disease pathogenesis. Here we show that stimulation of Fas can upregulate proteasomal activity *in vitro*, and that Fas-deficient *lpr* mice demonstrate decreased proteasomal activity compared with wild-type mice when exposed to a Parkinsonian-inducing toxin *in vivo*. Furthermore, Fas expression accelerated α-synuclein degradation *in vitro*, and Fas-deficient mice developed significantly greater behavioural deficits and midbrain neuropathology than wild-type mice after intranigral injection with adeno-associated viral vectors containing α-synuclein. These findings represent a new Fas function, and a novel mechanism of Fas-induced neuroprotection through upregulation of proteasomal activity and consequent decreased accumulation of toxic α-synuclein aggregates.

Keywords: Fas, CD95, proteasome, MPTP, α-synuclein, Parkinson's Disease, neuroprotection, midbrain, *lpr*

Introduction

Fas (CD95), known primarily as a death receptor due to its well-defined role in apoptosis (Nagata, 1997) has many diverse functions (Lambert et al., 2003). Fas is expressed throughout the central nervous system during development, and in the adult is mainly expressed in response to cellular stress or injury (Choi and Benveniste, 2004). Although the activation of Fas can induce neuronal apoptosis in some contexts (Martin-Villalba et al., 1999; Raoul et al., 1999; Raoul et al., 2002), apoptosis is not the only outcome of Fas signalling in the CNS. Fas can promote recovery after sciatic nerve crush and induce neurite outgrowth in dorsal root ganglion cells (Desbarats et al., 2003). Recently, we investigated the role of Fas signalling in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of Parkinson's Disease (PD). We demonstrated that reduced Fas expression significantly increased neuronal susceptibility of dopaminergic neurons to MPTP toxicity *in vivo*, supporting a neuroprotective role for Fas (Landau et al., 2005).

PD is a relentlessly progressive degenerative disease of the nigrostriatal system. Symptoms of PD include bradykinesia, or slowness of movement, tremor, postural instability and impaired motor coordination. PD results from the selective degeneration of dopamine neurons in the SN of the brain. The remaining neurons contain Lewy bodies, which are protein aggregates composed of α-synuclein, ubiquitin and parkin. The aggregation of modified proteins is a pathological hallmark present in many neurodegenerative diseases (Ciechanover and Brundin, 2003).

Defects in protein degradation by the ubiquitin proteasome system (UPS) have been reported in PD. In familial PD, rare mutations in *parkin* and *ubiquitin C-terminal hydrolase 1* are implicated in dysfunction of the UPS (Leroy et al., 1998; Shimura et al., 2001). Selective impairment of proteasome activity and reduced expression of proteasomal subunits have been reported in *postmortem* tissue from the SN pars compacta of patients with sporadic PD (McNaught et al., 2002a; McNaught et al., 2003). Proteasome inhibition has also been used to induce experimental PD in animal models (McNaught et al., 2004). A hypodysfunctional UPS may underlie abnormal protein accumulation, thereby facilitating the formation of toxic protein aggregates and increasing the vulnerability of nigral dopaminergic neurons to degeneration in sporadic PD.

α-synuclein, the main component of LBs, aggregates and can inhibit proteasome activity. Proteasome activity is decreased in cells overexpressing α-synuclein (Fujita et al., 2006). Overexpression of α-synuclein *in vivo* results in behavioural, neurochemical and neuropathological defects in mouse models (Masliah et al., 2000; Giasson et al., 2002; Richfield et al., 2002; Gomez-Isla et al., 2003), and in humans, duplications (Chartier-Harlin et al., 2004) and triplications (Singleton et al., 2003) of α-synuclein can lead to familial PD. Several lines of α-synuclein null mice have been found to be partially resistant to MPTP (Dauer et al., 2002; Schluter et al., 2003; Drolet et al., 2004), suggesting that α-synuclein is involved in the pathophysiology of MPTP-induced parkinsonism.

We have recently shown that Fas is neuroprotective in the MPTP-induced mouse model of PD, but the mechanism for Fas-induced neuroprotection was unknown. Here we show that Fas promotes proteasome enzyme activity, enhances α-synuclein degradation, and provides neuroprotection from α-synuclein-induced parkinsonism in mice.

Materials & Methods

MPTP treatment and midbrain dissection

We injected wt and Fas-deficient *lpr* mice with saline or 30 mg/kg MPTP-HCl (Sigma) 24 hours apart. We cervically dislocated mice two hours after the second injection and dissected the midbrain (Smeyne and Smeyne, 2002) for the proteasome enzyme assays.

Proteasome enzyme assays

We lysed midbrain tissue or SH-SY5Y cells in Tris/EDTA by 100 strokes of manual homogenization on ice, and cleared lysate by centrifugation. We adjusted the protein concentration of the supernatant to 0.1mg/ml and plated quadruplicates of each sample. We plated two extra replicates with the proteasomal inhibitor MG132 (15μM). We added fluorescent substrates (2.5μM) in proteasome assay buffer (8mM DTT, 10mM MgCl2, 4mM ATP) immediately prior to the first reading on an Analyst Fluorescence plate reader. We used substrates Z-LLE-AMC for caspase-like activity, Suc-LLVY-AMC for chymotrypsin-like activity, and Boc-LRR-AMC for trypsin-like activity. We measured fluorescence at 30 minute intervals for 5 hours for chymotrypsin and caspase activities, and for 1 hour for trypsin activity and maintained plates at 37°C/5% CO_2 in the dark between readings.

Fas Construct

We amplified the full-length human Fas sequence by PCR amplification of Invitrogen cDNA clone ID 4514272 (accession # BC012479) encoding human Fas isoform 1 (5' primer, GGAAGCGAATTCACTTCG; 3' primer, GTTTTTCGAATTCGACCAAGCTTTGG). We cloned the amplified sequence into the EcoR1 site of the pCMV-Tag4A vector (Stratagene), to generate Fas-C' terminal-FLAG-tagged Fas (Fas-FLAG) driven by the CMV promoter. We used the pCMV-luciferase-Tag4 control expression vector provided by Stratagene as a control.

Cell lines and transfections

We obtained SH-SY5Y cells from the American Type Culture Collection (ATCC) and maintained them in DMEM supplemented with 10% FCS. We performed transient transfections in 90-95% confluent cultures in 6-well tissue culture plates using 4.0 μg plasmid DNA and 10 μl Lipofectamine^{TM} 2000 (Invitrogen) in 2.5 ml DMEM / 10% FCS. We generated SH-SY5Y lines stably expressing Fas-FLAG by single cell sorting Fas positive cells after transient transfection, using a FACSAria cell sorter (BD Biosciences). We generated SH-SY5Y cells stably expressing α-synuclein-GFP by transfection with pEGFPC_2-α-synuclein (kindly provided by Dr. Edward A. Fon) followed by cell sorting for GFP. Stable transfectants were maintained in G418 (700 μg/ml). For experiments, we transiently transfected stable GFP-α-synuclein SH-SY5Y cells with luciferase-FLAG or Fas-FLAG. The transfection efficiency was 50 – 80% as determined by flow cytometry 48 hours after transfection.

Flow Cytometry

Eighteen hours after transfection, we fixed cells with cytofix/cytoperm (BD Biosciences). We stained Fas-transfected cells with PE-DX_2 (2μl antibody for 2 X 10^5 cells) and luciferase-transfected cells with α-FLAG (M_2, Sigma) and CY3 (Jackson Immunoresearch) for flow cytometry. We compared the GFP-α-synuclein fluorescence channel signal between Fas positive and luciferase positive cells.

Preparation of high-titer rAAV vector stocks

We cloned full length (423-bp) human α-synuclein into the pAAV-MCS plasmid (CMV promoter; Stratagene, La Jolla, CA) (pAAV- α -synuclein) as reported previously (Yamada et al., 2004). We prepared high-titer serotype-1 rAAV (rAAV1) vector stocks as described previously (Yasuda et al., 2007). In brief, we co-transfected pAAV-α-synuclein or control pAAV-GFP (humanized recombinant green fluorescent protein; Stratagene) with plasmids pHelper and Pack2/1 (kindly provided by Dr. Takashi Shimada, Nippon Medical School, Tokyo, Japan) into HEK293 cells using a standard calcium phosphate method. After 48 hours, we harvested the cells and obtained the crude rAAV1 solutions by repeated freeze/ thaw cycles. We concentrated virus particles by ammonium sulfate precipitation, then resuspended them in PBS, applied them to an OptiSeal centrifugation tube (Beckman Coulter, Inc., Fullerton, California), and overlayed them with OptiPrep solution (Axis-Shield PoC AS, Oslo, Norway). We processed the tube by GradientMaster (BioComp Instruments, Inc., New Brunswick, Canada) then ultracentrifuged it at 13,000 rpm for

18.5 hours. We collected the fractions containing high-titer rAAV1 vectors and used them for injection into animals. We semi-quantified the number of rAAV1 genome copies by PCR within the CMV promoter region using primers 5'-GACGTCAATAATGACGT ATG-3' and 5'-GGTAATAGCGATGACTAATACG-3'. The titer of the rAAV1 vector that we used in the injections was $6x10^{11}$ genomes/mL.

Stereotaxic injection of rAAV1 vectors

We anesthetized mice with sodium pentobarbital and positioned them in a stereotaxic frame. We exposed the skull and removed a small portion over the right side of the SN with a dental drill. Subsequently, we injected rAAV1-α-synuclein or rAAV1-hrGFP unilaterally into the SN (2 μL, 2.7 mm posterior and 1.3 mm lateral from bregma, 4.4 mm below the dural surface, tooth bar = 2 mm) through a 5-μL Hamilton microsyringe.

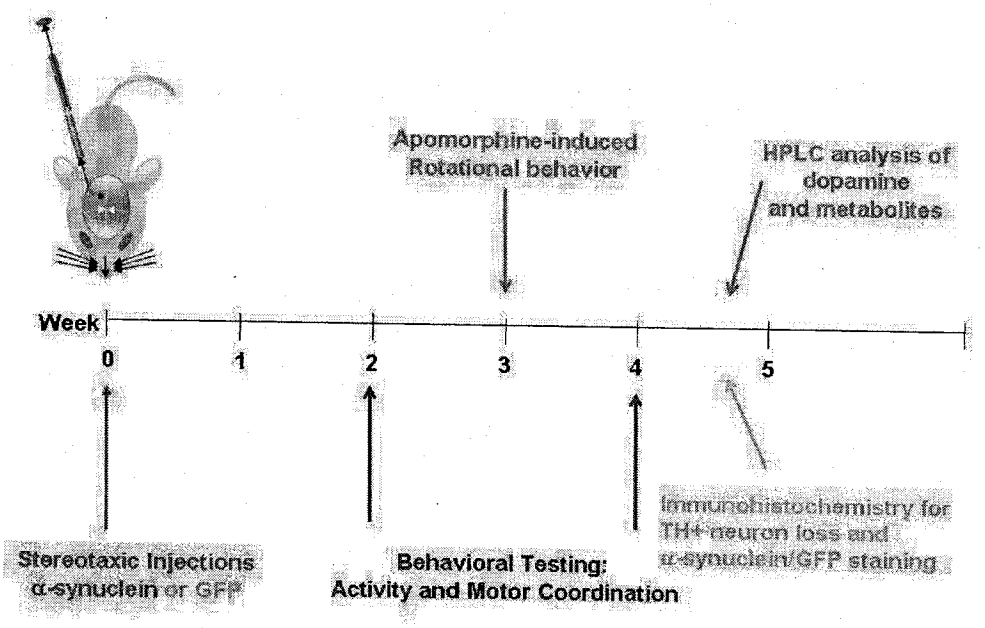


Figure 4.1: Dosing scheme for α-synuclein overexpression and analysis

Behavioural analysis

At two and four weeks after the injection of AAV-GFP or AAV-α-synuclein, we monitored mice for changes in spontaneous activity by an activity sensor (Neuroscience Inc., Tokyo, Japan) and for motor coordination by rotorod (UGO Basile, Varese, Italy). We assessed rotational behaviour induced by apomorphine (3mg/kg) three weeks after the injection for a 40 minute period. We videotaped the mice and an investigator blinded to the treatment groups counted the rotations.

Processing of brains for HPLC and immunohistochemistry

We sacrificed the rAAV1-injected mice at 4-weeks post-injection by cervical dislocation. We chose this time point because wt mice are not yet affected, allowing us to detect altered susceptibility to nigrostriatal degeneration in Fas-deficient *lpr* mice. We removed the brains, and cut coronally along an anterior tangent to the median eminence. We then dissected and immediately froze the striatal tissues on dry ice for analysis by HPLC. We fixed the posterior parts of the brain including the SN in 4% paraformaldehyde in PBS overnight, and immersed them in PBS containing 30% sucrose for 48 hours. We cut 20 μm coronal sections of the SN with a cryostat and stained every third section for tyrosine hydroxylase (TH) and Nissl compound and every sixth section for GFP or α-synuclein as previously described (Cao et al., 2006).

Results

We measured proteasome enzyme activities in the midbrain of wt and Fas-deficient *lpr* mice treated with MPTP or saline as a control. Wt and *lpr* mice had similar baseline proteasomal activities. However, in wt mice, MPTP exposure resulted in upregulation of proteasomal activity, whereas in *lpr* mice, MPTP treatment caused a decrease in chymotrypsin, caspase and trypsin proteasomal activities (Figure 4.2). Changes in midbrain proteasomal activity may reflect glial and neuronal proteasome function. We tested the direct consequence of Fas activation on neuronal proteasomal activity *in vitro* using SH-SY5Y neuroblastoma cells. SH-SY5Y cells transiently or stably transfected with Fas showed an upregulation of chymotrypsin and caspase-like proteasomal activity (Figure 4.3), indicating that Fas activation can enhance proteasomal activity in neuronal cells directly.

As Fas can regulate proteasome activity we investigated whether Fas could enhance the degradation of α-synuclein. We transiently transfected Fas-FLAG or control luciferase-FLAG into SH-SY5Y cells stably expressing GFP-tagged α-synuclein. We observed that cells transfected with Fas-FLAG had lower levels of α-synuclein GFP fluorescence than cells transfected with a control luciferase-FLAG, demonstrating that Fas expression promoted α-synuclein degradation *in vitro* (Figure 4.4).

To determine whether Fas is neuroprotective *in vivo* in an α-synuclein overexpression model of PD, we injected α-synuclein containing adeno-associated virus (AAV) vectors into the SN of Fas-deficient *lpr* and wt mice. As a control, we injected AAV vectors containing GFP. Only mice with clear α-synuclein staining or GFP fluorescence at the end of the study were included in the results (Figure 4.5 A). Both wt and *lpr* mice injected intranigrally with AAV-α-synuclein displayed behavioural deficits compared with AAV-GFP treated mice. Both strains demonstrated a decrease in motor coordination as assessed by rotorod performance two weeks after injection of AAV-α-synuclein and a decrease in spontaneous activity by 4 weeks following injection (Figure 4.5 B). Rotational behaviour elicited by injection of apomorphine, which detects upregulation of post-synaptic dopamine receptors in response to pre-synaptic dopamine deficiency, was observed in AAV-α-synuclein injected *lpr* but not wt mice (Figure 4.5 C). These results suggest that *lpr* mice exhibit dopamine deficiency in response to AAV-α-synuclein.

AAV-α-synuclein injected *lpr* mice, but not wt mice, demonstrated neuropathology characteristic of parkinsonism. *Lpr* mice exhibited greater than 20% loss of dopamine neurons (tyrosine hydroxylase $(TH)^+$ cells) in the injected SN compared with the contralateral uninjected side (Figure 4.6 A). No significant cell loss was found in AAV-GFP injected mice, and no significant change was observed in the number of TH^+ cells in the SN of wt mice injected with AAV-α-synuclein or GFP. Furthermore, *lpr* mice, but not wt mice, injected with AAV-α-synuclein showed decreased striatal dopamine and metabolites in the injected side compared with the

uninjected side and again, no differences were found in AAV-GFP treated mice (Figure 4.6 B). These data indicate that Fas deficiency increases the susceptibility to α-synuclein-induced PD-like behavioural changes and neuropathology *in vivo*.

Discussion

Here we show that Fas-deficient *lpr* mice were unable to upregulate proteasome activities in response to MPTP. We found that *lpr* mice injected with α-synuclein exhibited increased apomorphine-induced rotational behaviour compared to wt mice, which suggests a deficit in dopamine in the injected side of the brain. This was confirmed by HPLC analysis showing a significant reduction in striatal dopamine, DOPAC and HVA in the AAV-α-synuclein injected hemispheres of *lpr*, but not wt, mice. Additionally, we observed increased dopamine neuron death in the SN in the injected side versus the uninjected side of the brain in AAV-α-synuclein-treated *lpr* mice. None of these changes were observed in the AAV-α-synuclein-treated wt mice. These data indicate that the presence of Fas is neuroprotective for α-synuclein-induced neuropathology. We propose that when Fas cannot be upregulated, proteasome activity is not enhanced sufficiently to control the aggregation of α-synuclein which will be more severe and may cause more damage.

In PD patients, membrane-bound Fas and Fas Ligand (FasL) expression are reduced in the SN (Ferrer et al., 2000). Concomitantly, soluble Fas, which acts as a decoy receptor and blocks the binding of FasL to Fas, is elevated in PD (Mogi et al., 1996; Hartmann et al., 1998). In our previous work, we found that PD patients have a defect in Fas upregulation in their peripheral blood lymphocytes (Landau et al., 2005). Fas signalling in these patients may be diminished both by reduced cell surface Fas expression, and by the presence of soluble Fas.

Recent evidence indicates that inflammatory processes may play a major role in the pathogenesis of PD. The SN contains the highest concentration of resident microglia in the brain (Kim et al., 2000b) and the microglia are overactive in the SN of PD patients (McGeer et al., 1988). Activated microglia have been found to be present 16 years after MPTP exposure in humans (Langston et al., 1999) and after 14 years in primates (McGeer et al., 2003), indicating that the presence of microglia may perpetuate the disease progression long after the initial insult. The activation of microglia is thought to contribute to neurodegeneration through the release of pro-inflammatory factors. Inhibition of microglial activation by treatment with minocycline has been shown to protect against MPTP-induced neurotoxicity in mice (Wu et al., 2002). Studies show that levels of TNF-α, IL-1β and IFN-γ are elevated in the SN of PD patients (Mogi et al., 1994; Hirsch et al., 1998). IFN-γ can induce Fas mRNA by 20-fold in glial cells (Lee et al., 2000). Furthermore, IFN-γ on its own has the ability to regulate proteasome activity. In response to IFN-γ, the 20S proteasome has been shown to exchange proteasomal subunits and increase its epitope processing capacity (Groettrup et al., 1996).

Our findings describe a novel function for Fas as an inducer of proteasomal activity in neurons. The human Fas-associated factor 1 (hFAF1) has been proposed to serve as a regulatory protein in the UPS (Song et al., 2005), which further supports the potential of Fas as an inducer of proteasome activity. The increase in Fas-mediated proteasomal activity may protect dopaminergic neurons from degeneration

after exposure to Parkinsonian-inducing agents by slowing the accumulation of damaged proteins which would normally be processed through the UPS. This protection may be common to other neurodegenerative diseases which feature the accumulation of proteins as a pathological hallmark. Furthermore, we have provided evidence that Fas is neuroprotective in a model of α-synuclein toxicity in mice further confirming its role as a neuroprotective agent for dopaminergic neurons.

Figures

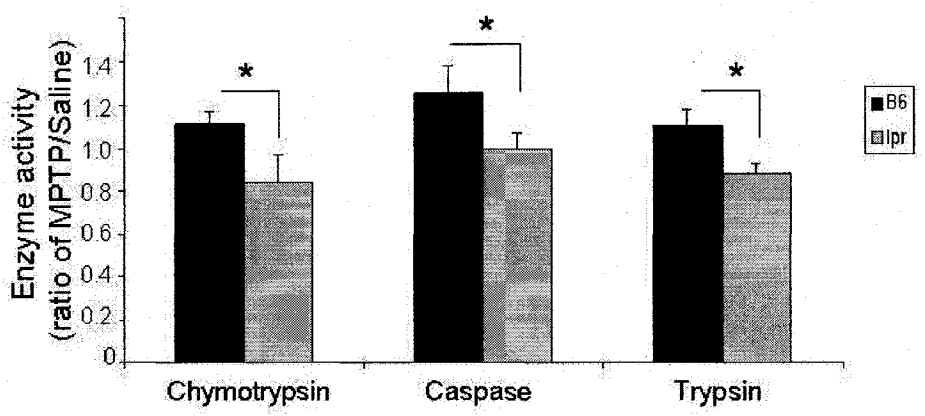


Figure 4.2: Fas-deficient *lpr* mice treated with MPTP demonstrate decreases in proteasomal activities. Chymotrypsin-like, caspase-like and trypsin-like activities in midbrain lysates of wt and *lpr* mice were measured fluorometrically. Data are expressed as a ratio of MPTP-treated mice to saline-treated mice for each strain in three experiments. (n=6 mice per group, error bars represent SE, *P<0.05)

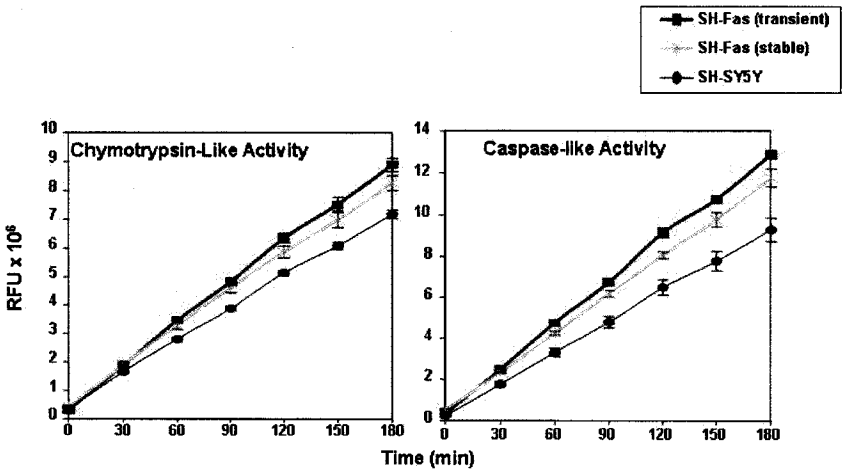


Figure 4.3: Fas directly upregulates proteasome activity in neurons. Transient transfection of SH-SY5Y cells with Fas-FLAG (red line) and stably Fas-FLAG expressing SH-SY5Y cells (yellow line) compared with untransfected SH-SY5Y cells (black line).

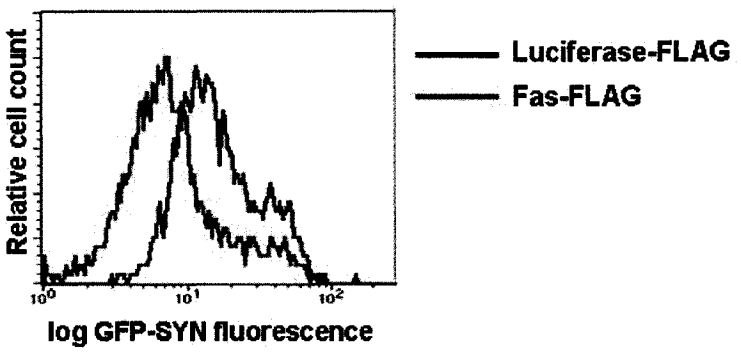
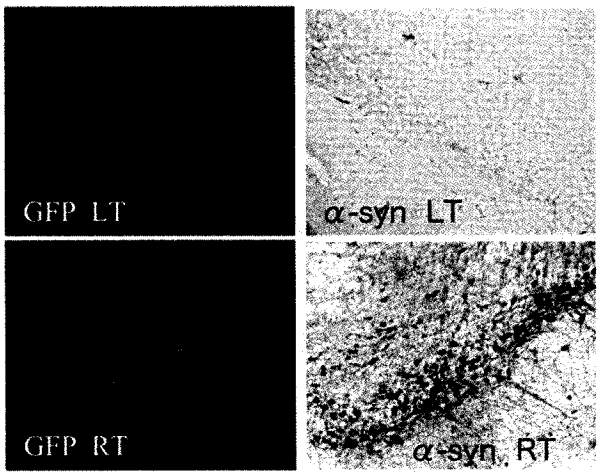
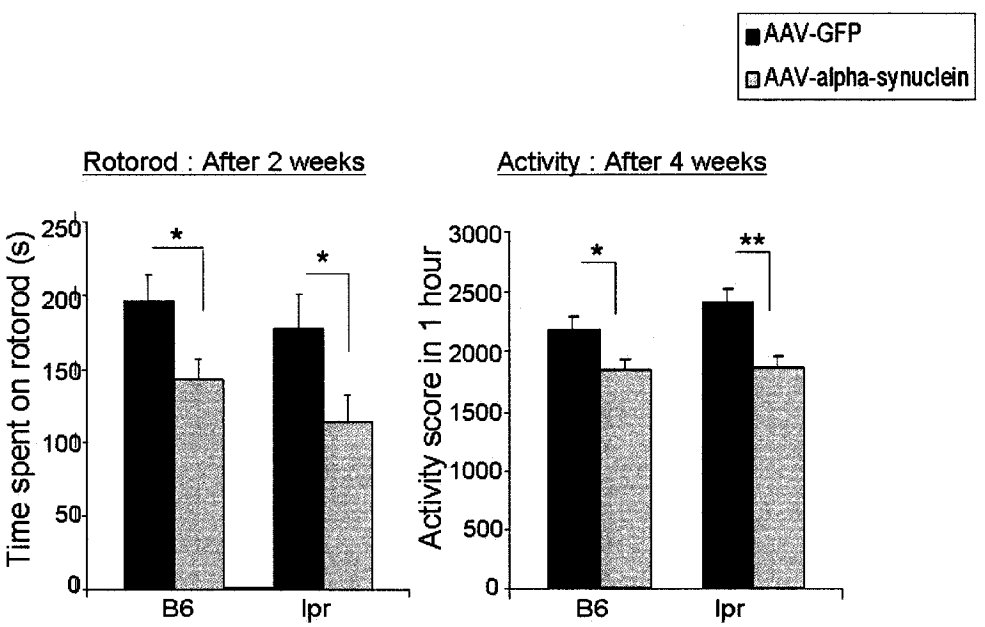


Figure 4.4: Fas expression reduces α-synuclein accumulation *in vitro*. Flow cytometry analysis demonstrates reduced levels of α-synuclein in SH-SY5Y cells stably expressing GFP-α-synuclein cells transfected with Fas-FLAG (red) compared with control luciferase-FLAG vector (black).

A


GFP LT
α-syn LT
GFP RT
α-syn RT

B


AAV-GFP
AAV-alpha-synuclein
Rotorod : After 2 weeks
Activity : After 4 weeks
Time spent on rotorod (s)
250
200
150
100
50
0
B6
lpr
*
*
Activity score in 1 hour
3000
2500
2000
1500
1000
500
0
B6
lpr
*
**

C

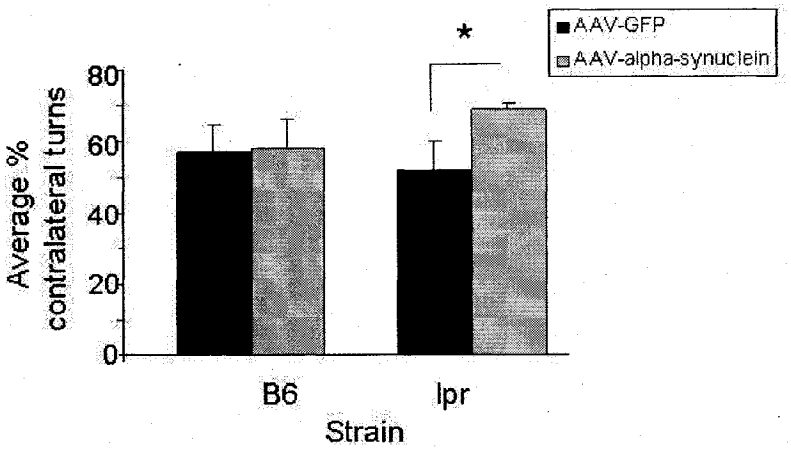


Figure 4.5: Mice injected with AAV-α-synuclein display behavioural deficits.

Only data from mice with clear α-synuclein staining or GFP fluorescence in the SN were included in this study. 28 mice in total were injected and 4 mice were discarded due to low expression of α-synuclein or GFP. A) GFP fluorescence and α-synuclein staining in the injected side (right) compared with the unlesioned side (left) (B) Wt and *lpr* mice displayed deficits in motor coordination (2wks after injection) and activity (4wks after injection). (C) Apomorphine-induced rotational behaviour was induced in *lpr* but not wt mice injected with AAV-α-synuclein. Data are expressed as percentage of contralateral turns over a 40 minute period. (n=4-7 mice per group, error bars represent SE, $^{**}P<0.01$, $^{***}P<0.001$.)

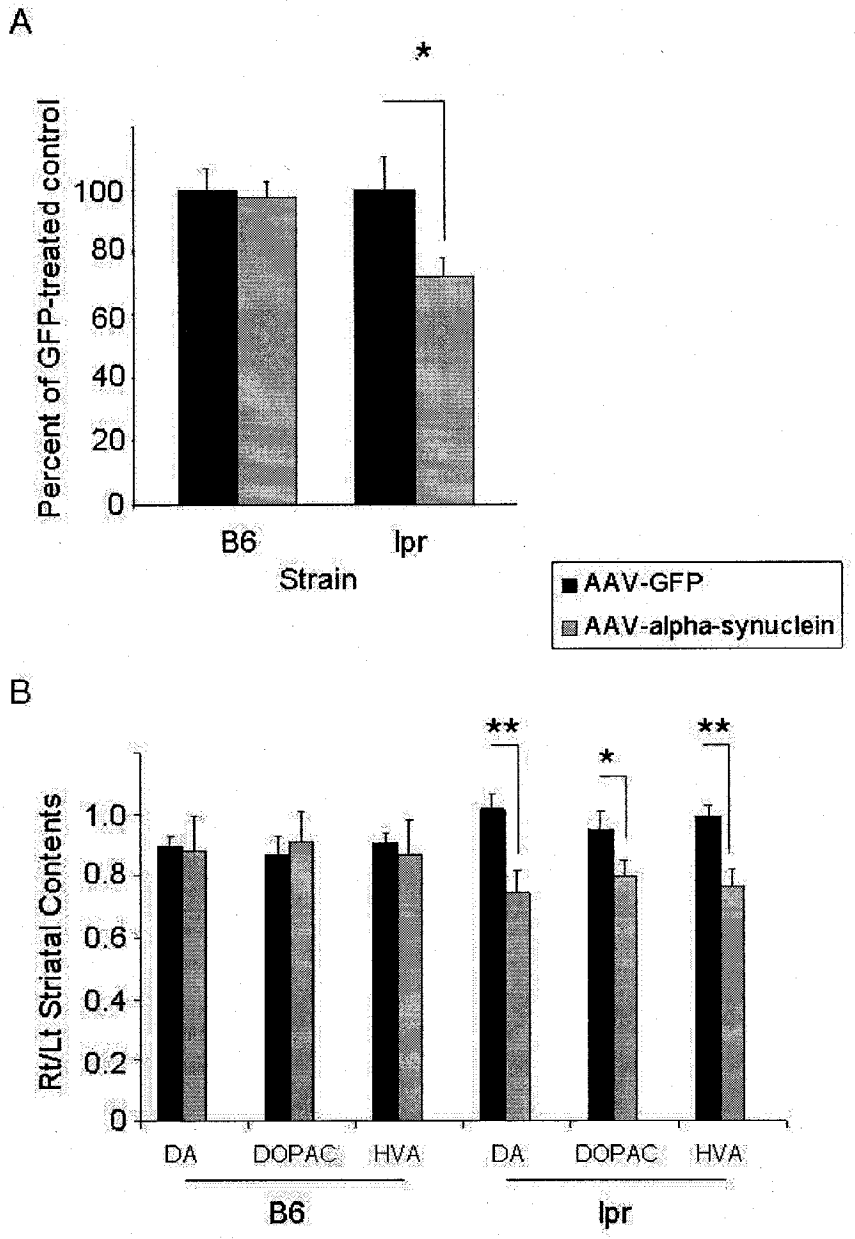


Figure 4.6: Intranigral overexpression of α-synuclein induces loss of dopamine neurons and reduced striatal dopamine and metabolites in Fas-deficient *lpr* mice. (A) Percent loss of dopaminergic TH^+ neurons in the SN pars compacta of α-synuclein compared with GFP injected mice is shown. Numbers of DA neurons in injected side/non-injected side were determined for each mouse and then data were

represented as a percentage of neurons in AAV-GFP control treated mice. (B) Striatal dopamine, DOPAC and HVA levels were compared in AAV-GFP and AAV-α-synuclein- injected B6 and *lpr* mice. Data are represented as injected side/uninjected side. (n=4-7 mice per group, error bars represent SE, *P<0.05, **$P<0.01$.)

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Chapter 5

Final Conclusions

The experiments presented in this thesis investigate the role of Fas in PD. Due to the historical emphasis of Fas-induced apoptosis over other functions, Fas was originally considered as a potential effector of apoptosis in PD. However, in this thesis, I demonstrate that Fas-deficient mice are acutely sensitive to PD induced by MPTP compared with wt mice, suggesting a neuroprotective role for Fas. We and others also found that another Fas mutant in which the Fas-DD is disrupted is resistant to MPTP-induced neurodegeneration (Hayley et al., 2004; Landau et al., 2005), implying that the neuroprotective signal is not transduced via the DD of Fas. Our findings suggest that lack of stimulatory Fas signals, rather than Fas-mediated apoptotic signals, are involved in the molecular pathogenesis of PD. Consistent with our studies on mice, we found that patients with PD manifest a defect in inducible Fas expression (Landau et al., 2005). Together, findings from PD patients and mice bearing mutations in *Fas* suggest that PD neurodegeneration is related to decreased Fas expression, and therefore that Fas is neuroprotective in PD. This work constitutes the first evidence for a neuroprotective role for Fas *in vivo*.

In addition, results from our PD patient study implicate Fas as a potential diagnostic tool to screen for PD. This would constitute a relatively non-invasive screen for a potential risk factor for PD since it would only require a blood sample. However, before this can even be considered, more experiments would be required to confirm this finding in a larger group of patients. It would also be interesting to see if this defect in Fas upregulation upon stimulation occurs in patients with other neurodegenerative diseases or if this is specific to PD patients. In a small preliminary

study, we found that the *Fas* regulatory region single nucleotide polymorphism (SNP) at -670, which can regulate inducible Fas expression (Huang et al., 1997), displays a skewed allele frequency in our small sample of PD patients. In our data analyzed to date (n = 12 PD patients), 50% of idiopathic PD patients are AA homozygotes at the -670 functional SNP, compared to a predicted value of 26% for a similar Caucasian population. This functional SNP in the *Fas* regulatory region may underlie our finding of defective Fas upregulation in PD patients, and may therefore represent a genetic risk factor in PD. Again, it would be very important to confirm these findings in a larger, well-controlled patient population.

We propose that Fas may be mediating neuroprotection via activation of the UPS leading to a more efficient clearance of damaged proteins in neurons. In PD, defects in the UPS have been implicated in both sporadic and familial forms of the disease. Furthermore, *in vivo* treatment with proteasomal inhibitors causes a parkinsonian syndrome in rats (McNaught et al., 2004). We attempted to use the PSI model of PD in mice to evaluate the effect of Fas on the UPS; however, due to the occurrence of neurotoxicity as a result of the ethanol vehicle, we had use alternate models.

We then turned back to the MPTP model in *lpr* mice in order to study the interaction of Fas and the UPS. We found that all three proteasome activities, chymotrypsin-like, caspase-like and trypsin-like, were decreased in *lpr* mice after exposure to MPTP, but not in wt mice. In wt mice, proteasome activities are

upregulated in response to MPTP, possibly as a means to increase the degradation of potentially toxic proteins. We found that *lpr* mice are unable to upregulate proteasome activities in response to MPTP which may account for their enhanced susceptibility to this neurotoxin. To further study the role of Fas and the UPS in PD, we injected wt and *lpr* mice with AAV containing α-synuclein. We hypothesized that if Fas cannot be upregulated, it will not stimulate proteasome activity and the aggregation of toxic proteins would be more severe and cause more damage to the dopaminergic neurons. We did in fact observe increased dopaminergic cell death in *lpr* mice overexpressing α-synuclein, compared with wt mice overexpressing α-synuclein. This is consistent with the lack of Fas in *lpr* mice increasing the susceptibility to dopaminergic neuron death through an inability to upregulate proteasomal activity.

We propose the following model to explain Fas-mediated neuroprotection (Figure 5.1). In the wt mouse, during exposure to cellular stressors (for example, MPTP), Fas is upregulated (Hayley et al., 2004) thus promoting proteasomal activity, which ensures the proper degradation of toxic or misfolded proteins. However, in the case where Fas is absent, (for example, in *lpr* mice), the activity of the proteasome may be decreased or simply unable to increase in response to stress, leading to the accumulation of abnormal proteins and eventually to cell death. Additionally, the decrease in proteasome activity may also destabilize mitochondrial function, increase oxidative stress, and contribute to cell death by those mechanisms as well.

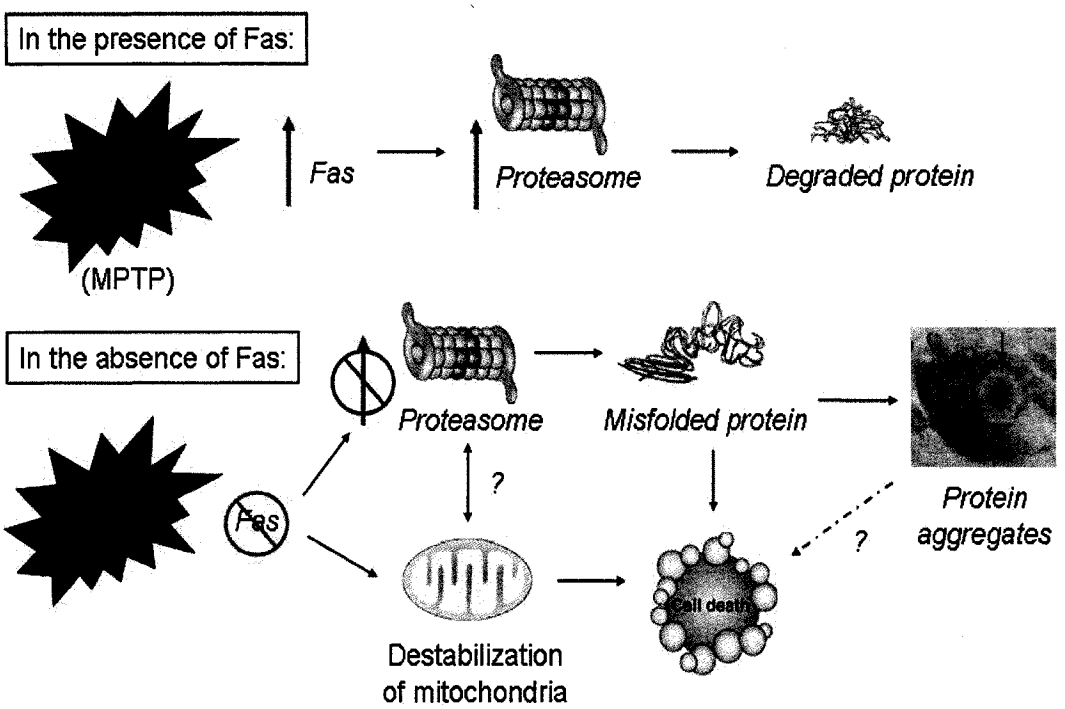

Figure 5.1: Proposed model of Fas induced neuroprotection.

On a separate note, the PSI proteasome inhibition studies, which did not shed light on the role of Fas in the UPS in PD as originally planned, did however lead to novel findings concerning the long-term effects of ethanol on striatal dopamine, serotonin and their metabolites, and the loss of TH^+ neurons. Ethanol on its own proved to be toxic to dopaminergic cells. We therefore went on to evaluate the possible neuroprotective effect of Fas in ethanol-induced dopaminergic neurotoxicity. We found no differences in striatal dopamine and metabolite levels in wt and Fas-deficient *lpr* mice when exposed to ethanol and therefore did not find any neuroprotection afforded by Fas in this model, suggesting that ethanol is toxic via a different mechanism from MPTP and α-synuclein.

Why is it that Fas is neuroprotective in the MPTP and α-synuclein-induced mouse models of PD, but not on ethanol-induced nigrostriatal degeneration? It is possible that since ethanol is not a specific nigrostriatal toxin and has been shown to cause damage in different areas in the brain, the damage to other brain areas can exacerbate the nigrostriatal neurotoxicity. Fas, like many of the members of the TNF-R superfamily, has wide array of functions depending on the environment. Certain factors, such as the presence of oxidative stress, the ratio of FLIP to caspase 8 and the presence of growth factors, cytokines, and neurotrophins and levels of Fas internalization can alter the outcome of Fas signalling. Further studies are necessary to determine what triggers are necessary to induce Fas-mediated neuroprotection over Fas-induced death or other roles of Fas.

There is no cure for PD and there is a great need for neuroprotective strategies to protect the remaining dopaminergic neurons from cell death. Some neuroprotective agents have been designed to reduce oxidative stress (rasagiline, selegiline, coenzyme Q10), boost mitochondrial function (coenzyme Q10, creatine), provide neurotrophic factor support (GDNF) and inhibit apoptosis (minocycline, rasagiline, selegiline) (reviewed in (Bonuccelli and Del Dotto, 2006)). In light of the new evidence of proteasomal involvement in PD, a new avenue for future research will be neuroprotective strategies that promote functional activity of the proteasome. As shown in this thesis, Fas can regulate proteasome activity *in vitro* and *in vivo* and therefore may eventually become a candidate neuroprotective agent in PD.

Given the data presented in this thesis, it would be worth further investigating the role of Fas as a neuroprotective factor in PD. One concern regarding the use of Fas would be the fact that in some contexts Fas signalling triggers cell death and therefore Fas stimulation cannot be used as a systemic therapy. Accomplishing molecular alterations to Fas or FasL, where signalling through the DD is impaired and only proliferative/regenerative signals are triggered, would be a good start to testing these strategies in the SN of animal models. However, even in this case, problems could arise since overstimulation may also disrupt neuronal circuitry. Thus, even the non-apoptotic/neuroprotective roles of Fas have to be well balanced. More studies will be necessary to further advance the knowledge of Fas-mediated neuroprotection and the contexts in which it may eventually become clinically useful.

PD is a heterogeneous and debilitating disease. Treatment can slow progression of the disease, but eventual deterioration is inevitable, and at present there is no cure. Together, the findings presented in my thesis from PD patients and mice bearing mutations in Fas suggest that susceptibility to PD neurodegeneration is related to a decrease in Fas, and therefore that Fas is neuroprotective in PD. Mechanistically, this may be explained, at least in part, due to the ability of Fas to upregulate proteasome activity in response to stress. Studies of Fas as a neuroprotective factor could lead to treatments which promote survival of dopamine neurons.

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Appendix