AN *IN VIVO* MODEL OF PARKINSON'S DISEASE INDUCED BY PROSTAGLANDIN J2 AND HSP105 AS A POTENTIAL THERAPEUTIC TARGET

by

Sha-Ron Pierre

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Date **Dr. MARIA E.FIGUEIREDO-PEREIRA**
(Hunter College, Chair of Examining Committee)

Date **Dr. Richard Chappell**
Executive Officer

Dr. Jesus Angulo
(Hunter College)

Dr. Anna Maria Cuervo
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Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

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Advisor: Dr. Maria E. Figueiredo-Pereira

Abstract

Chronic neuroinflammation is implicated in Parkinson's disease (PD), a disorder that exhibits accumulation of ubiquitinated proteins in neuronal inclusions (Lewy bodies), indicating a malfunction of the normal process of protein turnover in the affected areas. J2 prostaglandins (PGJ2) are neurotoxic products of inflammation that were shown in cellular models to impair the ubiquitin/proteasome pathway (UPP) causing the accumulation of ubiquitinated proteins into aggregates. J2 prostaglandins are derived from PGD2, the major prostanoid in the mammalian brain. Using PGJ2 as a prototype for a neuroinflammation toxicant product, we established an *in vivo* model of Parkinson's disease that mimics neuroinflammation. We administered PGJ2 into the *substantia nigra* and *striatum* of adult male FVB mice by microinjection. PGJ2 injections induced concentration-

dependent behavioral changes indicative of neuronal damage in the affected areas. Immunohistochemical analyses further supported and characterized the neuronal damage induced by the PGJ2 microinjections. This PD animal model involving PGJ2-induced toxicity is a valuable tool to address the pathogenic mechanisms that converge on the onset of PD neuroinflammation. In addition, we addressed the potential of increasing the levels of the molecular chaperone HSP105 to prevent the toxic effects of neuroinflammation. This approach could be tested as a protective strategy with our new PD model.

Acknowledgements

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List of Abbreviations

6-OHDA: 6-hydroxydopamine
15d-PGJ2 15-deoxy-Δ12,14-prostaglandin J2
AA: Arachidonic acid
AD: Alzheimer's disease
ADP: Adenosine diphosphate
APO: Apomorphine
ATP: Adenosine triphosphate
ALS Amyotrophic lateral sclerosis
BBB: Blood brain barrier
BSA: Bovine serum albumin
CNS: Central nervous system
CNS Central nervous system
COX-1 Cyclooxygenase-1
COX-2 Cyclooxygenase-2
Cys: Cysteine
DA: Dopamine
DAB: 3,3'-Diaminobenzidine
DAPI: 4',6-Diamidino-2-phenylindole
DMSO: Dimethl sulfoxide
DNA: Deoxyribonucleic acid
EDTA: Ethylenediamine tetra-acetic acid
Erk: Extracellular signal regulated kinase
FBS: Fetal bovine serum
FVB: Friend Leukemia Virus, strain B
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GFAP: Glial fibrillary acidic protein
HRP: Horseradish peroxidase
HSP: Heat shock protein
IB: Immunoblot
IP: Immunoprecipitation
IL: Interleukin
iNOS: inducible form of nitric-oxide synthase
IsoP: Isoprostanes
JNK: Jun N-terminal kinase
LB: Lewy body
LC-MS: Liquid chromatograpy-mass spectrometry
LDH: Lactate dehydrogenase
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MEM: Modified Eagle's medium
mRNA: messenger RNA
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NFκB: nuclear factor kappa binding
PBS: Phosphate buffer saline
PD: Parkinson's disease
PG: Prostaglandin
PPAR: Peroxisomal proliferator activator receptor
PBS Phosphate-buffered saline
ROS Reactive oxygen species
RTPCR: Reverse transcription polymerase chain reaction
SDS: Sodium dodecyl sulfate
SDS-PAGE: Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SN: *substantia nigra*
SNpc: *substantia nigra pars compacta*
SNpr: substantia nigra pars reticulata
SOD-1: Superoxide dismutase-1
SSA: Stress seventy A
SSB: Stress seventy B
Sse: *S. Cerevisiae* HSP105 homolog
TH: Tyrosine hydroxylase
TNFα: Tumor necrosis factorα
Ub: Ubiquitin
UCH-L1: Ubiquitin C-terminal hydrolase
UPP: Ubiquitin/Proteasome pathway
VTA: Ventral tegmental area
WT: Wild type
UCH: Ubiquitin-carboxyl terminal

CHAPTER I

INTRODUCTION

There have been tremendous strides to understand the molecular and cellular processes that cause aging-related neurodegenerative disorders such as Parkinson's disease (PD). Among the most plausible stochastic hypotheses of neurodegeneration associated with these disorders is the loss of quality control, i.e. our cells fail to eliminate abnormal proteins caused by environmental or/and genetic factors. Inflammatory processes in particular those attributed to chronic neuroinflammation, are also implicated as inducers and/or contributors to neurodegenerative conditions. Elucidation of the neurotoxic events associated with neuroinflammation and impaired protein turnover will open up new and important potential targets for treatment of neurodegenerative disorders, such as PD, that are associated with chronic inflammation and protein aggregation.

1.1 INFLAMMATION AND NEURODEGENERATION

The brain was long considered to be an immunologically privileged site, particularly because of the blood brain barrier and the lack of a lymphatic system. However, more recently it has been shown that the brain mounts an inflammatory response, as noted from the occurrence of edema, microglia and astrocyte activation, local invasion

of circulating immune cells and production of cytokines and other immune factors [reviewed in(Allan and Rothwell, 2003)]. There is abundant evidence supporting that an inflammatory reaction is mounted within the CNS following trauma, stroke, infection and seizures, all of which augment brain damage.

Chronic inflammation of the CNS has been implicated in a variety of neurodegenerative disorders. Notably, the spatial and temporal distribution of pro-inflammatory cyclooxygenase 2 (COX-2) correlates with neuropathological changes in a wide variety of disorders including AD, PD and ALS [reviewed in (Wyss-Coray and Mucke, 2002)]. These disorders that exhibit signs of inflammation are also associated with the accumulation of ubiquitinated proteins in neuronal inclusions [reviewed in (Li *et al.*, 2003)].

The brain expresses COX-1 and COX-2 under normal physiological conditions. However, the expression and activity of COX-2 are largely responsive to adverse stimuli, such as inflammation and physiologic imbalances (Yamagata *et al.*, 1993). COX-2 up-regulation following CNS injury is not restricted to neurons since COX-2 induction is also apparent in glia (Consilvio *et al.*, 2004). Although many studies support the notion that COX-2 is involved in neurodegeneration its contribution to the neurodegenerative

process remains poorly defined.

1.2 PROSTAGLANDINS OF THE J2 SERIES

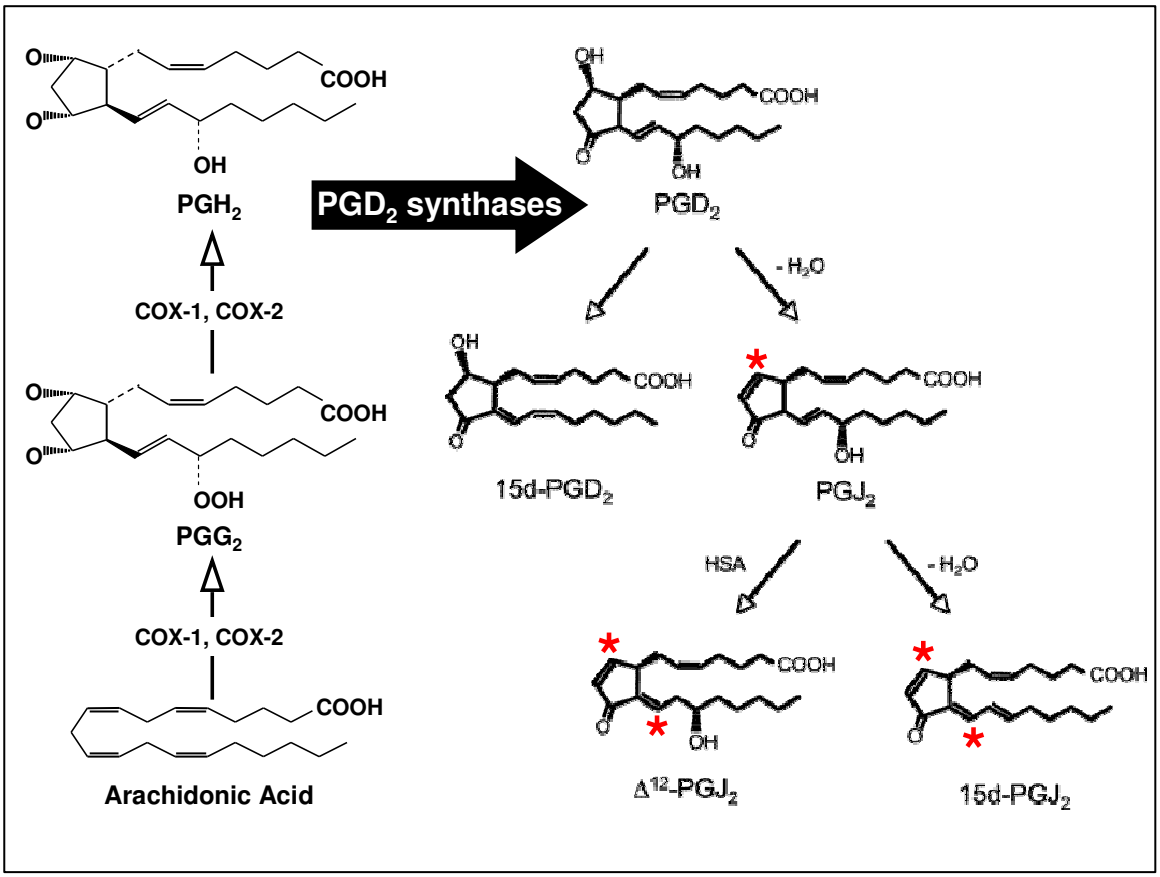


Figure A – Synthesis pathway of cyclopentenone prostaglandins of the J2 series. *Asterisks* (*) depict the electrophilic carbons, one in PGJ2 and two in Δ12-PGJ2 and 15d-PGJ2 (Modified from: Uchida K, Shibata T: Chem Res Toxicol. 2008 Jan;21:138-44).

SYNTHESIS: Prostaglandins in general are a family of structurally related molecules produced by cyclooxygenases in response to numerous extrinsic and intrinsic stimuli.

Cyclooxygenases are bifunctional proteins that catalyze the cyclooxygenation of arachidonic acid to PGG2 followed by hydroperoxidation of PGG2 to PGH2 (Kulkarni *et al.*, 2000). The coupling of PGH2 synthesis with the respective downstream enzymes (synthases) that produce the different types of prostaglandins is intricately orchestrated in a tissue and/or cell specific manner (Funk, 2001).

J2 prostaglandins are derived from PGD2, the major prostanoid synthesized in the mammalian CNS. PGD2 is produced by PGD2 synthases, which are enzymes that carry out the isomerization of PGH2 to PGD2 (Urade and Hayaishi, 2000). PGD2 is produced by two distinct types of prostaglandin D2 synthases (PGDS): *(i)* the lipocalin enzyme (L-PGDS) and *(ii)* the hematopoietic enzyme (H-PGDS) (Urade and Eguchi, 2002). In addition, PGD2 binds to G protein-coupled seven transmembrane receptors, which are DP1 and DP2 (Urade *et al.*, 2002).

PGD2 readily undergoes *in vivo* and *in vitro* non-enzymatic dehydration to generate the biologically active cyclopentenone J2 prostaglandins, which include PGJ2, Δ12-PGJ2 and 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) (Shibata *et al.*, 2002). PGJ2 and its metabolites are not stored in cells. Their production increases with diverse stimuli.

J2 prostaglandins are unique among the prostaglandin

family in that they have α,β-unsaturated carbonyl groups (*asterisks* in Fig. A), promoting Michael addition reactions with free sulfhydryl groups of cysteines in glutathione and cellular proteins (Straus and Glass, 2001). These cyclopentenone prostaglandins covalently modify several proteins, including the p50 subunit of NFκB, which may explain its anti-inflammatory effects (Cernuda-Morollon *et al.*, 2001). They also modify thioredoxin reductase, an enzyme that protects against oxidative damage (Moos *et al.*, 2003) and activate Ras, a small GTPase oncogene known to activate Erk signaling pathways (Oliva *et al.*, 2003).

IN VIVO CONCENTRATIONS: Physiological concentrations of prostaglandins in body fluids are found to be in the pico-nanomolar range, but their levels rise considerably under pathological conditions such as hyperthermia, infection and inflammation, reaching the micromolar range at the site of damage.

It is likely that PGJ2 is produced during chronic inflammation found to be associated with PD and that it exacerbates the neurodegenerative process. Recent studies demonstrated that the levels of 15d-PGJ2, a PGJ2 metabolite, were elevated in spinal cord motor neurons of ALS patients and that 15d-PGJ2 induces neuronal apoptosis

(Kondo *et al.*, 2002). In addition, J2 prostaglandins were detected *in vivo* in human body fluids (Hirata *et al.*, 1988) and human atherosclerotic plaques (Shibata *et al.*, 2002).

Any attempt to quantify PGJ2 levels in human tissues or fluids will be highly inaccurate and will not reflect its biological activity because of their high reactivity with thiol-containing intracellular compounds like glutathione or thiol-containing proteins via Michael addition (Uchida and Shibata, 2007). Similar to PGJ2, nitric oxide has a half life of just a few seconds, and yet is well accepted as a major signaling molecule in neurons and in the immune system (Murad, 1998). The same can be said for PGJ2, i.e. that it is a major signaling molecule/oxidative stress agent.

Since prostaglandins of the J2 series are formed from PGD2 by a non-enzymatic first order dehydration reaction, it is possible to estimate the rate of formation of the cyclopentenone J2 prostaglandins from accurate measurements of PGD2 tissue concentration. PGD2 levels in mouse brain were determined by LC-MS following head-focused microwave irradiation to heat inactivate all enzymes *in situ* (Golovko and Murphy, 2008b). Initially, it was established that microwave irradiation does not alter PGD2 and that, under basal conditions, mouse brain contained ~17 pmoles of

PGD2/g of brain (Golovko and Murphy, 2008a). Alternatively, decapitation of mice followed by removal of the brain and freezing in liquid nitrogen elevated brain PGD2 levels to ~102 pmoles/g of brain, a result of the about 30 seconds hypoxia occurring from decapitation till freezing. This clearly indicates the extraordinary dynamics of PGD2 synthesis under stress conditions leading to a six-fold elevation of PGD2 levels within 30 seconds. Dehydration of PGD2 to ultimately 15d-PGJ2 occurs with a half life of about 12 hours in the presence of albumin (Fitzpatrick and Wynalda, 1983).

ENTRY INTO CELLS: Once synthesized J2 prostaglandins are released or exported to the extracellular space (Fig. B). As they are unstable, they exert their effects near their sites of synthesis, acting as autocrine or paracrine hormones (Scher and Pillinger, 2005). These J2 cyclopentenone prostaglandins enter the cells by an active transport system (Narumiya and Fukushima, 1986;Harris *et al.*, 2002). An additional transport mechanism allows these PGs to enter the nucleus where they regulate gene transcription. Alternatively, PGD2 that enters cells by an anionic transporter can be dehydrated to J2 prostaglandins within the cytoplasm.

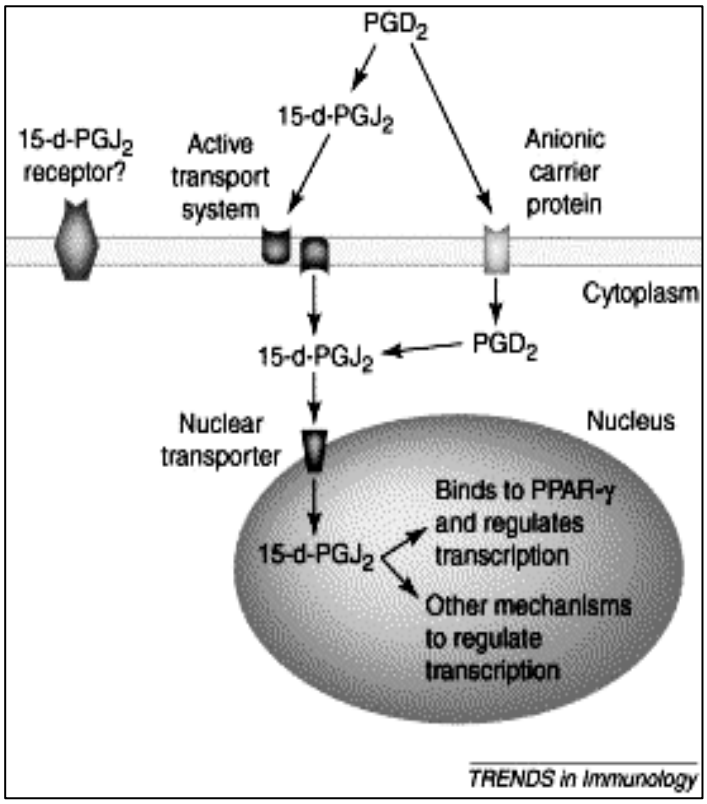


Figure B – Possible modes of action of prostaglandins of the J2 series including 15d-PGJ2, in the cell [from Harris, S.G., Padilla, J., Koumas, L., Ray, D. and Phipps, R.P. (2002) Trends Immunol 23, 144-150].

BIOLOGICAL ACTIVITIES: J2 prostaglandins display both protective and toxic effects. The effects of J2 prostaglandins appear to be cell type- and dose-dependent. Their biological activities include antiviral and antitumoral effects, modulation of the heat shock response, induction of oxidative stress, increase intracellular calcium fluxes and apoptosis (Straus *et al.*, 2001;Scher *et al.*, 2005). Although their anti-proliferative and pro-

apoptotic effects are most frequently described, these prostaglandins also induce the proliferation of different forms of cancer cells when used at nanomolar to low micromolar concentrations (Oliva *et al.*, 2003).

ROLE IN INFLAMMATION: The role of J2 prostaglandins in inflammation is complex (Harris *et al.*, 2002). On the one hand, 15d-PGJ2 has emerged as a key anti-inflammatory agent as it inhibits the production of pro-inflammatory mediators such as iNOS, TNFα and IL1β, suppresses microglia and astrocyte activation and induces apoptosis (Mrak and Landreth, 2004;Giri *et al.*, 2004;Eucker *et al.*, 2004). On the other hand, 15d-PGJ2 is a pro-inflammatory agent. It stimulates the production of pro-inflammatory mediators such as IL8 and expression of COX-2, and activates MAPK (Meade *et al.*, 1999;Zhang *et al.*, 2001). Furthermore, 15d-PGJ2 plays a role in the regulation of human autoimmune diseases and inhibits inflammation in models of arthritis, ischemia-reperfusion injury, inflammatory bowel disease, lupus nephritis and AD [reviewed in(Scher *et al.*, 2005)].

INTRACELLULAR TARGETS: 15d-PGJ2 and Δ12-PGJ2 (Fig. A) are endogenous ligands for the nuclear peroxisomal proliferator activator receptor (PPARγ) (Scher *et al.*, 2005).

PPARγ is an intranuclear receptor. PPARγ-agonists are linked to neuroprotection rather than neurotoxicity (Aoun *et al.*, 2003).

In addition, J2 prostaglandins act through PPARγ-independent mechanisms including activation of Erk (MAPK and JNK) pathways (Wilmer *et al.*, 2001;Li *et al.*, 2004a) and inhibition of the NFκB pathway (Rossi *et al.*, 2000;Straus *et al.*, 2000). This may account for the different effects of 15d-PGJ2 and other PPARγ ligands [reviewed in(Scher *et al.*, 2005)]. PGJ2 can also act via DP2 (Liang *et al.*, 2005) receptors. DP2 is a PGD2 seven transmembrane G-protein coupled receptor. DP2-activation by 15d-PGJ2 in eosinophils increases intracellular calcium fluxes with a potency nearly equal to that of PGD2 (Monneret *et al.*, 2002).

CONCLUSION: We focused on PGJ2 because it is potently neurotoxic and a highly reactive product of inflammation. J2 prostaglandins are endogenous ligands shown to be neurotoxic and pro-oxidant agents (Kondo *et al.*, 2001), to up-regulate the expression of COX-2 (Li *et al.*, 2004a), and to induce neuronal apoptosis (Kondo *et al.*, 2002). In addition, data from our laboratory and others clearly

demonstrate that PGJ2 impairs the UPP. These endogenous electrophiles *(1)* inhibit ubiquitin isopeptidase activity (Mullally *et al.*, 2001) as well as ubiquitin hydrolases UCH-L1 and UCH-L3 (Li *et al.*, 2004b), *(2)* induce the formation of cysteine-targeted thyolation of UCH-L1 (Ishii and Uchida, 2004) and PGJ2/proteasome conjugates (Shibata *et al.*, 2003), *(3)* trigger the oxidation of the S6 ATPase subunit of the 26S proteasome (Ishii *et al.*, 2004), and disrupt 26S proteasome assembly (Wang *et al.*, 2006). All of these effects on the proteasome cause a dramatic decrease in 26S proteasome activity, which together with inhibition of deubiquitinating activity promotes the build-up of pro-apoptotic and detrimental proteins, such as p53 and ubiquitinated proteins (Uchida *et al.*, 2007). *PGJ2 are thus optimal tools to address the relationship between inflammation and the neuropathology associated with UPP impairment in PD-neurodegeneration.*

1.3 PARKINSON'S DISEASE AND PROSTAGLANDIN D2, THE PRECURSOR OF PROSTAGLANDIN J2

Investigations on the role of inflammation in PD are quite recent (Rogers *et al.*, 2007). The involvement of inflammation in PD was first suggested by McGeer et al in 1988 (McGeer *et al.*, 1988a;McGeer *et al.*, 1988b). There are a few studies supporting the role of PGD2 in PD. For example, significant changes in L-PGDS isoforms, the lipocalin form of prostaglandin D2 synthase, were detected in the CSF of at least 20 idiopathic PD patients compared to 100 controls (Harrington *et al.*, 2006). These alterations reflected up/down regulation of L-PGDS isoforms and are likely to represent pathology at the cellular level that could have an impact on prostaglandin production thus establishing a correlation with PD symptoms (Harrington *et al.*, 2006). It was speculated that these altered isoforms could be candidate diagnostic biomarkers of PD and may have predictive value (Harrington *et al.*, 2006).

A number of studies suggest that α-synuclein plays a role in brain fatty acid metabolism including arachidonic acid, through modulation of ER-localized acyl-CoA synthetase activity (Castagnet *et al.*, 2005;Golovko *et al.*, 2006). In α-synuclein KO mice, exogenous addition of mutant (A30P, E46K, and A53T) forms of α-synuclein failed to

restore this activity, while wild-type mouse or human α-synuclein did (Golovko *et al.*, 2006). More recently, the levels of several prostaglandins in brains following a 30 second global ischemia were compared in wild type versus α-synuclein KO mice (Golovko *et al.*, 2008b). From all prostaglandins assayed (E2, D2, F2α, TxB2 and 6-ketoF1α) PGD2 showed the greatest increase (two-fold) in the α-synuclein KO mice versus wild type. The levels of PGD2 in brains of α-synuclein KO mice reached ~35ng/g following the 30s global ischemia. Under normal physiological conditions, α-synuclein ablation had no effect. Together these studies suggest that α-synuclein could play a role in brain inflammatory responses through modulation of arachidonic acid metabolism and downstream, PGD2 production. PGD2 in turn readily undergoes *in vivo* and *in vitro* non-enzymatic dehydration to generate the biologically active cyclopentenone J2 prostaglandins.

1.4 JUSTIFICATION FOR DEVELOPING A PROSTAGLANDIN J2-INDUCED MODEL OF PARKINSON'S DISEASE

In a recent review McGeer & McGeer (Klegeris *et al.*, 2007) discuss the evidence for the neuroinflammation hypothesis for PD. This evidence includes:

(1) Increased numbers of astrocytes and microglia in affected brain areas as well as continued presence of activated microglia in the *substantia nigra*. In one case, activated microglia were still detected 16 years after exposure of an individual to the neurotoxin MPTP; this suggests that once initiated, neuroinflammation may become self-sustaining.

(2) DNA polymorphisms in cytokines modify PD risk, in particular the age of onset (Wahner *et al.*, 2007).

(3) Protection by NSAIDs: 80,000 men and 90,000 women were involved in a study that concluded that the incidence of PD was lower among ibuprofen users (Chen *et al.*, 2005).

(4) Most *in vivo* and *in vitro* models of PD support involvement of inflammatory mechanisms. Many of the animal models for idiopathic PD are produced by neurotoxins, the four most popular being 6-hydroxydopamine, MPTP, rotenone and paraquat (Bove *et al.*, 2005). All of these models are associated with signs of neuroinflammation in the *substantia nigra*, supporting the view that inflammation and in particular glial cells including astrocytes and microglia, play a central role in PD (Klegeris *et al.*, 2007).

To directly test the hypothesis that inflammation in the brain can lead to selective loss of dopaminergic neurons, several groups used lipopolysaccharide (LPS) by:

(1) chronically infusing it into the *substantia nigra* (Gao *et al.*, 2002);

(2) an *in utero* exposure of developing fetuses to the endotoxin (Carvey *et al.*, 2003).

In the first paradigm, microglia activation preceded dopaminergic loss (Gao *et al.*, 2002) and in the second one selective degeneration of the nigrostriatal dopaminergic pathway was observed in the neonates (Carvey *et al.*, 2003). These studies provide a potential mechanistic link between inflammation in the brain and dopaminergic neurodegeneration (Liu *et al.*, 2003).

It is thought that the *substantia nigra* dopaminergic neurons are particularly sensitive to injury because they have mitochondrial defects (Greenamyre *et al.*, 1999), reduced anti-oxidant capacity (such as low reduced glutathione levels), and high content of dopamine, melanin and lipids, which are all prone to oxidation (Liu *et al.*, 2003). In addition, the *substantia nigra* is particularly rich in microglia (Liu, 2006), which once activated could provide for a highly damaging environment surrounding neurons (Liu *et al.*, 2003).

The LPS models of PD address the central role of inflammation in this disease, but they do not distinguish which of the factors produced by activated microglia and astrocytes induce neurodegeneration. This is a very important issue since the mechanisms by which activated microglia specifically target dopaminergic neurons remain critical missing links in the proof of a pathogenic role for activated microglia and astrocytes in PD.

Our new PGJ2-induced PD model addresses the hypothesis that localized production of highly reactive and neurotoxic cyclopentenone PGJ2 maybe one of such links.

1.5 CONCLUSIONS

Our studies focus on PGJ2 because they are endogenous products of inflammation that cause pleiotropic changes that mimic many of the pathological processes that occur in neurodegenerative disorders such as PD, that are associated with inflammation and protein aggregation. A recent review suggests that "formation of cyclopentenone eicosanoids [such as PGJ2] in the brain may represent a novel pathogenic mechanism that contributes to many neurodegenerative conditions (Musiek *et al.*, 2005). Moreover, PGJ2 up-regulate the expression and activity of COX-2, a prostaglandin synthesizing enzyme. PGJ2 thus have

potential to initiate a series of deleterious cascades leading to self-sustained progressive neurodegeneration.

To design therapies that prevent endangered neurons from dying it is critical to learn more about the *in vivo* effects of neuroinflammation and its products, which was the main goal of this project. Clearly, PGJ2 are optimal tools for dissecting the molecular pathology of inflammation linked to abnormal protein turnover and neuronal injury in PD. **Our new PGJ2-induced PD** model provides a means to address the pathological role for cytotoxic PGJ2 *in vivo*.

CHAPTER II

AN *IN VIVO* MODEL OF PARKINSON'S DISEASE INDUCED BY PROSTAGLANDIN J2

To be submitted to the Journal of Neuroscience

2.1 INTRODUCTION

The neuroinflammation hypothesis for Parkinson's disease (PD), first proposed by McGeer et al in 1988 (McGeer *et al.*, 1988a;McGeer *et al.*, 1988b), is supported by different kinds of evidence [reviewed in (Klegeris *et al.*, 2007)] such as *(1)* increased numbers of astrocytes and microglia in affected brain areas as well as continued presence of activated microglia in the *substantia nigra*; *(2)* DNA polymorphisms in cytokines modify PD risk, in particular the age of onset; *(3)* protection by non-steroidal anti-inflammatory drugs (NSAIDs) although this is highly controversial, and *(4)* most *in vivo* and *in vitro* models of PD support involvement of inflammatory mechanisms. Many of the animal models for idiopathic PD are produced by neurotoxins, the four most popular being 6-hydroxydopamine, MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine), rotenone and paraquat (Bove *et al.*, 2005). All of these animal models are associated with signs of neuroinflammation in the *substantia nigra*, supporting the view that inflammation and in particular glial cells including astrocytes and microglia, play a central role in PD (Klegeris *et al.*, 2007).

It is thought that the dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) are particularly

sensitive to injury because they have mitochondrial defects (Greenamyre *et al.*, 1999), reduced anti-oxidant capacity (such as low reduced glutathione levels), and high content of dopamine, melanin and lipids, which are all prone to oxidation (Liu *et al.*, 2003). In addition, the SN is particularly rich in microglia (Liu, 2006), which once activated could provide for a potentially damaging environment surrounding the neurons in that area of the brain (Liu *et al.*, 2003).

To directly test the hypothesis that inflammation in the brain can lead to selective loss of dopaminergic neurons, several groups used lipopolysaccharide (LPS) either *(i)* chronically infused into the *substantia nigra* (Gao *et al.*, 2002); or *(ii)* through an *in utero* exposure of developing fetuses to the endotoxin (Carvey *et al.*, 2003). In the first paradigm, microglia activation preceded dopaminergic loss (Gao *et al.*, 2002) and in the second one selective degeneration of the nigrostriatal dopaminergic pathway was observed in the neonates (Carvey *et al.*, 2003). These studies provide a potential mechanistic link between inflammation in the brain and dopaminergic neurodegeneration (Liu *et al.*, 2003). The LPS models of PD address the central role of inflammation in this disease but they do not distinguish which of the factors produced

by activated microglia and astrocytes induce neurodegeneration. This is a very important issue since the mechanisms by which activated glia specifically target dopaminergic neurons remain critical missing links in the proof of a pathogenic role for activated glia in PD.

Herein, we present *in vivo* evidence for the importance of prostaglandin J2 (PGJ2) in the death of dopaminergic neurons in the SNpc. We also provide *in vivo* evidence that PGJ2 induces the formation of ubiquitin-protein aggregates in this dopaminergic neuronal population and leads to microglial and astrocyte activation. This feed-forward cycle of glial activation and neuronal injury could be a driving force for progressive dopaminergic neurodegeneration observed in PD neurodegeneration.

2.2 MATERIALS AND METHODS

Animals – Male FVB mice, 11-week old weighing 33g to 39g, were obtained from Taconic Farms, Germantown, NY. Mice were singly housed on a 12-h light/dark cycle with food and water available *ad libitum*. The room temperature was maintained at 23°C and 50-70% humidity. Animals were habituated for two weeks before commencement of surgery, which was initiated at 13 weeks of age. All mice were treated in accordance with the guidelines for Animal Care

Use at NIH. All efforts were made to reduce animal suffering and the number of animals used.

A total of 23 mice received unilateral injections of PGJ2 or vehicle. The experiments were carried out in 4 groups of mice. One group received DMSO/PBS (n = 5) and three groups received PGJ2 concentration of 3.34μg, 6.7μg or 16.7μg PGJ2 in DMSO/PBS per injection (n = 6 per group), respectively (Fig. 1). Each mouse served as its own control, since there was no surgery on its left-side (contralateral to the lesion). In addition, mice injected with DMSO/PBS served as controls for PGJ2 infusion, since they received unilateral injections of DMSO/PBS solution. Upon microinjection some nonspecific damage (gliosis) was seen at the site of injection (Fig. 1, star indicates site of injection).

DMSO (dimethyl sulfoxide) was the solvent for PGJ2 and its final concentration was 17% for all microinfusions. The solutions were freshly prepared and stored for a maximum of 2h under cold (4^{o}C) and dark conditions.

Choice of PGJ2 dose and microinfusion schedule – In choosing the PGJ2 concentrations for infusion we had to consider that *(1)* the injected pool of PGJ2 would be in equilibrium via active transport (Bito *et al.*,

1976;Narumiya *et al.*, 1986) with the blood circulating through the injected area, and *(2)* that PGJ2 binds very quickly to blood albumin, leading to a continuous removal of PGJ2 from the site of injection. In one study it was estimated in cell cultures that ten percent fetal bovine serum reduces the concentration of PGJ2 available to cells by 150-fold, by directly and reversibly interacting with PGJ2 and sequestering it in the medium (Person et al., 2001). The same study demonstrated that human serum had similar PGJ2-sequestration properties. Moreover, due to slight hemorrhage at the site of injection, the released serum albumin will bind some of the injected PGJ2, leading to a further reduction of the available PJG2. Consequently, the amounts of PGJ2 that diffuse into to the brain tissue contiguous to the site of injection are bound to be significantly lower than those initially infused.

In addition, we wanted to establish a progressive PD model, one in which there is a progressive loss of SNpc dopaminergic neurons. In a recent study, 8μg of 6-OHDA were bilaterally infused into mouse striatum daily for 5 days (Richter et al., 2008). This subchronic paradigm provoked a moderate, but significant loss of nigral neurons while a single injection did not. All of these studies support our choice to infuse the mice unilaterally with three

concentrations (3.4μg, 6.7μg and 16.7μg per injection) of PGJ2, similar to the 6-OHDA concentration in the study described above.

All animals received four microinfusions per site of injection at the following ages: the initial injection was given at 13 weeks of age followed by individual injections at 17, 18 and 19 weeks of age (Fig. 1). Five animals were microinfused with DMSO (control, n = 5) while six animals per group were microinfused with PGJ2 (n = 6 per group). Our reasoning was that by giving a total of four injections, we would mimic, to a certain extent, subchronic inflammation.

Surgery and "subchronic" microinfusion – Mice were anesthetized with 2.5% isoflurane in oxygen (0.6L/min flow rate) and placed in a stererotaxic frame (Model 5000 Small Animal Stereotaxic Instrument; Kopf Instruments) fitted with a mouse anesthesia mask (Model 907, David Kopf instruments). A 2μl Hamilton microinjection syringe (7002KH) with a 25-gauge needle was attached to the stereotaxic frame via a Universal Holder (David Kopf Instruments). Holes were drilled into the skull and the needle moved to the injection site and inserted slowly. Unilateral (right side) injection sites were determined

using a mouse brain atlas (Paxinos and Franklin, 2001). The following coordinates were used relative to bregma: *substantia nigra*, RC −3.25mm; ML ±1.25mm; DV +4.13mm; *striatum*, RC +0.5mm; ML ± 2.0; DL +2.5mm. The needle was left in position for 5-min before two µl of the desired solution was injected into the right side over 10-min (~0.2µl/min). At the end of the injection, the incision was sutured. The mouse was removed from the stereotaxic apparatus, was given a subcutaneous injection of 1 ml of sterile saline, and was left in a warm place to recover.

Immunohistochemistry – For immunohistochemical analysis mice were perfused at 23 weeks of age, thus four weeks after the last injection. Immunohistochemical analyses were performed as described in (Thomas et al., 2007). Mice were anesthetized using a lethal dose of ketamine/Ace (100/3 mg/kg of body weight, intraperitoneally) and by perfusing them intracardially with 60ml of Tyrode (RT), followed by fixative (4% paraformaldehyde, 7% saturated picric acid, 0.1% glutaraldehyde in PBS) at 4°C. Brains were removed, postfixed for two hours in the same fixative at RT, and cryopreserved in 15% sucrose overnight at 4°C. They were then frozen in dry ice and stored in −80°C until further analysis. Free-floating coronal sections (30µm thick) from

both the *substantia nigra* and *striatum* were cut on a sliding microtome HM440E (Microm, Waldorf, Germany).

For immunostaining, sections were permeabilized by consecutive washing with TBS and TBS containing 0.5% Triton X-100 (TBS-T) for 30-min. Sections were then incubated with the primary antibodies in TBS containing 0.5% Triton X-100 (TBS-T) overnight at 4°C and washed with TBS and TBS-T successively for 30-min. Then the sections were incubated for 90-min with the first appropriate labeled secondary antibody (anti-mouse or anti-rabbit antibody, 1:100) in TBS-T and washed with TBS and TBS-T for 30-min. For sections that were triple stained, tissues were incubated with a second secondary antibody (anti-mouse or anti-rabbit, 1:100) for an additional 90-min, followed by incubation with Hoechst 33342 (a nuclear stain, 1:500) for 30-min. Sections were mounted on glass slides, using 80% glycerol in TBS. Cell staining was visualized with Leica TCS SP2 confocal microscope (Leica microsystems, Exton, PA). Symmetric areas from injected and control (contralateral, left side) sides as well as DMSO/PBS and PGJ2/PBS injected (ipsilateral) sides were compared.

Primary antibodies: dopaminergic neurons (tyrosine hydroxylase, 1:500, Abcam, ab6211, rabbit or 1:50, TOHA1.1, mouse, kindly supplied by Dr. C. Cuello, McGuill

University, Canada); neurons (NeuN, 1:50, Chemicon, mab377, mouse); GABAergic neurons (GAD67, 1:1,000, Chemicon, mab5406, mouse); ubiquitin inclusions (ubiquitinated proteins, 1:200, Dako Cytomation, Z0458, rabbit); microglia (IBA1, 1:500, Wako Chemicals, 019-19741, rabbit); astrocytes (GFAP, 1:500, G3893, mouse). Secondary antibodies: anti-rabbit Alexa 594, A21207, anti-mouse Alexa 488, A21206, and anti-mouse Alexa 350, S11249, all from Molecular Probes.

Image analysis - Stereological methods were employed to determine an unbiased estimate of total neurons (NeuN positive) and TH immunopositive neurons within the SNpc. Briefly, mice treated with DMSO/PBS or different concentrations of PGJ2/PBS were processed for TH and NeuN immunohistochemistry on every fourth SNpc section throughout the entire extent of SNpc. Neurons were counted using the optical fractionator, an unbiased method for cell counting that is not affected by either the volume of reference (SNpc) or the size of the counted elements (neurons). This method was carried out using a computer-assisted image analysis system, consisting of an Axiophot photomicroscope (Carl Zeiss Vision, Hallbergmoos, Germany) comprising of a Zeiss planapochromat 100 X oil objective

equipped with a computer-controlled motorized stage, a video camera, and the Stereo Investigator software (MicroBrightField, Williston, VT) as described in (Schmitz *et al.*, 2004;Schmitz and Hof, 2005). Cell counts were performed by counting the number of neurons on the right SNpc of every fourth section across the rostro-caudal axis throughout the entire extent of the SNpc using a standard mouse atlas (Paxinos *et al.*, 2001) as anatomical reference. The total number of TH-positive neurons was calculated using the formula previously described for this method (West et al., 1991).

Animal behavior – Simple behavioral tests in control and PGJ2-treated mice were performed. Asymmetry in body posture and gait abnormalities were tested with the curling test and the footprint test, respectively. The degree of nigrostriatal damage was assessed with the turning behavior test.

1) Curling: The curling test evaluates any asymmetry in body posture (Oehrn et al., 2007). The mouse was lifted gently 2cm above the bedding for 5-sec and any deviation from its vertical body axis of 10° or greater was recorded.

2) Footprint test: Mice were placed in a 5-cm wide, 85-cm long corridor. The floor of this corridor was covered with

white absorbing paper. The animals were first trained to pass straight forward through the corridor. After this training, the paws were colored with different colors (red for the forepaws and black for the hind paws), and the mice were then placed into the corridor (Richter et al., 2008). Step frequency was determined with the program Footprints version 1.22 (Klapdor et al., 1997).

3) Asymmetric circling motor behavior: This behavior is dependent on the degree of nigrostriatal damage. Rotation is more prominent after apomorphine (APO) due to imbalance between lesioned and unlesioned hemispheres (Bove et al., 2005). APO leads to a reversal of rodent asymmetry, in that the animals which otherwise have an ipsiversive asymmetry now turn contraversively. Furthermore, the induction of contraversive turning occurs rapidly within minutes after drug administration (Ungerstedt, 1971;Schwarting and Huston, 1996). APO was administered (1.5 mg/kg, intraperitoneally) to reverse animal asymmetry to determine whether this behavior is indeed due to SN damage and not disruption of a different sort of neighboring cell type.

Statistical analysis – Statistical significance was estimated using one-way ANOVA (Tukey-Kramer multiple

comparison test, Instat 2.0, Graphpad Software, San Diego, CA).

2.3 RESULTS

PGJ2-microinfused mice exhibit loss of dopaminergic neurons in the SNpc but the GABAergic neurons in the SNpr are spared – To assess the vulnerability of nigral dopaminergic neurons to the neurotoxic prostaglandin PGJ2, FVB mice received four injections of PGJ2 at 13, 17, 18 and 19 weeks of age. Mice were processed for immunohistochemical analysis four weeks after the last injection. At 23 weeks of age TH staining showed loss of SNpc dopaminergic neurons in a concentration-dependent manner (Fig.2, immunofluorescence staining). That the loss of TH staining was due to neuronal loss and not to TH down-regulation is asserted by the PGJ2 concentration-dependent loss of NeuN neuronal staining (Fig. 3). Preliminary unbiased stereolgic counts of NeuN-immunopositive and TH-immunopositive SNpc neurons are shown in Fig. 3 (table). Mice infused with 3.4µg, 6.7µg and 16.7µg exhibited 18%, 41% and 86% TH+ neuronal loss, respectively, which paralleled the loss in NeuN staining. These data corroborate the lesion in the SNpc induced by PGJ2.

One of the most challenging aspects of PD is to explain why the dopaminergic neurons in the SNpc are particularly vulnerable to neurodegeneration. One possibility is that the susceptibility to stress conditions is exacerbated by the pro-oxidant properties of dopamine. In fact, we observed that the GABAergic neurons of the *substantia nigra pars reticulata* (SNpr) were spared (Fig. 4) even in mice treated with the highest (16.7µg) PGJ2-concentration. These data support that SNpc dopaminergic neurons are indeed more sensitive to the neurotoxic effects of PGJ2 than the neighboring GABAergic neurons in the SNpr. To complement these findings, studies in our laboratory demonstrated that PGJ2 exacerbates dopamine toxicity in SK-N-SH cells (Ogburn et al., 2006).

To rule out the possibility that the prostaglandin did not reach the SNpr, we validated our infusion protocol by injecting 16.7µg of the dyes methylene blue or sudan black in 2µl of DMSO/PBS solution, to mimic the conditions used to inject PGJ2. The molecular weights of both dyes and PGJ2 are similar: 334.5 for PGJ2, 319.9 for methylene blue which is water soluble, and 456.55 for sudan black that, like PGJ2, is water insoluble. Sixty minutes post-injection the brains were extracted, frozen on dry ice and cut on a cryostat. The site and extent of the methylene blue (Fig.

5A) or sudan black (*not shown*) diffusion in the brain was determined. It was clear that both dyes reached the SNpc as well as the SNpr. Nissl staining (cresyl violet) of a contiguous section is shown in Fig. 5B. Together these data clearly establish that the dopaminergic neurons in the SNpc are more susceptible to PGJ2-neurotoxicity than the GABAergic neurons in the SNpr.

Detection of aggregates with ubiquitinated proteins in mice treated with PGJ2 - In mice infused with DMSO and 3.4μg of PGJ2 only low levels of ubiquitinated proteins were detected. The latter (*stained red*) exhibited a diffuse distribution throughout the cytoplasm of the TH-positive cells (*stained green*) in the SNpc (Fig. 6A). Infusion of 6.7μg and 16.7μg of PGJ2 caused the formation of intracellular aggregates of ubiquitinated proteins observed in the few spared TH-positive neurons in the SNpc (Fig. 6A and B). TH immunostaining (*green*) co-localized with the ubiquitin aggregates (*red*) suggesting that the enzyme is trapped in the aggregates containing ubiquitinated proteins.

Studies from some laboratories suggest that aggregates can be protective (Arrasate et al., 2004). Thus it is not clear if the few SNpc TH-positive neurons in the PGJ2-treated mice were spared because aggregate formation.

However, in cells containing protein aggregates (Fig. 6B, *arrows*) nuclei exhibit the typical morphology associated with apoptosis, i.e. a condensed or fragmented appearance (Fig. 6B, *arrowheads*) compared to normal nuclei. This nuclear morphology clearly indicates that cells exhibiting protein aggregates are committed to the apoptotic pathway. Most likely, the neurons that died also had aggregates prior to cell death, but we do not see them because they were already cleared. In other PD models such as the MPTP and the 6-OHDA models, no inclusions with ubiquitinated proteins were identified, pointing to an advantage of the PGJ2 model.

Increase microglia and astrocyte activation in the SNpc in response to PGJ2 – Increasing evidence suggests that prior occurrence of inflammation in the brain, due to either brain injury or infectious agents, may play a role in PD pathogenesis (Liu et al., 2003). It is proposed that a self-perpetuating cycle of inflammatory processes involving brain immune cells (microglia and astrocytes) may drive the slow progression of the neurodegenerative process. Prostaglandins (PGs) are largely produced by activated microglia and reactive astrocytes (less by neurons) in neuroinflammation. Prostaglandins act as potent local

regulators of the pathogenic processes associated with CNS inflammation. Our studies demonstrate that in mice infused with 6.7μg and 16.7μg of PGJ2 there is a potent activation of microglia (Fig. 7) as well as astrocytes (Fig. 8).

Microglia and astrocytes act as immune cells in the inflamed brain. Although activated microglia and astrocytes release trophic factors, most of their released products are pro-inflammatory and potently cytotoxic (Liu et al., 2003). The PGJ2-dependent glial activation may thus be instrumental in further exacerbating the neurodegenerative process observed in PD.

Behavioral changes induced by PGJ2 – To address the posture and walking difficulties associated with PD, we performed simple behavioral tests in control and PGJ2-treated mice (Fig. 9). It is clear that the PGJ2-treated mice exhibit gait impairment and severe postural instability, consistent with a unilateral lesion. Importantly, mice that were unilaterally microinfused with 16.7 μg of PGJ2 demonstrated a reversal of body asymmetry and slow, tight turning when administered apomorphine, a DA receptor agonist (turning behavior captured using videograpy). Induction of turning occurred within minutes after drug injection. Turning reached a peak frequency and gradually declined. Peaks in

turning frequency were evaluated over a span of 10 minutes. The apomorphine-induced turning was only elicited in the animals that received the highest dose of PGJ2 and consequently experienced the most substantial lesion to the nigrostriatal pathway. With moderate lesions (<80%), no asymmetry occurs (Schwarting *et al.*, 1996).

2. 4 DISCUSSION

Herein, we demonstrate *in vivo* that intracerebral infusion of PGJ2 into the *substantia nigra pars compacta* (SNpc) and the *striatum* induces a dose-dependent degeneration of dopaminergic neurons. Due to depletion of dopamine-producing neurons in the basal ganglia of the brain, individuals with PD experience deterioration in balance and postural control as well as a progressive reduction in the speed and amplitude of movements. The behavioral tests that were performed in control and PGJ2-treated mice clearly indicate that the PGJ2-treated mice exhibit gait impairment, severe postural instability and turning behavior, the latter upon apomorphine administration, supporting a basal ganglia lesion.

Notably, the GABAergic neurons in the neighboring *substantia nigra pars reticulata* (SNpr) were not affected by the PGJ2 microinfusions. It is thus likely that the

dopaminergic neurons of the SNpc are selectively vulnerable to the toxic actions of PGJ2. There are at least two reasons why the dopaminergic neurons may be more susceptible to PGJ2 toxicity. Firstly, PJG2 up-regulates the expression and increases the activity of COX-2 in cultured neuronal cells (Li *et al.*, 2004a). COX-2 readily uses dopamine as an electron donor and in so doing forms the highly cytotoxic dopamine quinone (DAQ) (Stokes *et al.*, 1999). Secondly, PGJ2 potentiates dopamine toxicity in neuronal cultures by attenuating catechol-*O*-methyltransferase activity (Ogburn *et al.*, 2006). In this way, PGJ2 increases the cytoplasmic availability of dopamine which, in excess of the buffering capacity of the cytosol, will further enhance the production of cytotoxic DAQ. DAQ covalently binds to intracellular proteins, including α-synuclein, leading to the accumulation of pathogenic protofibrils (Sulzer, 2001).

The ventral tegmental area (VTA), which is in the vicinity of the SN, also contains dopaminergic neurons. However, the VTA dopaminergic neurons were not affected by the PGJ2-treatment, most likely because they are further away from the site of injection and may not be exposed to the toxin. Prostaglandins of the J2 series are highly reactive lipid electrophiles that act as potent local

regulators in an autocrine or paracrine manner (Ueki et al., 2007). Assessment of the susceptibility of the VTA dopaminergic neurons to PGJ2-toxicity may require a similar microinjection paradigm to be applied to this site. The fact that some of the products of inflammation, such as PGJ2, exert their effects in a localized autocrine or paracrine manner, could explain why different forms of neurodegenerative disorders, such as AD and PD, share a common mechanism, i.e. neuroinflammation. The variety of disease manifestations associated with neuroinflammation could be correlated with the primary brain region affected by the injurious event, its severity and duration.

Distinct aggregates of ubiquitinated proteins were observed in the few detectable dopaminergic neurons in the SNpc of mice infused with the two highest concentrations of PGJ2 (6.7µg and 16.7µg). These dopaminergic neurons exhibit clear signs of apoptosis manifested by fragmented and/or highly condensed nuclei. These data support the view that protein aggregates are indicative of neurons committed to the death pathway and not of healthy neurons. Earlier studies from other laboratories suggested the contrary, i.e. that aggregates may protect cells by decreasing the levels of toxic diffuse forms of proteins (Arrasate et al., 2004). The reason for this discrepancy may reside on differences

in the methodology used in both studies. In the Arrasate et al study, cultured rat striatal neurons and PC12 cells were transiently transfected with different constructs expressing the N-terminal exon 1 of huntingtin with polyQ repeats of different lengths fused to GFP. The neurons were also co-expressed with a monomeric red fluorescent protein to reveal neurons independently of the GFP constructs. The level of co-expression of the two constructs in each individual cell is not regulated thus leading to unevenness in their levels of expression from cell to cell. In addition, the cell culture model system relies on the overexpression of exogenous proteins that have different propensities to aggregate. It is difficult to predict what happens to the reserves of endogenous ubiquitin and the ubiquitination machinery, including ubiquitin ligases responsible for ubiquitinating these foreign proteins that are being overexpressed. Intracellular ubiquitin and the respective ubiquitin ligase levels, for example, may be rate limiting factors in aggregate formation if the internal regulatory mechanisms are bypassed. Taking these considerations into account makes it difficult to interpret data on aggregate formation and cell viability that rely on overexpression of foreign proteins that bypass the endogenous regulatory mechanisms. On the other hand, in our

in vivo mouse model, protein aggregates were induced by treating mice with an endogenous cytotoxin, i.e. PGJ2. It is clear that in control mice and mice infused with the lowest dose of PGJ2 (3.4µg PGJ2) the levels of ubiquitinated proteins were low and appeared dispersed, with no apparent aggregation. The absence of aggregates coincided with non-significant neuronal loss if any. Aggregates were observed only in the apoptotic neurons that were still detectable in the SNpc but that were clearly committed to the death pathway. Many of the other SNpc dopaminergic neurons were already cleared in mice treated with the highest doses of PGJ2. Therefore, it is likely that in an *in vivo* setting protein aggregates are indicative of stressed cells.

It is not surprising that we observed ubiquitin-protein aggregates in mice infused with PGJ2. Data from our laboratory and others clearly demonstrate that PGJ2 impairs the UPP. These endogenous electrophiles *(1)* inhibit ubiquitin isopeptidase activity (Mullally et al., 2001) as well as ubiquitin hydrolases UCH-L1 and UCH-L3 (Li et al., 2004b), *(2)* induce the formation of cysteine-targeted thyolation of UCH-L1 (Ishii et al., 2004) and **of** PGJ2/proteasome conjugates (Shibata et al., 2003), *(3)* trigger the oxidation of the S6 ATPase subunit of the 26S

proteasome (Ishii et al., 2004), and disrupt 26S proteasome assembly (Wang et al., 2006). All of these effects on the proteasome impair proteasome activity, which together with inhibition of deubiquitinating activity promotes the build-up of pro-apoptotic and detrimental proteins, such as p53 and ubiquitinated proteins (Uchida et al., 2007).

It is surprising however that in the SNpc of mice infused with the two highest doses of PGJ2, ubiquitin-protein aggregates were observed only in dopaminergic neurons and not in astrocytes and microglia. We show that PGJ2 induces microglia and astrocyte activation in the SNpc. Microglia activation is linked to an increase in proteasome activity, which partially explains why microglia are resistant to the large amounts of oxygen free radicals that they produce once activated (Ullrich et al., 2001). It is possible that this same phenomenon of proteasome activation protects microglia from the toxic effects of PGJ2. Interestingly, microglia activation is required for their uptake of apoptotic material which is degraded by both the proteasomal and the lysosomal pathways with the proteasome being the major player (Stolzing and Grune, 2004).

The observation that PGJ2-infusion leads to a potent activation of microglia as well as astrocytes that act as immune cells in the inflamed brain, suggests that PGJ2

could be a driving force for progressive dopaminergic neurodegeneration. Although activated microglia and astrocytes release trophic factors, most of their released products are pro-inflammatory and potently cytotoxic (Liu et al., 2003). The PGJ2-dependent glial activation may thus be instrumental in further exacerbating the neurodegenerative process observed in PD.

It is clear that PGJ2 is not the only cytotoxic agent produced by activated glia (microglia and astrocytes) as a result of the chronic inflammatory process. Other factors, such as nitric oxide, IL1β, IL6, TNFα and reactive oxygen species (e.g. superoxide anion) are also produced under conditions of inflammation. All of these cytotoxic agents must work in concert to induce synergistic neurotoxicity leading to neurodegeneration (Liu et al., 2003;Klegeris et al., 2007).

We focused on PGJ2 because it is an endogenous product of inflammation that induces pleiotropic changes that mimic many of the pathological processes observed in neurodegenerative disorders that are associated with inflammation, such as in PD. PGJ2 is derived from PGD2, the major prostaglandin produced in the CNS (Narumiya et al., 1982;Ogorochi et al., 1984). PGD2 is very short lived and readily undergoes *in vivo* and *in vitro* non-enzymatic

dehydration to generate the biologically active cyclopentenone J2 prostaglandins, which include PGJ2, Δ12-PGJ2 and 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) (Shibata et al., 2002). A recent review suggests that "formation of cyclopentenone eicosanoids [such as PGJ2] in the brain may represent a novel pathogenic mechanism that contributes to many neurodegenerative conditions (Musiek et al., 2005). Because of their high reactivity with thiol-containing intracellular compounds like glutathione or thiol-containing proteins via Michael addition, any attempt to measure PGJ2 levels in human tissues or fluids will be highly inaccurate and will not reflect its biological activity (Uchida et al., 2007). Similar to PGJ2, nitric oxide has a half life of just a few seconds, and yet is well accepted as a major signaling molecule in neurons and in the immune system (Murad, 1998). The same can be said for PGJ2, i.e. that it is a major signaling molecule/oxidative stress agent.

The toxic effect of PGJ2 is concentration-dependent. This lipid electrophile is protective at low concentrations but toxic at higher concentrations (Uchida et al., 2007). Clinical intervention to lower PGJ2 levels with agents that dampen production of PGD2, the PGJ2 precursor, might be warranted in PD. For example, the synthetic tetrazole HQL-

79 is a selective inhibitor of the hematopoietic prostaglandin D2 synthase (H-PGDS). This compound was originally prepared as a possible anti-histamine to block the inflammatory signal mediated by both PGD2 receptors, DP1 and DP2 (Aritake et al., 2006). Selective inhibitors of H-PGDS are considered to be more useful to suppress inflammatory reactions than COX-1 and COX-2 inhibitors, because they will not halt production of all PGs, including the cytoprotective and anti-inflammatory ones (Aritake et al., 2006). These inhibitors do not alter the metabolic flow within the PG cascade, thus do not change the total amount of PGs (Aritake et al., 2006). Interestingly, HQL-79 was shown to suppress astrogliosis following stab-wounding brain injury (Aritake et al., 2006), suggesting that it could be an excellent anti-inflammatory lead compound against a variety of diseases associated with inflammation including PD.

2. 5 CONCLUSIONS

These studies underscore the role of inflammation in triggering neurodegeneration. A key question that remains unanswered is whether different forms of neurodegenerative disorders, such as AD and PD, share a common mechanism, i.e. neuroinflammation. If so, how can the enormously varied

etiology, presentation and time course of these devastating disorders be explained? For example, while head injury is a rapid, accidental event, PD is characterized by damage to specific brain regions resulting in motor disturbances and chronic degeneration. This variety of disease manifestations could be correlated with the primary brain region affected by the injurious event, its severity and duration. The selective vulnerability of distinct brain areas, such as the SNpc may contribute to the inability to sustain the initial damage from a proinflammatory event. Developing an *in vivo* endogenous neurotoxin model that recapitulates both the progressive nature of PD and the pathological consequences of the disease may help address some of the many issues involved in PD, including the development of drugs that may slow or stop progression of neuronal loss.

Overall, these studies aimed to characterize the *in vivo* effects of neuroinflammation. Developing an *in vivo* model of neuroinflammation is critical to determining the players involved in the pathogenesis of neurodegeneration. Since most cases of neurodegenerative disorders are sporadic, it will be interesting to determine the effect of a number of possible conditions that may synergistically cause the onset of disease. Other players, including age-

related decline of proteolytic systems, such as the UPP and the autophagic system [reviewed in (Martinez-Vicente *et al.*, 2005;Ventruti and Cuervo, 2007)], neurovascular dysfunction [reviewed in (Iadecola and Nedergaard, 2007)], environmental toxins, oxidative stress and mitochondrial impairment [reviewed in (Beal, 2007)] in conjunction with inflammatory mediators should all be taken into consideration. Future studies with our new PGJ2 neurotoxicity model can be undertaken to evaluate other factors that may increase the vulnerability to neuronal loss and protein aggregation in Parkinson's disease.

CHAPTER III

HSP105 AS A POTENTIAL THERAPEUTIC TARGET TO PREVENT NEURODEGENERATION

3.1 PROTEIN MISFOLDING AND HEAT SHOCK PROTEINS

Conditions that are damaging to proteins in cells can trigger the increased expression of several highly conserved proteins known as heat shock or stress proteins (HSPs). HSPs act as molecular chaperones preventing untoward interactions between unfolded proteins in several major cellular processes, such as protein transport, translation and folding. Additionally, chaperones can inhibit the irreversible aggregation of denatured proteins and in some instances, function in the refolding of denatured proteins (Fig. C).

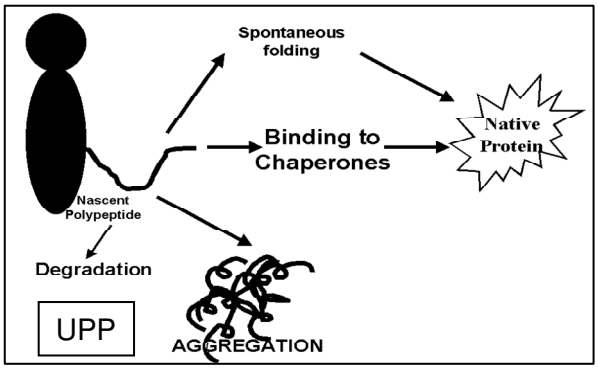


Fig. C - As a nascent polypeptide leaves the ribosome it can spontaneously fold, be degraded by the ubiquitin/proteasome pathway (UPP), or chaperones can bind to the hydrophobic regions of the polypeptide promoting its proper folding and preventing its aggregation.

Protein misfolding and a shortage of the adequate chaperones to prevent protein aggregation may play an important role in the pathogenesis of neurodegeneration. Our studies contributed to a better understanding of the function of HSP105, which due to its prevalence in the brain and its potential ability to protect neuronal cells from degeneration, may be an ideal therapeutic target for

neurodegenerative disorders associated with protein misfolding, such as PD.

In a variety of neurodegenerative disorders, such as AD and PD, aggregates of ubiquitinated proteins accumulate in neuronal inclusions [reviewed in (Figueiredo-Pereira ME and Rockwell, 2001)]. The cellular chaperone system is known to counteract the aggregation of non-native proteins, both during *de novo* folding and under conditions of stress, when some native proteins unfold (Muchowski and Wacker, 2005). Many chaperones, though constitutively expressed, are synthesized at significantly increased levels under stress conditions. A major chaperone in the mammalian brain, HSP105, is up-regulated under stress conditions, and may have an important function in counteracting the effect of stressors that cause the accumulation of ubiquitinated proteins (Saito *et al.*, 2007).

Molecular chaperones typically recognize and bind to hydrophobic amino acids exposed at the surface of unfolded polypeptides and release their substrates in a controlled manner, thereby preventing unproductive aggregation and promoting proper protein folding (Muchowski, 2002). These chaperones have been classified into several families based on their size: HSP105/HSP110, HSP 90, HSP70, HSP60, HSP40, and HSP28. Among these, the function of HSP105/HSP110

(herein designated as HSP105) is still poorly understood.

3.2 HSP105, A MAJOR STRESS PROTEIN IN THE MAMMALIAN BRAIN

HSP105 and its family members have only recently been cloned and were shown to be distant relatives of the HSP70 family (Easton *et al.*, 2000). HSP105α is a constitutively expressed 105kDa protein that is induced by a variety of stressors. A truncated version of HSP105 (HSP105β) can also exist in cells, but it is specifically induced by heat shock at 42°C. Regional and cellular localization of HSP105 in the brain using northern blot, western blot, and immunohistochemical analyses demonstrated that HSP105 is highly expressed in mouse and human brain regions with the exception of the cerebellum (Hylander *et al.*, 2000). Examination of the cerebral cortex indicated that HSP105 labeling is confined to the cytoplasm of neurons and extends into the apical dendrites of pyramidal neurons. The same widespread neuronal pattern of HSP105 expression was detected in subcortical structures such as the thalamus and the hippocampus. Neurons in other subcortical structures including the striatum and hypothalamus also show cytoplasmic localization of HSP105. The wide-spread distribution and abundance of HSP105 throughout the brain argues for an important role for this major stress protein

in the brain (Hylander *et al.*, 2000).

HSP105 cannot actively refold proteins but is able to hold thermally denatured model substrates such as luciferase in a protected state such that they can be more efficiently renatured by folding-competent chaperones such as HSP70. Because of this property, HSP105 is classified as a "holdase", similar to HSP90 and many of its co-chaperones, rather than a "foldase". Recently, overexpression of HSP105 in COS-7 cells was found to suppress cell toxicity caused by induction of the polyglutamine tract-containing truncated androgen receptor (Ishihara *et al.*, 2003). These findings are consistent with the general chaperone activity demonstrated *in vitro* but to date, no endogeneous cellular targets have been identified.

The HSP105 family is represented in *S. cerevisiae* by the Sse1 and Sse2 proteins. Like the mammalian HSP105, Sse1 acts as a holdase *in vitro*, binding to denatured substrates, preventing aggregation and enhancing renaturation by folding competent chaperones. HSP105 may act synergistically with HSP70. Co-immunoprecipitation and native gel electrophoretic approaches showed that Sse1 forms heterodimeric complexes with HSP 70 (SSA and SSB in yeast) *in vivo* and *in vitro* (Shaner *et al.*, 2005).

Furthermore, most of the cytosolic pool of yeast HSP105 (Sse) is found in complexes with either of the two major cytosolic HSP70s (SSA and SSB) (Yam *et al.*, 2005). The HSP105 yeast homolog, Sse1p was recently shown to modulate the HSP70–substrate interaction once it is established. HSP70 uses ATP binding and hydrolysis to protect polypeptides from aggregation and to facilitate their correct folding. HSP70 chaperones undergo ATP–induced conformational changes that determine their affinity for substrates. ATP bound to the N–terminal ATPase domain lowers the affinity of HSP70 for substrates. ADP release is rate–limiting for substrate release (Brehmer *et al.*, 2001). HSP105 has been reported to inhibit the ATPase activity of the nucleotide–loaded HSP70. Since nucleotide exchange (ATP hydrolysis) by HSP70 promotes polypeptide release, HSP105 may be a nucleotide exchange factor for HSP70. This plausible role as a nucleotide exchange modulator coincides with the enhanced association of nascent polypeptides with HSP70 chaperones (SSA and SSB) in Δ*sse1* cells (Yam *et al.*, 2005). HSP105 could thus act as a nucleotide exchange factor that promotes substrate release from HSP70 chaperones (Liu and Hendrickson, 2007). Additionally, HSP105 is essential for cell survival in eukaryotes.

Combined deletion in yeast cells of the SSE1 and SSE2 genes is lethal (Raviol et al, 2006).

Unlike HSP70, HSP105 is able to suppress the thermal aggregation of luciferase in the presence of ADP rather than ATP (Ishihara *et al.*, 2003). ATP is the main energy source used by cells to assume fundamental functions (e.g. respiration, proliferation, differentiation and apoptosis). However, cellular ATP levels decrease rapidly under stress conditions such as ischemia, infection and inflammation as well as heat shock. Furthermore, ATP depletion leads to the aggregation of intracellular proteins (in particular, actin cytoskeletal components), destabilization of the plasma membrane (blebbing), and necrotic cell death. Notably HSP105, but not HSP70, prevented the aggregation and/or denaturation of proteins *in vitro* and *in vivo* under ATP-depleted conditions (Ishihara *et al.*, 2003). Therefore, the HSP105 family of proteins may play important roles in protein disaggregation, substituting for the HSP70 family of proteins in cells under severe stress conditions, in which cellular ATP levels are decreased and ADP levels are increased (Ishihara *et al.*, 2003).

Consistent with the ability of HSP105 to maintain denatured proteins in a folding-competent state, HSP105 is able to suppress the formation of aggregates of mutant SOD1

in cultured cells (Yamashita *et al.*, 2007). Activation of the heat shock factor (HSF)-1, a transcription factor for heat-shock proteins, by administration of arimoclomol extended the life span of motor neuron disease mutant SOD1 (superoxide dismutase) transgenic mice (Kieran et al, 2004). Increases in the expression of agents such as HSP105 and arimoclomol that prevent protein aggregation may thus be of therapeutic value to neurodegenerative disorders such as PD.

3.3 INFLAMMATION, HSP105 AND PROTEIN AGGREGATES

Many of the neurodegenerative disorders, such as PD, which are associated with the accumulation of ubiquitinated proteins in neuronal inclusions also exhibit signs of inflammation [reviewed in (Li *et al.*, 2003)]. The relationship between the accumulation of ubiquitinated proteins and inflammation and their roles in neurodegeneration are not well defined. In these disorders, abnormal protein aggregates may, themselves, trigger the expression of inflammatory mediators, such as COX-2 [reviewed in (Wyss-Coray *et al.*, 2002)]. Inflammation is a defense reaction against diverse insults and is intended to remove damaging agents to prevent their detrimental effects [reviewed in (Wyss-Coray *et al.*, 2002)]. Postmortem studies have revealed a state of chronic inflammation in affected

regions of the brain in PD patients. Inflammation may play a deleterious role through the production of prostaglandins, such as J2 prostaglandins, that are products of COX-2 (McGeer and McGeer, 2001). Notably, J2 prostaglandins were shown to impair mitochondrial function (Martinez *et al.*, 2005), an effect that could lead to intracellular ATP-depletion. An up-regulation of HSP105 may therefore offset the effects of pro-inflammatory mediators by promoting proper protein folding and preventing protein aggregation under conditions of ATP-depletion (Fig. D).

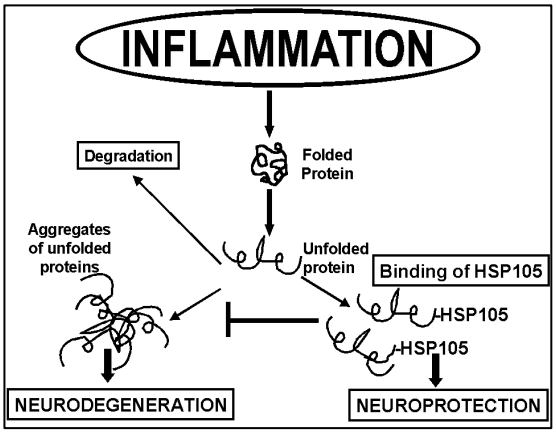


Fig. D **- Schematic indicating that under conditions of inflammation, an increase in the production of prostaglandins, in particular J2 prostaglandins, may cause folded proteins to loose their native conformation. The binding of HSP105 to the unfolded proteins may therefore, prevent the pathway leading to their aggregation and ultimately to neurodegeneration.**

Although stress proteins, such as HSP105, are up-regulated under stress conditions, they may become trapped as components of protein aggregates in neuronal inclusions and thus be in short-supply. To increase the expression of agents, such as HSP105, that prevent protein aggregation may thus be of therapeutic value to neurodegenerative

disorders. *This potential therapeutic strategy is addressed in the experiments described in this chapter.*

3.4 MATERIALS AND METHODS

Cells: SK-N-SH cells are a human neuroblastoma cell line derived from peripheral tissue (Biedler *et al.*, 1978). The cells are maintained at 37°C in MEM with Earle's salts containing 5% normal fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 0.4% MEM vitamins, 0.4% MEM nonessential amino acids, and 100 units/ml penicillin, 100 μg/ml streptomycin in 5% CO_2.

Western blotting: Following the indicated treatments, cell extracts were prepared and the proteins, 10-30 μg of protein/lane, were separated by SDS-PAGE. Identification of the proteins of interest was by western blotting and the antigens were visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent. Primary antibodies: HSP105, rabbit polyclonal, 1:1,000, from Santa Cruz; ubiquitin-conjugates, rabbit polyclonal, 1:1,500, from DAKO; HSP40, mouse monoclonal, 1:10,000, from Stressgen; Hsp70, mouse monoclonal, 1:1,000, from Stressgen; Hsp90, rabbit polyclonal, 1:2,000, Affinity Bioreagents. Secondary antibodies: donkey anti-rabbit-HRP (1:4,000, Santa Cruz) and goat anti-mouse-HRP (1:10,000, BioRad).

Semi-quantitative RT-PCR analysis: Using specific human *hsp105* PCR primers semi-quantitative RT-PCR analysis was performed to detect *hsp105* gene expression in SK-N-SH cells treated for 24h with vehicle (DMSO, control) or with increasing concentrations of PGJ2 (5 – 25μM). Total RNA was isolated with the RNAeasy Kit from Qiagen, Inc. (Valencia, CA). To perform each reverse-transcription-PCR, 1 *μ*g of RNA/sample was reverse transcribed in a 25-*μ*l reaction (RETROscript reverse transcription kit from Ambion, Austin, TX), and 2 *μ*l of the resultant cDNA was amplified with gene-specific primers using the SuperTaq polymerase kit from Ambion (Austin, TX). The human-specific PCR primers were for *hsp105:* TCAGTCCCCTCCTTCTTTACAG (forward) and AGATGCCGTAGAGATGGTGAAA (reverse) and for *gapdh: CCACCCATGGCAAATTCCATGGCA* (forward) and *TCTAGACGGCAGGTCAGGTCCACC* (reverse). All PCR products were run on 2% agarose gels and stained with ethidium bromide. PCR was performed for 5-min at 95°C, then 20 cycles of 94°C for 30s, 62°C for 60s, and 72°C for 60s with a final extension for 5-min at 72°C.

Glycerol density gradient centrifugation: Cells were lysed in 0.01M Tris-EDTA, pH 7.5 by sonication and the lysates were centrifuged for 10-min at 19,000Xg at 4°C. The cleared supernatants were subjected to centrifugation at

83,000xg for 24h in a Beckman SW41 rotor in a 10-40% glycerol gradient made in the same lysis buffer. Following centrifugation 20 fractions (500μl each) were collected and analyzed by western blotting.

Immunofluorescence: For immunofluorescence, cells were fixed in ice cold methanol:acetone (1:1) at -20°C and co-incubated with anti-Ub-conjugates (1:250, Zymed) and anti-HSP105 (1:100). The secondary antibodies (1:50) were Texas Red-labelled donkey anti-rabbit and fluorescein-labelled donkey anti-mouse (Jackson Laboratories, Inc.). Slides were mounted with Vectashield medium containing DAPI (Vector). Cell staining was always visualized with an OPTIPHOT-2 fluorescence microscope (NIKON).

Immunoprecipitation: Cells were grown to 90% confluence in four 100mm dishes/treatment. Cells were harvested in ice-cold 0.01M Tris/EDTA, pH 7.5 buffer containing the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, pepstatin A and aprotinin, to prevent unspecific proteolysis. The lysates were briefly vortexed and incubated on ice for 15-min to assure complete cell lysis. The lysates were cleared by centrifugation at 4°C and 19,000Xg for 10-min. The cleared lysates were incubated overnight at 4°C with the anti-HSP105 antibody. Protein A agarose beads were added to the lysate and incubated for

four hours at 4°C, to pull-down the HSP105-associated protein complexes. The sample pellets were washed and resuspended in Lamellae buffer after draining as much of the lysis buffer as possible and the samples were boiled for 5-min. After boiling the beads were removed by centrifugation. Eluted samples from control and PGJ2-treated cells were analyzed by SDS-PAGE followed by western blotting.

3.5 RESULTS

Effects of PGJ2 on HSP105 in human neuroblastoma SK-N-SH cells

In order to establish a relationship between PGJ2 and HSP105 we analyzed if the levels of HSP105 changed in response to increasing concentrations of PGJ2 in human SK-N-SH neuroblastoma cells (Fig 10). Our results indicate that *hsp105* mRNA levels are up-regulated in a concentration-dependent manner in response to PGJ2 treatment (Fig. 10D). The mRNA levels of the house keeping gene *gapdh* remained constant except in cells treated with the highest PGJ2-concentration at which *gapdh* levels decreased slightly. We further established that in human SK-N-SH neuroblastoma cells treated with PGJ2, the protein levels of HSP105 are significantly up-regulated in a time-

and dose-dependent manner (Fig. 10A). The HSP105 increase is dependent on *de novo* synthesis (Fig. 10C). These findings support the notion that HSP105 may have a neuroprotective role under pro-inflammatory conditions that cause an increase in the levels of ubiquitinated proteins, such as those induced by PGJ2. The levels of HSP70 and HSP90 also increased but those of HSP40 were not changed indicating that not all molecular chaperones respond in a similar manner to PGJ2-treatment (Fig. 10B).

HSP105 and HSP70 work in concert and are both highly up-regulated in response to PGJ2 treatment. This indicates that HSP105 and HSP70 may be associated under stress conditions induced by PGJ2. This premise is supported by our finding that HSP105 and HSP70 have very similar sedimentation patterns determined by a glycerol gradient sedimentation velocity analysis of SK-N-SH cell lysates treated overnight with 15μM of PGJ2 (Fig. 11).

Ubiquitinated proteins accumulate in cytosolic inclusions, such as Lewy bodies that are present in the *substantia nigra* of Parkinson's disease brains. For this reason, we investigated the distribution of ubiquitinated proteins and HSP105 in PGJ2-treated SK-N-SH cells (Fig. 12A). PGJ2-treated cells (15μM, 24h) exhibited large Ub-protein aggregates (*arrows*) detected with the anti-Ub

antibody (Zymed). In addition, immunofluorescence studies revealed that HSP105 was co-localized with the Ub-protein aggregates detected in the PGJ2-treated cells. Very low levels of ubiquitinated proteins and HSP105 were found in control, untreated cells (*not shown*).

We confirmed that HSP105 is associated with ubiquitinated proteins in PGJ2-treated cells. Normalized protein immunocomplexes obtained from pull-down assays (*IP*) with the anti-HSP105 antibody were resolved by SDS-PAGE followed by immunoblot analysis (*IB*) with the anti-ubiquitin antibody (Fig. 12B). These immunoprecipitation assays established that HSP105 is closely associated with the ubiquitinated proteins that accumulate in human neuroblastoma cells upon PGJ2 treatment.

The interaction between HSP105 and the ubiquitin-protein conjugates suggests that HSP105 may have a unique role in the brain as a neuroprotector under pro-inflammatory conditions associated with an increase in the levels of ubiquitinated proteins.

3.6 DISCUSSION

Our previous studies with neuronal cells indicate that J2 prostaglandins are neurotoxic products of inflammation that *(1)* induce the aggregation of ubiquitinated proteins

(Li *et al.*, 2004b), *(2)* inhibit the ubiquitin hydrolases UCH-L1 and UCH-L3 (Li *et al.*, 2004b) and *(3)* up-regulate cyclooxygenase-2 (COX-2), the inducible and pro-inflammatory form of cyclooxygenases (Li *et al.*, 2004a). We show now that PGJ2 up-regulates molecular chaperones, including an abundant brain chaperone, HSP105. These results indicate that J2 prostaglandins act as cellular damaging agents and may give rise to a "destructive" feedback mechanism that accelerates the neurodegenerative process, one of the devastating manifestations being ubiquitin-positive protein aggregates.

Although HSP105 levels are increased by PGJ2-treatment, this stress protein may be trapped in the inclusions and thus may be unable to protect the cells from additional protein misfolding induced by PGJ2. The sequestration of HSP105 into protein aggregates may thus lead to depletion of the available pool of HSP105. Less accessible HSP105 may contribute to the inability to maintain cellular homeostasis under stress conditions such as those induced by chronic inflammation. HSP105 may thus be a player in conditions of neuroinflammation where PGJ2 levels are greatly increased. We postulate that HSP105 overexpression prior to PGJ2-treatment may protect the cells from the neurotoxic effects of PGJ2 by preventing the

aggregation of misfolded proteins. One of the major events in PD pathology is the incidence of ubiquitin-positive neuronal aggregates. The inclusions may potentially lead to neuronal death. Thus, decreasing or preventing the formation of these aggregates may be an effective therapeutic strategy in slowing the progression of PD.

3.7 CONCLUSIONS

Our overall hypothesis is that products of inflammation including J2 prostaglandins contribute to the pathophysiological processes that underlie the degeneration of CNS neurons observed in neurodegenerative disorders such as PD.

Establishing how neuroinflammation mediates neuronal damage is crucial to developing disease prevention and treatment strategies. Increased intake of non-steroidal anti-inflammatory drugs which inhibit COX activity in all cells, is correlated with a decreased risk of developing PD (Chen *et al.*, 2003). However, inhibition of COX-1 and COX-2 have many undesirable side effects, therefore significant drug development efforts now focuses on even greater selectivity. Narrowing down molecules responsible for the deleterious effects that are caused by aberrant increased

COX-2 expression will prove valuable as prospects for therapeutic intervention.

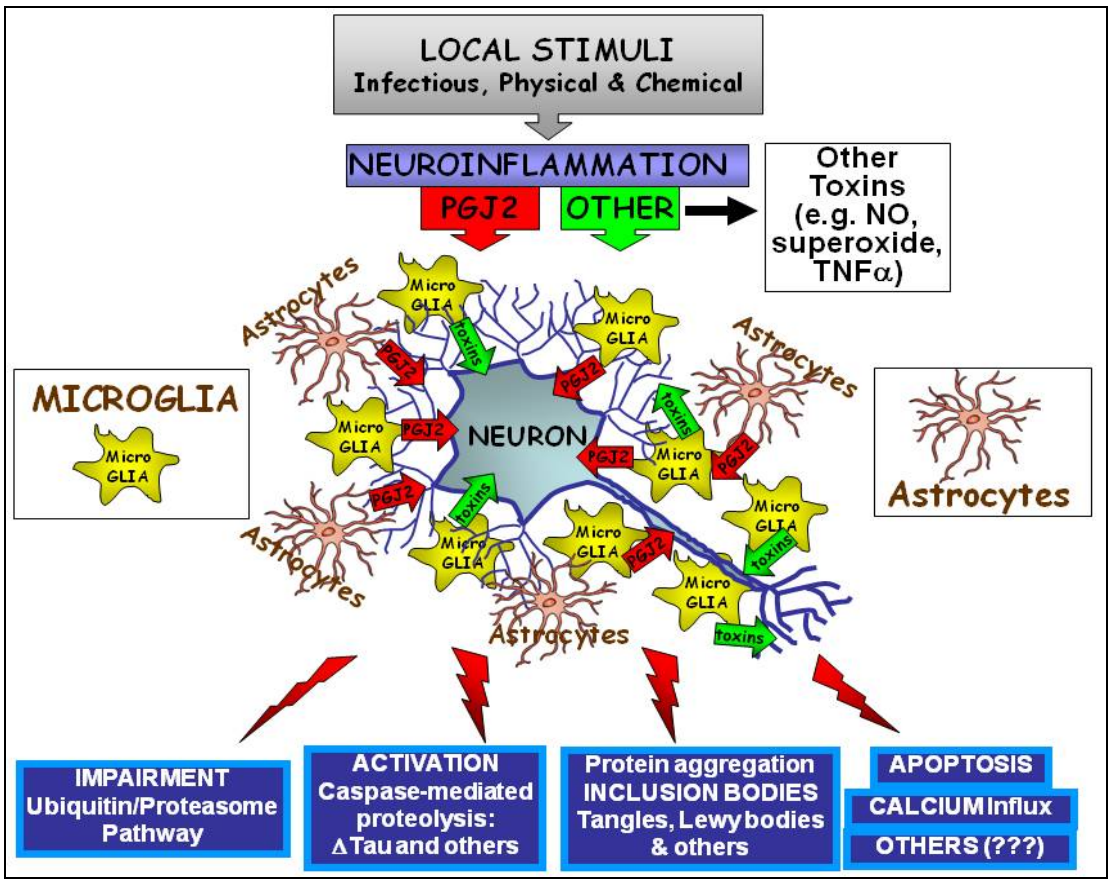
Heat shock proteins are attractive therapeutic candidates because they provide the first line of defense against misfolded proteins and probably function at the earliest stages of disease pathogenesis (Muchowski *et al.*, 2005). An exciting study by Kieran et al. showed that treatment of transgenic ALS mice with arimoclomol, a small molecule that acts as a co-inducer of the heat shock response, improved behavior phenotypes, prevented neuronal loss and extended survival rates by 22% (Kieran *et al.*, 2004). HSP105, an abundant brain chaperone is upregulated in response to PGJ2-treatment. Increasing the levels of available HSP105 may be a valid therapeutic approach for treating protein conformational disorders.

Increasing the levels of heat shock proteins, in particular HSP105 may assuage some of the protein aggregation induced in incidences of chronic neuroinflammation and mounting PGJ2 levels. Since PGJ2 is a very dynamic molecule and can exert its toxic effects in a plethora of ways, increasing chaperone availability, i.e. by arimoclomal administration, can be one weapon in the arsenal required to alleviate protein aggregation in the PD neurodegenerative process.

CHAPTER IV

MODEL AND CONCLUSIONS

OUR OVERALL MECHANISTIC MODEL



Prostaglandin J2 is clearly not the only cytotoxic agent produced by activated glia (microglia and astrocytes) as a result of the chronic inflammatory process. Other factors, such as nitric oxide, IL1β, IL6, TNFα and reactive oxygen species (e.g. superoxide anion) are also produced under conditions of inflammation. All of these cytotoxic agents must work in concert (*scheme above*) to induce synergistic neurotoxicity leading to neurodegeneration (Liu *et al.*, 2003;Klegeris *et al.*, 2007).

We propose that PGJ2 provides an optimal modeling tool to investigate the neurotoxic path by which mediators of inflammation lead to neurodegeneration because:

(1) it induces pleiotropic changes that mimic many of the pathological processes observed in neurodegenerative disorders related to inflammation, and

(2) due to its ability to up-regulate COX-2 activity, it can initiate a series of self-perpetuating deleterious cascades leading to self-sustained progressive neurodegeneration.

The **major goal** of these studies was to establish a novel progressive model of PD related to inflammation that recapitulated many of the hallmarks of PD. Indeed, due to its pleiotropic effects, PGJ2 triggers a multifactorial cascade of deleterious effects that target dopaminergic neurons and cause their demise.

Advantages of the PGJ2-induced model

1) We defined a means to deliver selective toxicity to dopaminergic neurons i.e. by direct infusion of PGJ2 into the nigrostriatal pathway. The dopaminergic neurons of the SNpc are suggested to be selectively vulnerable to the toxic actions of PGJ2 because COX-2 readily uses dopamine as an electron donor and in so doing forms the highly

cytotoxic dopamine quinone (DAQ)(Stokes *et al.*, 1999). DAQ covalently binds to intracellular proteins, including α-synuclein, leading to the accumulation of pathogenic protofibrils (Sulzer, 2001). PGJ2 activates COX-2 and drives this process (Li *et al.*, 2004a). We also found that PGJ2 potentiates dopamine toxicity in neurons by attenuating catechol-*O*-methyltransferase activity (Ogburn *et al.*, 2006). In this way, PGJ2 increases the cytoplasmic availability of dopamine which, in excess of the buffering capacity of the cytosol, will result in enhanced production of DAQ. This augmentation of dopamine-mediated toxicity raises the possibility that dopaminergic neurons might be more sensitive to PGJ2-induced toxicity than other neurons. Furthermore, PGJ2-induced activation of neurotoxic microglia (Minghetti and Levi, 1998) which are enriched in the SNpc (Kim *et al.*, 2000), may also confer selective vulnerability of this region to PGJ2.

2) PGJ2 models various mechanisms involved in PD neurodegeneration by multiple direct actions and by initiating cascades of vicious cycles of interactions between such processes. Those mechanisms include:

(a) dopaminergic degeneration in the SNpc;

(b) impairing the ubiquitin/proteasome pathway and causing the formation of intracellular aggregates of

ubiquitinated proteins;

(c) disrupting mitochondrial function, as revealed by a decrease in mitochondrial membrane potential, oxygen consumption and increased ROS production (Kondo *et al.*, 2001;Pignatelli *et al.*, 2005);

(d) potentially initiating a progressive neurodegenerative process. While COX-2 catalyses production of PGD2 and thus the production of PGJ2, PGJ2 in turn augments COX-2 activity (Li *et al.*, 2004a). PGJ2 can thus propagate a cycle of enhanced COX-2 activity and production of both itself and a multitude of prostanoids. Furthermore, COX-2 is degraded by the UPP (Mbonye *et al.*, 2008) and UPP impairment induced by PGJ2, is associated with a specific up-regulation of COX-2 activity (Rockwell *et al.*, 2000;Figueiredo-Pereira *et al.*, 2002). Thus, PGJ2 could initiate a self-perpetuating cycle in which UPP dysfunction leading to enhanced levels of COX-2, results in increased production of PGJ2, which in turn impairs UPP activity further. Such a cycle initiated by PGJ2 infusion, may result in a degenerative process progressing independently of the initiating factor as is likely to occur in idiopathic PD.

Limitations of this PGJ2-induced model

1) As with 6-hydroxydopamine, PGJ2 must be infused directly into the basal ganglia using stereotaxic surgery to induce selective neurodegeneration relevant to PD.

2) As with all toxin based models of PD some, but perhaps not all, pathological features of PD may be mirrored. For example, it is not clear whether this model will recapitulate PD losses to non-dopaminergic systems.

In conclusion, it is well established that PD is associated with multiple risk factors and that a synergy among these factors is required for the development of a progressive dopaminergic neuronal loss that is the major hallmark of PD. Because of its diverse biological effects, its ability to induce microglia and astrocyte activation and its positive feedback on COX-2 activity, we have demonstrated that PGJ2 has potential to establish a PD model with progressive loss of dopaminergic neurons.

Furthermore, we addressed the effect of PGJ2 on HSP105, a chaperone that is highly abundant in the brain. HSP105 is able to suppress protein aggregation in the absence of ATP. Since PGJ2 causes mitochondrial impairment, we postulate that up-regulation of HSP105 may offset the effects of pro-inflammatory mediators such as PGJ2, by promoting proper

protein folding and preventing protein aggregation under conditions of ATP-depletion. *In vivo* up-regulation of HSP105 may thus be an attractive therapeutic strategy to combat the proteotoxic effects of products of inflammation and prevent neurodegeneration in diseases like PD that are associated with both inflammation and protein aggregation.

CHAPTER V

FUTURE DIRECTIONS

Inflammation manifested by microglia and astrocyte activation is considered to be a major factor in PD (Liu *et al.*, 2003). Activated astrocytes and microglia make large quantities of prostaglandins such as PGE2 and PGD2 (Liu *et al.*, 2003) as well as PGJ2 (Bernardo *et al.*, 2003). Mohri and colleagues established that in culture, activated microglia produce large amounts of PGD2 synthesized by hematopoietic-prostaglandin D2 synthase (H-PGDS) increasing 23-fold following activation with a calcium ionophore (Mohri *et al.*, 2006). Furthermore, they demonstrated that H-PGDS expression was progressively upregulated in activated microglia in the mouse model of Krabbe's disease (the *twitcher* mouse) (Mohri *et al.*, 2006;Bosetti, 2007). When they knocked out H-PGDS or DP1 (a PGD2 receptor) in the *twitcher* mouse, astroglyosis was suppressed and so was demyelination, twitching and spasticity (Mohri *et al.*, 2006). This is the first example of a PGD2-mediated microglia/astrocyte interaction that enhances neuroinflammation and demyelination, and suggests that blockade of the HPGDS/PGD2 signaling pathway may be a useful strategy for other neurological conditions associated with inflammation.

Our new PGJ2-induced PD model provides an optimal tool to test different approaches to preventing PGJ2 toxicity.

We propose that in future studies two pharmacological approaches for preventing PGJ2-toxicty could be tested:

a) Inhibition of PGD2 synthase: the selective H-PGDS inhibitor HQL-79, a synthetic tetrazole compound originally prepared as a possible anti-histamine, blocks the inflammatory signal mediated by both PGD2 receptors, DP1 and DP2 (Aritake *et al.*, 2006). Selective inhibitors of H-PGDS are considered to be more useful to suppress inflammatory reactions than COX-1 and COX-2 inhibitors, because they will not halt production of all PGs, including the cytoprotective and anti-inflammatory ones (Aritake *et al.*, 2006). These inhibitors do not alter the metabolic flow within the PG cascade, thus do not change the total amount of PGs (Aritake *et al.*, 2006). Interestingly, HQL-79 was shown to suppress astrogliosis following stab-wounding brain injury, suggesting that it could be an excellent anti-inflammatory lead compound against a variety of diseases associated with inflammation including PD (Aritake *et al.*, 2006).

(b) Anti-oxidant: N-acetyl-cysteine (NAC) exhibits direct and indirect antioxidant properties (Dekhuijzen, 2004). Since PGJ2 binds to free thiols, we propose that NAC,

which binds to electrophilic groups, will prevent all of the PGJ2 effects tested in these studies, including its toxicity. NAC would exert its protective effect by acting as a scavenger for these cyclopentenone prostaglandins before they induce cell injury. NAC also increases intracellular reduced glutathione levels, which would add to its protective activity. We thus expect NAC to overcome overall PGJ2-toxicity as well as the build-up of ubiquitinated proteins.

In conclusion, these two different pharmacological approaches tested alone or in combination may prove to be useful for preventing PGJ2-toxicity and PD neurodegeration.

CHAPTER VI

FIGURES

Figure 1 (next page) – A. Schematic representation of the experimental design. Subchronic microinjections into the right *substantia nigra* and *striatum* were processed at 13, 17, 18 and 19 weeks of age. Mice (four groups) were microinjected with either DMSO/PBS (vehicle) or three concentrations of PGJ2/PBS (3.4µg, 6.7µg or 16.7µg) per injection. All animals were perfused intracardially for pathohistological examinations at 23 weeks of age, thus four weeks after the last injection. Behavioral tests were performed three weeks after the last microinjection.

B – Site of injection. Immunofluorescence analysis with anti-tyrosine hydroxylase (*red*, dopaminergic neuronal marker) and anti-IBA1 (*green*, activated microglia marker) antibodies revealed nonspecific damage at the site of injection in a mouse infused with DMSO/PBS (vehicle). The star indicates the site of injection.

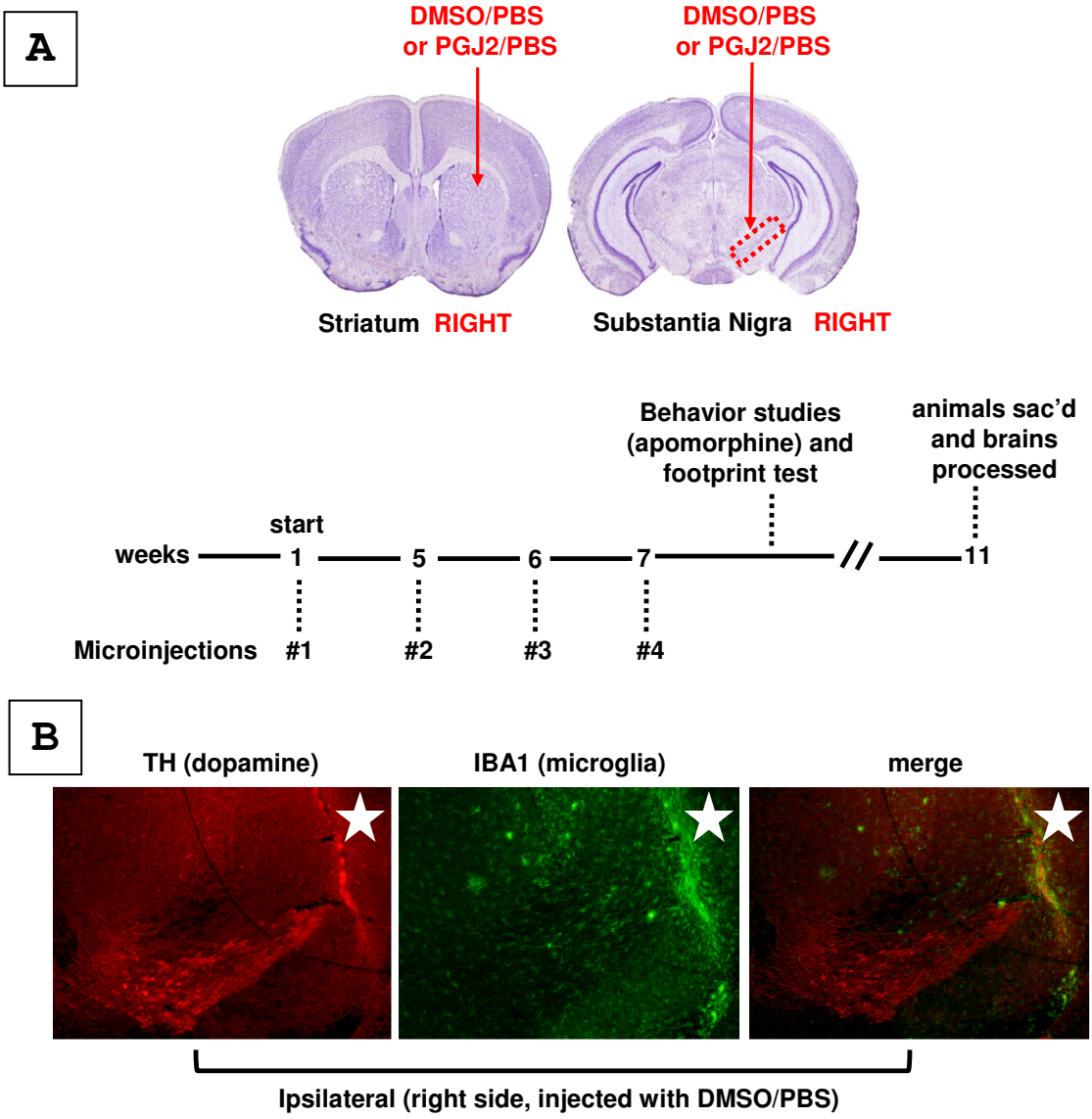

A
DMSO/PBS
or PGJ2/PBS
DMSO/PBS
or PGJ2/PBS
Striatum RIGHT
Substantia Nigra RIGHT
Behavior studies
(apomorphine) and
footprint test
animals sac'd
and brains
processed
start
weeks
1
5
6
7
11
Microinjections
#1
#2
#3
#4
B
TH (dopamine)
IBA1 (microglia)
merge
Ipsilateral (right side, injected with DMSO/PBS)

Figure 1

Figure 2 – Representative coronal sections of the ventral midbrain showing TH-immunoreactive neurons. TH-immunoreactivity was strong in VTA and SNpc of control (DMSO) mice and mice treated with 3.4µg PGJ2 (*two top panels*), but decreased in a concentration-dependent manner in the right SNpc (ipsilateral to the lesion) of mice treated with 6.7µg or 16.7µg of PGJ2 (*two bottom panels*).

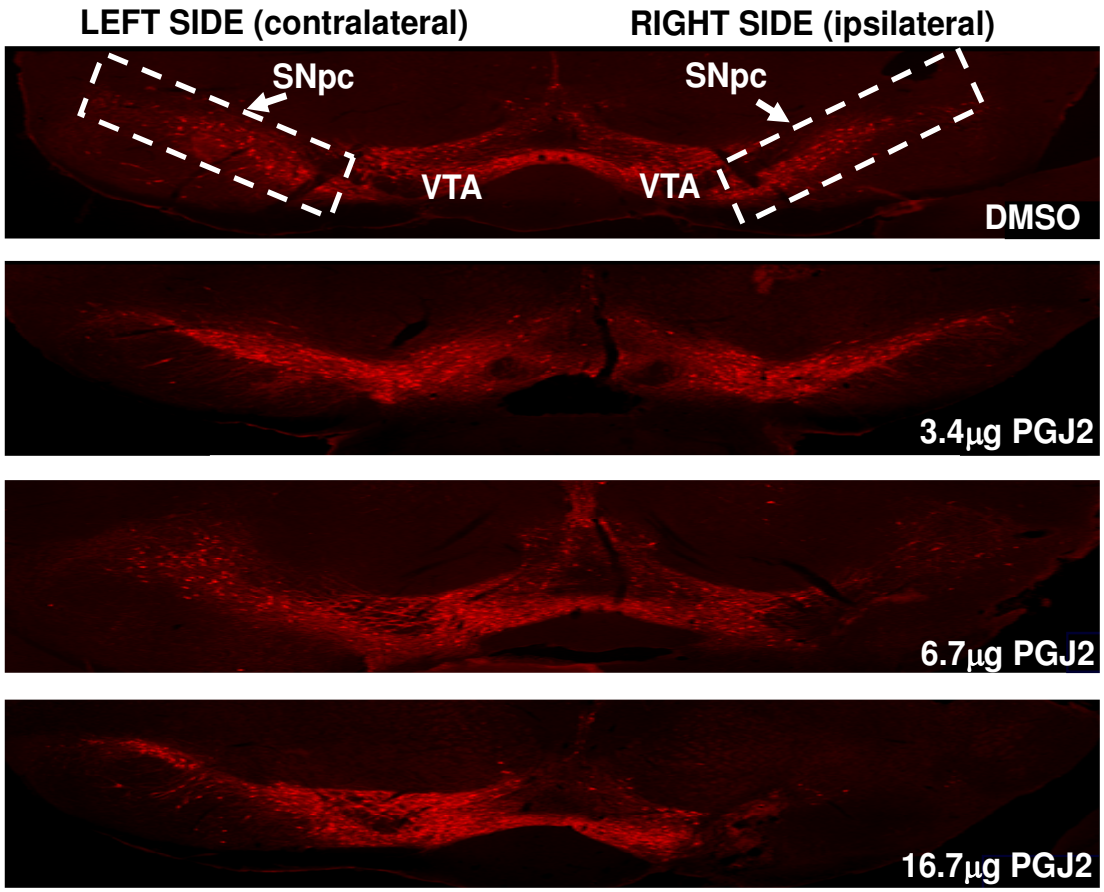
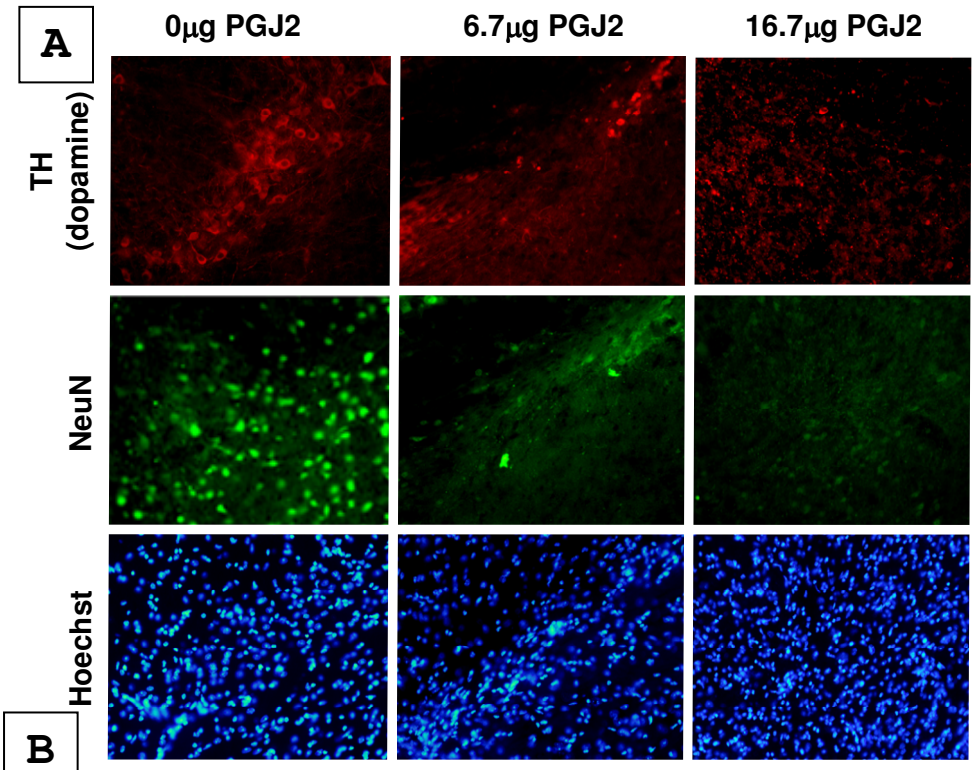


Figure 2

Figure 3 (next page) – PGJ2 dose-dependent loss of dopaminergic neurons in the SNpc. A – TH (*red*, dopaminergic) and NeuN (*green*, neuronal) immunostaining of SN shows a dose-dependent loss of dopaminergic neurons after microinjections with 6.7µg or 16.7µg of PGJ2. Nuclear staining (Hoechst) does not show a parallel loss because it stains neuronal as well as glial nuclei. **B –** Preliminary stereological neuronal counts of TH-immunopositive and total (NeuN-positive) neurons in control (DMSO) and PGJ2-treated mice were analyzed four weeks after the last microinjection. PGJ2 caused a dose-dependent reduction in TH-immunopositive and total neurons.



B

MOUSE #	TREATMENT	SLIDE #	TISSUE #	BREGMA COORD.	TH + cells	NeuN + cells
10	DMSO/PBS	I	I	-3.08	118	98
10		I	3	-3.64	127	121
10		I	5	-2.92	84	72
10		I	6	-3.16	134	89
10		2	I	-2.80	64	59
10		2	4	-3.64	114	117
				average	107	93
9	DMSO/PBS	I	4	-2.92	79	128
9		I	5	-2.92	62	112
9		2	2	-2.54	59	94
9		2	3	-3.08	102	79
9		2	4	-3.16	45	103
9		2	5	-3.28	119	49
9		2	6	-3.16	95	105
				average	80	96
22	3.4µg PGJ2/PBS	I	I	-3.08	138	48
22		I	4	-3.16	43	30
22		I	5	-3.16	181	100
22		I	6	2.92	20	26
22		I	8	-3.16	76	71
22		2	I	-3.16	27	20
22		2	2	-3.64	53	49
				average	77	49

MOUSE #	TREATMENT	SLIDE #	TISSUE #	BREGMA COORD.	TH + cells	NeuN + cells
18	6.7µg PGJ2/PBS	I	5	-3.64	51	46
18		2	2	-3.16	43	58
18		2	4	-2.80	27	13
18		2	5	-3.16	99	94
				average	55	53
3	16.7µg PGJ2/PBS	I	3	-3.16	18	21
3		I	4	-2.92	8	11
3		I	5	-3.16	3	2
3		2	1	-3.08	7	4
3		2	2	-3.16	12	10
3		2	3	-2.92	2	5
3		2	4	-3.16	8	7
3		2	5	-3.16	9	3
				average	8	8
6	16.7µg PGJ2/PBS	I	4	-2.80	11	14
6		I	5	-3.08	21	33
6		I	7	-3.16	28	34
6		I	8	-3.16	17	27
6		2	2	-3.08	6	8
6		2	3	-3.64	17	14
6		2	4	-3.16	32	30
6		2	5	-3.40	14	16
				average	18	22

Figure 3

Figure 4 – GABAergic neurons in the SNpr are spared in PGJ2-treated mice. TH (*green*, dopaminergic) and glutamic acid decarboxylase (GAD-67) staining (*red*, GABAergic) immunostaining of SN shows a dose-dependent loss of dopaminergic but not of GABAergic neurons after microinjections with 6.7µg or 16.7µg of PGJ2. The ellipse indicates the SNpr. The star indicates the site of injection.

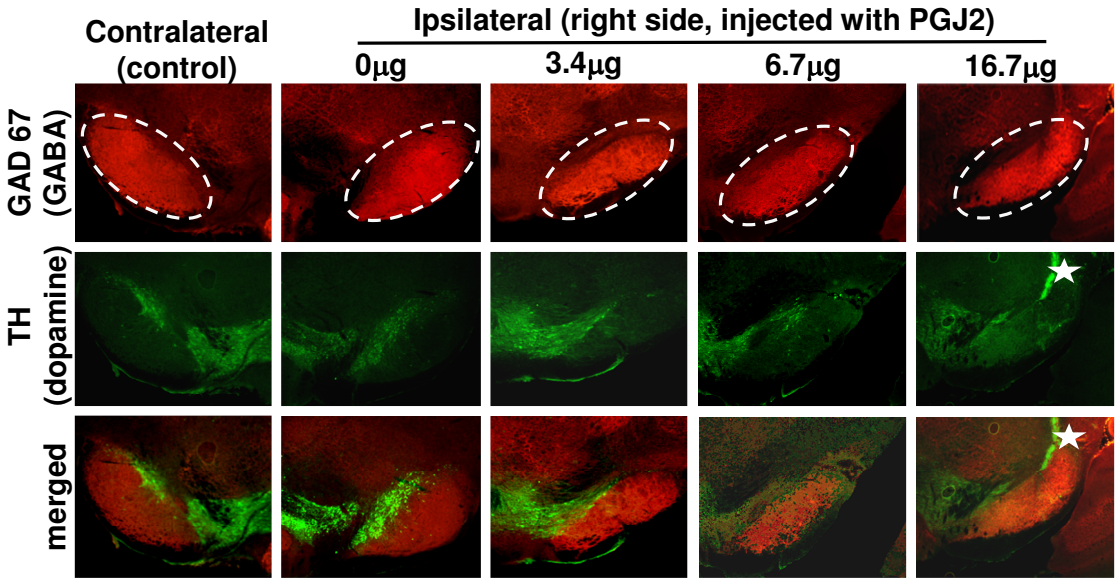


Figure 4

Figure 5 – Coronal section at the level of the midbrain SN and VTA showing the needle tract and methylene blue diffusion (A) one hour post-injection. A contiguous section was counterstained with cresyl violet (B). It is clear that one hour post-injection the dye reached both the SN *pars compacta* (SNpc) and *pars reticulata* (SNpr).

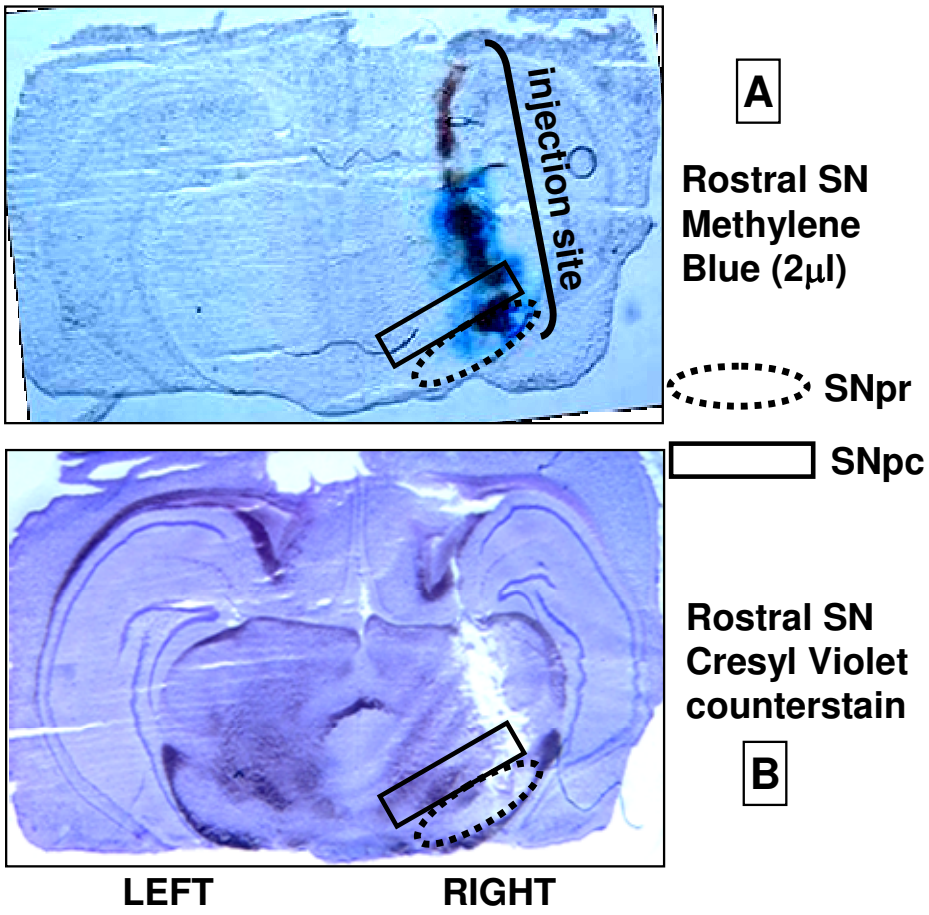


Figure 5

Figure 6 (next page) – Aggregates with ubiquitinated proteins are detected upon PGJ2 infusion. A & B – Immunostaining for ubiquitinated proteins (*red*) and TH-positive neurons (*green*) was performed four weeks after the last PGJ2 microinjection. Dopaminergic neurons in the SNpc of mice treated with 6.7µg or 16.7µg of PGJ2 exhibited clear aggregates (*arrows*) with ubiquitinated proteins. Nuclear staining (Hoechst) in the mice treated with the two highest doses of PGJ2 also exhibit an apoptotic morphology, i.e. a fragmented and/or condensed appearance (*arrowheads*). No difference was apparent between DMSO-treated mice and mice treated with the lowest PGJ2 concentration (3.4 µg). A – 10X magnification; **B –** 60X magnification.

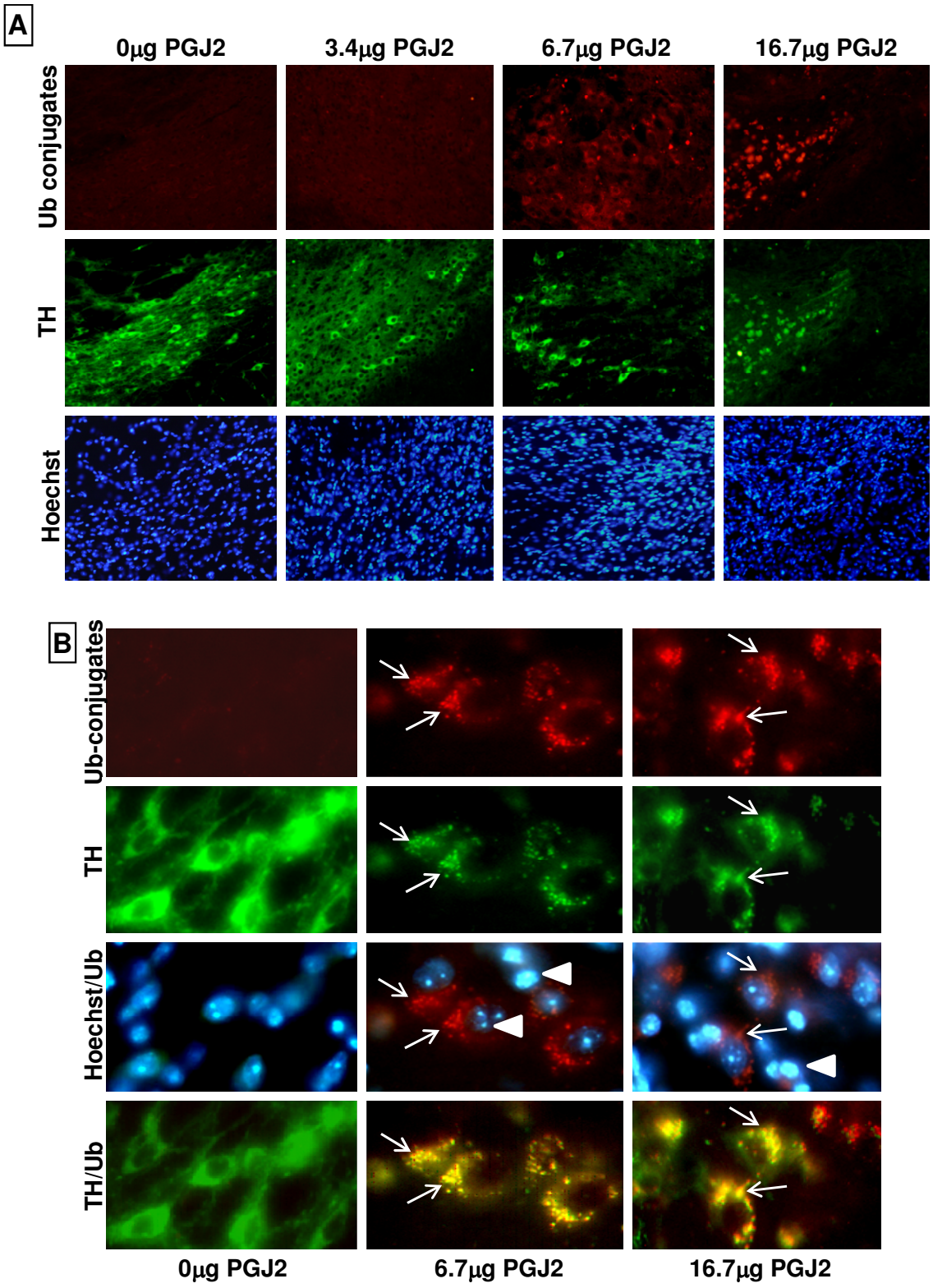

A
0µg PGJ2
3.4µg PGJ2
6.7µg PGJ2
16.7µg PGJ2
Ub conjugates
TH
Hoechst
B
Ub-conjugates
TH
Hoechst/Ub
TH/Ub
0µg PGJ2
6.7µg PGJ2
16.7µg PGJ2

Figure 6

Figure 7 – PGJ2 dose-dependent microglia activation in the SN. TH (*red*, dopaminergic) and IBA1 (*green*, activated microglia) immunostaining of SN shows a dose-dependent loss of dopaminergic neurons coinciding with microglia activation caused by treatment with the two highest PGJ2 concentrations (6.7µg or 16.7µg). No difference was apparent between DMSO-treated mice and mice treated with the lowest PGJ2 concentration (3.4 µg).

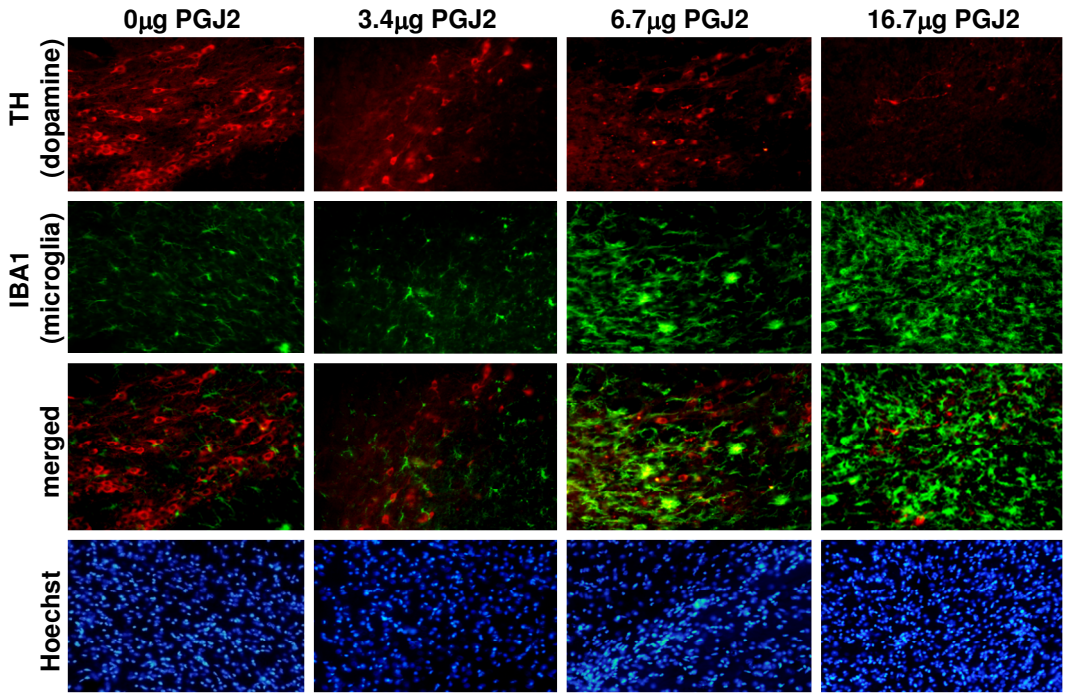


Figure 7

Figure 8 – PGJ2 dose-dependent astrocyte activation in the SN. TH (*red*, dopaminergic) and glial fibrillary acidic protein (GFAP, *green*, activated astrocytes) immunostaining of SN shows a dose-dependent loss of dopaminergic neurons concurring with astrocyte activation caused by treatment with the two highest PGJ2 concentrations (6.7µg or 16.7µg). No difference was apparent between DMSO-treated mice and mice treated with the lowest PGJ2 concentration (3.4 µg).

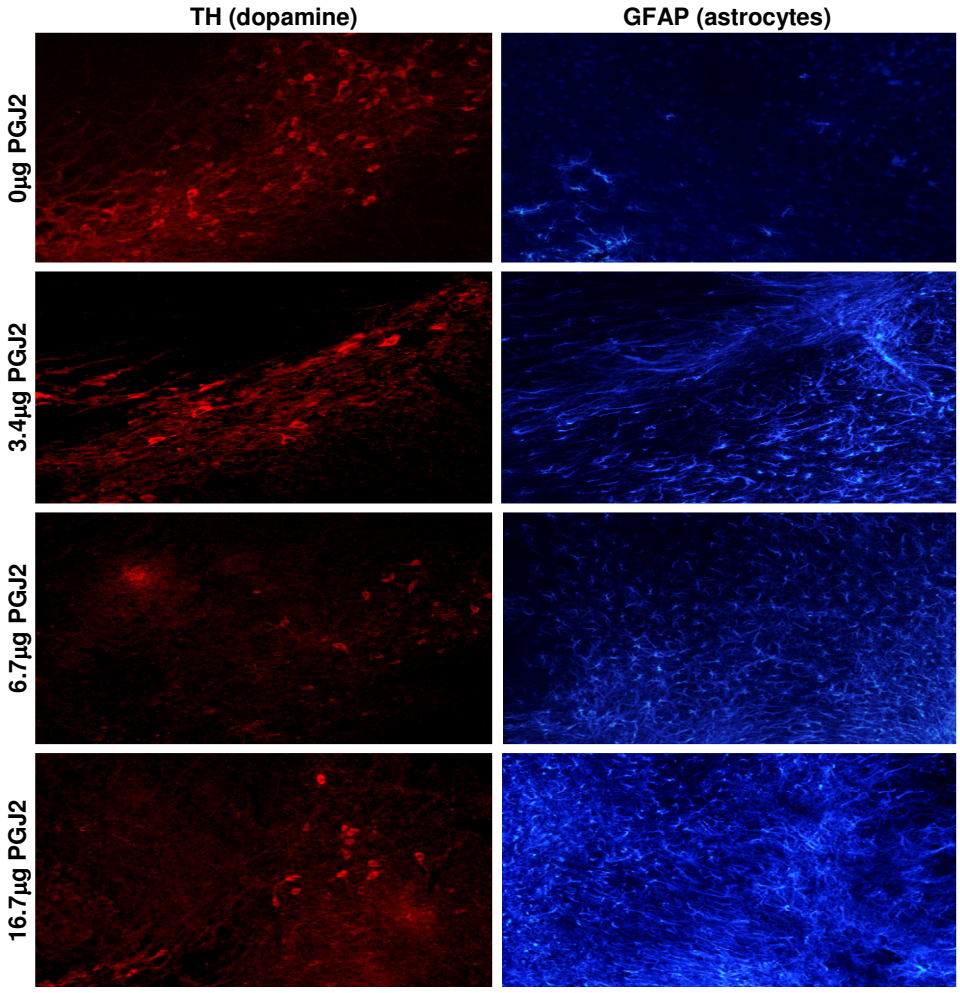


Figure 8

Figure 9 – Symptoms of motor dysfunction in mice treated with the highest PGJ2 concentration (16.7µg). PGJ2 causes severe postural instability (A) and gait impairment (B). The curling test evaluates any asymmetry in body posture. There is a severe deviation from the vertical body axis in PGJ2 treated animal. Representative walking footprint patterns display irregular step pattern and decrease in the step frequency in PGJ2 treated animal.

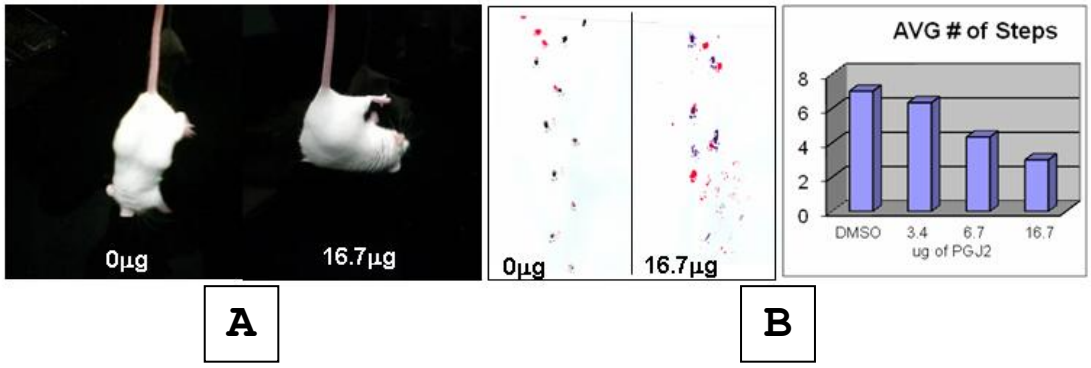


Figure 9

Figure 10 (next page) – PGJ2 up-regulates the expression of HSP105 in a dose- and time-dependent manner. SK-N-SH cells were incubated at 37°C for different times (4h, 8h, 16h and 24h) with the PGJ2-concentrations shown in the figure. Cells were harvested for western blot analyses (**A-C**) and semi-quantitative reverse transcription-PCR detection of *hsp105 and gapdh* gene expression **(D)**, as described under "Materials and Methods". Western blot analysis (30µg/lane) detected HSP105 in **(A)** and **(B)**. The blot in **(B)** was stripped and reprobed for HSP70, the levels of which also go up with increasing PGJ2 doses. The levels of HSP90 and HSP40 were not altered by the PGJ2-treatment.

(C) Up-regulation of HSP105 induced by PGJ2 is due to *de novo* synthesis. SK-N-SH cells were treated for one hour with inhibitors of transcription (actinomycin D, 5µg/ml) or translation (cycloheximide, 10µg/ml) prior to exposure to PGJ2 (15µM) for 24h.

(D) Semi-quantitative RT-PCR analysis was performed to detect *hsp105* and *gapdh* gene expression in SK-N-SH cells treated with different concentrations of PGJ2 for 24h. The PCR products for *hsp105* (1060bp) and *gapdh* (601bp) are shown.

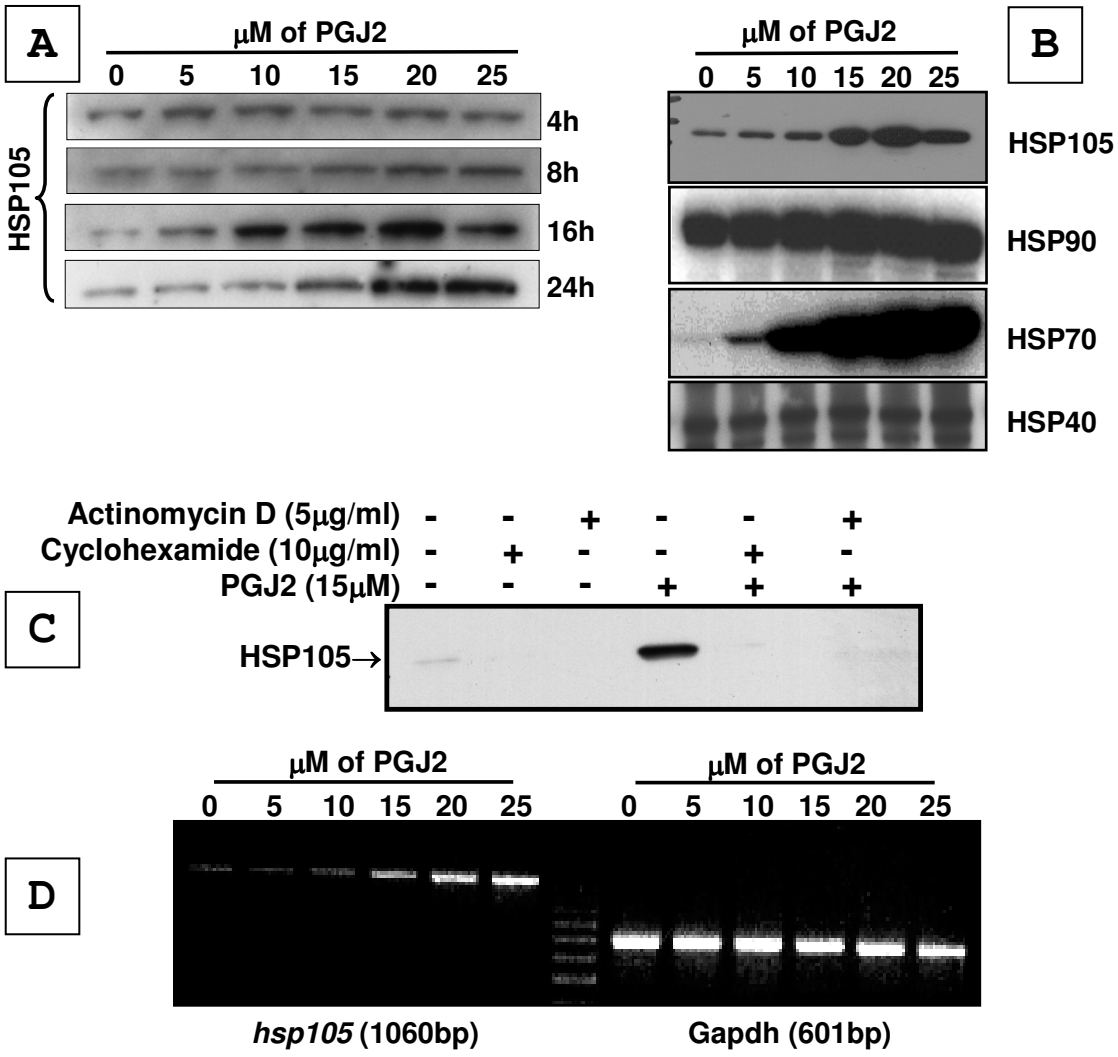

A
μM of PGJ2
0 5 10 15 20 25
HSP105
4h
8h
16h
24h
B
μM of PGJ2
0 5 10 15 20 25
HSP105
HSP90
HSP70
HSP40
C
Actinomycin D (5μg/ml) - - + - - +
Cyclohexamide (10μg/ml) - + - - + -
PGJ2 (15μM) - - - + + +
HSP105→
D
μM of PGJ2
0 5 10 15 20 25
μM of PGJ2
0 5 10 15 20 25
hsp105 (1060bp)
Gapdh (601bp)

Figure 10

Figure 11 – Sedimentation velocity analysis of cell lysates obtained from SK-N-SH cells incubated for 24h with 15µM PGJ2. The cleared supernatants (1mg of protein/sample) were fractionated by glycerol density gradient centrifugation (10-40% glycerol from fractions 1 to 19). Elution positions of the HSP105 and HSP70 are indicated. Proteins were precipitated with acetone from 300µl of each fraction obtained from cells incubated with vehicle (control, *top panels*) or with PGJ2 (*bottom panels*). Immunoblot analysis of each fraction probed for HSP105 and HSP70 established that HSP105 co-fractionates with HSP70 and that they may be associated especially upon PGJ2 treatment.

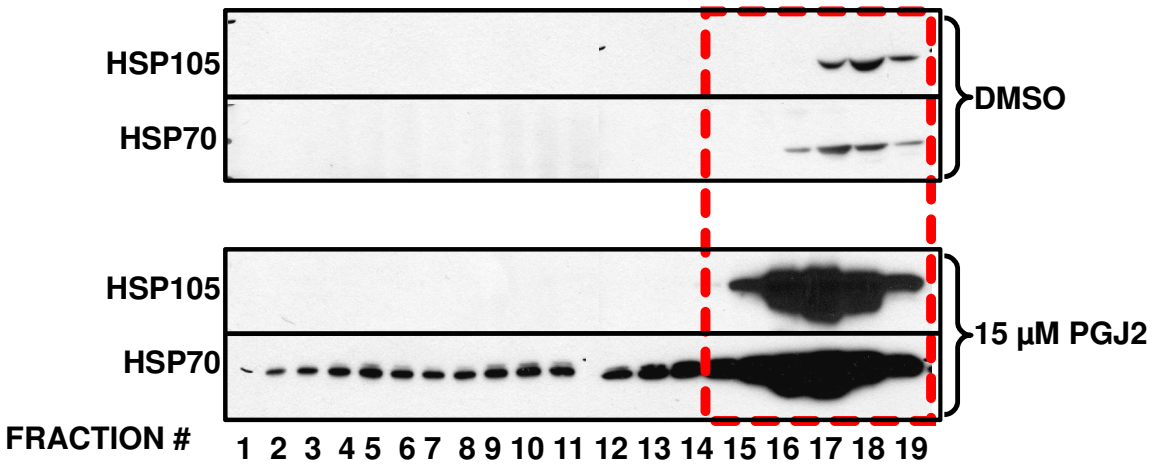
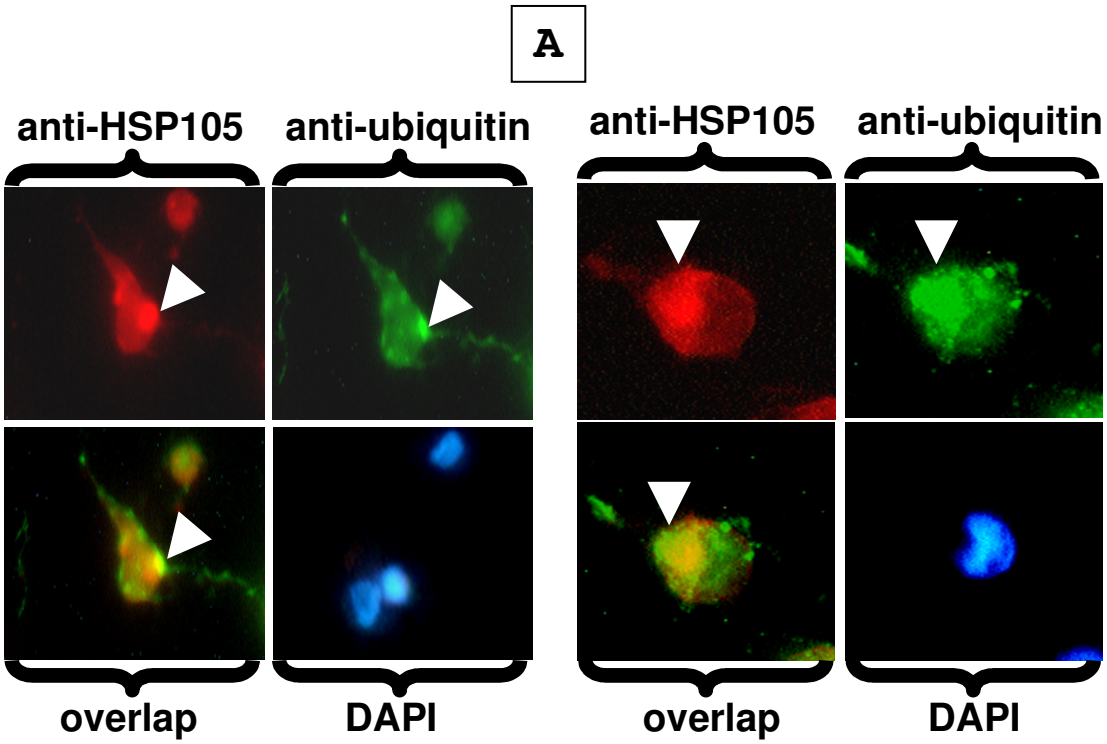


Figure 11

Figure 12 (next page) – (A)–HSP105 and ubiquitinated proteins are co–localized in aggregates in SK–N–SH cells treated with PGJ2. Double immunofluorescent staining of SK–N–SH cells treated with 15μM PGJ2 for 24h. HSP105 (*red*) and ubiquitinated proteins (*green*) are co–localized in aggregates (*arrowheads*). Overlapped images are also shown. Nuclei are stained with DAPI.

(B) HSP105 is closely associated with ubiquitinated conjugates – Immunoprecipitation of HSP105 from total SK–N–SH cell extracts. Normalized protein immunocomplexes were resolved on an 8% SDS gel, followed by Western blot analysis probed with anti–ubiquitin antibody. Cells were treated for 24h with vehicle (DMSO, lane 1) and 10μM, 15μM and 20μM PGJ2 (lanes 2–4, respectively).


A
anti-HSP105
anti-ubiquitin
anti-HSP105
anti-ubiquitin
overlap
DAPI
overlap
DAPI

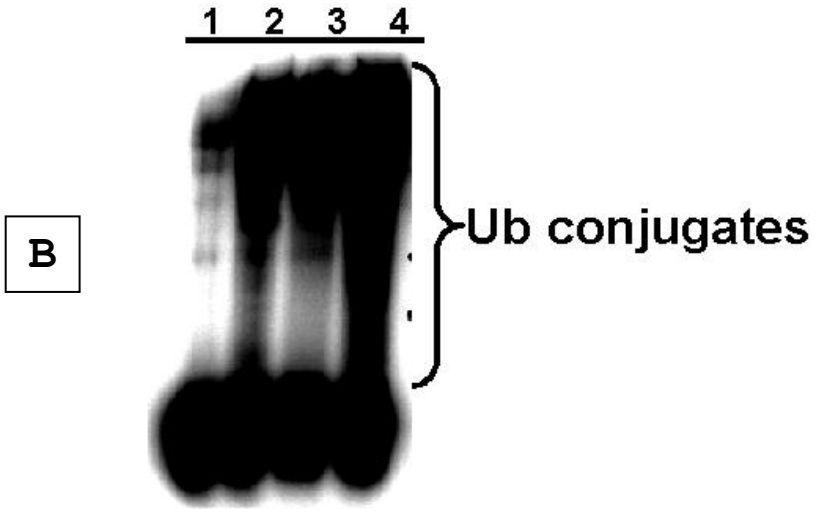

1 2 3 4
B
Ub conjugates

Figure 12

CHAPTER VII

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