Development of novel agonists for the orphan nuclear receptor, Nurr1 : a therapeutic strategy for Parkinson' s disease

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**NANYANG
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UNIVERSITY**

SINGAPORE

**DEVELOPMENT OF NOVEL AGONISTS FOR
THE ORPHAN NUCLEAR RECEPTOR, NURR1 –
A THERAPEUTIC STRATEGY FOR PARKINSON’S
DISEASE**

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**INTERDISCIPLINARY GRADUATE SCHOOL
NTU INSTITUTE FOR HEALTH TECHNOLOGIES**

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DISEASE**

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Interdisciplinary Graduate School
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A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of Doctor of Philosophy

2019

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List of Abbreviations

Abbreviation	Full Name
α -syn	Alpha Synuclein
AA	Arachidonic acid
AADC/DDC	Aromatic L-amino acid decarboxylase/DOPA decarboxylase
AF-1/2	Activation Function-1/-2
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
APC	Adenomatous Polyposis Coli
APS	Ammonium persulfate
AQ	Amodiaquine
BDC	β -catenin destruction complex
cAMP	Cyclic AMP
CNS	Central Nervous System
COX1/2	Cyclooxygenase 1/2
Cox5B	Cytochrome c oxidase subunit Vb
CQ	Chloroquine
CREB	cAMP Response Element Binding Protein
D1	Deutocerebral 1
DA	Dopaminergic
DAT	Dopamine Transporter
DBD	DNA-binding Domain
DGLA	Dihomo- γ -linolenic acid
DHA	Docosahexaenoic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
DVL	Dishevelled
FPLC	Fast Performance Liquid Chromatography
Foxa2	Foxhead box A2
FZD	Frizzled
GDNF	Glial Cell-line Derived Neurotrophic Factor

GSK3 β	Glycogen Synthase Kinase 3 β
HDAC	Histone Deacetylases
His-SUMO	Histidine-Small Ubiquitin-like Modifier
HSF	Heat Shock Transcription Factor
HSP	Heat Shock Protein
HSQC	Heteronuclear Single Quantum Correlation
IPTG	Isopropyl-beta-D-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
LAR II	Luciferase Assay Reagent II
LB	Luria-Bertani
LBP	Ligand Binding Pocket
Lmx1a	LIM homeobox transcription factor 1 alpha
LRP5/6	Low density Lipoprotein Receptor related Proteins 5 and 6
LRRK2	Leucine-rich Repeat Kinase 2
MAPK	Mitogen-Activated Protein Kinase
mDA	Midbrain Dopaminergic
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
Msx1	Msh homeobox homolog 1
NBRE	NGFI-B response element
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
NGFI-B	Nerve Growth Factor-Induced B
Ngn2	Neurogenin 2
NGS	Normal Goat Serum
NMR	Nuclear Magnetic Resonance
Nor1	Neuron-derived Orphan Receptor 1
NR	Nuclear Receptor
NRE	Nuclear Response Element
NSAIDs	Non-steroidal Anti-inflammatory Drugs
Nurr1	Nuclear Receptor Related-1
6-OHDA	6-hydroxydopamine
PAL	Protocerebral Arterial Lateral

PAM	Protocerebral Anterior Medial
PBS	Phosphate Buffered Saline
PBT	Phosphate Buffered Saline with Triton X-100
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PG	Prostaglandin
PI3K	Phosphoinositide 3-Kinase
PITX3	Paired-like Homeobox transcription factor 3
PKA	Protein Kinase A
PLA2	Phospholipase A2
PPAR	Peroxisome Proliferator-Activated Receptor
PPL	Protocerebral Posterior Lateral
PPM	Protocerebral Posterior Medial
PSF	PTB-associated Splicing Factor
RLU	Relative Light Unit
RT-qPCR	Reverse Transcription – Quantitative Polymerase Chain Reaction
RXR	Retinoid X Receptor
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SHH	Sonic Hedgehog
SMRT	Silencing Mediator of Retinoic acid and Thyroid hormone receptor
SN	Substantia Nigra
SOD1	Superoxide Dismutase 1
TH	Tyrosine Hydroxylase
TROSY	Transverse Relaxation Optimized Spectroscopy
UAS	Upstream Activator Sequence
UCH-L1	Ubiquitin carboxy-terminal hydrolase L1
VMAT2	Vesicular monoamine transporter 2
VUM	Ventral Unpaired Medial
Wnt1	Wingless-related MMTV Integration site 1
WT	Wild Type

Abstract

The increasing ageing population presages an increase in the number of cases for age-related diseases and would inadvertently place a significant burden on existing healthcare systems. One of such diseases is Parkinson's Disease (PD), the second most prevalent neurodegenerative disease among the elderly which remains incurable and current treatments only offer symptomatic relief. Pathologically, it is characterized by the demise of dopaminergic (DA) neurons in the substantia nigra region of the midbrain that results in the loss of motor functions and sometimes non-motor symptoms such as depression and dementia. The orphan nuclear receptor, Nurr1, has presented itself as an attractive drug target for PD due to its significant involvement in the development and maintenance of mid-brain DA neurons. Till date, Nurr1 remains an orphan nuclear receptor due to the lack of identified endogenous ligands. Preliminary high-throughput drug screening efforts via X-ray crystallization by our lab have revealed the binding of prostaglandins, PGA1 and PGA2 with Nurr1 ligand binding domain (LBD) forming a covalent Michael adduct with Cys566 sidechain sulphur atom in Nurr1 LBD. This is accompanied by the re-orientation of the helix H12, with the AF-2 transactivation domain, by $\sim 21^\circ$ in comparison to the apo structure. In this thesis, the binding site of these prostaglandins, and their precursors PGE1 and PGE2, was characterized on Nurr1 LBD using nuclear magnetic resonance (NMR) spectroscopy. All four prostaglandins induced perturbations in residues, predominantly around helices H4, H11 and H12. The conformational changes observed in helix H12 from our NMR data was consistent with the reorientation of helix H12 observed in the co-crystal structure of Nurr1 LBD. More importantly, the data validated that both PGE1/2 and PGA1/2 bind to the same site, which could not be revealed by crystallography. The binding of PGA2/PGE2 also increased the binding affinity of AQ to the LBD, suggesting possible allosteric regulation of the putative AQ binding site. Functionally, the prostaglandins were able to activate the transcriptional function of Nurr1 and were more potent than AQ, as shown in a luciferase reporter assay using SK-N-BE(2)C neuroblastoma cells. Using an *in vivo* transgenic fly model expressing LRRK2 G2019S exhibiting late-onset PD, these prostaglandins also exhibited the ability to rescue the locomotor deficits of these flies. Additional studies on PGA1 and PGA2 showed that they were able to mitigate LRRK2 G2019S-induced neurodegeneration and tyrosine hydroxylase positive (TH⁺) neuronal

loss and increase the mRNA expression of *Dhr38* (Nurr1 ortholog). Furthermore, PGA1 treatment significantly improved motor impairments and reduced lesions in the striatum and substantia nigra in the 6-hydroxydopamine (6-OHDA) neurotoxin mouse model of PD. The discovery that these prostaglandins could interact with Nurr1 LBD and activate its function, lay the groundwork for future development of neuroprotective and disease-modifying therapies for PD that should help slow down further progression of the disease.

Chapter 1: Introduction

1. Introduction

1.1 Parkinson's Disease – The past, present and future

“As slow as is the progress of the disease, so slow in all probability must be the period of the return to health.” -James Parkinson, An Essay on the Shaking Palsy, 1817

This poignant statement from James Parkinson in his historical account of “Shaking Palsy”, is a stark reminder of the degenerative and incurable condition subsequently named after him as Parkinson's Disease (PD) by renowned neurologist, Jean-Martin Charcot in the 19th century (Parkinson, 1817, Charcot, 1872). Prior to this seminal period, reports of PD were already scattered throughout history, traced as far back as 1000 BC in traditional Indian and Chinese texts (Zhang et al., 2006, Manyam, 1990) to the 17th century where resting tremor and gait were similarly described (Sylvius de la Boe, 1680, Sauvages, 1763). Parkinson also described the disease in his time as “the irremediable diminution of the nervous influence, naturally resulting from declining life; and remedies therefore are seldom sought for.” (Parkinson, 1817). One can only imagine the helplessness of sufferers especially during an era when medical treatments were not as advanced. Nevertheless, two centuries later, this devastating condition remains to be cured and current treatments only serve to alleviate symptoms which mainly affect motor functions.

PD is a movement disorder caused by the deficit of dopaminergic (DA) neurons chiefly in the substantia nigra (SN) pars compact region of the midbrain. Given the length of time for its development, its target is primarily the elderly over the age of 65, although it may also strike at any age (Fahn, 2008). Around 90 % of cases are sporadic, with 10 % accounted for by hereditary cases (Michel et al., 2016). A significant pathological hallmark of PD, besides the demise of DA neurons in the SN region of the brain, is the presence of Lewy bodies containing aggregated alpha synuclein (α -syn) proteins, other proteins and lipids in surviving neurons. Aggregated α -syn in their oligomeric and fibrillary forms (Masliah et al., 2000, Kirik et al., 2003) can damage DA neurons by interfering with oxidative stress protective mechanisms and mitochondrial integrity resulting in further damage to protein

homeostasis and synaptic transmission networks. As a neurodegenerative disease, the progressive loss of dopamine signalling leads to classical debilitating symptoms such as slowness in movement or bradykinesia, resting tremor and rigidity which progresses to freezing of gait and flexed posture later on (Fahn, 2008). During the course of the disease, non-motor symptoms may also be present such as dementia, depression and sleep disturbances (Fahn, 2008).

Being afflicted with mobility impairments and decreased mental abilities severely disrupts the quality of life for PD patients, particularly so when they might also suffer from other comorbidities. For now, early symptoms can be controlled through medications but would be difficult to control as the disease progresses. Regrettably, striatal DA neurons would have undergone severe damage by the time symptoms manifest, thereby making early detection and diagnosis a challenge.

Pivotal breakthroughs in PD therapeutics were made in the late 1950s when Arvid Carlsson and his team confirmed the presence of dopamine in rabbit brains and that reserpine depleted dopamine which can be rescued by L-DOPA treatment (Carlsson et al., 1958, Carlsson et al., 1957). Subsequently, Carlsson's students —Bertler and Rosengren — and a research group in Japan have also detected the presence of dopamine in human brain striatum (Bertler and Rosengren, 1959, Sano et al., 1959), strengthening the hypothesis that this new transmitter possessed an important function in motor control (Carlsson, 1959). Oleh Hornykiewicz, motivated by these findings, worked together with Herbert Ehringer and did studies on post-mortem human brains which showed a consistent lack of dopamine in the striatum of PD and post-encephalitic brains (Ehringer and Hornykiewicz, 1960). The first clinical trial of L-DOPA in humans was launched swiftly to which Hornykiewicz described the effects following L-DOPA injections as “spectacular”, seeing as patients were able to regain control of their motor function for a short amount of time that was never observed with any other treatment (Birkmayer and Hornykiewicz, 1961, Hornykiewicz, 2002). Another independent group in Montreal also performed human clinical trials by giving small L-DOPA doses orally and showed positive effects on patients (Barbeau et al., 1962, Hornykiewicz, 2002). It was only in 1967 that George Cotzias managed to develop an effective chronic, high-dose oral D,L-DOPA treatment still used today (Cotzias et al., 1967, Cotzias et al., 1969) although Yahr and group later established L-DOPA's superiority

in the first ever double-blind study (Yahr et al., 1969, Hornykiewicz, 2002). Nevertheless, their role in mitigating PD symptoms have proved to be indispensable.

60 years on, L-DOPA has remained the gold standard drug for treating PD due to its status as a dopamine precursor and the ability to cross the blood-brain barrier. A caveat that comes with the use of L-DOPA however, is that long-term administration might lead to further complications in motor functions and decreased efficacy (Lesser et al., 1979, Sweet and McDowell, 1975). Furthermore, peripheral side effects such as nausea and hypotension might also accompany L-DOPA treatment although it is currently managed by co-administration with Carbidopa, a decarboxylase inhibitor that prevents peripheral conversion of L-DOPA to dopamine (Muller et al., 2006). Other drugs are also available such as dopamine agonists which acts to activate dopamine receptors and monoamine oxidase B (MAO-B) inhibitors that serve to block dopamine breakdown (Collins et al., 2015). For advanced stage PD or when oral treatment becomes ineffective, deep brain stimulation is often contemplated if the risk from surgery is deemed relative low for the elderly patients (Collins et al., 2015). These treatments are so far only able to provide symptomatic relief, and there is still a need to develop neuroprotective and disease-modifying therapeutics for PD to slow down further damage to existing neuronal architectures. Recent reports have offered hopes to this predicament such as the development of a lead molecule named BRF110 that could activate Nurr1-RXR α heterodimer and exert both neuroprotective effects and symptomatic relief in neurons and preclinical mice models which could be used as a monotherapy in the future (Spathis et al., 2017). The implantation of DA neurons from induced pluripotent stem (iPS) cells derived from healthy people into PD patients is also a promising option as a report last year revealed positive results from a trial conducted in monkeys that lasted for two years (Kikuchi et al., 2017). The team led by Jun Takahashi, has also announced the launch of human clinical trials just recently (Normile, 2018).

The etiology of PD remains largely unknown but has been suggested to be caused by a complex interplay of environmental and genetic factors. Environmental factors such as chemical exposure to neurotoxins such as the street-drug MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), rotenone (insecticide) and paraquat (herbicide) was shown to affect mitochondrial function and induce oxidative stress leading to midbrain DA (mDA) degeneration (Langston et al., 1983, Hantraye et al., 1996, Henchcliffe and Beal, 2008, PhD thesis, IGS, NTU

Sherer et al., 2003). Genetic causes of PD account for about 5-10 % of cases and patients often develop the disease before they turn 50 (Mizuno et al., 2000, Van Den Eeden et al., 2003). Studies on the genetic causes of PD have contributed to our knowledge on the pathogenic mechanisms of PD as we examine the pathways disrupted by these mutations.

SNCA that encodes α -syn was the first gene associated with familial forms of PD (Polymeropoulos et al., 1997) and although it is a rare cause of PD, an increase in α -syn expression or in the mutant form is believed to result in a higher tendency for its aggregation and formation of higher-order amyloid fibrils (Savitt et al., 2006, Skovronsky et al., 2006). Its mutant A53T for instance, showed more rapid formation of fibrils compared to the wild-type (WT) form of α -syn (Conway et al., 1998, Krishnan et al., 2003). The autosomal dominant *PARK8* which encodes Leucine-Rich Repeat Kinase 2 is reported to account for most of the familial cases of PD (5-13 %) and also for sporadic PD (1-5 %)(Kumari and Tan, 2009). Out of the 20 mutations in the gene, G2019S is the most common mutation with an occurrence of 5-6 % in familial cases and 1-2 % in sporadic cases (Bardien et al., 2011). LRRK2 as a kinase, functions to phosphorylate other proteins of which this and its GTP-binding function has been suggested to be augmented in LRRK2 mutant forms (West et al., 2007). Most importantly, LRRK2 associated late-onset PD results in similar clinical pathologies as sporadic PD (Paisan-Ruiz et al., 2004, Zimprich et al., 2004) and explorations into LRRK2 associated mechanisms should therefore provide insights into the pathogenesis of PD. Genes that are autosomal-recessive leads to the loss-of-function of their encoded proteins such as *Parkin* (which leads to early-onset PD), *PINK1* (PTEN-induced putative kinase) and *DJ-1* genes. Collectively, these three proteins are important for the proper functioning of mitochondrial and oxidative stress regulatory pathways and their loss-of-function was shown to disrupt parts of these pathways leading to cellular damage (Mata et al., 2004, Clark et al., 2006, Gautier et al., 2008, Irrcher et al., 2010). Thus, the study of genetic mutations of PD in experimental models have allowed us to further connect these pathologies to the disease progression of PD.

1.2 The role of Nuclear Receptor Related-1 Protein in PD pathogenesis

As discussed above, the presence of aggregated α -syn in Lewy bodies is detrimental to the remaining lifespan of surviving neurons by interfering with critical cellular and molecular pathways. Experimental studies in rodent models and post-mortem examination of human PD brains have also associated the overexpression of α -syn with altered expression of important transcription factors involved in neuronal survival and maintenance (Chu et al., 2011, Desplats et al., 2009, Lin et al., 2012).

One very important transcription factor affected by α -syn overexpression is the Nuclear Receptor Related-1 (Nurr1) protein possessing critical roles in maintaining DA neuronal survival. In 2012, two different groups showed in experimental rodent models that α -syn overexpression resulted in a decrease in both Nurr1 mRNA and protein expression (Decressac et al., 2012, Lin et al., 2012). Lin and colleagues (Lin et al., 2012) found that when WT and A53T mutant α -syn was overexpressed in mice mDA neurons, a proteasome-dependent degradation of Nurr1 was induced which when inhibited could mitigate the loss of mDA neurons caused by α -syn overexpression. In the other study involving the viral vector delivery of human WT α -syn to nigral DA neurons in adult rats (Decressac et al., 2012), the expression of Nurr1 was found to be reduced which resulted in the disruption of one of its downstream target, Ret, a tyrosine-protein kinase receptor important for recognizing glial cell-line derived neurotrophic factor (GDNF), for protecting and preserving proper DA neuronal function. Disruption to the GDNF signalling pathway rendered the neurons more susceptible to α -syn related toxicity. Interestingly, Nurr1 overexpression was able to revive the intrinsic GDNF signalling and subsequent neuroprotective effect (Decressac et al., 2012) hinting that targeting Nurr1 activation is a viable option as a neuroprotective therapy for PD.

Nurr1 belongs to the nuclear receptor (NR) family of proteins, of which 49 are currently known in humans. NRs play vital roles in the transmission of extracellular signals to regulate the expression of genes and proteins involved in homeostasis, metabolism and inflammation. NRs are usually ligand-regulated and may be broadly classified into three categories: the class I receptors such as estrogen receptor and progesterone receptor that

bind as homodimers to their DNA recognition sequences and are transcriptionally inactive in the absence of ligands; the class II receptors such as thyroid hormone receptor and peroxisome proliferator-activated receptor (PPAR) bind as heterodimers with retinoid X receptor (RXR) and are able to repress basal promoter activity that can be reversed upon ligand binding; class III NRs are the orphan NRs, that Nurr1 is a part of, categorized based on the lack of known endogenous ligands at the point of discovery (Zaveri and Murphy, 2007). The orphan NRs may bind as monomers, homodimers or heterodimers to their NR response elements (NREs). Under the orphan NRs family tree, Nurr1 (nuclear receptor subfamily 4, group A, member 2; NR4A2) belongs to the NR4A subfamily that are structurally related, with two other members known as nerve growth factor-induced B (NGFI-B) or Nur77 (NR4A1) and neuron-derived orphan receptor 1 or Nor1 (NR4A3) (Kurakula et al., 2014).

Structural biology plays a fundamental role in understanding the molecular mechanism of the transcriptional activation of a NR, especially in the presence of its agonist. The structure of Nurr1 is organized into 5-6 regions, designated A-F from the N- to C- terminal (Figure 1.1) and can be functionally characterized by three domains that follows that of classical NRs. The N-terminal contains a transactivation domain called the activation function -1 (AF-1) domain (A/B) that takes part in ligand-independent transcriptional activity (Zaveri and Murphy, 2007). The DNA-binding domain (DBD; C) is located after the AF-1 domain and consists of two zinc finger motifs that can recognize NREs such as NGFI-B response element (NBRE) as monomers or homodimers (Paulsen et al., 1995, Maira et al., 1999); Nur-response element (NurRE) as homodimers or NR4A heterodimers (Forman and Evans, 1995); and also to DR5 motif with other NRs such as RXR (Perlmann and Jansson, 1995, Zetterstrom et al., 1996a). The C-terminal harbours the ligand-binding domain (LBD; E/F) that also contains the ligand-dependent activation function-2 (AF-2) domain. The DBD and LBD of members in the NR4A subfamily are highly-conserved (~95 % and 70 % homology respectively) while their N-terminal domains are highly variable with only ~30 % homology (Kurakula et al., 2014).

Figure 1.1 Structural organization of classical nuclear receptors

The typical primary structure is made up of 5-6 regions labeled from A-F. Regions A, B and D are highly variable regions while regions C and E/F which are the DBD and LBD, respectively, are highly conserved. The tertiary structure of the DBD usually consists of two zinc finger motifs that can recognize specific nuclear response elements. The tertiary structure of LBD is normally made up of 10-12 α -helices with a few short β -strands. Image adapted from Nuclear Receptor Resource (Heuvel, 2009).

The crystal structure of Nurr1 LBD (PDB: 1OVL) was solved in 2003 by Wang and colleagues (Wang et al., 2003) and they reported that the folded tertiary structure of Nurr1 LBD was in the active conformation in the absence of any ligand and this conformation resembled that of other ligand-bound NR LBDs. Its tertiary structure is typical of NR LBDs containing 12 antiparallel α -helices (H1-H12), two short strands of β -sheets (S1-S2 between H5-H6) with a few loop regions, all arranged in a three-layered sandwich (Germain et al., 2006) (Figure 1.2, Left). The putative ligand binding pocket (LBP) was shown to possess several hydrophobic and bulky residues bordered mainly by H3, H7, H10-H12 (Figure 1.2, Right). Most importantly, intramolecular interactions between Phe592 (H12) and Phe574 (H11) and a salt bridge between Lys577 (H11) and Asp589 (H12) was shown to be critical for maintaining the AF-2 helix in a transcriptionally active and stable conformation in the absence of a ligand (Wang et al., 2003). They also showed that the putative co-activator surface in classical NRs is occluded by the presence of van der Waals contacts between Arg418 and Phe439 preventing co-regulator interaction (Wang et al., 2003). This supposed canonical hydrophobic co-activator cleft (surface formed by H3-4 and H12) that can bind

LXXLL-containing co-activators was also shown to be hydrophilic rather than hydrophobic, backed by the lack of discovered co-activators (Wang et al., 2003). The “charge clamp” typically formed between a Glu residue from H12 and Lys residue from H3 was also shown to be reversed as shown by the presence of Lys590 from H12 and Glu422 from H3 although in this case, Lys590 is involved in a salt bridge with Glu440 from H4 and is not able to form the charge clamp (Wang et al., 2003). The authors have thus described Nurr1 as lacking the ability to recruit or interact with a coregulator through the canonical co-activator surface (Wang et al., 2003). However, the mutation of a few key residues located at H11 and H12 led to the impairment of transcriptional activity suggesting a possible co-activator surface at this region (Volakakis et al., 2006). Their findings further supported those from an earlier study which showed that replacing H11-H12 region of Nurr1 LBD with Nur77 LBD and mutations of hydrophobic surface residues in the H11-H12 region significantly affected the transcriptional function of Nurr1 (Flaig et al., 2005). As such, the identification of this novel co-activator surface is encouraging and should aid the subsequent identification of co-activators that can bind to Nurr1 LBD.

A recent paper by de Vera and colleagues demonstrated using solution NMR and hydrogen/deuterium exchange mass spectrometry (HDX-MS) that the canonical Nurr1 LBP is dynamic in solution and can exist between two or more conformations (de Vera et al., 2019). The conformational change induced following the binding of unsaturated fatty acids also increased the solvent accessibility of the LBP, allowing the physical expansion of the LBP to accommodate the ligand, similar to other NRs. This was further supported by molecular dynamics simulations, comparing the bound Nurr1 LBD structure with other ligand-binding NRs (de Vera et al., 2019). The estrogen-related receptor (ERR), for instance, possesses LBP volumes spanning a range of 40-1020 Å³ as shown in ERR crystal structures (Gallastegui et al., 2015). REV-ERB β , formerly an orphan NR, also previously showed a collapsed LBP from the crystal structure of the apo protein (Woo et al., 2007) but was later discovered to have expanded by 600 Å³ in the co-crystal structure with porphyrin heme (Pardee et al., 2009). The porphyrin heme was subsequently identified as the endogenous ligand for REV-ERB receptors, and they were therefore “de-orphanized” (Raghuram et al., 2007, Yin et al., 2007). NMR studies further supported that the LBP of the apo-REV-ERB β is dynamic (Matta-Camacho et al., 2014) and can expand to accommodate ligand binding. As such, it is possible that despite Nurr1 LBD showing a

collapsed LBP in its crystal structure (Wang et al., 2003), it could in fact be more dynamic than previously believed (de Vera et al., 2019), and allow the binding of an endogenous ligand.

Figure 1.2 Cartoon representation of Nurr1 LBD crystal structure and the LBP

Left: The crystal structure of Nurr1 LBD is shown (PDB: 1OVL) and its tertiary structure consists of a three-layered sandwich made up of antiparallel α -helices from H1-H12, a short β strand with two β -sheets labeled as S1 and S2. H1-H3 forms the first layer while H4-H9 constitutes the middle layer and H6-H12 makes up the third layer of the sandwich. H12 is also known as the AF-2 helix due to the involvement of many residues in this helix on the AF-2 function for transcriptional activation. The canonical hydrophobic co-activator surface covered by H3-4 and H12 is replaced with a hydrophilic surface instead and a novel co-activator surface was localized at the surface covered by H11-H12 above the LBP. **Right:** The putative LBP was shown to be occupied by several bulky and/or hydrophobic residues that occlude the binding of a ligand.

Nurr1 was first identified in 1992 and showed high expression in the brain (Law et al., 1992). Its expression was found to start as early as embryonic day 10 in the ventral midbrain of mice and is continually implicated in the development and differentiation of mDA neurons throughout the survival of adult neurons (Zetterstrom et al., 1996b, Zetterstrom et al., 1997). The mDA neurons are distributed into three discrete regions, namely the ventral tegmental area (VTA; A10 nucleus), substantia nigra pars compacta (SNpc; A9 nucleus) which is affected in PD, and retrorubral field (RRF; A8 nucleus) (Hong et al., 2014). The development of mDA neurons starts from the neural progenitor (NP) cells that goes through developmental phases carefully regulated by two major pathways, namely the SHH-Foxa2 (Sonic hedgehog-Foxhead box A2) and Wnt1-Lmx1a (wingless-related MMTV integration

site 1-LIM homeobox transcription factor 1 alpha) pathways (Arenas, 2014, Tang et al., 2010) (Figure 1.3).

Figure 1.3 Pathways and molecules involved in the specification, neurogenesis and differentiation of mDA neurons

The SHH-Foxa2 and Wnt1-Lmx1a are two important signaling pathways involved in the maturation and differentiation of mDA neurons from neural progenitor cells. SHH and Wnt1 induces the expression of Foxa2 and Lmx1a transcription factors and these two pathways converge to express *Ngn2* essential for neurogenesis (induction from Lmx1a is through the indirect expression of *Msx1*). Foxa2 and Lmx1a can also induce *Nurr1* expression to activate mDA neuronal differentiation. *Nurr1* activation leads to the expression of critical proteins involved in the dopaminergic phenotype.

Initial development begins at the ventral mesencephalon where the expression of *Foxa2* is stimulated by SHH at embryonic day 8 (Ye et al., 1998). Foxa2 then induces the expression of *Ngn2* (*Neurogenin 2*) important for the neurogenesis of mDA NP cells (Ferri et al., 2007, Roybon et al., 2008). Foxa2 and *Ngn2* both induces *Nurr1* expression that is critical for mDA neuronal differentiation (Ferri et al., 2007). Along the other axis, the expression of *Lmx1a* is induced by the activation of the β -catenin complex downstream from the Wnt1 signalling pathway at the midbrain-hindbrain border (Chung et al., 2009). *Lmx1a* then induces the expression of *Nurr1* and *Pitx3* (paired-like homeobox transcription factor 3) (Chung et al., 2009, Wurst and Prakash, 2014), of which the latter has been found to be expressed selectively in the SN and VTA in the midbrain (Smidt et al., 1997) and is an important terminal differentiation marker (Smidt and Burbach, 2007). *Lmx1a* can also induce the expression of *Ngn2* indirectly by activating *Msx1* (*Msh* homeobox homolog 1) expression and follows with the expression of *Nurr1*. (Andersson et al., 2006, Roybon et

al., 2008). Nurr1 and Pitx3 have been suggested to localize at the same transcriptional complex with the co-repressor PSF (PTB-associated splicing factor) and that Nurr1 alone cannot induce the DA phenotype, unless Pitx3 is present (Jacobs et al., 2009). The group reported that Nurr1 is usually kept in the repressed state by interacting with another co-repressor, SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), in the absence of Pitx3, of which SMRT recruits HDACs (Histone deacetylases) to maintain a repressed deacetylated state at promoter sites of Nurr1. This can be reversed by the addition of sodium butyrate (HDAC inhibitor) (Jacobs et al., 2009). In the presence of Pitx3, this SMRT/HDAC complex is released resulting in the transcription of target genes, resembling ligand activation of NRs (Jacobs et al., 2009).

As a critical nuclear transcription factor, the recognition of Nurr1 at the promoter site of various genes activates their transcription that regulates the development and maintenance of mDA neurons (Figure 1.4). A prime example is that of *tyrosine hydroxylase* (TH) which is a key enzyme involved in the synthesis of dopamine. Other important genes include the *dopamine transporter* (DAT) gene important for the reuptake of dopamine and manifestation of the DA phenotype, and also *vesicular monoamine transporter-2* (VMAT2) and *aromatic L-amino acid decarboxylase* or *DOPA decarboxylase* (AADC/DDC) genes that are transcribed and translated into proteins involved in the packaging and transporting of dopamine (Hermanson et al., 2003, Flames and Hobert, 2011, Lee et al., 1999). Nurr1 also regulates the tyrosine kinase receptor, Ret, and its recognition of GDNF contributes to the maintenance of mDA neuronal function by way of activating phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (Decressac et al., 2012). Nurr1 was also suggested to play a role in the regulation of the oxidative capacity of the mitochondria in mDA neurons by controlling a set of nuclear-encoded mitochondrial genes such as superoxide dismutase 1 (SOD1) and cytochrome c oxidase subunit Vb (COX5B) (Kadkhodaei et al., 2013). In addition to these, a novel anti-inflammatory role of Nurr1 was revealed by studies that reported the induction of Nurr1 expression in macrophages and T cells that could in turn control the expression of inflammatory response genes (Glass et al., 2010, Saijo et al., 2009, Pei et al., 2005). In microglia and astrocytes, Nurr1 was also shown to interact with CoREST corepressor complexes which exerted transrepressional control on

NF- κ B (nuclear factor kappa light chain enhancer of activated B cells) target genes that were pro-inflammatory (Saijo et al., 2009)(Figure 1.4).

Figure 1.4 The role of Nurr1 in the development and maintenance of mDA neurons

Nurr1 has been shown to play multiple roles in the maturation and maintenance of mDA neurons. Nurr1 as a nuclear transcription factors regulates the expression of multiple genes involved in maintaining dopaminergic phenotype, the Ret-GDNF signaling pathway, neuroinflammation as well as for mitochondria regulation.

Given the importance of Nurr1 in the development, survival and maintenance of the mDA neurons, its dysfunction or reduced expression has been linked to the pathogenesis of PD, although the exact mechanism has yet been established. The expression of *Nurr1* was found in the SNpc and VTA during development and throughout adulthood in rodents and correlated with *TH* expression (Zetterstrom et al., 1996b). Another study also revealed that Nurr1 mRNA was mainly expressed in the mDA neurons (A8-A10 nuclei) (Backman et al., 1999) and the genetic ablation of Nurr1 in mice specifically affected neuronal development in the ventral midbrain but not in other dopaminergic cell groups such as A11 and A13 (Zetterstrom et al., 1997). Mice that were heterozygous *Nurr1*-deficient showed enhanced susceptibility to toxins such as MPTP and lactacystin that destroy the DA neurons (Le et al., 1999, Pan et al., 2008). Conditional gene targeting on mature, differentiated mDA neurons that resulted in the loss of *Nurr1* also led to a variety of neuronal pathologies in mice (Kadkhodaei et al., 2009a, Kadkhodaei et al., 2013). These results suggest that the constitutive activation of Nurr1 is necessary for the development and subsequent

maintenance of DA neurons during adulthood and the loss of Nurr1 expression could lead to the progression of PD. Coming to human studies, ageing human brains have collectively showed a decrease in the number of Nurr1-expressing DA neurons and a reduction in *Nurr1* and *TH* expression (Chu et al., 2002, Chu and Kordower, 2007). The same group also found that in post-mortem brains of PD patients in sporadic cases, Nurr1 expression was reduced in nigral neurons containing α -syn inclusions but not in the hippocampal neurons, compared to age-matched controls (Chu et al., 2006). However, it remains unclear if this observation is a cause or effect of neurodegeneration (Chu et al., 2006). Le and group also showed that the level of *Nurr1* expression in the peripheral blood lymphocytes of PD patients especially of a familial nature, was lower than those found in healthy controls and patients suffering from other neurological diseases (Le et al., 2008). Taken together, the physiological importance of Nurr1 for DA neuronal development, maintenance during adulthood and its pathological associations with PD have highlighted its attractiveness as a therapeutic drug target for PD.

Though cognate ligands or co-regulators for the orphan Nurr1 LBD have yet been established, and was also suggested to be in the constitutively active and ligand-independent conformation (Kurakula et al., 2014, Pearen and Muscat, 2010, Wang et al., 2003), reports have emerged on drugs or small molecule compounds which could target and activate Nurr1. The anti-proliferative drug 6-mercaptopurine was shown earlier to be a Nurr1 agonist targeting the AF-1 domain of Nurr1 LBD (Ordentlich et al., 2003) while other groups have also developed or identified activators of high potency although their binding site on Nurr1 was not determined (Dubois et al., 2006, Hintermann et al., 2007). Zhang's group in collaboration with Hintermann et. al., reported anti-parkinsonian effects in PD mice models (Zhang et al., 2012) with the synthetic low molecular isoxazolo-pyridine compound they had earlier reported (Hintermann et al., 2007).

A study from our group in collaboration with Prof. Kwang-Soo Kim's group from Harvard Medical School revealed that existing FDA-approved anti-malarial drugs could be repurposed as perspective treatments for PD (Kim et al., 2015). More than 1000 FDA-approved compounds, natural products and their derivatives were screened for Nurr1 activators on cell-based assay systems. Three compounds were shown to induce Nurr1 transcriptional activation and possess identical 4-amino-7-chloroquinoline scaffold. These compounds are namely amodiaquine (AQ), chloroquine (CQ) currently used for malaria

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treatment and glafenine (a non-steroidal anti-inflammatory drug but was deemed to have weak activity) (Kim et al., 2015). AQ and CQ were further reported to activate Nurr1 LBD transcriptional function by approximately 15- to 10- fold respectively in SK-N-BE(2)C human neuroblastoma cells and both drugs were also shown to bind Nurr1 LBD detected using biophysical methods such as surface plasmon resonance and nuclear magnetic resonance (NMR). Furthermore, AQ and CQ were able to stimulate midbrain neurogenesis, offered neuroprotection against the neurotoxin 6-OHDA and reduce the expression of proinflammatory cytokine genes. AQ was also able to mitigate behavioural deficits in PD mice models (Kim et al., 2015).

In 2016, de Vera and colleagues have proposed the unsaturated fatty acid, docosahexaenoic acid (DHA) as a potential natural ligand of Nurr1 by interacting with its putative LBP although DHA was unable to increase Nurr1 transcriptional activity (de Vera et al.). Earlier reports have also proposed oxidized fatty acids as the natural ligands of another NR, PPAR γ (Itoh et al., 2008, Hughes et al., 2014). It is widely known that NR ligands are usually hydrophobic and are derived from retinoids, fatty acids and lipophilic hormones, to name a few (Sladek, 2011), and these can bind to hydrophobic cavities ranging from ~ 220 - $1600\text{\AA}$ (Ingraham and Redinbo, 2005, Benoit et al., 2004). There have also been cases in which orphan NRs became “adopted” or “de-orphanized” following the identification of their endogenous ligands such as the steroidogenic factor 1 and the liver receptor homolog-1 (Evans and Mangelsdorf, 2014, Lala et al., 1997, Krylova et al., 2005, Musille et al., 2012) and REV-ERB (Raghuram et al., 2007) as mentioned earlier. Therefore, the possibility remains that Nurr1 LBD can be stimulated through the binding of an endogenous ligand likely of a lipophilic nature.

1.3 Prostaglandins - biologically active lipids with a myriad of physiological functions

Prostaglandins (PGs) are bioactive lipids that are present in nearly all cells and tissues of which their secretion is usually through autocrine or paracrine mechanisms triggered by important cellular events such as inflammation, vasodilation/constriction and uterine contraction (Smith, 1989, Shimizu and Nakamura, 1985). PGs belong to a subfamily of eicosanoids that originate from the enzymatic oxidation of 20-carbon essential fatty acids.

Those of the 2-series have two double bonds in their long chains and are derived from arachidonic acid (AA) while those of the 1-series have one double bond in their long chains and are derived from Dihomo- γ -linolenic acid (DGLA) which is the precursor of AA.

The biosynthesis of PGs starts off with the intracellular release of AA from plasma membrane phospholipids with the help of phospholipase A2 (PLA2) (Straus and Glass, 2001). AA is sequentially converted first to PGG2 then to PGH2 by PGH synthase or cyclooxygenase through its cyclooxygenase and peroxidase activities. Two forms of cyclooxygenase exist, one being the constitutive form named COX-1 that possess specific housekeeping functions in the gastric mucosa (Dubois et al., 1998) and the other is an inducible form designated as COX-2. COX-2 expression is triggered by the presence of proinflammatory cytokines and is also a popular target for non-steroidal anti-inflammatory drugs (NSAIDs) (Herschman, 1999). Subsequent generation of the prostaglandins PGE2, PGD2, PGI2 and PGF2 α is mediated by their specific synthases (Figure 1.5).

Figure 1.5 Biosynthesis pathway of prostaglandins

AA is released from membrane phospholipids mediated by PLA2. AA is converted to PGH2 through the action of cyclooxygenases COX-1 and COX-2 (PGG2 before PGH2 is not shown). PGH2 is then converted to specific prostaglandins or prostacyclin by specific synthases. The various downstream receptors and sites of production are also indicated. PGE2 can bind to EP1-EP4 receptors that are G-protein coupled receptors and triggers intracellular cascades through secondary messenger molecules.

Prostaglandins of the E, D and F-series exert physiological effects by binding to their G-protein coupled receptors (Narumiya et al., 1999). In the context of PGE, there are four receptors designated EP₁-EP₄ and depending on the receptor type, the binding of PGE can trigger intracellular cascades leading to either increased or decreased cyclic AMP (cAMP) or the activation of phosphatidylinositol pathway (Narumiya et al., 1999). EP₂ and EP₄ leads to the increase in cAMP, while EP₃ inhibits cAMP generation and also increases intracellular Ca²⁺, EP₁ signalling leads to the activation of the phosphatidylinositol pathway (Ricciotti and FitzGerald, 2011).

The nomenclature of prostaglandins classifies them according to their cyclopentane ring type (such as. E, A, J) and number of double bonds in their hydrophobic tail (1, 2, 3). Numbering convention also begins from the carboxylic acid (Nelson, 1974) Cyclopentenone prostaglandins of the A and J-series such as PGA₂, PGA₁ and PGJ₂ are dehydrated metabolites of PGE₂, PGE₁ and PGD₂ (Straus and Glass, 2001)(Figure 1.6).

Figure 1.6 Dehydration of PGE₁ and PGE₂ to their metabolites PGA₁ and PGA₂

PGA₁ and PGA₂ share the same cyclopentenone ring structure containing the α,β unsaturated carbonyl group that allows the formation of a Michael adduct at C11. They differ by one double bond in the carboxyl-bearing tail and are also the dehydrated metabolites of PGE₁ and PGE₂. PGE₁ and PGE₂ also differ by one double bond in the carboxyl-bearing tail but their cyclopentane rings do not contain the reactive α,β unsaturated carbonyl. (*) indicate the position of electrophilic carbon atoms.

The unique feature of these prostaglandins that set them apart from their precursors is the presence of an α,β -unsaturated carbonyl group in their cyclopentenone ring. This chemically reactive group has an electrophilic center that is susceptible to Michael addition reactions with nucleophiles like the free sulfhydryl group from cysteine residues in reduced glutathione and cellular proteins (Honn and Marnett, 1985, Cagen et al., 1975, Parker and Ankel, 1992). A well-known example is illustrated by the prostaglandin 15d-PGJ2 (15-deoxy- $\Delta^{12,14}$ -PGJ2), the endogenous ligand of the NR, PPAR γ , that is able to exert anti-inflammatory and neuroprotective effects through the formation of a covalent adduct with the receptor (Forman et al., 1995, Kliewer et al., 1995, Bernardo et al., 2000). In addition, another cyclopentenone, PGA2, was found to activate the transcriptional function of Nor1 LBD although the binding mechanism remains unclear (Kagaya et al., 2005).

As highlighted, Nurr1 remains an orphan NR and multiple attempts have been made over the decade to identify its endogenous ligand. The only structure-based evidence of ligand binding to Nurr1 was presented by our group (Kim et al., 2015), identifying the binding of AQ and CQ using NMR titration. Following this, a recent report that the Nurr1 LBP is dynamic (de Vera et al., 2019) and can likely accommodate a ligand, is a promising finding contrary to the long standing belief that the LBP is in an active conformation (Wang et al., 2003). These studies show that Nurr1 can accommodate ligands and encouraged us toward identifying their probable endogenous ligand. Fatty acids such as prostaglandin 15d-PGJ2 was shown to bind PPAR γ , via a covalent Michael adduct with Cys285. In this direction, Nurr1 LBD also contains five cysteines and we predicted that one of them could be susceptible to Michael addition with the electrophilic centers of prostaglandins similar to PPAR γ . The ubiquitous presence of prostaglandins in the body and their involvement in various physiological processes such as inflammation that is also associated with Nurr1, promotes them as potential endogenous ligands for Nurr1.

1.4 The fruit fly – a valuable model organism for studying neurodegenerative diseases

Within the tiny body of the *Drosophila Melanogaster*, or the common fruit fly, holds a wealth of information that has provided valuable insights into areas such as genetics, neuroscience and behavioural science. The use of the fruit fly as a model for neurodegenerative diseases (and other human diseases) have gained popularity over the

years to not only attempt to elucidate neuronal functions/dysfunctions, but also to complement cellular studies and also serve as a platform for target validation and drug screening (Marsh and Thompson, 2006). There are also advantages that come with the use of flies as compared to animal models like rodents, such as rapid generation time and comparatively shorter lifespan, low cost of maintenance and they are not subjected to licensing laws as with animal models. Furthermore, their simple genetic makeup can be easily manipulated or engineered through genome-wide screenings to observe phenotypic changes linked to mutations. Genetic models that are challenging to reproduce in rodent models could at times prove more effective in flies as well (Xu and Rubin, 1993). Each system has their own advantages and disadvantages, and when used collectively, can provide valuable information and correlations for the observation and mitigation of disease progression that will only serve to strengthen the analyses.

~75 % of genes linked to human disease was found to have predicted orthologs in the fly and protein sequences were generally well-conserved (Reiter et al., 2001). In the context of PD, for instance, five out of six PD-related genes have a corresponding *Drosophila* ortholog such as *UCH-L1* (Ubiquitin carboxy-terminal hydrolase L1), *Parkin*, *DJ-1*, *PINK1* and *LRRK2* (*SNCA* coding for α -syn does not have a *Drosophila* ortholog) (Whitworth et al., 2006). Despite vast differences in the anatomy of the invertebrate *Drosophila* central nervous system (CNS) as compared to vertebrate CNS, many of their essential developmental pathways and functions were also conserved between them, allowing us to make use of the *Drosophila* system for studying physiological and pathological neuronal mechanisms (Whitworth et al., 2006).

The dopaminergic system in the *Drosophila* brain is made up of six major clusters (consisting of 200 neurons) and are arranged symmetrically from the midline of the brain with the cell bodies at the periphery and the axonal projections extending toward the center (Whitworth, 2011)(Figure 1.7). There are four clusters on the posterior side of the brain; the two medial clusters are named as the protocerebral posterior medial (PPM) 2 and 3, while the two lateral clusters are designated as the protocerebral posterior lateral (PPL) 1 and 2. On the anterior side, there are two clusters of DA neurons; one is a small cluster of ~five DA neurons called the protocerebral anterior lateral (PAL), while another larger cluster containing about 60 DA neurons with small cell bodies, is termed as the

protocerebral anterior medial (PAM). Aside from their clusters, a few DA neurons segregated from the major clusters are also identified such as PPM1, the deutocerebral 1 (D1) and the ventral unpaired medial (VUM) neurons (Whitworth, 2011)(Figure 1.7).

Figure 1.7 DA neuronal clusters in the *Drosophila* adult brain

Diagram showing the distribution of the DA neuronal clusters in the *Drosophila* adult brain. The neuronal clusters are arranged with bilateral symmetry and shown here is the posterior side of the brain with prominent clusters such as PPM, PPL, VUM; while PAL and PAM (not indicated) clusters are located on the anterior side of the brain.

Transgenic fly models can usually be generated using the binary system made up of the yeast transcription factor GAL4 and the yeast UAS (upstream activator sequence) to express the desired human gene. To create this system, transgenic flies harbouring a tissue-specific promoter fused to GAL4 are crossed with flies containing the desired gene fused to the UAS (Marsh and Thompson, 2006). This way, their F1 progeny will express the gene only in selected tissues through the recognition of GAL4 on UAS to which the downstream effects can then be monitored (Brand and Perrimon, 1993, Brand and Dormand, 1995). This will allow us to observe cellular changes upon either the knockdown or overexpression of a gene in a tissue-specific manner. For example, the expression of LRRK2 G2019S by the *Ddc*-GAL4 driver containing the promoter for *Ddc* gene would be mainly confined to DA neurons since *Ddc* is highly expressed in these neurons (Ng et al., 2009).

For this thesis, human LRRK2 GS transgenic flies were selected as a drug screening model since LRRK2 mutation is a major contributor to genetic cases of PD and these flies have also shown to recapitulate the features of late-onset PD in humans (Liu et al., 2008, Ng et al., 2009). The Nurr1 ortholog in flies was found to be DHR38 which is also the single fly ortholog for the NR4A family with highest sequence identity in the LBD with Nurr1 (61

%) compared with Nur77 and Nor1 (58 % and 57 % respectively). Structural determination of DHR38 using X-ray crystallography have also yielded a similar structure to the NR4A LBD consisting of 11 α -helices and a small three-stranded β -sheet organized in a triple-layer helical sandwich and is devoid of the classic LBP and coactivator site (Baker et al., 2003). Like the NR4A subfamily, DHR38 can bind to its specific NREs as a monomer or as heterodimers with ultraspiracle protein (USP; RXR ortholog) with the exception of the absence of a homodimer interface (Baker et al., 2003, Sutherland et al., 1995). DHR38 is important for the regulation of developmental transitions especially in later stages of metamorphosis through the ecdysteroid (androgens; moulting hormones) signalling pathway (Kozlova et al., 1998, Baker et al., 2003) as well as in carbohydrate metabolism (Ruaud et al., 2011). DHR38 also plays an important role in the expression of *Drosophila TH* and *Ddc* involved in dopamine synthesis (Sekine et al., 2011). Interestingly, it was earlier reported that the ectopic expression of DHR38 repressed the expression of *Ddc* in epidermal tissues while the opposite was observed in neuronal tissues (Davis et al., 2007), suggesting tissue-specific regulation of *Ddc* expression by DHR38. Since DHR38 LBD is structurally similar (RMSD of $\sim 0.7\text{\AA}$) to that of Nurrl LBD, the use of this LRRK2 GS transgenic fly model would allow us to examine the effects of drug treatment on a living system afflicted with late-onset PD and can provide further insights into the possible mechanisms that might also be affected in humans.

A possible question that might be raised with the use of this specific model to examine the effects on Nurrl/DHR38 activity is how LRRK2 mutation can affect Nurrl expression/activity. LRRK2 is a large protein consisting of 2527 amino acids and have two discrete enzymatic activities: the serine/threonine kinase activity and GTPase activity (Li et al., 2014). The presence of these domains is reflected by the functional role of LRRK2 in numerous signal transduction pathways such as JNK (c-Jun N-terminal kinase) (Hsu et al., 2010) and NF- κ B (Gardet et al., 2010) pathways. LRRK2 has also been shown to be involved in cellular functions like autophagy and endocytosis to which its overexpression and knockdown in various experimental models have also resulted in dysregulation in various cellular events like altered protein homeostasis and vesicle trafficking and mitochondrial dysfunction (Yue, 2009, Dawson et al., 2010).

Besides being implicated as a signalling protein, LRRK2 has also been suggested to be a scaffold protein in the canonical Wnt signalling pathway. It is at this pathway that LRRK2 could possibly be associated with Nurr1 expression/activity. Under basal conditions, β -catenin, a key protein in this pathway, is normally phosphorylated by glycogen synthase kinase 3 β (GSK3 β) in the β -catenin destruction complex (BDC) — which also harbours axin and APC (adenomatous polyposis coli) proteins — targeting it for proteasomal degradation (Berwick and Harvey, 2013). Upon stimulation with a Wnt ligand on FZD (frizzled) receptors and membrane co-receptors LRP5/6 (low density lipoprotein receptor related proteins 5 and 6), dishevelled (DVL) proteins are recruited to the plasma membrane where they will interact with the intracellular surface of the FZD receptor. DVL proteins are also thought to cause the inhibition of β -catenin phosphorylation by bringing them to juxtamembrane sites (Berwick and Harvey, 2013). LRRK2 was shown to be a scaffold protein by interacting with GSK3 β , LRP5/6 and DVL proteins (Lin et al., 2010, Sancho et al., 2009, Berwick and Harvey, 2012)(Figure 1.8). Berwick and Harvey have suggested that LRRK2 is part of the cytosolic BDC under basal state of which the loss of LRRK2 leads to BDC disruption and downstream pathway activation (Berwick and Harvey, 2013). However, upon Wnt ligand stimulation, LRRK2 relocates to the membrane and by interacting with DVL while the BDC interacts with LRP6, LRRK2 augments the activation of the pathway of which familial mutations have shown a decrease in Wnt signalling activity (Berwick and Harvey, 2012, Berwick et al., 2017). This would translate to a decrease in β -catenin which normally functions as a transcriptional co-regulator for transcription factors such as Nurr1 (Alves dos Santos and Smidt, 2011) (Figure 1.8). Therefore, the induction of Nurr1 activation by a ligand, could alleviate the effects associated with its reduced functionality in a LRRK2 G2019S condition.

The importance of Wnt signalling pathway in the development and differentiation of mDA neurons by activating Nurr1 has been discussed earlier. Mutations in *Wnt1* and *Lrp6* genes in mice resulted in failures in midbrain development and dopaminergic neuronal differentiation (Thomas and Capecchi, 1990, Castelo-Branco et al., 2010). The canonical Wnt signalling was also activated to promote neurogenesis from adult subventricular zone neuroprogenitor cells by reactive astrocytes and microglia as a form of protection against neuronal injury hinting at interactions between the inflammatory and Wnt signalling pathways (L'Episcopo et al., 2011a, L'Episcopo et al., 2011b, L'Episcopo et al., 2012).

Recently, LRRK2 GS mutation has also been shown to impair synaptic vesicle endocytosis in ventral mDA neurons which can be rescued by inhibiting LRRK2 kinase activity (Pan et al., 2017).

In a mouse model for LRRK2 GS mutation, a 6-fold increase in LRRK2 GS protein expression in the SN DA neurons was observed, compared to non-transgenic control mice (Liu et al., 2015). *Nurr1* and *Pitx3* mRNA expression was shown to be downregulated which led to a decrease in DA-related genes like *Vmat2* and *Aldh1a1* while *Th* and *Dat* expression showed a non-significant decrease (Liu et al., 2015). Protein levels of TH, VMAT2, DAT and ALDH1A1 (Aldehyde dehydrogenase 1 family, member A1), on the other hand, were shown to be drastically decreased in 18-month old GS mice compared to control and WT mice (Liu et al., 2015). However, these mice exhibited normal motor function without SN DA neurodegeneration and showed mainly age-related loss of DA axonal terminals, dopamine release and alterations to the genes and proteins mentioned in preceding sentences (Liu et al., 2015). Only one other mice model for LRRK2 GS exhibited significant neuron loss (Ramonet et al., 2011) while minimal DA neuron loss accompanied by decreased dopamine transmission and disrupted motor functions (Melrose et al., 2010, Herzig et al., 2011, Li et al., 2010) were observed in others. Although there is still much to explore on the association between LRRK2 and *Nurr1* in the Wnt and other signalling pathways, the happenstance that both exists in the same pathway and may regulate each other, holds promise for therapeutic interventions in this direction.

Figure 1.8 Proposed model connecting LRRK2 with the canonical Wnt pathway

The stimulation of Wnt ligand on FZD receptors and LRP5/6 co-receptors triggers a downstream cascade activated by DVL proteins which inhibits the BDCs containing GSK3 β , axin and APC. This is followed by the inhibition of the phosphorylation and repression of β -catenin by GSK3 β in the complex, allowing it to enter the nucleus and bind to its target transcription factors such as Nurr1. LRRK2 has been proposed to interact with GSK3 β , LRP5/6 and DVL proteins which leads to the activation of β -catenin upon Wnt ligand stimulation. Familial LRRK2 mutations have been shown to result in a decrease in Wnt signaling leading to β -catenin degradation.

Chapter 2:

Aims and Objectives

2. Aims and Objectives

Nurr1 has proved to be a significant therapeutic target for PD that may be activated by endogenous ligands that have yet been identified. We have previously reported, following the high-throughput screening of more than 1000 natural and synthetic compounds, that existing FDA-approved anti-malarial drugs, AQ and CQ, could be repurposed as perspective treatments for PD. Inspired by these results, we conducted another preliminary high-throughput screening for potential endogenous ligands of Nurr1. There are no Nurr1-agonist complex structures available to-date and furthermore, a collapsed LBP was observed in the crystal structure of Nurr1 LBD (Wang et al., 2003). Nevertheless, we believe that Nurr1 can be activated by an endogenous ligand. In fact, there have been classic examples of orphan NRs that have been “adopted” or “de-orphanized”. The recent report that the LBP of Nurr1 is dynamic and can expand from the collapsed conformation allowing for interaction with unsaturated fatty acids (de Vera et al., 2019), supports our hypothesis for the presence of an endogenous agonist of Nurr1.

Our screening efforts using X-ray crystallization revealed the covalent binding of prostaglandins PGA1 and PGA2 to Nurr1 LBD. Interestingly, the co-crystallization of Nurr1 LBD with its precursor PGE1, unveiled a structure containing the dehydrated metabolite, PGA1, which formed a covalent Michael adduct with Nurr1 LBD. Another cyclopentanone prostaglandin PGE2, was similarly co-crystallized with Nurr1 LBD but was shown to have dehydrated to PGA2 as well. Further confirmation through mass spectrometry established the presence of a peak corresponding to the complexes formed from Nurr1 LBD and the cyclopentenone PGA1 and PGA2, but not with PGE1 and PGE2, indicating that capturing the PGE1/2-bound Nurr1 crystal structure could thus be challenging.

Since we were not able to capture the interaction of Nurr1 LBD with PGE1 and PGE2 through X-ray crystallography, our next objective was to do so using another biophysical technique, NMR spectroscopy, which will allow us to examine any dynamic movements and conformational changes induced in Nurr1 LBD upon PGs binding. NMR was thus

carried out to verify the binding site on Nurr1 LBD that was shown in the co-crystal structures obtained with the cyclopentenone PGs.

We wanted to further elucidate the biological and biochemical functions of the PGs to determine if they have any functional relevance upon binding. The cytotoxicity effects of PGs on mammalian cells needed to be examined to assess a safe concentration to use on the cells before performing luciferase reporter assay. The luciferase reporter assay will allow us to test if the PGs could activate Nurr1 transcriptional function since they were able to bind to the LBD.

Besides cellular studies, we would also like to evaluate their efficacy in an *in vivo* fly model which has become a popular tool for drug screening on several diseases. The LRRK2 G2019S transgenic flies was selected as our drug screening model since they showed locomotor deficits and age-dependent TH⁺ neuronal loss that are classical symptoms of human PD. This model was used to assess if they were able to improve the survival rate, rescue climbing ability and TH⁺ neuronal loss as well as analyse the expression levels of a panel of PD-related genes. Their therapeutic potential would also be further explored by treating mice induced with the neurotoxin, 6-OHDA, and evaluating their rotational behaviour.

This thesis will discuss (a) the molecular interactions of Nurr1 LBD with PGs investigated through NMR spectroscopy followed by (b) cellular studies using PGs to first screen for cytotoxicity and then the transcriptional activation of Nurr1. The final part of the thesis will outline (c) the effects of PGs on the fly model of LRRK2 G2019S which results in late-onset PD as well as in the 6-OHDA mouse model for PD.

Chapter 3:

Materials and

Methods

3. Materials and Methods

3.1 Materials

3.1.1 Bacterial strains and mammalian cell lines

DH5 α *E. Coli* strain was selected for the maintenance and isolation of plasmid DNA whereas the BL21(DE3) *E. Coli* strain was used for expressing protein. HEK293T human embryonic kidney cells and SK-N-BE(2)C neuroblastoma cells were procured from American Type Culture Collection (Manassas, VA).

3.1.2 Vectors, primers and restriction enzymes for cloning

hNurr1 LBD (residues 356-598) was previously cloned in by Dr Toan into pE-SUMO plasmid (Lifesensors, PA) such that the protein has a His-SUMO tag at the N-terminal and can be captured by Ni²⁺-NTA beads. The tag confers enhanced expression and solubility to the protein which can then be cleaved off by SUMO protease enzyme, without affecting the native protein. For transfection into HEK293T and SK-N-BE(2)C cells, pcDNA3.1 Myc/His mouse Nurr1 full-length and LBD constructs were kindly gifted by Prof. Kwang-Soo Kim. pGL3-basic and pRL-null vectors were kind gifts from Prof. Valerie Lin.

NheI-HF (high fidelity) and XhoI for cloning the NL3 motif into pGL3-basic vector were purchased from New England Biolabs (MA). The oligo containing four copies of the NL3 motif (4xNL3-seq), encompassing the NBRE element that Nurr1 recognizes, was synthesized by Integrated DNA technologies (Singapore). Forward and reverse primers (4xNL3-amp) were designed for producing the complementary strand for 4xNL3, subsequent amplification and colony screening and were then synthesized by Integrated DNA technologies (Singapore). These sequences are shown in Table 3.1.1. *Pfu* and *Taq* DNA polymerases and T4 ligase were purchased from New England Biolabs (MA).

Table 3.1.1 Primer sequences for cloning 4xNL3 into pGL3-basic vector

Primer name	Sequence from 5' to 3'
4xNL3-seq	
(NheI site in red, XhoI site in blue, NL3 sequence underlined)	CTAGCTAGCGAAAAACAAAAGGTCACCTTACGAAAAACAA AGGTCACCTTACGAAAAACAAAAGGTCACCTTACGAAAAACA AAAGGTCACCTTACCTCGAGCGG
4xNL3-amp	
(Forward – NheI site in red)	CTAGCTAGCGAAAAACAAAAGGTCAC
4xNL3-amp	
(Reverse – XhoI site in blue)	CCGCTCGAGGTAAGTGACCTT

Primers used for qPCR were obtained from the FlyPrimerBank database (Hu et al., 2013) with the exception of TH which was adapted from Pathak and colleagues (Pathak et al., 2015) (Table 3.1.2).

Table 3.1.2 Primers used for RT-qPCR in LRRK2 G2019S fly model

Gene Symbol	Drosophila Ortholog	Primer sequences	T _m (°C)	Amplicon length (bp)
ACTB (β-Actin)	Act5C	FP: 5'– AGGCCAACCGTGAGAAGATG –3' RP: 5'– GGGGAAGGGCATAACCCTC – 3'	61.9 61.5	180
NR4A2 (Nurr1)	Dhr38	FP: 5'– GCAGCGGACAACTTCTACG –3' RP: 5'– ACCGAAGGGGAGGAGACTG –3'	60.8 62.3	182
TH	TH	FP: 5'– GTTGCAGCAGCCCCAAAAGAAC –3' RP: 5'– GAGACCGTAATCATTTGCCTTGC –3'	61.2 62.9	183
SLC18A2 (VMAT2)	Vmat	FP: 5'– GCCCATCATACCCGAGTTCC –3' RP: 5'– GTTGGACGTTAAAGGTGTGCG –3'	62.1 62	85

DDC	Ddc	FP: 5'– GGGTTTGATTCCCTTCTACGC –3' RP: 5'– CAAATTGTGCTTGTTTCCCACC –3'	60.7 60.7	103
DAT	DAT	FP: 5'– GTCAGCACAATCGTAAGGGTG –3' RP: 5'– CACGGCATATCCAATTCCTTTGA –3'	61 60.9	77
PITX3	Ptx1	FP: 5'– ATGACCGCCGTAGCAAATAGT –3' RP: 5'– GGTTTCGTTTACACTGCGATTG –3'	61.6 60.4	123

3.1.3 General chemicals and antibodies

Most of the chemicals mentioned in section 3.2 were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated. Table 3.1.3 presents all other chemicals used and their corresponding company and location.

Table 3.1.3 List of chemicals and their corresponding company and location

Chemical(s)	Company	Location
Bromophenol blue, Bacto yeast extract, Glycerol and Triton X-100	Affymetrix	Santa Clara, CA
Glycine	Axil Scientific	Singapore
Bacto tryptone and Bacto agar	BD Biosciences	San Jose, CA
Precision Plus Protein Unstained or All-Blue standards	Bio-Rad	Hercules, CA
Drosophila agar, active dry yeast and dextrose	Genesee Scientific	San Diego, CA
Kanamycin, fetal bovine serum, OPTI-MEM I reduced serum medium, 0.05 % trypsin-EDTA, penicillin and streptomycin	Gibco	Carlsbad, CA

Carbenicillin and Isopropyl-beta-D-thiogalactopyranoside (IPTG)	Goldbio	St. Louis, MO
Sodium dodecyl sulfate (SDS)	Hoefer Inc	Holliston, MA
Cornmeal	Hsin Lian Fa Trading Co	Singapore
Dulbecco's Modified Eagle Medium (DMEM)	Hyclone	Carlsbad, CA
UltraPure DEPC-Treated Water	Invitrogen	Carlsbad, CA
Normal goat serum	Jackson ImmunoResearch Inc	West Grove, PA
Absolute ethanol 99.5-99.8 %, isopropanol, sodium phosphate monobasic anhydrous, sodium phosphate dibasic anhydrous and sodium chloride	Merck Millipore	Whitehouse Station, NJ
Rabbit anti-Tyrosine Hydroxylase (TH) antibody	Pel-Freez Biologicals	Rogers, AR
Tris-base and Dual-Glo Luciferase reporter assay system	Promega	Madison, WI
WST-1 cell proliferation reagent	Roche	Basel, Switzerland
Chloroform, TRIzol Reagent, Lipofectamine 3000 and Goat anti-Rabbit IgG secondary antibody conjugated with Rhodamine Red-X	Thermo Fisher Scientific	Waltham, MA

3.1.4 Software and Equipment

ÄKTA FPLC system was fitted with HiLoad 16/60 Superdex 200 or 16/60 75 prep grade (GE healthcare; Buckinghamshire, UK) for protein purification by size-exclusion. Gel filtration curves were analysed using the associated UNICORN system control software.

NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to determine DNA or protein concentration. GeneAmp® PCR system 9700 (Applied Biosystems, MA) was used for PCR and reverse transcription reactions. CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was used for qPCR reactions. Cell viability and luciferase assays were measured using either Tecan Infinite® 200 Pro or Tecan Safire² microplate readers (Männedorf, Switzerland).

3.2 Methods

3.2.1 Competent bacterial cell preparation using CaCl₂

To prepare for competent *E. Coli* strains of DH5α or BL21(DE3), a single colony was first selected from a LB plate not harbouring any antibiotics to be inoculated into 5 ml of LB medium and incubated at 37 °C with shaking for an overnight duration. The next day, this culture was transferred to 100 ml of LB medium and incubated with shaking at 37 °C until the absorbance at 600 nm approached 0.4-0.5. The culture underwent centrifugation at 2000 g (≈3500 RPM) for 15 minutes at 4 °C after having chilled on ice for 10 minutes. The pellet was resuspended in 50 ml of ice-cold, autoclaved 0.1M CaCl₂ and kept on ice for a further 40 minutes to 2 hours and subjected to the same centrifugation procedure as before. Resuspension of the pellet with 0.1M CaCl₂ with 20 % glycerol followed and the bacterial cells were then stored in 50 µl aliquots at -80 °C after snap-freezing in liquid nitrogen.

3.2.2 Transformation of bacterial cells with plasmid DNA

The transformation of bacterial *E. Coli* cells entails the use of heat shock for the entry of plasmid DNA. This was first performed by means of incorporating 0.5 µl of purified plasmid DNA into 50 µl of competent *E. Coli* cells (DH5α and BL21(DE3)) in a chilled tube and kept on ice for 30 minutes. Heat shock was applied to the tube for 1 minute at 42 °C and was then chilled on ice for another 3 minutes. To this, 250 µl of LB medium (without antibiotic) was added which preceded further incubation at 37 °C with shaking at 200 RPM for 1 hour before it was plated on to a LB plate fortified with the relevant antibiotic and incubated at 37 °C for an overnight duration.

3.2.3 Cloning of 4xNL3 into pGL3-basic vector

Nurr1 recognizes the NBRE element (canonical sequence: AAAGGTCA) as a monomer and activates the transcription of target genes. Three NBRE-like motifs were earlier reported to be found on the TH promoter of which the NL3 motif had stronger affinity to Nurr1 and was also a more efficient transactivator compared to NL1 and NL2 (Kim et al., 2003). Hence, four copies of this sequence (GAAAACAAAAGGTCACTTAC) was cloned 52 bp upstream of the firefly *luciferase* gene on the pGL3-basic vector which is promoterless. Firstly, 3 µg of pGL3-basic vector was digested with NheI-HF restriction enzyme for 30 minutes at 37 °C. After performing PCR clean-up using the DNA gel extraction kit (Axygen Scientific, CA), the entire eluted sample was digested with XhoI for 30 minutes at 37 °C. The reaction mix was loaded onto a 1 % agarose gel and subjected to gel extraction using the same kit. Next, the complementary strand for the 4xNL3 DNA sequence was synthesized in a short reaction consisting of 2 minutes at 94 °C, 30 seconds at 50 °C and 3 minutes at 72 °C. The mix was first prepared by adding 8.5 µl of nuclease-free water into a 0.2 ml thin-wall PCR tube followed by 20 µM 4xNL3-seq oligo, 20 µM 4xNL3-amp reverse primer, 100 µM dNTP, 1x *Pfu* buffer with MgSO₄, 2.5 U of *Pfu* DNA polymerase. PCR clean-up was performed after this reaction and the entire eluted sample was subjected to PCR amplification that started with an initial denaturation at 95 °C for 5 minutes followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, extension at 72 °C for 60 seconds and a final extension step at 72 °C for 3 minutes. The mix was first prepared by adding 16 µl of nuclease-free water into a 0.2 ml thin-wall PCR tube followed by 0.2 µM 4xNL3-amp forward and reverse primers, 200 µM dNTP, 1X *Pfu* buffer with MgSO₄, 2.5 U of *Pfu* DNA polymerase and the DNA template. After the insert was amplified, 2 µg was double digested with NheI-HF and XhoI for 30 minutes at 37 °C followed by PCR clean up. Ligation was set up with 50 ng of vector together with the insert at 1:1 and 1:3 (vector:insert) ratios, 800 units of T4 DNA Ligase, 1X T4 DNA Ligase buffer and nuclease-free water topped up to 50 µl. The reaction was conducted at 25 °C for 1 hr. 20 µl of the mix was then transformed into DH5α *E. Coli* cells and plated on carbenicillin LB plates. The next day, colonies that grew on the plates were screened using PCR to check if any of the plasmids contain the 4xNL3 sequence. A typical reaction mix consists of 6.6 µl nuclease-free water, followed by 0.2 µM 4xNL3-amp forward and reverse primers, 200

μ M dNTP, 1X *Taq* buffer with 500 mM KCl, 2 mM MgCl₂, 1 U of *Taq* DNA polymerase. Individual colonies were picked and inoculated into each mix, the pipette tip was dropped into 100 μ l LB media with carbenicillin and grown at 37 °C in a shaking incubator for a few hours. The PCR reaction started with an initial denaturation at 95 °C for 5 minutes followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 60 seconds and a final extension step at 72 °C for 3 minutes. The reaction samples were then loaded into 1 % agarose gel to verify with the positive control. Positive clones were inoculated from the 100 μ l small culture into a 5 ml culture for plasmid extraction the next day. The isolated plasmids were sent for DNA sequencing (Axil Scientific, SG) to identify plasmids with the 4xNL3 sequence correctly inserted in.

3.2.4 Isolation and amplification of purified plasmid DNA

Following transformation of bacterial cells with the intended plasmid DNA to be amplified, a single colony can be isolated from the plate of bacterial cells and inoculated into 5 ml of LB medium containing the appropriate antibiotic. The culture was then incubated at 37 °C with shaking (200 RPM) overnight. The plasmid DNA can then be purified from the culture using AxyPrep Plasmid Miniprep Kit (Axygen Scientific, CA) following manufacturer's instructions. Quantification of DNA concentration was done using the NanoDrop 1000 Spectrophotometer. The ideal OD₂₆₀/OD₂₈₀ ratio is between 1.8-2.0 which indicates good sample purity. Any ratio beyond this range typically indicates the presence of contaminants and the need to isolate the DNA again.

3.2.5 Large scale protein expression of Nurr1 LBD

A single colony was selected from a plate of BL21(DE3) *E. Coli* cells transformed with pE-SUMO Nurr1 LBD for inoculation into a starter culture consisting of 10 ml LB medium with kanamycin (1:1000). After overnight incubation at 37 °C with shaking (200 RPM), the entire culture was added to 1 L LB medium containing kanamycin (1:1000) and this large culture was subjected to shaking at 180 RPM and at 37 °C for approximately 3 hours until the absorbance of the culture at 600 nm falls in the range of 0.6-1.0. Once the absorbance has reached the desired range, the culture was induced with 1 mM IPTG and allowed to

grow for another 3.5 hours at 25 °C with shaking at 180 RPM. The cells were then harvested by centrifugation at 9000 g at 4 °C for 15 minutes. The pellet was stored at -20°C if purification of the protein was not carried out immediately. The expression of ¹⁵N-labeled Nurr1 LBD follows a similar process, apart from the use of M9 media instead of LB media for bacterial growth. 2 L of M9 media could be prepared and split into 4 flasks of 500 ml media. 5 ml from each flask was then collected to make the starter culture. The next day, kanamycin was added into each 500 ml media and the overnight starter culture was added into each flask. They were incubated at 37°C with shaking at 180 RPM for 6 hours till their absorbance reached 0.5-0.6 before adding 1 mM IPTG and allowing the flasks to incubate overnight at 20°C with shaking. Subsequent collection of pellet and purification is the same as that for unlabeled protein.

3.2.6 Protein purification using Ni²⁺-NTA affinity chromatography

Resuspension of 1 L bacterial culture pellet was carried out using 25 ml of lysis buffer and the suspension underwent sonication for 20 – 30 minutes at a 23 % amplitude or until the lysate clears up. Another method to check if lysis is complete entails the use of the Bradford assay to confirm there is no further increase in the protein concentration.

The clarified lysate was further subjected to centrifugation at 48,000 g for 30 minutes at 4 °C. The supernatant was collected as the soluble fraction containing Nurr1 LBD protein. 2 ml final bed volume of Ni²⁺-NTA resin (50 % slurry) was packed into an Econo-column® glass chromatography column (Bio-Rad, CA) and the beads were equilibrated with 10 bed volumes of lysis buffer followed by a 1-hour incubation with the supernatant at 4 °C on a nutator to allow the His-SUMO tagged proteins to bind to the beads. After incubation, the flow-through was collected and the beads were washed with 25 ml of wash buffer to remove proteins that were non-specifically attached. Following a 15-minute incubation with 10 ml elution buffer, elution was carried out in fractions which were analyzed using SDS-PAGE to determine their yield and purity. The compositions of buffers used are provided in Appendix I.

3.2.7 Buffer exchange using PD-10 desalting column

After the purification process described above, the imidazole in the elution buffer should be removed before adding sumo protease as it could affect enzyme activity; other times, the protein buffer needs to be changed before other downstream experiments and, in both cases, PD-10 desalting columns (GE healthcare, CT) were used for buffer exchange. This method hinges on a size-exclusion basis that the higher molecular weight proteins would pass the column quickly and get eluted first while lower molecular weight solutes pass through slower and get eluted much later. By means of the gravity protocol, the column was equilibrated first with 25 ml of buffer (to be exchanged) before adding 2.5 ml of concentrated protein into the column. The flow-through following protein addition was discarded since the protein would remain in the column. Following the addition of 4-4.5 ml of buffer, the protein sample in the new buffer was then collected for subsequent experiments.

3.2.8 His-SUMO tag cleavage

The His-SUMO tag attached on the N-terminal side of the protein was cleaved following the first Ni^{2+} -NTA affinity purification and buffer exchange to remove imidazole from the buffer. Typically, 400 μg of purified sumo protease enzyme was used for approximately 40 mg of protein purified from 1 L of culture. After incubating the enzyme with the sample at 4 °C overnight, the sample was purified through another round of Ni^{2+} -NTA affinity purification using the same buffers as previously described. Purified Nurr1 LBD without the tag was collected from the flow-through fraction after 1 hour of incubation with the beads.

3.2.9 Protein purification using Fast Performance Liquid Chromatography (FPLC)

To improve the purity of protein obtained from Ni^{2+} -NTA affinity purification, further purification was carried out using the FPLC system fitted with a gel-filtration (size-exclusion) column. HiLoad 16/60 Superdex 200 or 16/60 75 prep grade (GE healthcare, Buckinghamshire, UK) columns were used, following equilibration with 1 bed volume of

FPLC buffer (Appendix I). Before injecting 2 ml of sample into the system, the sample loop was equilibrated with 4 ml of buffer. The system was able to detect the concentration of protein during the run based on its absorption of ultra-violet light at 280 nm. Flow rate was maintained at 0.5 ml/minute.

3.2.10 Ni²⁺-NTA agarose resin regeneration

The Ni²⁺-NTA agarose resin can be regenerated and recharged with fresh Ni²⁺ to preserve the efficiency of protein binding, although the efficiency will still deteriorate after numerous regenerations. Table 3.2.1 presents the process for the regeneration. Following the last step, the beads were kept as 50 % slurry in 20 % ethanol and stored at 4 °C.

Table 3.2.1 Steps involved in Ni²⁺-NTA agarose resin regeneration

Step	Reagent	Number of Column Volumes
1	8M Urea	2
2	Autoclaved ultrapure H ₂ O	5
3	25 % → 50 % → 75 % → 100 % Ethanol	1 each
4	75 % → 50 % → 25 % Ethanol	1 each
5	Autoclaved ultrapure H ₂ O	5
6	100 mM EDTA, pH 8.0	5
7	Autoclaved ultrapure H ₂ O	5
8	100 mM Ni ²⁺	2
9	Autoclaved ultrapure H ₂ O	5
10	20 % Ethanol	3

3.2.11 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein

SDS-PAGE analysis was carried out to assess the purity of the protein sample following purification. The percentage of acrylamide in the gel can be adjusted according to the size of the protein. Since Nurr1 LBD has an approximately molecular weight of 25-27 kDa, a 12 % gel was selected. 6X SDS loading dye was added into the sample which was heated

for 10 minutes at 95 °C to promote further denaturation. Precision Plus Protein unstained or all-blue standards (Bio-Rad, CA) was also loaded together with the samples as a reference for estimating protein size after staining. The electrophoresis run for the gel was done at a constant voltage of 90-100 V for 100-120 minutes, ensuring that the dye front did not run out of the gel. The gel was then stained with Coomassie staining solution for 30 minutes to overnight, before de-staining for 1 to 3 hours with De-staining solution. The bands on the gel could then be visualized on a white light box. The compositions of buffers and solutions used are provided in Appendix I.

3.2.12 2D ^1H - ^{15}N Heteronuclear Single Quantum Correlation (HSQC) NMR

NMR experiments were performed by Dr Shin Joon and Dr Ye Hong on Bruker Avance II 600/700MHz spectrometers equipped with a 5 mm triple resonance, z-axis-gradient cryoprobe at 298 K. The spectra acquired were processed by Topspin version 2.1 (Bruker BioSpin), converted to Sparky UCSF format with Sparky script bruk2ucsf and analysed using SPARKY (Goddard and Kneller, 2002)/ NMRFAM-SPARKY (Lee et al., 2015). The protein concentration tested was 0.2 mM (unless otherwise stated) while the ligand concentration varies and are indicated accordingly. The formula for calculating the chemical shift perturbation (CSP) for each assigned amino acid residue is $\Delta\delta = \sqrt{(\Delta\delta\text{H})^2 + 0.154(\Delta\delta\text{N})^2}$, where $\Delta\delta\text{H}$ is the difference in chemical shift for ^1H between free and bound protein and $\Delta\delta\text{N}$ is the difference in chemical shift for ^{15}N between free and bound protein (Williamson, 2013). The threshold value for determining significant peak shifts is calculated by adding average chemical shift values for all assigned residues with the standard deviation obtained. Any CSP value above the threshold was mapped onto the crystal structure of Nurr1 LBD (PDB: 1OVL) using PYMOL.

3.2.13 Mammalian cell culture maintenance

HEK293T and SK-N-BE(2)C cells were cultured in a humidified 5 % CO_2 enclosed incubator at a temperature of 37 °C. Their growth media consists of DMEM (high glucose with L-glutamine) enriched with 10 % (v/v) fetal bovine serum (FBS), 100 units/ml

penicillin and 100 µg/ml streptomycin. Upon their growth to a confluency of 70 – 80 %, they were passaged with 0.025 % trypsin-EDTA for the detachment of cells.

3.2.14 Cell viability assay

This colorimetric assay using the tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), enables the quantification of the water-soluble formazan dye, upon extracellular reduction of WST-1. This process occurs by means of the electron transport system across the plasma membrane (Berridge and Tan, 1998, Berridge et al., 1996) in the presence of an intermediate electron acceptor, such as mPMS (1-methoxy-5-methyl-phenazinium methyl sulfate) (Berridge et al., 2005). The amount of formazan dye produced, measured at 450nm, would therefore be associated with the number of metabolically active cells, allowing us to determine cell viability. To determine the toxicity of the compounds in HEK293T or SK-N-BE(2)C cells, 7.5×10^3 cells/well were seeded into a 96-well transparent plate (Nunc, NY) and incubated at 37 °C with 5 % CO₂ for 48 hours until the cells were 60-70 % confluent. Various doses of compounds were added into designated wells and the plate was incubated for another 24 hours under the same condition. Next, the media in all the wells was removed to exclude background signals from the compounds which could affect final readings. 10 µl of WST-1 Cell Proliferation Reagent (Roche, Basel) was then added to each well containing 100 µl of fresh media. The plate was further incubated for 30 minutes (HEK293T) or 2 hours (SK-N-BE2C) at 37 °C with 5 % CO₂ until the colour of the reagent turned from pink to orange. The absorbance of each well was then measured at 450 nm, with a reference wavelength of 600 nm. Each treatment was performed in triplicates and were averaged and the average absorbance reading from blank wells (media only) was subtracted from averaged readings from each treatment before further analysis.

3.2.15 Transfection of plasmid DNA into mammalian cells

Transfection of plasmid DNA into SK-N-BE(2)C cells involved the use of Lipofectamine 3000 reaction. OPTI-MEM and DMEM media (without antibiotics) had to be warmed to room temperature before transfection. For each sample well in a 24-well transparent plate,

different amounts of the plasmids (expression, firefly and renilla luciferase vectors; 8:1:1) totalling to 5 µg were added into OPTI-MEM media with P3000 reagent and incubated for 5 minutes. Another mix consisting of Lipofectamine 3000 reagent in a 1:1 ratio with P3000 reagent, was prepared with OPTI-MEM media. After the initial 5 minutes, the latter mix was added into the former and this reaction mix was left at room temperature for 15 minutes before adding 50 µl drop-wise to each of the well in the 24-well transparent plate (changed with fresh DMEM media). The plate was gently tapped to ensure homogeneity before incubating the plate for another 24 hours at 37 °C, 5 % CO₂.

3.2.16 Luciferase reporter assay

The luciferase reporter assay is a popular assay for determining NR transcriptional activity. The principle of this technique relies on the fact that ligand-activated NRs will recognize specific response element and trigger the expression of the target gene which in this case will be the firefly luciferase gene. The enzymatic activity of luciferase can then be quantified by adding its substrate into the reaction and measuring light emission (de Wet et al., 1987). The amount of light produced will equate to the level of activity of the NR. In other words, an increase in transcriptional activation caused by an increase in ligand concentration can be visualized by an increase in light emission. Luciferase reporter assay was performed using the Dual-Glo® Luciferase kit from Promega (Madison, WI). To start off, 1.5×10^5 SK-N-BE(2)C cells were seeded into a 24-well transparent plate and incubated for 24 hours at 37 °C, 5 % CO₂. Following a 24-hour transfection and 24-hour drug treatment, each tested well was lysed for 15 minutes with 75 µl of 1X passive lysis buffer from the same company and transferred to a Greiner CELLSTAR® 96-well white polystyrene plate. To each well of lysed cells, 75 µl of Dual-Glo® Luciferase Assay Reagent was added and incubated for 10 minutes at room temperature. The measurement was set with a 10-second integrated measurement period. This step determines the luminescence in relative light units (RLU) for the firefly luciferase. Then, 75 µl of Dual-Glo® Stop & Glo® reagent was added into each well and incubated for 10 minutes before commencing with the same parameters as before to determine the RLU for the *Renilla* luciferase. The averaged reading for wells corresponding to non-transfected cells was first subtracted from each firefly luminescence reading and the duplicate readings were then

averaged. Normalization of the luminescence reading for each well was carried out by dividing the firefly luciferase RLU with the corresponding *Renilla* luciferase RLU. Each drug-treated well was then normalized against the averaged DMSO-treated well reading.

3.2.17 Drug treatment and negative geotaxis (climbing) assay on flies

Transgenic human LRRK2 WT and G2019S (GS) flies driven by the *ddc*-GAL4 promoter were generated as previously described (Ng et al., 2009) by Ms. Adeline Henry Basil and Ms. Wang Ziyin from Prof. Lim Kah Leong's laboratory.

25-31 female flies that expressed human LRRK2 WT or GS were then randomly selected and aged-matched at the beginning of the experiment. The flies were fed with cornmeal-agar that were changed every 2-3 days. Drug treatment started at day 29-31 for a duration of 25 days and survival data was recorded with every change of food. One batch of LRRK2 GS flies were vehicle-treated (0.5 % EtOH) to compare with drug-treated flies while LRRK2 WT flies received normal fly food. Climbing assay was performed to assess the locomotor function of the flies upon drug treatment. Flies that remained at the end of the drug treatment (minimum of 10 flies) were anesthetized and placed in a vertical plastic column (25cm in length; 1.5cm in diameter). After recovering for 1.5 hours from the CO₂ exposure, flies were tapped to the bottom and the number of flies reaching the median line of the column after 1 minute was counted for a total of six times and averaged.

3.2.18 Immunohistochemical staining of adult fly brains for tyrosine hydroxylase (TH) neurons

Following drug treatment for the flies as described above, flies that remained till the end of the experiment were anesthetized with CO₂ and placed on a small agar dish containing 0.1 % phosphate buffered saline with Triton X-100 (PBT) buffer for dissection using two Dumont #3C forceps to retrieve intact whole brains. The brains were transferred to a 0.6 ml Eppendorf tube containing 400 µl of fixing solution for each batch and were fixed overnight at 4 °C on a tube roller. The next day, the fixing solution was rinsed away using 400 µl of wash buffer and the brains were washed with 400 µl of wash buffer for 1 hour at room

temperature. The brains were then blocked with 300 µl of blocking buffer and incubated at room temperature for another hour. 300 µl of rabbit anti-TH antibody (1:300) was then added to the brains and incubated at room temperature for 1 hour before transferring to 4 °C overnight. After overnight incubation, the primary antibody may be collected for re-use (2-3 more times) and any residual primary antibody was washed with 400 µl of wash buffer. A final wash with 300 µl of wash buffer for 1 hour at room temperature was carried out and 300 µl of secondary goat anti-rabbit conjugated with rhodamine (1:300) was then added in for incubation at room temperature for 1 hour before transferring to 4 °C overnight. Following this, the secondary antibody was discarded, and the brains were washed with 400 µl of wash buffer. 300 µl of wash buffer was added to the brains for incubation at room temperature for 1 hour. The brains were then arranged on coverslips and mounted with VECTASHIELD Antifade Mounting Medium (Vector Laboratories; Burlingame, CA). The slides were kept in the dark at 4 °C until required for confocal imaging. DA neurons from the PPL1 cluster were quantified accordingly as described by Whitworth and colleagues (2005), from the images taken with an Olympus Fluoview confocal microscope at 40X objective. The compositions of buffers used are provided in Appendix I.

3.2.19 RNA extraction from adult fly heads

10 flies were randomly selected at the end of drug treatment and their heads were removed following anaesthesia with CO₂. The heads were kept on ice in a 0.6 ml Eppendorf tube and 50 µl of TRIzol reagent was added into the tube. The samples were homogenized by crushing the heads for 2 minutes with the back of a sterile inoculating loop. 10 µl of chloroform was then added and the sample was mixed well for 15-20 seconds followed by incubation on ice for 5 minutes. The sample was centrifuged at 12,000g for 15 minutes at 4 °C and the upper phase was transferred into a new Eppendorf tube. 25 µl of isopropanol was then added to this aqueous phase to allow RNA to precipitate overnight at -20 °C. The following day, the sample was centrifuged at 12,000 g for 10 minutes at 4 °C and the supernatant was removed carefully. The RNA pellet was washed gently with 100 µl of 75 % ethanol by tilting the tube slightly. The sample was then centrifuged at 7,600 g for 5 minutes at 4 °C after which the supernatant was discarded, and the step was repeated another

time. The tube was then overturned to air-dry the pellet at room temperature for 1 hour. The dried pellet was then resuspended in 15 µl DEPC-treated water and stored at -20 °C.

3.2.20 Reverse Transcription-quantitative PCR (RT-qPCR)

First-strand cDNA synthesis was carried out by mixing 1 µg of total RNA, 5 µM of Oligo(dT)₇ primer, 12.5 µl nuclease-free water, 4 µl of 5X reaction buffer, 20 U of Ribonuclease Inhibitor, RiboLock (Thermo Fisher Scientific, Waltham, MA), 1 mM dNTP and 200 U of RevertAid Reverse Transcriptase (Thermo Fisher Scientific; Waltham, MA) to a total of 20 µl. The reaction mix was centrifuged briefly before incubation in the thermocycler for 1 hour at 42 °C. The reaction was terminated by heating at 70 °C for 10 minutes and the sample was kept at -20 °C, if not used immediately after.

Quantitative PCR (qPCR) following reverse transcription requires the use of either a double-stranded DNA dye or a dye-conjugated probe, which in this case is the former, using the iTaq Universal SYBR Green Supermix (Bio-Rad; Hercules, CA) on a 96-well semi-skirted PCR plate (4titude, Surrey). A master mix was prepared consisting of the supermix (2X), 200 nM of forward and reverse primers (each) and nuclease-free water topped up to 10 µl after accounting for the DNA template to be added last. The master mix was aliquoted to each well accordingly and 12.5 ng final concentration of cDNA was added in last. No template control (NTC) was also prepared in which the DNA template was replaced with nuclease-free water. The plate was sealed with a MicroAmp® Optical Adhesive Film (Life Technologies, CA) and centrifuged for 1000 RPM for 1 minute to collect the samples at the bottom and remove any air bubbles. The thermal cycling protocol began with polymerase activation and DNA denaturation at 95 °C for 30 seconds followed by 40 amplification cycles which consists of denaturation at 95 °C for 5 seconds and annealing/extension and plate read at 60 °C for 60 seconds. As the PCR proceeds, the increase in the amount of amplified DNA will reach a point where it enters the exponential/linear phase beyond the background fluorescence. The higher the amount of starting DNA, the earlier this linear phase is reached. A threshold line is determined close to where the fluorescence signals increase above the background. The cycle number that is matched to the threshold is also known as the threshold cycle or “C_t”. Following this, melt curve analysis was also set from

65-95 °C with 0.5 °C increments at 5 seconds/step to check for primer-dimer formation. Readings were performed in duplicates/triplicates for two independent runs involving two sets of 10 fly heads for each treatment. LinRegPCR program (Ramakers et al., 2003) was used to determine the primer efficiency to ensure that they were between 90-120 % before proceeding with data analysis. The averaged C_t values for the genes tested were normalized against the C_t value of the housekeeping gene *Act5C* (β -actin ortholog in flies) using the $2^{\Delta\Delta C_T}$ comparative C_T method (Livak and Schmittgen, 2001).

3.2.21 Statistical Analysis

All statistical analysis was carried out using Graphpad Prism 5.0 (La Jolla, CA). For the cell viability and luciferase assays, all drug-treated values were normalized to the drug-vehicle treated (control) values and one-way ANOVA was carried out followed by Dunnett's post-hoc analysis to determine which differences in the means between the treated and control groups were significant. Data related to fly survival, climbing and DA neuronal count experiments were subjected to one-way ANOVA followed by Tukey's multiple comparison test to compare the differences in means between each group. For fly qPCR data, one-way ANOVA was carried out followed by Dunnett's post-hoc analysis instead of Tukey's multiple comparison, to examine the effect of drug-treatment against G2019S drug-vehicle treated control flies. For the 6-OHDA mouse model, unpaired two-tailed t-test was used to determine the differences in the means between the drug- and vehicle-treated groups. The number of independent experiments (n) were also ≥ 2 . Statistical significance is presented accordingly by *: $0.01 < p < 0.05$, **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$.

Chapter 4:

Results

4. Results

4.1 Structural investigation of Nurr1 LBD with prostaglandins

4.1.1 Expression and purification of recombinant Nurr1 LBD

Recombinant human Nurr1 LBD was expressed from the pE-SUMO Nurr1 LBD (residues 356-598) construct that was cloned previously by Dr. Toan. There is a His-SUMO tag at the N-terminal of the protein which offered the benefit of first purifying the protein using Ni^{2+} -NTA affinity chromatography and subsequently removing the tag. The removal of the tag is a clean cut made by sumo protease enzyme which leaves the purified native protein free of any external sequences that may be left by the tag. Ni^{2+} -NTA affinity chromatography allows the histidine part of the tag to bind to Ni^{2+} -NTA beads and the protein can be eluted by competition from imidazole in the buffer. The construct was first transformed into BL21(DE3) *E. Coli* cells and after scaling up the bacterial culture, the protein was expressed following induction with 1 mM IPTG. The molecular weight of Nurr1 LBD is approximately 27 kDa and since the His-SUMO tag is around 12 kDa, the molecular weight of the fusion protein would therefore be 39 kDa (Figure 4.1A). Some proteins were observed in the wash 2 (W2) fraction (Figure 4.1A, lane 5) which might be due to a reduced efficiency of the resin or inadequate amount of resin used which led to premature elution of the proteins in the wash fraction. Hence, the W2 and elution fractions were combined and concentrated before going through the PD-10 desalting column to remove imidazole which could affect sumo protease activity.

The His-SUMO tag was then cleaved by overnight incubation at 4 °C with sumo protease enzyme. A second round of Ni^{2+} -NTA affinity chromatography was performed so that the His-SUMO tag can bind to the resin allowing the elution of the cleaved protein in the flow-through (FT) fraction (Figure 4.1B, lane 5). As observed from figure 4.1B, the molecular weight of the protein decreased from around 39 kDa (lane 2) to 27 kDa (lane 3) after removal of the tag which can be collected from the elution fraction (Figure 4.1B, lane 8).

Figure 4.1 Expression and purification of recombinant human Nurr1 LBD

Recombinant His-SUMO fusion Nurr1 LBD (356-598) protein was expressed in BL21(DE3) *E. Coli* cells and purified through Ni^{2+} -NTA affinity chromatography before the His-Sumo tag was removed from the protein. A second round of purification ensures the purity of the cleaved protein which was purified through the S-200 gel filtration column using FPLC system. **A:** SDS-PAGE analysis of His-SUMO Nurr1 LBD after first Ni^{2+} -NTA purification. Lane 1: Protein ladder; Lane 2: Supernatant of total cell lysate; Lane 3: Flow-through after incubation with beads; Lanes 4-5: Wash fractions; Lanes 6-12: Elution fractions **B:** SDS-PAGE analysis of Nurr1 LBD after cleavage of His-SUMO tag and second Ni^{2+} -NTA purification. Lane 1: Protein ladder; Lane 2: Protein before cleavage; Lane 3: Protein after cleavage; Lane 4: Pre-load sample before binding with resin; Lane 5: Flow-through containing cleaved Nurr1 LBD; Lane 6-7: Wash fractions; Lane 8: Elution fraction **C:** hNurr1 LBD purified further using S-200 gel filtration column. **Insert:** SDS-PAGE analysis of the fractions collected from the peak of the curve. Lane 1: Protein Ladder; Lane 2: Pre-load sample; Lane 3-9: Peak fractions.

To further improve the purity of the protein, the sample went through another round of purification using the FPLC system. The system was fitted with a Sephacryl S-200 gel filtration column which separates proteins by their size. In other words, larger proteins will be eluted first followed by smaller proteins. SDS-PAGE analysis was done after the FPLC run to check the purity of the fractions corresponding to the peak of the curve (Figure 4.1C) before using the protein for downstream experiments.

4.1.2 Expression and purification of ^{15}N -uniformly labeled recombinant Nurr1 LBD

^{15}N -uniformly labeled Nurr1 LBD protein was expressed by culturing BL21(DE3) E. Coli cells in M9 media containing ^{15}N -ammonium chloride as the sole nitrogen source. Since the growth of the bacteria are slower in M9 compared to LB media, the 2L culture was divided into 4 flasks of 500 ml culture for better growth. Protein expression was confirmed using SDS-PAGE following induction with 1 mM IPTG (Figure 4.2A). The purification steps are the same as that of unlabeled protein and a comparable yield was likewise obtained.

Figure 4.2 Expression and purification of ^{15}N -uniformly labeled Nurr1 LBD for NMR studies

Figure 4.2 (continued)

Expression and purification of ^{15}N -uniformly labeled recombinant His-SUMO fusion Nurr1 LBD (356-598) protein in BL21(DE3) *E. Coli* cells and purified using the same method for unlabeled protein **A:** SDS-PAGE analysis of 4 sets of 500 ml M9 culture of bacterial cells. Lane 1: Protein ladder; Lanes 2-9: Cultures before and after induction; I⁻: Before induction; I⁺: After induction **B:** SDS-PAGE analysis of ^{15}N -hNurr1 LBD after Ni^{2+} -NTA purification of 1L culture. Lane 1: Protein ladder; Lanes 2 and 6: Supernatant of total cell lysate; Lane 3 and 7: Flow-through after incubation with beads; Lanes 4 and 8: Wash fraction 2; Lanes 5 and 9: Elution fractions **C:** SDS-PAGE analysis of ^{15}N -Nurr1 LBD following cleavage of His-SUMO tag from 2L cultures combined. Lane 1: Protein before cleavage; Lane 2: Protein after cleavage; Lane 3: Protein ladder; Lane 4: Pre-load sample before binding with beads; Lane 5: Flow-through containing cleaved ^{15}N -Nurr1 LBD; Lane 6-7: Wash fractions; Lane 8: Elution fraction **D:** SDS-PAGE analysis of fractions containing purified ^{15}N -Nurr1 LBD using S-200 gel filtration column. Lane 1: Protein Ladder; Lane 2: Pre-load sample; Lane 3-10: Peak fractions.

Fractions that were most pure such as those from lanes 3-8 (Figure 4.2D) were pooled for subsequent NMR studies.

4.1.3 2D HSQC experiments for studying interaction of prostaglandins with Nurr1 LBD

After obtaining pure ^{15}N -Nurr1 LBD protein, 2D ^1H - ^{15}N transverse relaxation optimized spectroscopy (TROSY)-HSQC experiment for apo Nurr1 LBD was performed by Dr. Shin Joon. TROSY-HSQC is usually performed for large molecules or complexes due to shorter T_2 relaxation times that may lead to signal broadening and poor resolution from different relaxation rates. TROSY allows the cancelling of transverse relaxation and selects only the sharpest peak. The 2D spectrum comprised of peaks belonging to the backbone amide of all non-proline amino acid residues. The labeling of peaks in this and subsequent spectra followed the assigned TROSY spectrum previously done by Dr. Shin Joon. The spectrum for apo Nurr1 LBD (Figure 4.3) showed a good dispersion of the peaks indicating that the protein was properly folded. Subsequent 2D HSQC experiments involving prostaglandins were performed with the help of Dr. Ye Hong. For these titration experiments, HSQC instead of TROSY-HSQC was performed since the protein is not ^2H labelled; TROSY will also take a longer acquisition time compared to HSQC for the same S/N ratio and in our case, TROSY-HSQC also did not show any superiority over HSQC alone.

Figure 4.3 2D ^1H - ^{15}N TROSY-HSQC spectrum of ^{15}N uniformly labeled Nurr1 LBD

The TROSY-HSQC spectrum of 0.2 mM ^{15}N - Nurr1 LBD showed well dispersion of the peaks indicating that the protein is properly folded. Three peaks downfield on the ^1H axis, were omitted in the figure (but included in analysis) due to a large space upfield of the ^{15}N axis which as a result, shrank the size of majority of the peaks. Hence, only the main spectrum containing most of the peaks are shown. For the full spectrum, please refer to appendix III.

4.1.3.1 Interaction of PGA2 with Nurr1 LBD

The NMR spectra of Nurr1 LBD with PGA2 (1:1 and 1:10) when overlaid with the spectrum of apo Nurr1 LBD followed an intermediate exchange regime on the NMR time-scale which signified moderate affinity between PGA2 and Nurr1 LBD (Figure 4.4A). The weighted chemical shift perturbations (CSP) from assigned residues on the overlaid spectra (apo vs 1:10) were analysed (Figure 4.4B). The strongest CSP came from residues Phe592, Asp594, Thr595 and Leu596 found on helix H12 while moderate shifts came from residues nearby such as Lys590 on helix H12, Gly569 and Arg572 on helix H11 and Ser441 on helix H4 (Figure 4.4B and C). This data confirmed that PGA2 can bind to Nurr1 LBD, of which the region perturbed matched the location revealed from the crystallized structure of Nurr1

LBD with PGA2. Even though our crystallization data showed the presence of a Michael adduct between Cys566 of Nurr1 LBD and the cyclopentenone ring of PGA2 and PGA1, we were previously unable to assign this residue and hence, we are only able to observe chemical shifts of assigned residues in proximity. We also observed CSP from residues away from the binding site which could be due to ligand-induced conformational change.

When a protein undergoes conformational change due to direct or indirect interaction with a ligand, the measure of affinity can be determined by the rate of chemical exchange on the NMR timescale. The rate constants for the forward and backward reactions between a protein P and a ligand L defined by $P + L \leftrightarrow PL$ is depicted by K_{on} and K_{off} respectively. Upon ligand titration, if K_{off} is slower than the chemical shift difference between the free and bound protein, the peak corresponding to the free protein will slowly disappear while the bound protein peak will gradually appear. The intensities of the peaks are proportional to the concentrations of free or bound proteins. If the chemical exchange is fast (usually milliseconds), when K_{off} is greater than the chemical shift difference, the peak will show a gradual shift during the titration and the frequency of the signal is the weighted average of the shifts between the free and bound spectra (Williamson, 2013). Slow exchange occurs if the ligand is binding to the protein with high affinity, while fast exchange occurs if the binding affinity is weak. Sometimes, when the exchange rate is comparable to the chemical shift difference, the signals may broaden and shift, showing an intermediate exchange regime.

Figure 4.4 2D ${}^1\text{H}$ - ${}^{15}\text{N}$ HSQC experiment on Nurr1 LBD with PGA2

A: Above: Spectra overlay of 0.2mM apo Nurr1 LBD (red) and 0.2mM Nurr1 LBD with PGA2 (1:1, green; 1:10; blue) **Below:** Close-up of boxed sections on the spectra overlay showing peaks that shifted as a result of PGA2 binding. **B (next page):** Chemical shift perturbation plot revealing residues with perturbed resonances. Notable residues include F592, D594, T595 and K596 in H12, and G557 from H11, as indicated. The blue dotted line marks the threshold for $\Delta\delta > 0.025$ for mapping of the residues on the protein. **C (next page):** Mapping of perturbed residues on crystal structure of Nurr1 LBD (PDB: 1OVL) with a close-up section showing affected helices H11, H12, and H4 with amino acid residues indicated. Residues highlighted in red denotes $\Delta\delta > 0.025$.

Figure 4.4 (continued)

4.1.3.2 Interaction of PGE2 with Nurr1 LBD

The NMR spectra for Nurr1 LBD with PGE2 when overlaid (apo, 1:5 and 1:10) similarly revealed perturbations from residues Leu593, Asp594 and Thr595 on helix H12 and Leu444 on helix H4 (Figure 4.5A-C). Residues nearby such as Phe592 and Leu596 on helix H12 and Leu562 on H10 also showed moderate CSP. The localized region affected was similar to that upon PGA2 binding although the binding affinity instead followed a fast-exchange regime indicating a weaker affinity. This could be due to the lack of the covalent bond we observed between PGA2 and Cys566 on Nurr1 LBD which reduced the strength of the interaction.

Figure 4.5 2D ^1H - ^{15}N HSQC experiment on Nurr1 LBD with PGE2

A: Spectra overlay of 0.2mM apo Nurr1 LBD (red) and 0.2mM Nurr1 LBD with PGE2 (1:5, green; 1:10; blue)

B (next page): Chemical shift perturbation plot revealing residues with perturbed resonances. Notable residues include T595 in H12, G557 from H11, and L444 from H4 as indicated. The blue dotted line marks the threshold for $\Delta\delta > 0.024$ for mapping of the residues on the protein. **C (next page):** Mapping of perturbed residues on crystal structure of Nurr1 LBD (PDB: 1OVL) with a close-up section showing affected helices H11, H12, and H4 with amino acid residues indicated. Residues highlighted in red denotes $\Delta\delta > 0.024$.

Figure 4.5 (continued)

4.1.3.3 Interaction of PGA2 and AQ with Nurr1 LBD

As the binding site of PGA2 and PGE2 is near the putative AQ binding site previously reported (Kim et al., 2015), we hypothesized that the binding of PGA2 might facilitate or increase the binding affinity for AQ. This could also aid in our lead compound optimization by chemically linking PGA2/PGE2 and the 4-amino-7-chloroquinoline moiety. We first prepared the Nurr1 LBD-PGA2 saturated complex by incubating Nurr1 LBD with PGA2 (1:10) at 4 °C overnight before adding 1:1 and 1:5 AQ to the sample the next day. When AQ was added to Nurr1 LBD-PGA2 complex, two localized regions showed very strong perturbation as evidenced by intermediate-slow exchange regime observed (Figure 4.6A) indicating strong binding affinity. One of the regions was located at the observed PGA2 binding region on helices H12, H11 and H4 with residues Gln568, Gly569, Gln571 and Arg572 on H11, Ile588, Leu591 and F598 on H12 and Ser441 on H4 perturbed (Figure 4.6B-C). The second region greatly affected was the putative AQ binding site. Interestingly, the residues that were previously reported to have shifted (Kim et al., 2015), such as His402, Asp408 and L409 on helix H3, have now disappeared, suggesting stronger binding at this region compared to AQ binding alone on the LBD. The peaks for Ile403 and Gln404 have similarly disappeared while Gln405 also showed strong binding as with AQ binding alone.

Figure 4.6 2D ^1H - ^{15}N HSQC experiment on Nurr1 LBD-PGA2 complex with AQ

A: Above: Spectra overlay of 0.2mM Nurr1 LBD-PGA2 complex 1:10 (red) with AQ (1:1, green; 1:5; blue). **Below:** Close-up of boxed section on the spectra overlay showing peaks that shifted as a result of AQ binding on the Nurr1 LBD-PGA2 complex. **B (next page):** Chemical shift perturbation plot revealing residues with perturbed resonances. Notable residues include D594 and F598 in H12, Q405 from H3 and S441 from H4 as indicated. (*) denotes peaks (or peaks in the region) which have disappeared on the spectrum signifying strong binding. The blue dotted line marks the threshold for $\Delta\delta > 0.05$ for mapping of the residues on the protein. **C (two pages down):** Mapping of perturbed residues on crystal structure of Nurr1 LBD (PDB: 1OVL) showing affected regions with amino acid residues indicated. Residues highlighted in red denotes $\Delta\delta > 0.05$ while residues highlighted in blue denotes disappeared peaks on the spectrum.

B

C

Figure 4.6 (continued)

4.1.3.4 Interaction of PGE2 and AQ with Nurr1 LBD

As PGE2 does not form covalent bond with Nurr1 LBD and the binding affinity is weaker than PGA2 binding, we were also interested to investigate the changes on the Nurr1 LBD-PGE2 saturated complex upon the addition of 1:1 and 1:5 AQ. The condition and drug concentrations were the same as with Nurr1 LBD-PGA2 complex. Again, the binding affinity of AQ observed with the complex showed an intermediate-slow exchange regime (Figure 4.7A). Multiple residues in helix H12 (Ile587-Leu591, Asp594 and Phe598) as well as helix H3 at the putative AQ binding site (Gln401-Gln405, Asp408-Leu410) have also disappeared including other regions nearby such as Gln568, Gly569, Gln571, Arg572 and Ile574 in helix H11 indicating strong binding/perturbations at these regions (Figure 4.7B-C). Peaks with CSP values >0.05 were also located at these helices.

Figure 4.7 2D ^1H - ^{15}N HSQC experiment on Nurr1 LBD-PGE2 complex with AQ

A: Above: Spectra overlay of 0.2mM Nurr1 LBD-PGE2 complex 1:10 (red) with AQ (1:1, green; 1:5; blue). **Below (next page):** Close-up of boxed section on the spectra overlay showing peaks that shifted as a result of AQ binding on the Nurr1 LBD-PGE2 complex. **B (next page):** Chemical shift perturbation plot revealing residues with perturbed resonances. Notable residues include T595 from H12 and S441 from H4 as indicated. (*) denotes peaks (or peaks in the region) which have disappeared on the spectrum signifying strong binding. The blue dotted line marks the threshold for $\Delta\delta > 0.05$ for mapping of the residues on the protein. **C: Above (two pages down):** Mapping of perturbed residues on crystal structure of Nurr1 LBD (PDB: 1OVL) showing affected regions with amino acid residues indicated. Residues highlighted in red denotes $\Delta\delta > 0.05$ while residues highlighted in blue denotes disappeared peaks on the spectrum. **Below (two pages down):** Cartoon representation showing helices involved following addition of AQ to Nurr1 LBD-PGE2 complex. The regions perturbed were similarly affected

A (continued)

B

Figure 4.7 (continued)

c

Figure 4.7 (continued)

4.1.3.5 Interaction of PGA1 with Nurr1 LBD

The structure of PGA1 differs from that of PGA2 by one less double bond but retains the same cyclopentenone ring. As such, C11 of PGA1 is also able to form a Michael adduct with Cys566 of Nurr1 LBD that was observed from our crystallization data. When PGA1 binding on Nurr1 LBD was examined using NMR (apo, 1:5 and 1:10), the binding affected similar residues mainly on helices H10-H12 and helices H3 and H4 (Figure 4.8C). The binding affinity also showed an intermediate-slow exchange regime signifying strong binding between PGA1 and Nurr1 LBD (Figure 4.8A). Residues that were affected included Phe592, Leu593, Thr595 in H12, Ser553, Lys554, Gly557, Thr564, and Phe574 in helices H10 and H11. Peaks which disappeared included Ser375, Leu410, Lys530 and Leu578 (Figure 4.8B). In addition, there were also peaks that showed reduced intensity such as Leu562, Gln568, Asp580 and Ile588 from helices H10-H11 and also residues His402, Ile403, Leu409 and Thr411 on helix H3 at the putative AQ binding site signifying possible allosteric effect on this region upon PGA1 binding.

Figure 4.8 2D ^1H - ^{15}N HSQC experiment on Nurr1 LBD with PGA1

A: Above: Spectra overlay of 0.2mM apo Nurr1 LBD (red) and 0.2mM Nurr1 LBD with PGA1 (1:5, green; 1:10; blue) **Below:** Close-up of boxed section on the spectra overlay showing peaks that shifted as a result of PGA1 binding. **B (next page):** Chemical shift perturbation plot revealing residues with perturbed resonances. Notable residues include W420 in H3, L444 in H4, D484 and S485 in H7, as indicated. (*) denotes peaks (or peaks in the region) which have disappeared on the spectrum signifying strong binding. The blue dotted line marks the threshold for $\Delta\delta > 0.02$ for mapping of the residues on the protein. **C (next page):** Mapping of perturbed residues on crystal structure of Nurr1 LBD (PDB: 1OVL) with a close-up section showing affected helices H12, H4 and H11 with amino acid residues indicated. Residues highlighted in red denotes $\Delta\delta > 0.02$ while peaks which have disappeared are colored in blue.

Figure 4.8 (continued)

4.1.3.6 Effect of pH on the molecular interaction of PGA1 with Nurr1 LBD

In our continued efforts to optimize and accelerate the rate of covalent bond formation *in vitro* between Nurr1 LBD and PGA1, Mr. Yoo Jun Yeob and Dr. Serap Beldar, found that their interaction is more efficient at pH 8.0 compared to pH 7.5 (38 % and 30 %, respectively) at 18 °C and this was also after a three-day incubation. So far, the NMR samples were not incubated for a period of time prior to measuring, except to obtain the Nurr1 LBD-PGA2/PGE2 complexes which were incubated overnight at 4 °C. Following this observation, we wanted to determine the structural changes of Nurr1 LBD following the binding of PGA1 at pH 8.0 for three days as compared to pH 7.5 with no incubation. Prior to this, we first examined the structure and peaks for Nurr1 LBD at pH 8.0 and found that there were minimal shifts in a few peaks and in general, all the peaks were able to be labelled based on the spectrum at pH 7.5 (Figure 4.9A). We then proceeded with PGA1 titration on Nurr1 LBD at pH 8.0 (1:2, 1:4, 1:10) that was incubated for three days at 18 °C before 2D HSQC NMR was carried out. From the overlaid NMR spectra (Figure 4.9B), the binding of PGA1 on Nurr1 LBD at pH 8.0 showed a slow-exchange regime that indicated stronger binding than PGA1 on Nurr1 LBD at pH 7.5. Resonance line broadening was observed with increasing PGA1 concentration resulting in chemical shifts and reduction in peak intensity of multiple residues (Figure 4.9B). Besides plotting the CSP values of shifted peaks (Figure 4.9C), we also quantified changes in normalized peak intensity of all assigned residues (Figure 4.9D) and mapped the affected residues on the crystal structure of Nurr1 LBD (PDB: 1OVL; Figure 4.9F). Strong perturbations were observed from residues in nearly the entire helix H3 (Figure 4.9F, left) particularly at the putative AQ binding site consisting of residues from Gln401-Gln405, Asp408 and Leu 409 of which their corresponding peaks have mostly disappeared or shifted. On the other side of the protein (Figure 4.9F, right), there were intensity changes from residues such as Leu591, Phe592, Asp594 and Thr595 from helix H12, Gln568 and Gly569 from helix H11 and multiple residues stretching across helix H7 such as Asp484, Ser485, Glu484, Ser491 and Asn492. Residues such as Ala367, Leu368, Ala371, Asp374 and Asn376 from helix H1 were also perturbed, likely induced by motions from helix H3. Other perturbations that were scattered around the protein are also likely to be ligand-induced conformational changes. A higher

concentration of PGA1 was attempted (1:30) to reach saturation but the sample precipitated badly.

Figure 4.9 2D ^1H - ^{15}N HSQC experiment on Nurr1 LBD with PGA1 at pH 8.0

A: Spectra overlay of 0.2mM apo Nurr1-LBD at pH 7.5 (red) and pH 8.0 (blue). **B (next page): Above:** 0.2mM Nurr1-LBD with PGA1 after three-day incubation (apo, red; 1:2, green, 1:4, blue; 1:10; purple). **Below:** Close-up of boxed section on the spectra overlay showing peaks that shifted or show intensity changes as a result of PGA1 binding. **C (two pages down):** Chemical shift perturbation plot revealing residues with perturbed resonances. Notable residues include F427, I506 and E526 as indicated. (*) denotes peaks (or peaks in the region) which have disappeared on the spectrum signifying strong binding. The blue dotted line marks the threshold for $\Delta\delta > 0.05$ for mapping of the residues on the protein. **D (two pages down):** Quantification of normalized peak intensities of PGA1 titrations against apo Nurr1 LBD. The blue dotted line indicates the threshold of 0.27, which is the standard deviation of all peak intensities at 1:10 Nurr1-LBD:PGA. **E (three pages down):** Mapping of perturbed residues on crystal structure of Nurr1-LBD (PDB: 1OVL) showing affected regions with amino acid residues indicated. Residues highlighted in red denotes $\Delta\delta > 0.05$ while peaks which have disappeared are colored in blue. Peaks affected by intensity changes are highlighted in green while those that were affected by intensity and then disappeared are colored in purple.

Figure 4.9 (continued)

Figure 4.9 (continued)

E

Figure 4.9 (continued)

4.1.3.7 Interaction of PGE1 with Nurr1 LBD

Since PGE1 shares the same structure as PGE2 other than having one less double bond, we expected the binding of PGE1 to be similar to PGE2 binding on Nurr1 LBD. From our NMR data (apo, 1:4 and 1:10), we observed that PGE1 binding followed a fast-exchange regime as expected (Figure 4.10A) signifying weaker binding as compared to PGA1. Residues that were affected by PGE1 binding were those from helices H10-H12 such as Lys590, Phe592-Thr595 from helix H12, Ser553, Lys 554, Gly557, Thr564 and Q571 from helices H10- H11. The peak corresponding to Ser375 disappeared upon PGE1 binding (Figure 4.10B-C). This residue is found at the end of helix H1 before the loop, which could have accounted for its flexibility and strong perturbation.

Figure 4.10 2D ^1H - ^{15}N HSQC experiment on Nurr1 LBD with PGE1

A: Above: Spectra overlay of 0.2mM apo Nurr1-LBD (red) and 0.2mM Nurr1-LBD with PGE1 (1:4, green; 1:10; blue) **Below (next page):** Close-up of boxed section on the spectra overlay showing peaks that shifted as a result of PGE1 binding. **B (next page):** Chemical shift perturbation plot revealing residues with perturbed resonances. Notable residues include G426 in H3, K554 in H10, and L593, D594 and T595 from H12, as indicated. (*) denotes peaks (or peaks in the region) which have disappeared on the spectrum signifying strong binding. The blue dotted line marks the threshold for $\Delta\delta > 0.018$ for mapping of the residues on the protein. **C (two pages down):** Mapping of perturbed residues on crystal structure of Nurr1-LBD (PDB: 1OVL) with a close-up section showing affected helices H12, H4 and H11 with amino acid residues indicated. Residues highlighted in red denotes $\Delta\delta > 0.018$ while the peak which has disappeared is colored in blue.

A (continued)

Figure 4.10 (continued)

Figure 4.10 (continued)

4.1.4 Mass spectrometry analysis on the interaction of prostaglandins with Nurr1 LBD

Nurr1 LBD (residues 328-598) was purified by Dr. Sreekanth Rajan and sent to the School of Biological Sciences Mass Spectrometry facility for Top-Down Mass Spectrometry analysis. 300 μ M of protein was prepared in 0.1 % formic acid in water and analysed using a Dionex UltiMate 3000 UHPLC coupled to a linear quadrupole ion trap-Fourier transform Ultra apparatus (LTQ-FT Ultra, Thermo Scientific Inc, Germany). All the PGs were incubated overnight at 1:1.5 molar ratio (protein:ligand). A protein-ligand complex peak was present for PGA1/PGA2 bound Nurr1 LBD which also correlated with the combined molecular weight of PGA1/PGA2 and Nurr1 LBD (336.5/334.5 + 30500 Da) (Figure 4.11). This showed that PGA1/PGA2 formed a covalent bond with Nurr1 LBD. In contrast, the absence of a protein-ligand complex peak when PGE1/PGE2 was added to Nurr1 LBD demonstrated the lack of covalent bonding between them (Figure 4.11).

Figure 4.11 Mass spectrometry confirms covalent bonding of PGA1/PGA2 with Nurr1 LBD

The mass spectrometry data for apo (**Top**) and PGA1/PGA2 bound (**Bottom**) with Nurr1-LBD³²⁸⁻⁵⁹⁸ reveals the presence of the protein-ligand complex peak due to covalent bonding, with a combined molecular mass of 30862 Da (Nurr1-LBD³²⁸⁻⁵⁹⁸ is 30.5 kDa; PGA1 is 336.5 Da and PGA2 is 334.5 Da). On the contrary, PGE1/PGE2 that does not form a covalent bond with Nurr1 LBD, did not show any presence of the protein-ligand complex peak (**Middle**).

4.2 Cellular studies for Nurrl transcriptional activity

4.2.1 Cytotoxicity screening of prostaglandins on mammalian cell lines

Before embarking on the luciferase assay to determine the effect of PGs treatment on Nurrl transcriptional activity, their cytotoxicity profile must be assessed first. Human embryonic kidney HEK293T cells and the human neuroblastoma SK-N-BE(2)C cells were treated with varying concentrations of PGs for 24 hours before measuring absorbance using WST-1 reagent to assay cell viability (Figure 4.12). For PGA1 and PGA2, the concentrations used ranged from 100 nM to 100 μ M while for PGE1 and PGE2, the concentrations ranged from 100 nM to 1 mM. Our data showed that PGA1 and PGA2 treatment was more toxic to both types of cells as compared to PGE1 and PGE2 treatment as cell viability began to decrease when cells were treated with 50 μ M of PGA1 or PGA2 (Figure 4.12). However, it appears that the PGs were more toxic to SK-N-BE(2)C cells as compared to HEK293T (Figure 4.12B). 20 μ M PGA1 led to approximately 50 % cell viability which only occurred on HEK293T cells at around 50 μ M. PGA2 was also more toxic to SK-N-BE(2)C cells; 0.1 μ M of PGA2 resulted in close to 61 % cell viability which decreased further to 17 % at 50 μ M (Figure 4.12B). On the other hand, there was a decrease in cell viability in both cell lines only when PGE1 and PGE2 concentrations were 500 μ M (Figure 4.12).

Figure 4.12 Cytotoxicity profile of prostaglandins on HEK293T and SK-N-BE(2)C cells
 HEK293T cells (A) and SK-N-BE(2)C cells (B) were treated with varying doses of prostaglandins for 24 hours to examine cytotoxic effects on cells. Drug-vehicle treated (DMSO) wells are shown as the first bar on the graph (0 μM) while treated wells are indicated by their concentrations. Values represent means ± SEM from three independent experiments performed in triplicate measurements. One-way ANOVA followed by Dunnett's post-hoc analysis was used to compare treated with untreated cells. *: 0.01 < p < 0.05, **: 0.001 < p < 0.01, ***: 0.0001 < p < 0.001.

4.2.2 Luciferase assay to determine Nurr1 activity

To set up the luciferase assay, the first step was to co-transfect the three plasmids involved into SK-N-BE(2)C cells. One of them is the expression vector pcDNA 3.1 Myc/His containing full-length mouse Nurr1 (FL mNurr1; 99.5 % sequence similarity with human Nurr1), the second is the pGL3-basic vector with 4xNL3 containing the canonical NBRE sequence inserted 52 bp upstream of the firefly *luciferase* reporter gene; the last is the pRL-null vector that codes for Renilla luciferase as an internal control for correcting transfection efficiency. An initial optimization for the ratio of plasmids to be transfected was determined to be 8:1:1 (expression:reporter:control; data not shown). From Figure 4.13, treatment with 50 μ M of the control drug AQ showed an increase in Nurr1 transcriptional activity by approximately 2.3-fold ($0.01 < p < 0.05$) as compared to untreated cells. Treatment with 10 μ M of PGs showed significant increase in Nurr1 activity by 3.5-3.9-fold ($0.0001 < p < 0.001$).

Figure 4.13 Transcriptional activity of Nurr1 following prostaglandins treatment

Luciferase report assay was performed using full-length Nurr1 transfected into SK-N-BE(2)C cells followed by treatment with PGs and subsequent luciferase activity measurement. AQ was also used as control. Untreated wells with only drug-vehicle (DMSO) and shown as the first bar on the graph (0 μ M) while treated wells are indicated by their concentrations. Values represent means $\pm$ SEM from two independent experiments performed in duplicate measurements. One-way ANOVA followed by Dunnett's post-hoc analysis was used to compare treated with untreated cells. *: $0.01 < p < 0.05$, ***: $0.0001 < p < 0.001$.

4.3 *Drosophila Melanogaster* LRRK2 GS model for screening of prostaglandins

4.3.1 Prostaglandins pose minimal toxicity on LRRK2-GS and WT flies

Survival curves for LRRK2-GS treated flies showed that treatments with 50 μ M PGA2, PGE2 and AQ improved overall survival of the flies as compared to vehicle-treated LRRK2-GS flies (Figure 4.14). 50 μ M PGA1 and PGE1 showed slightly lower overall survival which could be attributed to the toxicity of the drugs on the flies as compared to PGA2 and PGE2. LRRK2-WT flies showed the lowest overall survival among the tested groups despite producing the best climbing score (Figure 4.15). Overall, the survival rate for all tested groups did not fall below 50 % at the end of the experiment. A one-way ANOVA analysis was also carried out followed by Tukey's multiple comparison test to compare the means between each tested group (data not shown). LRRK2 GS vehicle-treated and drug-treated groups showed no difference while there was significant difference between LRRK2 WT and LRRK2 GS flies (data not shown; $0.0001 < p < 0.001$).

Figure 4.14 Survival curves for LRRK2-WT and LRRK2-GS flies

Despite differing climbing scores, treatment with PGA2 and PGE2 was able to improve the survival of LRRK2-GS flies as compared to vehicle-treated flies. AQ treated flies also showed likewise, better survival compared to vehicle-treated flies. The overall survival of PGA1 and PGE1-treated LRRK2-GS flies showed a slightly lower trend compared to vehicle-treated flies. LRRK2-WT flies showed the lowest overall survival rate despite having the best climbing score. None of the tested groups had a survival rate below 50 % at the end of the experiment ($n=6$ independent experiments). One-way ANOVA analysis was carried out followed by Tukey's multiple comparison test to compare the means between each group. LRRK2 GS vehicle-treated and drug-treated groups showed no difference, while WT and GS vehicle-treated showed significant difference between the means (data not shown; $***:0.0001 < p < 0.001$).

4.3.2 Prostaglandins rescue the locomotor deficits of LRRK2-GS flies

Negative geotaxis assays (climbing assays) were performed to determine locomotor function of the flies upon drug treatment. The resulting climbing score for LRRK-WT flies was 53.1 % while the score for LRRK2-GS flies was 15 % indicating locomotor impairments associated with the GS mutation ($0.001 < p < 0.01$) (Figure 4.15). AQ served as a positive control for our assay and was able to increase the climbing score by a percentage difference of >30 % to achieve 48.7 % when compared with vehicle-treated LRRK2-GS flies ($0.01 < p < 0.05$). Climbing score for LRRK2-GS flies treated with PGA1 and PGA2 similarly increased by a percentage difference of >30 % to achieve 46.4 % and 48.2 % ($0.01 < p < 0.05$), respectively. PGE1 and PGE2 treatment was able to increase the score to 32.8 % and 29.1 % respectively, despite being a non-significant increase as compared to PGA1 and PGA2.

Figure 4.15 LRRK2-GS expressing flies treated with prostaglandins and amodiaquine

The climbing ability of LRRK2-GS vehicle-treated (0.5 % ethanol; white column) flies was greatly reduced as compared to LRRK2-WT flies (dark grey column). Treatment with 50 μ M PGA1, PGA2 and AQ was able to significantly rescue the climbing ability of LRRK2-GS flies (light grey columns) as compared to vehicle-treated LRRK2-GS flies. One-way ANOVA analysis was carried out followed by Tukey's multiple comparison test to compare the means between each group: *: $0.01 < p < 0.05$, **: $0.001 < p < 0.01$ and each column represents mean $\pm$ SEM (n=6 independent experiments).

4.3.3 PGA1 and PGA2 ameliorate LRRK2 GS-induced DA neuronal loss

Whole-mount adult fly brains from LRRK2-GS flies (vehicle treated; PGA1 or PGA2 treated) were subjected to immunohistochemical (IHC) analysis and were stained with rabbit anti-TH antibodies. The average neuronal count for LRRK2-GS vehicle-treated flies was 8.25 while treatment with 50 μ M PGA1 or PGA2 showed a higher neuron count of 10.5 and 10.31, respectively (Figure 4.16A), which were significant ($0.0001 < p < 0.001$). As illustrated in representative confocal images (Figure 4.16B), there were PPL1 clusters from the PGA1 or PGA2-treated group that showed considerable amelioration of DA degeneration which could have led to the rescue of their locomotor impairments.

Figure 4.16 PGA1 and PGA2 alleviates DA neuronal degeneration in LRRK2-GS expressing flies

A: DA neuronal count (PPL1 cluster) of LRRK2-GS PGA1 or PGA2-treated flies (grey columns) showed amelioration of the loss of DA neurons compared to LRRK2-GS vehicle-treated flies (0.5 % ethanol; white column). One-way ANOVA analysis was carried out followed by Tukey's multiple comparison test to compare the means between each group: ***: $0.0001 < p < 0.001$ (No. of PPL1 clusters indicated in each column). **B:** Representative confocal microscopy images of TH-positive neurons in the PPL1 cluster (from A) of LRRK2-GS flies that were vehicle-treated or treated with PGA1 or PGA2. Scale bar represents 50 μ m. For more images, please refer to

4.3.4 PGA1 and PGA2 mitigates the reduction in *Dhr38* gene expression

Following the observation that PGA1 and PGA2 were able to alleviate DA neuronal loss, it would be of interest to examine if this was associated with an increase in the expression of DA-related genes. The genes tested included *Dhr38* (*Nurr1* ortholog), *TH*, *Ptx1* (*Pitx3* ortholog), *Vmat*, *Ddc* and *DAT* with *Act5c* (β -*Actin* ortholog) as the reference housekeeping gene. From Figure 4.17, the data showed that in LRRK2 GS expressing flies, the level of *Dhr38* expressed was much lower by 3.6-fold ($0.0001 < p < 0.001$) than in LRRK2 WT expressing flies. Treatment with PGA1 and PGA2 was able to increase the expression by 2.8 and 2.9-fold respectively in LRRK2 GS flies compared to vehicle-treated flies ($0.01 < p < 0.05$). For *TH* expression, a decrease in expression was observed in LRRK2 GS flies as compared to WT (1.4-fold) although it was not deemed as a significant decrease. PGA1 and PGA2 treatment were not able to offer a significant increase in the *TH* expression as well (1.0-fold and 1.3-fold respectively). For *VMAT* expression, there was a 2.0-fold decrease in expression in LRRK2 GS flies as compared to WT flies ($0.01 < p < 0.05$). PGA1 treatment was able to increase the expression by 1.6-fold while PGA2 did not increase the expression further. As for *Ddc* expression, there was no significant difference in the expression between LRRK2 WT and GS. PGA1 and PGA2 treatment also did not lead to a significant increase in the expression. For *DAT*, the expression decreased by 1.6-fold in LRRK2 GS flies as compared to WT. There was only a slight and non-significant increase by 1.2 and 1.3-fold when LRRK2 GS flies were treated with PGA1 and PGA2, respectively. Finally, *Ptx1* expression decreased by 1.8-fold in LRRK2 GS flies compared to WT, although this was not significant. PGA1 and PGA2 treatment was able to increase the expression by 1.6 and 1.4-fold respectively, which were not significant as well.

Figure 4.17 Expression changes in selected genes following PGA1 and PGA2 treatment

The expression of selected genes related to PD were assessed following PGA1 and PGA2 treatment on LRRK-GS flies. LRRK2 GS mutation drastically decreased the expression of *Dhr38* (ortholog of *Nurr1* in flies) as compared to LRRK2 WT flies. Treatment with PGA1 and PGA2 was able to increase the expression of *Dhr38*. One-way ANOVA analysis was carried out followed by Dunnett's post-hoc analysis to compare the means of each group with G2019S group: *: 0.01 < p < 0.05; **: 0.001 < p < 0.01 and each column represents mean $\pm$ SEM (n=2 independent experiments with 10 fly heads/set).

4.4 PGA1 reduces neurotoxicity induced by 6-OHDA in mice

All animal-related work was carried out by Ms. Geraldine Goh Wan Ying with the approval and in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Neuroscience Institute (Protocol TNI 17-01-019). The following results are used with permission from Prof. Lim Kah Leong (NNI). 6-OHDA is a well-known neurotoxin that is widely used to generate unilateral lesions in the nigrostriatal pathway to examine motor deficits associated with PD (Thiele et al., 2012, Tieu, 2011). It is generally stereotactically injected into the substantia nigra, medial forebrain bundle or striatum (Tieu, 2011) to bypass the blood-brain barrier and induce lesions in these specific areas. In this case, the direct injection of 6-OHDA into the striatum on one hemisphere, results in a slower, progressive damage to the nigrostriatal structure (Sauer and Oertel, 1994) over a period of five weeks; the damage to dopaminergic neurons when 6-OHDA is injected into the nigra, for instance, may begin as early as 12 hours (Jeon et al., 1995). Following 6-OHDA induced damage to the nigrostriatal pathway, apomorphine (dopamine receptor agonist) was administered via intraperitoneal (IP) injection to induce asymmetrical rotational behavior and the extent of the damage is associated with the motor deficits (Ungerstedt and Arbuthnott, 1970, Przedborski et al., 1995). From Figure 4.18A, control 6-OHDA lesioned mice treated with drug-vehicle (ethanol) showed an increase in rotations over the 5-week period. The other group of 6-OHDA lesion mice treated with PGA1 via IP injection exhibited a significant reduction in the number of rotations at week 4 and 5 (Figure 4.18A). The immunohistochemical staining for TH in the brains of these mice showed a drastic loss of nigrostriatal TH⁺ dopaminergic neurons in the lesioned hemisphere that was alleviated following PGA1 treatment (Figure 4.18B). Collectively, these results highlighted the efficacy of PGA1 treatment in the mitigation of neurodegeneration in the dopaminergic pathway and the associated locomotor deficits in an established 6-OHDA induced mouse model of PD.

Figure 4.18 PGA1 diminishes rotational deficits and 6-OHDA induced nigrostriatal lesions in mice

A: Number of rotations per minute by 6-OHDA lesioned mice in different treatment groups with each data point representing mean $\pm$ S.E.M. 7.5 μ g of 6-OHDA was injected into the striatum and apomorphine was administered by IP injection (0.5 mg/kg) to induce rotational movements on a weekly basis. Unpaired two-tailed t-test was carried out between 6-OHDA ethanol-treated and PGA1-treated groups: *:0.01<p<0.05 **B:** Coronal sections of immunohistochemical staining for TH⁺ expression in the striatum and substantia nigra of C57BL/6 Tac mouse. The number of mouse brains in each group correspond accordingly to those in **A** and TH was detected using anti-TH antibody. Results presented with permission from Prof. Lim Kah Leong (NNI).

Chapter 5: Discussion

5. Discussion

5.1 Prostaglandins show direct interaction with Nurr1 LBD

When Nurr1 LBD was co-crystallized with the cyclopentanone prostaglandins PGE1/PGE2, they were spontaneously dehydrated under the crystallization conditions (MES buffer pH 5.5) to their dehydrated metabolites PGA1 and PGA2 (Figure 1.6). The cyclopentenone nucleus of PGA1/PGA2 contains an α,β -unsaturated carbonyl group which is prone to nucleophilic attack from deprotonated sulfhydryl group of Cys residues in proteins, to form a covalent bond known as Michael adduct (Honn and Marnett, 1985, Straus and Glass, 2001). The co-crystallized structure of Nurr1 LBD with PGA1/PGA2 was highly identical to the one obtained with PGE1/PGE2 that was dehydrated. From Figure 5.1 (using PGA1 as reference), PGA1/PGA2 was revealed to bind in a cavity flanked by helices H4, H11 and H12 with one of its hydrophobic tails inserted deeply into a narrow hydrophobic cavity while the other carboxylic-acid bearing tail (C1 end) is exposed to the solvent oriented toward the H9-H11 wedge. The hydrophobic cavity encompassing the tail (C20 end) is lined with residues Phe443, Leu444 from helix H4, Leu570 and Ile573 from helix H11 and Ile588, Leu591 and Phe592 from helix H12. PGA1/PGA2 is stabilized by hydrogen bonds made by the hydroxyl oxygen attached to C15 with Glu440 and Leu444, and the cyclopentenone ring ketone with Thr567 and Arg563 residues. The C20 end of PGA1/PGA2 also anchors itself through hydrophobic contacts with residues such as Leu440 and Ser441 from Helix H4, Leu570 and Ile573 from helix H11 and Leu591 and Thr595 from helix H12. The most striking observation was the presence of a covalent bond between carbon C11 in the cyclopentenone ring of PGA1/PGA2 with the sulfhydryl group of Cys566 through Michael addition which also flipped the side chain of Cys566 by $\sim 115^\circ$ as compared to the apo form (Figure 5.1). Mass spectrometry further confirmed the covalent conjugation of PGA1/PGA2 with Nurr1 LBD which was not observed with PGE1/PGE2 binding.

Furthermore, the AF-2 region on the H12 helix, was also found to be reoriented with respect to the protein core, by an angle of 21° which disrupted several charged interactions such as the salt-bridge between Glu440 (H4) and Lys590 (H12) and another salt-bridge between

Lys590 and Asp594 on helix H12. An expansion in the H9-H11 wedge region was observed that is partially concealed in the apo form due to the orientation of Glu514 and Arg563 sidechains toward each other but was disrupted due to PGA1/PGA2 binding. Structural expansions such as these have been known to occur in classical NR to facilitate the binding of co-regulators (Gronemeyer et al., 2004, Li et al., 2003, Rastinejad et al., 2013). Altogether, PGA1/PGA2 binding affected the electrostatic surface potential in the AF-2 region which led to dynamic motions in helix H12 but surprisingly, only three residues corresponding to the classical active site were found to interact with PGA1 (Leu444, Ile573 and Leu591) with only Leu591 undergoing conformational changes suggesting that Nurr1 activation is mainly regulated by the orientation of H12 (Figure 5.1).

Figure 5.1 Crystal structure of PGA1 bound Nurr1 LBD

A: Cartoon representation of Nurr1 LBD (green) with PGA1 shown in sphere mode. **B:** The 2Fo-Fc electron density map (in pink) contoured at 1 σ cut-off reveals the covalent bond (within black circle) between the sulphur of Cys566 and cyclopentenone C11 of PGA1, and the well-ordered PGA1 packed between helices H4, H11 and H12. **C:** Interactions between PGA1 and Nurr1 residues (labelled) through hydrophobic contacts (black broken lines) and hydrogen bonds (blue broken lines). Images kindly provided by Dr. Sreekanth Rajan.

NMR spectroscopy was thus performed on Nurr1 LBD following PGs binding to further corroborate our crystallization data. As mentioned, we were previously unable to assign Cys566 on apo Nurr1 LBD, therefore only the CSP or intensity changes on assigned residues in the vicinity were observed. When Nurr1 LBD was tested with PGA2, the strongest CSP were from residues found in helix H12, such as Phe592, Asp594-Leu596 followed by moderate shifts from Lys590 also on H12 which was shown to be disrupted from its salt-bridge interaction with Glu440 and Asp594. Arg515 was also perturbed and was shown to stabilize the carboxyl tail of PGA2 in the co-crystal structure while Ser441 from H4 which makes hydrophobic contact with PGA2 was similarly perturbed. As for

PGE2 binding on Nurr1 LBD, the binding affinity is weaker compared to PGA2 binding as the CSP values were lower and the exchange rate between free and bound was also fast. Despite this, the strongest perturbations were again from residues from helix H12 such as Leu593, Asp594 and Thr595 and also Leu444 from helix H4. Moderate shift was observed from Phe592 and Leu596 from helix H12 and Gly557 and Leu562 from helix H10. This suggests that PGE2 binding also led to H12 movements either due to direct interaction with PGE2 or ligand-induced conformational change.

The binding of PGA1 on Nurr1 LBD was shown to be stronger than PGA2 binding and followed an intermediate-slow exchange regime. There were more peaks which shifted or disappeared as compared to PGA2 binding even though both formed the Michael adduct at Cys566 shown on the crystallization data. The strongest CSP was from Trp420 and Leu444, of which the latter was shown to interact with PGA1 through hydrogen bonding between the main chain nitrogen and hydroxyl oxygen on PGA1. The regions/helices that were affected the most were helices H10-12 and H3-4. In particular, peaks corresponding to Leu578 and Leu410 have disappeared in the spectrum upon PGA1 binding. Peaks such as Leu578 is located at the end of H11 and could have been perturbed greatly owing to its flexibility and proximity to H12 which could be induced by its movement. Peaks corresponding to Leu562, Gln568, Asp580 and Ile588 from helices H10-H11 also showed intensity changes. Leu410 is located close to the putative AQ binding site; chemical shifts and intensity changes from residues His402-Gln404, Leu409-Thr411 at the AQ binding site were also observed, signifying that PGA1 binding could induce structural changes at this location since it is behind the putative LBP, or it could be an allosteric effect due to the major conformational changes occurring from ligand binding. As for PGE1, the binding is expected to follow that of PGE2 since they share the same cyclopentanone ring, except that PGE1 has one less double bond in its carboxyl-bearing tail. The NMR data for PGE1 showed that the strongest CSP was from the helix H12 of which nearly the entire helix was affected. Helix H10-11 was also affected from residues such as Ser553, Lys554, Gly557, and Thr564. The peak corresponding to Ser375 disappeared upon PGE1 binding and as this residue is located at the end of helix H1 before the loop, which is a flexible region in the crystal structure, it could have accounted for its flexibility.

Since the binding site of PGA1/PGA2 and PGE1/PGE2 consisting of helices H4, H11 and H12, is located very close to the putative AQ binding site, we hypothesized that the binding of PGA2 might affect the AQ binding site and could facilitate or increase AQ binding affinity. When AQ was added to Nurr1 LBD-PGA2 complex, two localized regions showed very strong perturbations. One region was located at the PGs binding region surrounded by helices H4, H11 and H12 with perturbed residues such as Ser441 (H4), Gln568, Gly569, Gln571 and Arg572 (H11) and Ile588, Leu591 and F598 (H12). The second region strongly affected was the putative AQ binding site which have now shown stronger binding affinity for AQ compared to AQ binding alone as previously reported (Kim et al., 2015). Peaks corresponding to His402, Asp408 and L409 on helix H3, have now disappeared rather than shifted. This suggests that PGA2 binding likely induced conformational changes on the nearby AQ binding site which in turn facilitated stronger AQ binding in the presence of PGA2.

AQ binding on the Nurr1 LBD-PGE2 saturated complex was also investigated since PGE2 binding is weaker than PGA2, it would be interesting to see if AQ binding might also in turn affect the existing interaction of PGE2 with the LBD. The binding affinity of AQ to the complex showed similar intermediate-fast exchange regime observed with AQ binding on Nurr1 LBD-PGA2 complex. Many peaks belonging to residues from helix H12 (Ile587-Leu591, Asp594 and Phe598) and helix H3 (Gln401-Gln405, Asp408-Leu410) have disappeared indicating strong perturbations in both PGE2 and AQ binding sites. Nearby residues such as Gln568, Gly56, Gln571, Arg572 and Ile574 from helix H11 were likely due to induced conformational change. Intriguingly, although PGE2 binding alone was not as strong as PGA2 binding, the addition of AQ to Nurr1 LBD-PGE2 complex induced stronger conformational changes in helix H12 and the AQ binding site as compared to AQ addition to Nurr1 LBD-PGA2 complex. It is tempting to postulate that AQ binding could indeed facilitate stronger binding of PGE2 that was otherwise weak, acting as a positive allosteric modulator. Since PGA2 is already covalently bonded to Nurr1 LBD, this might only have resulted in stronger binding of AQ to Nurr1 LBD without affecting greatly the existing PGA2 interaction with the LBD.

Finally, the interaction between Nurr1 LBD and PGA1 at pH 8.0 that was incubated for three days at 18 °C was also examined. The NMR data discussed so far were performed at pH 7.5 with no prior incubation period (individual PG) or were incubated overnight at 4 °C (for Nurr1 LBD-PGA2/PGE2 complexes). The spectrum of apo Nurr1 LBD at pH 8.0 was first compared with that for pH 7.5 and only a few peaks were slightly shifted which still allowed for the labelling of all the peaks at pH 8.0 with reference to assigned peaks from pH 7.5. When PGA1 was incubated with Nurr1 LBD under the conditions as mentioned, there was strong binding observed characterised by a slow-exchange regime. With increasing PGA1 concentration, there was resonance line broadening resulting in strong chemical shifts and reduction in peak intensity across many residues. Due to time constraint, the new peaks that appeared for PGA1-bound Nurr1 LBD were not assigned, therefore, only peaks affected from the apo form of Nurr1 LBD were analysed. Mapping of the affected residues on the crystal structure of Nurr1 LBD showed strong perturbations at almost the entire helix H3 especially residues such as Gln401-Gln405, Asp408 and Leu409 located at the putative AQ binding site. There were also strong perturbations on helices H11 (Gln568 and Gly569) and H12 (Leu591, Phe592, Asp594 and Thr595) based on the decrease in peak intensities. Multiple residues from helix H1 were also perturbed, most likely induced by movements from helix H3 since they are in proximity. Perturbations from scattered residues were also likely to occur due to ligand-induced conformational changes throughout the protein.

Taken collectively, our NMR and crystallization data showed that PGA1/PGA2 and PGE1/PGE2 can bind to the putative LBP of Nurr1 LBD, previously believed to be stabilized in the active conformation in the absence of a ligand through intramolecular interactions (Wang et al., 2003). The NMR data enabled us to confirm that both PGE1/PGE2 and PGA1/PGA2 occupy the same binding site in Nurr1-LBD, as crystallographic attempts could not capture the PGE1/PGE2-bound Nurr1, which was dehydrated to PGA1/PGA2 under crystallization conditions. We speculate that the binding mode of all these PGs would be similar, as the flexibility of Cys566 sidechain could accommodate the additional hydroxyl group in PGE1/PGE2 at the C11 position.

We showed that the interaction of PGs with Nurr1 LBD (a) disrupted the H12 helix positioning accompanied by several interactions of the nearby charged residues and (b) formed hydrophobic contacts and hydrogen bonding with residues in the cavity stabilizing the complex. The binding of PGA2/PGE2 also increased the binding affinity of AQ, suggesting possible allosteric regulation of the putative AQ binding site. Furthermore, there was also reorientation of helix H12 in the co-crystal structure of Nurr1 LBD with PGA1/PGA2 which was consistent with conformational changes observed in helix H12 from our NMR data with PGs. One of the possible modes of NR ligand activation is supported by a model known as H12 dynamic stabilization (Kallenberger et al., 2003, Rastinejad et al., 2015) in which the helix H12 in the apo form exists in a highly mobile, disordered and inactive state but upon ligand binding would lead to a more stable and rigid helical conformation. In a way, H12 acts as a “molecular switch” that also allows for a more exposed surface for co-activator binding (Kallenberger et al., 2003).

In addition, a “dock and lock” mechanism was suggested for the binding of 15d-PGJ2 to PPAR γ -LBD of which a Michael adduct was also formed between Cys285 sulphur of PPAR γ -LBD and 15d-PGJ2 (Shiraki et al., 2005). Shiraki’s group came up with a novel stopped-flow absorption spectrophotometric system and observed how 15d-PGJ2 would first enter the LBP (dock) followed by the covalent binding of the ligand to the protein (lock). They performed mutational analyses to show that the locking step rather than the docking step was the key to activating the protein. It is likely that the ligand moves into position in the pocket before the Michael addition reaction will occur which then leads to receptor activation (Shiraki et al., 2005). The mechanism for PGs binding to Nurr1 LBD could follow a combination of these two mechanisms in which the PGs would first enter the cavity and interact with surrounding residues followed by Michael addition, in the case of cyclopentenone prostaglandins, and the stabilization of H12 then turns on the “switch” for co-activator binding. Elucidating the mechanism for activation of Nurr1 LBD by PGs would be of interest for future research.

Site-directed mutagenesis was also carried out by Prof. Kim’s group on residues such as Thr567 (which formed hydrogen bond with cyclopentenone ketone), Leu570, Ile573 and Leu591 (non-polar interactions) that resulted in a reduction in PGA1-induced

transcriptional activation of Nurr1 LBD (manuscript under review). As for Cys566, mutating it to Phe or His rather than Ala or Ser, also diminished the PGA1-induced transcriptional activation of Nurr1, suggesting stereo-specificity of the interaction between PGA1 and particular residues.

5.2 Prostaglandins affect cell proliferation and Nurr1 transcriptional activity

In HEK293T and SK-N-BE(2)C cells, PGE1 and PGE2 were safely administered up to 100 μ M before there was any observable reduction in cell viability. Interestingly, there was a significant increase in cell viability beyond 100 % with 10 μ M PGE1 and 20 μ M PGE2 on HEK293T cells. As for PGA1 and PGA2, they were shown to be more cytotoxic as compared to their precursors. PGA2, in particular, was more harmful to SK-N-BE(2)C cells as compared to PGA1 as 0.1 μ M PGA2 led to approximately 50 % decrease in cell viability.

Earlier reports have highlighted the anti-proliferative roles of PGA1 and PGA2 in various human and mouse cell cultures (Bhuyan et al., 1986, Bregman et al., 1986, Fukushima et al., 1989) which have been attributed to their cyclopentenone moiety (Honn and Marnett, 1985, Kato et al., 1986). A study by Kondo and colleagues (2001) reported that cyclopentenone prostaglandins such as PGA2 and those derived from PGD2 in the following sequence: PGJ2, Δ^{12} -PGJ2 to 15d-PGJ2, were shown to induce oxidative stress in SH-SY5Y neuroblastoma cells and the terminal metabolite 15d-PGJ2 was the most potent among them. This finding was correlated with the cytotoxicity observed when tested on the cells. 25 μ M of those compounds caused a drastic decrease in cell viability of <30 % within 24 hours. Other prostaglandins tested such as 15-keto-PGA2, PGB2, PGE2 and 15-keto-PGE2, maintained cell viability at a basal level of 80-90 % (Kondo et al., 2001). Treatment with 15d-PGJ2 showed a significant decrease in GSH peroxidase activity and was suggested to be due to the formation of a Michael adduct between the α,β -unsaturated ketone and the selenocysteine residue in GSH peroxidase (Kondo et al., 2001). Other cellular events induced by these cyclopentenone PGs include decreased mitochondrial membrane potential by direct interaction with mitochondrial GSH causing the release of apoptotic factors; increase in the level of lipid peroxidation products that are cytotoxic to cells and also the accumulation of ubiquitinated proteins (Kondo et al., 2001).

On the other hand, the cyclopentanone PGE₂ has been shown to inhibit apoptosis caused by a COX-2 inhibitor and induces Bcl-2 expression through MAPK activation in human colon carcinoma cells (Sheng et al., 1998). The activation of EP₂ receptor in the PC3 prostate cancer cell line by PGE₂ also promoted cell proliferation and invasion (Jiang and Dingledine, 2013). Another study similarly reported that PGE₁ and PGE₂ stimulated cell proliferation in rat osteoblast-like cells (Conconi et al., 2002). These studies effectively showed that PGE₁ and PGE₂ were able to increase cell proliferation or inhibit apoptosis in certain cell types.

Furthermore, data from Prof. Kim's group showed that 1 μ M PGE₁ and 5 μ M PGA₁ were able to exert neuroprotective effects through Nurr1 on SK-N-BE(2)C cells treated with MPP⁺, a precursor of the neurotoxin MPTP, by mitigating the increase in oxidative stress levels (unpublished data). Thus, our results suggest that PGA₁ and PGA₂ shows a biphasic dose-dependent relationship between anti-proliferative and neuroprotective effects. High concentrations of PGA₁ and PGA₂ reduced cell viability after 20 μ M which is possibly due to an increase in oxidative stress (Kondo et al., 2001, Amici et al., 1992) while their use at lower concentrations was shown to be safe and could offer neuroprotective effects in neuroblastoma cells. The neuroprotective potential of PGA₁ has also been highlighted by others as shown by administration in the nano-molar range on rodent models (Zhang et al., 2005, Qin et al., 2001, Xu et al., 2006, Wang et al., 2002). Their neuroprotective ability has been suggested to be mediated through the induction of heat shock proteins in multiple nervous system injurious events (Sato et al., 1996, Amici et al., 1992, Xu et al., 2006). PGA₂ treatment, for instance, has been shown to increase the level of HSP70 expression through the activation of the heat shock transcription factor (HSF) (Holbrook et al., 1992) while PGA₁ has also shown similar activation of HSP70 as well as HSP90 (D'Onofrio et al., 1995, Amici et al., 1992).

Following the cytotoxicity screen which provided insights on the concentration-dependent effects of PGs on cells, luciferase assay was performed to test Nurr1 transcriptional activation. To that end, four copies of the NL3 motif containing the canonical NBRE element (Kim et al., 2003) was cloned upstream of the firefly *luciferase* gene on the reporter construct which Nurr1 will recognize and activate the transcription of *luciferase*. Our data showed that the positive control drug 50 μ M AQ was able to increase Nurr1 transcriptional activity by 2.3-fold compared to untreated cells. Treatment with 10 μ M of all the PGs was

able to increase Nurr1 activation by close to 4-fold which was significant and more potent than 50 μ M of AQ. Earlier studies from Holla et. al. (2006) have shown that PGE2 was able to induce Nurr1 mRNA and protein expression in colon cancer cells, and this regulation is through the cAMP/PKA-dependent pathway (Holla et al., 2006). In 2012, Ji and colleagues (Ji et al., 2012) found that PGE2 stimulation of the G protein-coupled EP₁ receptor led to a cAMP-independent activation of PKA (protein kinase A) that upregulated Nurr1 expression through CREB (cAMP response element binding protein) and NF- κ B in HEK293 and SH-SY5Y neuroblastoma cells. Our study showed for the first time that Nurr1 can be activated through direct interaction of PGs with the LBD. As Nurr1 is an essential regulator of multiple target genes in the DA neuronal system, the increase in its transcriptional activity mediated by PGs should also increase the expression of its downstream targets, serving a disease-modifying function.

5.3 Prostaglandins can mitigate LRRK2 GS-induced neurodegeneration in flies

Since LRRK2 GS mutation accounts for the highest percentage of genetic cases of PD and transgenic *Drosophila* overexpressing LRRK2 GS has been shown to recapitulate the features of late-onset PD in humans (Liu et al., 2008, Ng et al., 2009), the LRRK2 GS *Drosophila* model was selected for our drug screening. Liu et. al. (2008) observed earlier in their *Drosophila* model that the expression of LRRK2 WT was toxic to the flies although lesser than that of LRRK2 GS. Their LRRK2 WT flies showed the same DA neurodegeneration and locomotor deficits as LRRK2 GS flies also at a lesser degree. Contrastingly, our transgenic *Drosophila* model overexpressing LRRK2 GS did exhibit loss of DA neurons and locomotor deficits but this was not observed with LRRK2 WT flies, which is consistent with published results from our collaborators whom we are using the system from (Ng et al., 2009). This variation was suggested to be ascribed to different fly strains or artificial constructs that have been optimized for protein expression (Ng et al., 2009, Liu et al., 2008).

In cells, AQ was shown to have transcriptional effects at 20 μ M; the dose was increased to 50 μ M in flies due to a difference in the system used. At this dose, the flies showed improvement in their climbing ability and this concentration was maintained with the testing of PGs. We assessed the survival rate of LRRK2 GS and WT flies during the course of the experiment and found that AQ, PGA2 and PGE2 were able to show improvements to

the overall survival of the flies as compared to LRRK GS vehicle-treated flies. PGA1 and PGE1 treatment only reduced the overall trend slightly but was not a significant difference from vehicle-treated flies. Intriguingly, LRRK2 WT flies showed the lowest survival trend among all the groups which was significantly lower than LRRK2 GS flies. It is unclear why their survival rate was lower than LRRK2 GS, but a possibility could be that the overexpression of LRRK2 WT led to other cellular events independent from LRRK2 GS-induced events leading to its toxicity.

Drug treatment for the flies began from day 29-31 post-eclosion for a period of 25 days, and negative geotaxis assays were performed on the flies to determine their locomotor function. The climbing ability of LRRK2 GS vehicle-treated flies were significantly reduced as compared to LRRK2 WT flies. Although there was a reduced survival rate of LRRK2 WT flies, those that survived till the end of the experiment showed good climbing ability. Upon AQ treatment, which was our positive control, the climbing ability of the LRRK2 GS flies was rescued which was expected. For flies tested with PGs, a rescue by more than 30 % from PGA1 and PGA2 against vehicle-treated flies which reached a comparable level with AQ treatment was observed. As for PGE1 and PGE2, there was also an improvement in the climbing score although it was not deemed to be significant.

Following these findings that the PGs were able to improve locomotor impairments in the flies, it would be of interest to assess if this improvement was associated with a prevention of the loss of DA neurons in one of the six DA clusters, the PPL1 cluster, previously reported to be affected in *Ddc*-GAL4 LRRK2-GS expressing flies (Ng et al., 2012). Whole-mount adult fly brains from LRRK2-GS flies were dissected from the vehicle-treated, PGA1 and PGA2-treated groups and stained with rabbit anti-TH antibodies. DA neurons from the PPL1 cluster were imaged and quantified and the average neuron count for LRRK2-GS vehicle-treated was found to be around 8.25 which was consistent with published results (Ng et al., 2012). As IHC was not performed on WT flies, the count was referenced from published result which was close to 10 (Ng et al., 2012). Upon PGA1 and PGA2 treatment (for the same amount of time as climbing assays), there was a significantly higher neuron count that was close to that of WT. This suggests that PGA1 and PGA2 could ameliorate DA neuron degeneration associated with locomotor deficits in LRRK2 GS flies.

The development and survival of SN DA neurons has been shown to be regulated by *Nurr1* and *Pitx3* (Smidt et al., 2009, Decressac et al., 2013) and previous studies have highlighted how *Nurr1* and *Pitx3*-deficient mice show progressive loss of DA neurons as a result of decreased expression in DA-related genes such as *Th*, *Dat* and *VMAT2* (Smidt et al., 2004, Kadkhodaei et al., 2009b, Zetterstrom et al., 1997). The selective expression of LRRK2 GS in transgenic mice, showed an age-related loss of mDA axon terminals, dopamine content and release, although they seem to display normal motor function without SN DA neurodegeneration (Liu et al., 2015). While attempting to understand the molecular mechanisms of these observations, the group found that the expression of DA-related proteins such as TH, VMAT, DAT and ALDH1A1 were significantly reduced in the GS mice. More importantly, they found that the gene expression of *Pitx3* and *Nurr1* was also downregulated in these mice (Liu et al., 2015), suggesting that these genes could be downstream targets of LRRK2 GS.

In light of these findings, the level of expression of DA-related genes in this *Drosophila* model was also assessed to observe if PGA1 and PGA2 treatment could affect their expression levels. RT-qPCR was performed to determine the level of gene expression of *Dhr38*, *TH*, *Ptx1*, *Vmat*, *Ddc* and *DAT* with reference to the housekeeping gene *Act5c*. The sequence alignment of *Nurr1* with *DHR38* showed that the Cys566 in *Nurr1* is replaced with Ser in *DHR38*. As mentioned in section 5.1, the mutation of Cys566 to Ser, did not diminish the transcriptional activation of *Nurr1* in cells. As the Cys566 in *Nurr1* is replaced with Ser in *DHR38*, it is therefore still possible for PGs to bind to *DHR38* and activate its transcriptional activity in flies. *Ptx1* is the single fly ortholog for *Pitx1*, *Pitx2* and *Pitx3* showing highest similarity with *Pitx2* followed by *Pitx3* and *Pitx1* (Hu et al., 2013). *Act5c* is the fly ortholog for β -actin while the other DA-related genes have the exact or similar names with their human orthologs.

Our data showed that in LRRK2 GS flies, *Dhr38* expression was significantly lower compared to the expression in LRRK2 WT flies. The other genes tested except for *Ddc* also showed reduced expression in GS flies compared to WT, albeit deemed to be non-significant. When the LRRK2 GS flies were treated with PGA1 and PGA2, *Dhr38* expression was significantly increased compared to vehicle-treated flies. For other genes tested, PGA1 and PGA2 treatment did not significantly increase their expression. To our knowledge, this is the first report showing a reduction in *Dhr38* expression in LRRK2 GS

flies which could be alleviated by treatment with PGA1 and PGA2. This is a novel discovery that provides further opportunities to examine deeper into other mechanisms/pathways that may crosstalk with the DHR38 signalling pathway as there was no significant changes to DA-related genes supposedly activated by DHR38 following PGA1 and PGA2 treatment. Other genes such as those involved in synaptic transmission or inflammatory pathways, for example, could also be examined. Furthermore, the expression of these DA-related genes can also be analysed at different time-points as these readings were taken at the final time-point. In *Drosophila*, prostaglandins have been reported to be important during oogenesis (Spracklen and Tootle, 2015) such as eggshell formation and temporal regulation of actin cytoskeleton (Spracklen et al., 2014, Tootle and Spradling, 2008, Tootle et al., 2011). Further studies to delineate the activation of DHR38 expression by PGA1 and PGA2 could uncover other physiological roles of prostaglandins in flies.

Finally, in the 6-OHDA mouse model of PD by Prof. Lim's group, treatment with PGA1 was able to improve motor deficits and mitigate the loss of TH⁺ dopaminergic neurons in the nigrostriatal pathway in these mice as compared to vehicle-treated group. The corroboration of our data in two different *in vivo* models, further strengthen our findings that PGs possess neuroprotective effects, can function to rescue motor impairments, mitigate the loss of TH⁺ neurons and increase the expression of DA-related genes, slowing down DA neurodegeneration.

The search for endogenous ligands of Nurr1 has been an arduous journey spanning over a decade. The results here have, for the first time, presented PGA1/PGA2 and PGE1/PGE2 as potential endogenous ligands of Nurr1. Similar to the covalent binding between PPAR γ with its endogenous ligand 15d-PGJ2, PGA1 and PGA2 were able to bind covalently to Cys566 on Nurr1 LBD. Their precursors, PGE1 and PGE2 were also able to interact in the same site, as demonstrated by NMR spectroscopy. The interaction of these PGs with Nurr1 LBD showed conformational changes particularly in the H12 helix, which supports recent findings that the Nurr1 LBD could expand to accommodate unsaturated fatty acids (de Vera et al., 2019). More importantly, the PGs were able to activate the transcriptional function of Nurr1 and was shown to be more potent than AQ, previously identified to bind at another site near the LBP. In LRRK2 GS flies, the PGs were also able to mitigate motor impairments and TH⁺ neuronal loss. Also, for the first time, the expression of *Dhr38* was shown to be drastically downregulated which could be rescued by PGA1 and PGA2 treatment.

Furthermore, an *in vivo* mouse model where 6-OHDA induced locomotor deficits and TH⁺ neuronal loss, exhibited significant improvement upon PGA1 treatment. Altogether, these results paint a well-rounded picture strongly promoting these PGs as endogenous ligands of Nurr1, allowing it to finally be “adopted”.

Chapter 6:

Future Work

6. Future Work

As PGs have short half-lives, it would be crucial to design and develop analogues of the PGs studied to enhance their potency and at the same time reduce their toxicity, as shown with PGA1 and PGA2. For a start, since PGA2/PGE2 binding was shown to be able to increase the binding affinity of AQ, combinatorial chemistry can be carried out to combine the 4-amino-7-chloroquinoline scaffold with PGA2/PGE2 or modify the structure of PGA2/PGE2 to improve its stability or slow its breakdown. The same could be done with PGA1/PGE1.

As for our luciferase reporter assay, we are currently working on creating a GAL4-based assay system in which the GAL4-DBD is cloned in place of Nurr1-DBD resulting in a GAL4DBD-Nurr1LBD construct. This is a versatile system because the Nurr1 LBD can be replaced by other NR LBD and will be useful for studying NR specificity. DHR38 LBD could also be cloned in to assess the activation of its transcriptional function by PGs. Other cell lines could also be examined for cellular specific regulation of Nurr1. So far, the cells have been treated with exogenous PGs and examining their effects. It would be critical to also capture the interaction between endogenous PGA1/PGA2 (based on their covalent bond) and Nurr1 in cells. Immunoprecipitation using anti-Nurr1 antibodies can be carried out to serve such a purpose. It would also be of interest to observe if PGs binding have any effect on Pitx3 interaction with Nurr1 since Pitx3 appears to be essential for Nurr1 function.

For *Drosophila* studies, the protein expression level of DHR38 and other PD-related proteins could also be measured besides looking at the mRNA expression as performed here. The downstream effect of *Dhr38* (or *Nurr1*) gene expression and the interplay between LRRK2 and Nurr1 pathways also remains to be explored. Furthermore, it would be critical to expand our testing for the mitigation of TH⁺ neuronal loss to PGE1 and PGE2 and evaluate their effects on the gene expression profile of relevant genes.

Since prostaglandins are found naturally in our body and are involved in numerous cellular events, it is highly plausible that they could be endogenous ligands for Nurr1. Besides acting on DA neuronal development through Nurr1 activation, another possible pathway in which they could interact would be during an inflammatory event. Nurr1 has previously been shown to be involved in modulating inflammation induced injury/death in cells and mice

(Kim et al., 2015, Saijo et al., 2009) and could also interact with the p65 subunit of the transcription factor NF- κ B and inhibit the expression of inflammatory genes (Saijo et al., 2009). The inhibition of I κ B kinase (IKK) activity through the formation of Michael adduct between IKK β subunit with cyclopentenone prostaglandins that resulted in decreased NF- κ B activity and inflammation has also been reported (Rossi et al., 2000). In the case of PPAR γ -LBD and its natural ligand 15d-PGJ2, their covalent interaction was also shown to be important for the transcriptional activity of PPAR γ which in turn downregulates NF- κ B (Ricote et al., 1998, Shiraki et al., 2006). It would thus be of immense relevance to investigate further the pathways in which PGs and Nurr1 can interact such as the inflammatory pathway that both play major roles in. The pharmacokinetics (ADME/PK) of PGs would also be examined to assess for possible side effects such as heightened immune and inflammatory responses.

Chapter 7:

Conclusion

7. Conclusion

Proper development and maintenance of the dopaminergic system in the brain is critical for supporting motor functions in the body. The progressive loss of DA neurons triggered by a multitude of factors, slowly leads to the manifestation of PD. This devastating condition severely affects the quality of life for patients due to motor impairments and non-motor symptoms. There is, therefore, a pressing need to develop neuroprotective and disease-modifying therapies with the hope of slowing down further damage to existing neuronal networks.

Nurr1 has been deemed as an attractive therapeutic target for PD due to its role as a master regulator of the DA neuronal phenotype and its dysfunction has also been linked to the pathogenesis of PD. The crystal structure of Nurr1 LBD has revealed a narrow cavity filled with bulky and hydrophobic residues which occludes the binding of any ligand. Since its endogenous ligand has yet been identified, it is hence, grouped under the orphan nuclear receptor subfamily. In spite of these findings, it is still possible for small lipophilic ligands to bind to the LBD and activate its function as evidenced by reports of other NRs that have since been “adopted” or “de-orphanized”.

This thesis has presented evidence from NMR spectroscopy that PGE1, PGE2 and their cyclopentenone metabolites PGA1 and PGA2 were able to bind to the same region in Nurr1 LBD through dynamic conformational change of the helix H12. Characterization of the residues involved in the binding of the PGs with Nurr1 LBD, revealed interactions with helices H4, H11 and H12 which corroborated with data from crystallization studies of PGA1/PGA2 bound Nurr1 LBD.

Following these structural investigations, functional cell-based studies revealed that these PGs showed neuroprotective effects at low doses and were also able to activate the transcriptional function of Nurr1. In the transgenic LRRK2 GS fly model, these PGs were able to rescue motor impairments associated with late-onset PD. LRRK2 GS-induced neurodegeneration and TH⁺ neuronal loss was also alleviated by PGA1 and PGA2

treatment, possibly through the increase in mRNA expression of *Dhr38*. The decrease in *Dhr38* expression induced by the LRRK2 GS mutation was also a novel observation in flies. PGA1 similarly rescued motor deficits and TH⁺ neuronal loss exhibited in a 6-OHDA mouse model of PD.

The natural presence of PGs in cells and tissues together with the convergence of parallel pathways involving Nurr1 and PGs, support the possibility of their interaction and brings us a step closer to “adopting” Nurr1. These promising findings pave the way for future development of novel agonists of Nurr1 for PD and other Nurr1-related pathologies.

Chapter 8:

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8. References

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Appendix I:

Buffer compositions and media used in this thesis

Appendix I - Buffer compositions and media used in this thesis

1. Experimental buffers

1.1 Buffers for Ni²⁺-NTA affinity chromatography

The composition of the buffers used for Ni²⁺-NTA affinity chromatography are listed in Table I and the amount prepared is mentioned in the corresponding methods section. The buffer used for PD-10 desalting column to remove imidazole is the same as the lysis buffer listed (Table I).

Table I Composition of buffers used for Ni²⁺-NTA affinity chromatography

Buffers	Composition
Lysis	50 mM Tris-HCl (pH 7.8) and 150 mM sodium chloride
Wash 1	50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride and 20 mM imidazole
Wash 2	50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride and 40 mM imidazole
Elution	50 mM Tris-HCl (pH 7.0), 150 mM sodium chloride and 500 mM imidazole

1.2 Buffers for SDS-PAGE

5X Tris-Glycine running buffer

5 L of 5X running buffer was made by mixing 25 mM Tris base, 200 mM glycine, 0.5 % (w/v) SDS and topped up with distilled water. This was diluted to 1X for SDS-PAGE.

6X SDS loading dye

6X SDS loading dye was made by dissolving 12 % (w/v) SDS in 300mM Tris (pH 6.8) and 50 % glycerol and heating it at 42 °C before adding in 600 mM Dithiothreitol (DTT) and 0.2 % Bromophenol blue.

10 % ammonium persulfate (APS)

10 % APS was made by adding 1 g of APS into 10 ml of autoclaved ultrapure water and stored at 4 °C.

Coomassie blue staining solution

Coomassie blue staining solution was made by first preparing 50 % methanol (v/v) and 10 % glacial acetic acid (v/v) with 40 % distilled water before dissolving 0.25 % of Coomassie-Brilliant Blue R-250 powder into the solution.

De-staining solution

De-staining solution was made by mixing 45 % (v/v) methanol with 10 % (v/v) glacial acetic acid and topped with 45 % distilled water.

1.3 Buffer for FPLC and 2D ^1H - ^{15}N - HSQC NMR

For FPLC use, 2 L of 20 mM phosphate buffer (pH 7.5 or 8.0), 50 mM NaCl, 0.02 % NaN_3 was prepared using autoclaved ultrapure water and vacuum filtered with a 0.2 μm filter (Sartorius, Goettingen), sufficient for 2 rounds of equilibration and sample run. This buffer used for subsequent HSQC NMR which involved the addition of 10 % D_2O .

Phosphate buffer stock

The desired pH of phosphate buffer stock was prepared according to the formulation by Sorensen (Sorensen, 1909). x ml of 0.2 M monobasic sodium phosphate and y ml of 0.2 M dibasic sodium phosphate solution may be adjusted accordingly to prepare 100ml of 0.2M phosphate buffer of the required pH (Table II).

Table II Formulation for phosphate buffer stock

pH	x	y	pH	x	y
5.7	93.5	6.5	6.9	45.0	55.0
5.8	92.0	8.0	7.0	39.0	61.0
5.9	90.0	10.0	7.1	33.0	67.0
6.0	87.7	12.3	7.2	28.0	72.0
6.1	85.0	15.0	7.3	23.0	77.0
6.2	81.5	18.5	7.4	19.0	81.0
6.3	77.5	22.5	7.5	16.0	84.0
6.4	73.5	26.5	7.6	13.0	87.0
6.5	68.5	31.5	7.7	10.5	90.5
6.6	62.5	37.5	7.8	8.5	91.5
6.7	56.5	43.5	7.9	7.0	93.0
6.8	51.0	49.0	8.0	5.3	94.7

1.4 Buffers for immunohistochemical staining of adult fly brains

0.1 %/0.2 % phosphate buffered saline with Triton X-100 (PBT)

0.1 % or 0.2 % PBT was made by adding 0.1 or 0.2 ml Triton X-100 respectively, into 100 ml of 1X phosphate buffered saline (PBS). This was used as wash buffer.

4 % paraformaldehyde fixing solution

400 µl of fixing solution was made by adding 80 µl of 20 % paraformaldehyde (in PBS) and 40 µl of 1M HEPES (pH 7.3) to 280 µl of 1X PBS. It was made fresh for each dissection.

5 % normal goat serum (NGS) in 0.2 % PBT

50 µl of NGS was added to 950 µl of 0.2 % PBT as blocking buffer.

2. Media for Bacteria culture, Mammalian cell lines and *Drosophila melanogaster*

2.1 Media for Bacteria culture

Luria-Bertani (LB) media

1 L of LB media was made by mixing 5 g sodium chloride, 5 g yeast extract and 10 g Tryptone followed by topping up with distilled water to 1 L before sterilizing the media by autoclaving. This media was used to grow bacteria for the expression of unlabeled Nurr1 protein.

M9 minimal media

For the expression of ¹⁵N-uniformly labeled Nurr1, M9 minimal media was used for bacterial growth. 1 L of M9 media was made up of the following components: 105.2 ml of 0.5 M disodium hydrogen phosphate, 26.6 ml of 1 M potassium dihydrogen phosphate, 2 ml of 5 M sodium chloride, 1.2 ml of 1 M magnesium sulfate, 1.2 ml of 0.5 % Thiamine-Cl, 22.6 ml of 20 % glucose, 1 g of ¹⁵N-ammonium chloride and lastly 1.2 ml of 0.1M calcium chloride. Each component was either autoclaved or sterile-filtered before adding into 840 ml of autoclaved ultrapure water.

LB agar plates

17 g Bacto agar powder was added into the formulation for 1 L LB media and autoclaved before adding the required antibiotic once the media cools to 50-60 °C. The media was distributed into petri dishes and left to solidify at room temperature before storing in 4 °C.

Antibiotic formulations

Kanamycin stock (30 mg/ml) and Carbenicillin stock (100 mg/ml) were made by adding 0.3 g or 1 g powder respectively to 10ml autoclaved ultrapure water. They were sterile filtered through a 0.2 µm filter and kept at 4 °C or -20 °C respectively.

2.2 Media for mammalian cell lines

Dulbecco's Modified Eagle Medium (DMEM) containing high glucose and 4 mM L-glutamine was supplemented with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. OPTI-MEM I reduced serum medium was used for preparing the transfection mix containing plasmid DNA and transfection reagent.

2.3 Cornmeal agar for *Drosophila melanogaster*

A large batch of 15 L cornmeal agar was made by mixing 12 g of agar, 873 g cornmeal, 763.5 g dextrose, 354 g yeast with 18 L of distilled water (more water was used to account for water loss during cooking) that was cooked to a low boil and stirred constantly. 10 % Nipagin (in absolute ethanol) was added after cooling the food to about 65°C and after mixing well, about 2-3 ml of agar was then aliquoted to each drosophila vial.

Appendix II:

Confocal images for IHC
analysis in flies

Appendix II - Confocal images for IHC analysis in flies

1. Confocal images of vehicle-treated (0.5 % EtOH) LRRK2-G2019S adult fly brains stained with anti-TH antibodies

Scale bar: 20µm; number of neurons in yellow

2. Confocal images of PGA1-treated (50 μ M) LRRK2-G2019S adult fly brains stained with anti-TH antibodies

Scale bar: 20 μ m; number of neurons in yellow

3. Confocal images of PGA2-treated (50 μ M) LRRK2-G2019S adult fly brains stained with anti-TH antibodies

Scale bar: 20 μ m; number of neurons in yellow

Scale bar: 20 μ m; number of neurons in yellow

Appendix III:

2D TROSY-HSQC full
spectrum for Nurr1 LBD

Appendix III -2D TROSY-HSQC full spectrum for Nurr1 LBD