Systems regulating and inducing dopaminergic cell death in Parkinson's disease: an analysis of signalling associated with Parkinson's disease models.

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Abstract

Parkinson's disease (PD) is characterized by the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc). Mechanisms regulating this neurodegeneration, however, are unclear. Evidence from PD pathology and models of PD, indicate mitochondrial disfunction triggers several death signalling pathways. Accordingly, *in vivo* and *in vitro* mitochondrial stress models of PD were employed to explore the role of two divergent molecular influences on dopaminergic neuronal survival. We examined neuroinflammatory and death signalling pathways arising from MPTP-induced mitochondrial stress.

Interferon-gamma (IFN-ɣ) is a cytokine known to activate cellular components of inflammation, including microglia of the central nervous system (CNS). Results of a screen for cytokines in PD patient plasma revealed elevated levels of IFN-ɣ, suggesting a correlation between IFN-ɣ and PD associated DA cell death. In an MPTP mouse model of PD, germline deletion of IFN-ɣ improved survival of DA neurons and the nigrostriatal system, along with a reduction in microglia activation. Employing a survival co-culture system of neurons and microglia, it was found that neutralizing IFN-ɣ reduced DA cell loss induced by the mitochondrial complex I inhibitor, rotenone. DA cell death required localized microglia, activated through the IFN-ɣ-receptor (IFN-ɣ-R), with DA survival inversely proportional to IFN-ɣ expression, found to be up-regulated following rotenone.

Investigation of the calpain-Cdk5-MEF2 signalling pathway in the MPTP model of DA cell death, motivated an examination of the nuclear orphan receptor, Nur77, following a review of potential MEF2 regulatory targets. MPTP induced a reduction in Nur77 mRNA from basal

levels in SNc tissue, further regulated by ectopic Nur77 expression. These results strengthened our new model of MEF2 Nur77 regulation in DA neurons. In MPP^+/MPTP DA survival experiments, loss in germline Nur77 expression presented an elevation in DA neuronal death both *in vitro* and *in vivo*, with a greater impairment in the nigrostriatal circuitry in comparison with normal expressing animals and cells. Dopaminergic supersensitivity related to Nur77 deficiency was attenuated with the ectopic expression of AV-Nur77 *in vivo*.

These opposing mediators of survival yield new mechanisms by which DA neurons die, suggesting a mutitargeting approach to halt the progression of DA cell death.

For the Joy, Love and Strength Provided by

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Ursula and Jim

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List of Manuscripts

I) **Mount MP**, Lira A, Grimes D, Smith PD, Faucher S, Slack RS, Anisman H, Hayley SP, Park DS (2007) Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons. *Journal of Neuroscience* 27:3328–3337.

II) **Mount, MP**, Zhang, Y., Amini, A., Callaghan, S., Mao, Z., Slack, R., Anisman, H., Park, D.S. (2013) Perturbation of transcription factor Nur77 expression mediated by myocyte enhancer factor 2D (MEF2D) regulates dopaminergic neuron loss in response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Journal of Biological Chemistry*, 288(20): 14362-71.

Co-Authored Articles

I) Smith PD, Crocker SJ, Jackson-Lewis V, Jordan-Sciutto KL, Hayley SP, **Mount MP**, O'Hare MJ, Callaghan S, Slack RS, Przedborski S, Anisman H, Park DS (2003) Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci USA 100:13650–13655.

II) Hayley SP, Crocker SJ, Smith PD, Shree T, Jackson-Lewis V, Przedborski S, **Mount M**, Slack RS, Anisman H, Park DS (2004) Regulation of dopaminergic loss by Fas in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. J Neurosci 24:2045–2053.

III) Kim RH, Smith PD, Aleyasin H, Hayley SP, **Mount MP**, Pownall S, Wakeham A, You-Ten AJ, Kalia SK, Horne P, Westaway D, Lozano AM, Anisman H, Park DS, Mak TW (2005) Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine (MPTP) and oxidative stress. Proc Natl Acad Sci USA 102:5215–5220.

IV) Smith PD, **Mount MP**, Shree R, Callaghan S, Slack RS, Anisman H, Vincent I, Wang X, Mao Z, Park DS (2006) Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. J Neurosci 26:440–447.

V) Crocker SJ, Hayley SP, Smith PD, **Mount MP**, Lamba WR, Callaghan SM, Slack RS, Park DS (2006) Regulation of axotomy-induced dopaminergic neuron death and c-Jun phosphorylation by targeted inhibition of cdc42 or mixed lineage kinase. J Neurochem 96:489–499.

VI) Qu D, **Mount MP,** Rashidian J, Aleyasin H, Parsanejad M, Lira A, Haque E, Zhang Y, Callaghan S, Daigle M, Rousseaux MWC, Slack RS, Albert PR, Vincent I, Woulfe JM, Park DS (2007) Role of Cdk5-mediated phosphorylation of Prx2 in MPTP toxicity and Parkinson's disease. Neuron 55:37–52.

VII) Haque ME, **Mount MP**, Safarpour F, Abdel-Messih E, Callaghan S, Mazerolle C, Kitada T, Slack RS, Wallace V, Shen J, Anisman H, Park DS (2012) Inactivation of Pink1 gene in vivo sensitizes dopamine-producing neurons to 1-methyl-4-

phenyl-1,2,3,6-tetrahydropyridine (MPTP) and can be rescued by autosomal recessive Parkinson disease genes, Parkin or DJ-1. Journal of Biological Chemistry 287:23162–23170.

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List of Abbreviations

6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
ANOVA	Analysis of variance
BG	Basal ganglia
BBB	Blood brain barrier
BP	Butylidenephthalide
CDCrel-1	Cell division control related protein 1
CDK	Cyclin-dependant kinase
Cdk5	Cyclin-dependant kinase 5
ChIP	Chromatin immunoprecipitation
CNS	Central Nervous System
COMT	Catechol-*O*-methyltransferase
CTL	Cytotoxic T lymphocytes
Ctr	Control
CV	Cresyl violet
DA	Dopamine
DAT	Dopamine transporter
DBS	Deep-brain stimulation

DLB	Dementia with Lewy Bodies
GI	Gastrointestinal
iNOS	Inducible nitric oxide
IL-1	Interleukin-1
IFN-ɣ	Interferon-gamma
IFN-ɣ-R	Interferon-gamma-receptor
KO	Knock-out
LB	Lewy bodies
IL	Interleukin
iRNA	Inhibitor RNA
LC	*Locus coeruleus*
LPS	Lipopolysaccharides
MAO-B	Monoamine oxidase B
mRNA	Messenger RNA
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MEF2	Myocyte enhancer factor 2
MTN	Medial terminal nucleus
NE	Norepinephrine

NK	Natural killer
NO	Nitric oxide
p	Probability
PET	Position Emission Tomography
PBS	Phosphate buffered saline
PD	Parkinson’s disease
PFA	Paraformaldehyde
PINK1	PTEN induced kinase-1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SGZ	Subgranular zone
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SPECT	Single Photon Emission Computed Tomography
STN	Subthalamic nucleus
SVZ	Subventricular zone
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
TNF	Tumor necrosis factor

VTA	Ventral tegmental area
WT	Wild-type

Thesis format

Thesis guidelines from the Departments of Cellular and Molecular Medicine and Neurosciences at the University of Ottawa, include a general introduction reviewing Parkinson's disease (PD) research, followed by two manuscripts, and finally a general discussion, laying this research within a context of the current body of knowledge.

Chapter 1 presents an overview of PD, along with a rational for the research undertaken in these studies. This section includes PD etiology and pathology, models employed in PD research and cellular systems underlying the disease for which the following chsysapters develop from.

Chapter 2, entitled: "Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons" was published in the *Journal of Neuroscience* (2007), 27:3328–3337, and examines neuroinflammation and the role for interferon-γ and microglial activation in PD associated dopaminergic cell death.

Chapter 3 presents our manuscript: "Perturbation of transcription factor Nur77 expression mediated by myocyte enhancer factor 2D (MEF2D) regulates dopaminergic neuron loss in response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)." This article was published in the *Journal of Biological Chemistry* (2013) 288(20): 14362-71, and evaluates modulation of Nur77 expression on dopaminergic survival in an MPTP model of PD.

Chapter 4 presents a summary of the findings presented in chapters 2 and 3 with a discussion of the results in the context of research literature along with an analysis of future experimental relating to these findings.

CHAPTER 1: Introduction

1. Parkinson's Disease

Parkinson's disease (PD) was first described by British physician, James Parkinson in 1817 (Parkinson, 1817; 2002). Clinical manifestations of the disease are characterized by its motor hallmark phenotypes, which include bradykinesia, postural instability, resting tremor and slowness of movement (Jankovic, 2008). Knowledge of the disorder has grown since Parkinson's initial report, however, this time has not provided a definitive link nor understanding between reported symptoms and the neuropathology observed in PD brain tissue. Consequently, there are still no treatments to halt and reverse the symptoms of PD.

The principle risk factor for PD is age (Collier et al., 2011). In Western developed countries, normal life expectancy is over 70 years (Mathers et al., 2001), while the prevalence of PD is approximately 300 per 100,000 individuals (Rajput and Birdi, 1997). However, in developing countries, PD displays a lower life expectancy, of below 50 years (Mathers et al., 2001), with a prevalence of approximately 50 per 100,000 individuals (Nicoletti et al., 2003; Dotchin et al., 2011). Therefore, its prevalence is highest in countries where the aging population is increasing. In these affected populations, males are more commonly affected than females (Baldereschi et al., 2000).

1.1 Clinical presentation

Clinically, PD is characterized by presence of two or more of the following groups of classical motor symptoms: bradykinesia, postural instability, resting tremor and slowness of movement. These features are thought to be the product of a loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNc) and a resulting disruption in basal ganglia

function. The correlation between SNc neuron loss and motor symptom progression can be indirectly evaluated by imaging techniques such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT). These sensitive, functional imaging techniques can also be used to identify early stages of PD, with associated dopaminergic neurodegeneration, before clinical symptoms present (Brooks, 1997).

In addition to primary motor symptoms, patients exhibit significant alterations in non-motor systems as secondary symptoms. Depression is a common feature in PD patients, affecting 30-40% of the PD population (Leentjens, 2004; Aarsland et al., 2011). In this same broad category, one also finds apathy and anxiety as emotional symptoms of the disease (Starkstein et al., 1992). Disruption in the latter symptoms, although not as frequent as depression, may negatively impact cognitive function more than emotion itself thereby making the depression/apathy combination seen in PD patients a difficult therapeutic target (Jones et al., 2014; la Riva et al., 2014).

In late stage PD, a higher incidence of dementia is observed. In fact, there is a six fold increased risk of dementia in an individual suffering from PD(Aarsland et al., 2001; 2011). In addition, there appears to be a correlation between disease duration and onset of cognitive decline (Aarsland et al., 2004). This correlation may be explained in part by the progression of synucleopathies to the neocortical region of the brain, according to the Braak hypothesis (Braak et al., 2003a).

Another clinical presentation in PD patients is sleep disturbance, due to disruption of their rapid eye movement (REM) sleep patterns, possibly as a result of altered muscle tone or noradrenergic (NE) dysfunction at the level of the *locus coeruleus (LC)* (Tan et al., 1996; Goldstein et al., 2012; del Tredici and Braak, 2013). In addition, a key side effect of dopamine (DA) replacement therapy is vivid dreams and hallucinations due to overstimulation of certain dopaminergic systems.

Two early symptoms are present in pre-clinical PD: anosmia (loss of sense of smell) (Attems et al., 2014; López Hernández et al., 2014)) and gastrointestinal (GI) troubles (principally constipation) (Braak et al., 2006; Plouvier et al., 2014). Anosmia and GI-disturbances are thought to be indicators of PD years before disease onset, although they are not specific to PD. As a result, it has been hypothesized that a foreign body (toxin or virus) enters the body by these two ports of entry and selectively propagates through the nervous system, selectively targeting catecholaminergic neurons and resulting in the pathogenesis and progression of PD.

1.2 Treatment

The development of effective therapeutic options for PD is hindered by limited understanding of the pathogenesis of the disorder. Given the observation that parkinsonian symptoms emerged as a result of dramatic depletion of DA in the brain, replenishment of dopamine has been employed for therapeutic symptom management for over 50 years (Cotzias et al., 1969). Currently, the most widely used approach to treat parkinsonian symptoms is administration of levodopa, the generic name for the dopamine precursor, L-

dihydroxyphenylalanine (L-DOPA) (Hely et al., 2000; Di Stefano et al., 2011). Although, oral systemic administration of levodopa results in marked improvement in some parkinsonian symptoms, long term treatment is limited by instability of motor responses following treatment, as well as development of other peripheral side effects (Goetz, 1997; Di Stefano et al., 2011). Side effects caused by peripheral dopamine include GI symptoms, such as vomiting, nausea and diarrhea (Goetz, 1997). The major motor side effect of levodopa treatment is the development of dyskinesias. Dyskinesias are defined as a general impairment in movement control, characterized by involuntary, repetitive motions or lack of coordination. These side effects generally develop with long term use of levodopa (Obeso et al., 2000a; 2000b). While the exact cause of levodopa-induced dyskinesias is still not known, elevated concentration of DA and the effects initiated in the striatum to postsynaptic targets, may contribute to development of such effects (Doucet et al., 1996; Gaillard et al., 2009). Therefore, understanding how the BG responds to these changes will be critical to understanding why dyskinesias develop. Given that the current therapy for PD is accompanied by debilitating side effects, one of the major challenges in therapeutic management of PD are therefore the need to treat therapy-related complications and the efficacy of treatment. In this regard, the search for more effective treatment options for PD has prompted much research geared toward understanding the etiology and pathogenesis of the disorder.

1.3 Idiopathic PD

The majority of PD is thought to be idiopathic (of unknown cause) in origin. Several studies have identified environmental susceptibility factors for the disease and these have been tested in a laboratory setting. These studies are correlative and it remains difficult to establish a direct causal link to PD. Nevertheless, they include, but are not limited to age, exposure to heavy metals (manganese, iron), pesticide exposure (rotenone, paraquat, maneb) and head trauma.

Understanding how environmental factors interact with an individual's genetic make-up have thus far yielded a better understanding of the etiology of PD, although pathogenesis of PD is still far from being fully understood. Taken together, environmental factors likely contribute to the pathogenesis of PD and, therefore, have been used frequently in a laboratory setting because of their easy manipulation and globally reproducible results.

1.4 Familial PD

Although PD was originally thought to be entirely sporadic in origin, a seminal study indicated a familial linkage for the disease (Polymeropoulos et al., 1996). A year later, the first PD gene, α-synuclein was identified (Polymeropoulos et al., 1997a; 1997b). Several PD genes have been linked to autosomal dominant and autosomal recessive traits since this first identification. Functional analysis of these genes within the cell and the effect that their perturbation poses on the cell is potentially important as this understanding could yield new drug targets through better understanding of the disease pathogenesis.

Advances in technologies allowing for sequencing and high-throughput genome-wide studies, has led to an increasing number of genetic variants postulated to confer susceptibility to the disease (Hardy, 2010; Peeraully and Tan, 2012). These include genes previously identified to be linked to PD such as LRRK2 (Hardy, 2010; Tsuji, 2010; Pihlstrøm and Toft, 2011). With this broad technical arsenal, the research field can attempt to combine both familial PD genes and PD-associated genes with the sporadic components (environmental exposure to toxicants) with the goal of recapitulating drug-targetable, clinically relevant models (Gao and Hong, 2011).

1.4.1 α-synuclein

The first gene to be implicated in PD pathogenesis was α-synuclein. Mutations in *α-synuclein* were first identified in 1997, when an Italian family showed autosomal dominant inheritance of PD, with a point mutation resulting in substitution of an alanine to threonine residue at position 53 (A53T) in the protein product of the *α-synuclein* gene (Polymeropoulos et al., 1997a). Another mutation resulting in a substitution of an alanine to proline at site 30 (A30P) in the translated protein, was later implicated in PD pathogenesis (Krüger et al., 1998). These two missense mutations, A53T and A30P, resulted in a dominantly inherited form of PD. While the normal physiological function of α-synuclein is beginning to be uncovered, the mechanisms by which mutant α-synuclein induces cell death processes associated with familial PD remains unclear.

Clues with regard to normal function of α-synuclein suggest potential mechanisms that might be involved in the DA terminal region. Notably, α-synuclein is widely expressed in the

nervous system and is prevalent at the presynaptic nerve terminal in close association with synaptic vesicles (Maroteaux et al., 1988). In striatal dopaminergic terminals, α-synuclein is thought to regulate synaptic function by negatively regulating DA release and neurotransmission (Abeliovich et al., 2000). However, while no data presently supports a role for α-synuclein in regulation of sporadic forms of PD, accumulation of α-synuclein in LBs does occur in sporadic PD brains (Spillantini et al., 1998). Further, mutant forms of α-synuclein are prone to misfolding and formation of protofibrils (Conway et al., 2000a; 2000b), contributing to associated PD neuropathology. The importance of α-synuclein in regulation of DA cell loss is supported by the observation that α-synuclein deficient mice are resistant to toxin induced degeneration of dopaminergic cells *in vivo* (Dauer et al., 2002), suggesting that α-synuclein mutations in PD may acquire a pathogenic function. A functional role pre-synaptically was highlighted by Südhof's group (Chandra et al., 2004), showing *α*-synuclein interacting with multiple components of pre-synaptic exocytosis machinery (Abeliovich et al., 2000; Chandra et al., 2004; Wakamatsu et al., 2007; Burré et al., 2010).

1.4.2 DJ-1

Mutations in the *DJ-1* gene have been implicated in the development of a rare autosomal recessive form of PD (Bonifati et al., 2002; 2003) with deletion and missense mutations being described in DJ-1 forms of PD. While the normal physiological function of DJ-1 is presently not clear, there are some clues from the crystal structure of the protein (Tao and Tong, 2003; Tao et al., 2003). The crystal structure of the protein suggests that specific missense mutations, implicated in familial PD, abolish protein function (Moore et al., 2003). One such missense mutation, resulting in substitution of a lysine residue at position 166 to a

proline residue (L166P mutant) suggest a potential abnormality in protein function. The secondary structure of DJ-1 predicts that this proline mutation occurs in an alpha helical region, potentially resulting in compromised protein function, due to conformational abnormality (Moore et al., 2003). Previous reports, indicate DJ-1 function as a monitor of oxidative stress (Mitsumoto et al., 2001), a process suggested to be involved in PD pathogenesis. While the role of DJ-1 in regulation of DA cell loss is presently unclear, work in our lab indicates that loss of DJ-1 confers greater sensitivity to oxidative stress *in vitro* and toxic insults in an *in vivo* model of PD (Aleyasin et al., 2007). This research points to the involvement of DJ-1 in regulating of dopaminergic cell death processes. However, a greater understanding of the normal function of DJ-1 is still required to understand the mechanisms involved in regulation of dopaminergic loss induced by DJ-1 mutation in familial forms of PD.

1.4.3 Parkin

The identification of α-synuclein mutations as a cause of inherited forms of PD, lead to an explosion in attempts to identify other potential PD genes. Mutations in the *parkin* gene have since been implicated in the development of an autosomal recessive early onset form of parkinsonism (Kitada et al., 1998; Klein et al., 2000). Various deletions and point mutations have been described in the gene that encodes the parkin protein (Kitada et al., 1998). Mutations in parkin account for about 50% of early onset PD cases (Lücking et al., 2000), indicating that it plays an important role in protecting from PD. The neuropathological characteristics of parkin-related PD include selective loss of DA neurons in the substantia nigra (SN), without formation of LBs (Mizuno et al., 2001). The parkin protein is normally

expressed diffusely throughout neurons, but mutant forms of the protein are suggested to be absent from parkin-related PD brains (Solano et al., 2000). The role of parkin mutations in DA cell death has been further advanced by examining its physiological function. Parkin functions primarily as a ubiquitin E3 ligase and is involved in facilitating polyubiquitination of select proteins targeted for degradation by the ubiquitin proteasome system (Lim et al., 2006; Chin et al., 2010; Sha et al., 2010). Several parkin substrates have been identified, including the synaptic vesicle associated protein, CDCrel-1 (cell division control related protein 1), a glycosylated form of α-synuclein and cyclin E, a protein previously implicated in cell death processes (Zhang et al., 2000; Shimura et al., 2001; Staropoli et al., 2003; Sha et al., 2010). While this evidence supports a role for parkin mediated processes in modulation of protein handling, and cell death, the specific mechanisms that regulate dopaminergic neurodegeneration remains to be identified.

1.4.4 PINK1

PINK1 (PTEN-Induced Kinase 1) was first identified in 2001 as a gene responsive to the tumor suppressor PTEN (Unoki and Nakamura, 2001). Around the same time, the *PARK6* locus was associated with PD (Valente et al., 2001). These two findings were later merged when a direct link between PINK1 and autosomal recessive early onset PD was generated. In this case, two homozygous mutations (G309D and W437X) were observed in three Italian families (Valente et al., 2004). In this same study, PINK1 subcellular localization was noted to be mitochondrial and this associated function has since been the focus of intense research. For instance, in *Drosophila*, it was noted that PINK1 deficient flies exhibit mitochondrial cristae disruption and display muscle and dopaminergic neuron degeneration (Clark et al.,

2006; Park et al., 2006; Yang et al., 2006). Interestingly, in all three studies, this phenotype was rescued by Parkin, whereas PINK1 could not rescue Parkin deficiency. This suggests that both parkin and PINK1 share a common biochemical pathway, and that parkin may be downstream from PINK1 signalling.

Recent findings have attributed a functional role for the PINK1-Parkin genetic interaction with regard to mitochondrial quality control. PINK1 is believed to recruit Parkin to damaged mitochondria, where Parkin can target these defective organelles for a specific degradation by autophagy, a process termed mitophagy (McBride, 2008; Geisler et al., 2010; Narendra et al., 2010; Sha:2010bv Van Humbeeck et al., 2011). Though this mechanistic finding is interesting and plausible, it remains controversial as to whether this process occurs in more physiologically representative settings (Van Laar et al., 2011; Sterky et al., 2012).

1.4.5 LRRK2

Mutations in leucine-rich repeat kinase 2 (LRRK2), are identified in autosomal dominant late onset familial form of PD, and has relatively unknown function (Zimprich et al., 2004). Mutations in the gene encoding LRRK2 have been linked to multiple diseases, including a prominent association with familial and sporadic PD, as well as inflammatory bowel disorders, including Crohn's disease (Hakimi et al., 2011; Kabi et al., 2012). The LRRK2 protein possesses both kinase and GTPase signalling domains, as well as multiple protein interaction domains (Zimprich et al., 2004; Taymans, 2012). Experimental studies in both *in vitro* and *in vivo* models on mutant LRRK2-induced neurodegeneration have given insight into potential LRRK2 functions. Recent evidence indicates that intact kinase and GTPase

activity are required for mutant forms of the protein to trigger cell death, but their specific targets are not known (Yamano et al., 2014).

Mutations within the kinase domain of LRRK2 (G2019S, I2020T) seem to be most prevalent in PD patients, althoughmutations have been found in other domains, including the ROC (Ras of complex) domain (R1441C), the COR (C-terminal of ROC) domain (Y1699C) as well as the LRR (Leucine Rich Repeat) domain (I1122V) (Mata et al., 2006). Primary cellular examination of human mutations of the LRRK2 kinase domain suggest that they might be gain-of-function in nature, as they increase its kinase function (West et al., 2005; Gloeckner et al., 2006).

Since these initial findings, however, multiple cellular roles have been attributed to LRRK2 and most research has thus been focused on elucidating the cellular function of LRRK2 under pathophysiological conditions. It has been suggested that the kinase activity of LRRK2 mediates its neurotoxicity, although the validity of this finding remains unclear (West et al., 2005; Haque et al., 2008; Saha et al., 2009). One notable finding has been the capacity of LRRK2 to interact with other cellular components. For instance, LRRK2 interacts with the PD gene Parkin (Gloeckner et al., 2006). In addition, it forms complexes with neurite outgrowth machinery {Gillardon:2009ce, Heo:2010id, Lee et al., 2010, MacLeod et al., 2006, Piccoli et al., 2011, Shin et al., 2008}, which has a potential involvement in PD pathogenesis (Simón-Sánchez et al., 2009; Gan-Or et al., 2012). In addition, recent studies have indicated a putative role for LRRK2 in the regulation of autophagy (Alegre-Abarrategui et al., 2009; Ramonet et al., 2011). Its endogenous function, pro-survival or pro-death, remains unclear.

1.5 Pathology and Pathophysiology of Parkinson's disease

Parkinson's disease can be distinguished from other similar neurodegenerative diseases principally on its pathological features. Its hallmark features are that of specific neurodegeneration in the SNc, as well as the appearance of cytoplasmic inclusions termed Lewy Bodies (LB) in surviving neurons. Primarily highlighted as a disorder of the nigrostriatal pathway, PD pathology expands to various other areas of the brain thereby accounting for its multitude of clinical phenotypes.

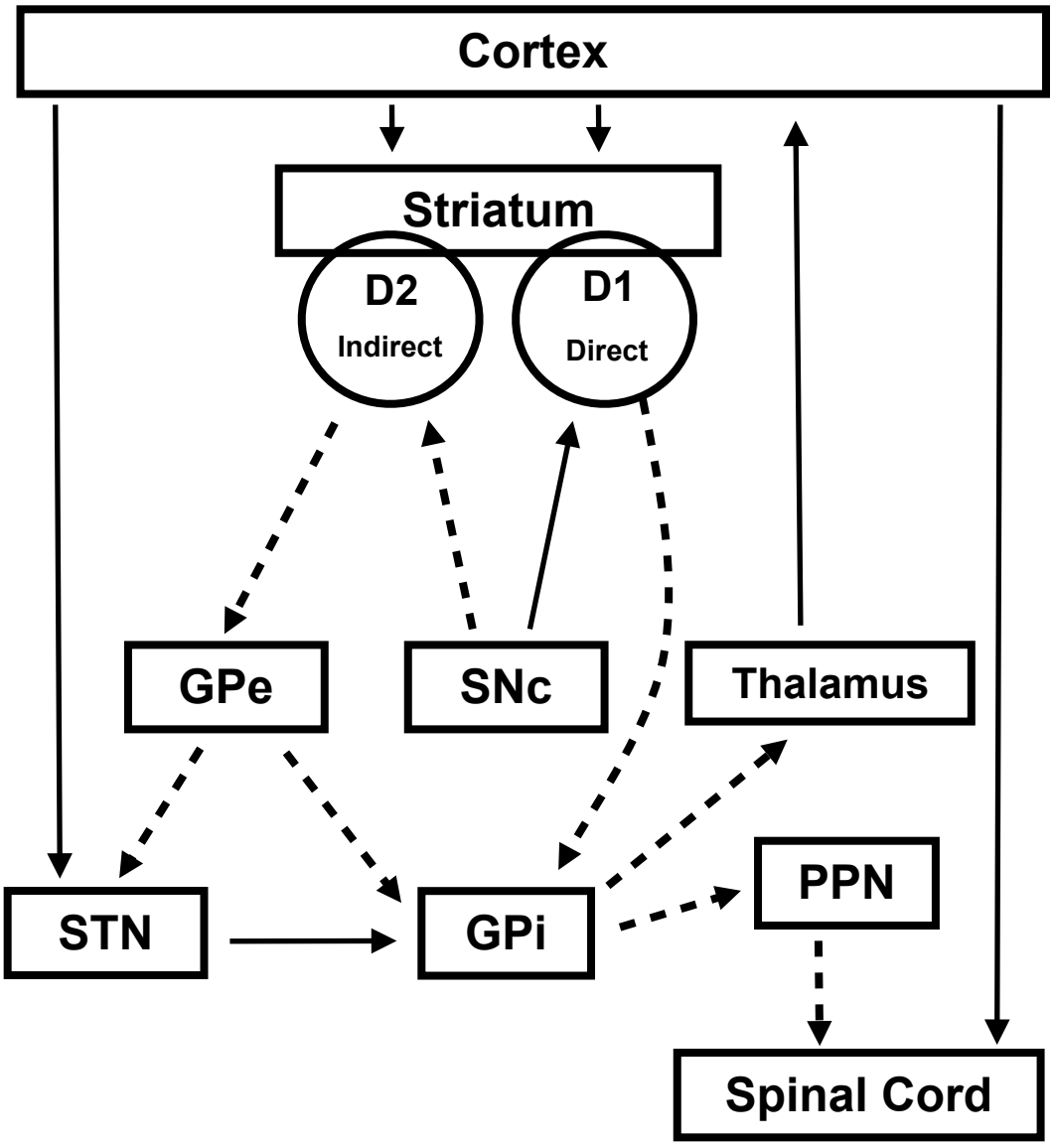
1.5.1 SNc degeneration

PD pathology is primarily classified with degeneration of the dopaminergic neurons in the midbrain SNc, which result in the principal motor symptoms observed in PD. In humans, degeneration of SNc neurons is apparent on the gross examination with a clear absence of black appearance of the structure in the midbrain (substantia nigra, latin for "Black Substance").

This natural black staining in the SNc results from the presence of neuromelanin in the cell bodies of dopaminergic neurons. The association between SNc neuronal degeneration and clinical behaviour correlates with the disruption of the nigro-striatal pathway in the basal ganglia circuitry (Figure 1.1) (reviewed by (Blandini et al., 2000)). The reduction in SNc releasing DA neurons results in a reduction in striatal synptic DA, affecting both the direct and indirect pathways of the basal ganglia. This neuronal degeneration ultimately results in reduction in activity of the D1 receptor dependent, direct pathway, facilitating movement.

FIGURE 1.1. Basal ganglia-thalamocortical circuitry.

Representation of the basal ganglia circuitry in normal healthy patients. Dopaminergic projections from the SNc terminate in the the striatum where dopamine (DA) activates D1 receptors (direct pathway) and inhibits D2 receptors (indirect pathway). At this point, the striatum (medium spiny neurons primarily) tonically inhibit thalamocortical function through interaction with the internal and external segments of the globus pallidus (*GPi* and GPe, respectively), creating activation of the motor cortex. The indirect pathway consists of projections from the striatum to the GPe, inhibiting the subthalamic nucleus (STN) from activating the GPi. In contrast, the direct pathway consists of the striatum directly inhibiting the GPi to tonically inhibit the ventral anterior and ventral lateral nuclei of the thalamus. In PD, neurons of the SNc die and therefore there is decreased striatal innervation. As a result, there is an increase in thalamocortical inhibition (via the GPi) resulting in a decreased in activation in the motor cortex. Solid arrows indicate increased neural activity; dashed arrows indicate decreased neural activity. PPN = pedunculopontine nucleus. Adapted from Kandel 2012 (Kandel et al., 2012).


Cortex
Striatum
D2
Indirect
D1
Direct
GPe
SNc
Thalamus
STN
GPi
PPN
Spinal Cord

1.5.2 Locus coeruleus (LC)

The locus coeruleus (LC) is a pontine nucleus principally responsible for physiological responses to stress or panic. Like the SNc, the LC is pigmented by melanin giving it a blue appearance (hence the Latin name meaning “blue spot”). The brains of post-mortem PD patients, demonstrate degeneration in the LC. This pathological finding is thought to primarily account for sleep disturbance in PD patients, as itcorrelates strongly with the REM sleep abnormalities, as well as anxiety symptoms, in PD patients (Richard, 2005; García-Lorenzo et al., 2013). The LC has been suggested to provide trophic support to the SNc and therefore its loss may account for increased susceptibility of death to the SNc neurons (Gesi et al., 2000). Finally, disruptions in the synaptic connections emanating from the LC may also play a part in the postural instability seen in PD (Grimbergen et al., 2009).

1.5.3 Ventral tegmental area (VTA)

The ventral tegmental area (VTA) is a cluster of DA neurons adjacent and medial to the SNc. These DA neurons give rise to the mesolimbic and mesocortical pathways that are responsible for reward and motivation systems under physiological conditions. This area of the brain is the target of many drugs of abuse including cocaine and amphetamines (Wise and Bozarth, 1985; Giros et al., 1996). In PD patients, however, the VTA can be hyper-activated by dopaminergic precursors and agonists. This may account for pathological gambling and impulse buying reported in certain medicated PD cases (Vilas et al., 2012). Additionally, in postmortem brains of PD patients, LBs accumulate in the cells of the VTA. Interesting is their relative resistance to death compared to the SNc neurons. This divergence may be due

to the oxidant stress evoked by the Ca^{2+} autonomous pacemaking in SNc neurons relative to the Na^{2+} pacemaking in VTA neurons, however, extensive work remains to be done to confirm this. Ultimately, understanding how VTA neurons are resistant to death versus those in the SNc may aid in the development of therapeutics for PD.

1.5.4 Lewy Bodies and the Braak Hypothesis

Lewy body (LB) structures have been reported in rostral nuclei in the brain stem such as the dorsal motor nucleus, intermediate reticular zone and raphe nucleus (Müller et al., 2005). These may individually have an effect on the clinical presentation of the disease though it has been difficult to tease out specific pathways thus far. Nevertheless, the anatomical and temporal appearance of LBs in PD patients has brought forth a disease progression hypothesis pioneered by Dr. Heiko Braak (Braak et al., 2003b).

The Braak hypothesis stems from a staging system that suggests that the SNc is only affected during mid-stage disease whereas more caudal components of the brain are affected earlier (Braak et al., 2003a; 2003b). From this staging, Braak and colleagues went on to suggest that PD-pathology may originate through a pathogen at two principal points of entry to the body: the olfactory and the gastrointestinal (GI) route. In support of this notion are the findings that over 95% of PD patients exhibit anosmia (loss of the sense of smell) prior to the onset of symptoms (Haehner et al., 2011). Half of PD patients report constipation and general GI disturbances long before the clinical manifestation of the disease (Braak et al., 2006; Hawkes et al., 2007).

Interestingly, new findings in α-synuclein research suggests this protein may act to form LBs by propagating from cell to cell in a cell-autonomous fashion, similarities observed in prion protein diseases (Luk et al., 2009; Angot et al., 2010; Volpicelli-Daley et al., 2011; Lema Tomé et al., 2013). It is possible an initial environmental insult or a genetic susceptibility may cause a misfolding process in α-synuclein, accelerated and propagated through time in a rostral fashion leading to LBs not only in the nigrostriatal axis, but also progressing to the cortex, resulting in dementia symptoms.

1.6 Neurochemical models

Damage to the nigrostriatal DA system, with a resulting DA-deficiency syndrome, has been produced by mechanical lesion of ascending nigrostriatal nerve fibers (Brecknell et al., 1995), electrolytic lesion to the SN (Donaldson et al., 1976), and administration of various toxins such as reserpine (to deplete brain catecholamines) (Carlsson, 1996), methamphetamine (Sonsalla et al., 1996), 6-hydroxydopamine (either into the SN, striatum, or medial forebrain bundle) (Deumens et al., 2002), rotenone (Betarbet et al., 2000), paraquat (McCormack et al., 2002), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston and Ballard, 1983; Chiba et al., 1984; Heikkila et al., 1984; Langston et al., 1984a). While all of these models have merit, none is ideal nor are any of these employed models universally suited for all types of studies. For a particular animal model to be valuable, it should possess certain qualities: 1) accurately model the disease or pathology of interest; 2) allow for inferences to be drawn concerning the human counterpart of the model;

and 3) the system under study should have the necessary biochemical and anatomical organization in order make the model a reliable substitute for the target human.

Evidence supports a role for genetic factors in PD pathogenesis, however, the cause of the vast majority of PD cases is not known (idiopathic in nature). Consequently, it has been suggested that environmental toxins may play a role in pathogenesis of PD. In support of this notion, previous evidence indicates that dopamine levels decrease markedly with age, more significantly so past the age of 60 years, with nigrostriatal loss averaging about 1.4% per decade between 15 and 65 years of age (Calne and Peppard, 1987). This indicates that increase in age provides a risk factor for the development of Parkinsonism and suggests that there might be accumulation of environmental cues that signal degeneration of the nigrostriatal pathway over time.

Many epidemiological studies support a link between environmental toxins and PD pathogenesis. In addition to the evidence that the vast majority of PD cases lack any identifiable mutations in PD genes, it is probable that the environment may play a significant role in development of PD. Supporting a potential role for environmental factors is the observation of increased prevalence of PD in farming communities, with several reports indicating a correlation between exposure to pesticides, herbicides and fungicides and development of PD {Semchuk and Love, 1995, Lai et al., 2003}. Given the potential that environmental factors may contribute to pathogenesis of PD, several toxin based models of the disorder have been described.

Mice and rats are the most widely used tools to understand the pathogenesis of the disease. The applicability of these models relies on the breadth of genetic tools that are available, as well as their response to toxins and viral-mediated gene delivery. The anatomical, physiological and behavioural correlates between mice/rats and humans are fairly similar and suggest an accurate assessment of the disease process as a whole.

Several neurotoxins induce dopaminergic degeneration, which include lipopolysaccharide (LPS), 6-hydroxydopamine (6-OHDA treatment), pesticide/herbicide (eg. rotenone and paraquat), and MPTP (1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine). These models have been instrumental in identification of potential molecular mechanisms that may contribute to PD pathogenesis. Of these models, only the MPTP model has been shown to develop parkinsonism in humans, and as such has been suggested to be the most relevant model of PD (Dauer and Przedborski, 2003). Experiments in this thesis have focused on the use of the MPTP and rotenone models, therefore, discussion will be limited to the more commonly used neurochemical models of PD: 6-OHDA, rotenone, and MPTP, with particular focus on their mode of action and potential utility in identification of pathogenic mechanisms associated with PD.

1.6.1 Rotenone

The contribution of environmental factors in PD was initially identified with the observation that administration of the pesticide rotenone in rats resulted in degeneration of SN DA neurons (Betarbet et al., 2000). In addition to nigral degeneration, there is some suggestion that rotenone treatment is accompanied by the histopathological formation of LB-like

inclusions, abundant in α-synuclein (Betarbet et al., 2000). Similar to other dopaminergic neurotoxin models, rotenone has been suggested to inhibit complex I of the mitochondrial respiratory chain. The specificity of rotenone to dopaminergic neurodegeneration is presently a controversial issue due to inconsistencies in experimental results from different groups (Ferrante et al., 1997). While rotenone provides support for the potential epidemiological correlation between PD and pesticide use, previous evidence suggests that agricultural chemicals were unable to elicit dopaminergic toxicity , and therefore this model (and its relevance to PD) must be utilized with some degree of caution (Perry et al., 1986).

Clinically, the incidence of PD has been found to increase 5-10 times with the exposure to agricultural pesticides (Hancock et al., 2008). Case-controlled studies further associate rotenone pesticide use in agricultural environments as a major risk factor for PD (Dhillon et al., 2008). Nevertheless, rotenone was used previously to bridge the relationship between these environmental associations and neurodegeneration in the lab. Betarbet *et al.* (Betarbet et al., 2000), treated rats with continual intravenous rotenone or vehicle control and demonstrated presence of LB inclusions, dopaminergic degeneration and associated behavioural characteristics.

1.6.2 6-OHDA

The 6-hydroxy-dopamine (6-OHDA) model was the first described PD animal model (Ungerstedt, 1968). 6-OHDA is a neurotoxic derivative of dopamine that is thought to be generated as a result of autooxidation of dopamine. Since 6-OHDA does not cross the blood brain barrier, the toxin is generally injected stereotactically into the SN or striatum to

selectively target the nigrostriatal pathway (Ungerstedt, 1968; Sauer and Oertel, 1994). 6-OHDA is taken up into dopaminergic cells, where it is thought to inhibit complex I of the mitochondrial respiratory chain and induce formation of reactive oxygen species (Glinka et al., 1996). While it is not clear whether 6-OHDA death mechanisms are relevant in development of Parkinsonism in humans, it is clear that this model does recapitulate the loss of nigral DA neurons associated with the disorder. The major benefit of this model is the possibility for unilateral lesioning (internal control and associated rotational behaviour) that has been suggested as an effective means of evaluating functional efficacy of new drug targets (Schwarting and Huston, 1996). However, this model is limited due to the challenges associated with drug delivery and inconsistencies in the degree of dopaminergic degeneration observed.

1.6.3 MPTP

The discovery of MPTP constitutes a major milestone in understanding the pathogenesis of PD. MPTP is a synthetic meperidine byproduct that selectively damages dopamine neurons and induces PD symptoms in humans (Langston and Ballard, 1983; Langston et al., 1983). MPTP-induced clinical PD symptoms are almost indistinguishable from those observed in sporadic PD (Przedborski and Vila, 2006). Although clinical cases of PD have been suggested to develop progressively, MPTP induces massive degeneration within a matter of days (Langston et al., 1983; Jackson-Lewis et al., 1995). Pathological analysis clearly confirms that the administration of MPTP results in selective loss of the nigrostriatal dopamine system in humans, non-human primates and select rodent strains (Burns et al., 1983; Langston et al., 1999; Sedelis et al., 2000).

The MPTP model of PD has been instrumental in the identification of pathogenic mechanisms involved in dopaminergic degeneration. While other experimental models of PD are available, the MPTP model of PD remains the most widely used and arguably the best animal model of PD (Dauer and Przedborski, 2003). Several MPTP animal models have been proposed over the years, including acute (Aubin et al., 1998; Jackson-Lewis and Przedborski, 2007; Pain et al., 2013), subchronic and chronic models (Tatton and Kish, 1997; Jackson-Lewis and Przedborski, 2007). The application of a continuous brain infusion model, in contrast to previously described models, has revealed the presence of LB-like inclusions in the brain of MPTP-treated animals (Fornai et al., 2005). The time course and morphology of neuronal death differs significantly in each of these different models (Jackson-Lewis et al., 1995; Schmidt and Ferger, 2001). These models differ in ability to induce either rapid degeneration of dopamine neurons or more progressive loss of dopaminergic cells. To mimic a more progressive neurodegenerative process, a subchronic MPTP mouse model of PD, which induces delayed degeneration of dopaminergic cells (Tatton and Kish, 1997), was utilized in this thesis research effort.

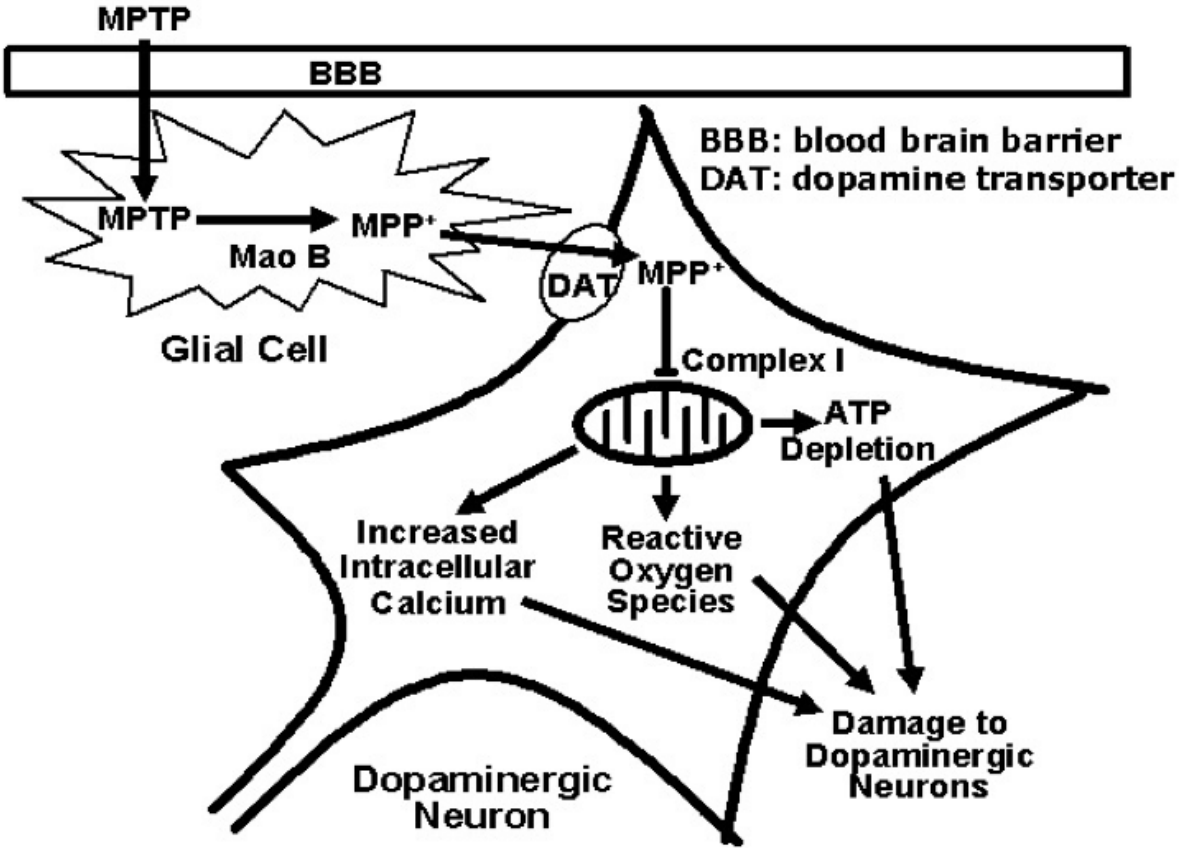
In order to be an effective neurotoxin, MPTP requires conversion to it active metabolite, 1-methyl-4-phenylpyridinium (MPP^+), which mediates the neurotoxic effects of MPTP (Markey et al., 1984; Langston et al., 1984a; 1984b). As illustrated in Figure 1.2, MPTP neurotoxic activity relies on a series of cellular processes. MPTP is able to cross the blood brain barrier; once in the brain, MPTP is converted to its active metabolite, MPP^+ by monoamine oxidase B (MAO-B) (Przedborski and Jackson-Lewis, 1998). The metabolism of MPTP has been suggested to occur in glial cells, with MPP^+ release mediated by the

extraneuronal monoamine transporter (Russ et al., 1996). MPP^+ is then actively taken up by the dopamine transporter (DAT) (Mayer et al., 1986). MPTP dopaminergic toxicity requires DAT as evidenced by resistance to MPTP-induced toxicity in DAT null mice (Bezard et al., 1999). The inverse results are produced in DAT over expressing transgenic mice, displaying increased sensitivity to MPTP-induced neurotoxicity (Donovan et al., 1999). In the dopamine neuron, MPP^+ is thought to induce cell death through targeted inhibition of complex I of the mitochondrial respiratory chain (Nicklas et al., 1985; Mizuno et al., 1987).

The first link between mitochondrial dysfunction and Parkinson's disease came from understanding of potential mechanisms governing the toxic effects of MPP^+ (Nicklas et al., 1985). MPTP is suggested to inhibit NADH ubiquinone oxidoreductase (Complex I) of the mitochondrial respiratory chain. While mitochondrial impairment in the MPTP model has been reported, a direct link between mitochondrial dysfunction and clinical PD is less clear. Interestingly, previous observations of reduced Complex I activity in the SN of PD brains (Schapira et al., 1989). A potential mechanism by which Complex I inhibition may contribute to cell death in PD is through ATP depletion. In mouse brain, MPTP induces a significant decrease in ATP synthesis resulting in depletion of cellular ATP (Scotcher et al., 1990) and generation of free radicals (Beal, 1996), suggesting potential oxidative damage.

FIGURE 1.2. MPTP metabolism and toxicity.

MPTP must be converted to its active metabolite MPP^+ in order to induce neurotoxic effects on dopaminergic neurons. MPTP is converted by MAO-B enzyme to form MPP^+ in glial cells. MPP^+ is then released from glial cells into the extracellular space. The DAT facilitates uptake of MPP^+ into dopaminergic neurons, where the neurotoxin targets and inhibits the mitochondrial Complex I.


MPTP
BBB
BBB: blood brain barrier
DAT: dopamine transporter
MPTP
MPP+
Mao B
DAT
MPP+
Glial Cell
Complex I
ATP
Depletion
Increased
Intracellular
Calcium
Reactive
Oxygen
Species
Damage to
Dopaminergic
Neurons
Dopaminergic
Neuron

MPTP-induced models in rodents are also not without their problems. The mouse MPTP model has been used primarily to study pathogenic mechanisms contributing to PD and for development of neuroprotective strategies. Many iterations of the mouse MPTP model have been produced in last two decades, but the most commonly used model is one that uses "acute" administration of toxin (multiple MPTP doses in a single day) (Jackson-Lewis and Przedborski, 2007). Other models employ subchronic administration of toxin (typically 1 dose per day over a 5-day period) or chronic administration (doses over approximately 1 month) (Jackson-Lewis and Przedborski, 2007). What is rarely taken into account in studies employing these models is that the cell death processes are likely quite different depending on a number of variables, including the frequency and duration of toxin exposure, and may or may not have relevance to what occurs in the brain of a PD patient. A number of different mechanisms contributing to cell death have been suggested to occur in the acute mouse MPTP model. However, despite numerous reports developing neuroprotective strategies employing this model, success in translating these findings to the clinic has not always produced similar results. One possible reason for this is that the acute MPTP model employed, in which rapid cell death ensues, may not reflect the time-dependent, complex sequence or cascade of events that occurs in a slowly evolving neurodegenerative disease such as PD. Thus, using this model alone for development of putative neuroprotective strategies may not be productive.

1.7 Reactive oxygen species

Extensive studies have attributed a significant role of reactive oxygen species (ROS) and one of their greatest generators, mitochondria, in the pathogenesis of PD. ROS are a natural by-product of various cellular processes, which, upon deregulation, can lead to neuronal death (Mattson et al., 1995; Beal, 1996; Dawson and Dawson, 1996; Dehmer et al., 2000; Wu et al., 2002; Abramov and Scorziello, 2007). One of the largest sources of ROS in the cell is generated by mitochondria whilst performing cellular respiration. As electron transfer progresses to generate a hydrogen ion gradient inside the mitochondrion to subsequently form ATP, superoxide radicals are created which can react with other small molecules to create various reactive oxygen/nitrogen species (ROS/RNS) and can subsequently damage cellular macromolecules (nucleic acids, proteins and lipids).

These oxygen radicals, in low concentrations, seem harmless to the cell and often serve a physiological function (Chandel et al., 1998; Dada et al., 2003; Hamanaka and Chandel, 2010); however, their overproduction in a pathological context may lead to cell death (Simon et al., 2000). This is thought to occur during DA cell loss in PD. This idea stems from a multitude of studies relating both ROS and mitochondrial dysfunction to PD. For example, markers of mitochondrial complex I deficiency (Schapira et al., 1989; 1990; Blandini et al., 1998), and accumulation of mitochondrial genome mutations have been demonstrated in these patients (Tanaka et al., 1996). Furthermore, dopaminergic neurotoxins such as MPTP and rotenone, both of which have been highly linked to Parkinsonism, likely exert their toxic effects through direct inhibition of mitochondrial complex I (LINDAHL and OBERG, 1961;

OBERG, 1961; Mizuno et al., 1987). Extensive evidence in cellular and animal models of PD link ROS to the pathogenesis of PD (Jenner, 2003; Ekstrand et al., 2007; Vila et al., 2008).

1.8 Microglia and neuroinflammation in Parkinson's disease

As much as the brain is isolated itself from the rest of the system through the blood brain barrier, the immune system nevertheless remains functionally intertwined with it. Under physiological conditions, this function serves effectively to clear debris or aggregates when needed or to signal an infection to the rest of the body. However, under pathological conditions, the interplay between nervous system and immune system may result in exacerbation of the cell death phenotype.

Work from our lab, as well as other groups, indicates roles for both the innate and adaptive responses of immunity in PD (Orr et al., 2005; Lira et al., 2011). Recent attention has been given to PD genes, particularly PINK1 and LRRK2, with regards to modulation of the immune response in the context of neurodegeneration (Gardet et al., 2010; Hakimi et al., 2011; Liu et al., 2011; Moehle et al., 2012). Though the understanding of the interplay between immune and nervous systems in the context of neurodegeneration is still in its infancy, better comprehension of the pathways involved, particularly with PD genes, may lead to better treatment symptoms by anti-inflammatory agents.

Activation of the brain's resident microglia occurs during normal aging, and is associated with many neurodegenerative diseases such as PD and Alzheimer's disease (AD). This may drive a self-propagating toxic cycle promoted by the release of pro-inflammatory and loss of

protective mediators (Aarsland et al., 2001; Akiyama et al., 2002; Hobson and Meara, 2004; Block and Hong, 2005; Griffin et al., 2006; Whitton, 2007; Griffin, 2008; Cribbs et al., 2012; Bardou et al., 2014). When these processes are triggered within vulnerable brain regions, they may lead to the loss of cholinergic neurons in the nucleus basalis magnocellularis (Willard et al., 1999; Whitton, 2007), as well as DA neurons in the SNc, NE neurons in the LC and, all regions that show significant early cell loss in the brains of patients with PD and AD (Braak et al., 2003a; Szot et al., 2006; Grudzien et al., 2007; Rudow et al., 2008).

Under normal physiological conditions, neuroinflammatory-associated microglial activation is carefully controlled. However, unregulated activation of microglia through exogenous toxins or endogenous signalling proteins, may result in production of pro-inflammatory cytokines, gradually initiating neurodegenerative processes (Block and Hong, 2005; Colton and Wilcock, 2010; Smith et al., 2012). Microglia can assume varying phenotypes associated with the release of potentially destructive, pro-inflammatory cytokines or the expression of cytokines that promote repair, recovery, and growth. Microglia in various states of activation are detectable many years before the onset of neuropathological changes (Imamura et al., 2003; Cagnin et al., 2006; Gerhard et al., 2006; Tai et al., 2006). Vulnerable brain regions, such as the SNc in PD, are likely exposed for many decades to complex combinations of varying states of microglial activation with accompanying changes in expression levels of pro-inflammatory cytokines (Bilbo, 2010; Heneka et al., 2010; Herrup, 2010).

1.8.1 Interferon-ɣ

Inflammatory mediators are central to microglial activation (McGeer and McGeer, 2002). However, the nature of the inflammatory response, as it relates to microglia and the loss of DA neurons, is not clear. Various cytokines, including interleukin (IL)-1ß, IL-2, IL-4, IL-6, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) are increased in the brains of PD patients (Nagatsu et al., 2000). Likewise, cDNA microarray revealed that MPTP-treated mice displayed alterations in genes for numerous pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) as well as the anti-inflammatory cytokine IL-10 (Mandel et al., 2000). However, the function of these cytokines in dopaminergic neuronal loss is less clear.

There is recent evidence that another critical inflammatory cytokine, interferon-ɣ (IFN-ɣ), may be important in PD. IFN-ɣ is believed to play an important role in early immunological responses to viral and tumor insults, as well as slow developing adaptive responses to infections (Mamane et al., 1999; Hiscott et al., 2006). The only member of type II class of interferons, it is a soluble dimerizing cytokine (Nathan et al., 1983) which is critical for both the innate and the adaptive immune system against viral, bacterial and protozoan infection (Karupiah et al., 1993; Mosovsky et al., 2014; Smith and Denning, 2014). The importance of IFN-ɣ in the immune system is in its function in inhibiting viral replication, and stimulating and regulating immune effects. It is primarily produced in natural killer (NK), natural killer T (NKT) cells, and cytotoxic T lymphocytes (CTL) (Scharton and Scott, 1993). Evidence also suggests IFN-ɣ is produced by microglia in the CNS (De Simone et al., 1998) and can activate microglia, as observed *in vitro* (Badie et al., 2000; Kraft et al., 2009). Through the Janus kinase/signal transducers and activators of transcription-1 (Jak/STAT1) pathway, IFN-ɣ

mediates the induction of microglial iNOS, resulting in elevated levels of nitric oxide (NO) (Delgado, 2003). Levels of IFN-ɣ mRNA have been shown to increase following MPTP treatment in mice (Ciesielska et al., 2003). Evidence for a relationship between IFN-ɣ and PD is suggested by the fact that allelic differences between early and late-onset PD patients were reported for the *IFN*-ɣ gene (Mizuta et al., 2001). Chapter 2 provides evidence to indicate that DA neuron survival in models of PD, is mediated by IFN-ɣ and microglia activation.

1.9 Death mechanisms in dopamine neurons

1.9.1 Calpain-induced neurodegeneration

Increasing evidence indicates that impairment in protein handling may contribute to the pathogenesis of several neurodegenerative disorders, such as PD. Protein aggregation in the form of LB inclusions have been observed in a many PD cases (Olanow and Tatton, 1999; Olanow et al., 2004). Calpains are calcium activated proteolytic enzymes that are involved in regulation of many physiologically important processes. However, evidence indicate that calpains may play a role in the pathogenesis of neurological disorders such as ischemia (Neumar et al., 2001) and AD (Lee et al., 2000; Adamec et al., 2002). With regard to PD, previous immunohistochemical analysis revealed an increase in calpain expression in the midbrain of PD brains (Mouatt-Prigent et al., 1996). However, the functional consequence of such an increase on PD associated DA cell death processes was not evaluated. Of further interest, p35, an activator of cyclin dependent kinase 5 (Cdk5), is predominantly expressed in the brain, and has been identified as a proteolytic target of calpain (Kusakawa et al., 2000).

1.9.1.1 Cyclin-dependant kinase 5 (Cdk5)

Cdk5 is a serine/threonine kinase that was originally identified based on sequence homology with the family of cyclin dependent kinases (CDKs) (Xiong et al., 1992). Despite ubiquitous expression, previous evidence indicates that Cdk5 activity is predominantly restricted to the nervous system (Tsai et al., 1993). This activation of Cdk5 was attributed to the expression of co-activators p35 and p39, and their respective, calpain cleaved, non-membrane bound forms, p25 and p29 (Tsai et al., 1994; Ko et al., 2001). The importance of Cdk5 in development of the nervous system was later recognized, when Cdk5 deficient mice displayed severe cortical lamination defects and perinatal death (Ohshima et al., 1996).

The mechanism by which p25 may contribute to death is highlighted by observations that p25/Cdk5 complexes may be targeted to the nucleus, a potential regulation of death/survival related to the regulation of transcription factors. In support of this, oxidative stress induces nuclear localization of p25/Cdk5 activity, with a resulting inhibition of myocyte enhancing factor 2 (MEF2) survival activity (Gong et al., 2003). Supporting a role for cdk5 in PD pathology is the observation that Cdk5 is highly accumulated in LBs observed in postmortem PD dopaminergic neurons (Brion and Couck, 1995). In support of a role for Cdk5 in PD, use of a dominant negative (DN) expression of Cdk5 in the SNc, attenuated MPTP-induced DA cell death (Smith et al., 2003).

1.9.1.2 Myocyte enhancer factor 2 (MEF2)

Myocyte enhancer factor 2 (MEF2) is a transcription factor that has been implicated in neuronal development (Shalizi and Bonni, 2005). Our research moved towards examining the

potential involvement of MEF2 as a survival factor and as a Cdk5 phosphorylation target in our model. Evidence indicated MEF2 hyper-phosphorylation was associated with neuronal death *in vitro* (Mao and Wiedmann, 1999). This evidence suggested the potential pathogenic effect of MEF2 phosphorylation in cell death signalling, the upstream mechanisms regulating MEF2 phosphorylation events. *In vitro* results established Cdk5 as a regulator of MEF2 phosphorylation following neurotoxic insult (Gong et al., 2003). Results show that Cdk5 mediates phosphorylation of the isophorm MEF2D on the inactivating residue, Serine 444, with resulting inhibition of its survival function (Gong et al., 2003). Subject to this, the phosphorylation and inhibition of MEF2 in the MPTP model was performed (Smith et al., 2006). Results found an increase in phosphorylation of MEF2 at the Cdk5 site. Further, ectopic expression of a non-phosphorylatable form of MEF2 (MEF2D-S444A) reduced MPTP-induced DA cell death (Smith et al., 2006).

1.9.1.3 Nur77

Recent evidence suggests Nur77 as a potential MEF2 transcriptional target gene (Youn et al., 1999; Liu et al., 2001; Ananieva et al., 2008). The Nur77 promoter contains a putative binding sites for MEF2 (Woronicz et al., 1995). Originally, Nur77 was identified as an inducer of apoptosis in thymocyte selection (Lee et al., 1995b; Youn et al., 1999; Fujii et al., 2008). Nur77 is a member of the Nur family of nuclear hormone receptor, specifically the orphan NR4A subgroup, which includes Nur77 (NR4A1, nerve growth factor-inducible gene B, NGFI-B), Nurr1 (NR4A2) and Nor1 (neuron-derived receptor-1, NR4A3). In the CNS, Nurr1 is expressed in DA neurons of the SNc and VTA. This nuclear receptor is essential for the development and maintenance of mesencephalic DA neurons (Zetterström et al., 1997).

Reports have found Nor-1 and Nur77 expression localized to regions of DA terminal neurons, including the striatum, nucleus accumbens, olfactory tubercle, and prefrontal and cingulate cortices (Zetterström et al., 1996; Werme et al., 2000). In contrast to these reports, Nur77 has shown functional versatility acting as a survival factor in several apoptotic paradigms outside the CNS (Brás et al., 2000; Suzuki et al., 2003). Interestingly, recent evidence also suggests Nur77 expression regulates dopaminergic cell biochemistry and dopamine metabolism (Gilbert et al., 2006).

These reports did not provide substantial data with regard to the functional relevance of Nur77 in regulation of dopaminergic survival and neuropathology. Consequently, research performed in Chapter 3 evaluated the role of Nur77 in MPTP-induced DA cell death, with attention to its regulation by calpain-Cdk5-MEF2.

1.10 Hypotheses and Experimental Approach

1.10.1 Thesis Aims

Advances in the understanding of PD etiology and pathophysiology have been driven by the development and use of experimental models closely mimicking the clinical symptoms, pathology and signalling mechanisms observed in patients. Using the MPTP model of PD, experiments toward this thesis were designed to evaluate roles for IFN-ɣ and Nur77 in mechanisms mediating DA cell loss.

1.10.2 Hypotheses

Expression of IFN-ɣ alters dopaminergic cell death, mediated by IFN-ɣ activated microglia in stressed DA neurons.

We examined whether IFN-ɣ modulated the survival of DA neurons both *in vivo* and *in vitro* models of PD. Employing a neuronal/microglia co-culture in vitro survival model, we evaluate the interactive effects on DA neuron survival.

Nur77 expression and regulation alters survival of microglial stressed neurons.

Following our model of DA neuron survival regulated by the calpain-Cdk5-MEF2 pathway (Smith et al., 2003; 2006) in a PD mouse model, we evaluated the transcription factor Nur77 as a regulatory target. DA neuron survival in the MPTP mouse was assessed following down and up-regulation of Nur77 expression.

CHAPTER 2: Involvement of Interferon-ɣ in Microglial-Mediated Loss of Dopaminergic Neurons.

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STATEMENT OF AUTHOR CONTRIBUTION

This manuscript includes both *in vivo* and *in vitro* experiments supporting the resulting conclusions. *In vivo* experiments were developed and executed by MP Mount with A Lira performing the *in vitro* experiments. D Grimes provided the Parkinson's disease patient samples, which were analyzed by S Faucher. MPP^+ and amine HPLC analysis was performed in the laboratory of H Anisman. Experiments were developed by MP Mount with guidance from S Hayley and DS Park, with scientific input from R Slack. All *in vivo* figures and text for this manuscript was prepared by MP Mount while *in vitro* figures were prepared by A Lira. All experiments were made possible with the guidance and scientific assistance of DS Park.

Involvement of Interferon-ɣ in Microglial-Mediated Loss of Dopaminergic Neurons

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Abbreviated Title: Interferon-ɣ and Parkinson's disease

Key words: Interferon-ɣ (IFN-ɣ), Parkinson's disease, neurodegeneration, dopamine, cytokine, microglia, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

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ABSTRACT

Growing evidence implicates microglia in the loss of dopaminergic neurons in Parkinson's disease (PD). However, factors mediating microglial activation in PD are poorly understood. Pro-inflammatory cytokines, such as interferon-ɣ (IFN-ɣ), orchestrate the actions of microglia. We report here that PD patients express significantly elevated levels of IFN-ɣ in their blood plasma. After this initial finding, we found that *IFN-ɣ*-deficient mice displayed attenuated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced substantia nigra pars compacta dopaminergic cell loss along with reduced loss of striatal tyrosine hydroxylase and dopamine transporter fiber density. MPTP-induced depletion of striatal dopamine and its metabolite DOPAC (3,4-dihydroxyphenylacetic acid), as well as ΔFosB, a marker of postsynaptic dysfunction, were also attenuated in these knock-out mice. Consistent with the role for IFN-ɣ in microglial activation, MPTP-induced morphological activation of microglia was abrogated compared with wild-type mice. To examine more mechanistically the role of IFN-ɣ in microglial activation, we evaluated the interactions between microglia and dopaminergic neurons in an *in vitro* mixed microglia/midbrain neuron rotenone-induced death paradigm. In this *in vitro* paradigm, dopaminergic neurons are selectively damaged by rotenone. Exogenous IFN-ɣ ligand alone and without rotenone resulted in dopaminergic cell loss, but only in the presence of microglia. The addition of an IFN-ɣ message neutralizing antibody attenuated neuronal loss as a result of rotenone treatment. The presence of only wild-type microglia and not those deficient in IFN-ɣ receptor elicited significant dopaminergic cell loss when exposed to rotenone. Neurons deficient in IFN-ɣ receptor, however, did not display increased resistance to death. Finally, levels of IFN-ɣ message

increased in microglia in response to rotenone. Together, these data suggest that IFN-ɣ participates in death of dopaminergic neurons by regulating microglial activity.

Key words: interferon-ɣ (IFN-ɣ); Parkinson's disease; neurodegeneration; dopamine; cytokine; microglia; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative condition characterized by progressive motor deficits including tremor, bradykinesia, rigidity, and postural instabilities (Przedborski and Vila, 2001)(Przedborski et al., 2001). Many of the cardinal symptoms of PD are attributed to depletion of dopamine (DA) in the brain resulting from the loss of dopaminergic neurons of the substantia nigra pars compacta (SNc) (Smeyne and Jackson-Lewis, 2005). Current therapies, such as L-DOPA (L-3,4-dihydroxyphenylalanine), are mainly directed toward replacing DA levels in the brain and, as such, provide only symptomatic relief. These drugs do not modify the progressive neurodegenerative cell loss process associated with PD that, in many cases, results in debilitating side effects (Kostic et al., 1991). Attenuation of the underlying degeneration in PD may provide opportunities for more effective therapeutic intervention. However, the mechanism(s) controlling dopaminergic loss is not completely clear.

Administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces a syndrome in mice and primates resembling that of clinical PD (Langston et al., 2000; Fukuda, 2001). Similar to the histopathological features of PD, MPTP treatment provokes a selective loss of dopaminergic neurons in the SNc coupled with a profound reduction in striatal DA with little alteration in other monoamine neurotransmitter systems (Smeyne and Jackson-Lewis, 2005)(Smeyne and Jackson-Lewis, 2005). This model is frequently used to examine dopaminergic cell loss in an *in vivo* setting.

A common characteristic of PD and the MPTP model of degeneration is increased microglial activation (Członkowska et al., 1996; Gao et al., 2002b; Barcia et al., 2004)(Czlonkowska et al., 1996; Gao et al., 2002b; Barcia et al., 2004). Several lines of evidence support the importance of this activation in the deterioration of dopaminergic neurons. The use of the pharmacological anti-inflammatory agent minocycline attenuates activation of microglia as well as MPTP-induced dopaminergic cell loss (Du et al., 2001; Wu et al., 2002). Direct administration of lipopolysaccharide (LPS) induces microgliosis and has been correlated with the loss of dopaminergic neurons *in vivo* (Arai et al., 2004; de Pablos et al., 2005). Activation of this innate immunity has also been implicated in neurodegeneration with the specific triggering of Toll-like receptor-4-dependent pathways (Lehnardt et al., 2003). Furthermore, low concentrations of rotenone require microglia to induce death of dopaminergic neurons (Gao et al., 2002a). One potential mechanism underlying the pro-death effects of microglia on dopaminergic neurons involves increased oxidative insult. For example, microglia are known to activate proteins that increase oxidative load, including NADPH oxidase and inducible nitric oxide synthase (iNOS) (Almer et al., 1999; Liberatore et al., 1999; Wu et al., 2003). Indeed, mice deficient in both of these signals display attenuated MPTP-induced loss of dopaminergic neurons. Although this evidence implicates microglial activation, the functional relevance of microgliosis is still controversial. Specifically, it is unclear, particularly *in vivo*, whether microglial activation is causative or correlative to dopaminergic neuron loss. In addition, other types of glial activation (i.e., astrocytes) might also be important in dopaminergic cell death (Suzumura et al., 2006). Accordingly, questions of (1) whether microglia may only be a secondary effect to initial dopaminergic degeneration and (2) how

important microglial activation may be to dopaminergic degeneration *in vivo* remain. For example, agents such as minocycline may protect by means other than microglial activation (Wu et al., 2002; Yang et al., 2003).

Inflammatory mediators are central to microglial activation (McGeer and McGeer, 2002). However, the required nature of the inflammatory response, as it relates to microglia and dopaminergic death, is not completely clear. Various cytokines including interleukin (IL)-1ß, IL-2, IL-4, IL-6, transforming growth factor-ß (TGFβ), and TNFα are increased in the brains of PD patients (Nagatsu et al., 2000). Likewise, cDNA microarray revealed that MPTP-treated mice displayed alterations in genes for numerous pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) as well as the anti-inflammatory cytokine IL-10 (Mandel et al., 2000). However, the function of these cytokines in dopaminergic neuronal loss is less clear.

There is recent evidence that another critical inflammatory cytokine, interferon-ɣ (IFN-ɣ), may be important in PD. IFN-ɣ is believed to play an important role in early immunological responses to viral and tumor insults, as well as slow developing adaptive responses to infections (Mamane et al., 1999). In the brain, IFN-ɣ is produced by microglia (De Simone et al., 1998) and can be a strong activator of microglia *in vitro* (Badie et al., 2000). IFN-ɣ, through the Janus kinase/signal transducers and activators of transcription-1 (Jak/STAT1) pathway, mediates the induction of iNOS (Delgado, 2003). Recently, Ciesielska et al. (Ciesielska et al., 2003) have shown IFN-ɣ transcripts increase after MPTP treatment in mice. Evidence for a relationship between IFN-ɣ and PD is suggested by the fact that allelic differences between early- and late-onset PD patients were reported for the *IFN-γ* gene

(Mizuta et al., 2001). However, to date, there is no clear evidence to indicate that IFN-ɣ is central to dopaminergic cell loss. Consequently, we examined whether IFN-ɣ may be required in the loss of dopaminergic neurons in both *in vivo* and *in vitro* models of PD. We present evidence for the importance of IFN-ɣ in the death of dopaminergic neurons induced by MPTP (*in vivo*) and rotenone (*in vitro*). We also provide evidence that IFN-ɣ can act through microglia to promote dopaminergic loss and suggest that this may be critical in the pathogenesis of PD.

MATERIALS AND METHODS

Patient IFN-ɣ plasma analysis. Human plasma was examined using a multiplex bead-based immunoassaying system as described previously (Hulse et al., 2004). The patients' sera were diluted fourfold in PBS containing 1% BSA and 0.05% NaN_3. **Fifty microliters** (50 μm)of each sample were incubated for 2 h at room temperature with the antibody-coupled beads in a filter-bottomed 96-well plate (Nunc, Naperville, IL; VWR International, West Chester, PA). The biotinylated anti-cytokine antibody conjugate (1:500) was added to the well and incubated for 1 h at room temperature. Bound antibodies were revealed with a streptavidin–phycoerythrin conjugate. After washing and resuspension in 2% paraformaldehyde in PBS, the beads were analyzed on a luminex-100 system (Luminex, Austin, TX) equipped with Luminex Data Collector software, version 1.7, using the median fluorescence intensity as the read out. Standard curves were established using recombinant IFN-ɣ, and sample IFN-ɣ concentrations were derived from the linear portion of the curve. Patient diagnosis was assessed by David Grimes after clinical history and examination. Blood samples from 13 PD patients and 7 disease control patients were analyzed in this study (supplemental Table 1, available at www.jneurosci.org as supplemental material). The Ottawa Hospital Research Ethics Board sanctioned approval for this study. Samples were drawn and immediately centrifuged with serum drawn off and frozen. Patient confidentiality was maintained using number coding, and samples were blinded to condition. The mean age of the PD patients was 66 years (range, 44–77 years), the duration of the disease was 7.5 ± 1.3 years, and all patients had been medicated as specified (supplemental Table 1, available at www.jneurosci.org as

supplemental material). The mean age of the disease control patients was 66 years (range, 61–76 years).

Experimental animals. Male *IFN*-ɣ –/– knock-out (KO), *IFN*-ɣ receptor –/– KO, and +/+ wild-type (WT) mice (all on a C57BK/6J background; 12 generations of KO mice) were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals were maintained on a 12 h light/dark cycle with lights on at 6:00 A.M., the room temperature was maintained at 21°C, and the mice were permitted an *ad libitum* diet of Ralston Purina (St. Louis, MO) mouse chow. For the *in vivo* experiments, two separate groups of animals were analyzed. In the first, *IFN*-ɣ KO and WT mice were treated with both MPTP and saline. To corroborate data from these findings, the *IFN*-ɣ KO and WT mice were interbred for two generations to produce littermate controls, which were then used in a second set of experiments. All experimental procedures were approved by the University of Ottawa Committee for Animal Care and met the guidelines set out by the Canadian Council on Animal Care.

MPTP administration. N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP-HCl; 25 mg/kg, i.p., measured as free base; Sigma, St. Louis, MO) was administered to mice (8–10 weeks old) intraperitoneally once per day for 5 consecutive days (Crocker et al., 2003b). Mice used as controls received an equivalent volume of saline (0.9%). Mice were killed 14 d after initial MPTP/saline treatment.

Immunohistochemistry. After overnight postfixation in 4% paraformaldehyde, brains were cryoprotected in 10% sucrose before cryosectioning into 14 or 40 μm (used for stereology)

free-floating sections, obtained as described previously (Smith et al., 2003). Immunostaining was performed as described previously using mouse anti-tyrosine hydroxylase (TH; 1:10,000; ImmunoStar, Hudson, WI), rat anti-DA transporter (DAT; 1:2000; Millipore, Bedford, MA), rat anti-CD11b (1:200; Serotec, Oxford, UK), or rabbit anti-ΔFosB (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were visualized using diaminobenzidine, as described previously (Crocker et al., 2003b).

Assessment of neuronal survival. Dopaminergic neurons in the SNc of treated mice were assessed for survival using the dopaminergic cell marker TH. Assessment of survival was performed using two different counting methodologies. In the first set of animals, dopaminergic neurons in the SNc of WT and *IFN*-ɣ KO mice were determined by serial section analysis of the total number of TH-positive (TH+) neurons as described previously (Smith et al., 2003; Crocker et al., 2003b) at 14 d after initial MPTP treatment. Briefly, midbrain tissue sections were stained for TH. At least two sections were obtained from each animal representing each of five levels from –2.92 to –3.52 relative to bregma. Sections from each level and animal were averaged for the number of TH+ neurons. A total of six to eight animals per group were analyzed. Estimates of total TH+-stained neurons in the SNc were calculated using Abercrombie's correction for the previously stated bregma levels, centering on the central region of the SNc, the medial terminal nucleus (MTN) (Hayley et al., 2004). Adjacent tissue was also stained using anatomical stain, cresyl violet, to validate survival as assessed by TH analysis. These results were subsequently corroborated, by examining a second group of animals interbred to produce littermate controls, as described above. The SNc region of this second group of animals was analyzed by unbiased stereological estimates.

The total number of TH+ neurons in the SNc was obtained by applying optical fractionation (Gundersen et al., 1988) using Stereo Investigator (version 6; MicroBrightField, Williston, VT), as described previously (Rymar et al., 2004). In brief, 40 μm brain sections were examined within the rostal and caudal limits of the SNc (bregma –2.54 to –3.88) (Paxinos and Franklin, 2001). For each brain, six coronal sections were examined. After immunohistochemistry, mounting, defatting, and coverslipping, the mean section thickness, as measured with a *z*-axis microcator, was 16 μm. Sections were analyzed using a 100x lens. Total number of TH+ neurons was determined using the optical fractionator.

Quantification of striatal immunohistochemistry. Quantification of striatal dopaminergic density fiber staining and striatal ΔFosB-positive nuclei were analyzed using Eclipse densitometry analysis (Crocker et al., 2003a). In all cases of immunohistochemical quantification, analyses were performed by an individual unaware of the experimental treatments. Each tissue quantified was compared relative to its own non-stained background.

N-*methyl-4-phenylpyridinium ion measurements*. Striatal concentrations of *N*-methyl-4-phenylpyridinium ion (MPP^+) were measured 90 min after a single dose of MPTP using HPLC measurements, as described previously (Crocker et al., 2003a).

Neurochemical analyses. After decapitation, brains were sectioned into a series of coronal slices using a dissecting block with adjacent slots spaced 2 mm apart. The SNc and striatum were obtained by micropunch using a 1-mm-diameter biopsy needle. Brain punches were taken according to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin,

2001; Smith et al., 2003). HPLC analysis was performed 14 d after initial MPTP treatment. Levels of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were determined by HPLC using methods described previously (Seegal et al., 1986; Smith et al., 2003).

Primary neuron-enriched mesencephalic cultures. The entire midbrain, without meninges and blood vessels, was collected from the brains of embryonic day 13.5 (E13.5) mice, as described previously (Liu et al., 2000). The midbrain tissue was removed and subjected to trypsin (T-4549; 1 mg/ml; Sigma) for 25 min at 37°C. After dissociation, the cells were treated with trypsin inhibitor (0.92 mg/ml dissolved in 1 mM HCl; catalog #10137100; Roche, Indianapolis, IN) and DNase (1 mg/ml; catalog #1284-932; Roche) and were counted using a hemacytometer (Bright-Line; Hausser Scientific, Horsham, PA). Midbrain cells were seeded (5 x 10^5 per well) in 24-well culture plates precoated for 1 h with poly-D-lysine (20 μg/ml). Cultures were maintained in 0.5 ml per well of Neurobasal medium supplemented with 2% B27, 1% N2, 416 μM L-glutamine, 41.6 U/ml penicillin, and 41.6 μg/ml streptomycin and kept in a 37°C incubator-humidified atmosphere of 5% CO_2 and 95% air.

Microglia harvest. Postnatal day 2–3 mice were used for mixed glia cultures. Brains were dissociated using a tissue grinder kit and plated in minimum essential medium supplemented with 5% heat-inactivated fetal bovine plasma, 5% heat-inactivated horse plasma, and 0.05 mg/ml gentamycin. Two days later, the medium was replenished, and the culture reached confluency ~8 d later. Confluent mixed-glia cultures were shaken for 5 h at 180 rpm at 37°C to separate microglia from astrocytes. This produces a yield of >94% enriched microglia and is comparable with similar harvesting protocols (Gao et al., 2003).

Co-culturing. Harvested microglia were added (5 x 10^4 per well) to 7-d-old neuron-enriched mesencephalic cultures. The following day, rotenone was added (20–25 nM), and 7 d later the cultures were fixed and stained for TH (1:2500) and Hoescht (0.24 μg/ml; Sigma). TH immunoreactivity was quantified by counting the total number of all live cells (assessed by Hoescht staining) that stained positive for TH. All live cells typically appear intact with uncondensed nuclei, whereas dead cells displayed fragmented with a condensed morphology. Rotenone treatment has been shown previously to diminish the total number of TH+ live cells in this culture model (Gao et al., 2002a). The results were performed in triplicate and show the average total number of live TH-immunoreactive cells per well. This was compared with untreated control wells that were plated identically and obtained from the same neuronal culture preparation as the experimental wells. For neutralizing antibody studies, IFN-ɣ-neutralizing IgG (rIFN-ɣ-purified goat IgG; R & D Systems, Minneapolis, MN) or control nonspecific IgG (normal goat IgG; R & D Systems) was added (15 μg/ml) 30 min before rotenone application. In our ligand application studies, IFN-ɣ ligand (recombinant mouse IFN-ɣ; R & D Systems) was added 24 h after co-culturing, and the cultures were fixed 7d later. IFN-ɣ receptor KO neurons and microglia were cultured in the same way as WT cells.

Detection of IFN-ɣ mRNA using reverse transcription-PCR. One day after plating, WT microglia-enriched cultures were treated with rotenone (20 nM) for 0, 1, or 3 d. RNA was extracted using Trizol (Invitrogen, San Diego, CA). After RNA purification and determination of RNA concentration, reverse transcription (RT)-PCR analysis for IFN-ɣ mRNA was conducted using the following primers: forward, ACT GGC AAA AGG ATG GTG AC; reverse, GAC CTG TGG GTT GTT GAC CT. As an internal control, S12 RNA

was amplified using the following primers: forward, GGA AGG CAT AGC TGC TGG; reverse, CCT CGA TGA CAT CCT TGG. Cycle conditions were as follows: 94°C (30 s), 59°C (30 s), and 72°C (1 min). Positive control samples were extracted from CD4+ T-cells after being stimulated in the presence of T_H1 (T helper cell 1) cytokines for 6 d. After amplification, the PCR products were run on a 2.25% agarose electrophoretic gel in 1x Tris–acetate–EDTA buffer for 30 min and visualized by ethidium bromide staining. Densitometry analysis revealed an increase in IFN-ɣ mRNA after exposure with rotenone for 1 and 3d.

Statistical analysis. All data analyses were performed by using one-way ANOVA, followed by the Newman–Keuls *post hoc* test (unless otherwise stated).

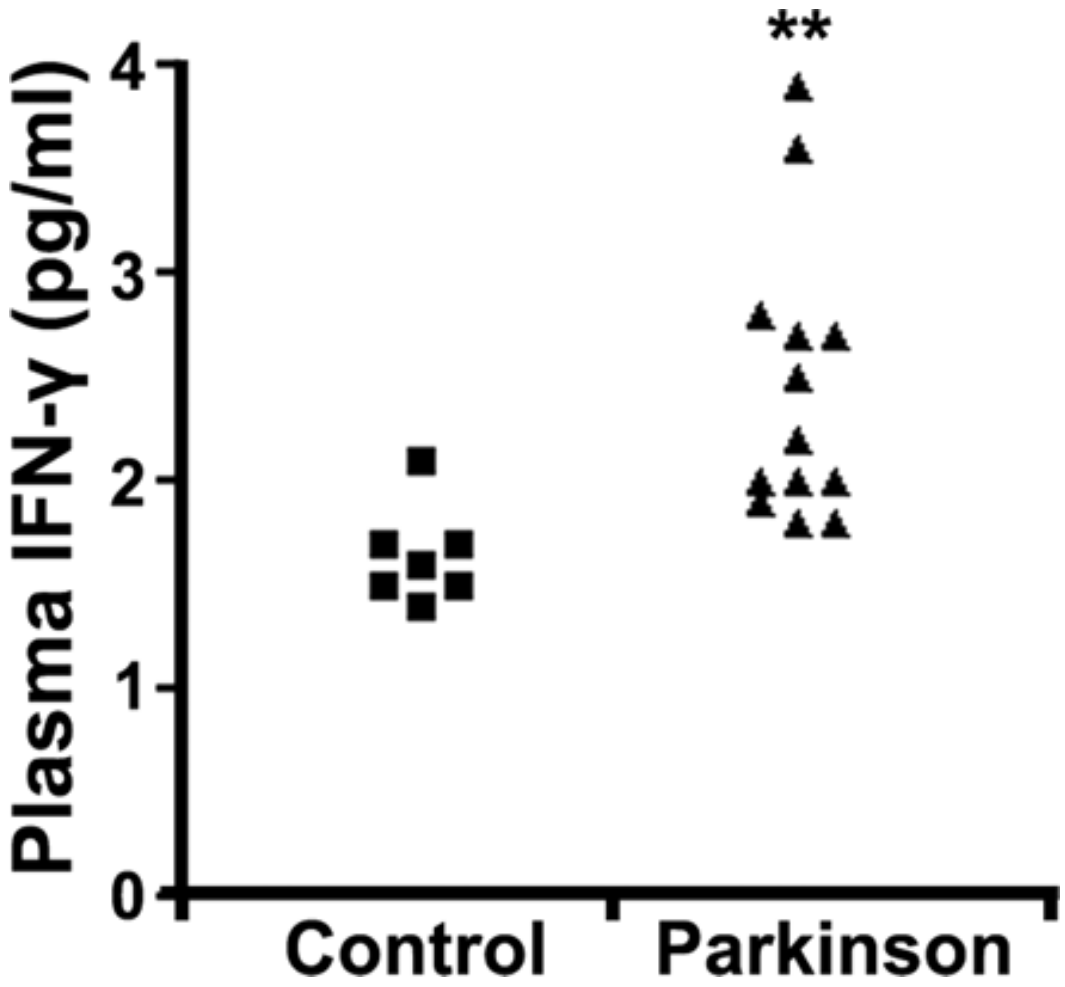
RESULTS

Elevated levels of plasma IFN-ɣ in PD patients

We initially examined the levels of several cytokines [IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, and TNF-α] by multiplex bead-based immunoassaying analyses in blood plasma from 13 PD patients compared with 7 normal control individuals. As shown in the Figure 2.1 dot plot, immunoassay analysis revealed a significant elevation of IFN-γ in blood plasma from PD patients over that in control subjects (33.0 ± 4.1% increase; $p < 0.01$), as was revealed previously by Gribova et al. (Gribova et al., 2003). Other cytokines, including GM-CSF, TNF-α, IL-2, IL-4, and IL-5, were not detectable, whereas no significant differences in IL-6 and IL-10 were detected (supplemental Table 2, available at www.jneurosci.org as supplemental material). Accordingly, combined with results by Ciesielska et al. (Ciesielska et al., 2003), displaying elevated IFN-γ transcripts in the SNc of mice treated with MPTP, we hypothesized that IFN-γ may be central to dopaminergic loss occurring in PD and MPTP-induced neurotoxicity. Accordingly, we proceeded to examine whether IFN-γ may be important in an *in vivo* animal model of dopaminergic loss.

FIGURE 2.1. PD patients display elevated IFN-ɣ plasma levels relative to non-PD patients.

Blood plasma from 13 PD and 7 control patients was analyzed by a multiplex bead assay system for several cytokines. Results for IFN-*ɣ* concentrations are provided here in dot plot representation (two-tailed *t* test, $^{**}p < 0.01$).


**
Plasma IFN-γ (pg/ml)
4
3
2
1
0
Control
Parkinson

IFN-γ-deficient mice exhibit attenuated MPTP-induced degeneration of dopaminergic cell bodies in the SNc

We first assessed dopaminergic neuron survival after saline or MPTP treatment of separate groups of WT and *IFN-γ* KO mice on a C57BK/6J background (Fig. 2.2A–*C*). No statistical difference in the number of TH+ neurons in the SNc could be detected between saline-treated WT and *IFN-γ* KO mice (Fig. 2.2B). Similarly, no statistical difference in striatal TH or DAT immunopositive fiber density was detected by immunohistochemistry (Fig. 2.2D–G). DAT is critical for the reuptake of DA and MPP^+, the active MPTP metabolite in dopaminergic neurons (Meissner et al., 2003). Finally, no differences in levels of striatal DA or its metabolite DOPAC were detected in these mutant mice under basal conditions (Fig. 2.3C, D). After MPTP administration, there was a significant reduction (42%; $p < 0.01$) in TH+ SNc neurons in WT mice (Fig. 2.2E). Interestingly, *IFN-γ*-deficient mice displayed significantly more TH+ cells than WT mice treated with MPTP ($p < 0.05$).

FIGURE 2.2. IFN-γ-deficient mice display protection of dopaminergic cell bodies and striatal terminals of MPTP-induced degeneration of SNc dopaminergic neurons.

A, Representative photomicrographs illustrating TH immunoreactivity in the ventral midbrain SNc of the indicated non-littermate treatment groups, as indicated in Materials and Methods. ***B***, Quantification of TH+ neurons using Abercrombie correction, as described in Materials and Methods. ***C***, Quantification of cresyl violet-stained cells of the SNc (MTN level). ***D***, Representative photomicrographs of striatal sections stained with TH from the animal groups as described in ***A***. Magnified images are included in the insets of ***D–J***. ***E***, Quantification of optical density of TH striatal fibers. ***F***, Representative photomicrographs illustrating DAT immunoreactivity in the striatum of indicated treatment groups as in ***A***. ***G***, Quantification of optical density of striatal DAT-stained fiber density. ***H***, Representative photomicrographs illustrating TH immunoreactivity in the SNc of the indicated littermate treatment animal groups. Staining is more intense here than in ***A*** because of increased section thickness required for stereological analyses. ***I***, Quantification of TH+ neurons, in littermate animals by stereology, as described in Materials and Methods. ***J***, Representative photomicrographs of striatal TH fiber density of indicated littermate treatment groups. ***K***, Quantification of OD of striatal TH-stained fiber density of indicated littermates. Error bars represent mean ± SEM. ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; $n = 6–8$ animals per group. OD, Optical density; Sal, saline.

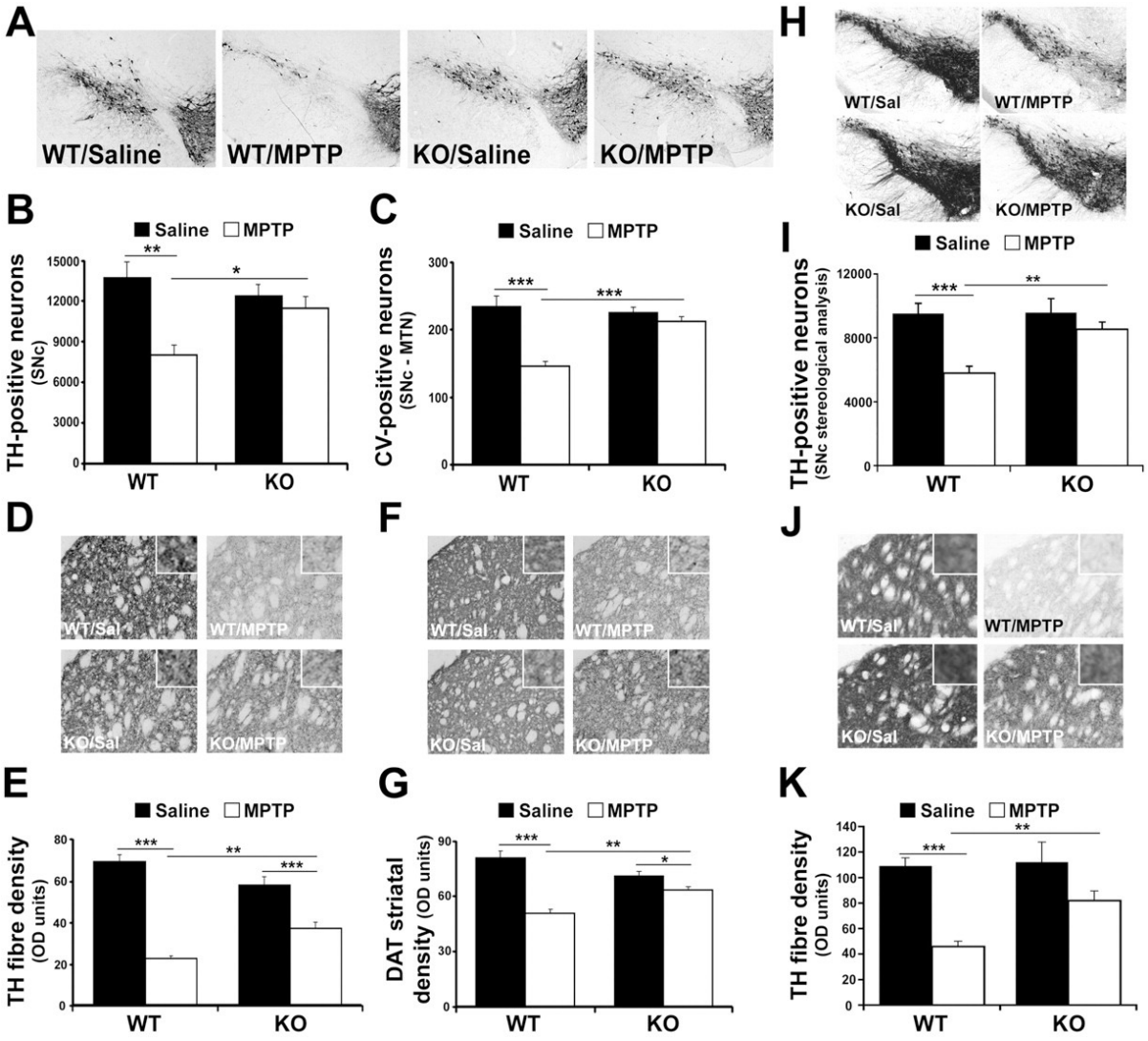
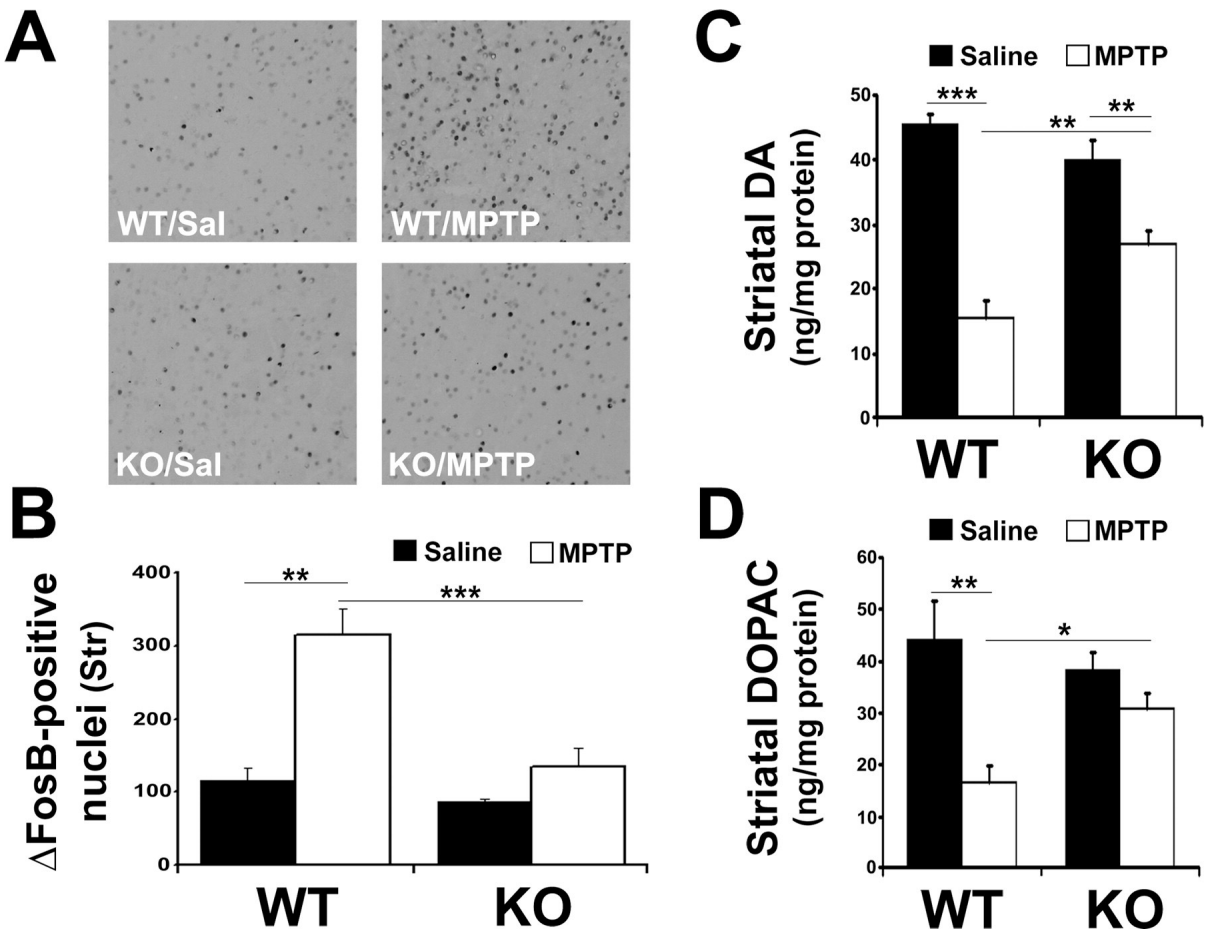

A
WT/Saline
WT/MPTP
KO/Saline
KO/MPTP
H
WT/Sal
WT/MPTP
KO/Sal
KO/MPTP
B
Saline
MPTP
TH-positive neurons
(SNc)
WT
KO
C
CV-positive neurons
(SNc - MTN)
I
TH-positive neurons
(SNc stereological analysis)
D
F
J
E
TH fibre density
(OD units)
G
DAT striatal
density (OD units)
K

FIGURE 2.3. IFN-ɣ KO mice display reduced striatal ΔFosB, a marker for postsynaptic changes in the denervated striatum, as well as attenuated DA and DOPAC.

A, Representative photomicrographs showing ΔFosB staining in the striatum of mice treated as indicated. Sal, Saline. ***B***, Quantification of ΔFosB-positive cells/nuclei. Str, striatum. ***C***, ***D***, Levels of DA (***C***) and its metabolite DOPAC (***D***) in the striatum were analyzed using HPLC on 14-d-old tissue after saline/MPTP injection. Error bars represent mean ± SEM. ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; $n = 6$ animals per group.


A
WT/Sal
WT/MPTP
KO/Sal
KO/MPTP
B
ΔFosB-positive nuclei (Str)
Saline
MPTP
400
300
200
100
0
**
***
WT
KO
C
Striatal DA (ng/mg protein)
Saline
MPTP
50
40
30
20
10
0
***
**
**
WT
KO
D
Striatal DOPAC (ng/mg protein)
Saline
MPTP
60
50
40
30
20
10
0
**
*
WT
KO

To further substantiate these findings, we repeated the experiment using WT and *IFN-γ* KO mice interbred to produce littermate controls. These animals were analyzed by stereological assessment for surviving TH+ neurons over the entire nigral region. Identical results were obtained in comparison to the initial dopaminergic cell survival experiment (Fig. 2.2*A–C*). MPTP-treated *IFN-γ* KO mice exhibited a significant dopaminergic cell-body survival ($p < 0.001$) compared with WT littermate control animals also treated with MPTP (Fig. 2.2*H*,*I*).

To corroborate TH analysis of dopaminergic neuron survival, the number of neurons at the level of the MTN (bregma –3.16) within the SNc of all groups was determined using cresyl violet staining (Fig. 2.2*C*). Analysis was consistent with that of TH survival, showing no difference between saline-treated groups, a significant loss of TH+ neurons in WT MPTP-treated mice ($p < 0.001$), and a significant reduction in cells lost in IFN-γ-deficient MPTP-treated animals compared with WT mice ($p < 0.001$).

MPTP derives its toxic effects once it crosses the blood–brain barrier (BBB) and is converted to MPP^+, the active dopaminergic neural toxin, by monoamine oxidase-B. The protective effects observed in *IFN-γ*-deficient animals may be attributed to altered MPTP metabolism and therefore reduced MPP^+. To examine this possibility, striatal tissue was evaluated from mice 90 min after a single injection of MPTP, and levels of MPP^+ were determined using HPLC. It was observed that MPP^+ levels did not significantly differ between WT and *IFN-γ*-deficient animals (data not shown), suggesting that the protection afforded by *IFN-γ* deficiency was not because of impaired MPTP metabolism.

IFN-γ-deficient mice exhibit attenuated MPTP-induced degeneration of the DA axon terminal nigrostriatal dopaminergic pathway

Previous results indicated that MPTP-induced loss of dopaminergic cell bodies in the SNc is attenuated by *IFN-γ* deficiency. To determine whether the striatal projections of the SNc neurons were also protected in *IFN-γ*-deficient mice, striatal dopaminergic terminal fiber density was assessed initially in non-littermate animals. This analysis is important because this is the region where dopaminergic neurons express their activity through DA release. Densitometry analysis of TH striatal fiber staining showed a significant reduction (68%; $p < 0.001$) in WT MPTP-treated animals compared with saline controls (Fig. 2.2*D*,*E*). Similar to that observed in the SNc, there was no statistical difference in the TH striatal density of saline-treated WT or *IFN-γ*-deficient mice. *IFN-γ* KO animals exhibited an attenuation of MPTP-induced reduction in striatal TH density compared with MPTP-treated WT animals (32 vs 68%). This suggests striatal terminal fibers were protected by *IFN-γ* deficiency similar to the dopaminergic cell bodies. Results using littermate control animals paralleled these findings, with *IFN-γ* KO mice displaying attenuation in TH-fiber density ($p < 0.01$) (Fig. 2.2*J*,*K*).

Additional corroboration of these findings included examining density levels of striatal DAT. Densitometric analysis of DAT staining of striatal fibers showed a significant reduction (38%; $p < 0.001$) in WT MPTP-treated animals compared with saline controls (Fig. 2.2*F*,*G*). *IFN-γ* KO animals exhibited an attenuation of MPTP-induced reduction in striatal DAT density compared with WT treated mice ($p < 0.01$).

Postsynaptic functional effects of IFN-*γ* deficiency

Treatment with MPTP has been demonstrated previously to induce expression of the transcription factor ΔFosB within the striatum, which is thought to reflect postsynaptic changes (Smith et al., 2003). ΔFosB is suggested to mediate the supersensitivity of striatal DA receptors after denervation (Dragunow et al., 1995). Consistent with these reports, WT MPTP-treated mice displayed a significant increase in the number of ΔFosB-positive cells (Fig. 2.3*A*,*B*) compared with saline controls. Importantly, MPTP-treated *IFN-γ*-deficient mice showed a significantly reduced increase in FosB staining.

Effect of *IFN-γ* deficiency on amine levels

Finally, we examined how DA levels are affected in IFN-*γ*-deficient mice after MPTP treatment. Previous analyses report diminished DA and its metabolite, DOPAC, in the striatum 14 d after MPTP treatment (Crocker et al., 2003a). The effects of *IFN-γ* deficiency parallel the dopaminergic cell survival results observed previously (Fig. 2.3C, *D*). In both the striatum and the SNc, MPTP significantly reduced the levels of DA and DOPAC in WT mice. However, there was a significant decrease in the loss of DA in the MPTP-treated *IFN-γ* KO mice.

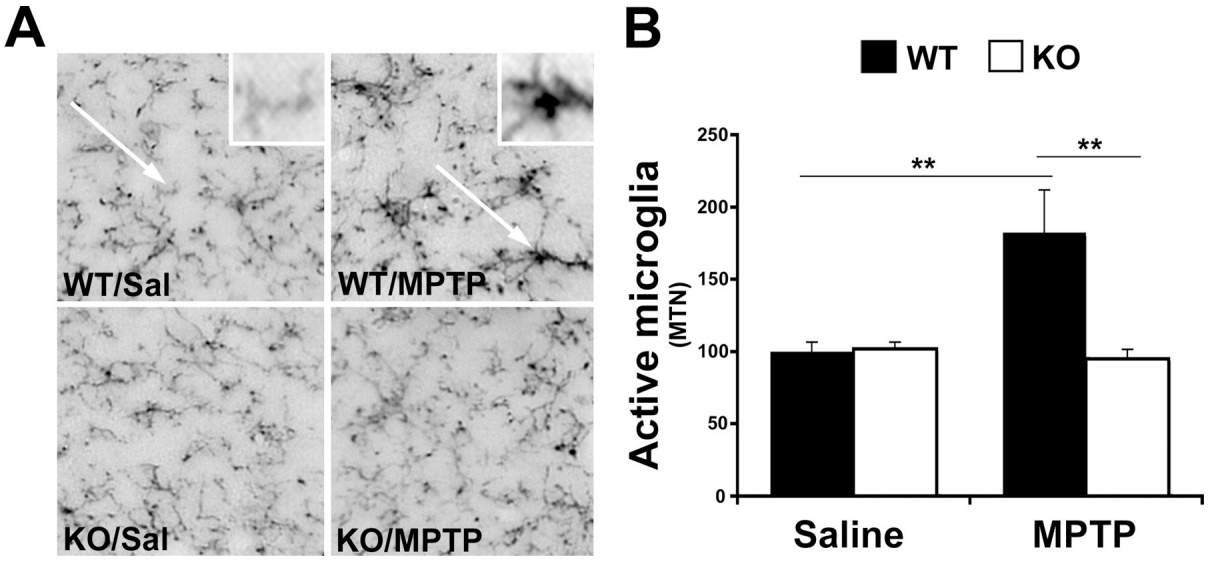
***IFN-γ*-deficient mice display attenuated microglial activation in the SNc in response to MPTP**

In WT mice, MPTP has been shown to initiate microgliosis, the morphological and pathological activation of microglia, within the SNc where the highest concentration of microglia resides (Członkowska et al., 1996; 2002). Because IFN-*γ* has also been reported to

activate microglia, we examined whether microgliosis was altered in *IFN-γ*-deficient mice treated with MPTP compared with WT animals. Microglia were stained for CD11b, the α-subunit of the $\alpha_M\beta_2$ integrin (CD11b/CD18, complement 3bi receptor) that plays an important role in leukocyte adhesion and whose expression in the brain is confined to microglial cells (Zeng et al., 2005). Activated microglia appear more compact and rounded with cellular thickening and complexing (Fig. 2.4A, WT/MPTP, arrow) (Soltys et al., 2001). Although normal resident microglia express basal levels of CD11b, they appear smaller and have a ramified shape with thin processes (Fig. 2.4*A*, WT/Sal, arrow). Quantification of microgliosis yielded a significant difference ($p < 0.01$) with twice the number of activated microglia in MPTP WT mice than in saline-treated mice (Fig. 2.4*B*). Basal activity of microglia in *IFN-γ* KO mice treated with saline displayed no difference in microgliosis compared with WT saline-treated mice (Fig. 2.4B). The increase in microgliosis exhibited with MPTP treatment, as seen in WT mice, was not observed in *IFN-γ*-deficient mice. There was no significant difference in active microglia between *IFN-γ*-deficient saline- versus MPTP-treated mice. However, there was a significant difference between WT and *IFN-γ* KO mice treated with MPTP ($p < 0.01$) (Fig. 2.4*B*).

FIGURE 2.4. Analysis of active microglia using anti-CD11b.

A, Representative photomicrographs illustrating CD11b immunoreactivity in the SNc (MTN) of WT and KO animals treated with either saline (Sal) or MPTP. Arrows indicate resting CD11b-positive microglia (WT/Sal) and activated microglia (WT/MPTP). ***B***, Quantification of morphologically active microglia. Magnified images of each are provided in the insets of ***A*** (WT/Sal and WT/MPTP). Error bars represent mean ± SEM (ANOVA, $^{**}p < 0.01$; $n = 6$ animals per group).


A
WT/Sal
WT/MPTP
KO/Sal
KO/MPTP
B
WT
KO
Active microglia
(MTN)
250
200
150
100
50
0
**
**
Saline
MPTP

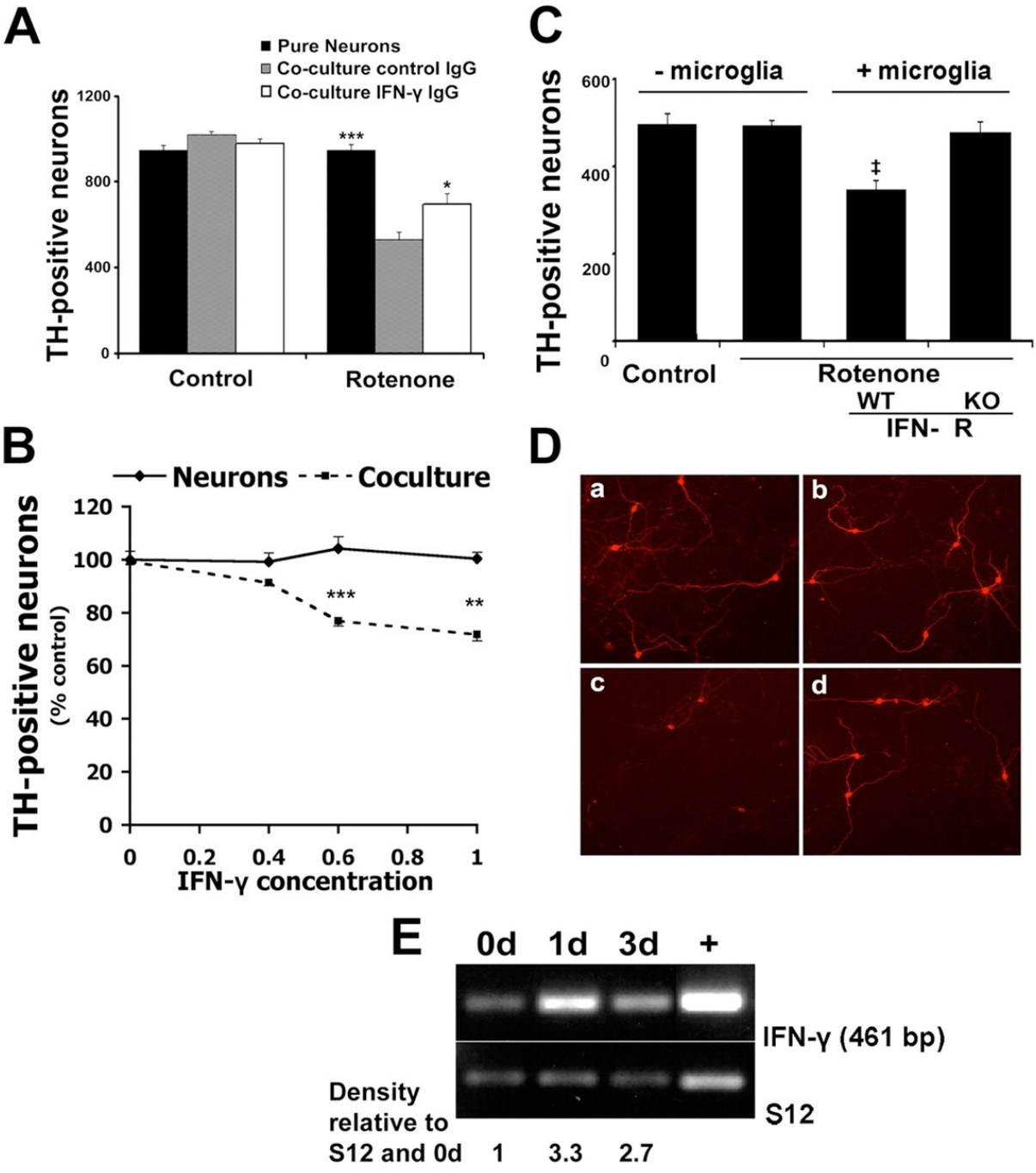
Role of IFN-γ in dopaminergic cell loss in an *in vitro* microglia/neuron co-culture system

Our *in vivo* results indicate that IFN-γ is important in MPTP-induced dopaminergic cell death. The protection associated with *IFN-γ* deficiency is accompanied with reduced microglial activation. However, the mechanism by which IFN-γ deficiency may be protective and whether it involves microglial activation is difficult to assess in an *in vivo* setting. This is particularly true in this present situation in which reduced microglial activation could be a secondary effect of reduced neuronal loss. Accordingly, we used an *in vitro* model of dopaminergic cell loss in which effects of microglia on dopaminergic neurons can be studied more directly. Therefore, to address whether IFN-γ may promote dopaminergic loss through activation of microglia and to more closely study the interaction between these two populations of cells, we used a microglia/midbrain neuron co-culture system, similar to that reported previously (Gao et al., 2002a). In this system, midbrain cultures enriched for neurons, obtained from E13.5 embryos, are cultured in defined Neurobasal medium. In these cultures, dopaminergic neurons constitute <1% of all the neurons in culture. All neurons, including TH+ cells, in these cultures are resistant to 20–25 nM rotenone, another complex I inhibitor similar to MPP^+ (Kotake and Ohta, 2003). No dopaminergic cell death is observed even after 7 d of rotenone treatment in the absence of microglia (Fig. 2.5*A*). The addition of microglia to these cultures alone, in the absence of rotenone, does not significantly affect survival of total neurons or the more sensitive dopaminergic neurons. However, with the addition of 20–25 nM rotenone, a significant loss of TH+ neurons is observed only in the presence of microglia (Fig. 2.5*A*). These findings indicate that death of TH+ neurons in this system, at a defined rotenone concentration, is dependent on microglia. It is important to note

that this effect is concentration specific; at higher concentrations, rotenone will begin to kill non-dopaminergic cell types as well, even in the absence of microglia (data not shown).

FIGURE 2.5. IFN-ɣ requirement in a co-culture model of dopaminergic cell loss induced by rotenone *in vitro*.

A, Neutralizing IFN-*ɣ* antibody protects dopaminergic neurons from rotenone-induced death in the presence of microglia. Seven-day-old WT neuron-enriched midbrain cultures were co-cultured with and without WT microglia in the presence or absence of rotenone and with control or IFN-*ɣ* neutralizing antibody, as described in Materials and Methods and as shown. The number of live TH+ neurons was assessed as described in Materials and Methods. *$p <$ 0.05, ***$p < 0.001$ relative to co-culture with control IgG (two-tailed *t* test). ***B***, Seven-day-old neuron-enriched midbrain cultures supplemented with or without microglia were subjected to IFN-*ɣ* ligand (nanograms per milliliter) applications. The cultures were fixed 7 d later and stained with TH and Hoechst. ***$p < 0.001$ compared with control, **$p < 0.01$ (two-tailed *t* test; $n = 3$). ***C***, Dopaminergic neurons treated with rotenone are resistant to death in the presence of microglia lacking IFN-*ɣ* receptor. Dopaminergic neurons used were similar to those in ***A***, except WT neurons were cultured in the presence of either WT or IFN-*ɣ* receptor (IFN-R)-deficient microglia. ‡$p < 0.05$ relative to IFN-*ɣ* receptor KO (two-tailed *t* test; $n = 3$). ***D***, Representative immunofluorescence of co-cultures quantified in ***C***, stained with TH antibody are as follows: ***a***, WT mesencephalic neurons; ***b***, WT mesencephalic neurons treated with rotenone; ***c***, WT mesencephalic neurons and microglia treated with rotenone; ***d***, WT mesencephalic neurons and IFN-*ɣ* receptor KO microglia treated with rotenone. ***E***, WT microglia-enriched cultures were treated with rotenone for 0, 1, and 3 d. One week after plating, microglia were collected, RNA was extracted, and RT-PCR was performed for IFN-*ɣ* and S12 control. Densitometric analysis was performed with values normalized to each respective S12 signal and further normalized to 0 d value. +, IFN-*ɣ*-positive RNA control sample obtained from CD4+ T-cells. Error bars represent mean ± SEM.


A
Pure Neurons
Co-culture control IgG
Co-culture IFN-γ IgG
TH-positive neurons
1200
800
400
0
***
*
Control
Rotenone
C
- microglia
+ microglia
TH-positive neurons
600
400
200
0
‡
Control
Rotenone
WT
KO
IFN-
R
B
Neurons
Coculture
TH-positive neurons
(% control)
120
100
80
60
40
20
0
***
**
0
0.2
0.4
0.6
0.8
1
IFN-γ concentration
D
a
b
c
d
E
0d
1d
3d
+
IFN-γ (461 bp)
S12
Density
relative to
S12 and 0d
1
3.3
2.7

Using this system, we first asked whether inhibition of IFN-γ would affect rotenone-induced dopaminergic death as was observed *in vivo*. As shown in Figure 2.5*A*, a neutralizing antibody to IFN-γ but not control IgG significantly improved dopaminergic survival. This indicates that IFN-γ can modulate the rotenone-induced dopaminergic cell death, which occurs only in the presence of microglia. To further support the importance of IFN-γ in microglia-induced sensitivity of dopaminergic neurons, we exposed midbrain neurons with and without co-cultured microglia to varying concentrations of exogenous IFN-γ ligand. As shown in Figure 2.5*B*, 0.6 and 1 ng/ml concentrations of exogenous IFN-γ caused significant loss of dopaminergic neurons. This, however, only occurred in the presence of microglia and not in their absence. This indicates that IFN-γ is an important mediator of dopaminergic neuron loss, but only in the presence of microglia.

To determine more clearly whether IFN-γ may act on microglia to control rotenone-induced dopaminergic cell loss in this system, we cultured microglia from *IFN-γ* receptor KO or WT control mice. These WT or *IFN-γ* receptor-deficient microglia were then co-cultured with normal neurons with or without rotenone treatment. Death of dopaminergic neurons only occurred in the presence of WT microglia with rotenone treatment (Fig. 2.5*C*,*D*). Cultures treated with rotenone but containing IFN-γ receptor-deficient microglia displayed no death. Conversely, we also examined whether midbrain neurons cultured from *IFN-γ* receptor-deficient animals were resistant to rotenone in the presence of WT microglia. There was no difference in death of IFN-γ receptor-deficient neurons compared with WT neurons exposed to rotenone in the presence of WT microglia (data not shown). Finally, if rotenone-mediated death of dopaminergic neurons occurs through IFN-γ, we would predict that IFN-γ should be

present in microglia. Accordingly, we isolated microglia, exposed them to rotenone, and determined the IFN-γ message at certain times after treatment by RT-PCR. As shown in Figure 2.5*E*, there was a basal level of IFN-γ message present in untreated microglia that increased on exposure to rotenone. Together, these results indicate that IFN-γ plays a crucial role in modulating microglial responses *in vitro*. In addition, it suggests that IFN-γ may promote MPTP-induced dopaminergic loss *in vivo*, at least partially through activation of microglia.

DISCUSSION

Previous work has suggested the importance of the inflammatory response and microglial activation in dopaminergic loss in animal models of PD as well as in PD patients (McGeer et al., 1988; Banati et al., 1998; Wu et al., 2002; Barcia et al., 2004). However, the required nature of the inflammatory mediators or their mechanisms of action in PD is not fully understood. Our findings are of significance because they directly demonstrate that a central inflammatory mediator, IFN-γ, plays a critical role in dopaminergic loss, both *in vitro* and *in vivo*. We found that (1) levels of IFN-γ are elevated in the serum of PD patients, (2) *IFN-γ* deficiency attenuates the degeneration of the nigrostriatal system and significantly reduces microgliosis in a model of *in vivo* dopaminergic loss, and (3) IFN-γ is involved in microglial-mediating dopaminergic neurodegeneration.

Our analysis of human blood serum for possible elevated cytokines yielded results with only levels of IFN-γ displaying a significant increase from those of control patients. It must be noted that cytokine detection in this assay may be hampered because of a lack in cytokine stability (Noguchi et al., 1996). In addition, it should also be stressed that cytokine blood levels do not necessarily reflect actions in the CNS. However, systemic IFN-γ-producing inflammatory cells have been reported to infiltrate the brain (Perry, 2004). In addition, Banks (Banks, 2005) has recently suggested that cytokines circulating in the blood may cross into the CNS through direct cytokine transport across the BBB. Therefore, we also examined the role of IFN-γ in dopaminergic neuronal loss.

Our results, in an *in vivo* mouse model of dopaminergic neurodegeneration, reveal that mice deficient in *IFN-γ* show reduced loss of dopaminergic cell bodies and striatal terminals compared with WT animals treated with MPTP. Germline loss of *IFN-γ* did not appear to grossly perturb the dopaminergic system, including possible postsynaptic signalling changes (FosB), as assessed in saline-treated animals. In addition, DA and DOPAC levels, as well as markers of postsynaptic function (FosB), are significantly attenuated in *IFN-γ*-deficient animals treated with MPTP. Interestingly, microglial activation is also reduced in these deficient mice.

Consistent with our *in vivo* findings, we observed that IFN-γ is required for the death of dopaminergic neurons cultured in the presence of microglia with rotenone. Importantly, reduced cell loss was only observed when microglia deficient in IFN-γ receptor, but not WT microglia, were added to the dopaminergic neurons. In contrast, deficiency of IFN-γ receptors in dopaminergic neurons did not result in protection in our co-culture death paradigm. IFN-γ was also detected in cultured microglia that increased on rotenone exposure. Together, the data not only provide evidence for direct relevance of the microglial response in dopaminergic cell loss induced by mitochondrial toxins but also define IFN-γ as a critical inflammatory mediator in this process.

Our results are consistent with the view that IFN-γ is a primary mediator of microglial activation (Moss and Bates, 2001; Platten et al., 2001; Abbas et al., 2002; Delgado, 2003). There are a number of possible effects of IFN-γ on microglia that may contribute to dopaminergic loss, both *in vivo* and *in vitro*. First, IFN-γ, through binding to its receptor and

activation of the Jak/STAT pathway, can activate iNOS. Alternatively, IFN-γ has also been shown to up-regulate the death receptor Fas and its ligand, FasL (Badie et al., 2000). Accordingly, IFN-γ could mediate its effects on microglia through direct activation of the death receptor on dopaminergic neurons. Indeed, Fas has been shown to be localized to dopaminergic neurons and is implicated in MPTP-induced dopaminergic cell death (Hayley et al., 2004).

Our model that IFN-γ promotes dopaminergic cell death through the activation of microglia is consistent with growing evidence that the inflammatory pathway is central to dopaminergic loss in *in vivo* and in *in vitro* models. Indeed, microglial activation has been noted in PD animal models and postmortem brains (McGeer et al., 1988; Banati et al., 1998). Direct activation of microglia through administration of the endotoxin LPS was found to induce loss of dopaminergic neurons in mice (Arai et al., 2004), while also stimulating the production of iNOS and subsequent reactive oxygen species (ROS) (Li et al., 2005). In fact, the nigra appears to be exquisitely sensitive to microglia activation because equivalent LPS injections in alternate brain regions (hippocampus, cortex) did not have the same pattern of degeneration (Xie et al., 2003). Finally, LPS and MPTP can synergistically increase dopaminergic death (Gao et al., 2003).

However, most functional evidence implicating microglia as a direct cause of dopaminergic cell degeneration is controversial. Minocycline, a tetracycline derivative known to inhibit microglia activation/inflammation and protect dopaminergic neurons in an MPTP model of PD, can also inhibit classic death molecules, such as caspases (Chen et al., 2000; Wei et al.,

2005). Moreover, others have reported that minocycline can exacerbate MPTP-induced toxicity (Yang et al., 2003). In addition, the role of microglia may also be dependent on the mode of activation. For example, activation of microglia in the hippocampus by IL-4- and IFN-γ-expressing T-cells is neuroprotective (Butovsky et al., 2005). Our evidence presented here is important because it provides clear evidence for the central death-promoting nature of the inflammatory response in dopaminergic neuronal loss. Finally, it is important to note that microglial activation is likely a graded response, and the degree of activation may depend on the type and severity of insult.

Although our evidence indicates the important nature of IFN-γ activation of microglia *in vitro*, the exact mechanism by which IFN-γ promotes dopaminergic death *in vivo* and whether this is strictly reliant on microglial activation is unclear. Indeed, IFN-γ receptors are expressed ubiquitously (Blanck, 2002). In addition, it is important to note that our results do not imply that inflammatory processes are the exclusive mediators of neuronal damage. Indeed, although IFN-γ deficiency effectively blocks microglial activation, it does not completely eliminate the reduction in striatal DA/DOPAC. This is an interesting observation that suggests that microglial activation, although important, is not the only mechanisms by which dopaminergic neuron damage occurs. This notion is supported by the fact that MPTP (MPP^+ metabolite) can directly target mitochondria in neurons and lead to increased ROS and damage (Liberatore et al., 1999; Dehmer et al., 2000; González-Polo et al., 2004; Chen et al., 2006).

There are some intriguing links with our present IFN-ɣ studies to that of human PD. First, microglial activation in PD patients is well characterized (Ouchi et al., 2005). Second, we and others have shown that plasma levels of IFN-ɣ are elevated in PD patients (Nakamura et al., 1992). The exact significance of this is unknown, particularly because the relationship, if any, between peripheral events and events in the CNS is unclear. However, increased plasma IFN-ɣ levels could reflect a systemic increase in IFN-*ɣ* production by lymphocytes and infiltrates capable of entering the CNS. In support of this, T-cells are known to circulate in the brain with lymphocyte infiltration, and release of pro-inflammatory cytokines, such as TNF-α and IFN-ɣ, have been shown at the site of injury (Raivich et al., 2003). Third, IFN-ɣ levels have also been shown to be increased in CSF and in the SN of PD patients (Widner et al., 2002). Fourth, IFN-ɣ has been shown to up-regulate iNOS, by binding the IFN-ɣ receptor and by up-regulating expression of astrocyte CD23 (Okuno et al., 2005). Interestingly, CD23 is found in glia from the SNc of PD patients, yet it is not detected in control subjects (Hunot et al., 1999). Finally, genetic studies have linked a dinucleotide CA repeat polymorphism within the first intron of the *IFN-ɣ* gene of patients with late onset of PD (Håkansson et al., 2005). This polymorphism leads to a higher production of IFN-ɣ. Together, this evidence suggests the potential importance of IFN-ɣ in PD pathogenesis and provides a link to our animal studies presented here.

In conclusion, we have shown that mice deficient in *IFN-ɣ* display an attenuation of MPTP-induced dopaminergic cell death and maintenance of DA functional activity. Our data suggest that one mechanism by which this is observed involves the activation of microglia, aiding in

the damage to dopaminergic neurons. Accordingly, targeting microgliosis by modulating levels of IFN-ɣ may be a candidate therapeutic strategy for treatment of PD.

CHAPTER 3: Perturbation of Transcription Factor Nur77 Expression Mediated by Myocyte Enhancer Factor 2D (MEF2D) Regulates Dopaminergic Neuron Loss in Response to 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

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STATEMENT OF AUTHOR CONTRIBUTION

The experiments for this manuscript were predominantly performed by MP Mount. All in vivo experiments, which included MPTP administration, adenoviral injections, tissue preparation, immunohistochemistry and analysis, were carried out by MP Mount. Y Zhang performed ChIP and cortical survival culture assays. Mesencephalic embryonic survival cell cultures were carried out by A Amini. Adenoviruses were generated by S Callaghan. MPP^+ and amine HPLC analysis was performed in the laboratory of H Anisman. Experiments were developed by MP Mount with guidance from DS Park and scientific input and expertise from Z Mao and R Slack. All figures and text for this manuscript were prepared by MP Mount along with guidance and editorial assistance from DS Park.

Perturbation of transcription factor Nur77 expression mediated by myocyte enhancer factor 2D (MEF2D) regulates dopaminergic neuron loss in response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).*

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***Running title:** *Nur77 expression in dopaminergic neuron survival*

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Keywords: Parkinson's disease; dopamine neurons; Nur77; MEF2; Cdk5; MPTP; SNc

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FOOTNOTES

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Background: Nur77 expression is regulated by MEF2D, found to be associated with the calpain-Cdk5-MEF2D neuronal death pathway.

Results: Nur77 deficiency results in hypersensitivity to neuronal toxicity, with Nur77 expression rescuing this loss.

Conclusion: Nur77 reduces toxic neuronal insult, regulated by the calpain-Cdk5-MEF2 pathway.

Significance: Previously reported calpain-Cdk5-MEF2 signalling is now further elucidated with regulation of Nur77 in dopaminergic neuronal loss.

ABSTRACT

We have earlier reported the critical nature of calpain-Cdk5-MEF2 signalling in governing dopaminergic neuronal loss *in vivo*. Cdk5 mediates phosphorylation of the neuronal survival factor myocyte enhancer factor 2 (MEF2) leading to its inactivation and loss. However, the downstream factors that mediate MEF2 regulated survival are unknown. Presently, we define Nur77 as one such critical downstream survival effector. Following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment *in vivo*, Nur77 expression in the nigrostriatal region is dramatically reduced. This loss is attenuated by expression of MEF2. Importantly MEF2 constitutively binds to the Nur77 promoter in neurons under basal conditions. This binding is lost following MPP^+ treatment. Nur77 deficiency results in significant sensitization to dopaminergic loss following MPP^+/MPTP treatment, *in vitro* and *in vivo*. Furthermore, Nur77-deficient MPTP-treated mice displayed significantly reduced levels of dopamine (DA) and DOPAC in the striatum as well as elevated post synaptic FosB activity, indicative of increased nigrostriatal damage when compared to WT MPTP treated controls. Importantly, this sensitization in Nur77-deficient mice was rescued with ectopic Nur77 expression in the nigrostriatal system. These results indicate that the inactivation of Nur77, induced by loss of MEF2 activity, plays a critical role in nigrostriatal degeneration *in vivo*.

INTRODUCTION

Parkinson's disease (PD) is characterised by progressive clinical hallmarks including tremor, bradykinesia, rigidity, and postural instability (Przedborski and Vila, 2001) and loss of dopamine neurons in the substantia nigra pars compacta (SNc). Current therapies are mainly directed toward replacing dopamine levels in the brain and, as such, provide only symptomatic relief (Kostic et al., 1991). Attenuation of the underlying degeneration in PD is a potentially more effective therapeutic strategy, the mechanism(s) controlling this loss is not clear.

Cdk5, a member of the cyclin-dependent kinase family (CDKs) (Pines, 1993) is primarily involved in regulation of neuronal function (Tsai et al., 1993; 1994). Cdk5 is involved in regulation of normal physiological function, evidence suggests that this protein also mediates death signalling (Smith et al., 2003; 2006; Qu et al., 2007; Huang et al., 2010). Importantly, we have previously provided evidence for the importance of Cdk5 in MPTP-induced dopaminergic death (Smith et al., 2003). For example CDK inhibitors, dominant negative (DN) Cdk5 expression, and deficiency of the Cdk5 regulatory activating partner p35 (Ko et al., 2001), blocks dopaminergic loss *in vivo*. MPTP induces calpain-dependent cleavage of p35 to form p25, resulting in increased Cdk5 activity in the SNc (Smith et al., 2006).

We identified a number of potential Cdk5 substrates that mediate dopaminergic cell loss. The first substrate identified was myocyte enhancer factor 2 (MEF2), a transcription factor with a critical role in muscle development (McKinsey et al., 2001), neuronal differentiation (Heidenreich and Linseman, 2004), and neuronal survival (Mao et al., 1999; Wang et al.,

2005; She et al., 2011). In this regard, we observed calpain-mediated cleavage of p35 and consequent activation of Cdk5 increased phosphorylation and inactivation of MEF2D in an MPTP model of dopamine degeneration *in vivo* (Smith et al., 2006). In addition, expression of constitutively active MEF2D protects dopamine neurons from MPTP-induced insult. However, the critical question of how MEF2 regulates survival is not completely clear.

Recent evidence suggests Nur77 as a potential MEF2 transcriptional target gene (Youn et al., 1999; Liu et al., 2001; Ananieva et al., 2008). The Nur77 promoter contains a putative binding sites for MEF2 (Woronicz et al., 1995). Nur77 was originally identified as an inducer of apoptosis in thymocyte selection (Lee et al., 1995b; Youn et al., 1999; Fujii et al., 2008). However, it is now known to show remarkable functional versatility. Nur77 has also been found to act as a survival factor in several apoptotic paradigms outside the CNS (Brás et al., 2000; Suzuki et al., 2003). Interestingly, recent evidence also suggests Nur77 expression regulates dopaminergic cell biochemistry and dopamine metabolism (Gilbert et al., 2006).

A potential link between MEF2D and Nur77 in dopaminergic cell loss and the critical need to understand how MEF2 may regulate dopaminergic survival, we examined whether Nur77 might be the mechanistic link driving a calpain-Cdk5-MEF2D mediated pathway of death. We provide evidence that Nur77 is both regulated by Cdk5-MEF2D and plays a critical in the survival response to dopaminergic cell death.

EXPERIMENTAL PROCEDURES

Animal − Male Nur77 knock-out (KO) mice (generously provided by Dr. Jeff Milbrandt, University of Washington, St. Louis, Missouri) (Lee et al., 1995a; 1995b) on a C57BK/6J background were further backcrossed for 6 generations with C57BK/6J strain mice (Jackson Laboratories). Animals were maintained on a 12 hour light/dark cycle with lights on between 06:00 and 18:00 and were permitted an ad libitum diet of Ralston Purina mouse chow. The room temperature was kept at 21^{o} C. All experimental procedures meet the Guidelines set out by the Canadian Council on Animal Care and were approved by the University of Ottawa Committee for Animal Care.

MPTP administration − Intraperitoneal injections (ip) of MPTP (Sigma; 30/mg/kg, measured free base) were administered to mice (8-10 weeks old) once a day for five consecutive days (Mount et al., 2007). Control animals received an equivalent volume of saline (0.9%). Mice were sacrificed 14 days following initial MPTP/saline treatment or at indicated time points.

Immunohistochemistry − Following overnight post-fixation in 4% paraformaldehyde, brains were cryoprotected in 10% sucrose with free-floating sections obtained as previously described (Crocker et al., 2003a; Mount et al., 2007). Immunolabelling was performed as previously described using anti-tyrosine hydroxylase (TH)(ImmunoStar, 1:10,000), rat anti-DA transporter (DAT)(Millipore, 1:2000), or rabbit anti-delta fosB (Santa Cruz, 1:1000). Primary antibodies were visualized using diaminobenzidine (DAB).

Assessment of neuronal survival − Dopaminergic neurons in the SNc of treated mice were assessed for survival using the dopaminergic cell marker TH. A total of six to eight animals per group were analyzed. Estimates of total TH+ stained neurons in the SNc were analyzed by unbiased stereological estimates, applying optical fractionation (Gundersen et al., 1988) using Stereo Investigator (version 6; MicroBrightField, Williston, VT), as described previously (Rymar et al., 2004; Mount et al., 2007). In brief, 40 nm brain sections were examined within the rostral and caudal limits of the SNc (bregma -2.54 to -3.88) (Paxinos and Franklin, 2001). For each brain, six coronal sections were examined. After immunohistochemistry, mounting, defatting, and coverslipping, the mean section thickness, as measured with a z-axis microdissector, was 16 um. Sections were analyzed using a 100X lens.

Quantification of Striatal immunohistochemistry − Striatal dopaminergic (TH and DAT) fiber density and FosB-positive nuclei were quantification using ImageJ (NIH) densitometry analysis (Mount et al., 2007). Each tissue quantified was initialed to its own non-stained background.

Adenoviruses delivery − Adenoviral constructs expressing MEF2D-S444A (Gong et al., 2003; Smith et al., 2006) and Nur77 (pcDNA generously provided by Dr. Jeff Milbrandt, University of Washington, St. Louis, Missouri) (Milbrandt, 1988) were constructed using the pAdEasy system, as previously described (Sedarous et al., 2003; O'Hare et al., 2005; Qu et al., 2007). Adenoviruses were delivered unilaterally to the right striatum using coordinates as previously described (Smith et al., 2006), seven days prior to initiation of MPTP/saline

treatment. A GFP-containing construct was used as controls for all adenovirus experiments. A single unilateral injection of virus was given to each animal (3ul, 1 X 10^7 particles per µl), delivered to the right striatum (0.5 mm rostral, 2.2 mm right of Bregma, and 3.4 mm below the skull surface). Each adenovirus injection was given at a constant rate of 0.5 µl/min using a syringe pump system (Harvard Apparatus). Brains were extracted at indicated times following the first MPTP injection.

Brain microdissection − Following decapitation, brains were sectioned into series 2.0 mm coronal slices using a plastic dissecting block. The SNc and striatum were obtained by micropunch using a 2 mm diameter biopsy needle. Brain punches were taken according to the Franklin and Paxinos mouse brain atlas(Mount et al., 2007).

Amine analyses − HPLC analysis was used to evaluate the levels of DA and its metabolite DOPAC in 14 day following first MPTP treatment brain microdissections. Levels were determined by HPLC as previously described (Mount et al., 2007).

Real-time PCR − RNA was extracted from mouse SNc microdissections using TRIzol reagent (Invitrogen). The primer sequences were as follows: Nur77, (forward) 5'-TGATGTTCCCGCCTTTGC-3' and (reverse) 5'-CAATGCGATTCTGCAGCTCTT-3'; GAPDH, (forward) 5'-CTGCACCACCAACTGCTTAG-3' and (reverse) 5' -GGGCCATCCACAGTCTTCT-3'. Nur77 and GAPDH primers used for gene expression and PCR conditions have been previously described (Tsukahara and Haniu, 2011), with cycling

parameters: 55°C for 10 min, 95°C for 5 min, and 40 cycles of 95°C for 30 s and 60°C for 60s.

Mesencephalic embryonic survival cell culture−Mesencephalic neurons were collected from day 13.5 embryos, adapted as previously described (Cheung et al., 1997). 7 DIV cultures were treated with 20 μM 1-methyl-4-phenylpyridinium (MPP^+; Sigma) for 24 and 36 hours. Cultures were fixed and stained for TH (1:2500) and Hoescht. Survival was assessed by quantifying live TH+ cells.

RNA interference − To disrupt Nur77 expression, 3 days after plating, siRNA, against Nur77 (Santa Cruz, sc-36109), as well as control siRNA (Santa Cruz, sc-37007) was transfected in cortical neuron cultures 1 day prior to MPP^+ (20 uM) treatment, as previously described (Zhang et al., 2006). At select time points, cells were lysed and assessed for survival (Zhang et al., 2006).

In Situ Hybridization - Performed at previously described (Gilbert et al., 2006).

Western blot analysis − Performed as previously described (Zhang et al., 2010) using antibodies against Nur77 (1:1000; BD Pharmingen) and b-actin (1:5000; Sigma) as a loading control.

Chromatin immunoprecipitation − Performed as previously described with embryonic day 15 mouse cortical neurons, following a 0, 24, 36, and 48 hour MPP^+ time course (Zhang et al.,

2010).The primers used for PCR amplification of the mouse Nur77 promoter region are 5'-CTGCGGGCACGGATTTACAACACC-3' and 5'-GGCGAGCCCGACCCACATCTT-5'.

Behavioural analysis − Behavioural analysis was carried out by utilizing the computer-assisted beam break, MicroMax system (Accuscan, Columbia, Ohio, USA). Animals were monitored for a 24 hour at 8 weeks of age (previously described by (Hayley et al., 2004)).

Statistical analysis−Histochemical and monoamine data analysis was carried out by one-way ANOVA, followed by Newman Keul's post hoc.

RESULTS

Nur77 expression following MPTP and relationship with MEF2. Our previous evidence indicated that loss of MEF2 activity mediated by Cdk5 was critical for dopaminergic loss induced by MPTP *in vivo* (Smith et al., 2006). This evidence suggested that loss of MEF2 mediated expression of a downstream transcriptional targets may lead to neuronal loss. Several reports suggested Nur77 as a candidate for MEF2 regulation (Youn and Liu, 2000; Liu et al., 2001; Dequiedt et al., 2003). Therefore, we initially ascertained whether loss of Nur77 expression could be detected in normal WT animals with MPTP treatment. We first assessed the levels of Nur77 transcripts from nigral extracts from an MPTP time course by quantitative real-time PCR (qPCR). Previous reports have shown Nur77/TH+ colocalization in the SNc following haloperidol treatment, while quantitative analysis by *in situ* hybridization did not display detectable levels under basal conditions (Gilbert:2006eq; Mahmoudi et al., 2009). However, employing qPCR, we observe a dramatic loss of Nur77 transcript expression in comparison to basal levels at 6 hours to 7 days post initial MPTP-treatment, ranging from a 44 to 80% reduction in Nur77 mRNA (Fig. 3.1A). These results were corroborated via *in situ* hybridization analysis in the dorsolateral and ventrolateral striatum (Fig.3.1B-D). We next assessed whether MEF2D binding on the endogenous Nur77 promoter may be affected by conditions which lead to neuronal loss using chromatin immunoprecipitation (ChIP) analyses. Because of the need for a more homogenous population of neurons, we utilized the MPP^+-treatment paradigm in cultured cortical neurons. We and others have shown that MPP^+ can induce death in non dopaminergic neurons, such as ceberebellar granule and cortical neurons, by non dopamine transporter related mechanisms

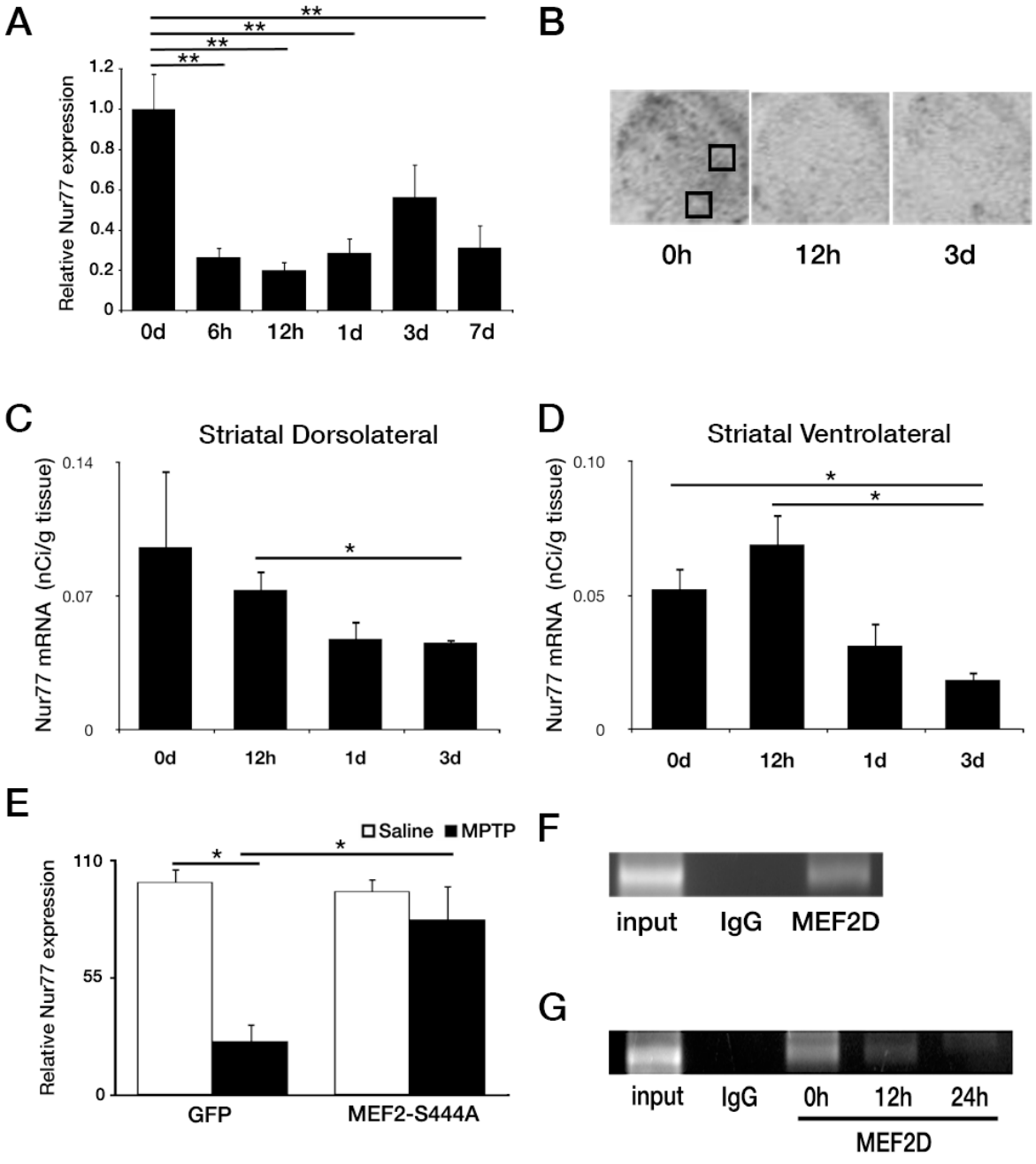
(Qu:2007gx; Huang:2010ic; Harbison:2011gk; Sheline et al., 2013). Importantly, we observed that there was detectable MEF2D binding at the Nur77 promoter basally in neurons (Fig. 3.1F) and that this binding diminished following MPP^+ exposure (Fig. 3.1G).

We next examined whether the reduction in Nur77 might relate to loss of MEF2 as previously reported to occur following MPTP treatment. In this regard, Smith et al. (Smith et al., 2006) showed that expression of non-phosphorylatable MEF2D attenuate DA cell death. Accordingly, we determined whether expression of this construct might also attenuate the loss of Nur77 expression in the SNc *in vivo*. Nur77 levels were examined in WT mice virally expressing MEF2D-S444A or a GFP control as previously reported (Smith et al., 2006). AV-MEF2D-S444A significantly attenuated the decrease in Nur77 at 1 day post-MPTP (Fig. 3.1E). Interestingly, similar patterns of rescue was observed with p35 deficiency (data not shown).

Nur77 siRNA elevates neuronal cell death in vitro. The above results indicate that Nur77 expression decreases in a MEF2D dependent manner following MPTP-treatment *in vivo*. However, whether the loss of Nur77 plays any functional role in DA loss is unknown. Accordingly, we examined this question and reasoned that if our logic was true, loss of Nur77 should reduce neuronal survival either basally or in the presence of stress.

FIGURE 3.1. Nur77 expression and regulation following MPTP and MPP^+ treatment.

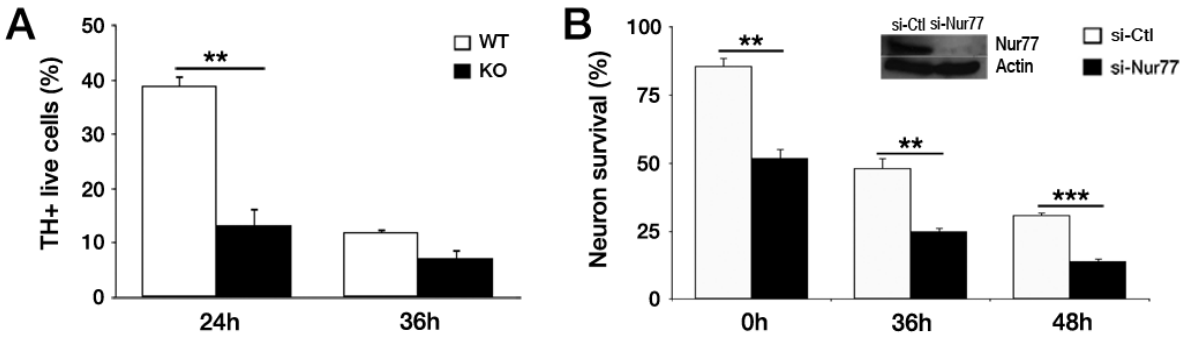
A. Expression of Nur77 mRNA in an MPTP time course, at 0, 6, 12, 24 hours, 3 and 7 days, post-initial MPTP treatment. B. Representative photomicrographs illustrating Nur77 *in situ* hybridization of striatal tissue, following 0, 12 hours, and 3 days MPTP. Black boxes designate dorsolateral and ventrolateral striatal quantified regions. C-D. Quantification of striatal Nur77 *in situ* hybridization following no treatment, 12 hours, 1, and 3 days post-initial MPTP treatment; C. striatal dorsolateral; D. striatal ventrolateral. E, Attenuation of Nur77 expression following 1 day of MPTP by AV-MEF2-S444A expression. F, ChIP assay for MEF2 Nur77 promoter bind in cortical cultures with (G) MPP^+ for 0, 12 and 24 hours. Error bars represent mean ± SEM. ANOVA, *$p < 0.05$, **$p < 0.01$; $n = 3$-4 animals per group.


A
Relative Nur77 expression
1.2
1.0
0.8
0.6
0.4
0.2
0
0d
6h
12h
1d
3d
7d
**
B
0h
12h
3d
C
Striatal Dorsolateral
Nur77 mRNA (nCi/g tissue)
0.14
0.07
0
0d
12h
1d
3d
*
D
Striatal Ventrolateral
Nur77 mRNA (nCi/g tissue)
0.10
0.05
0
0d
12h
1d
3d
*
E
Saline
MPTP
Relative Nur77 expression
110
55
0
GFP
MEF2-S444A
F
input
IgG
MEF2D
G
input
IgG
0h
12h
24h
MEF2D

We first evaluated whether Nur77-deficient mesencephalic tyrosine hydroxylase-positive (TH+) neurons might display sensitivity to MPP^+ treatment in culture. Midbrain neurons from wild-type (WT) and Nur77-deficient mice were cultured and treated with MPP^+. Survival of TH+ neurons was then evaluated. Nur77-deficient TH+ neurons showed a significant hypersensitivity at 24 hours exposure to MPP^+ (13% survival with Nur77 deficiency versus vs 39% survival in WT; Fig 3.2A). As an interesting side note, we also examined how other neuron types might respond to loss of Nur77. In this case, we examined cortical neurons treated with siRNA for Nur77. Importantly, treatment of cultured neurons to control siRNA to Nur77 siRNA induced significant toxicity even in the absence of any stress (91% survival with control siRNA vs 49% survival with Nur77 siRNA)(Fig 3.2B). MPP^+ treatment also diminished survival. However, the survival ratio remained relatively stable between the control and Nur77 siRNA treated cultures at the various time points. This suggested that acute loss in Nur77 expression in neurons produces a basal detrimental effect that can be further exaggerated by toxin exposure. Nur77 siRNA downregulation was confirmed by western blot analysis (Fig. 3.2B). These results indicated that Nur77 can play a role in neuronal loss and provided the rational to proceed further to examine the role of Nur77 *in vivo*.

FIGURE 3.2. In vitro neuronal cultures display sensitization to toxic insult with loss in Nur77 expression.

A. Mesencephalic WT and Nur77-deficient neuronal cultures treated with MPP^+ for 24 and 36 hours. B. Cortical neuronal cultures treated with Nur77 and control siRNA and MPTP for 36 and 48 hours. Error bars represent mean ± SEM. ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n = 3 embryos per group.


A
TH+ live cells (%)
50
40
30
20
10
0
**
WT
KO
24h
36h
B
Neuron survival (%)
100
75
50
25
0
si-Ctl si-Nur77
Nur77
Actin
si-Ctl
si-Nur77
**
**
***
0h
36h
48h

Nur77-deficient mice exhibit exacerbated MPTP-induced degeneration of dopaminergic cell bodies in the SNc. We next assessed dopaminergic neuron survival following saline or MPTP treatment of WT and Nur77 KO mice on a C57BK/6J background following a subchronic MPTP dosing regime (Fig. 3.3). These animals were analysed by stereological assessment for surviving TH+ neurons over the entire SNc region. Interestingly, unlike *in vitro,* with acute downregulation of Nur77 in cortical neurons, no statistical difference in the number of TH+ neurons in the SNc could be detected between saline-treated WT and Nur77 KO mice (Fig. 3.3A-C). Similarly, no statistical difference in basal striatal TH or DAT fiber density was detected by immunohistochemistry in saline treated animals (Fig. 3.3d-G). DAT is critical for the reuptake of DA and MPP^+, the active MPTP metabolite in dopaminergic neurons (Meissner et al., 2003). Finally, no differences in levels of striatal DA or its metabolites DOPAC and HVA were detected in these mutant mice under basal conditions (Fig. 3.4C-D). Furthermore in our hands, Nur77-deficient mice displayed no significant differences in 24 hour home-cage activity (Fig. 3.3H). Similar groups of animals were also treated and assessed for potential differences in DA cell survival following MPTP. After MPTP administration, there was a significant reduction (36.7%; $p < 0.001$) in TH+ SNc neurons in WT mice (Fig. 3.3B). Interestingly and consistent with results observed in culture, Nur77-deficient mice displayed a significantly greater loss of TH+ cells than WT mice treated with MPTP (68.6%; $p < 0.001$). This observation of increased dopaminergic neuron loss was corroborated morphologically using cresyl violet staining, assessing neurons at the level of the MTN (medial terminal nucleus; bregma -3.16) within the SNc (Fig. 3.3C). Analysis was consistent with that of TH survival, showing no difference between saline-treated groups, a

significant loss of TH+ neurons in WT MPTP-treated mice (28.7%; $p < 0.001$), and a significant increased loss in DA neurons in Nur77-deficient MPTP-treated animals (56.7%; $p < 0.001$).

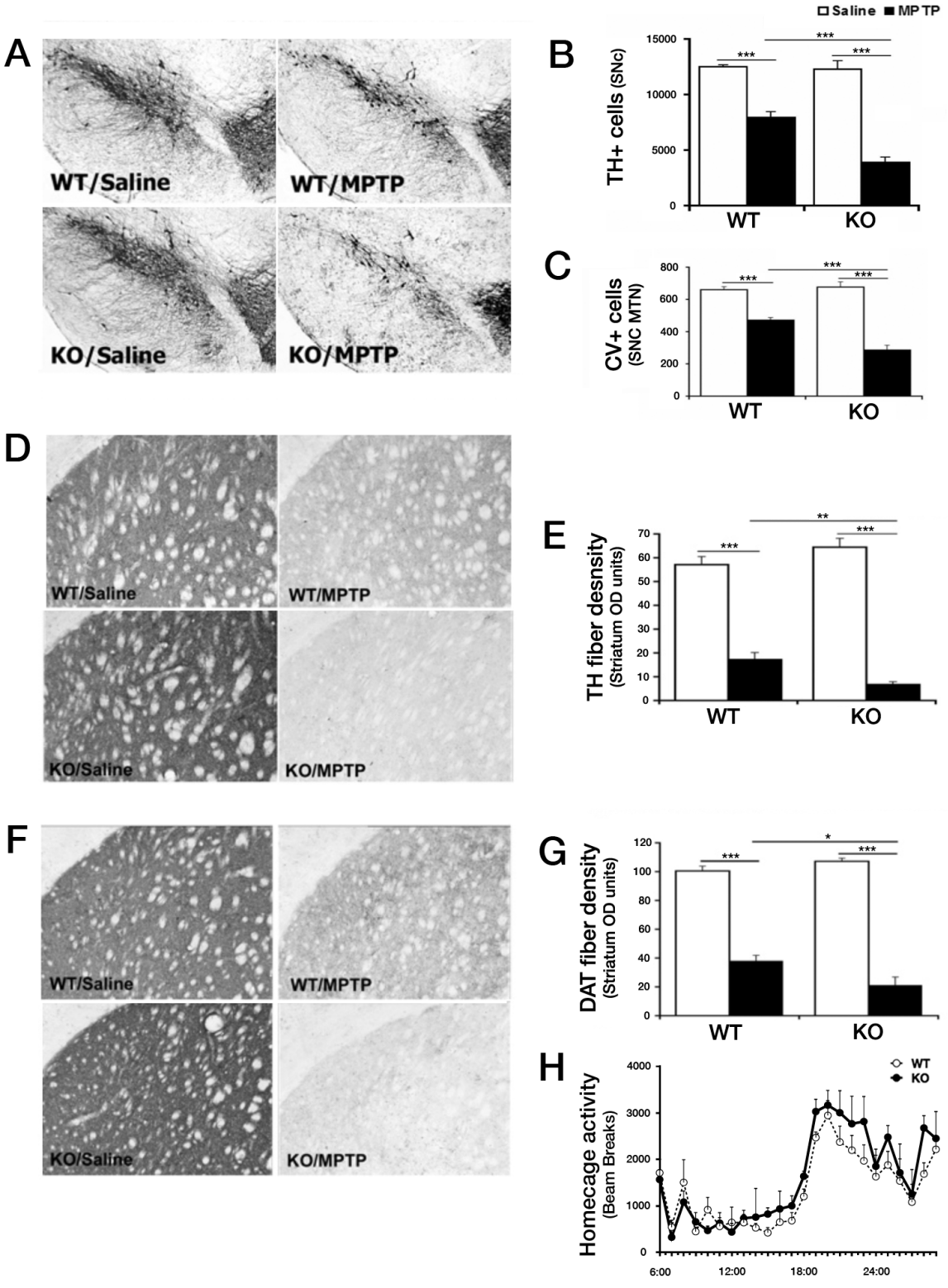
MPTP derives its toxic effects once it crosses the blood–brain barrier (BBB) and is converted to MPP^+, the active dopaminergic neural toxin, by monoamine oxidase-B (Jackson-Lewis and Przedborski, 2007). The hypersensitivity observed in Nur77-deficient animals may be attributed to altered MPTP metabolism and therefore significant increased MPP^+. To examine this possibility, striatal tissue was evaluated from mice 90 min after a single injection of MPTP, and levels of MPP^+ in the striatum were determined using HPLC. It was observed that MPP^+ levels did not significantly differ between WT and Nur77-deficient animals, (66.0 ± 5.4 and 74.5 ± 3.2, respectively, $p = 0.225$), suggesting that the increased sensitivity afforded by Nur77 deficiency was not because of altered MPTP metabolism.

Nur77-deficient mice exhibit increased degeneration of DA terminals, loss of amines, and markers of deregulated basal ganglia following MPTP treatment. Our results above indicate that MPTP-induced loss of dopaminergic cell bodies in the SNc is increased in Nur77-deficient mice. To determine whether the striatal projections of the SNc neurons also exhibit hypersensitivity in Nur77-deficient mice, striatal dopaminergic terminal fiber density was assessed in response to MPTP. This analysis is important because this is the region where dopaminergic neurons express their activity through DA release. Densitometric analysis of TH striatal fiber staining showed a significant reduction in WT MPTP-treated animals compared with saline controls (69.9% vs. 89.6%; $p < 0.001$)(Fig. 3.3D-E) Examination of

DAT levels also confirmed these findings. Densitometric analysis of striatal DAT stained fibers showed a significant reduction (62%; $p < 0.001$) in WT MPTP-treated animals compared with saline controls (Fig. 3.3). Nur77 KO animals exhibited a hypersensitivity to MPTP-induced reduction in striatal DAT density compared with WT treated mice (80.2%; $p < 0.001$). These results indicate that striatal terminal fibers were further sensitized by Nur77 deficiency similar to that exhibited by dopaminergic cell bodies.

FIGURE 3.3. Nur77-deficient mice display increased dopamine cell bodies and striatal terminals of MPTP-induced degeneration of SNc dopaminergic neurons.

A. Representative photomicrographs illustrating TH immunoreactivity in the ventral midbrain SNc following indicated treatments. B. Quantification of TH+ neurons using stereological analysis, as described in Materials and Methods. C. Quantification of cresyl violet-stained cells of the SNc (MTN level). D. Representative photomicrographs of striatal TH immunoreactive sections from treated animal groups in A. E. Quantification of striatal TH fiber optical density. F, Representative photomicrographs of striatal DAT immunoreactive sections from treated animal groups in A. G. Quantification of striatal DAT fiber optical density. H. Analysis of 24 hour home cage locomotor activity for WT and Nur77-deficient mice. Error bars represent mean ± SEM. ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 6$ – 8 animals per group. OD, Optical density.

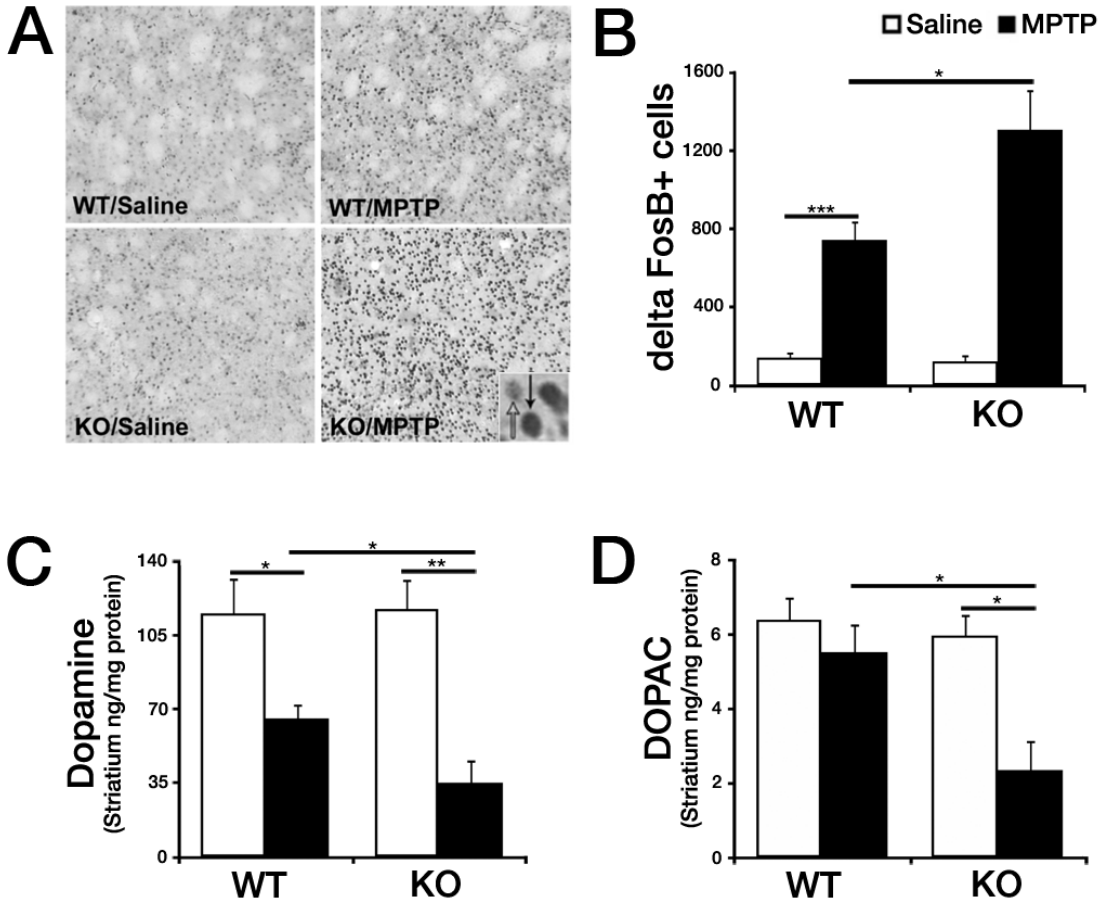

A
WT/Saline
WT/MPTP
KO/Saline
KO/MPTP
B
Saline
MPTP
TH+ cells (SNc)
15000
10000
5000
0
***
***
***
WT
KO
C
CV+ cells (SNC MTN)
800
600
400
200
0
***
***
***
WT
KO
D
WT/Saline
WT/MPTP
KO/Saline
KO/MPTP
E
TH fiber desnsity (Striatum OD units)
70
60
50
40
30
20
10
0
**
***
***
WT
KO
F
WT/Saline
WT/MPTP
KO/Saline
KO/MPTP
G
DAT fiber density (Striatum OD units)
120
100
80
60
40
20
0
*
***
***
WT
KO
H
Homecage activity (Beam Breaks)
4000
3000
2000
1000
0
WT
KO
6:00
12:00
18:00
24:00

Treatment with MPTP previously has been demonstrated to induce expression of the transcription factor ΔFosB post-synaptically within the striatum (Smith et al., 2003). ΔFosB is suggested to mediate the supersensitivity of striatal DA receptors after denervation (Dragunow et al., 1995). Consistent with these reports, WT MPTP-treated mice displayed a significant increase in the number of ΔFosB+ cells (422.1%; p , 0.001)(Fig. 3.4A-B) compared with saline controls. Importantly, MPTP-treated Nur77-deficient mice showed consistent hypersensitivity (929.3%; $p < 0.001$), a significant increase in ΔFosB staining over that observed in WT mice ($p < 0.05$).

We next examined how DA levels are affected in Nur77-deficient mice after MPTP-treatment. Previous analyses report diminished DA and its metabolite DOPAC in the striatum 14 days following MPTP-treatment (Smith:2006ga; Mount et al., 2007). The effects of Nur77 deficiency parallel the dopaminergic cell survival results observed previously. In the striatum, MPTP significantly reduced the levels of DA in WT mice (43.1%; $p < 0.05$) with a greater diminishment in the KO animals than WT (70.0; $p < 0.001$)(Fig. 3.4C). Striatal DOPAC was significantly reduced in Nur77 KO mice treated with MPTP (Fig. 3.4D). However, this significant loss was greater in the Nur77 KO MPTP-treated mice than WT(60.5% vs 13.6%; $p < 0.05$).

FIGURE 3.4. Nur77 KO mice treated with MPTP display increased striatal FosB, a marker for postsynaptic changes in the denervated striatum, as well as further reduced DA and DOPAC in comparison to WT MPTP-treated mice.

A. Representative photomicrographs showing FosB staining in the striatum of mice treated as indicated (insert, black arrow positive FosB; white arrow negative FosB. B. Quantification of FosB+ cells/nuclei striatal photomicrographs. C-D. Levels of DA (C) and its metabolite DOPAC (D) in the striatum were analyzed using HPLC on 14d-old tissue after saline/MPTP injection. Error bars represent mean ± SEM. ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n = 5-8 animals per group.


A
WT/Saline
WT/MPTP
KO/Saline
KO/MPTP
B
Saline
MPTP
delta FosB+ cells
1600
1200
800
400
0
*
***
WT
KO
C
Dopamine
(Striatium ng/mg protein)
140
105
70
35
0
*
*
**
WT
KO
D
DOPAC
(Striatium ng/mg protein)
8
6
4
2
0
*
*
WT
KO

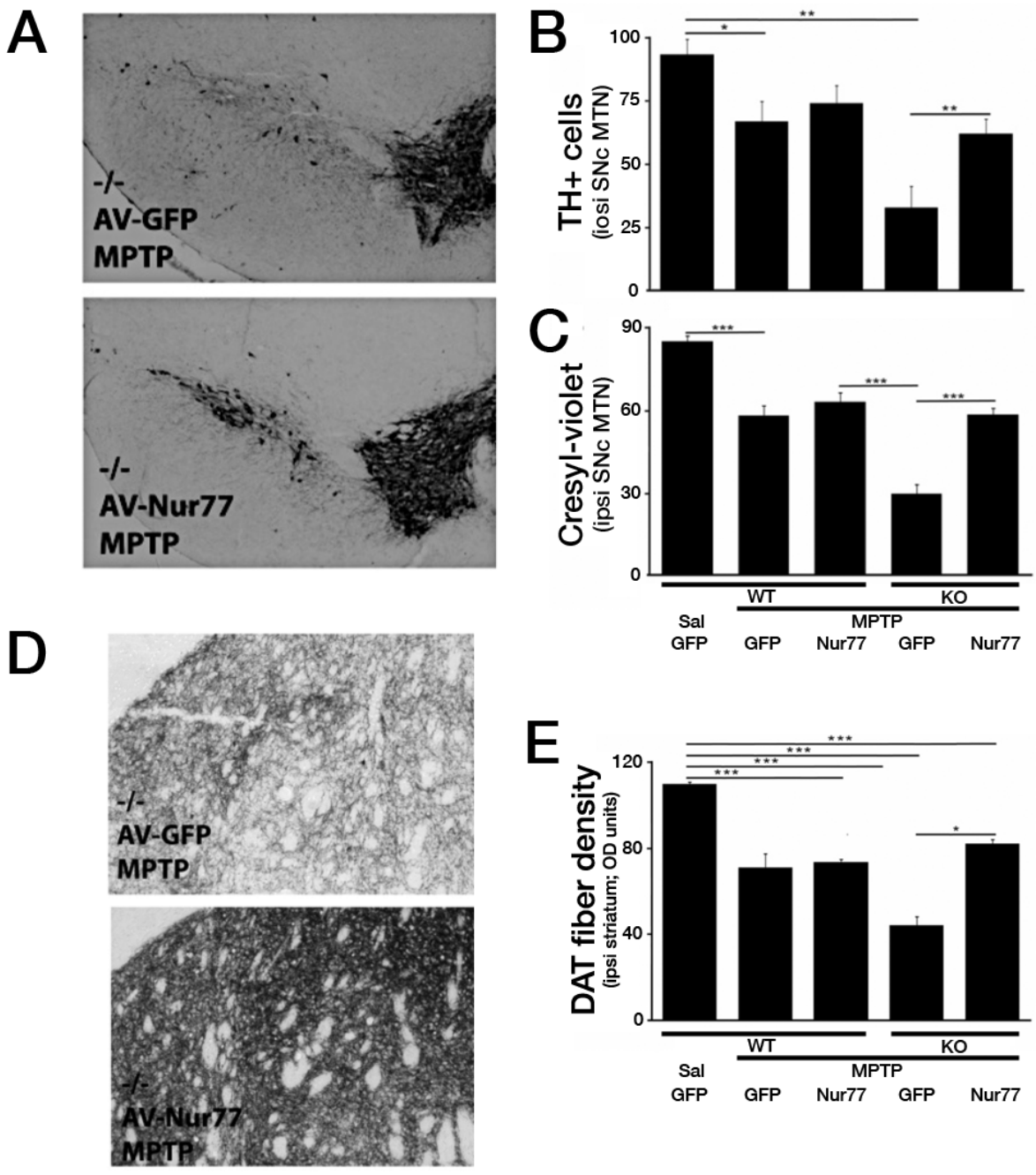
Ectopic expression of Nur77 rescues Nur77-deficient hypersensitivity to MPTP. The results described above demonstrate that germline loss of Nur77 does not lead to degeneration of DA basally, but increases these neurons sensitivity to MPTP-induced degeneration *in vivo*. Because of the germline nature of Nur77 loss in the model system examined, we included some controls for potential confounding compensatory factors which might account for the sensitization observed with chronic Nur77 loss. To this end, we explored whether we could rescue the sensitivity observed with Nur77 with ectopic WT Nur77 expression. We expressed Nur77 and control GFP in the SNc using an adenoviral (AV) system, in both Nur77-deficient and WT mice. Seven days following intracranial viral injection, the animals followed the same MPTP paradigm as previously employed. GFP expression in WT and Nur77-deficient mice produced similar results to those earlier exhibited in the *in vivo* survival experiments described above (Fig. 3.5A-C). WT, MPTP treated, GFP expressing mice showed a 28.4% reduction in TH+ neurons in comparison with the experimental control WT, saline treated, GFP expressing mice ($p < 0.05$). Nur77-deficient, GFP expressing, MPTP treated mice showed a similar heightened hypersensitivity as observed in the initial *in vivo* experiments, with a 65.0% reduction in TH+ neurons in comparison to control mice. Ectopic Nur77 expression in Nur77-deficient animals dramatically and almost completely reversed the sensitivity observed in caparison to WT animals. These results were corroborated using the morphological cresyl violet analysis (Fig. 3.5C). Interestingly, Nur77 expression in WT animals produced a slight but non-significant increase in DA neuron numbers.

Examination of striatal DAT density produced similar results (Fig. 3.5D-E). Ectopic expression of Nur77 in Nur77-deficient animals almost completely reversed the sensitization

induced by germline loss of Nur77. Interestingly, Nur77 expression again did not significantly attenuate fiber loss in the WT mouse. This suggest that levels of Nur77 expression was insufficient to promote dramatically increased protection in WT cells but was sufficient to prevent the sensitization observed with full Nur77 deficiency.

FIGURE 3.5. Nur77-deficient hypersensitivity to MPTP can be attenuated with ectopic expression of Nur77 in the nigrostriatal system.

A. Representative photomicrographs illustrating TH immunoreactivity in the ventral midbrain SNc following indicated treatments. B. Quantification of TH+ neurons at the MTN level of the SNc. C. Quantification of cresyl violet-stained cells at the MTN level of the SNc. D. Representative photomicrographs of striatal DAT immunoreactive. E. Quantification of striatal DAT fiber optical density. Error bars represent mean ± SEM. ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 5 – 8$ animals per group. OD, Optical density.


A
-/-
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MPTP
-/-
AV-Nur77
MPTP
B
TH+ cells
(iosi SNc MTN)
100
75
50
25
0
*
**
**
C
Cresyl-violet
(ipsi SNc MTN)
90
60
30
0
***
***
***
WT
KO
Sal
GFP
MPTP
GFP
Nur77
GFP
Nur77
D
-/-
AV-GFP
MPTP
-/-
AV-Nur77
MPTP
E
DAT fiber density
(ipsi striatum; OD units)
120
80
40
0
***
***
***
***
*
WT
KO
Sal
GFP
MPTP
GFP
Nur77
GFP
Nur77

DISCUSSION

Previous work has suggested the importance of a calpain-Cdk5-MEF2 signalling cascade in the adult *in vivo* MPTP model of dopaminergic loss (Gong et al., 2003; Smith et al., 2003; Verdaguer et al., 2005; Smith et al., 2006; She et al., 2011). How Cdk5 mediated repression of MEF2 activity leads to DA loss is unclear. Our findings are of significance as they delineate a novel player, Nur77, in DA loss and link this activity to the calpain regulated pathway of death. We found that 1) Nur77 is rapidly lost following MPTP treatment and that this loss is attenuated by expression of the transcription factor MEF2D; 2) Nur77 loss results in hypersensitization of the nigro-striatal system to exogenous toxic stress and degeneration; and 3) this hypersensitization can be rescued by re-expression of exogenous Nur77.

Our observations of MEF2 mediated Nur77 regulation is consistent with previous reports suggesting a relationship between the two transcription factors in other systems. For example, MEF2 has previously been shown to regulate Nur77 expression in thymocyte differentiation (He, 2002). In this context, MEF2 regulation of Nur77 is mediated by molecular transcription associating proteins including HDAC7 (Dequiedt et al., 2003; Verdin et al., 2004) and Cabin1 (Youn and Liu, 2000; Liu et al., 2001). In neurons, depolarization induced up-regulation of MEF2 has also been shown to increase Nur77 levels (Tian et al., 2010). In addition, CREB mediated induction of Nur77 is also regulated by MEF2 in PC12 cells (Lam et al., 2010). Importantly, MEF2 sites exist in the Nur77 promoter (Woronicz et al., 1995) with several groups reporting MEF2 binding to the Nur77 promoter (Youn: 2000um; Liu et al., 2001). Importantly, we presently observed that MEF2 does constitutively

occupy these sites endogenously in neurons. Taken together we have proposed a model by which neuronal MEF2, known to act as a survival signal, mediates a constitutive level of Nur77 expression. This basal level of Nur77 activity is rapidly lost when calpain mediated Cdk5 activation leads to phosphorylation and inactivation of MEF2.

Several observations support a role for Nur77 loss in facilitating DA loss. First, we showed that Nur77-deficient animals are hypersensitive to MPTP-induced degeneration. Importantly, we provide support, although not definitive proof, that this sensitivity relates to deficiency of Nur77 in neurons themselves. First, TH+ Nur77 deficient neurons cultured from neuronal midbrain cultures, under conditions where other contaminating cell types are limited, are more sensitive to MPP^+ than their WT counterparts. Second, acute suppression of Nur77 by siRNA in cortical neurons leads to their demise even in the absence of stress. It is important to note that this Nur77 basal neuronal loss does not appear to occur with germline Nur77 loss, suggesting some compensatory events with germline deficiency. Indeed, there are other Nur family members including Nor1 and Nurr1 (Chao et al., 2008; Zhao and Bruemmer, 2010). Nevertheless Nur77-deficient neurons are sensitive to exogenous stress and this sensitivity can be rescued by ectopic Nur77 expression.

These results are consistent with other reports indicating a role for Nur77 in survival, as seen in TNFa (Suzuki et al., 2003) and ceramide-induced cell death (Brás et al., 2000), and Nur77-NFkB inhibition of apoptosis (de Léséleuc and Denis, 2006). However, the role of Nur77 appears context dependent. In other cases such as T cell selection (Woronicz et al., 1995; Green et al., 2003) and cancer cell death induction (Cho et al., 2007; Cheng et al., 2011),

Nur77 appears to induce death. Interestingly, Nur77 can localize to the mitochondria where it can interact and inhibit Bcl-2, inducing apoptosis (Lin et al., 2004; Thompson and Winoto, 2008). Adding to the complexity is that Nur77 can have function activity outside of death regulation. In neurons for example, Nur77 has been associated with synaptic remodelling, response to L-Dopa, and behavioural modifications (St-Hilaire et al., 2006; Lévesque and Rouillard, 2007; Mahmoudi et al., 2009). Taken together, these observations belie the complexity of Nur77 function. At least in DA neurons, however, in response to MPTP, we provide evidence for a unique MEF2 dependent function in DA survival.

What are potential targets of Nur77 which might promote survival? In high metabolic cells such as muscle, Nur77 expression appears to regulate genes involved in glycolysis, glycogenolysis, and the glycerophosphate shuttle (Chao et al., 2007). Over-expression and shRNA inhibition of Nur77 in muscle cells respectively increased and decreased expression of glucose transporter 4 (GLUT4), muscle phosphofructokinase (Phkka1), and glycogen phosphorylase (Pygm). Furthermore, Nur77 was found to bind to the promoter regions of these metabolic controlling genes. Therefore, one attractive hypotheses is that interruption in expression of these metabolic controlling genes under conditions of elevated energy requirements, such as in neurons, might be detrimental. In this regard, maintenance of proper bioenergentics in neurons is critical for their survival and loss of this capacity may sensitize to exogenous stresses which pose additional metabolic stress onto the cell as observed with MPTP-treatment.

While our present results suggest Nur77 as one critical effector downstream of MEF2, other MEF2 regulated pathways may also play a role in regulating dopaminergic loss following MPTP-treatment. For example, She et al. (She et al., 2011) recently discovered MEF2 localized to the mitochondria where it is bound to its consensus site on mitochondrial DNA containing and regulated the ND6 gene. ND6, a component of the mitochondrial complex I, controlling oxidative phosphorylation, is key to maintaining energy requiring homeostasis. MPTP-induced MEF2 degradation disrupted the expression of NG6 along with increased hydrogen peroxide concentration, reduced ATP production, and increased DA cell death. MEF2 has also been shown to be degraded in other toxic insult models, including the 6-hydroxydopamine (Kim et al., 2011). Further inhibition of MEF2 contributing to neuronal toxicity is also presented by GSK3beta phosphorylation (Wang et al., 2009b). Finally, MEF2D itself acts alongside other regulatory targets of Cdk5 to mediate DA loss following MPTP-treatment. These additional Cdk5 targets include both cytoplasmic and nuclear factors, Prx2 and APE1, respectively (Qu et al., 2007; Huang et al., 2010). Prx2 is critical for handling oxidative stress in this paradigm while APE1 acts to facilitate DNA repair. With all three targets, Cdk5 mediated phosphorylation of these substrates leads to down-regulation of their respective activities promoting death. In the case of MEF2, however, we have identified an additional critical downstream effector of this transcription factor.

In conclusion, we have shown that mice deficient in Nur77 display a hypersensitization to MPTP-induced dopaminergic cell death and that levels of Nur77 are regulated by MEF2. Our data suggest a new candidate in the calpain-Cdk5-MEF2 pathway involving the demise of the nigro-striatal dopaminergic system. We propose that targeting and specifically

inhibiting the calpain-Cdk5-MEF2-Nur77 pathway may be effective in protecting dopamine neurons in PD.

CHAPTER 4: Discussion

4.1 General Discussion

This work has revealed two opposing influences on dopaminergic neuronal survival. In particular, we have demonstrated an IFN-ɣ induced neurotoxic influence that is mediated through microglial activation, exacerbating dopaminergic neurotoxicity in mitochondrial stress models of PD. We have also uncovered a novel neuroprotective capacity for the transcription factor Nur77 in dopaminergic neurons. Earlier research from our group delineated a model where calpain-mediated activation of Cdk5, inducing phosphorylation-inactivation of MEF2, regulated dopaminergic MPTP-induced neuron degeneration. In this model, the inactivation of MEF2D was in turn found to reduce neuronal expression of Nur77. These effects, of IFN-ɣ microglia activation and of neuronal Cdk5-MEF2D-induced Nur77 down-regulation, shed new light on mechanisms that underlie dopaminergic cell death under conditions of mitochondrial distress.

The MPTP and rotenone treatments we used to explore these signalling pathways, constitutes well-characterized experimental models of idiopathic PD. However, hereditary forms of PD are also linked to mitochondrial dysfunction and the relevance of these pathways to human PD is further supported by our discovery of IFN-ɣ up-regulation in Parkinson's patients. In this regard, since our IFN-ɣ publication was in press further results have implicated IFN-ɣ in PD pathogenesis.

Barcia et al. (Barcia et al., 2011) evaluated a synergistic signalling model with IFN-ɣ and TNF-α displaying a required glial activation for neuronal cell death (Barcia et al., 2011). MPTP-treated monkeys showed an elevation in blood serum IFN-ɣ, a result we observed in

PD patients, corroborating our results and strengthening the use of the MPTP PD model. Further, expression of IFN-ɣ-R was highly up-regulated in the monkey SN with sustained IFN-ɣ and TNF-α (also elevated in serum) expression in microglia and astrocytes. However, we did not observe increase in TNF-α in our human PD samples, suggesting some differences with the monkey PD model. Examination of phosphorylated STAT1 (pSTAT1), downstream required signalling, was localized to the SNc in both glial cell types.

Our work focused on the cell specific role of microglia in IFN-ɣ mediated DA cell death. However, results have suggested astrocytes also are involved. In culture model of neuronal cell death, medium from astrocytes stimulated with IFN-ɣ stimulated significant toxicity to differentiated human neuroblastoma SH-SY5Y cells (Lee et al., 2013). In order to understand what IFN-ɣ may have stimulated to cause cell death, several inhibitors were employed to block specific IFN-ɣ mediated compounds. Inhibition of IL-6, NADPH oxidase, prostaglandin, and glutamate, each individually significantly reduced synergistic toxicity. Of importance, these IFN-ɣ mediated toxic stimuli were from astrocytes, implicating both cell types in neuronal death. Here we see IFN-ɣ inducing cellular toxic insult through several inflammatory mediators, each of which can be targeted to reduce cell death. However, as discussed, in combination, a greater synergistic effect can yield the greatest survival. Appropriately, our evaluation of an inflammatory mediator and intracellular cell death signalling mediator supports an approach of targeting multiple systems to reduce and halt cell death.

4.2 Future Directions

This work employed the use of genetic molecular manipulation to down-regulate or up-regulate expression of select genes and proteins in order to gain further understanding for their impact within specific signalling pathways following mitochondrial stress. With an interest in moving these findings towards a potential therapeutic use, an examination in the efficacy employing pharmacological antagonists and inhibitors would recapitulate these results and move towards treatments.

Our experiments with IFN-ɣ-deficient and IFN-ɣ-R-deficient models have established the microglial source of the IFN-ɣ and that the cytokine kills dopamine neurons at least in part through interaction with IFN-ɣ-R on microglia. It is possible that this toxicity is caused directly. However, another possibility is that that IFN-ɣ interacts with dopamine neurons through direct activation of the Fas and Fas-associated death domain (FADD) and activation of caspases. Carmona et al. (Carmona et al., 2006) successfully employed the use of a pharmacological Fas inhibitor inhibiting dendritic cell activation. To evaluate the potential interaction between IFN-ɣ and Fas and whether they function in parallel or in sequence, the Fas/FasL antagonist Kp7-6 (Calbiochem) could be used in our rotenone and MPP+ model of DA cell death. Insights could be evaluated with regard to cell specific interactions, cellular toxicity, and how these two pro-inflammatory signalling molecules are required. An astrocytic role could also be examined as activation of astrocytes with IFN-ɣ induced up-regulation of several toxic insults (Lee et al., 2013). These experiments would look closer at specific regulatory events associated with the larger pro-inflammatory system. Attenuation of

microglia and pro-inflammatory stimuli has been reported to reduce neurotoxicity with the use of the pharmacological anti-inflammatory agent minocycline (Du et al., 2001). However, these results are not entirely clear with further results displaying opposite effects (Wu et al., 2002). Therefore, a further detailed understand could produce a direct pharmacological targeting.

We have also described a neuroprotective capacity for Nur77 in MPTP/MPP+ stressed DA neurons. This work further contextualizes and strengthens the importance of the calpain-Cdk5-MEF2 pathway in DA cell survival. We could evaluate new molecular mediators within this system, survival genes regulated by Nur77 such as glucose transporter 4 (GLUT4), muscle phosphofructokinase (Phkka1), and glycogen phosphorylase (Pygm) (Chao et al., 2007) as Nur77 was found to regulate genes that regulate high metabolic systems. However, several pharmacological tools have recently been used to successfully regulate points within our calpain-Cdk5-MEF2-Nur77 system.

Inhibition of calpain would halt downstream signalling, including both the down-regulation of MEF2-Nur77 signalling as well as the Cdk5 phosphorylation inhibition of Prx2 (Qu et al., 2007) induced by MPTP. The agent SNJ-1945 has recently been used to inhibit calpain activity in several models. Knaryan et al (Knaryan et al., 2013) were able to inhibit calpain, protecting SH-SY5Y cells against MPP+ and rotenone. SNJ-1945 was used *in vivo* study murine neurodegeneration using an oral administration (Trager et al., 2014). Although targeting calpains may have side effects in other systems, the benefit of neuroprotection may be greater, particular in PD.

Using these system, we could evaluate expression and activity of the downstream Cdk5-MEF2-Nur77 pathway. In terms of therapeutic use, SNJ-1945 provides a simple and direct means to inhibit calpain in patients. Employed in conjunction with early detection of neurodegeneration of the SNc (Brooks, 1997), may yield therapeutic benefits of staving off classical PD symptoms, rather than simply treating the symptoms.

Reduction in Nur77 expression increased MPTP-induced neuronal cell death. With potential to induce Nur77 expression pharmacologically, new insights into increasing survival with increasing Nur77 expression. The naturally occurring compound butylidenephthalide (BP), isolated from *Angelica sinensis* chlorophorm protected DA neurons and the accumulation of α-synuclein in a *C. elegans* model of PD protect DA neurodegeneration and α-synuclein accumulation in PD C. elegans model (Fu et al., 2014). Chemotherapy research in treatment of glioblastoma multiform (GBM) brain tumors in in vitro and in in vivo models used BP to induce Nur77 expression (Chen et al., 2008; Lin et al., 2008). Interestingly, BP selectively up-regulated Nur77 without modifying the expression pattern of related Nor-1 and Nurr1. Up-regulated Nur77 was found to move from the nucleus to the cytoplasm and mitochondria with downstream activation of caspase-3. With reference to our model and goal to attenuate cell death caspase activation would not be a desired outcome. However, in the *C. elegans* PD model, Nur77 expression attenuates neurodegeneration. The role for Nur77 is still unclear with both evidence for induction and inhibition of cell death. One mechanism to elucidate some questions would be to evaluate Nur77 subcellular localization with regard to survival or cell death in our mitochondrial cell model. Evaluation of Nur77 in T cell selection and GI cancers, have found translocation of Nur77 to the mitochondria mediates apoptosis (Wang et

al., 2009a; Cheng et al., 2011; Han and Cao, 2012). Select signalling cascades have been found to induce this translocation, including members of the MAPK system, found to phosphorylate Nur77 (Wang et al., 2009a). Once localized to the mitochondria, Nur77 has been found to couple with Bcl-2, inhibiting its protective function, initiating apoptotic signalling. However, our report observe a potential survival function for Nur77 in the MPTP PD model. This suggests Nur77 is not travelling to the mitochondria, but playing a significant survival role at the nucleus.

4.3 Conclusion

The nature of PD is multifactorial and was exhibited in our research of the inflammatory system and cell death signalling regulating PD pathogenesis. Hope for therapeutic intervention therefore will follow a similar nature, combining different synergistic treatments. However, this is one of several modes of therapeutic treatment to reduce and eliminate the symptoms and pathology of PD. In combination with early detection biomarkers and therapies to replace lost neurons, new strategies continue to develop. It is my hope that with this work, new insights may aid in development of effective therapeutic interventions for the treatment of PD.

Appendices

Appendix A: References Cited

Aarsland D, Andersen K, Larsen JP, Perry R, Wentzel-Larsen T, Lolk A, Kragh-Sørensen P (2004) The rate of cognitive decline in Parkinson disease. Arch Neurol 61:1906–1911.

Aarsland D, Ballard C, Larsen JP, McKeith I (2001) A comparative study of psychiatric symptoms in dementia with Lewy bodies and Parkinson's disease with and without dementia. Int J Geriatr Psychiatry 16:528–536.

Aarsland D, Påhlhagen S, Ballard CG, Ehrt U (2011) Depression in Parkinson disease—epidemiology, mechanisms and management. Nature Reviews ….

Abbas N, Bednar I, Mix E, Marie S, Paterson D, Ljungberg A, Morris C, Winblad B, Nordberg A, Zhu J (2002) Up-regulation of the inflammatory cytokines IFN-gamma and IL-12 and down-regulation of IL-4 in cerebral cortex regions of APP(SWE) transgenic mice. Journal of Neuroimmunology 126:50–57.

Abeliovich A, Schmitz Y, Fariñas I, Choi-Lundberg D, Ho WH, Castillo PE, Shinsky N, Verdugo JM, Armanini M, Ryan A, Hynes M, Phillips H, Sulzer D, Rosenthal A (2000) Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron 25:239–252.

Abramov AY, Scorziello A (2007) Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. The Journal of ….

Adamec E, Mohan P, Vonsattel JP, Nixon RA (2002) Calpain activation in neurodegenerative diseases: confocal immunofluorescence study with antibodies specifically recognizing the active form of calpain 2. Acta Neuropathol 104:92–104.

Akiyama Y, Radtke C, Kocsis JD (2002) Remyelination of the rat spinal cord by transplantation of identified bone marrow stromal cells. J Neurosci 22:6623–6630.

Alegre-Abarrategui J, Christian H, Lufino MMP, Mutihac R, Venda LL, Ansorge O, Wade-Martins R (2009) LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model. Hum Mol Genet 18:4022–4034.

Aleyasin H, Rousseaux MWC, Phillips M, Kim RH, Bland RJ, Callaghan S, Slack RS, During MJ, Mak TW, Park DS (2007) The Parkinson's disease gene DJ-1 is also a key regulator of stroke-induced damage. Proc Natl Acad Sci U S A 104:18748–18753.

Almer G, Vukosavic S, Romero N, Przedborski S (1999) Inducible nitric oxide synthase up-regulation in a transgenic mouse model of familial amyotrophic lateral sclerosis. J Neurochem 72:2415–2425.

Ananieva O, Macdonald A, Wang X, McCoy CE, McIlrath J, Tournier C, Arthur JSC (2008) ERK5 regulation in naïve T-cell activation and survival. Eur J Immunol 38:2534–2547.

Angot E, Steiner JA, Hansen C, Li J-Y, Brundin P (2010) Are synucleinopathies prion-like disorders? Lancet neurology 9:1128–1138.

Arai H, Furuya T, Yasuda T, Miura M, Mizuno Y, Mochizuki H (2004) Neurotoxic effects of lipopolysaccharide on nigral dopaminergic neurons are mediated by microglial activation, interleukin-1beta, and expression of caspase-11 in mice. J Biol Chem 279:51647–51653.

Attems J, Walker L, Jellinger KA (2014) Olfactory bulb involvement in neurodegenerative diseases. Acta Neuropathol 127:459–475.

Aubin N, Curet O, Deffois A (1998) Aspirin and salicylate protect against MPTP−induced dopamine depletion in mice. Journal of ….

Badie B, Schartner J, Vorpahl J, Preston K (2000) Interferon-gamma induces apoptosis and augments the expression of Fas and Fas ligand by microglia in vitro. Exp Neurol 162:290–296.

Baldereschi M, Di Carlo A, Rocca WA, Vanni P, Maggi S, Perissinotto E, Grigoletto F, Amaducci L, Inzitari D (2000) Parkinson's disease and parkinsonism in a longitudinal study: two-fold higher incidence in men. ILSA Working Group. Italian Longitudinal Study on Aging. Neurology 55:1358–1363.

Banati RB, Daniel SE, Blunt SB (1998) Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. Mov Disord 13:221–227.

Banks WA (2005) Blood-brain barrier transport of cytokines: a mechanism for neuropathology. Curr Pharm Des 11:973–984.

Barcia C, Ros CM, Annese V, Gómez A, Ros-Bernal F, Aguado-Yera D, Martínez-Pagán ME, de Pablos V, Fernandez-Villalba E, Herrero MT (2011) IFN-γ signaling, with the synergistic contribution of TNF-α, mediates cell specific microglial and astroglial activation in experimental models of Parkinson's disease. Cell Death Dis 2:e142.

Barcia C, Sánchez-Bahillo A, Fernández-Villalba E, Bautista V, Poza Y Poza M, Fernández-Barreiro A, Hirsch EC, Herrero M-T (2004) Evidence of active microglia in substantia nigra pars compacta of parkinsonian monkeys 1 year after MPTP exposure. Glia 46:402–409.

Bardou I, Kaercher RM, Brothers HM, Hopp SC, Royer S, Wenk GL (2014) Age and duration of inflammatory environment differentially affect the neuroimmune response and catecholaminergic neurons in the midbrain and brainstem. Neurobiol Aging 35:1065–1073.

Beal MF (1996) Mitochondria, free radicals, and neurodegeneration. Curr Opin Neurobiol 6:661–666.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3:1301–1306.

Bezard E, Gross CE, Fournier MC, Dovero S, Bloch B, Jaber M (1999) Absence of MPTP-induced neuronal death in mice lacking the dopamine transporter. Exp Neurol 155:268–273.

Bilbo SD (2010) Early-life infection is a vulnerability factor for aging-related glial alterations and cognitive decline. Neurobiol Learn Mem 94:57–64.

Blanck G (2002) Components of the IFN-gamma signaling pathway in tumorigenesis. Arch Immunol Ther Exp (Warsz) 50:151–158.

Blandini F, Nappi G, Greenamyre JT (1998) Quantitative study of mitochondrial complex I in platelets of parkinsonian patients. Mov Disord 13:11–15.

Blandini F, Nappi G, Tassorelli C, Martignoni E (2000) Functional changes of the basal ganglia circuitry in Parkinson's disease. Prog Neurobiol 62:63–88.

Block ML, Hong JS (2005) Microglia and inflammation-mediated neurodegeneration: Multiple triggers with a common mechanism. Prog Neurobiol 76:77–98.

Bonifati V et al. (2002) Autosomal recessive early onset parkinsonism is linked to three loci: PARK2, PARK6, and PARK7. Neurol Sci 23 Suppl 2:S59–S60.

Bonifati V, Rizzu P, Squitieri F, Krieger E, Vanacore N, van Swieten JC, Brice A, van Duijn CM, Oostra B, Meco G, Heutink P (2003) DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. Neurol Sci 24:159–160.

Braak H, del Tredici K, Rüb U, de Vos RAI, Jansen Steur ENH, Braak E (2003a) Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 24:197–211.

Braak H, Rüb U, del Tredici K (2006) Cognitive decline correlates with neuropathological stage in Parkinson's disease. J Neurol Sci 248:255–258.

Braak H, Rüb U, Gai WP, del Tredici K (2003b) Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. Journal of neural transmission (Vienna, Austria : 1996) 110:517–536.

Brás A, Albar JP, Leonardo E, de Buitrago GG, Martínez-A C (2000) Ceramide-induced cell death is independent of the Fas/Fas ligand pathway and is prevented by Nur77 overexpression in A20 B cells. Cell Death Differ 7:262–271.

Brecknell JE, Dunnett SB, Fawcett JW (1995) A quantitative study of cell death in the substantia nigra following a mechanical lesion of the medial forebrain bundle. Neuroscience 64:219–227.

Brion JP, Couck AM (1995) Cortical and brainstem-type Lewy bodies are immunoreactive for the cyclin-dependent kinase 5. Am J Pathol 147:1465–1476.

Brooks DJ (1997) PET and SPECT studies in Parkinson's disease. Baillieres Clin Neurol 6:69–87.

Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin IJ (1983) A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Proc Natl Acad Sci USA 80:4546–4550.

Burré J, Sharma M, Tsetsenis T, Buchman V (2010) α-Synuclein promotes SNARE-complex assembly in vivo and in vitro. Science.

Butovsky O, Talpalar AE, Ben-Yaakov K, Schwartz M (2005) Activation of microglia by aggregated beta-amyloid or lipopolysaccharide impairs MHC-II expression and renders them cytotoxic whereas IFN-gamma and IL-4 render them protective. Mol Cell Neurosci 29:381–393.

Cagnin A, Kassiou M, Meikle SR, Banati RB (2006) In vivo evidence for microglial activation in neurodegenerative dementia. Acta Neurol Scand, Suppl 185:107–114.

Calne DB, Peppard RF (1987) Aging of the nigrostriatal pathway in humans. … journal of neurological sciences Le journal ….

Carlsson A (1996) Neurotransmitter Interactions Important for Schizophrenia. In: Implications of Psychopharmacology to Psychiatry, pp 1–12. Berlin, Heidelberg: Springer Berlin Heidelberg.

Carmona EM, Vassallo R, Vuk-Pavlovic Z, Standing JE, Kottom TJ, Limper AH (2006) Pneumocystis cell wall beta-glucans induce dendritic cell costimulatory molecule expression and inflammatory activation through a Fas-Fas ligand mechanism. J Immunol 177:459–467.

Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci USA 95:11715–11720.

Chandra S, Fornai F, Kwon H-B, Yazdani U, Atasoy D, Liu X, Hammer RE, Battaglia G, German DC, Castillo PE, Südhof TC (2004) Double-knockout mice for alpha- and beta-synucleins: effect on synaptic functions. Proc Natl Acad Sci USA 101:14966–14971.

Chao LC, Bensinger SJ, Villanueva CJ, Wroblewski K, Tontonoz P (2008) Inhibition of adipocyte differentiation by Nur77, Nurr1, and Nor1. Molecular Endocrinology 22:2596–2608.

Chao LC, Zhang Z, Pei L, Saito T, Tontonoz P, Pilch PF (2007) Nur77 coordinately regulates expression of genes linked to glucose metabolism in skeletal muscle. Mol Endocrinol 21:2152–2163.

Chen J, Tang XQ, Zhi JL, Cui Y, Yu HM, Tang EH, Sun SN, Feng JQ, Chen PX (2006) Curcumin protects PC12 cells against 1-methyl-4-phenylpyridinium ion-induced apoptosis by bcl-2-mitochondria-ROS-iNOS pathway. Apoptosis 11:943–953.

Chen M, Ona VO, Li M, Ferrante RJ, Fink KB, Zhu S, Bian J, Guo L, Farrell LA, Hersch SM, Hobbs W, Vonsattel JP, Cha JH, Friedlander RM (2000) Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med 6:797–801.

Chen Y-L, Jian M-H, Lin C-C, Kang J-C, Chen S-P, Lin P-C, Hung P-J, Chen J-R, Chang W-L, Lin S-Z, Harn H-J (2008) The induction of orphan nuclear receptor Nur77 expression by n-butylenephthalide as pharmaceuticals on hepatocellular carcinoma cell therapy. Mol Pharmacol 74:1046–1058.

Cheng Z, Völkers M, Din S, Avitabile D, Khan M, Gude N, Mohsin S, Bo T, Truffa S, Alvarez R, Mason M, Fischer KM, Konstandin MH, Zhang X-K, Heller Brown J, Sussman MA (2011) Mitochondrial translocation of Nur77 mediates cardiomyocyte apoptosis. Eur Heart J 32:2179–2188.

Cheung NS, Hickling YM, Beart PM (1997) Development and survival of rat embryonic mesencephalic dopaminergic neurones in serum-free, antioxidant-rich primary cultures. Neurosci Lett 233:13–16.

Chiba K, Trevor A, Castagnoli N (1984) Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. Biochem Biophys Res Commun 120:574–578.

Chin L-S, Olzmann JA, Li L (2010) Parkin-mediated ubiquitin signalling in aggresome formation and autophagy. Biochem Soc Trans 38:144–149.

Cho SD, Yoon K, Chintharlapalli S, Abdelrahim M, Lei P, Hamilton S, Khan S, Ramaiah SK, Safe S (2007) Nur77 agonists induce proapoptotic genes and responses in colon cancer cells through nuclear receptor-dependent and nuclear receptor-independent pathways. Cancer Res 67:674–683.

Ciesielska A, Joniec I, Przybyłkowski A, Gromadzka G, Kurkowska-Jastrzebska I, Członkowska A, Członkowski A (2003) Dynamics of expression of the mRNA for cytokines and inducible nitric synthase in a murine model of the Parkinson's disease. Acta Neurobiol Exp (Wars) 63:117–126.

Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, Yoo SJ, Hay BA, Guo M (2006) Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature 441:1162–1166.

Collier TJ, Kanaan NM, Kordower JH (2011) Ageing as a primary risk factor for Parkinson's disease: evidence from studies of non-human primates. Nat Rev Neurosci 12:359–366.

Colton CA, Wilcock DM (2010) Assessing activation states in microglia. CNS Neurol Disord Drug Targets 9:174–191.

Conway KA, Harper JD, Lansbury PT (2000a) Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. Biochemistry 39:2552–2563.

Conway KA, Lee SJ, Rochet JC, Ding TT, Harper JD, Williamson RE, Lansbury PT (2000b) Accelerated oligomerization by Parkinson's disease linked alpha-synuclein mutants. Ann N Y Acad Sci 920:42–45.

Cotzias GC, Papavasiliou PS, Gellene R (1969) L-dopa in parkinson's syndrome. N Engl J Med 281:272.

Cribbs DH, Berchtold NC, Perreau V, Coleman PD, Rogers J, Tenner AJ, Cotman CW (2012) Extensive innate immune gene activation accompanies brain aging, increasing vulnerability to cognitive decline and neurodegeneration: a microarray study. J Neuroinflammation 9:179.

Crocker SJ, Liston P, Anisman H, Lee CJ, Smith PD, Earl N, Thompson CS, Park DS, Korneluk RG, Robertson GS (2003a) Attenuation of MPTP-induced neurotoxicity and behavioural impairment in NSE-XIAP transgenic mice. Neurobiol Dis 12:150–161.

Crocker SJ, Smith PD, Jackson-Lewis V, Lamba WR, Hayley SP, Grimm E, Callaghan SM, Slack RS, Melloni E, Przedborski S, Robertson GS, Anisman H, Merali Z, Park DS (2003b) Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease. J Neurosci 23:4081–4091.

Członkowska A, Kohutnicka M, Kurkowska-Jastrzebska I, Członkowski A (1996) Microglial reaction in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced Parkinson's disease mice model. Neurodegeneration 5:137–143.

Członkowska A, Kurkowska-Jastrzebska I, Członkowski A, Peter D, Stefano GB (2002) Immune processes in the pathogenesis of Parkinson's disease - a potential role for microglia and nitric oxide. Med Sci Monit 8:RA165–RA177.

Dada LA, Chandel NS, Ridge KM, Pedemonte C, Bertorello AM, Sznajder JI (2003) Hypoxia-induced endocytosis of Na,K-ATPase in alveolar epithelial cells is mediated by mitochondrial reactive oxygen species and PKC-zeta. J Clin Invest 111:1057–1064.

Dauer W, Kholodilov N, Vila M, Trillat A-C, Goodchild R, Larsen KE, Staal R, Tieu K, Schmitz Y, Yuan CA, Rocha M, Jackson-Lewis V, Hersch S, Sulzer D, Przedborski S, Burke R, Hen R (2002) Resistance of alpha -synuclein null mice to the parkinsonian neurotoxin MPTP. Proc Natl Acad Sci USA 99:14524–14529.

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889–909.

Dawson VL, Dawson TM (1996) Free radicals and neuronal cell death. Cell Death Differ.

de Léséleuc L, Denis F (2006) Inhibition of apoptosis by Nur77 through NF-kappaB activity modulation. Cell Death Differ 13:293–300.

de Pablos RM, Herrera AJ, Villarán RF, Cano J, Machado A (2005) Dopamine-dependent neurotoxicity of lipopolysaccharide in substantia nigra. FASEB J 19:407–409.

De Simone R, Levi G, Aloisi F (1998) Interferon gamma gene expression in rat central nervous system glial cells. Cytokine 10:418–422.

Dehmer T, Lindenau J, Haid S, Dichgans J, Schulz JB (2000) Deficiency of inducible nitric oxide synthase protects against MPTP toxicity in vivo. J Neurochem 74:2213–2216.

del Tredici K, Braak H (2013) Dysfunction of the locus coeruleus-norepinephrine system and related circuitry in Parkinson's disease-related dementia. J Neurol Neurosurg Psychiatr 84:774–783.

Delgado M (2003) Inhibition of interferon (IFN) gamma-induced Jak-STAT1 activation in microglia by vasoactive intestinal peptide: inhibitory effect on CD40, IFN-induced protein-10, and inducible nitric-oxide synthase expression. J Biol Chem 278:27620–27629.

Dequiedt F, Kasler H, Fischle W, Kiermer V, Weinstein M, Herndier BG, Verdin E (2003) HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. Immunity 18:687–698.

Deumens R, Blokland A, Prickaerts J (2002) Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. Exp Neurol 175:303–317.

Dhillon AS, Tarbutton GL, Levin JL, Plotkin GM, Lowry LK, Nalbone JT, Shepherd S (2008) Pesticide/environmental exposures and Parkinson's disease in East Texas. J Agromedicine 13:37–48.

Di Stefano A, Sozio P, Cerasa LS, Iannitelli A (2011) L-Dopa prodrugs: an overview of trends for improving Parkinson's disease treatment. Curr Pharm Des 17:3482–3493.

Donaldson I, Dolphin A, Jenner P, Marsden CD, Pycock C (1976) The roles of noradrenaline and dopamine in contraversive circling behaviour seen after unilateral electrolytic lesions of the locus coeruleus. Eur J Pharmacol 39:179–191.

Donovan DM, Miner LL, Perry MP, Revay RS, Sharpe LG, Przedborski S, Kostic V, Philpot RM, Kirstein CL, Rothman RB, Schindler CW, Uhl GR (1999) Cocaine reward and MPTP toxicity: alteration by regional variant dopamine transporter overexpression. Brain Res Mol Brain Res 73:37–49.

Dotchin C, Jusabani A, Walker R (2011) Three year follow up of levodopa plus carbidopa treatment in a prevalent cohort of patients with Parkinson's disease in Hai, Tanzania. J Neurol 258:1649–1656.

Doucet JP, Nakabeppu Y, Bedard PJ, Hope BT, Nestler EJ, Jasmin BJ, Chen JS, Iadarola MJ, St-Jean M, Wigle N, Blanchet P, Grondin R, Robertson GS (1996) Chronic alterations in dopaminergic neurotransmission produce a persistent elevation of deltaFosB-like protein(s) in both the rodent and primate striatum. Eur J Neurosci 8:365–381.

Dragunow M, Butterworth N, Waldvogel H, Faull RL, Nicholson LF (1995) Prolonged expression of Fos-related antigens, Jun B and TrkB in dopamine-denervated striatal neurons. Brain Res Mol Brain Res 30:393–396.

Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, Triarhou LC, Chernet E, Perry KW, Nelson DL, Luecke S, Phebus LA, Bymaster FP, Paul SM (2001) Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proc Natl Acad Sci USA 98:14669–14674.

Ekstrand MI, Terzioglu M, Galter D, Zhu S, Hofstetter C, Lindqvist E, Thams S, Bergstrand A, Hansson FS, Trifunovic A, Hoffer B, Cullheim S, Mohammed AH, Olson L, Larsson N-G (2007) Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. Proc Natl Acad Sci USA 104:1325–1330.

Ferrante RJ, Schulz JB, Kowall NW, Beal MF (1997) Systemic administration of rotenone produces selective damage in the striatum and globus pallidus, but not in the substantia nigra. Brain Res.

Fornai F, Schlüter OM, Lenzi P, Gesi M, Ruffoli R, Ferrucci M, Lazzeri G, Busceti CL, Pontarelli F, Battaglia G, Pellegrini A, Nicoletti F, Ruggieri S, Paparelli A, Südhof TC (2005) Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and alpha-synuclein. Proc Natl Acad Sci USA 102:3413–3418.

Fu R-H, Harn H-J, Liu S-P, Chen C-S, Chang W-L, Chen Y-M, Huang J-E, Li R-J, Tsai S-Y, Hung H-S, Shyu W-C, Lin S-Z, Wang Y-C (2014) n-Butylidenephthalide Protects against Dopaminergic Neuron Degeneration and α-Synuclein Accumulation in Caenorhabditis elegans Models of Parkinson's Disease Hart AC, ed. PLoS ONE 9:e85305.

Fujii Y, Matsuda S, Takayama G, Koyasu S (2008) ERK5 is involved in TCR-induced apoptosis through the modification of Nur77. Genes Cells 13:411–419.

Fukuda T (2001) Neurotoxicity of MPTP. Neuropathology 21:323–332.

Gaillard A, Decressac M, Frappé I, Fernagut PO, Prestoz L, Besnard S, Jaber M (2009) Anatomical and functional reconstruction of the nigrostriatal pathway by intranigral transplants. Neurobiol Dis 35:477–488.

Gan-Or Z, Bar-Shira A, Dahary D, Mirelman A, Kedmi M, Gurevich T, Giladi N, Orr-Urtreger A (2012) Association of sequence alterations in the putative promoter of RAB7L1 with a reduced parkinson disease risk. Arch Neurol 69:105–110.

Gao HM, Hong JS (2011) Gene-environment interactions: key to unraveling the mystery of Parkinson's disease. Prog Neurobiol 94:1–19.

Gao HM, Hong JS, Zhang W, Liu B (2002a) Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. J Neurosci 22:782–790.

Gao HM, Jiang J, Wilson B, Zhang W, Hong JS, Liu B (2002b) Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. J Neurochem 81:1285–1297.

Gao HM, Liu B, Zhang W, Hong JS (2003) Synergistic dopaminergic neurotoxicity of MPTP and inflammogen lipopolysaccharide: relevance to the etiology of Parkinson's disease. FASEB J 17:1957–1959.

García-Lorenzo D, Longo-Dos Santos C, Ewenczyk C, Leu-Semenescu S, Gallea C, Quattrocchi G, Pita Lobo P, Poupon C, Benali H, Arnulf I, Vidailhet M, Lehericy S (2013) The coeruleus/subcoeruleus complex in rapid eye movement sleep behaviour disorders in Parkinson's disease. Brain 136:2120–2129.

Gardet A, Benita Y, Li C, Sands BE, Ballester I, Stevens C, Korzenik JR, Rioux JD, Daly MJ, Xavier RJ, Podolsky DK (2010) LRRK2 is involved in the IFN-gamma response and host response to pathogens. J Immunol 185:5577–5585.

Geisler S, Holmström KM, Treis A, Skujat D, Weber SS, Fiesel FC, Kahle PJ, Springer W (2010) The PINK1/Parkin-mediated mitophagy is compromised by PD-associated mutations. Autophagy 6:871–878.

Gerhard A, Pavese N, Hotton G, Turkheimer F, Es M, Hammers A, Eggert K, Oertel W, Banati RB, Brooks DJ (2006) In vivo imaging of microglial activation with [11C](R)-PK11195 PET in idiopathic Parkinson's disease. Neurobiol Dis 21:404–412.

Gesi M, Soldani P, Giorgi FS, Santinami A, Bonaccorsi I, Fornai F (2000) The role of the locus coeruleus in the development of Parkinson's disease. Neuroscience and biobehavioral reviews 24:655–668.

Gilbert F, Morissette M, St-Hilaire M, Paquet B, Rouillard C, Di Paolo T, Lévesque D (2006) Nur77 gene knockout alters dopamine neuron biochemical activity and dopamine turnover. Biol Psychiatry 60:538–547.

Giros B, Jaber M, Jones SR, Wightman RM, Caron MG (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379:606–612.

Glinka Y, Tipton KF, Youdim MB (1996) Nature of inhibition of mitochondrial respiratory complex I by 6-Hydroxydopamine. J Neurochem 66:2004–2010.

Gloeckner CJ, Kinkl N, Schumacher A, Braun RJ, O'Neill E, Meitinger T, Kolch W, Prokisch H, Ueffing M (2006) The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. Hum Mol Genet 15:223–232.

Goetz CG (1997) New strategies with dopaminergic drugs: modified formulations of levodopa and novel agonists. Exp Neurol 144:17–20.

Goldstein DS, Holmes C, Sewell LT, Park MY (2012) Sympathetic noradrenergic before striatal dopaminergic denervation: relevance to Braak staging of synucleinopathy. Clinical Autonomic ….

Gong X, Tang X, Wiedmann M, Wang X, Peng J, Zheng D, Blair LAC, Marshall J, Mao Z (2003) Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuron 38:33–46.

González-Polo RA, Soler G, Rodríguezmartín A, Morán JM, Fuentes JM (2004) Protection against MPP+ neurotoxicity in cerebellar granule cells by antioxidants. Cell Biol Int 28:373–380.

Green DR, Droin N, Pinkoski M (2003) Activation-induced cell death in T cells. Immunol Rev 193:70–81.

Gribova IE, Gnedenko BB, Poleshchuk VV, Morozov SG (2003) [Level of interferon-gamma, tumor necrosis factor alpha, and antibodies to them in blood serum from Parkinson disease patients]. Biomed Khim 49:208–212.

Griffin R, Nally R, Nolan Y, McCartney Y, Linden J, Lynch MA (2006) The age-related attenuation in long-term potentiation is associated with microglial activation. J Neurochem 99:1263–1272.

Griffin WST (2008) Perispinal etanercept: potential as an Alzheimer therapeutic. J Neuroinflammation 5:3.

Grimbergen YAM, Langston JW, Roos RAC, Bloem BR (2009) Postural instability in Parkinson's disease: the adrenergic hypothesis and the locus coeruleus. Expert Rev Neurother 9:279–290.

Grudzien A, Shaw P, Weintraub S, Bigio E, Mash DC, Mesulam MM (2007) Locus coeruleus neurofibrillary degeneration in aging, mild cognitive impairment and early Alzheimer's disease. Neurobiol Aging 28:327–335.

Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B (1988) The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96:857–881.

Haehner A, Hummel T, Reichmann H (2011) Olfactory loss in Parkinson's disease. Parkinsons Dis 2011:450939.

Hakimi M, Selvanantham T, Swinton E, Padmore RF, Tong Y, Kabbach G, Venderova K, Girardin SE, Bulman DE, Scherzer CR, LaVoie MJ, Gris D, Park DS, Angel JB, Shen J, Philpott DJ, Schlossmacher MG (2011) Parkinson's disease-linked LRRK2 is expressed in circulating and tissue immune cells and upregulated following recognition of microbial structures. J Neural Transm 118:795–808.

Hamanaka RB, Chandel NS (2010) Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends Biochem Sci 35:505–513.

Han Y-F, Cao G-W (2012) Role of nuclear receptor NR4A2 in gastrointestinal inflammation and cancers. World J Gastroenterol 18:6865–6873.

Hancock DB, Martin ER, Mayhew GM, Stajich JM, Jewett R, Stacy MA, Scott BL, Vance JM, Scott WK (2008) Pesticide exposure and risk of Parkinson's disease: a family-based case-control study. BMC Neurol 8:6.

Haque ME, Thomas KJ, D'Souza C, Callaghan S, Kitada T, Slack RS, Fraser P, Cookson MR, Tandon A, Park DS (2008) Cytoplasmic Pink1 activity protects neurons from dopaminergic neurotoxin MPTP. Proc Natl Acad Sci U S A 105:1716–1721.

Hardy J (2010) Genetic analysis of pathways to Parkinson disease. Neuron 68:201–206.

Hawkes CH, del Tredici K, Braak H (2007) Parkinson's disease: a dual-hit hypothesis. Neuropathol Appl Neurobiol 33:599–614.

Hayley SP, Crocker SJ, Smith PD, Shree T, Jackson-Lewis V, Przedborski S, Mount M, Slack RS, Anisman H, Park DS (2004) Regulation of dopaminergic loss by Fas in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. J Neurosci 24:2045–2053.

Håkansson A, Westberg L, Nilsson S, Buervenich S, Carmine A, Holmberg B, Sydow O, Olson L, Johnels B, Eriksson E, Nissbrandt H (2005) Investigation of genes coding for inflammatory components in Parkinson's disease. Mov Disord 20:569–573.

He Y-W (2002) Orphan nuclear receptors in T lymphocyte development. J Leukoc Biol 72:440–446.

Heidenreich KA, Linseman DA (2004) Myocyte enhancer factor-2 transcription factors in neuronal differentiation and survival. Mol Neurobiol 29:155–166.

Heikkila RE, Hess A, Duvoisin RC (1984) Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. Science 224:1451–1453.

Hely MA, Fung VS, Morris JG (2000) Treatment of Parkinson's disease. J Clin Neurosci 7:484–494.

Heneka MT, O'Banion MK, Terwel D, Kummer MP (2010) Neuroinflammatory processes in Alzheimer's disease. J Neural Transm 117:919–947.

Herrup K (2010) Reimagining Alzheimer's disease--an age-based hypothesis. J Neurosci 30:16755–16762.

Hiscott J, Lacoste J, Lin R (2006) Recruitment of an interferon molecular signaling complex to the mitochondrial membrane: disruption by hepatitis C virus NS3-4A protease. Biochem Pharmacol 72:1477–1484.

Hobson P, Meara J (2004) Risk and incidence of dementia in a cohort of older subjects with Parkinson's disease in the United Kingdom. Mov Disord 19:1043–1049.

Huang E, Qu D, Zhang Y, Venderova K, Haque ME, Rousseaux MWC, Slack RS, Woulfe JM, Park DS (2010) The role of Cdk5-mediated apurinic/apyrimidinic endonuclease 1 phosphorylation in neuronal death. Nat Cell Biol 12:563–571.

Hulse RE, Kunkler PE, Fedynyshyn JP, Kraig RP (2004) Optimization of multiplexed bead-based cytokine immunoassays for rat serum and brain tissue. Journal of Neuroscience Methods 136:87–98.

Hunot S, Dugas N, Faucheux B, Hartmann A, Tardieu M, Debré P, Agid Y, Dugas B, Hirsch EC (1999) FcepsilonRII/CD23 is expressed in Parkinson's disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-alpha in glial cells. J Neurosci 19:3440–3447.

Imamura K, Hishikawa N, Sawada M, Nagatsu T, Yoshida M, Hashizume Y (2003) Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. Acta Neuropathol 106:518–526.

Jackson-Lewis V, Jakowec M, Burke RE, Przedborski S (1995) Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurodegeneration 4:257–269.

Jackson-Lewis V, Przedborski S (2007) Protocol for the MPTP mouse model of Parkinson's disease. Nat Protoc 2:141–151.

Jankovic J (2008) Parkinson's disease: clinical features and diagnosis. J Neurol.

Jenner P (2003) Oxidative stress in Parkinson's disease. Ann Neurol 53 Suppl 3:S26–S36; discussionS36–discussionS38.

Jones JD, Butterfield LC, Song W, Lafo J, Mangal P, Okun MS, Bowers D (2014) Anxiety and Depression Are Better Correlates of Parkinson's Disease Quality of Life Than Apathy. J Neuropsychiatry Clin Neurosci.

Kabi A, Nickerson KP, Homer CR, McDonald C (2012) Digesting the genetics of inflammatory bowel disease: insights from studies of autophagy risk genes. Inflamm Bowel Dis 18:782–792.

Kandel E, Schwartz J, Jessell T, Siegelbaum S, Hudspeth AJ (2012) Principles of Neural Science, Fifth Edition. McGraw Hill Professional.

Karupiah G, Xie Q, Buller RM, Nathan C, Duarte C (1993) Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. Science.

Kim M-K, Kim S-C, Kang J-I, Hyun J-H, Boo H-J, Eun S-Y, Park D-B, Yoo E-S, Kang H-K, Kang J-H (2011) 6-Hydroxydopamine-induced PC12 cell death is mediated by MEF2D down-regulation. Neurochem Res 36:223–231.

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392:605–608.

Klein C, Schumacher K, Jacobs H, Hagenah J, Kis B, Garrels J, Schwinger E, Ozelius L, Pramstaller P, Vieregge P, Kramer PL (2000) Association studies of Parkinson's disease and parkin polymorphisms. Ann Neurol 48:126–127.

Knaryan VH, Samantaray S, Park S, Azuma M, Inoue J, Banik NL (2013) SNJ-1945, a calpain inhibitor, protects SH-SY5Y cells against MPP +and rotenone. J Neurochem 130:280–290.

Ko J, Humbert S, Bronson RT, Takahashi S, Kulkarni AB, Li E, Tsai LH (2001) p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. J Neurosci 21:6758–6771.

Kostic V, Przedborski S, Flaster E, Sternic N (1991) Early development of levodopa-induced dyskinesias and response fluctuations in young-onset Parkinson's disease. Neurology 41:202–205.

Kotake Y, Ohta S (2003) MPP+ analogs acting on mitochondria and inducing neuro-degeneration. Curr Med Chem 10:2507–2516.

Kraft AD, McPherson CA, Harry GJ (2009) Heterogeneity of microglia and TNF signaling as determinants for neuronal death or survival. Neurotoxicology 30:785–793.

Krüger R, Kuhn W, Müller T, Woitalla D, Graeber M, Kösel S, Przuntek H, Epplen JT, Schöls L, Riess O (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 18:106–108.

Kusakawa G, Saito T, Onuki R, Ishiguro K, Kishimoto T, Hisanaga S (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J Biol Chem 275:17166–17172.

la Riva de P, Smith K, Xie SX, Weintraub D (2014) Course of psychiatric symptoms and global cognition in early Parkinson disease. Neurology.

Lam BYH, Zhang W, Ng DC-H, Maruthappu M, Roderick HL, Chawla S (2010) CREB-dependent Nur77 induction following depolarization in PC12 cells and neurons is modulated by MEF2 transcription factors. J Neurochem 112:1065–1073.

Langston JW, Ballard P, Tetrud JW, Irwin I (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219:979–980.

Langston JW, Ballard PA (1983) Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N Engl J Med 309:310.

Langston JW, Forno LS, Rebert CS, Irwin I (1984a) Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyrine (MPTP) in the squirrel monkey. Brain Res 292:390–394.

Langston JW, Forno LS, Tetrud J, Reeves AG, Kaplan JA, Karluk D (1999) Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann Neurol 46:598–605.

Langston JW, Irwin I, Langston EB, Forno LS (1984b) Pargyline prevents MPTP-induced parkinsonism in primates. Science 225:1480–1482.

Langston JW, Quik M, Petzinger G, Jakowec M, Di Monte DA (2000) Investigating levodopa-induced dyskinesias in the parkinsonian primate. Ann Neurol 47:S79–S89.

Lee M, McGeer E, McGeer PL (2013) Neurotoxins released from interferon-gamma-stimulated human astrocytes. Neuroscience 229:164–175.

Lee MS, Kwon YT, Li M, Peng J, Friedlander RM, Tsai LH (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 405:360–364.

Lee SL, Tourtellotte LC, Wesselschmidt RL, Milbrandt J (1995a) Growth and differentiation proceeds normally in cells deficient in the immediate early gene NGFI-A. J Biol Chem 270:9971–9977.

Lee SL, Wesselschmidt RL, Linette GP, Kanagawa O, Russell JH, Milbrandt J (1995b) Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). Science 269:532–535.

Leentjens A (2004) Depression in Parkinson's disease: conceptual issues and clinical challenges. Journal of geriatric psychiatry and neurology.

Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T (2003) Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proc Natl Acad Sci USA 100:8514–8519.

Lema Tomé CM, Tyson T, Rey NL, Grathwohl S, Britschgi M, Brundin P (2013) Inflammation and α-synuclein“s prion-like behavior in Parkinson”s disease--is there a link? Mol Neurobiol 47:561–574.

Lévesque D, Rouillard C (2007) Nur77 and retinoid X receptors: crucial factors in dopamine-related neuroadaptation. Trends Neurosci 30:22–30.

Li F-Q, Wang T, Pei Z, Liu B, Hong JS (2005) Inhibition of microglial activation by the herbal flavonoid baicalein attenuates inflammation-mediated degeneration of dopaminergic neurons. Journal of neural transmission (Vienna, Austria : 1996) 112:331–347.

Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, Dawson VL, Dawson TM, Przedborski S (1999) Inducible nitric oxide synthase stimulates

dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat Med 5:1403–1409.

Lim K-L, Dawson VL, Dawson TM (2006) Parkin-mediated lysine 63-linked polyubiquitination: a link to protein inclusions formation in Parkinson's and other conformational diseases? Neurobiol Aging 27:524–529.

Lin B, Kolluri SK, Lin F, Liu W, Han Y-H, Cao X, Dawson MI, Reed JC, Zhang X-K (2004) Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. Cell 116:527–540.

Lin P-C, Chen Y-L, Chiu S-C, Yu Y-L, Chen S-P, Chien M-H, Chen K-Y, Chang W-L, Lin S-Z, Chiou T-W, Harn H-J (2008) Orphan nuclear receptor, Nurr-77 was a possible target gene of butylidenephthalide chemotherapy on glioblastoma multiform brain tumor. J Neurochem 106:1017–1026.

LINDAHL PE, OBERG KE (1961) The effect of rotenone on respiration and its point of attack. Exp Cell Res 23:228–237.

Lira A, Kulczycki J, Slack R, Anisman H, Park DS (2011) Involvement of the Fc{gamma} receptor in a chronic MPTP mouse model of dopaminergic loss. Journal of Biological Chemistry.

Liu B, Du L, Hong JS (2000) Naloxone protects rat dopaminergic neurons against inflammatory damage through inhibition of microglia activation and superoxide generation. J Pharmacol Exp Ther 293:607–617.

Liu W, Acín-Peréz R, Geghman KD, Manfredi G, Lu B, Li C (2011) Pink1 regulates the oxidative phosphorylation machinery via mitochondrial fission. Proc Natl Acad Sci U S A 108:12920–12924.

Liu W, Youn HD, Liu JO (2001) Thapsigargin-induced apoptosis involves Cabin1-MEF2-mediated induction of Nur77. Eur J Immunol 31:1757–1764.

López Hernández N, García Escrivá A, Shalabi Benavent M (2014) Diagnostic value of combined assessment of olfaction and sustantia nigra hyperechogenicity for Parkinson's disease. Neurologia.

Luk KC, Song C, O'Brien P, Stieber A, Branch JR, Brunden KR, Trojanowski JQ, Lee VM-Y (2009) Exogenous alpha-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells. Proc Natl Acad Sci U S A 106:20051–20056.

Lücking CB, Dürr A, Bonifati V, Vaughan J, de Michele G, Gasser T, Harhangi BS, Meco G, Denèfle P, Wood NW, Agid Y, Brice A, French Parkinson's Disease Genetics Study Group, European Consortium on Genetic Susceptibility in Parkinson's Disease (2000) Association between early-onset Parkinson's disease and mutations in the parkin gene. N Engl J Med 342:1560–1567.

Mahmoudi S, Samadi P, Gilbert F, Ouattara B, Morissette M, Grégoire L, Rouillard C, Di Paolo T, Lévesque D (2009) Nur77 mRNA levels and L-Dopa-induced dyskinesias in MPTP monkeys treated with docosahexaenoic acid. Neurobiol Dis 36:213–222.

Mamane Y, Heylbroeck C, Génin P, Algarté M, Servant MJ, LePage C, DeLuca C, Kwon H, Lin R, Hiscott J (1999) Interferon regulatory factors: the next generation. Gene 237:1–14.

Mandel S, Grünblatt E, Youdim M (2000) cDNA microarray to study gene expression of dopaminergic neurodegeneration and neuroprotection in MPTP and 6-hydroxydopamine models: implications for idiopathic Parkinson's disease. J Neural Transm Suppl:117–124.

Mao Z, Bonni A, Xia F, Nadal-Vicens M, Greenberg ME (1999) Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science 286:785–790.

Mao Z, Wiedmann M (1999) Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons. J Biol Chem 274:31102–31107.

Markey SP, Johannessen JN, Chiueh CC, Burns RS, Herkenham MA (1984) Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. Nature 311:464–467.

Maroteaux L, Campanelli JT, Scheller RH (1988) Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. J Neurosci 8:2804–2815.

Mata IF, Wedemeyer WJ, Farrer MJ, Taylor JP (2006) LRRK2 in Parkinson's disease: protein domains and functional insights. Trends in ….

Mathers CD, Sadana R, Salomon JA, Murray CJ, Lopez AD (2001) Healthy life expectancy in 191 countries, 1999. Lancet 357:1685–1691.

Mattson MP, Barger SW, Begley JG, Mark RJ (1995) Calcium, free radicals, and excitotoxic neuronal death in primary cell culture. Methods in cell biology.

Mayer RA, Kindt MV, Heikkila RE (1986) Prevention of the nigrostriatal toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by inhibitors of 3,4-dihydroxyphenylethylamine transport. J Neurochem 47:1073–1079.

McBride HM (2008) Parkin mitochondria in the autophagosome. J Cell Biol.

McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, Di Monte DA (2002) Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neurobiol Dis 10:119–127.

McGeer PL, Itagaki S, Boyes BE, McGeer EG (1988) Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson“s and Alzheimer”s disease brains. Neurology 38:1285–1291.

McGeer PL, McGeer EG (2002) Inflammatory processes in amyotrophic lateral sclerosis. Muscle Nerve 26:459–470.

McKinsey TA, Zhang CL, Olson EN (2001) Control of muscle development by dueling HATs and HDACs. Curr Opin Genet Dev 11:497–504.

Meissner W, Prunier C, Guilloteau D, Chalon S, Gross CE, Bezard E (2003) Time-course of nigrostriatal degeneration in a progressive MPTP-lesioned macaque model of Parkinson's disease. Mol Neurobiol 28:209–218.

Milbrandt J (1988) Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron 1:183–188.

Mitsumoto A, Nakagawa Y, Takeuchi A, Okawa K, Iwamatsu A, Takanezawa Y (2001) Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic Res 35:301–310.

Mizuno Y, Hattori N, Mori H, Suzuki T, Tanaka K (2001) Parkin and Parkinson's disease. Curr Opin Neurol 14:477–482.

Mizuno Y, Sone N, Saitoh T (1987) Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on activities of the enzymes in the electron transport system in mouse brain. J Neurochem 48:1787–1793.

Mizuta I, Nishimura M, Mizuta E, Yamasaki S, Ohta M, Kuno S, Nishimura M, Ota M (2001) Relation between the high production related allele of the interferon-gamma (IFN-gamma) gene and age at onset of idiopathic Parkinson's disease in Japan. J Neurol Neurosurg Psychiatr 71:818–819.

Moehle MS, Webber PJ, Tse T, Sukar N, Standaert DG, DeSilva TM, Cowell RM, West AB (2012) LRRK2 inhibition attenuates microglial inflammatory responses. J Neurosci 32:1602–1611.

Moore DJ, Zhang L, Dawson TM, Dawson VL (2003) A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization. J Neurochem 87:1558–1567.

Mosovsky K, Silva E, Troyer R, Propst-Graham K, Dow S (2014) Interaction of IFN-γ Induced Reactive Oxygen Species with Ceftazidime Leads to Synergistic Killing of Intracellular Burkholderia. Antimicrob Agents Chemother.

Moss DW, Bates TE (2001) Activation of murine microglial cell lines by lipopolysaccharide and interferon-gamma causes NO-mediated decreases in mitochondrial and cellular function. Eur J Neurosci 13:529–538.

Mouatt-Prigent A, Karlsson JO, Agid Y, Hirsch EC (1996) Increased M-calpain expression in the mesencephalon of patients with Parkinson's disease but not in other neurodegenerative disorders involving the mesencephalon: a role in nerve cell death? Neuroscience 73:979–987.

Mount MP, Lira A, Grimes D, Smith PD, Faucher S, Slack RS, Anisman H, Hayley SP, Park DS (2007) Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons. J Neurosci 27:3328–3337.

Müller CM, de Vos RAI, Maurage C-A, Thal DR, Tolnay M, Braak H (2005) Staging of sporadic Parkinson disease-related alpha-synuclein pathology: inter- and intra-rater reliability. J Neuropathol Exp Neurol 64:623–628.

Nagatsu T, Mogi M, Ichinose H, Togari A (2000) Cytokines in Parkinson's disease. J Neural Transm Suppl:143–151.

Nakamura S, Nagano I, Yoshioka M, Onodera J, Kogure K (1992) [Detection of interferon-gamma and IL-6 producing cells in cerebrospinal fluid cells in the central nervous system infectious diseases using immunocytochemistry]. Rinsho Shinkeigaku 32:143–147.

Narendra DP, Jin SM, Tanaka A, Suen D-F, Gautier CA, Shen J, Cookson MR, Youle RJ (2010) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol 8:e1000298.

Nathan CF, Murray HW, Wiebe ME (1983) Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. The Journal of ….

Neumar RW, Meng FH, Mills AM, Xu YA, Zhang C, Welsh FA, Siman R (2001) Calpain activity in the rat brain after transient forebrain ischemia. Exp Neurol 170:27–35.

Nicklas WJ, Vyas I, Heikkila RE (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci 36:2503–2508.

Nicoletti A, Sofia V, Bartoloni A, Bartalesi F, Gamboa Barahon H, Giuffrida S, Reggio A (2003) Prevalence of Parkinson's disease: a door-to-door survey in rural Bolivia. Parkinsonism Relat Disord 10:19–21.

Noguchi Y, Yoshikawa T, Matsumoto A, Svaninger G, Gelin J (1996) Are cytokines possible mediators of cancer cachexia? Surg Today 26:467–475.

O'Hare MJ, Kushwaha N, Zhang Y, Aleyasin H, Callaghan SM, Slack RS, Albert PR, Vincent I, Park DS (2005) Differential roles of nuclear and cytoplasmic cyclin-dependent kinase 5 in apoptotic and excitotoxic neuronal death. J Neurosci 25:8954–8966.

OBERG KE (1961) The site of the action of rotenone in the respiratory chain. Exp Cell Res 24:163–164.

Obeso JA, Olanow CW, Nutt JG (2000a) Levodopa motor complications in Parkinson's disease. Trends Neurosci 23:S2–S7.

Obeso JA, Rodríguez-Oroz MC, Rodriguez M, DeLong MR, Olanow CW (2000b) Pathophysiology of levodopa-induced dyskinesias in Parkinson's disease: problems with the current model. Ann Neurol 47:S22–32–discussionS32–4.

Ohshima T, Ward JM, Huh CG, Longenecker G, Veeranna, Pant HC, Brady RO, Martin LJ, Kulkarni AB (1996) Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. Proc Natl Acad Sci USA 93:11173–11178.

Okuno T, Nakatsuji Y, Kumanogoh A, Moriya M, Ichinose H, Sumi H, Fujimura H, Kikutani H, Sakoda S (2005) Loss of dopaminergic neurons by the induction of inducible nitric oxide synthase and cyclooxygenase-2 via CD 40: relevance to Parkinson's disease. J Neurosci Res 81:874–882.

Olanow CW, Perl DP, DeMartino GN, McNaught KSP (2004) Lewy-body formation is an aggresome-related process: a hypothesis. The Lancet Neurology 3:496–503.

Olanow CW, Tatton WG (1999) Etiology and pathogenesis of Parkinson's disease. Annu Rev Neurosci 22:123–144.

Orr CF, Rowe DB, Mizuno Y, Mori H, Halliday GM (2005) A possible role for humoral immunity in the pathogenesis of Parkinson's disease. Brain 128:2665–2674.

Ouchi Y, Yoshikawa E, Sekine Y, Futatsubashi M, Kanno T, Ogusu T, Torizuka T (2005) Microglial activation and dopamine terminal loss in early Parkinson's disease. Ann Neurol 57:168–175.

Pain S, Gochard A, Bodard S, Gulhan Z, Prunier-Aesch C, Chalon S (2013) Toxicity of MPTP on neurotransmission in three mouse models of Parkinson's disease. Exp Toxicol Pathol 65:689–694.

Park J, Lee SB, Lee S, Kim Y, Song S, Kim S, Bae E, Kim J, Shong M, Kim J-M, Chung J (2006) Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nat Cell Biol 441:1157–1161.

Parkinson J (1817) An essay on the shaking palsy.

Parkinson J (2002) An essay on the shaking palsy. 1817.

Paxinos G, Franklin K (2001) [CITATION][C]. San Diego: Academic.

Peeraully T, Tan EK (2012) Genetic variants in sporadic Parkinson's disease: East vs West. Parkinsonism Relat Disord 18 Suppl 1:S63–S65.

Perry TL, Yong VW, Wall RA, Jones K (1986) Paraquat and two endogenous analogues of the neurotoxic substance N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine do not damage dopaminergic nigrostriatal neurons in the mouse. Neurosci Lett 69:285–289.

Perry VH (2004) The influence of systemic inflammation on inflammation in the brain: implications for chronic neurodegenerative disease. Brain, Behavior, and Immunity 18:407–413.

Pihlstrøm L, Toft M (2011) Genetic variability in SNCA and Parkinson's disease. Neurogenetics 12:283–293.

Pines J (1993) Cyclins and cyclin-dependent kinases: take your partners. Trends Biochem Sci 18:195–197.

Platten M, Wick W, Wischhusen J, Weller M (2001) N-[3,4-dimethoxycinnamoyl]-anthranilic acid (tranilast) suppresses microglial inducible nitric oxide synthase (iNOS) expression and activity induced by interferon-gamma (IFN-gamma). Br J Pharmacol 134:1279–1284.

Plouvier AOA, Hameleers RJMG, van den Heuvel EAJ, Bor HH, Olde Hartman TC, Bloem BR, van Weel C, Lagro-Janssen ALM (2014) Prodromal symptoms and early detection of Parkinson's disease in general practice: a nested case-control study. Fam Pract 31:373–378.

Polymeropoulos MH et al. (1997a) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276:2045–2047.

Polymeropoulos MH, Higgins JJ, Golbe LI, Johnson WG, Ide SE, Di Iorio G, Sanges G, Stenroos ES, Pho LT, Schaffer AA, Lazzarini AM, Nussbaum RL, Duvoisin RC (1996) Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. Science 274:1197–1199.

Polymeropoulos MH, Hurko O, Hsu F, Rubenstein J, Basnet S, Lane K, Dietz H, Spetzler RF, Rigamonti D (1997b) Linkage of the locus for cerebral cavernous hemangiomas to human chromosome 7q in four families of Mexican-American descent. Neurology 48:752–757.

Przedborski S, Jackson-Lewis V (1998) Mechanisms of MPTP toxicity. Mov Disord 13 Suppl 1:35–38.

Przedborski S, Vila M (2001) The last decade in Parkinson's disease research. Basic sciences. Adv Neurol 86:177–186.

Przedborski S, Vila M (2006) The 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model. Ann N Y Acad Sci 991:189–198.

Qu D, Rashidian J, Mount MP, Aleyasin H, Parsanejad M, Lira A, Haque E, Zhang Y, Callaghan S, Daigle M, Rousseaux MWC, Slack RS, Albert PR, Vincent I, Woulfe JM, Park DS (2007) Role of Cdk5-mediated phosphorylation of Prx2 in MPTP toxicity and Parkinson's disease. Neuron 55:37–52.

Raivich G, Bohatschek M, Werner A, Jones LL, Galiano M, Kloss CUA, Zhu X-Z, Pfeffer K, Liu ZQ (2003) Lymphocyte infiltration in the injured brain: role of proinflammatory cytokines. J Neurosci Res 72:726–733.

Rajput AH, Birdi S (1997) Epidemiology of Parkinson's disease. Parkinsonism Relat Disord.

Ramonet D et al. (2011) Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2. PLoS ONE 6:e18568.

Richard IH (2005) Anxiety disorders in Parkinson's disease. ADVANCES IN NEUROLOGY-NEW YORK-RAVEN ….

Rudow G, O'Brien R, Savonenko AV, Resnick SM, Zonderman AB, Pletnikova O, Marsh L, Dawson TM, Crain BJ, West MJ, Troncoso JC (2008) Morphometry of the human substantia nigra in ageing and Parkinson's disease. Acta Neuropathol 115:461–470.

Russ H, Staust K, Martel F, Gliese M, Schomig E (1996) The extraneuronal transporter for monoamine transmitters exists in cells derived from human central nervous system glia. Eur J Neurosci 8:1256–1264.

Rymar VV, Sasseville R, Luk KC, Sadikot AF (2004) Neurogenesis and stereological morphometry of calretinin-immunoreactive GABAergic interneurons of the neostriatum. J Comp Neurol 469:325–339.

Saha S, Guillily MD, Ferree A, Lanceta J, Chan D, Ghosh J, Hsu CH, Segal L, Raghavan K, Matsumoto K, Hisamoto N, Kuwahara T, Iwatsubo T, Moore L, Goldstein L, Cookson M, Wolozin B (2009) LRRK2 modulates vulnerability to mitochondrial dysfunction in Caenorhabditis elegans. J Neurosci 29:9210–9218.

Sauer H, Oertel WH (1994) Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and **and immunocytochemical study in the rat.** Neuroscience 59:401-415.

Schapira A, COOPER JM, Dexter D (1990) Mitochondrial complex I deficiency in Parkinson's disease. Journal of Neurochemistry 54:823-827.

Schapira AH, COOPER JM, Dexter D, Jenner P, Clark JB, Marsden CD (1989) Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1:1269.

Scharton TM, Scott P (1993) Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. J Exp Med. 178:567.

Schmidt N, Ferger B (2001) Neurochemical findings in the MPTP model of Parkinson's disease. Journal of neural transmission (Vienna, Austria : 1996) 108:1263–1282.

Schwarting RK, Huston JP (1996) The unilateral 6-hydroxydopamine lesion model in behavioral brain research. Analysis of functional deficits, recovery and treatments. Prog Neurobiol 50:275–331.

Scotcher KP, Irwin I, DeLanney LE, Langston JW, Di Monte D (1990) Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on ATP levels of mouse brain synaptosomes. J Neurochem 54:1295–1301.

Sedarous M, Keramaris E, O'Hare M, Melloni E, Slack RS, Elce JS, Greer PA, Park DS (2003) Calpains mediate p53 activation and neuronal death evoked by DNA damage. J Biol Chem 278:26031–26038.

Sedelis M, Hofele K, Auburger GW, Morgan S, Huston JP, Schwarting RK (2000) MPTP susceptibility in the mouse: behavioral, neurochemical, and histological analysis of gender and strain differences. Behav Genet 30:171–182.

Seegal RF, Brosch KO, Bush B (1986) High-performance liquid chromatography of biogenic amines and metabolites in brain, cerebrospinal fluid, urine and plasma. J Chromatogr 377:131–144.

Sha D, Chin L-S, Li L (2010) Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling. Hum Mol Genet 19:352–363.

Shalizi AK, Bonni A (2005) Brawn for brains: the role of MEF2 proteins in the developing nervous system. Current topics in developmental biology.

She H, Yang Q, Shepherd KR, Smith Y, Miller GW, Testa C, Mao Z (2011) Direct regulation of complex I by mitochondrial MEF2D is disrupted in a mouse model of Parkinson disease and in human patients. J Clin Invest.

Sheline CT, Zhu J, Zhang W, Shi C, Cai A-L (2013) Mitochondrial Inhibitor Models of Huntington“s Disease and Parkinson”s Disease Induce Zinc Accumulation and Are Attenuated by Inhibition of Zinc Neurotoxicity in vitro or in vivo. Neurodegener Dis 11:49–58.

Shimura H, Schlossmacher MG, Hattori N, Frosch MP, Trockenbacher A, Schneider R, Mizuno Y, Kosik KS, Selkoe DJ (2001) Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science 293:263–269.

Simon HU, Haj-Yehia A, Levi-Schaffer F (2000) Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 5:415–418.

Simón-Sánchez J et al. (2009) Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nat Genet 41:1308–1312.

Smeyne RJ, Jackson-Lewis V (2005) The MPTP model of Parkinson's disease. Brain Res Mol Brain Res 134:57–66.

Smith JA, Das A, Ray SK, Banik NL (2012) Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. Brain Res Bull 87:10–20.

Smith NL, Denning DW (2014) Clinical implications of interferon gamma genetic and epigenetic variants. Immunology.

Smith PD, Crocker SJ, Jackson-Lewis V, Jordan-Sciutto KL, Hayley SP, Mount MP, O'Hare MJ, Callaghan S, Slack RS, Przedborski S, Anisman H, Park DS (2003) Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci USA 100:13650–13655.

Smith PD, Mount MP, Shree R, Callaghan S, Slack RS, Anisman H, Vincent I, Wang X, Mao Z, Park DS (2006) Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. J Neurosci 26:440–447.

Solano SM, Miller DW, Augood SJ, Young AB, Penney JB (2000) Expression of alpha-synuclein, parkin, and ubiquitin carboxy-terminal hydrolase L1 mRNA in human brain: genes associated with familial Parkinson's disease. Ann Neurol 47:201–210.

Soltys Z, Ziaja M, Pawlínski R, Setkowicz Z, Janeczko K (2001) Morphology of reactive microglia in the injured cerebral cortex. Fractal analysis and complementary quantitative methods. J Neurosci Res 63:90–97.

Sonsalla PK, Jochnowitz ND, Zeevalk GD, Oostveen JA, Hall ED (1996) Treatment of mice with methamphetamine produces cell loss in the substantia nigra. Brain Res 738:172–175.

Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M (1998) alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc Natl Acad Sci USA 95:6469–6473.

St-Hilaire M, Bourhis E, Lévesque D, Rouillard C (2006) Impaired behavioural and molecular adaptations to dopamine denervation and repeated L-DOPA treatment in Nur77-knockout mice. Eur J Neurosci 24:795–805.

Starkstein SE, Mayberg HS, Leiguarda R, Preziosi TJ, Robinson RG (1992) A prospective longitudinal study of depression, cognitive decline, and physical impairments in patients with Parkinson's disease. J Neurol Neurosurg Psychiatr 55:377–382.

Staropoli JF, McDermott C, Martinat C, Schulman B, Demireva E, Abeliovich A (2003) Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. Neuron 37:735–749.

Sterky FH, Hoffman AF, Milenkovic D, Bao B, Paganelli A, Edgar D, Wibom R, Lupica CR, Olson L, Larsson N-G (2012) Altered dopamine metabolism and increased vulnerability to MPTP in mice with partial deficiency of mitochondrial complex I in dopamine neurons. Hum Mol Genet 21:1078–1089.

Suzuki S, Suzuki N, Mirtsos C, Horacek T, Lye E, Noh S-K, Ho A, Bouchard D, Mak TW, Yeh W-C (2003) Nur77 as a survival factor in tumor necrosis factor signaling. Proc Natl Acad Sci USA 100:8276–8280.

Suzumura A, Takeuchi H, Zhang G, Kuno R, Mizuno T (2006) Roles of glia-derived cytokines on neuronal degeneration and regeneration. Ann N Y Acad Sci 1088:219–229.

Szot P, White SS, Greenup JL, Leverenz JB, Peskind ER, Raskind MA (2006) Compensatory changes in the noradrenergic nervous system in the locus ceruleus and hippocampus of postmortem subjects with Alzheimer's disease and dementia with Lewy bodies. J Neurosci 26:467–478.

Tai YF, Pavese N, Gerhard A, Tabrizi SJ, Barker RA (2006) Imaging activated microglia in presymptomatic Huntington's disease gene carriers: An 11C-PK11195 PET study.

Tan A, Salgado M, Fahn S (1996) Rapid eye movement sleep behavior disorder preceding Parkinson's disease with therapeutic response to levodopa. Mov Disord 11:214–216.

Tanaka M, Kovalenko SA, Gong JS, Borgeld HJ, Katsumata K, Hayakawa M, Yoneda M, Ozawa T (1996) Accumulation of deletions and point mutations in mitochondrial genome in degenerative diseases. Ann N Y Acad Sci 786:102–111.

Tao X, Tong L (2003) Crystal structure of human DJ-1, a protein associated with early onset Parkinson's disease. J Biol Chem 278:31372–31379.

Tao X, Yang Z, Tong L (2003) Crystal structures of substrate complexes of malic enzyme and insights into the catalytic mechanism. Structure 11:1141–1150.

Tatton NA, Kish SJ (1997) In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. Neuroscience 77:1037–1048.

Taymans J-M (2012) The GTPase function of LRRK2. Biochem Soc Trans 40:1063–1069.

Thompson J, Winoto A (2008) During negative selection, Nur77 family proteins translocate to mitochondria where they associate with Bcl-2 and expose its proapoptotic BH3 domain. J Exp Med 205:1029–1036.

Tian X, Kai L, Hockberger PE, Wokosin DL, Surmeier DJ (2010) MEF-2 regulates activity-dependent spine loss in striatopallidal medium spiny neurons. Mol Cell Neurosci 44:94–108.

Trager N, Smith A, Wallace G IV, Azuma M, Inoue J, Beeson C, Haque A, Banik NL (2014) Effects of a novel orally administered calpain inhibitor SNJ-1945 on immunomodulation and neurodegeneration in a murine model of multiple sclerosis. J Neurochem 130:268–279.

Tsai LH, Delalle I, Caviness VS, Chae T, Harlow E (1994) p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. Nature 371:419–423.

Tsai LH, Takahashi T, Caviness VS, Harlow E (1993) Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Development 119:1029–1040.

Tsuji S (2010) Genetics of neurodegenerative diseases: insights from high-throughput resequencing. Hum Mol Genet 19:R65–R70.

Tsukahara T, Haniu H (2011) Nanoparticle-mediated intracellular lipid accumulation during C2C12 cell differentiation. Biochem Biophys Res Commun 406:558–563.

Ungerstedt U (1968) 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. Eur J Pharmacol 5:107–110.

Unoki M, Nakamura Y (2001) Growth-suppressive effects of BPOZ and EGR2, two genes involved in the PTEN signaling pathway. Oncogene 20:4457–4465.

Valente EM et al. (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304:1158–1160.

Valente EM, Bentivoglio AR, Dixon PH, Ferraris A, Ialongo T, Frontali M, Albanese A, Wood NW (2001) Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. Am J Hum Genet 68:895–900.

Van Humbeeck C, Cornelissen T, Hofkens H, Mandemakers W, Gevaert K, De Strooper B, Vandenberghe W (2011) Parkin interacts with Ambra1 to induce mitophagy. J Neurosci 31:10249–10261.

Van Laar VS, Arnold B, Cassady SJ, Chu CT, Burton EA, Berman SB (2011) Bioenergetics of neurons inhibit the translocation response of Parkin following rapid mitochondrial depolarization. Hum Mol Genet 20:927–940.

Verdaguer E, Alvira D, Jiménez A, Rimbau V, Camins A, Pallàs M (2005) Inhibition of the cdk5/MEF2 pathway is involved in the antiapoptotic properties of calpain inhibitors in cerebellar neurons. Br J Pharmacol 145:1103–1111.

Verdin E, Dequiedt F, Kasler H (2004) HDAC7 regulates apoptosis in developing thymocytes. Novartis Found Symp 259:115–129; discussion129–discussion131, 163–169.

Vila M, Ramonet D, Perier C (2008) Mitochondrial alterations in Parkinson's disease: new clues. J Neurochem 107:317–328.

Vilas D, Pont-Sunyer C, Tolosa E (2012) Impulse control disorders in Parkinson's disease. Parkinsonism Relat Disord 18 Suppl 1:S80–S84.

Volpicelli-Daley LA, Luk KC, Patel TP, Tanik SA, Riddle DM, Stieber A, Meaney DF, Trojanowski JQ, Lee VM-Y (2011) Exogenous α-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. Neuron 72:57–71.

Wakamatsu M, Ishii A, Ukai Y, Sakagami J, Iwata S, Ono M, Matsumoto K, Nakamura A, Tada N, Kobayashi K, Iwatsubo T, Yoshimoto M (2007) Accumulation of phosphorylated alpha-synuclein in dopaminergic neurons of transgenic mice that express human alpha-synuclein. J Neurosci Res 85:1819–1825.

Wang A, Rud J, Olson CM, Anguita J, Osborne BA (2009a) Phosphorylation of Nur77 by the MEK-ERK-RSK cascade induces mitochondrial translocation and apoptosis in T cells. J Immunol 183:3268–3277.

Wang X, She H, Mao Z (2009b) Phosphorylation of neuronal survival factor MEF2D by glycogen synthase kinase 3beta in neuronal apoptosis. Journal of Biological Chemistry 284:32619–32626.

Wang X, Tang X, Li M, Marshall J, Mao Z (2005) Regulation of neuroprotective activity of myocyte-enhancer factor 2 by cAMP-protein kinase A signaling pathway in neuronal survival. J Biol Chem 280:16705–16713.

Wei X, Zhao L, Liu J, Dodel RC, Farlow MR, Du Y (2005) Minocycline prevents gentamicin-induced ototoxicity by inhibiting p38 MAP kinase phosphorylation and caspase 3 activation. Neuroscience 131:513–521.

Werme M, Olson L, Brené S (2000) NGFI-B and nor1 mRNAs are upregulated in brain reward pathways by drugs of abuse: different effects in Fischer and Lewis rats. Brain Res Mol Brain Res 76:18–24.

West AB, Moore DJ, Biskup S, Bugayenko A, Smith WW, Ross CA, Dawson VL, Dawson TM (2005) Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. Proc Natl Acad Sci USA 102:16842–16847.

Whitton PS (2007) Inflammation as a causative factor in the aetiology of Parkinson's disease. Br J Pharmacol 150:963–976.

Widner B, Leblhuber F, Fuchs D (2002) Increased neopterin production and tryptophan degradation in advanced Parkinson's disease. Journal of neural transmission (Vienna, Austria : 1996) 109:181–189.

Willard LB, Hauss-Wegrzyniak B, Wenk GL (1999) Pathological and biochemical consequences of acute and chronic neuroinflammation within the basal forebrain cholinergic system of rats. Neuroscience 88:193–200.

Wise RA, Bozarth MA (1985) Brain mechanisms of drug reward and euphoria. Psychiatr Med 3:445–460.

Woronicz JD, Lina A, Calnan BJ, Szychowski S, Cheng L, Winoto A (1995) Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis. Mol Cell Biol 15:6364–6376.

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi D-K, Ischiropoulos H, Przedborski S (2002) Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. J Neurosci 22:1763–1771.

Wu DC, Teismann P, Tieu K, Vila M, Jackson-Lewis V, Ischiropoulos H, Przedborski S (2003) NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Proc Natl Acad Sci USA 100:6145–6150.

Xie Z, Morgan TE, Rozovsky I, Finch CE (2003) Aging and glial responses to lipopolysaccharide in vitro: greater induction of IL-1 and IL-6, but smaller induction of neurotoxicity. Exp Neurol 182:135–141.

Xiong Y, Zhang H, Beach D (1992) D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71:505–514.

Yamano K, Fogel AI, Wang C, van der Bliek AM, Youle RJ (2014) Mitochondrial Rab GAPs govern autophagosome biogenesis during mitophagy. Elife 3:e01612.

Yang L, Sugama S, Chirichigno JW, Gregorio J, Lorenzl S, Shin DH, Browne SE, Shimizu Y, Joh TH, Beal MF, Albers DS (2003) Minocycline enhances MPTP toxicity to dopaminergic neurons. J Neurosci Res 74:278–285.

Yang Y, Gehrke S, Imai Y, Huang Z, Ouyang Y, Wang J-W, Yang L, Beal MF, Vogel H, Lu B (2006) Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. Proc Natl Acad Sci USA 103:10793–10798.

Youn HD, Liu JO (2000) Cabin1 represses MEF2-dependent Nur77 expression and T cell apoptosis by controlling association of histone deacetylases and acetylases with MEF2. Immunity 13:85–94.

Youn HD, Sun L, Prywes R, Liu JO (1999) Apoptosis of T cells mediated by Ca2+-induced release of the transcription factor MEF2. Science 286:790–793.

Zeng H-Y, Zhu X-A, Zhang C, Yang L-P, Wu L-M, Tso MOM (2005) Identification of sequential events and factors associated with microglial activation, migration, and cytotoxicity in retinal degeneration in rd mice. Invest Ophthalmol Vis Sci 46:2992–2999.

Zetterström RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T (1997) Dopamine neuron agenesis in Nurr1-deficient mice. Science 276:248–250.

Zetterström RH, Williams R, Perlmann T, Olson L (1996) Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. Brain Res Mol Brain Res 41:111–120.

Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, Dawson TM (2000) Parkin functions as an E2-dependent ubiquitin- protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc Natl Acad Sci USA 97:13354–13359.

Zhang Y, Parsanejad M, Huang E, Qu D, Aleyasin H, Rousseaux MWC, Gonzalez YR, Cregan SP, Slack RS, Park DS (2010) Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage. J Neurochem 112:497–510.

Zhang Y, Qu D, Morris EJ, O'Hare MJ, Callaghan SM, Slack RS, Geller HM, Park DS (2006) The Chk1/Cdc25A pathway as activators of the cell cycle in neuronal death induced by camptothecin. J Neurosci 26:8819–8828.

Zhao Y, Bruemmer D (2010) NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology. Arterioscler Thromb Vasc Biol 30:1535–1541.

Zimprich A et al. (2004) Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 44:601–607.

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